

# 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  $\text{Ca}^{2+}$  liberation starts as a circumscribed subplasma membrane hotspot, which is followed by a flash of  $\text{Ca}^{2+}$  increase restricted to the cortical layer.  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  at the initial hotspot or the cortical flash, but abolishes the centripetal spreading of the wave and the  $\text{Ca}^{2+}$  oscillations. In  $\text{Ca}^{2+}$ -free medium, LAT-A also initiates  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ , somehow promoted by the depolymerization of cortical (microvillar) actin. The  $\text{Ca}^{2+}$  response to spermatozoa (i.e., peripheral hotspot, cortical flash, globalization of the signal) closely mimics that promoted by LAT-A. Thus, the initial cortical release of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  wave or repetitive  $\text{Ca}^{2+}$  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 ( $\text{InsP}_3$ ) and ryanodine/cyclicADP-ribose receptors (2, 3) since the injection of either  $\text{InsP}_3$  or cyclic ADPr-ribose (cADPr) induces a massive increase in intracellular  $\text{Ca}^{2+}$ , leading to cortical granules exocytosis and elevation of the fertilization envelope (4). It has been suggested that  $\text{InsP}_3$  would initiate  $\text{Ca}^{2+}$  release at fertilization of sea urchin eggs whereas cADPr would be the endogenous regulator of the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) (5, 6). However, a recent report indicates that cyclic GMP and cADPr stimulate  $\text{InsP}_3$  pro-

duction (7). Sea urchin eggs and starfish oocytes also respond to the newly discovered  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$ , which produces a cortical flash when the injected caged messenger is liberated (10, 11). This confirms the suggestion that the  $\text{Ca}^{2+}$  store sensitive to NAADP is independent of those that respond to cADPr and  $\text{InsP}_3$  (12). The activation of the putative cortical NAADP receptors is preliminary to the propagation of the  $\text{Ca}^{2+}$  wave by activation of the  $\text{InsP}_3$  receptors (13). The type of  $\text{Ca}^{2+}$  response and the nature of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  increase restricted to the cortical layer of the oocytes.  $\text{Ca}^{2+}$  then propagates rapidly from these peripheral regions to the center of the oocyte. Globalization of the  $\text{Ca}^{2+}$  signal results in rapid discharge of the cortical granules and in the elevation of the fertilization envelope. The  $\text{Ca}^{2+}$  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  $\text{InsP}_3$ -mediated globalization of the signal. We propose that the initial cortical release of  $\text{Ca}^{2+}$  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 CaCl<sub>2</sub>, 35.9 mM MgCl<sub>2</sub>, 17.5 mM MgSO<sub>4</sub>, 2.5 mM NaHCO<sub>3</sub>, 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  $\mu$ M. For fertilization experiments, 1  $\mu$ L of dry sperm was suspended in 2 mL of artificial sea water, and 40  $\mu$ L 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  $\mu$ m) 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 Ca<sup>2+</sup> changes were measured using either a cooled CCD camera (MicroMax, Princeton Instruments, Inc., Trenton, NJ) mounted on a Zeiss Axiovert 200 microscope with a Plan-Neofluar 20 $\times$ /0.50 objective or a confocal laser scanning microscope Olympus FVX-ZM-IL (Olympus Optical Co., Ltd., Japan), an UplanApo 20 $\times$ /0.70 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 (F<sub>0</sub>). 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 Ca<sup>2+</sup>-free sea water (CaFSW) containing 6  $\mu$ M 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 the Ca<sup>2+</sup> measurement acquisition. The concentration of DMSO in the experimental bath did not exceed 0.2%. For experiments on oocytes injected with OGBD, cells were washed twice in a solution containing 470 mM NaCl, 10.1 mM KCl, 35.9 mM MgCl<sub>2</sub>, 17.5 mM MgSO<sub>4</sub>, 2.5 mM NaHCO<sub>3</sub>, pH 8.0, supplemented with 2 mM EGTA. The oocytes were transferred to CaFSW containing 1 mM EGTA. A 50 mg/mL solution of heparin (MW=6000) (Sigma) in the injection buffer was prepared just before the injection experiment. The 8NH<sub>2</sub>cADPr antagonist of the cADPr-sensitive channel (Sigma) was prepared as a 1 mM stock solution in IB and kept frozen. For microinjection experiments, the stock solution of 8NH<sub>2</sub>cADPr was dissolved at a final concentration of 250  $\mu$ M.

