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Review

Calcium and actin in the saga of awakening oocytes

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This review is dedicated to Professor Ernesto Carafoli.

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ABSTRACT

The interaction of the spermatozoon with the egg at fertilization remains one of the most fascinating mysteries of life. Much of our scientific knowledge on fertilization comes from studies on sea urchin and starfish, which provide plenty of gametes. Large and transparent, these eggs have served as excellent model systems for studying egg activation and embryo development in seawater, a plain natural medium. Starfish oocytes allow the study of the cortical, cytoplasmic and nuclear changes during the meiotic maturation process, which can also be triggered *in vitro* by hormonal stimulation. These morphological and biochemical changes ensure successful fertilization of the eggs at the first metaphase. On the other hand, sea urchin eggs are fertilized after the completion of meiosis, and are particularly suitable for the study of sperm–egg interaction, early events of egg activation, and embryonic development, as a large number of mature eggs can be fertilized synchronously. Starfish and sea urchin eggs undergo abrupt changes in the cytoskeleton and ion fluxes in response to the fertilizing spermatozoon. The plasma membrane and cortex of an egg thus represent "excitable media" that quickly respond to the stimulus with the Ca^{2+} swings and structural changes. In this article, we review some of the key findings on the rapid dynamic rearrangements of the actin cytoskeleton in the oocyte/egg cortex upon hormonal or sperm stimulation and their roles in the modulation of the Ca^{2+} signals and in the control of monospermic fertilization.

1. Starfish and sea urchin eggs as suitable model systems for studying maturation and fertilization

For more than one hundred years, eggs of marine animals have provided favored materials with which to explore the mechanisms of egg activation and embryonic development. When extracted from the gonad during the breeding season, nearly all immature oocytes contain the characteristic large nucleus (termed germinal vesicle, GV) (Fig. 1). Upon interaction with the sperm, these oocytes increase their intracellular Ca^{2+}, but are penetrated by numerous spermatozoa. It was thought that the polyspermic fertilization of immature oocytes was because of their incapability of cortical granules exocytosis and the consequent lack of the elevation of the fertilization envelope, which would mechanically block the entry of supernumerary spermatozoa [1–3]. Successful monospermic fertilizability develops in the maturation process, during which the morphological changes in the surface of the eggs coincide with the breakdown of the germinal vesicle (GVBD) (Fig. 1B). After the first descriptions of oocyte maturation [4,5], the control of starfish maturation has been extensively studied thanks to the identification of the maturing hormone 1-methyladenine (1-MA), which can be easily added to the seawater in which the immature oocytes are suspended [6,7].

A few minutes after hormonal stimulation, a rapid reorganization of the actin cytoskeleton occurs at the oocyte surface, which is accompanied by a Ca^{2+} increase in the cortex and in the GV [8–12]. Later events in the maturation process include the GVBD and the intermixing of the nucleoplasm with cytoplasm, which is essential to trigger the events of cortical maturation that are critically conducive to the successful egg activation by the sperm. The optimal period for fertilizing starfish eggs is between the GVBD and the extrusion of the first polar body. At variance with other egg types, which have a very limited time window during which fertilization is possible, starfish eggs can still be fertilized several hours after the addition of 1-MA. This allows them to be used for studying the cytoplasmic changes that misguide the aging eggs during the fertilization process [13–15].

Unlike starfish that are fertilized as primary oocytes, sea urchin eggs are shed into the seawater after the completion of meiosis i.e., they are fully matured and thus more stable. Much of the knowledge

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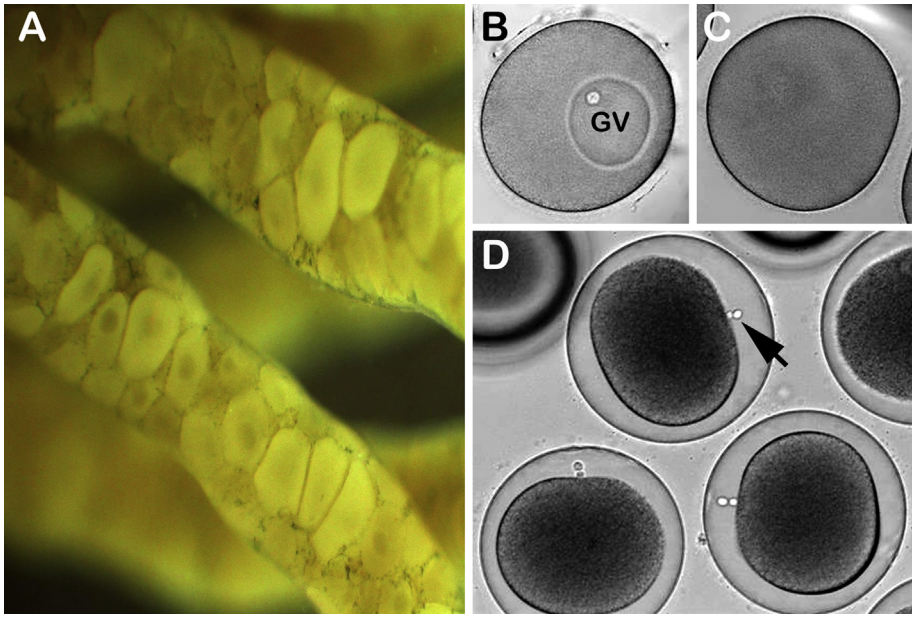

