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The role of the actin cytoskeleton in calcium signaling in starfish oocytes

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ABSTRACT Ca^{2+} is the most universal second messenger in cells from the very first moment of fertilization. In all animal species, fertilized eggs exhibit massive mobilization of intracellular Ca^{2+} to orchestrate the initial events of development. Echinoderm eggs have been an excellent model system for studying fertilization and the cell cycle due to their large size and abundance. In preparation for fertilization, the cell cycle-arrested oocytes must undergo meiotic maturation. Studies of starfish oocytes have shown that Ca^{2+} signaling is intimately involved in this process. Our knowledge of the molecular mechanism of meiotic maturation and fertilization has expanded greatly in the past two decades due to the discovery of cell cycle-related kinases and Ca^{2+}-mobilizing second messengers. However, the molecular details of their actions await elucidation of other cellular elements that assist in the creation and transduction of Ca^{2+} signals. In this regard, the actin cytoskeleton, the receptors for second messengers and the Ca^{2+}-binding proteins also require more attention. This article reviews the physiological significance and the mechanism of intracellular Ca^{2+} mobilization in starfish oocytes during maturation and fertilization.

KEY WORDS: *cell activation, sperm-egg interaction, meiosis, germinal vesicle, cyclic ADP ribose, NAADP*

Starfish oocytes and maturation-promoting factor

Starfish oocytes have contributed greatly to our understanding of the molecular mechanisms controlling the cell cycle (maturation) and fertilization. During oogenesis, the oocytes undergo cell growth and differentiation bringing them to the end of the first prophase of meiosis where they remain synchronously arrested until spawning (Fig. 1). At this stage of maturation, the oocytes are characterized by a very large nucleus (approximately 60 μm in diameter) termed the germinal vesicle (GV) (Fig. 2). The starfish oocyte has several advantages as an experimental model system. First, the cell is large and nearly transparent, making it suitable for imaging experiments after microinjection of fluorescent markers. Second, maturation (or meiosis re-initiation) can be induced *in vivo* and *in vitro* by the hormone 1-methyladenine (1-MA), rendering the oocytes successfully fertilizable (Kanatani *et al.,* 1969; Meijer and Guerrier, 1984). The first visible sign of oocyte maturation is the germinal vesicle breakdown (GVBD) in which the large nucleus breaks down to release its nucleoplasm into the cytoplasm 20-30 minutes after the application of the hormone. This is how the starfish oocyte is conspicuously different from sea urchin eggs, which are already mature at the time of spawning. In sea urchins, the haploid egg pronucleus has a complete nuclear envelope, and the chromatin is decondensed in the interphase of the first mitosis.

One of the most dramatic events that follows hormonal stimulation of starfish oocytes is protein phosphorylation, which is associated with the increase of protein kinase activity (Guerrier *et al.,* 1977). Indeed, the idea that the cyclin-dependent kinase Cdc2/CDK1-cyclin B plays a pivotal role in the control of the G2/M transition phase of the cell cycle also came from the physiological studies of the starfish oocytes (Dorée and Hunt, 2002). Following the discovery that the progesterone-matured frog oocytes produce a cytoplasmic factor that causes maturation (hence, named “Maturation-Promoting Factor” or MPF) (Masui, 2001), similar results were obtained from the starfish oocytes. The cytosol of 1-MA-treated starfish oocytes can induce GVBD and polar body formation when injected into immature oocytes. Addi-

Abbreviations used in this paper: cADPr, cyclic-ADPribose; CaMKII, calmodulin dependent kinase II; CICR, calcium induced calcium release; GV, germinal vesicle; GVBD, germinal vesicle breakdown; InsP3, inositol 1,4,5-trisphosphate; LAT-A, latrunculin-A; MPF, maturation promoting factor; NAADP, nicotinic acid adenine dinucleotide phosphate; PIP2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; RyR, ryanodine receptor.

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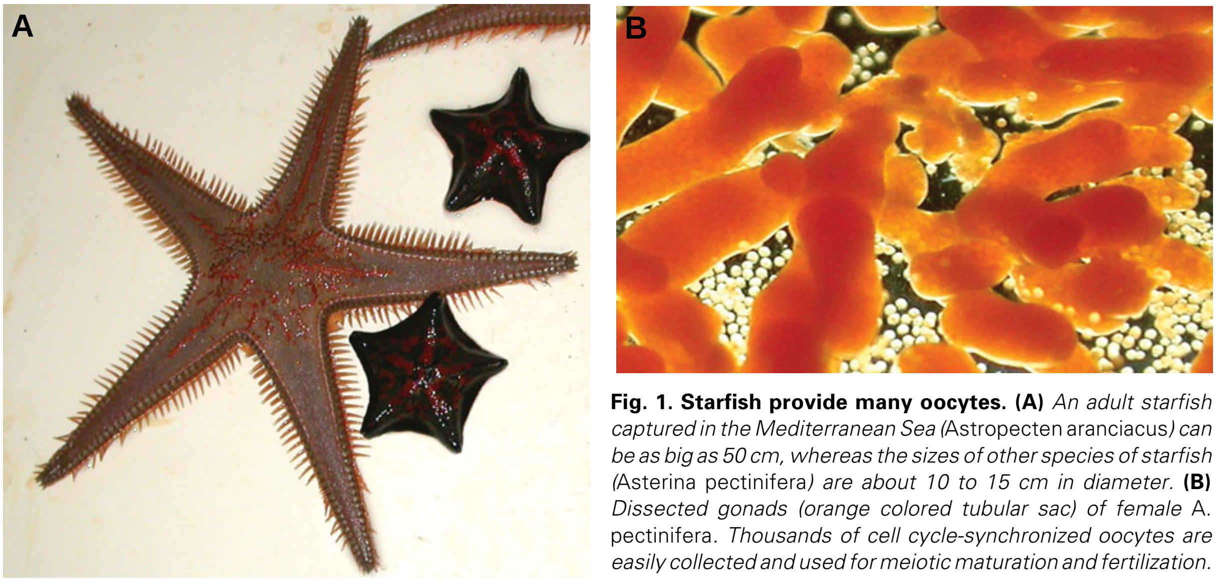

Fig. 1. Starfish provide many oocytes. (A) *An adult starfish captured in the Mediterranean Sea (*Astropecten aranciacus*) can be as big as 50 cm, whereas the sizes of other species of starfish (*Asterina pectinifera*) are about 10 to 15 cm in diameter.* **(B)** *Dissected gonads (orange colored tubular sac) of female* A. pectinifera*. Thousands of cell cycle-synchronized oocytes are easily collected and used for meiotic maturation and fertilization.*

tional evidence that MPF is a Cdc2/CDK1-cyclin heterodimer was also demonstrated in starfish oocytes (Prigent and Hunt, 2004). Recently, the signaling pathway leading to MPF activation has been unraveled in several experimental systems. 1-MA activates the kinase Akt which phosphorylates and down-regulates Myt1, a membrane-associated inhibitory kinase that in turn phosphorylates Cdc2 on both threonine-14 and tyrosine-15 (Mueller *et al.,* 1995; Okumura *et al.,* 2002). Cdc25 is a tyrosine phosphatase that activates MPF by dephosphorylating the tyrosine residues. The activity of Cdc25 is in turn regulated by autophosphorylation and dephosphorylation, which is sensitive to protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Masui, 2001; Prigent and Hunt, 2004). In starfish MPF is inactive in the cytoplasm of immature starfish oocytes, but it accumulates in the nucleus after being activated (Ookata *et al.,* 1992). The translocation of MPF into the nucleus along with Cdc25 is thought to be essential for the final MPF activation. Studies in a number of cell types have indicated that the perinuclear/centrosomal region is the site of MPF accumulation (Bechelling *et al.,* 2000). Recently, using cyclin B-GFP, it was shown that MPF enters the starfish nucleus starting from the animal pole side, which is the location of the centrosomes (Terasaki *et al.,* 2003). The timing of its accumulation in the nucleus is consistent with its proposed role in disassembling the nuclear envelope, as the event takes place after the phosphorylation of the nuclear pore complexes (Macaulay et *al.,* 1995). MPF may also play a role in chromosome condensation as was demonstrated by its periodic appearance during the mitotic cycles in the blastomere cells of amphibian embryos (Wasserman and Smith, 1978).

