Activation of oocytes by latrunculin A

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ABSTRACT Actin depolymerization by latrunculin A (LAT-A) in mature starfish oocytes induces a massive calcium mobilization that results in the discharge of the cortical granules and in the elevation of the fertilization envelope. The Ca2+ liberation starts as a circumscribed subplasma membrane hotspot, which is followed by a flash of Ca2+ increase restricted to the cortical layer. Ca2+ propagates rapidly from these peripheral regions to the center of the oocyte, initiating calcium oscillations. Blockade of the inositol 1,4,5-trisphosphate receptors with heparin does not affect the liberation of Ca2+ at the initial hotspot or the cortical flash, but abolishes the centripetal spreading of the wave and the Ca2+ oscillations. In Ca2+-free medium, LAT-A also initiates Ca2+ release at a discrete cortical point, but then propagates throughout the cell without first forming the uniform cortical flash. The latter is thus linked to the influx of external Ca2+, somehow promoted by the depolymerization of cortical (microvillar) actin. The Ca2+ response to spermatozoa (i.e., peripheral hotspot, cortical flash, globalisation of the signal) closely mimics that promoted by LAT-A. Thus, the initial cortical release of Ca2+ promoted by the sperm may be due to the depolymerization of actin.—Lim, D., Lange, K., Santella, L. Activation of oocytes by latrunculin A. *FASEB J.* 16, 1050–1056 (2002)

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Changes in intracellular calcium are essential to egg activation. Although a single Ca2+ wave or repetitive Ca2+ spikings are routinely detected just after fertilization in a spectrum of species ranging from marine invertebrate to mammals, the mechanism by which the sperm triggers the calcium signal is obscure (see ref 1 for a recent discussion of the problem). In echinoderms, the increase in intracellular calcium has been linked to the activation of inositol 1,4,5-trisphosphate (InsP3) and ryanodine/cyclicADP-ribose receptors (2, 3) since the injection of either InsP3 or cyclic ADP-ribose (cADPr) induces a massive increase in intracellular Ca2+, leading to cortical granules exocytosis and elevation of the fertilization envelope (4). It has been suggested that InsP3 would initiate Ca2+ release at fertilization of sea urchin eggs whereas cADPr would be the endogenous regulator of the Ca2+-induced Ca2+ release (CICR) (5, 6). However, a recent report indicates that cyclic GMP and cADPr stimulate InsP3 pro-

duction (7). Sea urchin eggs and starfish oocytes also respond to the newly discovered Ca2+-mobilizing messenger nicotinic acid adenine nucleotide phosphate (NAADP) (8, 9). One peculiar aspect of NAADP first documented in starfish oocytes is its linkage to the influx of external Ca2+, which produces a cortical flash when the injected caged messenger is liberated (10, 11). This confirms the suggestion that the Ca2+ store sensitive to NAADP is independent of those that respond to cADPr and InsP3 (12). The activation of the putative cortical NAADP receptors is preliminary to the propagation of the Ca2+ wave by activation of the InsP3 receptors (13). The type of Ca2+ response and the nature of the Ca2+ stores active in starfish oocytes at fertilization are thus the subject of this contribution. Given the importance of the actin cytoskeleton in the cortical region of the oocytes (14), we decided to explore its role in the initial Ca2+ mobilization event in starfish oocytes. Here we show that actin depolymerization by latrunculin A (LAT-A), which is more effective than cytochalasin B (15), induces an intracellular Ca2+ mobilization that initiates at a circumscribed subplasma membrane hotspot in mature oocytes (16) suspended in sea water. This initial liberation is followed by a sudden flash of Ca2+ increase restricted to the cortical layer of the oocytes. Ca2+ then propagates rapidly from these peripheral regions to the center of the oocyte. Globalization of the Ca2+ signal results in rapid discharge of the cortical granules and in the elevation of the fertilization envelope. The Ca2+ response after the interaction of spermatozoa with the oocytes suspended in sea water closely mimics that promoted by LAT-A: it begins with an initial circumscribed peripheral release, followed by a cortical flash and by the InsP3-mediated globalization of the signal. We propose that the initial cortical release of Ca2+ promoted by the sperm may be due to the depolymerization of actin.

