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Early events of fertilization in sea urchin eggs are sensitive to actin-binding organic molecules

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ABSTRACT

We previously demonstrated that many aspects of the intracellular Ca^{2+} increase in fertilized eggs of starfish are significantly influenced by the state of the actin cytoskeleton. In addition, the actin cytoskeleton appeared to play comprehensive roles in modulating cortical granules exocytosis and sperm entry during the early phase of fertilization. In the present communication, we have extended our work to sea urchin which is believed to have bifurcated from the common ancestor in the phylogenetic tree some 500 million years ago. To corroborate our earlier findings in starfish, we have tested how the early events of fertilization in sea urchin eggs are influenced by four different actin-binding drugs that promote either depolymerization or stabilization of actin filaments. We found that all the actin drugs commonly blocked sperm entry in high doses and significantly reduced the speed of the Ca^{2+} wave. At low doses, however, cytochalasin B and phalloidin increased the rate of polyspermy. Overall, certain aspects of Ca^{2+} signaling in these eggs were in line with the morphological changes induced by the actin drugs. That is, the time interval between the cortical flash and the first Ca^{2+} spot at the sperm interaction site (the latent period) was significantly prolonged in the eggs pretreated with cytochalasin B or latrunculin A, whereas the Ca^{2+} decay kinetics after the peak was specifically attenuated in the eggs pretreated with jasplakinolide or phalloidin. In addition, the sperm interacting with the eggs pretreated with actin drugs often generated multiple Ca^{2+} waves, but tended to fail to enter the egg. Thus, our results indicated that generation of massive Ca^{2+} waves is neither indicative of sperm entry nor sufficient for cortical granules exocytosis in the inseminated sea urchin eggs, whereas the structure and functionality of the actin cytoskeleton are the major determining factors in the two processes.

1. Introduction

One of the first changes taking place in the fertilized eggs of nearly all animal species is an increase of intracellular Ca^{2+}, which is utilized as a second messenger to initiate a cascade of subsequent egg activation procedures [1–3]. In fertilized sea urchin eggs, the Intracellular Ca^{2+} increase has two components: the rapid synchronized influx of external Ca^{2+} via L-type Ca^{2+} channels and the subsequent global Ca^{2+} wave that propagates from the sperm interaction site to the antipode [4–8]. A similar pattern of Ca^{2+} signaling has also been observed in fertilized eggs of starfish, and the intracellular Ca^{2+} increases in echinoderm eggs have been recapitulated in large part by the second messengers that induce Ca^{2+} release from the internal stores, e.g. $InsP_3$, cADPr, and NAADP [9–11].

During the course of our studies on the molecular mechanisms of Ca^{2+} signaling in starfish oocytes, we have found that many aspects of the intracellular Ca^{2+} increases in the oocytes undergoing meiotic maturation, as well as in the eggs fertilized or microinjected with the second messengers, were significantly influenced by the actin cytoskeleton. This has been demonstrated in various approaches by microinjecting the actin-binding protein cofilin [12], the function-blocking antibody against depactin [13], inhibitor of G-protein [14], or the PIP2-sequestering domain of PLC-δ1 [15], each of which changes the state of the actin cytoskeleton by directly or indirectly altering the activity or availability of the actin-binding proteins, as well as by using actin-binding drugs [16,17]. In these studies with starfish eggs, we have demonstrated that the actin cytoskeleton plays comprehensive roles at fertilization by modulating not only Ca^{2+} signaling, but also cortical granules exocytosis and sperm entry.

Abbreviations: PIP2, phosphatidylinositol-4,5-bisphosphate; $InsP_3$, inositol trisphosphate; cADPr, cyclic ADP ribose; NAADP, nicotinic acid adenine dinucleotide phosphate; RFU, relative fluorescence unit; CYT-B, cytochalasin B; LAT-A, latrunculin A; JAS, jasplakinolide; PHAL, phalloidin; FE, fertilization envelope.

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In one striking case, incubation of the eggs from a certain species of starfish (*Astropecten aranciacus*) with an actin-binding drug latrunculin A (LAT-A) caused a 'spontaneous' rise of intracellular Ca^{2+} waves without an aid of exogenous factors such as sperm or second messengers [18], but this extraordinary phenomenon has not been observed in the eggs of other echinoderm species. Thus, the aforementioned roles of the actin cytoskeleton might be restricted to a certain species of echinoderm, and herein we have tested whether or not those effects that we had observed in the fertilized eggs of starfish could be also demonstrated with sea urchin eggs. To this end, we have used two different actin-binding drugs that promote net depolymerization of actin (cytochalasin B and latrunculin A) and two others that rather stabilize actin filaments (jasplakinolide and phalloidin), and their effects on the pattern of Ca^{2+} signaling, cortical granules exocytosis, and sperm entry were compared in one place.

2. Materials and methods

2.1. Preparation of gametes

Adult animals of sea urchin (*Paracentrotus lividus*) were captured from the Gulf of Naples during the breeding season, and kept in the circulating seawater (16 °C). Eggs were collected from females by an intracoelomic injection of 0.5 M KCl, and dry sperm were surgically removed from the male gonad. Fertilization was performed at 3.7×10^6 spermatozoa/ml (final density) in fresh seawater. To determine the number of egg-incorporated spermatozoa, sperm were stained with Hoechst 33342 (Sigma–Aldrich) as described previously [19].