Roles of calcium in the maturation process of starfish oocytes

Starfish oocytes have also been an excellent model system for studying the initial events of meiotic maturation. All observations indicated that 1-MA becomes effective in inducing oocyte maturation only if the hormone was applied outside the oocyte, suggesting that the binding of the hormone to the cell surface receptor is an indispensable step (Kanatani and Hiramoto, 1970). After that, interest converged on understanding how the hormonal message could be transduced through the plasma membrane toward the effectors. The important role of Ca^{2+} in this maturation process had already been demonstrated in many animal species based on the external Ca^{2+} requirements, or an increased Ca^{2+} concentration in the oocyte (Guerrier *et al.,* 1982; Meijer and Guerrier, 1984). In starfish, microinjection of Ca^{2+} indicator aequorin showed that the hormonal stimulation immediately results in a transient increase of intracellular Ca^{2+} during the first 2 min (Moreau *et al.,* 1978). The Ca^{2+} increase occurred even in the absence of the external Ca^{2+}, indicating that Ca^{2+} was released from the intracellular stores. These results were confirmed with the *in vitro* experiments showing that 1-MA could trigger a Ca^{2+} release from the isolated plasma membrane-enriched fraction (Dorée *et al.,* 1978; Meijer and Guerrier, 1984). However, the possibility that Ca^{2+} could control the MPF activity was subsequently questioned by other researchers. Indeed, studies in a number of species failed to detect changes in Ca^{2+} levels after hormonal stimulation of the oocytes, and they suggested that Ca^{2+} signaling is not absolutely required for all oocyte maturation (Eisen and Reynolds, 1984; Witchel and Steinhardt, 1990).

Over the last 13 years, our laboratory has been studying calcium signaling in the cytoplasm and the nucleus of starfish oocytes during the meiotic maturation. We have used either confocal microscopy or a sensitive CCD (charge-coupled device) camera, which are connected to the computer programs analyzing the Ca^{2+} mobilization patterns between the cytoplasm and the nucleus. We found that 1-MA induces a cytosolic Ca^{2+} increase which is then followed by Ca^{2+} elevation in the nuclear compartment several seconds later. The injection of the calcium chelator BAPTA directly into the nucleus completely blocked GVBD and the continuation of the maturation process, indicating that Ca^{2+} plays a crucial role in triggering meiosis re-initiation (Santella and Kyozuka, 1994; Santella, 1998). The delayed rise of Ca^{2+} in nucleus suggests that the nuclear envelope may serve as a diffusion barrier for Ca^{2+}, and that the nucleus may have a distinct mechanism of Ca^{2+} release. Later studies provided additional evidence in favor of the idea that the nucleus of starfish oocytes is insulated from the cytosolic Ca^{2+} transients (Santella *et al.,* 2003). The Ca^{2+} permeability of the nuclear envelope and the mode of Ca^{2+} rise in the nucleus has been a subject of a vigorous debate in the literature (Santella, 1996; Santella and Carafoli, 1997; Bootman *et al.,* 2000; Gerasimenko and Gerasimenko, 2004).

The very large size of the nucleus of the arrested oocytes has made it easier to inject directly into the nucleus the Ca^{2+}-linked second messengers and the inhibitors of the calcium signaling pathway. In line with a role of nuclear Ca^{2+} in the activation of the maturation process, the delivery of the Ca^{2+}-linked second messenger inositol 1,4,5-trisphosphate ($InsP_3$) into the nucleus did release starfish oocytes from the I-prophase block (Santella and Kyozuka, 1997). These findings were at variance with the previous results provided by others showing that Ca^{2+} release induced by cytoplasm-injected $InsP_3$ does not trigger the meiotic process (Picard *et al.,* 1985). Taken together, these results added weight to the hypothesis that "nuclear" calcium signaling is indeed essential for the re-initiation of maturation.

On the other hand, the way the nucleus-injected $InsP_3$ elicits Ca^{2+} increase raises intriguing questions as to the molecular mechanism of Ca^{2+} release from the internal stores. After the microinjection of $InsP_3$ into the nucleus, a very evident Ca^{2+} increase started at the point of the $InsP_3$ delivery and propagated to the entire nucleoplasm. The nuclear Ca^{2+} increase declined approximately after 20 sec, but the Ca^{2+} level failed to reach the baseline and remained elevated for the entire duration of the experiment (Santella *et al.,* 2003). The fact that the Ca^{2+} concentration in the nucleoplasm did not return to the normal level for a long time indicates that there is no free diffusion of Ca^{2+} through the nuclear pore complexes (NPCs) as was suggested by others (Brini *et al.,* 1993; Gerasimenko *et al.,* 1995; Perez-Terzic *et al.,* 1997). The criticism that the injection of $InsP_3$ may have brought contaminating Ca^{2+} into the nucleus was proven invalid by the different kinetics of the Ca^{2+} increase following the delivery of 1 mM Ca^{2+} instead of $InsP_3$. In this case, the Ca^{2+} signal declined and disappeared completely in about 40 sec. Then, it is evident that $InsP_3$, which is known to act on its receptors on the endoplasmic reticulum, liberates Ca^{2+} in the nucleoplasmic compartment independent of the cytosolic Ca^{2+} increase (Santella *et al.,* 2003). In line with a nucleoplasmic Ca^{2+} storage and release, the nucleus of the epithelial cells was shown to contain a reticular network that is continuous with the endoplasmic reticulum and the nuclear envelope. Photoactivation of caged $InsP_3$ in the nucleoplasmic reticular structures resulted in small increases of Ca^{2+}, suggesting that the nuclear network expresses functional $InsP_3$ receptors (Echevarría *et al.,* 2003). These results are in agreement with the previous data showing that Ca^{2+} can be released from the nuclear tubular structures in several cell types (Lui *et al.,* 1998), and that the antibodies against $InsP_3R2$ interact with them (Laflamme *et al.,* 2002). Besides the $InsP_3$ receptors, functional ryanodine receptors (RyRs) were observed on the invaginations of the nuclear envelope and the nucleus-penetrating endoplasmic reticulum of the striated muscle cells (Marius *et al.,* 2006). The type 1 RyR is also expressed on intranuclear extensions of the sarcoplasmic reticulum of a skeletal muscle derived cell line (Marius et *al.,* 2006). In the immature oocytes of starfish, however, the GV generally lacks $InsP_3R$ (Iwasaki *et al.,* 2002), and the anti-$InsP_3R$ immunogold staining did not decorate the inner membrane of the GV in starfish oocytes (Santella and Kyozuka, 1997). The GV of immature starfish nonetheless released Ca^{2+} in response to the injected $InsP_3$ (Santella *et al.,* 2003). Hence, the molecular detail of Ca^{2+} release in the nucleus of starfish is still an open question, and it is even conceivable that $InsP_3$ might have an alternative target or pathway to release Ca^{2+}.

$InsP_3$ is not the only second messenger that releases Ca^{2+} inside the nucleus. The nucleus of starfish oocyte can also respond to cyclic ADP-ribose (cADPr) and liberate Ca^{2+}. cADPr is an endogenous metabolite of β-NAD^+ with a potent Ca^{2+}-mobilizing activity. The Ca^{2+} releasing activity of NAD^+ was first discovered in sea urchin homogenates, but the response required a characteristic delay. Later studied showed that this delay was attributable to enzymatic conversion of β-NAD^+ by ADP-ribosyl cyclase (Lee, 2002). Cross-desensitization studies with ryanodine and caffeine indicated that cADPr releases Ca^{2+} via a ryanodine-sensitive calcium-induced-calcium-release (CICR) mechanism through the activity of a soluble protein (Clapper *et al.,* 1987). Caged cADPr injected into starfish nuclei can elevate nuclear Ca^{2+} after its activation with UV light (Santella and Kyozuka, 1997). The nuclear Ca^{2+} transients induced by cADPr often showed an oscillatory character, and the peak-to-peak intervals of the repetitive spikes ranged 5 to 10 min. This observation implies that functional RyRs may reside in the nucleus of immature oocytes. The presence of functional RyRs in the nucleus was proven true more recently in other cell types (Marius *et al.,* 2006).

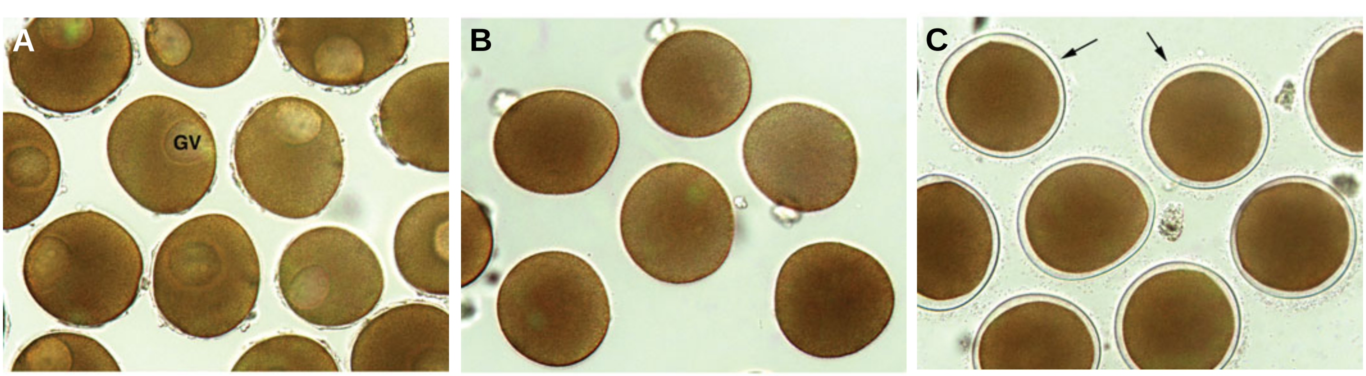

Fig. 2. Starfish oocytes exhibit reliable *in vitro* fertilization. (A) *Immature oocytes are characterized by the big nucleus called the germinal vesicle (GV) near the animal pole.* **(B)** *In the presence of 1-methyladenine, oocytes undergo meiotic maturation. The GV is now mixed with the cytoplasm and no longer visible.* **(C)** *Successful fertilization induces elevation of the vitelline envelope (arrows), thus preventing polyspermy.*

Aside from the Ca^{2+} releasing intracellular channels, it has long been noted that nucleus contains phospholipase C (PLC) and other components for producing $InsP_3$ (Cocco *et al.,* 1994; Divecha *et al.,* 1991), as well as and the cADPr-producing enzymes in the inner membrane of the nuclear envelope (Adebanjo *et al.,* 1999). Taken together, these observations suggest that the nucleus contains intrinsic and autonomous capacity for intranuclear mobilization of Ca^{2+}.