MATERIALS AND METHODS

Preparation of gametes

Starfish (*Astropecten aurantiacus*) were collected during the breeding season in February-June in the gulf of Naples and kept in running natural sea water (16°C). Immature oocytes

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(containing the germinal vesicle, nucleus) were dissected from the ovaries and kept in artificial sea water (ASW; 460 mM NaCl, 10.1 mM KCl, 9.2 mM CaCl₂, 35.9 mM MgCl₂, 17.5 mM MgSO₄, 2.5 mM NaHCO₃, pH 8.0) for 30 min before use. Oocytes in which the breakdown of the germinal vesicle occurred spontaneously were discarded. Maturation was promoted by adding the hormone 1-methyladenine (Sigma Chemical Co., St. Louis, MO) at a final concentration of 5 μM. For fertilization experiments, 1 μL of dry sperm was suspended in 2 mL of artificial sea water, and 40 μL of this suspension was added to 1 mL of the oocyte suspension to obtain a final sperm dilution of 1:50,000.

Microinjections and calcium measurements

The calcium fluorescent dye OR Green 488 BAPTA-1 coupled to a 10 kDa dextran (OGBD; Molecular Probes, Eugene, OR) was injected into the cytoplasm of mature oocytes. The concentration of the dye in the pipette (diameter of the tip 1 μm) was adjusted to 5 mg/mL with injection buffer (IB; 450 mM potassium chloride, 10 mM HEPES, pH 7.0). The volume of injected dye corresponded to 1–2% of the total cell volume; thus, the final concentration of injected substances in the cellular environment was 50- to 100-fold lower than in the micropipette. Cytosolic Ca²⁺ changes were measured using either a cooled CCD camera (MicroMax, Princeton Instruments, Inc., Trenton, NJ) mounted on a Zeiss Axiovert (Olympus Optical Co., Ltd., Japan), an UplanApo 20× objective, laser power 20%, and confocal aperture no. 2. Fluorescence images were processed with a MetaMorph Imaging System software (Universal Imaging Corporation, West Chester, PA). To exclude variations of fluorescence intensity, the signals were corrected for variations in dye concentration by normalizing fluorescence (F) against baseline fluorescence (F₀). The region of interest to measure the fluorescence level was positioned as shown in the scheme of the figures.

Chemicals

LAT-A was purchased from Molecular Probes. A 3 mM stock solution in dimethyl sulfoxide (DMSO) was prepared and kept frozen. ASW or Ca²⁺-free sea water (CaFSW) containing 6 μM LAT-A was prepared just before the experiment. Mature oocytes (70 min after 1-MA addition) were gently transferred to LAT-A containing ASW and kept in a free position during maturing hormone 1-methyladenine (1-MA). Immediately afterward, LAT-A was added to the chamber and the Ca²⁺ response monitored by a cooled CCD camera. Figure 1A shows that the response initiated 6 min and 14 s after the addition of LAT-A, which was the time evidently necessary for it to depolymerize actin, at a circumscribed cortical site (see the second fluorescent image). 24 s after the first local Ca²⁺ increase, a uniform fluorescent ring suddenly appeared in the entire cortical region of the oocyte (cortical flash) (see the fourth fluorescent image). The cortical flash failed to decay, but spread instead centripetally to the entire oocyte in approximately 60 s at a rate of 5 μm/s, strongly resembling the rate of spreading of the Ca²⁺ wave at fertilization calculated for the nemertean worm Cerebratulus lacteus (17). The graph of the relative fluorescence of the Ca²⁺

Transmission and scanning electron microscopy

For transmission electron microscopy, control mature oocyte and oocytes treated with LAT-A were fixed first with 1% glutaraldehyde-90% sea water for 1 h at room temperature. Oocytes were rinsed several times in ASW and postfixed in 1% osmium tetroxide for 0.5–1 h. The samples were dehydrated in a graded alcohol series and embedded in EPON 812. Sections were stained with 2% uranyl acetate and 0.2% lead citrate and examined with a Philips 400 transmission electron microscope. For scanning electron microscopy, the microvilli on the surface of the oocyte were visualized by removing the follicle cells and the vitelline envelope with 0.01% actinase-E (Kaken Pharmaceutical Co., LTD., Japan) for 10 min. The treated oocytes were then washed several times in ASW. After fixation in glutaraldehyde, the samples were dehydrated in the ethanol series and critical point dried from carbon dioxide, cemented to specimen stubs, and sputtered with gold. A Philips 505 scanning electron microscope was used for the observations.