2.2. Microinjection, confocal microscopy, and video imaging

The method of microinjection regarding injection buffer, transjector, and volumetric calibration was previously described in detail [15,19]. Fresh intact eggs were microinjected with a gentle tapping of the apparatus without additional steps of removing jelly coats. For Ca^{2+} imaging, Calcium Green 488 conjugated to 10 kD dextran (500 μM) was mixed with Rhodamine Red (35 μM) in the injection buffer. To visualize F-actin in a living egg, 10 μM AlexaFluor568–phalloidin was injected into eggs as previously described [17], and the eggs were monitored with a CrEST X-Light spinning disk confocal microscope. The bright field view and the fluorescence images of Ca^{2+} were detected with a cooled CCD camera (MicroMax, Princeton Instruments, Inc) in about 2 s time resolution, and were analyzed with MetaMorph (Universal Imaging Corporation). The quantified Ca^{2+} signal at a given time point was normalized to the baseline fluorescence (F_0) following the formula $F_{rel} = [F - F_0]/F_0$, where F represents the average fluorescence level of the entire egg. Thus, F_{rel} was defined as RFU (relative fluorescence unit) for plotting Ca^{2+} trajectories in Figs. 2 and 3. To compensate batch-to-batch variability of Ca^{2+} signals, the peak amplitudes of the Ca^{2+} wave and the cortical flash of each egg were normalized with the averages of the corresponding values in the control eggs from the same batch. Thus, the data presented in reference to the batch-matching control (taken as 100%) was pooled together and subjected to statistical analyses. To visualize the momentary increase of Ca^{2+} signals in subcellular regions, incremental changes of the Ca^{2+} rise were analyzed by applying the formula $F_{inst} = [(F_t - F_{t-1})/F_{t-1}]$ and used for illustrations in Figs. 2 and 3 (top panels).

2.3. Chemicals and statistical analysis

All fluorescent probes and the actin-binding drugs, except phalloidin (Sigma–Aldrich), were purchased from Molecular Probes. The average and variation of the data were reported as 'mean ± SD (standard deviation)' in all cases. The paired and unpaired *t*-tests and the one-way ANOVA were performed by use of Prism 3.0 (GraphPad Software, La Jolla, USA), and the *P*-values smaller than 0.05 ($P < 0.05$) were considered statistically significant.

3. Results

3.1. Effects of the actin-binding drugs on structure dynamics of the actin cytoskeleton in sea urchin eggs

The actin cytoskeleton is a dynamical structure that undergoes constant changes even in a quiescent egg. Thus, actin-binding drugs are expected to cause structural changes to the actin cytoskeleton by interfering with the natural process of treadmilling. To demonstrate the efficacy of the actin-binding drugs and to compare their effects on the structure of the actin cytoskeleton in sea urchin eggs, we microinjected individual eggs with a small amount of AlexaFluor568–phalloidin that does not interfere with biological processes by itself [15,17]. Five minutes later, the eggs were treated with the four different actin-binding drugs or their vehicles as a control, and the structural changes of the actin cytoskeleton in the living eggs were monitored by confocal microscopy at five minutes' intervals. As shown in Fig. 1A, the control eggs treated with 0.4% DMSO retained a myriad of fluorescently stained actin filaments in the cytoplasm over the 20 min period. However, in the eggs treated with 2 μM cytochalasin B (CYT-B), the actin filaments began to disappear at 5 min and were hardly visible in the cytoplasm by 15 min. Likewise, the eggs treated with 3 μM latrunculin A (LAT-A) displayed reduction of the heavily labeled actin filaments by 5 min, but some of them lingered on in the cytoplasm for the 20 min period. Nonetheless, the actin cytoskeleton in these eggs appeared to be globally altered, as judged by the loss of the perfectly spherical cell shape by 5 min, which reflects a substantial change in cell rigidity. In contrast to CYT-B and LAT-A that tended to shift actin filaments towards net depolymerization, the other two drugs known to stabilize actin filaments strongly enhanced the actin meshwork underneath the plasma membrane. In the eggs exposed to 12 μM jasplakinolide (JAS), the layer of the subplasmalemmal actin filaments became visibly thicker by 5 min (Fig. 1A, arrow). Likewise, the eggs microinjected with 1 mM phalloidin (PHAL) exhibited intense hyperpolymerization of actin in the subplasmalemmal region of the cortex by 5 min (Fig. 1B, arrow), with the concomitant depolymerization in the inner cytoplasm. Thus, all the four actin drugs at given doses displayed appreciable structural changes in the actin cytoskeleton in specific subcellular regions.

3.2. Effects of cytochalasin B and latrunculin A on the intracellular Ca^{2+} increase in the sea urchin eggs responding to fertilizing sperm

We then examined how fertilization is affected by the two actin-binding drugs that promote net depolymerization. First, we monitored their effects on the Ca^{2+} response at fertilization. As shown in Fig. 2, CYT-B and LAT-A exhibited similar effects in many aspects. The most conspicuous thing about the Ca^{2+} response in the eggs pretreated with CYT-B (2 μM, 10 min) was that the time interval between the cortical flash and the detection of the first Ca^{2+} spot at the sperm interaction site, which was defined as the 'latent period' in this work, was distinctively increased (12.3 ± 4.9 s, $n = 47$) in comparison with the control eggs (7.8 ± 3.3 s, $n = 36$; $P < 0.0001$), although the amplitude of the cortical flash itself remained virtually unchanged: 111.0 ± 30.8% in CYT-B-pretreated eggs vs. 100.0 ± 19.4% in the control eggs treated with 0.1% DMSO, $P = 0.2223$ (Fig. 2A). Likewise, the latent period in the eggs