Ca^{2+} increases during the maturation of starfish oocytes are temporally and spatially associated with the GVBD. Besides the initial Ca^{2+} burst, our laboratory has detected Ca^{2+} signals for the first time in the perinuclear area a few minutes before the breakdown of nuclear envelope (Lim *et al.,* 2003). The spatiotemporal pattern of this Ca^{2+} release coincided with the increased sensitivity of the Ca^{2+} stores to $InsP_3$, which always starts at the animal hemisphere (where the nucleus is located) about 12 or 17 minutes after the addition of 1-MA, depending on the species used (see below). Starfish oocytes have been well suited for the visualization of the perinuclear Ca^{2+} changes by analyzing the ratio between the fluorescence of the Ca^{2+}-sensitive dye (Oregon Green 488 BAPTA-1) and that of the Ca^{2+}-insensitive internal control dye (rhodamine). Both fluorescent dyes are conjugated to dextran (Mw=70,000 Da, so that they cannot cross the intact nuclear envelope) and co-injected prior to the addition of 1-MA. Since the rhodamine control distinguishes nonspecific contributions such as the ones provoked by the contraction of the oocytes that may occur before GVBD, this analysis allowed precise evaluation of the real changes in the intracellular Ca^{2+} concentration. The evidence that the Ca^{2+} increase just precedes GVBD was provided by the subsequent entry of the dyes into the nucleoplasm as a result of the breakdown of the nuclear envelope (Lim *et al.,* 2003). These findings were in line with the data from the early sea urchin embryos, where the entry into the mitosis required triggering of perinuclear Ca^{2+} transient before the nuclear envelope breakdown (Poenie *et al.,* 1985; Wilding *et al.,* 1996). Regarding the initial Ca^{2+} burst, a recent contribution has suggested that the early Ca^{2+} transient increase following 1-MA application might directly trigger MPF activation. The inhibition of the Ca^{2+} increase with a chelator TMB-8 also inhibited MPF, GVBD and the subsequent chromosome condensation in *Asterina pectinifera* oocytes (Tosuji *et al.,* 2007). The experimental evidence for the two Ca^{2+} signals occurring in the first few minutes of 1-MA stimulation, and before the GVBD, supports the idea that Ca^{2+} is indeed a universal activator of both the mitotic and the meiotic cell cycles.

Calcium targets during oocyte maturation

Experiments were then performed to identify potential calcium targets among the calcium-activated proteins that may play a role in the maturation of starfish oocyte. Calmodulin (CaM) is a Ca^{2+}-binding protein with EF hand motif and is considered as the key molecule to transduce Ca^{2+} signals either by activation of target enzymes or by modulation of protein/protein interactions in the cytoplasm and the nucleus (Carafoli *et al.,* 2001). Nuclear Ca^{2+} also plays an important role in regulating gene expression by several distinct pathways. While the trans-activating properties of transcription factors such as CREB (cAMP-responsive element binding protein) are regulated by Ca^{2+}-dependent kinases and phosphatases (Hardingham *et al.,* 2001), Ca^{2+} can also directly bind to a transcription regulator such as DRE-antagonist modulator (DREAM) and modulate gene expression (Carrión *et al.,* 1999).

In starfish, following the identification of CaM and several CaM-binding proteins, the role of CaM in the regulation of the meiotic cycle has been investigated using CaM antagonists. Several inhibitors and antagonists could suppress 1-MA-induced maturation (Mejier and Guerrier, 1984). Experiments were performed to establish whether a nuclear CaM pool is relevant to the progression of the meiotic cycle. CaM antagonists, antibodies, and the inhibitory peptide corresponding to the CaM-binding domain of myosin-light-chain kinase were directly injected into the nucleus of prophase-arrested starfish oocytes. While the CaM antagonists only delayed GVBD, the peptide inhibitor and the antibodies completely inhibited it. The antibodies also suppressed the nuclear Ca^{2+} spikes that were induced by photoactivation of caged cADPr in the nucleus. Immunofluorescence staining of isolated starfish oocyte nuclei with CaM antibodies showed that CaM is localized in the nuclear envelope and in the nucleolus, while immunogold labeling studies revealed that the aggregates of 36-kDa protein and CaM are present in the nuclear matrix as heterogeneous ribonucleoprotein particles (hnRNP). 1-MA treatment made these hnRNP disappear from the nucleoplasm and caused translocation of CaM and its associated 36-kDa protein to the cytoplasm before the breakdown of the nuclear envelope (Santella and Kyozuka, 1997a). Taken together, these observations strongly suggested that a CaM-dependent step in the nucleus is involved in the initiation of the maturation process. It is now known that the nuclear activation of MPF is mediated by translocated Cdc25 which in turn is activated by a calmodulin-dependent protein kinase II (CaMKII) in the nucleus (Kishimoto, 1999; Lim *et al.,* 2003). A role for CaMKII in promoting GVBD and the metaphase-anaphase transition has also been demonstrated with the maturing mammalian oocytes (Su and Eppig, 2002).

Development of the Ca^{2+}-releasing systems during maturation of starfish oocytes

During the meiotic maturation, a starfish oocyte develops its ability to be successfully activated by a fertilizing spermatozoon. Data have been documented on the morphological changes that accompany maturation, and it has been reported that the electrophysiological properties of the oocyte are also changed in this process (Meijer and Guerrier, 1984).

The immature starfish oocyte manifests polarized cell morphology with its large nucleus located closer to the plasma membrane of the animal hemisphere. The cytoskeletal organization of the animal pole is also different from that of the other hemisphere. In addition, the cortical region of the oocyte differs from the inner cytoplasm in that the F-actin filaments are orderly clustered beneath the plasma membrane and form a visibly distinct cortical layer. The 1-MA treatment induces morphological changes at both cortical and nuclear regions. Scanning EM and immunofluorescence microscopic studies have established that 1-MA stimulates the transient appearance of prominent spike-like protrusions on the oocyte surface due to the rapid assembly of the actin filaments in the inner-core bundles of microvilli (Schroeder and Stricker, 1983; Otto and Schroeder, 1984). Following these early events, 1-MA subsequently induces more massive reorganization of the endoplasmic reticulum (ER), the major cytoplasmic Ca^{2+} store that contributes to the development of a normal Ca^{2+} response at fertilization. The purpose of this process, hallmarked by the breakdown of nuclear envelope and the intermixing of the nucleoplasm with the cytoplasm, is to adapt the cells to the subsequent fertilization event. In the end of the maturation process, the intracellular Ca^{2+} release is facilitated, and the Ca^{2+}-induced exocytosis of cortical granules leads to elevation of the vitelline layer (fertilization envelope) to prevent polyspermy (Longo *et al.,* 1995; Santella *et al.,* 1999).