RESULTS

Latrunculin A experiments

Oocytes incubated in sea water were injected with the calcium fluorescent dye Oregon green 488 BAPTA-1 coupled to an OGBD 70 min after the addition of the maturing hormone 1-methyladenine (1-MA). Immediately afterward, LAT-A was added to the chamber and the Ca²⁺ response monitored by a cooled CCD camera. Figure 1A shows that the response initiated 6 min and 14 s after the addition of LAT-A, which was the time evidently necessary for it to depolymerize actin, at a circumscribed cortical site (see the second fluorescent image). 24 s after the first local Ca²⁺ increase, a uniform fluorescent ring suddenly appeared in the entire cortical region of the oocyte (cortical flash) (see the fourth fluorescent image). The cortical flash failed to decay, but spread instead centripetally to the entire oocyte in ~60 s at a rate of 5 μm/s, strongly resembling the rate of spreading of the Ca²⁺ wave at fertilization calculated for the nemertean worm Cerebratulus lacteus (17). The graph of the relative fluorescence of the Ca²⁺

Figure 1. Ca²⁺ release induced by the addition of LAT-A to mature oocytes suspended in sea water. A) The Ca²⁺ increase, monitored with a cooled CCD camera, initiated in one cortical site of the oocyte and yielded a uniform cortical flash (fourth fluorescent image, arrow). The Ca²⁺ wave then spread from the cortex in a centripetally directed wave. B) Graph of the relative fluorescence offers a numerical equivalent of the colors visualized in the fluorescent images of panel A. The regions of interest (ROIs) were positioned as shown.

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indicator (Fig. 1B) offers a numerical equivalent of the colors shown in the fluorescent images in the two sites analyzed in the cortical region (violet and green circles; blue circle for the central area). After the initial increase at a distinct point (time 0 on the graph), the relative fluorescence of the cortical flash reached an amplitude of $0.5 \pm 0.15$ arbitrary units. The $Ca^{2+}$ increase in the center of the cell peaked at a relative fluorescence value of $0.7 \pm 0.1$ ($n=35$) 75 s after the initial $Ca^{2+}$ elevation.

The increase of $Ca^{2+}$ induced by LAT-A in the cortical region of the cell could have been due to the influx of external $Ca^{2+}$ through calcium channels in the oolemma. LAT-A was thus applied to oocytes kept in $Ca^{2+}$-free sea water containing 1 mM EGTA (Fig. 2A). As in the experiments in $Ca^{2+}$-containing sea water, an initial circumscribed cortical site of fluorescence was observed 6 min and 19 s after LAT-A addition (see the second fluorescent image). However, after this initial increase, the $Ca^{2+}$ wave spread throughout the cell without first generating a cortical flash (see the third fluorescent image), showing that the latter was generated by the influx of external $Ca^{2+}$. The graph of the relative fluorescence $1.1 \pm 0.15$ ($n=15$) (Fig. 2B) shows the $Ca^{2+}$ increase at the initial point in the cortex (violet circle in the scheme) propagating at the same rate measured in the experiment in the presence of external $Ca^{2+}$ (5 $\mu$m/s), and reaching the opposite pole of the cell in $\sim$60 s.