### Transmission and scanning electron microscopy

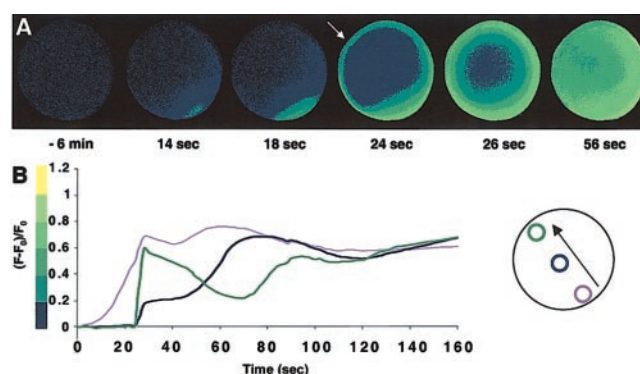
For transmission electron microscopy, control mature oocyte and oocytes treated with LAT-A were fixed first with 1% glutaraldehyde-90% sea water for 1 h at room temperature. Oocytes were rinsed several times in ASW and postfixated in 1%

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 Ca<sup>2+</sup> 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 Ca<sup>2+</sup> 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  $\sim$ 60 s at a rate of 5  $\mu$ m/s, strongly resembling the rate of spreading of the Ca<sup>2+</sup> wave at fertilization calculated for the nemertean worm *Cerebratulus lacteus* (17). The graph of the relative fluorescence of the Ca<sup>2+</sup>

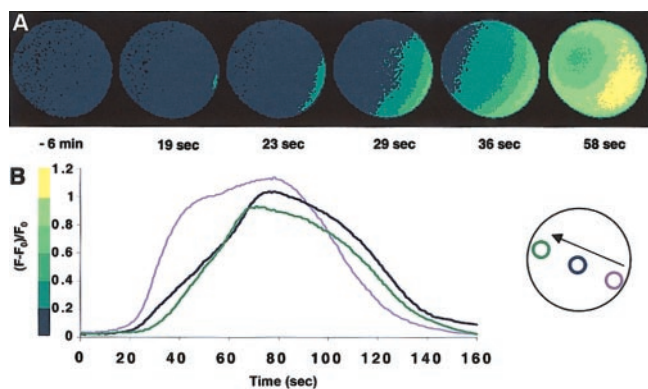


**Figure 1.** Ca<sup>2+</sup> release induced by the addition of LAT-A to mature oocytes suspended in sea water. *A*) The Ca<sup>2+</sup> increase, monitored with a cooled CCD camera, initiated in one cortical site of the oocyte and yielded a uniform cortical flash (fourth fluorescent image, arrow). The Ca<sup>2+</sup> 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.

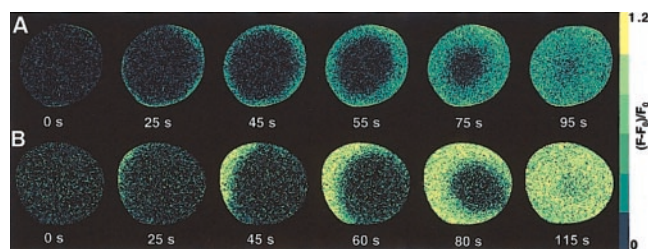
indicator (Fig. 1B) offers a numerical equivalent of the colors shown in the fluorescent images in the two sites analyzed in the cortical region (violet and green circles; blue circle for the central area). After the initial increase at a distinct point (time 0 on the graph), the relative fluorescence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

The increase of  $\text{Ca}^{2+}$  induced by LAT-A in the cortical region of the cell could have been due to the influx of external  $\text{Ca}^{2+}$  through calcium channels in the oolemma. LAT-A was thus applied to oocytes kept in  $\text{Ca}^{2+}$ -free sea water containing 1 mM EGTA (Fig. 2A). As in the experiments in  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ . The graph of the relative fluorescence  $1.1 \pm 0.15$  ( $n=15$ ) (Fig. 2B) shows the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  ( $5 \mu\text{m/s}$ ), 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  $\text{Ca}^{2+}$  flash seen in normal sea water with the cooled CCD camera was well visible, but the initial circumscribed site of the  $\text{Ca}^{2+}$  increase was visible only with the CCD camera (see second and third images of Fig. 1A). This was appar-