With starfish oocytes, it was first documented that the maturation-induced structural reorganization of the ER is linked to the facilitated Ca^{2+} signaling in fertilization. The visualization of the ER membranes by injecting an oil drop saturated with the fluorescent lipophilic dye DiI (Jaffe and Terasaki, 1994) has made it possible to easily observe the changes of the ER structures during the 1-MA-induced meiotic maturation. The dramatic structural changes of ER could explain why sperm entry produces more Ca^{2+} release in the mature eggs than in the immature oocytes (Chiba *et al.,* 1990). The ability of an egg cell to release more Ca^{2+} after maturation was also found in other species. Maturation-induced formation of ER clusters in marine worm eggs was shown to be associated with the ability to elicit a proper Ca^{2+} response at fertilization (Stricker *et al*., 1998). Similar ER clusters, which were not present at the GV-stage, also appeared in the mouse egg cortex following the meiotic maturation (Mehlmann *et al.,* 1995). Since the available evidence at that time indicated that the Ca^{2+} release at fertilization was mediated by $InsP_3$, (Swann and Whitaker, 1986), the Ca^{2+}-releasing effect by $InsP_3$ was investigated before and after the maturation process. It was found that the response of the Ca^{2+} stores to the same amount of $InsP_3$ was much higher in the oocytes after maturation (Chiba *et al.,* 1990; Chun and Santella 2007). Such increased sensitivity of the $InsP_3$ receptors to $InsP_3$ was not due to the increased charge of the Ca^{2+} stores during the maturation process nor to overexpression or redistribution of the $InsP_3$ receptors (Iwasaki *et al.,* 2002). The $InsP_3$-sensitive Ca^{2+} stores were already fully replenished in immature starfish oocytes so that $InsP_3$ would have induced comparable Ca^{2+} release from immature and mature oocytes (Chiba *et al.,* 1990; Lim *et al.,* 2003). Subsequent works in other species were aimed at the differential expression and redistribution of the $InsP_3$ receptors during the maturation process. Indeed, the increased sensitivity to $InsP_3$ between the GV stage and prometaphase of the first meiosis in mammalian oocytes (Fujiwara *et al.,* 1993) correlated with the increased number of the cortical $InsP_3$ receptors (Mehlmann *et al.,* 1996). In addition, heterogeneity and the differential expression level of $InsP_3R$ isoforms during maturation and fertilization may add to the functional fine tuning of the $InsP_3$ receptor complex (Parrington *et al.,* 1998; Fissore *et al.,* 1999; Malcuit *et al.,* 2005).

In starfish, RyR Ca^{2+} channels also produce characteristically distinct Ca^{2+} release patterns after the oocyte maturation process. While RyR agonists include ryanodine, cADPr and caffeine, cADPr is the major endogenous agonist of the receptor (Lee and Aarhus, 1991). The pre-injection of the starfish oocytes with specific antagonist of the cADPr/ryanodine receptors $8NH_2cADPr$ completely blocked the Ca^{2+} response following the cADPr uncaging, confirming that RyR mediates the Ca^{2+} releasing effect of cADPr. RyR mediates CICR, and it modulates the Ca^{2+} signals following sea urchin fertilization (Galione *et al.,* 1993). The spatiotemporal aspects of the cADPr-dependent Ca^{2+} release have been also explored in immature and 1-MA-matured starfish oocytes. In immature *Astropecten aranciacus* oocytes, uncaging of the injected cADPr produced multiple patches of Ca^{2+} release in the cortical region. The Ca^{2+} signals then spread centripetally from these initial points of increase to the entire cell. Both the cortical Ca^{2+} patches and the global Ca^{2+} wave induced by cADPr are due to the Ca^{2+} release from the intracellular stores, as indicated by the lack of effect of external Ca^{2+}. In mature oocytes, the photoactivation of cADPr initiates Ca^{2+} release in the cortex from fewer spots (one or two at most), but the Ca^{2+} release was greatly enhanced in comparison with the immature oocytes. The mature oocytes manifested clearly defined cortical flash in response to the uncaged cADPr. The Ca^{2+} burst is then followed by subsequent globalization of the wave before the elevation of the fertilization envelope. In both mature and immature oocytes, it is worth noting that cADPr initiates Ca^{2+} responses preferentially in the cortical areas. These results imply that the cortical region beneath the plasma membrane is generally more sensitive to cADPr than the inner cytoplasm. However, the precise role of cADPr in shaping the intracellular Ca^{2+} waves during fertilization is not clear. The sequence of Ca^{2+} responses induced by cADPr in mature oocytes strongly mimicked those seen at fertilization, suggesting that cADPr and ryanodine receptors might play a role in the initial Ca^{2+} signal at fertilization. However, the pre-injection of the cADPr antagonist did not inhibit the propagation of the sperm-induced Ca^{2+} wave, indicating that cADPr is not involved in the CICR mechanism in these oocytes species. This suggestion is further supported by the lack of Ca^{2+} elevation following the uncaging of cADPr in the Japanese species (*Asterina pectinifera*) of starfish oocytes (Nusco *et al.,* 2006).

Very recently, the development of the RyR- or $InsP_3$ receptor-dependent Ca^{2+}-releasing systems was investigated during the *in vitro* maturation of sea urchin oocytes. By comparing the Ca^{2+} response induced by cADPr, $InsP_3$ or the sperm, it has been demonstrated that the sensitivity to cADPr (RyR pathway) is much higher than that of $InsP_3$ whose response is established considerably later (Miyata *et al.,* 2006). By contrast, in mammalian oocytes, the density of the RyR is 30- to 100-fold lower than that of the $InsP_3R$, implying that the Ca^{2+} signals in meiotic maturation is mostly attributed to $InsP_3R$. These results agree with the suggestion that, although ryanodine receptors are present and functional in mammalian oocytes, the release of Ca^{2+} from this store is not essential for the sperm-induced egg activation (He *et al.,* 1997).

We have found that a newly established second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) also contributes to the intracellular Ca^{2+} release in starfish oocytes (Santella *et al.,* 2000). In NAADP, the nicotinamide ring of nicotinamide adenine dinucleotide phosphate (NADP) is replaced by nicotinic acid. Ever since its discovery as a Ca^{2+}-mobilizing molecule in sea urchin egg homogenates, NAADP has exhibited distinct pharmacological properties (Clapper *et al.,* 1987). Depletion of Ca^{2+} stores from endoplasmic reticulum (ER) in sea urchin egg homogenates using thapsigargin, a sarcoplasmic/ER calcium ATPase (SERCA) pump inhibitor, does not prevent NAADP-mediated release of Ca^{2+} from these preparations. Hence, the

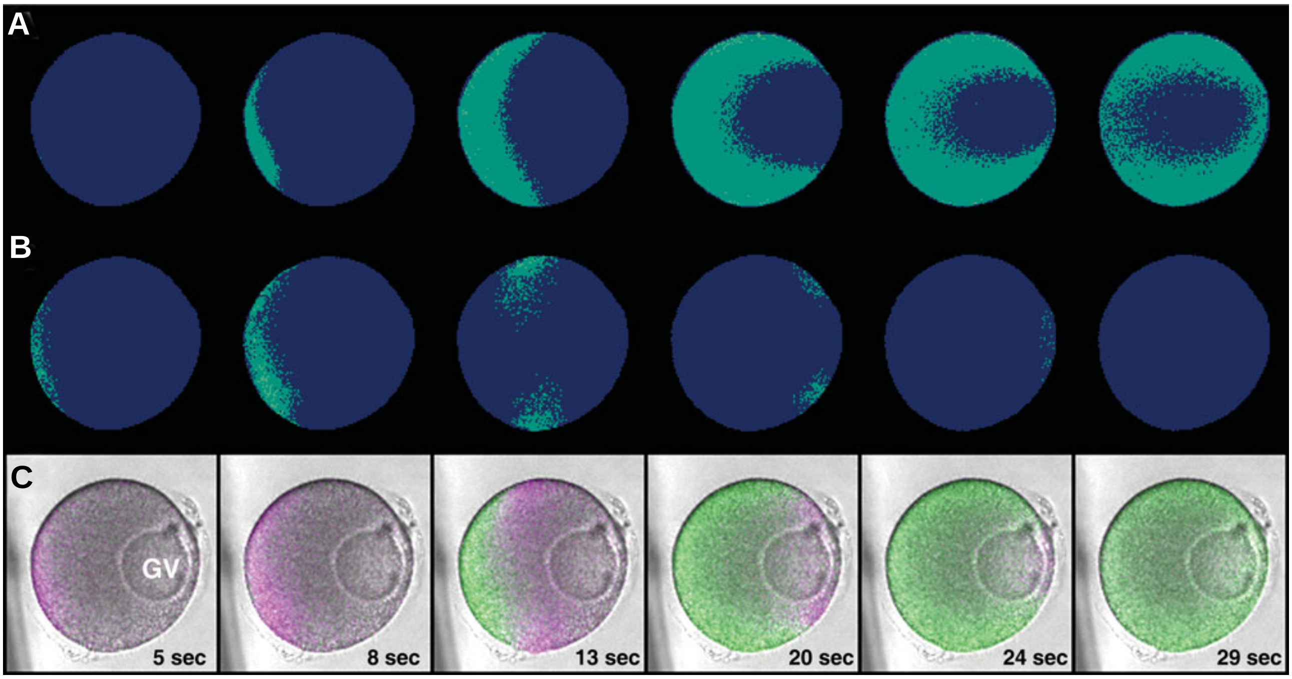

Fig. 3. Meiotic maturation of starfish oocytes is signalled by quick mobilization of intracellular Ca^{2+}. *Within 1-2 min after the addition of 1-methyladenine, intracellular Ca^{2+} starts to be released from the vegetal hemisphere of the oocytes.* **(A)** *The relative fluorescence pseudo-colored images of the Ca^{2+} wave.* **(B)** *The relative pseudo-colored images of the instantaneous Ca^{2+} release represented by the formula $F_{inst}=[(F_t-F_{t-1})/F_{t-1}]$.* **(C)** *The merged views of the transmission micrograph and the two relative pseudo-colored images acquired in (A) and (B). Green fluorescent Ca^{2+} dye delineates diffusion of intracellular Ca^{2+}, whereas the momentary increment of Ca^{2+} was depicted in pink to show the actual sites of instantaneous Ca^{2+} release.*

NAADP-sensitive Ca^{2+} stores are not located in the ER. These findings are also in line with the distribution profile of NAADP-mediated Ca^{2+} stores in cell fractionation experiments, which did not co-migrate with the ER fraction (Genazzani and Galione, 1996). In starfish oocytes, one peculiar aspect of the NAADP-induced Ca^{2+} response is that the cortical Ca^{2+} mobilization by uncaged NAADP is linked to the extracellular Ca^{2+} (Nusco *et al.,* 2002). The NAADP-induced Ca^{2+} mobilization becomes more dependent on the external Ca^{2+} as the starfish oocyte is matured by 1-MA, raising the possibility that the NAADP-sensitive Ca^{2+} pool might be shifted toward the plasma membrane during this process (Santella *et al.,* 2000; Santella, 2005).