The effect of LAT-A was also analyzed using confocal microscopy, monitoring two sites within the cortical region and in the center of an equatorial plane of the oocyte. The confocal images in Fig. 3A confirmed the findings made with the cooled CCD camera (see Fig. 1A for comparison). The cortical $Ca^{2+}$ flash seen in normal sea water with the cooled CCD camera was well visible, but the initial circumscribed site of the $Ca^{2+}$ increase was visible only with the CCD camera (see second and third images of Fig. 1A). This was apparently due to the difficulty of detecting a $Ca^{2+}$ increase in a small region that could be out of the confocal plane of the oocyte. The $Ca^{2+}$ increase shown in Fig. 3B was identical to that observed with the cooled CCD camera in oocytes kept in $Ca^{2+}$-free sea water.

The cortical flash of $Ca^{2+}$ induced by LAT-A and subsequent spreading of the wave were followed by the elevation of the fertilization envelope, as imaged by transmitted light microscopy (Fig. 4). Transmission electron microscopy of mature control oocytes (data not shown) showed the cortical granules in close association with the plasma membrane. Figure 4 shows the fertilization envelope of an oocyte 9 min after LAT-A addition. As with fertilization, the vitelline layer lifted from the oocyte’s surface after the fusion and the release of the cortical granule material to the perivitelline space.

The $Ca^{2+}$ response to the addition of spermatozoa to mature oocytes observed with the cooled CCD camera strikingly resembled that induced by LAT-A. The sperm induced an initial small elevation of $Ca^{2+}$ at the site of

Figure 3. Confocal laser scanning imaging of $Ca^{2+}$ release induced by LAT-A. A) In sea water, the $Ca^{2+}$ release induced by LAT-A treatment yielded a uniform cortical flash and then spread from the cortex in a centripetally directed wave. B) In $Ca^{2+}$-free sea water, the cortical $Ca^{2+}$ increased from one cortical point, then propagated over the entire cell surface without generating a cortical flash.

Figure 4. Transmission electron micrograph of a mature oocyte treated with LAT-A for 9 min. The fertilization envelope elevated from the surface of the oocyte (arrow) after the extrusion of the cortical granules. Bar = 1 $\mu$m. The inset shows the elevation of the fertilization envelope (arrow) imaged by transmitted light microscopy.
interaction, which expanded to a uniform cortical flash and then spread to the entire oocyte (Fig. 5). The cortical flash after exposure to spermatozoa had already been observed, for example, in the nemertean worm C. lacteus (17), in whose eggs "the cortical flash became visible after the sperm attached to the oolemma and eventually yielded a rise in [Ca^{2+}], throughout the entire ooplasm in a manner suggestive of a centripetally directed wave". The cortical flash in C. lacteus has been claimed to involve calcium influx from outside based on experiments on the blockade of oolemmal calcium channels with cobalt (17). Ca^{2+} influx at fertilization also occurs in sea urchin eggs due to rapid depolarization of the plasma membrane produced by the insertion of sperm cation channels (18), amplified by the influx of calcium through voltage-dependent (L-type) channels (19). However, no reports available in the literature had so far shown a distinct point source pattern of propagation. As mentioned above, this was probably due to the difficulty of detecting a Ca^{2+} increase restricted to a very limited region when using only a confocal plane of the oocyte instead of imaging the whole cell. Figure 5A shows that in all imaged oocytes (n=20), the cortical flash started 6 s after the first Ca^{2+} elevation at the initial cortical point (second fluorescent image), reaching a relative fluorescence amplitude of ~0.3 ± 0.1 and decaying afterward in ~7 s. The wave spread from the same point throughout the oocyte reaching an amplitude of 0.9 ± 0.1 arbitrary units in ~3 min at a rate of 1.65 μm/s. The graph of the relative fluorescence in Fig. 5B shows the Ca^{2+} response to the sperm in different regions of the oocytes (see the scheme). The cortical flash is well visible at the opposite oocyte pole (small spike of the green line in the graph), although it initiates at the point of sperm fusion. In this region (violet circle and line), the cortical ring was difficult to detect because of the superposition of the propagating wave.

Repetitive Ca^{2+} spikings induced by latrunculin A

After the first Ca^{2+} wave induced by LAT-A, the overall Ca^{2+} level declined toward the baseline in ~2 min, but was followed ~1 min later by a second wave that swept centripetally from the periphery of the cell. More cycles followed, repetitive waves occurring with decreasing frequency and amplitude for ~150 min, which may correspond to the time required to completely disassemble F-actin (Fig. 6A).