Fig. 1. Starfish oocytes as an experimental model system for studying meiotic maturation and fertilization. (**A**) Dissected female gonads of the Mediterranean starfish *Astropecten aranciacus* in seawater. During the breeding season (February–May), the female gonad provides fully grown immature oocytes that are arrested in prophase I of meiosis. (**B-D**) Meiotic maturation and fertilization of the eggs from *Asterina pectinifera*. (**B**) A transmitted light image of immature oocytes. Note the large nucleus (germinal vesicle, GV) and the small nucleolus inside it. (**C**) Immature oocytes become mature eggs after the treatment with 1-MA: at this stage of maturation, GV breakdown has taken place, and the nucleoplasm is intermixed with the cytoplasm. (**D**) A transmitted light image of the fertilized eggs. Note the elevation of the fertilization envelope. Regardless of fertilization, the first and second polar bodies are extruded from the animal pole respectively 70 and 90 min after the stimulation with 1-MA (arrow).

on the fast events of sperm–egg interaction originated from the studies of sea urchin eggs. Within seconds after the addition of active spermatozoa, rapid structural changes take place in the egg following the liberation of Ca^{2+} and the elevation of the fertilization envelope, the latter of which is the consequence of cortical granules exocytosis. The importance of Ca^{2+} during the process of oocyte maturation and fertilization has been confirmed in numerous animal species, and the detailed signal transduction pathways that lead to the intracellular Ca^{2+} liberation during hormonal and sperm stimulation have been most extensively studied with starfish and sea urchin. This review article will focus on these two marine animals to describe the roles of the cortical actin cytoskeleton in modulating sperm–egg interaction and Ca^{2+} signals [16–18].

2. Changes of the actin cytoskeleton during the maturation of starfish oocytes

In the starfish ovary, immature oocytes are ensheathed with the vitelline and jelly coats over the plasma membrane, and are associated with a thin layer of follicle cells. The application of 1-MA induces detachment of the follicle cells from the oocytes surface and their migration and concentration in one side of the mature egg. The starfish ovary undergoes a marked contraction at spawning due to the operation of the smooth muscles present in the ovarian wall. As expected of a muscle contraction process, this contraction does not occur in the absence of Ca^{2+} in seawater [7]. The average diameter of fully grown oocytes is about 250 μm for the Mediterranean species *Astropecten aranciacus*, and 180 um for the Japanese species *Asterina pectinifera*. As aforementioned, these oocytes contain a large GV (80–100 μm in diameter) with a nucleolus (Fig. 1C). The position of the GV defines the animal pole of the oocyte where polar bodies are extruded during meiosis (Fig. 1D, arrow). Immunofluorescence using anti-α-tubulin antibody has shown that the anchorage of the GV to the animal cortex is controlled by microtubular structures, which is essential for the normal course of meiotic events [19]. However, in addition to the microtubule-mediated nuclear positioning, we have found that actin filaments are also intimately associated with the surface of the GV, which may play a role in anchoring the GV at the animal pole (Fig. 2B).

1-MA acts on the surface of the oocyte from outside, as it is inactive when injected into the cytoplasm [20]. It is assumed that 1-MA interacts with the cell surface receptor belonging to the G-protein-coupled receptor family [21,22]. This triggers the maturation process through the activation of the Maturation Promoting Factor (MPF) which turned out to be the cyclin B-Cdc2 kinase (Cdk1) complex that serves as the universal activator of the meiotic and mitotic cycles [23,24]. The intracellular mediator of 1-MA stimulation has been identified as the phosphorylated (and activated) protein kinase AKT (protein kinase B, PKB) which in turn phosphorylates and suppresses the inhibitory activity of Myt1. As a member of the Wee1 family of protein kinases, Myt1 upregulates the Ca^{2+}-dependent Cdc25 phosphatase which in turn activates cyclin B-Cdc2 [25,26]. The GVDB of the starfish oocyte requires at least 8 min exposure to 1 μM 1-MA, and its onset varies depending on the species. The length of this so-called 'hormone-dependent period (HDP)' is not dependent on the hormone concentration, but the removal of the hormone during HDP interrupts maturation. Beyond the HDP, however, the maturation process irreversibly proceeds even in the absence of 1-MA [27–30]. Curiously, oocyte maturation in starfish can also be induced by disulfide-reducing agents (e.g. dithiothreitol). They mimicked 1-MA in inducing morphological changes and an increase of the sulfhydryl contents in the proteins of the oocyte cortex [31].

Light and electron microscopic studies have revealed cortical changes as early as 1 or 2 min after 1-MA addition that are linked to the structural reorganization of the actin cytoskeleton: microvilli retraction, alignment of the cortical granules with their longer axis perpendicular to the plasma membrane, and the formation of surface spikes that contain bundles of actin filaments. These structures, which are generated by rapid, reversible polymerization

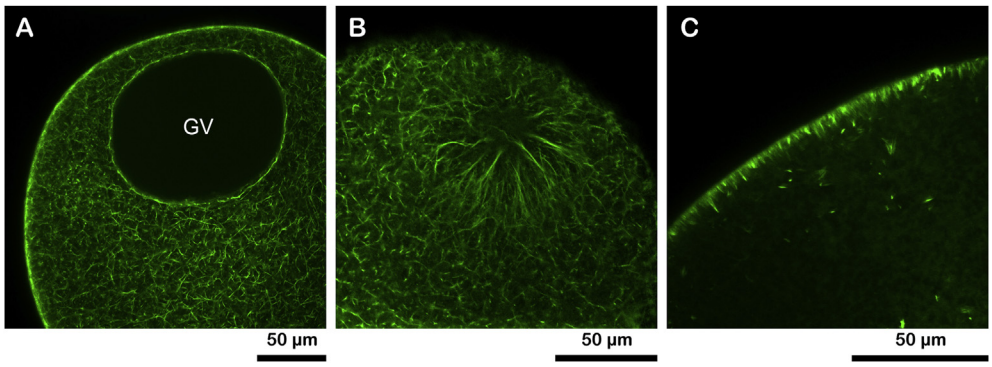

Fig. 2. Changes of the actin cytoskeleton in the *A. aranciacus* oocytes during meiotic maturation. Immature oocytes were microinjected with Alexa-Fluor488-conjugated phalloidin, and the changes of the F-actin in the living cell were monitored with confocal microscopy during the 1-MA-induced meiotic maturation. (**A**) Dense networks of actin filaments are visible near the plasma membrane and in the cytoplasm of the immature oocyte. F-actin is also abundantly present around the nuclear membrane, but not inside the GV. (**B**) An image at the oblique confocal plane tangent to the curvy surface of the GV near the animal pole of the immature oocyte. Note the radial arrangement of the F-actin fibers over the surface of the GV. (**C**) A mature egg manifests dramatic changes in the structural organization of the actin cytoskeleton. The actin fibers are now visible mainly in the subplasmalemmal areas of the egg cortex, and are oriented perpendicularly to the plasma membrane.