Maturation-promoting factor and the increased sensitivity of $InsP_3$ receptors

In starfish, the whole process leading to GVBD in the 1-MA-treated oocytes can be divided into three steps with regard to the changes in MPF activities. In the first stage, the activation by 1-MA produces a small amount of active MPF in the cytoplasm which increases due to its autocatalytic amplification. Then, MPF accumulates in the nucleus before GVBD takes place (Picard and Dorée, 1984). Finally, the MPF activity is boosted further by Cdc25 inside the GV. Indeed, MPF is completely absent in the GV of the starfish oocytes before the 1-MA treatment as determined by confocal microscopy. After the onset of GVBD, however, higher intensity of the anti-cyclin B immunofluorescence was observed in the nucleus than in the cytoplasm (Ookata *et al.,* 1992). It has been suggested that MPF plays a role in the modification of the centrosome, which eventually leads to the formation of the mitotic spindle (Bailly *et al.,* 1989).

Apart from the role of MPF as a decisive factor that liberates the oocytes from the prophase block and induces the complete meiotic maturation, MPF appears to modulate ER reorganization and Ca^{2+} signaling in both maturing and fertilized oocytes (Chun and Santella, 2007). Pharmacological and biochemical assays combined with *in vivo* confocal imaging have demonstrated a functional relationship between MPF activities and the ER cluster formation. While maturing oocytes manifested higher MPF activity and more extensive clusters formation, fertilized eggs undergoing ER cluster disassembly showed concomitant drop of MPF activity (Stricker and Smythe, 2003). On the other hand, metaphase II-arrested oocytes maintain high levels of MPF activity. At fertilization, the transition to anaphase is stimulated by the sperm-induced increase in intracellular Ca^{2+}. This increase of Ca^{2+} results in the destruction of cyclin B1 (the regulatory subunit of MPF) and the kinase inactivation, which eventually leads to egg activation (Marangos and Carroll, 2004). Hence, in large part, the downstream effect of intracellular Ca^{2+} signaling may be mediated through MPF.

Conversely, MPF activity can also affect the intracellular mobilization of Ca^{2+}. It has been shown that MPF activity can influence the sperm-triggered calcium oscillations in ascidian and mammalian eggs (Levasseur and McDougall, 2000; Deng and Shen, 2000; Marangos and Carroll, 2004). In starfish oocytes, the experiments with the MPF inhibitor roscovitive have demonstrated that the increased $InsP_3$ response during meiotic maturation is strongly correlated with the MPF activity. While roscovitine inhibited GVBD and 1-MA-induced Ca^{2+} signals, injection of active MPF into immature oocytes produced Ca^{2+} signals and frequent GVBD. Furthermore, the global propagation of Ca^{2+} signals takes place only when $InsP_3$ was photoactivated well after the full establishment of MPF activity, e.g. 30 min after hormonal stimulation (Lim *et al.,* 2003). While the nuclear amplification of MPF may be mediated by the CaMKII–linked activation of Cdc25 (Patel *et al.,* 1999), it is not known whether the increased $InsP_3$-sensitivity of the Ca^{2+} pool is due to the direct (or indirect) phosphorylation of $InsP_3$ receptors by MPF. For this reason, search for the other MPF targets have been made (see below).

F-actin and actin-binding proteins modulate the release of Ca^{2+} during oocyte maturation

In the past few years, additional information has been accumulated on the spatiotemporal pattern of the 1-MA-induced Ca^{2+} signals. We have observed that the Ca^{2+} signal always initiated at the certain side of the oocytes, the vegetal hemisphere (Fig. 3). The Ca^{2+} wave propagates to the cytoplasm in a shape of cortical half moon and eventually reaches the nucleus at the opposite side within 15-20 sec (Santella *et al.,* 2003). By analyzing the incremental changes of the Ca^{2+} rise to map the actual site of instantaneous Ca^{2+} release, we have noticed that the 1-MA-induced Ca^{2+} increase is exclusively localized to the cortex (Moreau *et al.,* 1978; Kyozuka *et al.,* 2008). Combined with the fact that the Ca^{2+} wave specifically starts from the vegetal hemisphere, where $InsP_3$ receptors are less concentrated, these observations raised a possibility that additional factors intrinsic to the cortex may also contribute to the Ca^{2+} releasing process in response to 1-MA.

One of the elements that add to the asymmetry of the oocyte is the actin cytoskeleton. In oocytes, actin filaments are not randomly distributed inside the cell. First of all, dense networks of actin filaments are preferentially concentrated in the subplasmalemmal zone of the oocyte, exactly the same area where 1-MA-induced Ca^{2+} signals propagate. This cortex-specific F-actin layer is peculiarly absent in the space between the GV and the plasma membrane, the animal pole. Indeed, it is through this “corridor” that the two polar bodies are extruded at the later stage of meiosis. Hence, it is possible that such asymmetric organization of the actin cytoskeleton may be accountable for the vegetal hemisphere-specific initiation of the Ca^{2+} signals and their cortex-mediated propagation. In support of the idea that the subplasmalemmal actin cytoskeleton may play a role in Ca^{2+} signaling, the 1-MA-induced Ca^{2+} release takes place exactly at the same moment when the actin bundles on the cell surface (microvilli) are undergoing dynamic remodeling (Schroeder and Stricker, 1983; Otto and Schroeder, 1984).

In line with this, we have recently observed that the rearrangement of the actin cytoskeleton can strongly modulate Ca^{2+} signals during the maturation and fertilization processes of starfish oocytes (Lim *et al.,* 2002; Lim *et al.,* 2003: Nusco *et al.,* 2006). The first evidence that cortical actin plays a role in starting and propagating Ca^{2+} came from the experiments performed in our laboratory. In matured oocytes of starfish, incubation with the actin-depolymerizing drug latrunculin-A (LAT-A) induced a massive calcium mobilization and the consequential discharge of the cortical granules, which led to the elevation of the fertilization envelope (Lim *et al.,* 2002). These surprising results were observed even without the addition of Ca^{2+}-inducing second messengers. The initial pattern of the LAT-A-induced Ca^{2+} release was reminiscent of the fertilization process. The Ca^{2+} release starts at a circumscribed site on the oocyte surface, and then expanded to the cortical layer before propagating rapidly to the center of the oocyte and eventually producing Ca^{2+} oscillations. Initially, it was suggested that the Ca^{2+} spreading from the cortex to the remainder of the oocyte was mediated by $InsP_3$ receptors because the classical $InsP_3$ receptor inhibitor, heparin, can completely abolish the LAT-A-induced Ca^{2+} mobilization (Lim *et al.,* 2002). Whatever may be its molecular mechanism, LAT-A-induced Ca^{2+} release also depends on the maturation-related cytological changes of the oocyte. In immature oocytes, LAT-A can only induce a gradual increase of Ca^{2+}. In this regard, it is worth noting that starfish undergoes drastic rearrangement of actin filaments during 1-MA-induced meiotic maturation (Fig. 4). Interestingly, the LAT-A-induced Ca^{2+} release always starts at the animal hemisphere at the time when the increased sensitivity to $InsP_3$ was established in response to 1-MA. While these results implicate the $InsP_3$-sensitive stores into the LAT-A-induced Ca^{2+} signaling, it was also suggested that the rearrangement of the actin cytoskeleton may be the downstream target of MPF-mediated phosphorylation events (Lim *et al.,* 2003). In support of the idea that reorganization of cytoskeleton may be caused by active MPF, MPF is associated with microtubules through microtubule-associated proteins (MAPs) (Ookata *et al.,* 1993). Rendering more significance to the role of cytoskeleton in regulating the signal transduction by 1-MA, immunofluorescence data have shown that the G-protein $\beta\gamma$ subunits are associated not only with the plasma membrane but also with the cytokeratin intermediate filaments in the cytoplasm. The activation of MPF and oocyte maturation by injected $\beta\gamma$ subunits in the perinuclear region gives them functional significance as cytoplasmic effectors transducing 1-MA signal (Chiba *et al.,* 1995).