Experiments were performed to establish whether the increase in intracellular Ca^{2+} was linked to the increased metabolism of phosphoinositides, as suggested by the cytoskeletal dependence of InsP_{3}-generating phospholipases (20). Oocytes were incubated for 20 min with the phospholipase inhibitor U73122 (10 μM) before the addition of LAT-A. Although under the experimental conditions the height of the Ca^{2+} spikes was slightly lower, the elevation of the fertilization envelope and the oscillatory pattern occurred normally (data not shown). It was then decided to investigate whether the initial local Ca^{2+} liberation induced by LAT-A elicited the Ca^{2+} oscillations by activating InsP_{3}-sensitive receptors, as would have been expected from the accepted ability of Ca^{2+} to promote InsP_{3} channel

**Figure 5.** Intracellular Ca^{2+} increase after fertilization of a control mature oocyte. A) An overlay of the relative fluorescence of the intracellular Ca^{2+} increase visualized with OGBD in a mature control oocyte (upper panel) induced by the sperm. Relative fluorescence of the Ca^{2+} increase induced by the sperm (lower panel). B) The graph shows the Ca^{2+} increase in the cortical region and in the center of the oocyte (circles). The cortical flash is highly visible at the opposite oocyte pole (arrow).

**Figure 6.** Repetitive Ca^{2+} spikings induced by LAT-A. A) The Ca^{2+} oscillations propagated across the entire ooplasm, the repetitive waves occurring with decreasing frequency and amplitude for ~150 min. B) Heparin preinjected into the oocytes completely inhibited the Ca^{2+} waves but failed to block the cortical Ca^{2+} elevation. C) Fluorescent image of the cortical Ca^{2+} flash in the inset of panel B sandwiched between images in which Ca^{2+} was at baseline level.
opening (21). The InsP₃ receptor antagonist heparin was preinjected into mature oocytes before treatment with LAT-A. Figure 6B shows that the Ca²⁺ response was radically altered in all experiments (n=15), but the long acquisition times used to follow the late-onset oscillations prevented the detection of the initial cortical Ca²⁺ liberation event. The uniform cortical Ca²⁺ flash occurred normally, reaching a relative fluorescence peak of 0.15 ± 0.1; n = 15 (Fig. 6B). However, the centripetal spreading of the Ca²⁺ wave failed to follow it. The inset in Fig. 6C shows the fluorescent image of the cortical Ca²⁺ rise sandwiched between images in which Ca²⁺ was at baseline level. Thus, in line with previous observations (22, 23), the spreading of the Ca²⁺ wave from the cortex to the remainder of the oocyte was indeed mediated by the InsP₃ receptors. The possible involvement of ryanodine/cADPr receptors was also probed using the specific antagonist 8NH₄-cADPr (24). Its preinjection failed to inhibit the LAT-A-induced Ca²⁺ oscillations, which actually reached significantly higher peak amplitudes 1.2 ± 0.08 (n=7) than in the controls 0.98 ± 0.1 (n=13).

The hypothesis was tested that LAT-A promoted the influx of extracellular Ca²⁺ by inducing the depolymerization of actin in the microvilli. Figure 7A shows the surface of a mature oocyte examined with the scanning electron microscope 70 min after the addition of I-MA. The oocytes were pretreated at the immature stage with actinase (10 min, 0.01%) to remove the follicle cells and the vitelline coat (14). The elongated microvilli are distributed over the surface in regular form and length in a nondensely packed pattern. After 10 min in the presence of LAT-A, the length of the microvilli was clearly reduced and their shape became vesicular (Fig. 7B), reminiscent of that prevailing in unfertilized sea urchin eggs treated with cytochalasin B (25).