of actin, represent the earliest morphological response of starfish oocytes to the maturation-inducing hormone [8,32,33]. The structural modifications in the cortex are also reflected in the changes of the mechanical properties of the oocytes and the stiffness of the endoplasm. A transient decrease in oocyte stiffness *(A. pectinifera)* starts soon after 1-MA application, and reaches the minimum at the time of the breakdown of the germinal vesicle (20–25 min after 1-MA stimulation), but again exhibits a transient rise and fall during the polar body formation [34]. The cyclic change of surface stiffness during the emission of polar bodies is linked to the reorganization of the actin filaments in such a way that it forms the contractile ring at the animal pole and alleviates the unequal distances between the centrosomes and the contractile ring on the other parts of the starfish oocyte during the extremely asymmetric meiotic division [35]. The contractile nuclear actin network appears to be essential for the delivery of chromosomes to the microtubule spindle during the first nuclear division of meiosis [36].

The mechanical changes of the maturing oocyte take place both in the cortex and in the inner cytoplasm, and are sensitive to cytochalasin, which disrupts F-actin-based structures [37]. The characterization of changes in F-actin during starfish maturation has led to the identification of two populations of cortical actin filaments: spikes and non-spikes F-actin [38]. The structural reorganization of the oocyte cortex appears to be correlated with the changes of the electrical property of the plasma membrane. The addition of 1-MA progressively depolarizes the oocyte membrane potential (0.5 mV/min) until it induces abrupt depolarization at the time of GVBD. This maturation-related transition of ion permeability requires intermixing of the GV contents with cytoplasm [39,40]. It was suggested that selective closure of K^+ channels is responsible for the increase in membrane resistance and the shift of the membrane potential to a less negative voltage [39,41]. The exact cause of the gradual changes in the electrical property of the plasma membrane during the meiotic maturation is not known, but it is conceivable that the structural reorganization of microvilli and the subplasmalemmal actin meshwork may have affected microvillar ion channels either by relocating them or by rendering a diffusion barrier against Ca^{2+} near the channel openings [42].

3. Intracellular calcium increase during the meiotic maturation of starfish oocytes

When immature oocytes are injected with Ca^{2+} sensitive dyes and exposed to 1-MA, a transient increase of Ca^{2+} occurs in the cytoplasm within few minutes [10,43]. At variance with other species in which an increased Ca^{2+} influx has been documented [44,45], the 1-MA-induced Ca^{2+} increase in starfish oocytes can also take place in Ca^{2+}-depleted seawater [7]. In *A. pectinifera* oocytes, the Ca^{2+} increase represents the sum of cytosolic and nuclear Ca^{2+} elevations [11,12]. Interestingly, the Ca^{2+} chelator EGTA inhibited the 1-MA-triggered maturation when it was microinjected into the nucleus, but not into the cytoplasm [11]. The microinjection of EGTA into the nucleus prior to 1-MA addition completely abrogated the GVBD and the continuation of the maturation process. These results have highlighted the importance of nuclear Ca^{2+} and its increase in the regulation of meiotic maturation [11,12,46–50].

According to the spatio-temporal analyses, the 1-MA-induced Ca^{2+} wave the 1-MA-induced Ca^{2+} wave may be propagated in the oocytes in a mechanism that is distinct from that of the Ca^{2+} wave at fertilization. First, the 1-MA-induced Ca^{2+} increase always originates at the vegetal hemisphere of the oocyte, although the entire oocyte surface is exposed to 1-MA. Second, the 1-MA Ca^{2+} wave arrives at the antipode much faster (within 20 s) than the sperm-induced Ca^{2+} wave (80 s) [51]. Third, NAADP (nicotinic acid adenine dinucleotide phosphate) is not likely to contribute to the 1-MA-induced Ca^{2+} increase since NAADP promotes a Ca^{2+} influx in starfish oocytes whereas 1-MA can liberate intracellular Ca^{2+} in Ca^{2+}-free seawater [7,52]. Similarly, the contribution of cADPr (cyclic ADP ribose) was ruled out because *A. pectinifera* oocytes do not respond to uncaged cADPr [51]. Finally, the experimental evidence suggesting the involvement of the inositol 1,4,5-trisphosphate ($InsP_3$) receptors became questionable because both U73122 (inhibitor of $InsP_3$ synthesis) and heparin (inhibitor of $InsP_3$ receptor) severely altered the structure of the cortical actin cytoskeleton while the actin cytoskeleton itself significantly affects intracellular Ca^{2+} increase [51]. Interestingly, the Ca^{2+} increase is followed by the changes in the mechanical properties of the cytoplasm (decrease in stiffness) which is detected 5–9 min after 1-MA treatment. The sensitivity of oocyte stiffness to cytochalasin B (see above) had indicated the involvement of the cortical microfilaments [37]. As to a role of the actin cytoskeleton in the Ca^{2+} release, it is noteworthy that the 1-MA-induced Ca^{2+} increase always starts at the vegetal hemisphere [51], while the oocyte actin cytoskeleton is polarized along the animal–vegetal axis [53].