As mentioned earlier, starfish oocytes begin to respond to 1-MA with two visible changes: the Ca^{2+} burst and dynamic reorganizations of actin bundles in microvilli. Since these two events are simultaneously taking place a few minutes after adding hormone, these phenomena provided us with an optimal opportunity to study the relationship between Ca^{2+} signaling and the cortical actin cytoskeleton. This is even before any significant structural changes takes place in ER. Based on the observation that the classical agents blocking either $InsP_3$-producing enzyme (U-73122) or $InsP_3$ receptor (heparin) can completely suppress the 1-MA-induced intracellular Ca^{2+} release, this process was initially presumed to be mediated by $InsP_3$. However, further analysis of the oocyte revealed that both U-73122 and heparin produce drastic rearrangement of cortical actin layers, raising the possibility that the inhibitory effect of these agents might have been caused by the changes of actin cytoskeleton at the very site where Ca^{2+} signal is produced. In support of this idea, all the tested agents that promote unidirectional disassembly (LAT-A) or assembly of actin filaments (jasplakinolide) at the cortical layer of oocytes have severely inhibited the 1-MA-induced Ca^{2+} release (Kyozuka *et al.,* 2008). Conversely, the 1-MA-induced Ca^{2+} release can be also facilitated by the agent that reorganizes actin cytoskeleton. Cofilin, a member of the actin-depolymerizing factor (ADF) family, can enhance the Ca^{2+} signals in the 1-MA-treated starfish oocytes by nearly two-fold (Nusco *et al.,* 2006). Taken together, these results indicate that the actin cytoskeleton is a key player in modulating the Ca^{2+} release in response to 1-MA.

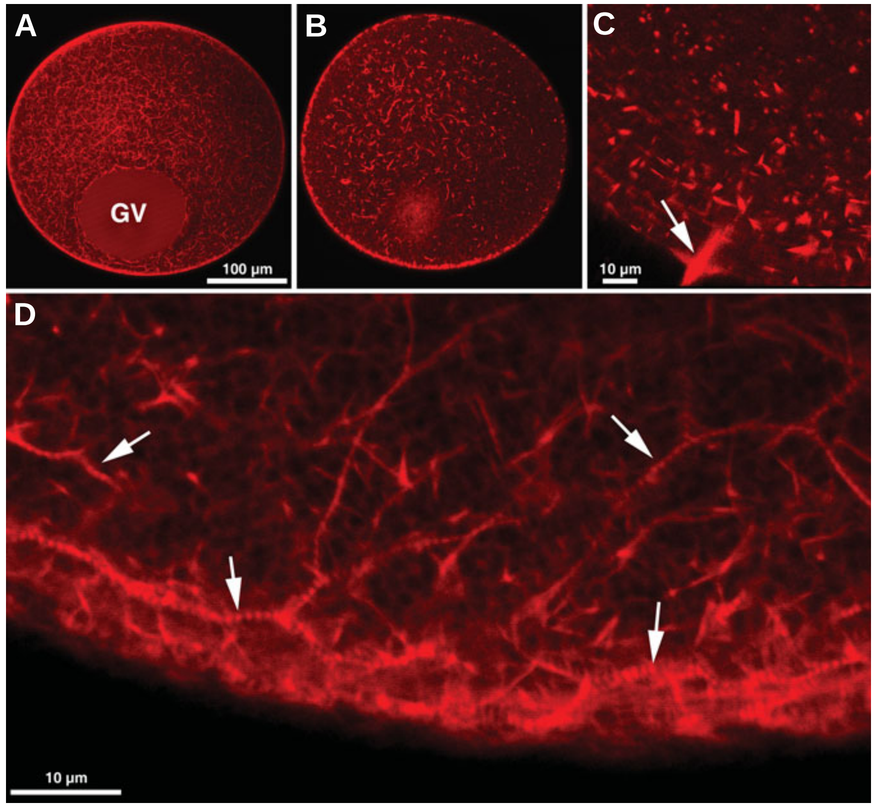

Fig. 4. Changes of the actin cytoskeleton during the meiotic maturation and fertilization of starfish oocytes. *Confocal miscroscopic images of live oocytes pre-injected with Alexa-568-conjugated phalloidin.* **(A)** *Immature oocytes manifest a dense network of actin filaments underneath the plasma membrane and inside the cytoplasm. The nucleus (germinal vesicle, GV) is phalloidin-negative at this stage.* **(B)** *Actin cytoskeleton in the same oocyte after 1h treatment with 1-methyladenine. It is noticeable that the actin cytoskeleton in the cortical region and the cytoplasm is now drastically reduced.* **(C)** *Fast reorganization of the actin cytoskeleton at the very moment of sperm internalization. The sperm is pulled into the egg by the actin fibers from the fertilization cone (arrow).* **(D)** *Higher magnification view of the cortical region of an immature oocyte shows occasional occurrence of striated actin fibers (arrows), which are presumably due to the periodic association of actin-binding proteins.*

Besides regulating Ca^{2+} release, actin may play additional role in the nucleus especially during the meiotc cell division. Very recently, it has been shown that nuclear actin network is instrumental in bringing chromosomes to the proximity of the nascent meiotic spindle in starfish oocyte. The mechanism by which spindle captures chromosomes has been exclusively attributed to microtubules. However, the finding that chromosomes move on actin filaments and not microtubules indicated a novel role for the actin cytoskeleton in the long-range transport (Lénárt *et al.,* 2005).

Sperm-egg interaction at fertilization

Echinoderm eggs have been a useful model for studying the ultrastructural changes at fertilization. A prerequisite for the entry of the sperm into eggs is the acrosome reaction because the egg plasma membrane can only fuse with the newly formed membrane of the acrosomal process. In sea urchin, sulfated fucose-rich compounds in the jelly coat of the egg provide species-specificity in inducing the acrosome reaction (Hirohashi *et al.,* 2002). Within 2-4 sec following the attachment of the fertilizing spermatozoon to the vitelline coat, a step-like depolarization occurs across the egg plasma membrane, probably due to a factor released from the acrosome in the proximity of the egg plasma membrane (Longo *et al.,* 1986). This is followed by the fertilization potential, which is accompanied by the cortical reaction (Vacquier, 1975). In starfish, the acrosome reaction-regulated exocytotic process involves the secretion of the acrosomal content upon the contact of the sperm with the sugar components of the outer layer of the jelly coat (Nakachi *et al.,* 2006). The first sign of sperm activation is the morphological changes of the spermatozoon that extends a long thin acrosomal process, owing to the polymerization of the acrosomal globular actin (Fig. 5). The acrosomal process could be as long as 20 μm, protruding from the outer border of the plasma membrane (Dan, 1960; Dale *et al.,* 1981). At fertilization, Ca^{2+} signals initiate well before the sperm is physically incorporated into the egg, raising the possibility that a mode of remote control through the acrosomal process might play a role in this process (Chun and Santella, 2007). At variance with sea urchin eggs, the Ca^{2+} wave produced by the sperm was not correlated with the changes in the electrical properties of the plasma membrane in starfish oocytes (Dale *et al.,* 1981). The first detectable electrical change of the egg plasma membrane is the fertilization potential, whose rise is slower than that of the sea urchin egg. The cortical reaction initiates simultaneously with the fertilization potential before the spermatozoon is completely inside the oocyte (Dale *et al.,* 1981). It has been a matter of continued debate in the past whether the activation of the egg requires sperm attachment or penetration into the egg (Shapiro and Eddy, 1980). Starfish oocytes have allowed us to determine the precise location of the acrosome reaction and to carefully monitor the molecular events underlying successful attachment of the spermatozoon to the plasma membrane and the initiation of the Ca^{2+} wave (Fig. 5).

Calcium signaling during fertilization

At fertilization, in all the species studied so far, a transient increase in the intracellular Ca^{2+} appears essential for reinitiating protein and DNA synthesis (Epel, 1990). The Ca^{2+} elevation begins at the site of sperm-egg interaction and crosses the egg as a wave (Fig. 5). This Ca^{2+} increase induces cortical granule exocytosis and the consequent elevation of the vitelline layer, which also begins at the point of sperm entry. Several observations have led to the conclusion that the phospholipase C (PLC)-induced hydrolysis of the PIP_2 and the resulting formation of $InsP_3$ were responsible for the liberation of Ca^{2+} from intracellular stores (Santella *et al.,* 2004). An increase in $InsP_3$ and DAG (the other product of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis) had been detected in the first 15 sec after fertilization (Whitaker, 2006). Supporting the idea that $InsP_3$ is the messenger that promotes Ca^{2+} mobilization at fertilization, microinjection of neomycin (an inhibitor of PIP_2 hydrolysis) prevented the sperm-induced calcium transient and egg activation (Swann and Whitaker 1986). In agreement with this hypothesis, microinjection of $InsP_3$ induced cortical granule exocytosis and the membrane elevation as a result of a massive release of Ca^{2+} from intracellular stores. Thus, the role of $InsP_3$ and its receptors in the onset of the Ca^{2+} response at fertilization was reinforced by the observation that the fertilization Ca^{2+} waves in most species were strongly inhibited by the antagonist of the $InsP_3$ signaling pathway such as heparin, anti-$InsP_3R$ monoclonal antibodies and $InsP_3$-sequestering peptides (McDougall *et al.,* 2000; Iwasaki *et al.,* 2002; Miyazaki *et al.,* 1992).