**Figure 2.**  $\text{Ca}^{2+}$  release induced by the addition of LAT-A to mature oocytes suspended in  $\text{Ca}^{2+}$ -free sea water. A) The  $\text{Ca}^{2+}$  wave spread throughout the cell from the initial point without first generating a cortical flash. The cortical  $\text{Ca}^{2+}$  then propagated from one point over the entire cell surface. B) A graph of the relative fluorescence shows that the initial  $\text{Ca}^{2+}$  increase in one point of the cortex propagated throughout the oocyte, reaching the opposite pole.

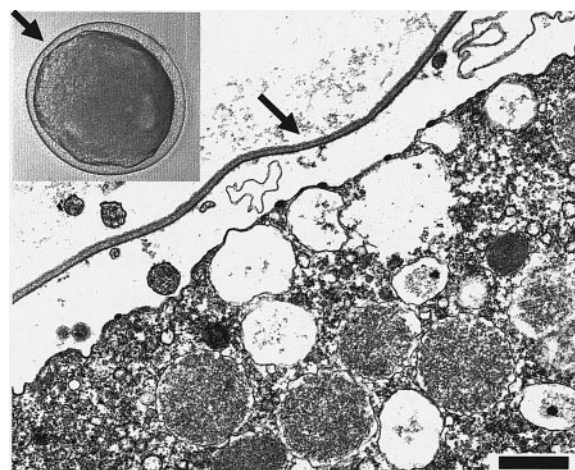


**Figure 3.** Confocal laser scanning imaging of  $\text{Ca}^{2+}$  release induced by LAT-A. A) In sea water, the  $\text{Ca}^{2+}$  release induced by LAT-A treatment yielded a uniform cortical flash and then spread from the cortex in a centripetally directed wave. B) In  $\text{Ca}^{2+}$ -free sea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

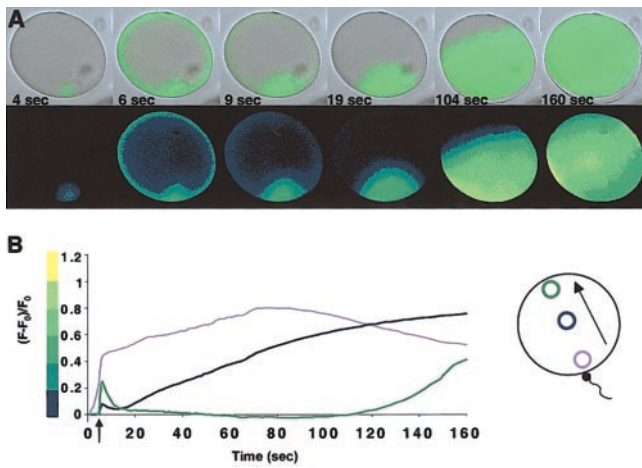
ently due to the difficulty of detecting a  $\text{Ca}^{2+}$  increase in a small region that could be out of the confocal plane of the oocyte. The  $\text{Ca}^{2+}$  increase shown in Fig. 3B was identical to that observed with the cooled CCD camera in oocytes kept in  $\text{Ca}^{2+}$ -free sea water.

The cortical flash of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  at the site of



**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 oocytes (arrow) after the extrusion of the cortical granules. Bar =  $1 \mu\text{m}$ . The inset shows the elevation of the fertilization envelope (arrow) imaged by transmitted light microscopy.



**Figure 5.** Intracellular  $\text{Ca}^{2+}$  increase after fertilization of a control mature oocyte. *A*) An overlay of the relative fluorescence of the intracellular  $\text{Ca}^{2+}$  increase visualized with OGBD in a mature control oocyte (upper panel) induced by the sperm. Relative fluorescence of the  $\text{Ca}^{2+}$  increase induced by the sperm (lower panel). *B*) The graph shows the  $\text{Ca}^{2+}$  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).