During the meiotic maturation of starfish oocytes, the intracellular Ca^{2+} release mechanism also matures in order to produce the appropriate Ca^{2+} response at fertilization. Thus, in comparison with

the immature oocytes at the GV-stage, mature eggs in their optimal period for fertilization release more Ca^{2+} in response to the same amount of $InsP_3$. This is not because the endoplasmic reticulum (ER) of the immature oocytes are loaded with less Ca^{2+}, for the oocytes can release the same amount of Ca^{2+} if 100 times more $InsP_3$ is microinjected into the cytoplasm [54–56]. Interestingly, the sensitization of the Ca^{2+}-releasing mechanism proceeds together with the structural reorganization of the actin cytoskeleton during the meiotic maturation. Firstly, the 1-MA-induced sensitization of the oocytes to $InsP_3$ is successfully negated by the actin-sequestering agent latrunculin-A (LAT-A) [56]; and secondly, pre-injection of GDPßS not only blocked the 1-MA-induced Ca^{2+} increase and GVBD, but also strikingly altered the structure of the cortical actin cytoskeleton [57]. The enhanced $InsP_3$-dependent Ca^{2+} release during maturation correlates with the modulation of the actin cytoskeleton by MPF, which in turn is activated by the 1-MA-induced Ca^{2+} increase in the nucleus [56]. In a number of species, including mammals, the ER in mature eggs is organized in clusters whose formation and disassembly are under the control of MPF. These clusters are accountable for the shaping of the Ca^{2+} oscillations at fertilization, as their disassembly terminated the oscillations [58–60].

That the actin cytoskeleton may play a role in regulating the intracellular Ca^{2+} liberation in starfish eggs is further supported by the finding that the *Astropecten aranciasuc* eggs exposed to LAT-A display increases of Ca^{2+} in the cortex which then propagated throughout the entire cytoplasm as a centripetal wave. These repetitive Ca^{2+} spikes were observed only in the mature eggs and continued for 3 h until the plasma membrane microvilli core collapsed, suggesting that the phenomenon may be linked to the actin-based cortical structures that have been altered during maturation [61]. These results imply that the responsiveness to LAT-A may be related to the sensitization of the oocytes to $InsP_3$ during maturation, or to the structural and functional differences of the actin cytoskeletons in the oocytes and mature eggs [56,61]. This Ca^{2+} wave was indeed blocked by heparin, but its interpretation became complicated because heparin hyperpolymerizes cortical actin [51,62]. It is possible that the LAT-A treatment rendered the mature eggs hypersensitive to the background level of $InsP_3$, or alternatively the increased rate of net depolymerization induced by LAT-A may have released Ca^{2+} from the dissociating actin monomers that had been charged with Ca^{2+}, as was first suggested by K. Lange based on biochemical properties of actin [63–65].

4. Correlation between the electrical events and Ca^{2+} changes at fertilization

Egg activation by sperm or other agents reflects the general phenomenon of cell excitation [66]. Numerous studies on the stimulated eggs of marine animals underlined the necessity of the adequate physical organization of the egg cortex, as it represents the most sensitive and excitable portion of the cell [13,67–69]. Sea urchin and starfish eggs have provided essential experimental evidence underscoring the importance of the cortex in initiating a chain of reactions related to electrical events and Ca^{2+} signaling. Mazia (1937) was the first to describe an increase in free Ca^{2+} upon fertilization of sea urchin eggs [70], and the increase was directly visualized by Ridgway (1977) in medaka eggs [71]. The acrosome-reacted spermatozoa trigger a wave of Ca^{2+} that traverses the egg of starfish and sea urchin from the sperm interaction site to the opposite pole. The Ca^{2+} increase induces exocytosis of the cortical granules beneath the plasma membrane, and the release of their contents into the perivitelline space leads to the elevation of the fertilization envelope (Fig. 3).

A precise proposal on the sequence of events that block the supernumerary sperm entry in sea urchin eggs was made by E. E. Just, who wrote " *With membrane-separation the eggs undergo some change and it is this change – not its result, membrane-separation – which constitutes the block to the entrance of additional spermatozoa. Thus this block, which is more subtle than the mechanical obstacle interposed by the presence of a separated membrane, is established before membrane-separation occurs*" ([13], pag. 199). He observed that sea urchin eggs in their optimal physiological conditions undergo a rapid structural change which spreads from the contact point of the first sperm to the entire egg surface and rejects the second sperm. He wrote "*At least for the normally monospermic ova of the marine forms which I have studied, it is certainly true that if they are in optimum condition, polyspermy is difficult if not impossible. Such ova in order to become polyspermic must undergo treatment which impairs their cortices … …Doubtless the very instant that contact between spermatozoa and ova is made, polyspermy is blocked*" ([72], pag. 337).

The idea that the block to polyspermy can be quickly established within 1–3 s upon sperm interaction led to the suggestion that the fast block to the polyspermy is due to the quick changes in the electrical potential of the egg plasma membrane. Following the contact of the first sperm, a small initial step-like depolarization takes place in the egg, which is accompanied by a decrease in resistance and increase in membrane noise. About 13 s later, a slower and larger bell-shaped fertilization depolarization (the fertilization potential) follows. The time between the two electrical events is called the "latent period" [73,74]. The establishment of a fast block to polyspermy as a result of the abrupt depolarizing shift of the egg's membrane potential was largely accepted [75], but was also disputed by others [13,76–78].