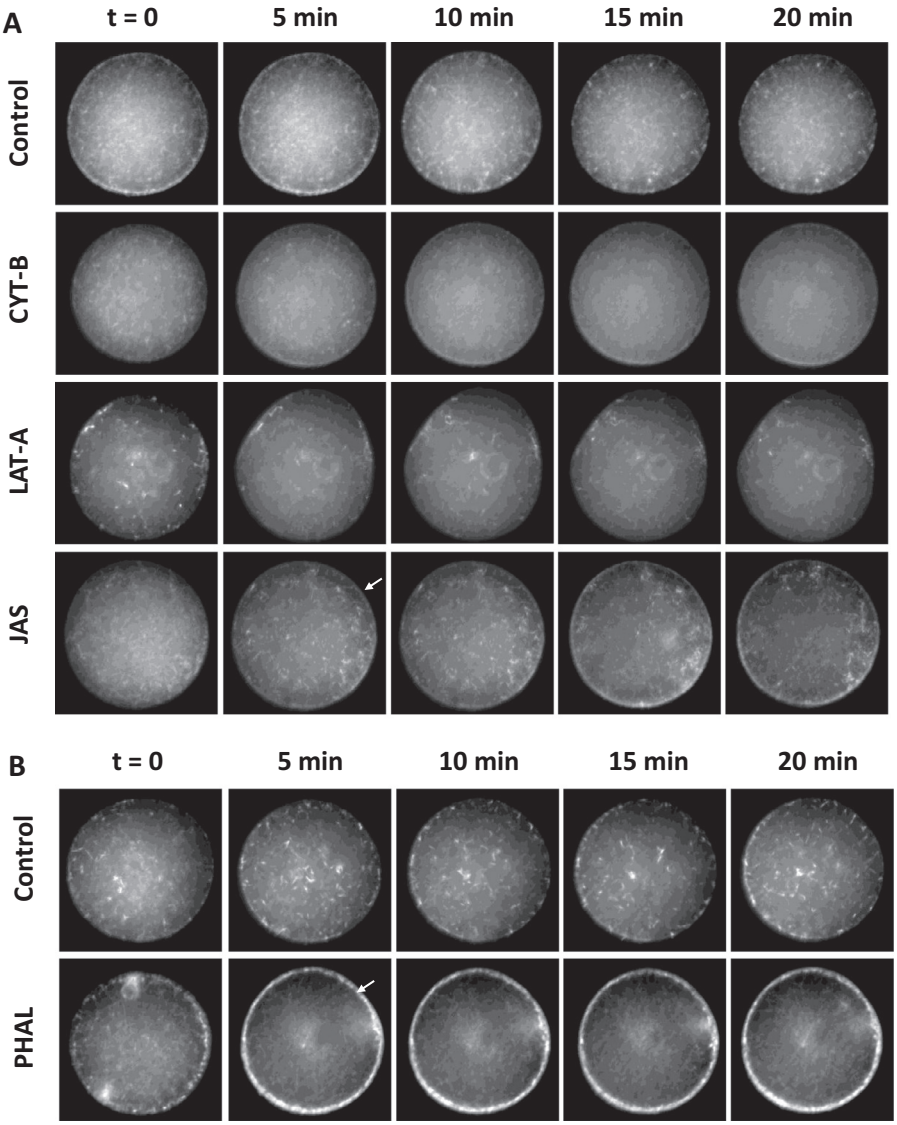

Fig. 1. Alteration of the actin cytoskeleton in the sea urchin eggs treated with the actin-binding drugs. *P. lividus* eggs were microinjected with AlexaFluor568–phalloidin (10 μM in pipette) and subsequently treated with four different actin-binding drugs to compare their effects. (A) The eggs loaded with the fluorescent probe for F-actin were then incubated in seawater containing either 0.4% DMSO (control) or one of the actin-binding drugs: CYT-B (2 μM cytochalasin B), LAT-A (3 μM latrunculin A), and JAS (12 μM jasplakinolide). For each treatment, the moment immediately before drug addition was taken as $t = 0$, and the time-lapse images of the actin cytoskeleton were obtained with confocal microscopy at the equatorial plane. (B) Being membrane-impermeant, phalloidin (PHAL, 1 mM in pipette) was delivered to the eggs by a second microinjection after 10 min recovery, whereas the control eggs were microinjected with DMSO. Arrows indicate the subplasmalemmal layer where actin was readily hyperpolymerized following the treatment.