**DISCUSSION**

This study has dissected the temporal sequence of Ca²⁺ responses that follow the interaction of oocytes with the sperm, documenting for the first time that a spatially restricted calcium increase at the point of sperm–egg interaction preceded the cortical flash previously observed by others. The initial Ca²⁺ increase at a restricted cortical point induced by the fertilizing sperm strikingly resembled that promoted by LAT-A, suggesting that the initial trigger of Ca²⁺ release beneath the plasma membrane could in both cases be linked to the depolymerization of actin. The uniform cortical flash of Ca²⁺ apparently was produced by Ca²⁺ penetration from the outside, as its absence was the only difference observed in the Ca²⁺ response to LAT-A in the absence of external Ca²⁺. Since the only known action of LAT-A is the depolymerization of F-actin, the results are compelling in suggesting that the actin cytoskeleton is involved in the regulation of Ca²⁺ channels. This would be in line with observations that cation exchangers and ion channels are modulated by actin effectors or by the interaction with G- or F-actin (26). The problem, then, becomes that of understanding 1) why LAT-A, which evidently is available to the entire surface of the oocyte, only induced the initial Ca²⁺ liberation in a very restricted cortical area, and 2) the nature of the latrunculin-sensitive cortical Ca²⁺ store. One possibility is that polymerized actin itself was the Ca²⁺ store, as previously claimed in the literature (27, 28). The disassembly of the Ca²⁺-rich actin filament in a localized domain in the oocyte cortex (29) could set free a limited amount of Ca²⁺ and, at the same time, trigger the opening of plasma membrane channels, eventually leading to the globalization of the signal via the previously described canonical InsP₃-dependent pathway (30). Alternatively, disassembly of the actin cytoskeleton could promote the liberation of Ca²⁺ from conventional membrane-enclosed stores. One could mention previous findings of morphological changes during the maturation of starfish oocytes that are linked to the polymerization and depolymerization of cortical actin. I-MA induces a rapid reorganization of the oocyte surface that culminates after 10 min in the formation of long spikes and in the elimination of microvilli as spikes form (14). Long after microvilli are eliminated, the number of microvilli is restored, especially around the animal pole (31).

In summary, it could be suggested that the initial Ca²⁺ response by LAT-A would be produced by acting on the microvilli at the animal hemisphere. Ca²⁺ could likely be directly liberated from depolymerized microvillar actin, or, indirectly, by neighboring membrane-enclosed deposits controlled by the actin cytoskeleton (27). The initial local Ca²⁺ liberation would be followed by a series of Ca²⁺ release and reuptake events involving the InsP₃-sensitive stores. Such a role for Ca²⁺ has been proposed, for example, in *Xenopus laevis* oocytes, where the close apposition of neighboring ‘puff sites’ is important for the generation of Ca²⁺ waves (30, 32). Results have appeared showing that cortical structures such as actin and the endoplasmic

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**Figure 7.** Scanning electron micrograph of the surface of a mature oocyte. A) Microvilli on the surface on a mature control oocyte 70 min after hormonal stimulation. B) Microvilli of a mature oocyte treated for 9 min with LAT-A. After the drug treatment, the microvilli collapsed onto the oocyte surface. Bar = 1 μm.
reticulum are involved in initiating the repetitive Ca\(^{2+}\) waves in fertilized ascidian oocytes (33). Thus, in addition to having a role in the activation of oocytes, actin reorganization in the cortex may be the initial event that eventually leads to the sperm-induced globalization of the signal and the Ca\(^{2+}\) oscillations that may arise from the periodic opening of plasma membrane calcium channels (membrane oscillator) (34). Repetitive Ca\(^{2+}\) waves originating at the site of sperm entry have been shown in different species. In ascidian oocytes, Ca\(^{2+}\) waves start from the site of injection of a sperm extract in the peripheral region and propagate across the ooplasm. If the injection of the sperm extract is performed in the central region of the oocyte (35), the Ca\(^{2+}\) response is significantly delayed, indicating that the sensitivity to the extract is higher in the cortex of the cell (33, 35–37) and dependent on the continuous Ca\(^{2+}\) entry by a capacitative mechanism (38, 39). Changes in the state of cortical actin induced by the sperm may play a pivotal role in the generation of Ca\(^{2+}\) waves at fertilization.

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