The sperm-induced Ca^{2+} signal is a biphasic event that mirrors the voltage changes at the plasma membrane. In sea urchin, the first detectable Ca^{2+} change (cortical flash), which precedes the larger Ca^{2+} wave, lasts about 8 s [79]. As the time resolution of the Ca^{2+} detecting system is lower than that of electrophysiology, this time gap between the two modes of Ca^{2+} signals is in agreement with the aforementioned latent period of 13 s (Fig. 2 in Ref. [80]). Given its rapid synchronous occurrence at the entire egg surface, the cortical flash in the fertilized eggs of sea urchin and starfish is thought to be caused by the Ca^{2+} influx through the voltage-sensitive Ca^{2+} channels, which is inhibited by nifedipine [81,82]. The cortical flash in fertilized starfish eggs is much smaller and short-lived (about 2–5 s), and is sometimes difficult to detect even in electrophysiological method. Thus, differences exist in the electrical events and Ca^{2+} responses in sea urchin and starfish eggs, which were attributed to their differences in the location of the acrosome reaction, and not to their differences in the meiotic stages at the time of fertilization [80]. Alternatively, the differences could result from the intrinsic nature of the egg cortices, which show different structural organization in the two species [62,79].

5. Mechanisms of the intracellular Ca^{2+} liberation induced by the sperm

Ca^{2+} is universally accepted as the essential trigger of egg activation, but the pathways leading to its increase within the egg are still vigorously debated. A number of hypotheses have been put forward: i) a spermatic ligand binds to a specific receptor on the egg surface, ii) spermatozoa inject a soluble factor or Ca^{2+} itself into the egg after gamete fusion [83–86]. Studies using pharmacological inhibitors or dominant negative mutant proteins in echinoderm, ascidian, fish and frog eggs have indicated that the sperm triggers activation of a Src family kinase. In sea urchin and starfish eggs, it was suggested that the global Ca^{2+} wave after the cortical flash is

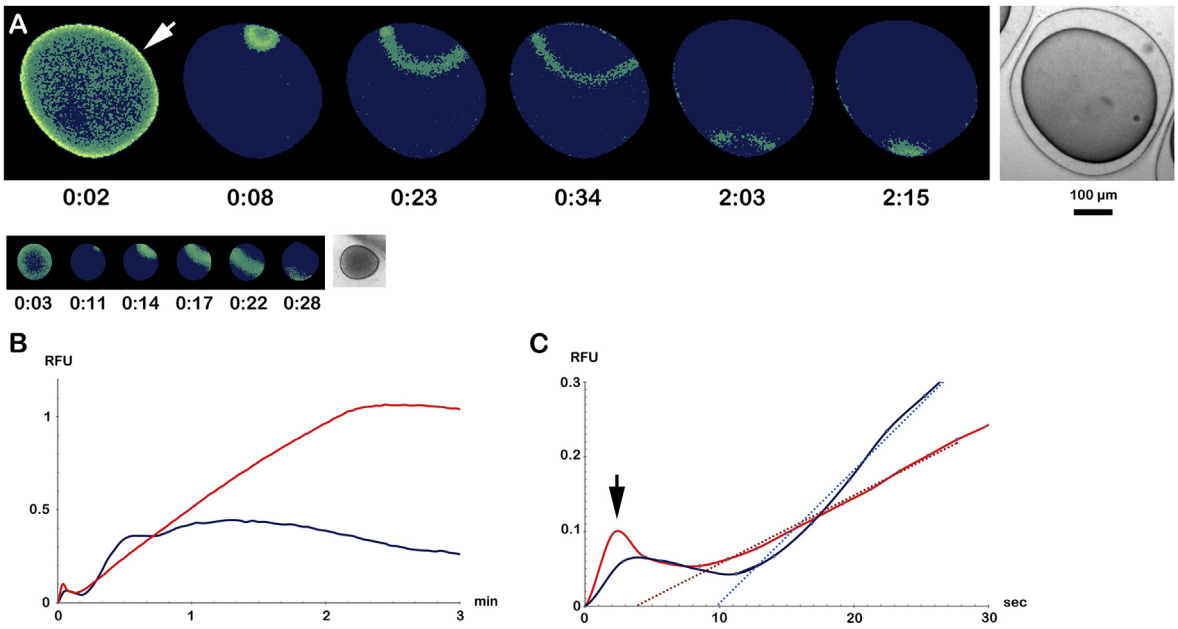

Fig. 3. Intracellular Ca^{2+} increases in the fertilized eggs of starfish and sea urchin. Eggs of *A. aranciacus* and *P. lividus* were microinjected with calcium dyes and fertilized to monitor the changes of the intracellular Ca^{2+} levels. (**A**) Pseudo-color images correspond to the momentary increase of the Ca^{2+} levels at the key time points. The images in transmission view represent the shape of the fertilized eggs with the elevation of the fertilization envelope. To portray the size difference, the eggs from starfish (top) and sea urchin (bottom) were depicted on the same scale. (**B**) The trajectories of the Ca^{2+} increase in the fertilized eggs of starfish (red) and sea urchin (blue). (**C**) The same Ca^{2+} trajectories were plotted on a different time scale to illustrate the initial Ca^{2+} rises. The onset of the global Ca^{2+} wave at the sperm interaction was estimated by extrapolating the linear portions of the curves to the time axis (dotted lines). Note that the initial Ca^{2+} spot takes place much earlier in starfish than in sea urchin in reference to the cortical flash (arrows).

propagated as a direct or indirect result of the activation of phopholipase Cγ (PLCγ) which hydrolyzes PIP2 to produce $InsP_3$ to release Ca^{2+} through the $InsP_3$ receptors on the ER [87–91]. In the fertilized eggs of sea urchin, however, it was postulated that another Ca^{2+}-releasing second messenger is at work, i.e., cADPr, to sustain the calcium induced calcium release (CICR) mechanism through ryanodine receptors (RyRs) [92,93]. At variance with sea urchin eggs, a role for cADPr in sustaining the Ca^{2+} wave at fertilization of starfish eggs is excluded since the blockage of the cADPr-promoted Ca^{2+} increase had no effect on the propagation of the sperm-induced Ca^{2+} wave [82].