pretreated with LAT-A (100 nM, 15 min) was also significantly increased (9.1 ± 2.6 s, $n = 18$) in comparison with the control (7.4 ± 2.5 s, $n = 22$; $P < 0.01$). The amplitude of the cortical flash in these eggs displayed high variability, but was not significantly different from that of the control (90.9 ± 56.0% vs. 100.0 ± 22.2%; $P = 0.4400$) (Fig. 2B). On the other hand, the peak amplitude of the global Ca^{2+} increase was significantly decreased in both cases. The Ca^{2+} peak in the CYT-B-pretreated eggs scored 90.8 ± 22.0% ($n = 47$, $P < 0.05$) of the level in the control (100.0 ± 11.4%, $n = 36$), and the Ca^{2+} peak in the LAT-A-pretreated eggs was merely 74.1 ± 14.8% ($n = 18$, $P < 0.0001$) of the level in the control eggs (100.0 ± 14.6%, $n = 22$). Another remarkable observation in the eggs pretreated with LAT-A was that the Ca^{2+} wave initiating at the sperm interaction site failed to propagate to the antipode in 72.2% of the cases ($n = 18$), whereas the Ca^{2+} wave in the control egg always displayed complete propagation ($n = 22$). The LAT-A-pretreated eggs often displayed irregular Ca^{2+} increases that had apparently initiated at multiple sites but faded out before being developed as a coordinated wave (Fig. 2B, red arrowheads). In addition to these localized erratic Ca^{2+} increases, insemination of the LAT-A-pretreated eggs led to production of multiple Ca^{2+} peaks on a larger time scale, which usually started at different areas of the egg surface and developed into at least a partial wave (Fig. 2B, Ca^{2+} trajectories in bold lines). To a lesser extent, the Ca^{2+} wave in the CYT-B-pretreated eggs failed to reach the antipode in 23.4% of the cases ($n = 47$). Interestingly, even in cases where a Ca^{2+} wave successfully traversed the CYT-B-pretreated eggs, the time span from the initial Ca^{2+} spot to the fading one in the antipode (defined here as the 'traverse time') was significantly increased (22.3 ± 4.4 s, $n = 36$) in comparison with that in the control (17.8 ± 3.1 s, $n = 35$; $P < 0.0001$). Accordingly, the planar speed of the Ca^{2+} wave was significantly reduced in the CYT-B-treated eggs (circa 3.6 μm/s, as opposed to 4.5 μm/s in the control). To a lesser degree, the traverse time of the Ca^{2+} wave in