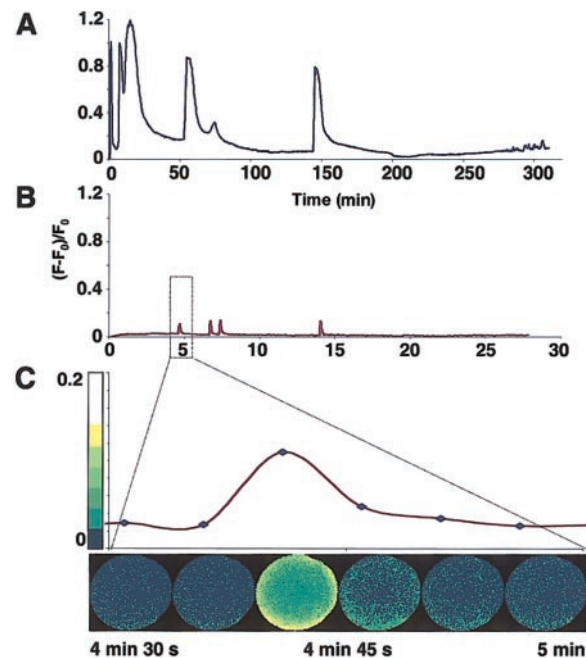
interaction, which expanded to a uniform cortical flash and then spread to the entire oocyte (**Fig. 5**). The cortical flash after exposure to spermatozoa had already been observed, for example, in the nemertean worm *C. lacteus* (17), in whose eggs “the cortical flash became visible after the sperm attached to the oolemma and eventually yielded a rise in  $[\text{Ca}^{2+}]_i$  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).  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  increase restricted to a very limited region when using only a confocal plane of the oocyte instead of imaging the whole cell. Figure 5*A* shows that in all imaged oocytes ( $n=20$ ), the cortical flash started 6 s after the first  $\text{Ca}^{2+}$  elevation at the initial cortical point (second fluorescent image), reaching a relative fluorescence amplitude of  $\sim 0.3 \pm 0.1$  and decaying afterward in  $\sim 7$  s. The wave spread from the same point throughout the oocyte reaching an amplitude of  $0.9 \pm 0.1$  arbitrary units in  $\sim 3$  min at a rate of  $1.65 \mu\text{m}/\text{s}$ . The graph of the relative fluorescence in Fig. 5*B* shows the  $\text{Ca}^{2+}$  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 $\text{Ca}^{2+}$ spikings induced by latrunculin A

After the first  $\text{Ca}^{2+}$  wave induced by LAT-A, the overall  $\text{Ca}^{2+}$  level declined toward the baseline in  $\sim 2$  min, but was followed  $\sim 1$  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  $\sim 150$  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  $\text{Ca}^{2+}$  was linked to the increased metabolism of phosphoinositides, as suggested by the cytoskeletal dependence of  $\text{InsP}_3$ -generating phospholipases (20). Oocytes were incubated for 20 min with the phospholipase inhibitor U73122 (10  $\mu\text{M}$ ) before the addition of LAT-A. Although under the experimental conditions the height of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  liberation induced by LAT-A elicited the  $\text{Ca}^{2+}$  oscillations by activating  $\text{InsP}_3$ -sensitive receptors, as would have been expected from the accepted ability of  $\text{Ca}^{2+}$  to promote  $\text{InsP}_3$  channel



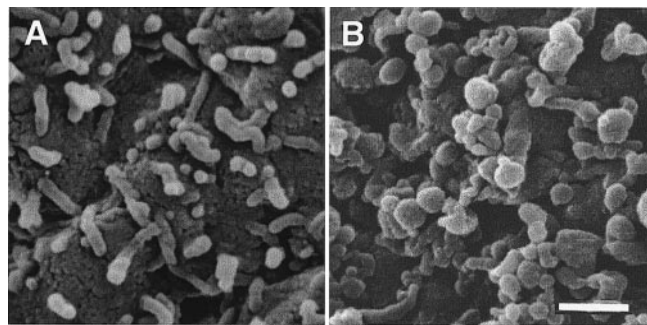
**Figure 6.** Repetitive  $\text{Ca}^{2+}$  spikings induced by LAT-A. *A*) The  $\text{Ca}^{2+}$  oscillations propagated across the entire ooplasm, the repetitive waves propagated with decreasing frequency and amplitude for  $\sim 150$  min. *B*) Heparin preinjected into the oocytes completely inhibited the  $\text{Ca}^{2+}$  waves but failed to block the cortical  $\text{Ca}^{2+}$  elevation. *C*) Fluorescent image of the cortical  $\text{Ca}^{2+}$  flash in the inset of panel *B* sandwiched between images in which  $\text{Ca}^{2+}$  was at baseline level.