More recently, another Ca^{2+}-linked second messenger NAADP was discovered from the sea urchin egg homogenates. NAADP triggers Ca^{2+} release from the stores that are insensitive to $InsP_3$ and cADPr, thus distinct from the ER [94]. The possibility that NAADP could work as a Ca^{2+}-releasing sperm factor was suggested by the finding that seawater-activated spermatozoa were able to synthesize NAADP, and that sperm extracts contain micromolar level of NAADP [95]. The latter study suggested that the sperm may deliver NAADP to the egg to induce the cortical flash as a result of the depolarization-induced activation of L-type Ca^{2+} channels. It was suggested that NAADP acts on lysosomes-like organelles to release Ca^{2+}, and their proximal location to the ER would amplify the Ca^{2+} wave [96]. The acidic organelles on which NAADP acts would contain a new class of Ca^{2+} permeable channels. Three isoforms of two-pore channels (TPCs) were suggested as NAADP receptors, and were shown to be localized in the cortex of starfish oocytes and eggs [97]. Even if TPCs may be associated with endosomes and lysosomes, however, their involvement in the release of Ca^{2+} under the influence of NAADP has been recently questioned [98]. In line with this, the knocking down of the TPCs in starfish has failed to significantly influence the sperm-induced Ca^{2+} response [97]. Support for the idea that lysosomes are Ca^{2+}-signaling organelles has been provided by the findings that the β and γ isoforms of ADP-ribosyl cyclase (ARC), which synthesize cADPr from NAD+ and NAADP from NADP, localize to the cortical granule lumen in sea urchin eggs. cADPr was shown to be locally synthesized in the lumen of acidic exocytotic granules and released to cytosol to create Ca^{2+} signals and drive exocytosis [99].

Observations in starfish eggs have originally suggested a triggering role for NAADP in the sperm-induced Ca^{2+} response at the egg surface [100]. Uncaging of NAADP in an immature oocyte or in a mature egg induced a Ca^{2+} increase which was uniformly distributed at the periphery of the cells. This cortical flash, which then spread centripetally, was strictly dependent on the presence of Ca^{2+} in the surrounding seawater. In line with a role of the NAADP-sensitive stores in initiating the sperm-induced Ca^{2+} response, NAADP induced a Ca^{2+} influx through the activation of an inward current with biophysical properties typical of Ca^{2+}-mediated currents [52]. This current was blocked by verapamil, SKF 96356, and thapsigargin, but was not affected by the impairment of lysosomes with Bafilomycin A1 or GPN, or by agents blocking the activation of the $InsP_3$ and RyR receptors [101]. Interestingly, the Ca^{2+} response induced by NAADP in mature oocytes was nearly abolished by the absence of external Ca^{2+}, but the same condition had only a minor effect on immature oocytes, adding weight to the aforementioned electrophysiological changes during the maturation process [41,80,102]. The removal of the GV prior to the application of 1-MA heavily affected the spreading of the Ca^{2+} wave in response to the sperm, but not the initial NAADP dependent Ca^{2+} response. These results have led to the proposal that NAADP receptors may play a role in initiating the Ca^{2+} release in the egg cortex, while $InsP_3$ receptors mediate the globalization of the Ca^{2+} wave. Those eggs matured without the GV failed to elevate the vitelline layer upon $InsP_3$ uncaging [100]. The mechanisms by which the nuclear contents contribute to the changes of the electrical properties of the plasma membrane during the maturation process (see above), decrease the excitability of the egg with a delayed mobilization of Ca^{2+} at fertilization are unknown.

The search for the mechanism by which the sperm generates the Ca^{2+} signals in mammalian eggs led to the identification of PLCζ as a plausible sperm factor. Injected into the egg, PLCζ mimics the effect of sperm at fertilization, generating the same pattern of Ca^{2+} release. It has been suggested that PLCζ is released by the sperm into the egg during the gamete fusion to produce $InsP_3$ from the PIP2 localized on the intracellular vesicles, as the PIP2 level in the plasma membrane is rather increased at fertilization [103].

6. Roles of the actin cytoskeleton in the starfish and sea urchin eggs at fertilization

Evidence that egg actin filaments are involved in sperm incorporation has been available for decades. In sea urchin eggs, actin is polymerized around the sperm binding site to form a fertilization cone, a specialized structure to facilitate sperm incorporation [104–106]. The internalization of the sperm occurs concomitantly with the translocation of actin fibers towards the center of the fertilized eggs [107]. In the marine worm Annellida (*Pseudopotamilla occelata*), the spermatozoon interacts with the long microvilli that traverse the egg envelope owing to the actin polymerization at egg activation, and this leads to the formation of a cortical cytoplasmic protrusion (the fertilization cone) which incorporates the sperm [108]. The formation of the cytoplasmic protrusion is preceded by a Ca^{2+} increase in the cortical cytoplasm at the sperm interaction site, but the Ca^{2+} signal does not propagate to the remainder of the egg. Only after sperm incorporation, a second Ca^{2+} increase takes place at the periphery of the egg in the form of the cortical flash that propagates towards the center of the cell. The interesting finding here is that the local Ca^{2+} increase precedes the Ca^{2+} influx which usually represents the earliest Ca^{2+} response upon egg activation in many species, or in some cases the sole manifestation of Ca^{2+} increase [44]. The cytoplasmic Ca^{2+} increase in the fertilized eggs of *P. occelata* was inhibited by U73122 and heparin, which also blocked the microfilaments-linked sperm incorporation. Since U73122 and heparin have been shown to affect the organization of the cortical F-actin in starfish [51,62], it would be interesting to verify whether the inhibition of the initial cytoplasmatic Ca^{2+} by these inhibitors of the PLC/$InsP_3$R pathways are in part ascribed to the perturbation of the actin cytoskeleton in the fertilized eggs of *P. occelata* [108].