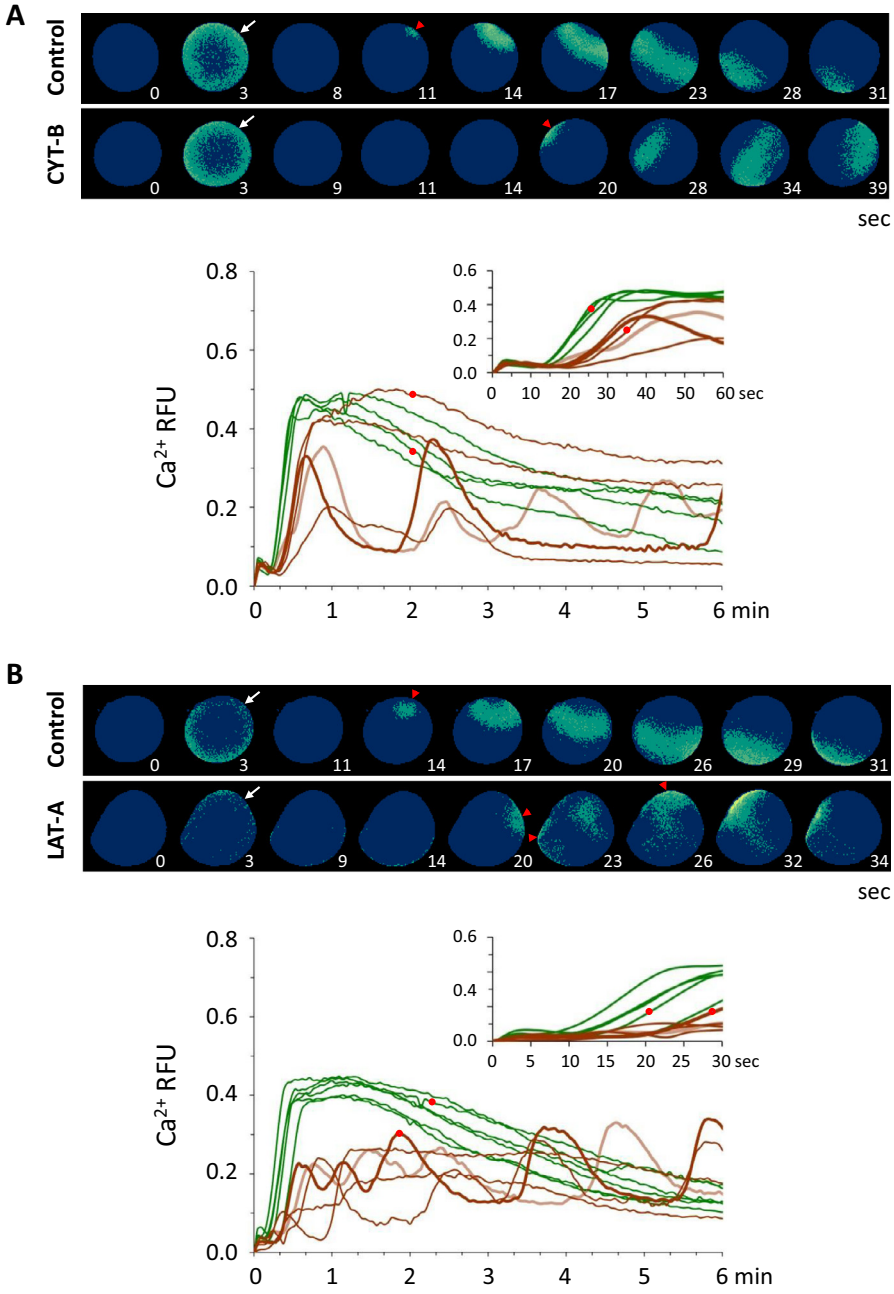

Fig. 2. Effects of cytochalasin B and latrunculin A on the intracellular Ca^{2+} increase in the sea urchin eggs responding to fertilizing sperm. (A) *P. lividus* eggs were microinjected with calcium dyes and incubated in seawater containing 2 μM CYT-B or 0.1% DMSO (control). Ten minutes later, the eggs were thoroughly rinsed and inseminated in fresh seawater. The changes of the intracellular Ca^{2+} level were monitored after sperm addition in the control (green curves) and CYT-B-pretreated eggs (brown curves). The pseudo-colored relative fluorescence images at the top represent the sites of momentary Ca^{2+} increases at the key time points (numbers refer to 'seconds' after $t = 0$), and the Ca^{2+} trajectories in the graph trace the global changes of the Ca^{2+} levels in reference to the background. In this and other figures, the time frame immediately before the first detectable Ca^{2+} signal in the CCD camera recording was set to time zero ($t = 0$). Here, results from one of the five independent experiments were illustrated. For visual distinction, two Ca^{2+} trajectories displaying repetitive patterns were marked with bold lines with different shades. In the inset is the same Ca^{2+} trajectories plotted on a different time scale to depict the initial Ca^{2+} changes. The cortical flash and the initial Ca^{2+} spots at the presumed sites of significant sperm contact were marked by arrows and arrowheads, respectively. (B) Results of a comparable experiment with the 15 min pretreatment with 100 nM LAT-A or 0.1% DMSO (control). Out of three independent experiments, data from one representative batch of eggs were presented here. The Ca^{2+} trajectories of the eggs whose Ca^{2+} waves were visualized in panel A were marked with small red circles.

the LAT-A-pretreated eggs was also increased (20.0 ± 5.3 s, $n = 5$), but its difference from the values in the control (18.2 ± 2.2 s, $n = 22$) did not reach statistical significance. Overall, the eggs pretreated with CYT-B or LAT-A shared similarities in nearly all aspects of the Ca^{2+} response at fertilization (Fig. 2) in agreement with their comparable effects on the morphology of the actin cytoskeleton (Fig. 1).

3.3. Effects of jasplakinolide and phalloidin on the intracellular Ca^{2+} increase in the sea urchin eggs responding to fertilizing sperm

We then examined how fertilization of sea urchin eggs would be affected by the other class of actin drugs, i.e. JAS and PHAL, which tended to stabilize actin filaments in the subplasmalemmal regions (Fig. 1). Unlike the cases with CYT-B and LAT-A, the eggs