However, the suggested "receptor-G-protein–PLC hypothesis" was questioned due to the lack of evidence for a sperm receptor in the plasma membrane. Complicating results were also obtained from the experiments manipulating G-proteins with toxins (cholera and pertussis toxins), in which G-protein signaling pathway may be involved in the cortical granule exocytosis, but not in the intracellular Ca^{2+} liberation (Turner *et al.,* 1987; Jaffe *et al.,* 1988). Subsequently, a pathway not involving a canonical G-protein-linked cascade, but instead tyrosine kinases, was proposed following the finding that an increase in protein-tyrosine phosphorylation was detected 1 min after the activation of sea urchin eggs with sperm. Since the same increase was detected upon activation by ionomycin, it was suggested that Ca^{2+} itself could reciprocally stimulate the tyrosine kinase activity, completing a positive feedback loop. It was then investigated whether phospholipase Cγ (PLCγ) is involved in Ca^{2+} release. Western blot and immunocytochemistry indicated that PLCγ is present in cortical regions, suggesting that PLCγ may be a part of the cascade of events leading to the calcium release in sea urchin fertilization (de Nadai *et al.,* 1998). In starfish oocyte, PLCγ is activated when its two src-homology (SH2) domains bind to an activated tyrosine kinase. The sperm-induced Ca^{2+} signal was delayed or completely blocked by the injection of PLCγSH2 domain-fusion proteins that suppress PLCγ activation (Carroll *et al*., 1997). Recently, an oocyte cDNA encoding PLCγ has been isolated from starfish, supporting that the Ca^{2+} response at fertilization in this species is regulated by endogenous PLCγ (Runft *et al.,* 2004). It being understood that $InsP_3$ is the main actor in Ca^{2+} mobilization at fertilization, the proposal that the sperm introduces a cytosolic factor into the eggs that triggers $InsP_3$-dependent Ca^{2+} mobilization has been controversial for many years due to the failure to identify the molecular nature of such a "sperm factor" (Dale *et al.,* 1985; Swann, 1990; Stricker, 1997; Kyozuka *et al.,* 1998; Parrington *et al.,* 2007). However, recent work in mammalian oocytes has significantly advanced our knowledge on this matter. A sperm-specific PLC ζ (zeta) isoform with distinctive enzymatic properties has been proposed as a possible sperm factor candidate. This suggestion was supported by the finding that the injection of mRNA encoding PLC ζ into mouse eggs induced Ca^{2+} signals indistinguishable from those at fertilization, and that the removal of PLC ζ from sperm extracts abolished Ca^{2+} release in eggs (Saunders *et al.,* 2002). A similar response was obtained by microinjecting purified PLC ζ protein

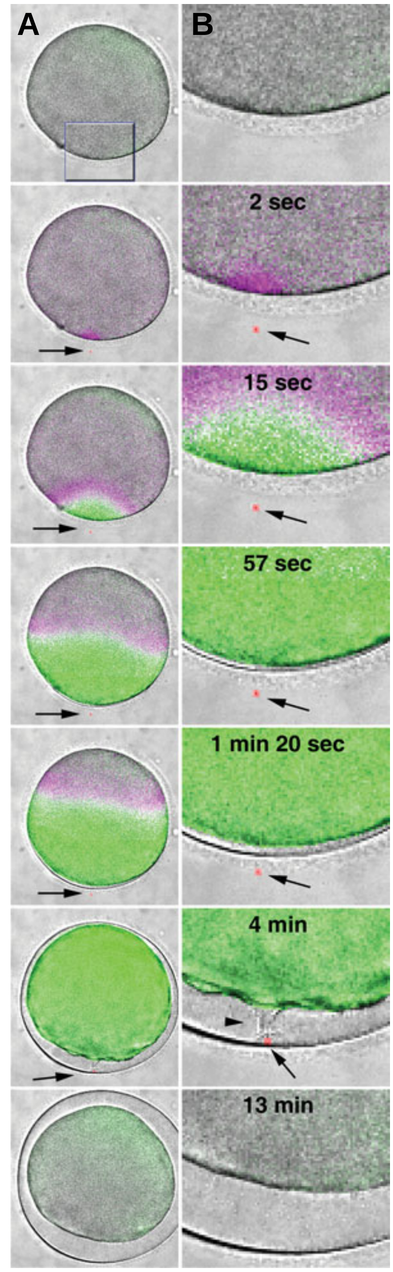

Fig. 5. Elevation of Ca^{2+} at the time of sperm-egg interaction. *After the cortical flash quickly subsides (not shown), a small elevation of Ca^{2+} starts at the point of sperm interaction, and the Ca^{2+} wave gradually propagates through the cytoplasm. The process was represented in the merged views of the transmission micrograph and the two relative pseudo-colored images depicting either diffusing Ca^{2+} (in green) or its momentary increment (in pink). The actual release of Ca^{2+} (pink) obviously precedes its diffusion (green).* **(A)** *The whole-cell view of the fertilization process. The cortical region where the sperm meets the egg is indicated with the rectangular box and magnified in the adjacent panels.* **(B)** *The magnified views of the same images. It is noticeable that the sperm (the red spot marked with an arrow) induces Ca^{2+} release before being physically incorporated into the egg. When the fertilization envelope is being elevated, the sperm is apart from the egg surface but connected by the actin-rich acrosomal process and the fertilization cone (arrow head). The sperm enters the egg only when the fertilization envelope is fully elevated.*

that was readily activated by the resting level of the cytoplasmic Ca^{2+} (Kouchi *et al.,* 2004). In line with these results, reduction of sperm PLC ζ by transgenic RNAi approach significantly perturbed the number of Ca^{2+} oscillations, suggesting that the sperm-derived PLC ζ may be solely responsible for the signature Ca^{2+} spiking pattern (Knott *et al.,* 2005). However, what still remains to be understood about PLCζ is the exact mode of its action. Indeed, it is not clear how this isoform interacts with plasma membranes since PLC ζ lacks the PH domain that anchors PLC δ1 to PIP_2. Very recently, it has been demonstrated that a cluster of basic amino acid residues in the X-Y linker region of mouse PLC ζ, which is not present in PLC δ1, can laterally sequester PIP_2 and concentrate it in the region of the catalytic domain (Nomikos *et al.,* 2007). In mouse, however, the expected loss of PIP_2 for producing $InsP_3$ was not detected by a fluorescent probe (PH-GFP) that binds specifically to PIP_2 in the plasma membrane. Taken together with the observation that the presence of PIP_2 is not evident in the cytoplasmic membranes, these results raise further questions on the location of the polyvalent phoshoinositides to which PLC ζ may bind for the enzymatic reaction (Halet *et al.,* 2002). Very recently, analysis of the Ca^{2+} oscillatory pattern in eggs overexpressing PLCβ1 have shown a decrease in the amount of total Ca^{2+} liberated inside fertilized eggs, implying that egg PLCβ1 may be somehow involved in the modulation of the sperm-derived PLC ζ (Igarashi *et al.,* 2007). Hence, the exact roles of the different isoforms in the PLC family need to be further elucidated in the future study.