In starfish, a long acrosomal filament emerges from the sperm head upon its interaction with the outer layer of the egg jelly coat [109,110]. This morphological event has allowed us to follow the spatio-temporal patterns of the sperm-induced Ca^{2+} release and sperm incorporation. The sperm increases Ca^{2+} in the egg when its head is far away from the plasma membrane [62]. It was found that, in addition to preventing the entry of the sperm, the alteration of the structural organization of the cortical actin cytoskeleton by the agents interfering with F-actin dynamics affected nearly all other events associated with the fertilization process, including those involving the $InsP_3$ receptors. To better understand the involvement of the latter in the generation of the Ca^{2+} wave during the Ca^{2+} response at fertilization, eggs pre-injected with heparin were used. It was found that the surface of these eggs slowly responded to the first sperm, but failed to produce a cortical flash and to prevent polyspermy. Furthermore, the amplitude of the later Ca^{2+} wave was reduced, and the propagation of the Ca^{2+} wave was halted. Many fertilized eggs were polyspermic; and even so, the spreading of the multiple Ca^{2+} waves was much slower and took longer time to cover the entire areas of the egg. The shape of the fertilization cones was abnormal, suggesting that heparin may have influenced the functional state of the actin cytoskeleton in the cortex of the mature eggs. The visualization of the F-actin showed that heparin indeed induced a hyperpolymerization of the cortical actin. Based on these observations, an intriguing question was raised whether heparin suppresses the Ca^{2+} signals solely as a pharmacological inhibitor of $InsP_3$ receptors *per se*, or as a result of the alteration of the actin cytoskeleton. The reduction or loss of the cortical flash in many heparin-injected eggs lends a support to the latter idea [62].

A more recent study on the roles of PIP2 at fertilization of starfish eggs used fluorescently tagged pleckstrin homology (PH) domain of PLC-δ1, which binds specifically to PIP2. Similar to the mammalian eggs, the PIP2 level in the plasma membrane was on the rise after fertilization except for the short stretch of time (for 20 s) when the intracellular Ca^{2+} is steeply increased [111–113]. Moreover, sequestration of PIP2 by the PH domain at higher doses promoted changes of the cortical F-actin network in such a way to delay cortical maturation and the sperm-induced intracellular Ca^{2+} increase with a significantly increased rate of polyspermic fertilization [113]. Since PIP2 itself is a key docking point that harbors and thereby regulates the activity of a number of actin-binding proteins involved in the assembly of actin microfilaments [114,115], these results underlined the importance of a proper organization of the F-actin in the egg cortex for a successful Ca^{2+} response and monospermic fertilization. This idea has been tested and sustained by microinjection of the actin-binding protein cofilin (depactin in starfish) [116], and the function-blocking antibody against depactin [117].

In the discussion of intracellular Ca^{2+} signaling in starfish oocytes, it may be worthy to mention the effect of calcium ionophore ionomycin. Ionomycin is often used upon intracytoplasmic sperm injection (ICSI) during *in vitro* fertilization of animal species [118], and occasionally in humans [119]. A brief exposure of immature starfish oocytes to ionomycin raises intracellular Ca^{2+} levels by facilitating the transport of Ca^{2+} across the plasma membrane and from the intracellular stores. Alarmingly, however, we found that ionomycin also induced drastically rapid and irreversible ultrastructural changes in the cortex of the oocyte, which include microvilli retraction and fusion of the cortical granules with other vesicles. Similarly, *A. aranciacus* eggs activated with LAT-A [61] also displayed reduction of microvilli in the perivitelline space (Fig. 4), suggesting that the extension of microvilli in perivitelline space may require physiological egg activation signals [120]. At fertilization, the ionomycin-pretreated eggs experienced a lower Ca^{2+} increase, and failed to elevate the fertilization envelope and to spread the cortical actin fibers centripetally. These structural alterations also led to a higher rate of abnormal development, even when ionomycin was added after the fertilization [121].

The *in vitro* preparation of the fractured egg cortices of sea urchin (cortical lawn) has contributed a great deal in establishing the importance of Ca^{2+} signals in triggering exocytosis of cortical granules [122]. Early experiments with the cortical lawn of sea urchin eggs suggested that Ca^{2+} was “the only secretory modulator” on the basis of the lack of the effects of the pharmacological agents disrupting actin and tubulin polymers [123]. However, recent studies with starfish and sea urchin eggs have indicated that Ca^{2+} is necessary but not sufficient in triggering exocytosis of cortical granules, as the intact eggs with deregulated actin cytoskeleton failed to undergo cortical granules exocytosis [51,62,113,121]. Thus, as was demonstrated also in the neuroendocrine cells [124], Ca^{2+} requires exquisite control of the cortical actin cytoskeleton to extrude exocytotic vesicles.

The importance of the structural and functional integrity of the actin cytoskeleton was demonstrated also with sea urchin eggs at fertilization. To put to the test the idea that proper physiological state of the cortical actin cytoskeleton is a prerequisite for successful fertilization, *Paracentrotus lividus* eggs were incubated with four different actin drugs that promote either polymerization