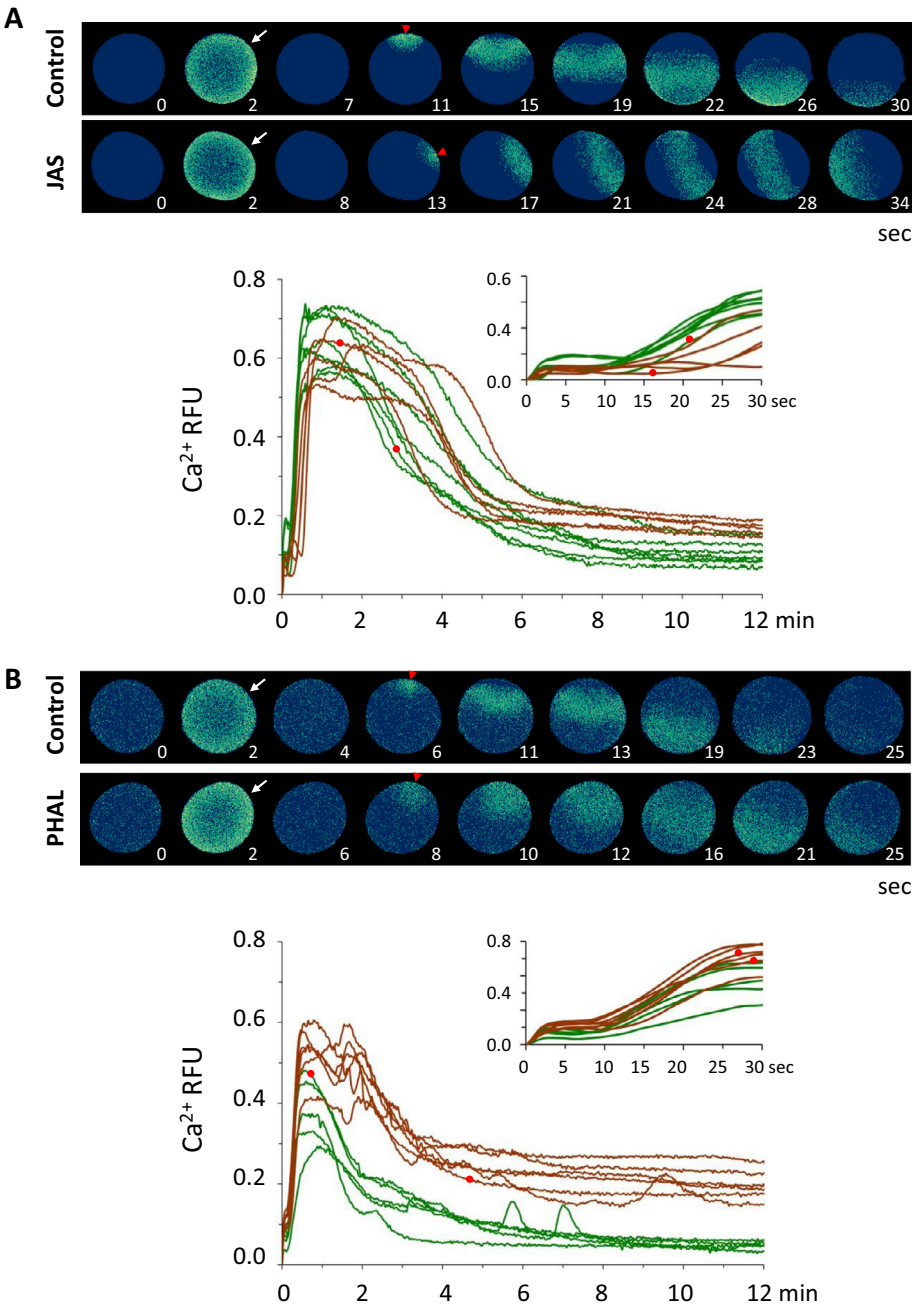

Fig. 3. Effects of jasplakinolide and phalloidin on the intracellular Ca^{2+} increase in the sea urchin eggs responding to fertilizing sperm. (A) *P. lividus* eggs were microinjected with calcium dyes and incubated in seawater containing 12 μM JAS or 0.4% DMSO (control). Twenty minutes later, the eggs were extensively rinsed in fresh seawater and sperm were added. The changes of the intracellular Ca^{2+} level were monitored after sperm addition in the control (green curves) and JAS-pretreated eggs (brown curves), and presented in pseudo-colored relative fluorescence images and in Ca^{2+} trajectory graphs, as described in the legend of Fig. 2. Results from one of the three independent experiments were illustrated. (B) *P. lividus* eggs were microinjected with the calcium dye mixed with either 1 mM PHAL (concentration in the pipette) or DMSO (control). After 20 min incubation, the eggs were inseminated, and the Ca^{2+} response was monitored and presented as described in the legend of Fig. 2. The Ca^{2+} trajectories in the control and the PHAL-microinjected eggs were depicted in green and brown curves, respectively. Data from one of the four independent experiments were presented here.

treated with JAS (12 μM, 20 min) or microinjected with 1 mM PHAL (20 min incubation) displayed no significant difference from their control eggs with respect to the latent period. JAS-pretreated eggs scored 8.8 ± 4.0 s (n = 24) which was similar to 8.1 ± 1.7 s of the control eggs exposed to 0.4% DMSO (n = 22, P = 0.2485). PHAL-injected eggs also scored 7.5 ± 2.4 s (n = 29) which was not much different from 7.0 ± 1.3 s of the DMSO-injected control eggs (n = 24, P = 0.3975) (Fig. 2). However, these two drugs had significant effects on the cortical flash, but in opposite ways. Whereas JAS slightly repressed the amplitude of the cortical flash (82.9 ± 22.3% vs. 100 ± 22.5% of the level in the control, P < 0.05), PHAL strongly enhanced it (162.7 ± 100.3% vs. 100 ± 27.6% of the level in the control, P < 0.01). As to the global Ca^{2+} increase at fertilization, pretreatment of sea urchin eggs with JAS did not make a significant difference to the peak amplitude, which was 90.6 ± 23.8% of the level in the control (100 ± 19.4%, P = 0.2142) (Fig. 3A). At variance with this, the Ca^{2+} peak in the eggs preinjected with 1 mM PHAL (n = 30) exhibited a strong enhancement: 140.6 ± 59.3% in comparison with the control, 100.0 ± 13.3% (n = 26, P < 0.01) (Fig. 3B). Despite these subtle differences, however, the pretreatment of

sea urchin eggs with JAS or PHAL manifested strikingly similar effects at the phase of Ca^{2+} decay. Whereas the Ca^{2+} signals in the entire field of the DMSO-treated control eggs continued to decline after the peak (Fig. 3A and B, green curves), the diminishing Ca^{2+} levels in the JAS- and PHAL-treated eggs apparently came to a virtual halt at about 5 and 3 min, respectively (Fig. 3A and B, brown curves). As a result, the fading Ca^{2+} signals in these eggs arrived at a plateau, which was distinctively higher than the Ca^{2+} trajectories of their corresponding control eggs. To quantify this, we extrapolated the two linear stretches of the declining Ca^{2+} trajectories before and after the sharp curvature at the beginning of the plateau, and obtained the Ca^{2+} level at the crossing time point and normalized it with the amplitude of the Ca^{2+} peak. The ratio of plateau to peak thus defined was significantly higher in the eggs pretreated with JAS (0.406 ± 0.086, $n = 17$) than in the control eggs (0.175 ± 0.039, $n = 22$; $P < 0.0001$), as well as in the PHAL-preinjected eggs (0.455 ± 0.089, $n = 30$) in comparison with their control eggs (0.231 ± 0.102, $n = 26$; $P < 0.0001$). Hence, it appeared that the Ca^{2+} ions mobilized at fertilization was reabsorbed from cytosol with much less efficiency or capacity in those eggs that had been pretreated with the drugs inducing actin hyperpolymerization in the cortex and subplasmalemmal regions.