opening (21). The  $\text{InsP}_3$  receptor antagonist heparin was preinjected into mature oocytes before treatment with LAT-A. Figure 6B shows that the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  liberation event. The uniform cortical  $\text{Ca}^{2+}$  flash occurred normally, reaching a relative fluorescence peak of  $0.15 \pm 0.1$ ;  $n = 15$  (Fig. 6B). However, the centripetal spreading of the  $\text{Ca}^{2+}$  wave failed to follow it. The inset in Fig. 6C shows the fluorescent image of the cortical  $\text{Ca}^{2+}$  rise sandwiched between images in which  $\text{Ca}^{2+}$  was at baseline level. Thus, in line with previous observations (22, 23), the spreading of the  $\text{Ca}^{2+}$  wave from the cortex to the remainder of the oocyte was indeed mediated by the  $\text{InsP}_3$  receptors. The possible involvement of ryanodine/cADPr receptors was also probed using the specific antagonist 8NH<sub>2</sub>-cADPr (24). Its preinjection failed to inhibit the LAT-A-induced  $\text{Ca}^{2+}$  oscillations, which actually reached significantly higher peak amplitudes  $1.2 \pm 0.08$  ( $n=7$ ) than in the controls  $0.98 \pm 0.1$  ( $n=13$ ).

The hypothesis was tested that LAT-A promoted the influx of extracellular  $\text{Ca}^{2+}$  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 1-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  $\text{Ca}^{2+}$  responses that follow the interaction of oocytes



**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  $\mu\text{m}$ .

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  $\text{Ca}^{2+}$  increase at a restricted cortical point induced by the fertilizing sperm strikingly resembled that promoted by LAT-A, suggesting that the initial trigger of  $\text{Ca}^{2+}$  release beneath the plasma membrane could in both cases be linked to the depolymerization of actin. The uniform cortical flash of  $\text{Ca}^{2+}$  apparently was produced by  $\text{Ca}^{2+}$  penetration from the outside, as its absence was the only difference observed in the  $\text{Ca}^{2+}$  response to LAT-A in the absence of external  $\text{Ca}^{2+}$ . 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  liberation in a very restricted cortical area, and 2) the nature of the latrunculin-sensitive cortical  $\text{Ca}^{2+}$  store. One possibility is that polymerized actin itself was the  $\text{Ca}^{2+}$  store, as previously claimed in the literature (27, 28). The disassembly of the  $\text{Ca}^{2+}$ -rich actin filament in a localized domain in the oocyte cortex (29) could set free a limited amount of  $\text{Ca}^{2+}$  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  $\text{InsP}_3$ -dependent pathway (30). Alternatively, disassembly of the actin cytoskeleton could promote the liberation of  $\text{Ca}^{2+}$  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. 1-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  $\text{Ca}^{2+}$  response by LAT-A would be produced by acting on the microvilli at the animal hemisphere.  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  liberation would be followed by a series of  $\text{Ca}^{2+}$  release and reuptake events involving the  $\text{InsP}_3$ -sensitive stores. Such a role for  $\text{Ca}^{2+}$  has been proposed, for example, in *Xenopus laevis* oocytes, where the close apposition of neighboring 'puff sites' is important for the generation of  $\text{Ca}^{2+}$  waves (30, 32). Results have appeared showing that cortical structures such as actin and the endoplasmic

reticulum are involved in initiating the repetitive  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  oscillations that may arise from the periodic opening of plasma membrane calcium channels (membrane oscillator) (34). Repetitive  $\text{Ca}^{2+}$  waves originating at the site of sperm entry have been shown in different species. In ascidian oocytes,  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  waves at fertilization. **[FJ]**

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