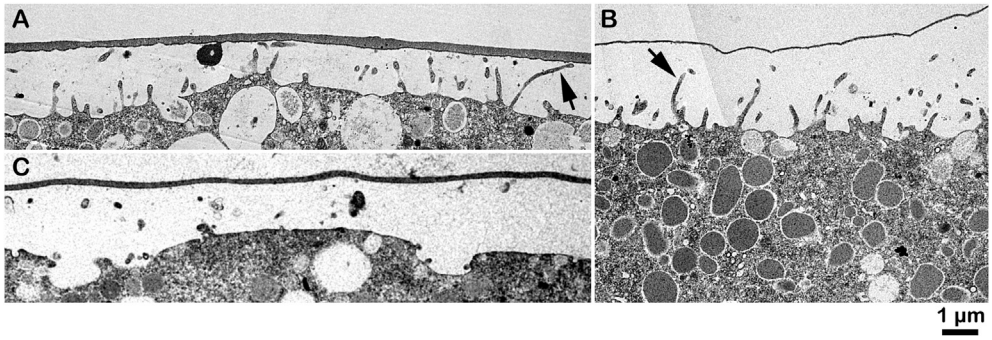

Fig. 4. Ultrastructure of the egg surface and cortex after fertilization. *A. aranciacus* (**A**,**C**) and *P. lividus* (**B**) eggs were analyzed with transmission electron microscopy after fertilization. (**C**) *A. aranciacus* eggs artificially activated with 6 µM LAT-A. Note that the microvilli, which are normally extended in the perivitelline space of the fertilized egg (arrows [120]), are largely missing in the eggs activated by LAT-A [61]. Eggs activated with ionomycin exhibited similar reduction of microvilli [121].

(jasplakinolide and phalloidin) or depolymerization (cytochalasin B and LAT-A) of actin filaments [79]. At fertilization, the eggs pretreated with cytochalasin B displayed much longer time lag between the cortical flash and the first localized Ca^{2+} increase at the sperm interaction site, which was in line with the previous electrophysiological observation of prolonged latent period [125]. Cytochalasin B increased the incident of polyspermy at a low dose, but inhibited sperm entry at high doses as with the other actin drugs. On the other hand, some other findings in the sea urchin eggs pre-treated with actin drugs defy our intuition on the role of Ca^{2+} signals at fertilization. The sea urchin eggs pre-incubated with jasplakinolide did not undergo cortical granules exocytosis at fertilization despite the seemingly normal increase of intracellular Ca^{2+}. Without the elevation of fertilization envelope, these eggs were expected to be polyspermic, but they engulfed less than one spermatozoon. On the other hand, the eggs pretreated with LAT-A often displayed multiple Ca^{2+} waves, but the eggs were entered by no sperm, whereas some other eggs had displayed one single Ca^{2+} wave but incorporated multiple sperm. Thus, the fertilization envelope is not the major deciding factor for polyspermy, and the global Ca^{2+} signal at fertilization is not indicative of sperm entry in echinoderm eggs [79,121]. A final comment in the last portion of this contribution proposes that the perturbation of the cortical flash may reflect the non-physiological conditions of the actin cytoskeleton of the egg cortex, which also affects many aspects of intracellular Ca^{2+} signaling and leads to multiple sperm interactions. An intimate relationship between the cortical flash and the Ca^{2+} wave was suggested by those who showed that the prevention of the cortical flash, by preventing the influx of Ca^{2+} with nifedipine, affected the increase and decay phases of the global Ca^{2+} wave [81]. On the other hand, it is well known that the successful sperm attached to the plasma membrane of sea urchin eggs generates a normal step depolarization event and a cortical flash. About 13 s later, the sperm stops its gyrating behavior and stiffens its tail. At this time, the Ca^{2+} wave initiates and the sperm is engulfed in the egg [78]. Thus, the major part of the mystery in fertilization still lies in the first few seconds of sperm–egg interaction.

7. Conclusions

A normal increase of Ca^{2+} is essential for egg activation and monospermic sperm entry, but the signal transduction pathway leading to it appears to be quite diverse depending on the animal species, and its molecular mechanism is still poorly understood. Nonetheless, so far the general consensus has been that the Ca^{2+} increase derives mainly from the ER, which is the major Ca^{2+} store of the cytoplasm. This store releases Ca^{2+} through $InsP_3$ receptors that are activated when $InsP_3$ is produced by the hydrolysis of the plasma membrane PIP2.

In sea urchin eggs, $InsP_3$ independent Ca^{2+} release pathways have also been suggested to operate, and two additional second messengers, i.e., NAADP and cADPr, have been identified to have roles in the activation process. NAADP appears to be the initiator of the Ca^{2+} increase and may operate by releasing Ca^{2+} from an acidic organelle containing its cognate channels (TPCs), whereas cADPr could generate the Ca^{2+} wave by a conventional mechanism of a CICR involving RyRs. Acidic organelles different from lysosomes (cortical granules) would also synthesize cADPr and donate it to the neighboring RyRs to promote the global Ca^{2+} release. The issue has controversial aspects since NAADP has been claimed to induce a Ca^{2+} influx in starfish oocytes by the activation of a plasma membrane Ca^{2+} channel, and because cADPr does not seem to play any role in sustaining the Ca^{2+} wave in starfish eggs. Interestingly, it has also been suggested that mammalian intracellular vesicles may play a role in producing $InsP_3$, as PIP2 has been found to be located in them as well.

The main point in this contribution has been the role of the cortical actin cytoskeleton in mediating the most important portion of the fertilization process of starfish and sea urchin eggs. The first event affected by the organization of the cortical actin cytoskeleton is the Ca^{2+} increase in the form of a cortical flash. Perturbation of the structural organization of the actin cytoskeleton in the cortex significantly alters its amplitude and duration. Moreover, the shape of the global Ca^{2+} wave is also affected. The next key events in the fertilization process, the exocytosis of the cortical granules and the elevation of the fertilization envelope, which so far had been assumed to be dependent solely on the Ca^{2+} increase, are also heavily influenced by the cortical actin cytoskeleton: they are abolished even in the presence of the conventional Ca^{2+} increase, once the dynamic organization of the cytoskeletal actin in the cortex has been perturbed. A proper elevation of the fertilization envelope is not sufficient to prevent the entry of supernumerary spermatozoa. Finally, the possibility that actin cytoskeleton plays a role as a Ca^{2+} store during the maturation and fertilization of starfish and sea urchin eggs cannot be discounted.

Conflict of interest

None.

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