3.4. Effects of the actin-binding drugs on sperm entry and elevation of the fertilization envelope

In normal experimental conditions, insemination of *P. lividus* eggs almost always leads to monospermic fertilization [20] and full elevation of the fertilization envelope (FE), the latter of which results from cortical granules exocytosis. We tested whether the sperm entry and FE elevation would be influenced by actin-binding drugs in a dose-dependent manner. To this end, *P. lividus* eggs pretreated with increasing concentrations of actin drugs were extensively rinsed and inseminated with Hoechst 33342-stained sperm. The number of egg-incorporated sperm was then scored in each egg (Fig. 4A), and the frequency of the eggs displaying full elevation of the FE at each drug dose was averaged from the results of 4–6 independent experiments comprising 20–40 eggs. As shown in Fig. 4B (brown dots and lines), the average percentage of the eggs displaying full elevation of the FE dropped progressively with the increasing doses of the four actin-binding drugs. In parallel, the number of the egg-incorporated sperm also diminished (Fig. 4B, green bars), suggesting that the four actin-drugs pre-administered to the eggs generally inhibited sperm entry. However, we noted that a lower dose of CYT-B (0.4 μM, 10 min) contrarily increased the number of egg-incorporated sperm (1.2 ± 0.51 per egg, $n = 60$) in comparison with the control eggs (1.03 ± 0.18 per egg, $n = 87$; $P < 0.01$). This small but significant increase of polyspermy in the CYT-B-pretreated eggs cannot be attributed to the incomplete elevation of the FE, as all the eggs displaying polyspermy (9 out of 60) incorporated 2–3 sperm in the face of full elevation of the FE (Fig. 4A, see the three internalized sperm in a CYT-B-pretreated egg). Conversely, all the eggs that exhibited incomplete elevation of the FE (14 out of 60) ended up with monospermic fertilization. A similar trend of increased polyspermy at a low dose was also found in the eggs microinjected with PHAL (3 μM, estimated concentration in cytosol), which scored 1.31 ± 1.27 sperm per egg ($n = 80$, $P < 0.05$) as opposed to 1.01 ± 0.11 ($n = 80$) for the control eggs microinjected with DMSO (Fig. 4B, PHAL). It is noteworthy that PHAL was administered to the eggs by microinjection unlike the other three drugs, which were added by bath incubation, and that the microinjection of the vehicle itself (DMSO) significantly lowered the frequency of the eggs displaying full elevation of the FE (only $80.0 \pm 24.8\%$, $N = 4$). Nonetheless, only 1 out of 80 DMSO-microinjected eggs exhibited entry of multiple sperm, suggesting that the presence or absence of a fully elevated FE is not the major determinant of polyspermy in sea urchin eggs. Despite the lack of the mechanical barrier and apparent adherence of the sperm (Fig. 4A), the eggs pretreated with the highest given doses of the four actin-binding drugs were able to incorporate sperm only scarcely, as the average number of egg-incorporated sperm was drastically reduced in the eggs pretreated with CYT-B (0.05 ± 0.22, $n = 60$), LAT-A (0.11 ± 0.34, $n = 120$), JAS (0.0 ± 0.0, $n = 60$), and PHAL (0.013 ± 0.11, $n = 80$) in comparison with their corresponding control eggs ($P < 0.0001$ in all cases) (Fig. 4B). Taken together, these results indicated that the actin-binding drugs used in this study had dose-dependent effects on sperm entry. At higher doses, they commonly inhibited the sperm incorporation mechanism of the egg; but at low doses, CYT-B and PHAL increased incidents of supernumerary sperm entry.

4. Discussion

In this communication, we have reported that the actin-binding drugs pre-administered to sea urchin eggs have profound effects on Ca^{2+} signaling, cortical granules exocytosis, and sperm entry at fertilization. Sea urchin eggs pretreated with CYT-B or LAT-A, which generally promotes net depolymerization of cortical actin (Fig. 1), manifested several common features of Ca^{2+} increases at fertilization. First, the time span between the cortical flash and the onset of the first detectable Ca^{2+} spot at the sperm interaction site (the 'latent period') was significantly prolonged by 57% and 23% when the eggs were pretreated with CYT-B and LAT-A (Fig. 2). Although much lower in time resolution, our results are in line with the previous electrophysiological data reporting that the interval between the step potential and the fertilization potential increased nearly twofold by cytochalasin [21]. Thus, the cortical flash and the initial Ca^{2+} spot at the sperm interaction site may correspond to the step potential and the onset of the fertilization potential, respectively. However, the exact physiological significance of the latent period and its prolongation by cytochalasin is not clear [21,22]. In view of the historical theories on how sperm activates the eggs [9,23,24], the latent period may reflect the time needed by the successful sperm either to physically infuse a substance into the egg via a conduit or to transmit a cascade of signals through a receptor on the egg surface. Whichever may be the pathway, CYT-B and LAT-A appeared to delay it significantly (Fig. 2). Thus, the egg subplasmalemmal actin cytoskeleton is thought to be implicated in this yet unclear process that leads from sperm interaction to the generation of the initial Ca^{2+} spot.

The other characteristic feature often found in the eggs pretreated with CYT-B or LAT-A was the generation of multiple Ca^{2+} peaks at fertilization (Fig. 2). During the first 6 min of the Ca^{2+} response, the LAT-A-pretreated eggs exhibited as many as 3.8 ± 2.1 peaks ($n = 18$) in comparison with their control eggs (1.05 ± 0.21, $n = 22$; $P < 0.0001$). To a lesser extent, the CYT-B-pretreated eggs displayed 1.8 ± 0.95 ($n = 47$) Ca^{2+} peaks in the same period, as opposed to 1.19 ± 0.47 scored by the control eggs ($n = 36$, $P < 0.01$). The occurrence of the multiple Ca^{2+} peaks at fertilization was extremely sensitive to LAT-A, as the minimal dose to induce the phenomenon was as low as 1 nM. This supernumerary Ca^{2+} increases facilitated by LAT-A was somewhat reminiscent of the 'spontaneous' Ca^{2+} increase in *A. aranciacus* eggs bathed in the presence of LAT-A [18], but was clearly different from it. First, LAT-A alone could not induce Ca^{2+} increase without sperm in *P. lividus eggs*, and the similar experiment with LAT-A and a Ca^{2+}-ionophore produced only a single Ca^{2+} peak (data not shown). Thus, the multiple Ca^{2+} increase that usually start from different areas of the egg surface and develop into a partial wave are likely to represent multiple sperm interaction. Nonetheless, in the same