Ca^{2+}-linked second messengers other than $InsP_3$ may play a role at fertilization

The results described above have dealt with the role of $InsP_3$ in the generation of sperm-induced Ca^{2+} release. On the other hand, different second messengers may play a subtly different role during the same process, as exemplified by cADPr that contributes to the propagation of the Ca^{2+} wave. To facilitate the process, the sensitivity of its receptor, ryanodine-sensitive calcium-release channels, is enhanced by Ca^{2+} through a CICR phenomenon (Galione *et al.,* 1993). The report suggesting cADPr as a primary egg activator raised a question about any specialized roles played by different Ca^{2+}-releasing second messengers. Of particular interest was to discover which second messenger plays a primary role in triggering the first Ca^{2+} response at fertilization. A clue was provided by the activation pathway of nitric oxide (NO), a signaling molecule acting through the elevation of cellular cGMP. The cGMP-dependence of the sea urchin ADP-ribosyl cyclase has prompted the proposal that NO may be the activating stimulus of the cADPr-signaling pathway (Galione *et al.,* 1993a). The presence of NO in the sperm during acrosome reaction and in the fertilized eggs could be accountable for the activation of ADP-ribosyl cyclase by cGMP-dependent protein kinase (PKG) and the consequent production of cADPr (Kuo *et al.,* 2000). Indeed, direct measurements of cGMP, cADPr and $InsP_3$ contents of sea urchin eggs and the comparison of their levels with the Ca^{2+} rise during the early stage of sperm activation indicated that cGMP began to rise first and then cADPr followed. By contrast, the major rise in $InsP_3$ occurred after the Ca^{2+} signal (Kuroda *et al.,* 2001), weakening its role as an inducer of Ca^{2+} signals. Recently, using a fluorescence indicators of NO and Ca^{2+} dyes, both NO and Ca^{2+} increase have been measured simultaneously at fertilization. The results have showed that NO levels rise after the Ca^{2+} wave is initiated (Leckie *et al.,* 2003). Again, this observation implies that NO and cADPr pathway may not be the very first initiating factor of Ca^{2+} release. Although NO pathway is still needed to regulate the duration of the Ca^{2+} wave (Leckie *et al.,* 2003), others have shown that the synthesis of cADPr during sea urchin fertilization is not necessary because inhibitors of either PKG or ADP-ribosyl cyclase activities did not prevent the transient rise in intracellular Ca^{2+} in heparin-loaded eggs during fertilization (Lee *et al.,* 1996).

The involvement of cADPr/RyRs in the sperm-triggered Ca^{2+} response is also uncertain in starfish. While the Ca^{2+} response induced by the uncaged cADPr in mature oocytes was completely blocked by the specific antagonist of the cADPr/ryanodine receptors ($8NH_2cADPr$), the same antagonist failed to block the sperm-induced Ca^{2+} increase at fertilization (Nusco *et al.,* 2002). Taken together, these observations cast doubt on the role of cADPr as the first initiator of Ca^{2+} release at the fertilization of echinoderm eggs.

A recently discovered Ca^{2+}-releasing second messenger, NAADP, seems to act on distinct targets that are pharmacologically and physically different from the Ca^{2+} stores activated by $InsP_3$ and cADPr. NAAPD is a universal Ca^{2+}-releasing second messenger that mobilizes Ca^{2+} from intact oocytes and eggs of starfish, ascidians and sea urchin (Lee, 2002). Observations on starfish oocytes matured for 50 min with 1-MA have shown that the Ca^{2+} response to NAADP consists of a cortical flash that is inhibited in Ca^{2+}-free seawater. The Ca^{2+} signal then spread centripetally to the center of the oocyte as a wave (Santella *et al.,* 2000; Lim *et al.,* 2001). Since the Ca^{2+} signal generated by NAADP closely resembles that induced by the sperm, NAADP and $InsP_3$ receptors in starfish oocytes have been explored in detail as determinants of the spatiotemporal pattern of Ca^{2+} signals at fertilization. The striking difference between $InsP_3$ and NAADP was observed in the enucleated oocytes in the presence of 1-MA. Whereas NAADP was potent enough to support cortical granule exocytosis following Ca^{2+} release in enucleated oocytes, massive $InsP_3$-induced Ca^{2+} response failed to produce vitelline envelope elevation. On the other hand, the sperm can still fertilize the enucleated oocytes and produce a normal cortical exocytosis even with a slowed Ca^{2+} wave. Hence, Ca^{2+} release is not sufficient for the vitelline envelope elevation. In line with this, massive $InsP_3$-induced Ca^{2+} release in the matured oocytes failed to produce cortical granule exocytosis in the presence of the agents perturbing the organization of cortical actin cytoskeleton (Kyozuka *et al.*, 2008). Based on these observations, it was postulated that NAADP is more responsible for the initiation of Ca^{2+} waves at the cortex, while $InsP_3$-sensitive Ca^{2+} stores may mediate the propagation of the waves initiated by NAADP (Lim *et al.,* 2001). The NAADP-induced Ca^{2+} response is closely preceded by the activation of a membrane current which is responsible for the Ca^{2+} entry from the extracellular space and for the triggering of the global Ca^{2+} wave in the egg (Moccia *et al.,* 2003). Interestingly, modification of cortical actin layers by LAT-A or jasplakinolide led to severe reduction of the NAADP-mediated membrane current. Hence, the main action of NAADP takes place at the cell surface, and the idea that NAADP may initiate the intracellular Ca^{2+} wave at fertilization has been further reinforced by the similarities between the biophysical and pharmacological properties of the sperm-elicited depolarization of membrane potentials and the NAADP-activated Ca^{2+} current (Moccia *et al.,* 2004). The tentative conclusion is that the NAADP-dependent Ca^{2+} entry may contribute to the stimulation of $InsP_3$-producing PLCγ in starfish oocytes (Runft *et al.,* 2004), which requires micromolar concentration of priming Ca^{2+} (Rhee, 2001). Alternatively, the NAADP-induced Ca^{2+} may help sensitize the $InsP_3Rs$ through a process of CICR (Moccia *et al.,* 2006). At any rate, these results suggest that Ca^{2+} signaling during starfish fertilization is initiated by NAADP, although it has been assumed to be exclusively under the control of the $InsP_3$.

In intact sea urchin eggs, NAADP evoked long-lasting Ca^{2+} oscillations in the absence of extracellular Ca^{2+}, suggesting that the mobilized Ca^{2+} may be from the intracellular stores (Churchill and Galione, 2001). More recent findings, however, have indicated that the response involves, as in starfish oocytes, a cortical flash produced by Ca^{2+} influx. It was reported that such Ca^{2+} burst was inhibited by the desensitization of NAADP receptors by pre-injection of sub-threshold concentrations of NAADP, which presumably inactivates the channel (Lee, 2002). The finding that NAADP desensitization also prevented the onset of the fertilization potential in starfish eggs corroborated the involvement of NAADP in the fertilization process (Moccia *et al.,* 2006a). In line with this idea, it has been reported that sea urchin sperm contains large amounts of NAADP, which could be delivered directly into the eggs (Billington *et al.,* 2002; Churchill *et al.,* 2003). What remains to be clarified is the nature of the Ca^{2+} store activated by NAADP. In sea urchin eggs, it has been suggested that the sperm-activated NAADP-sensitive Ca^{2+} store is located on lysosome–like organelles. However, in other cell types, agents that disrupt lysosomes failed to block or reduce the NAADP-induced Ca^{2+} response, which could be mediated through ryanodine receptors (Gerasimenko and Gerasimenko, 2004; Steen *et al.,* 2007). In starfish oocytes, neither drugs which disrupt acidic compartments nor inhibitors of RyRs affected the NAADP-induced depolarization and the cortical Ca^{2+} influx (Moccia *et al.,* 2006a). Hence, as neither the identity of the functional receptors, nor the exact location of its action is known for NAADP, the molecular detail of the Ca^{2+}-releasing action by NAADP is still an open question.

Conclusion

Aside from its pivotal role in muscle contraction, Ca^{2+} has been known to be essential to egg activation since nearly 90 years ago. Complementing the electrophysiological tools, the technical advancement of luminescent Ca^{2+} sensors and ionophores has visually demonstrated that fertilization starts with massive mobilization of intracellular Ca^{2+}, and that the instantaneous Ca^{2+} signaling is crucial for the formation of fertilization membrane and the catalysis of other processes related to egg activation. The multidisciplinary studies in the past two decades have revealed some of the key mechanisms in which Ca^{2+} is released from the intracellular stores. The spotlight has been focused on the three major second messengers that release Ca^{2+} inside the cell, namely $InsP_3$, cADPr and NAADP. Among the three, $InsP_3$ is the best characterized one, and it may play a central role in generating Ca^{2+} waves in many cell types. Indeed, an isoform of the $InsP_3$-producing enzyme designated PLC-ζ has been recently recognized as a strong candidate for a long-sought "sperm factor" that transduces sperm-borne signals in the fertilized eggs. Intensive studies on the corresponding receptors of these second messengers, e.g. $InsP_3R$ and RyR, have elucidated how Ca^{2+} can diffuse and propagate as a wave with the concept of CICR. Hence, these three and possibly other second messengers may play distinct but concerted roles in shaping the characteristic Ca^{2+} response in the egg. However, many questions are yet to be answered on this subject. First of all, the identity of the NAADP receptor is not fully established, and another fundamental question is whether or not the Ca^{2+}-releasing actions of these second messengers are mediated exclusively by those corresponding receptors. Whichever may be the answer, it is now becoming evident that the efficacy of the ligand-gated ion channels is significantly influenced by the surrounding cytoskeletal microenvironment. Actin filaments in the oocyte cortex undergo rapid re-organization and turnover during maturation and fertilization. Recent studies have indicated that actin filaments may play direct or indirect roles in intracellular Ca^{2+} mobilization and exocytosis, as well as in chromosome sorting. There is no doubt that actin cytoskeleton has structural roles, but it requires further studies to understand whether and how these dynamically re-organizing actin filaments play highly regulated functional roles in egg activation.

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