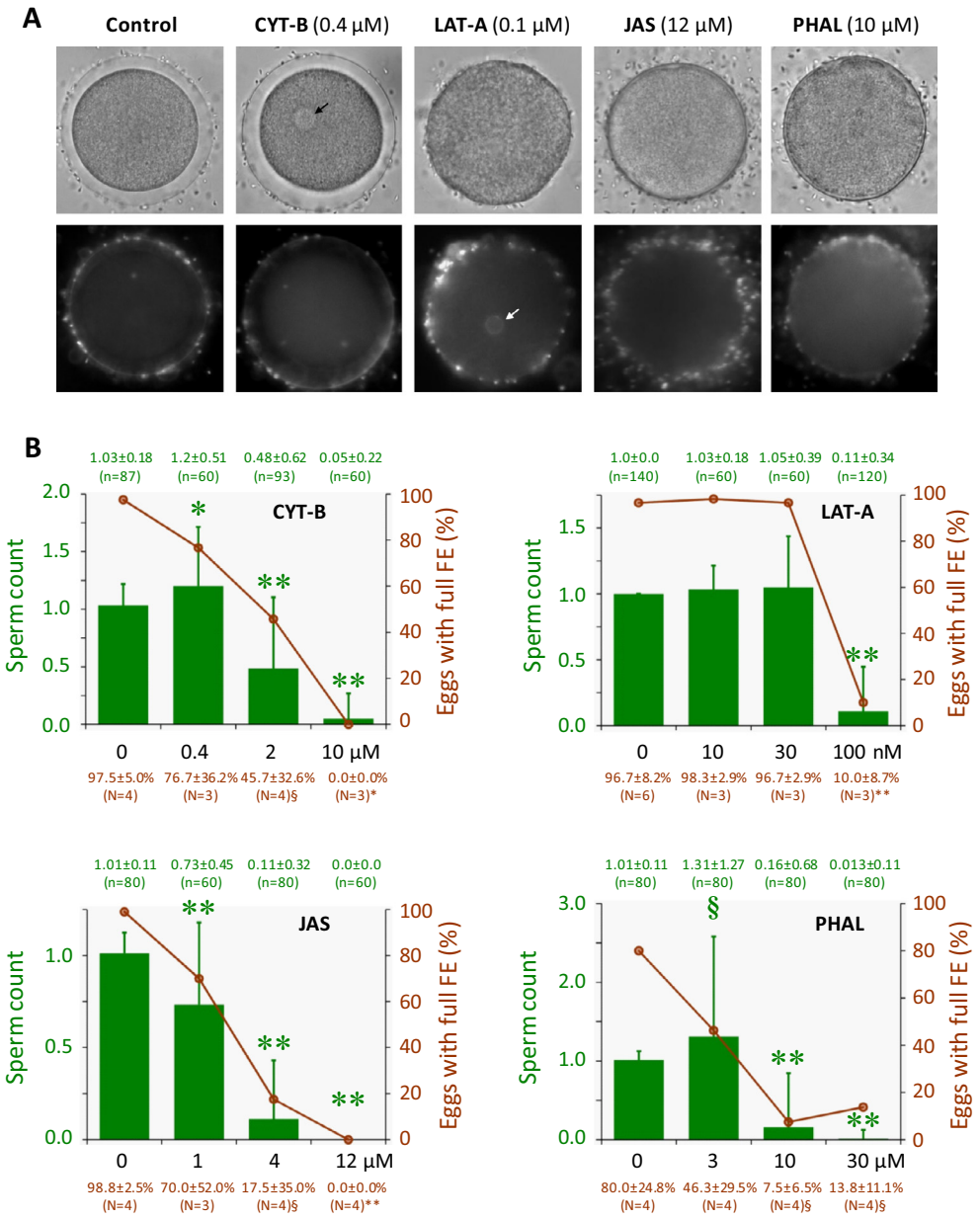

Fig. 4. Effects of the actin-binding drugs on sperm entry and elevation of the fertilization envelope. *P. lividus* eggs were treated with increasing doses of the actin-binding drugs and inseminated with Hoechst 33342-stained sperm, and their effects on fertilization was monitored by assessing the number of egg-incorporated sperm and the percentage of the eggs displaying full elevation of the FE. Incubation time: CYT-B, 10 min; LAT-A, 15 min; JAS, 20 min; PHAL, 20 min. (A) About 5 min after insemination, the elevation of the FE and the number of egg-incorporated sperm were respectively examined in the bright field view (top) and in epifluorescence microscopy (bottom). Arrows indicate egg pronuclei that happened to be in focus. (B) Graphic summaries of the dose-dependent effects of the actin-binding drugs on fertilization. The average frequency of the eggs displaying full elevation of the FE was presented in brown dots and lines, and the exact Mean ± SD and the number (*N*) of independent batches were indicated below the corresponding drug concentration. The average number of egg-incorporated sperm was calculated from the pooled data at the given dose of each drug, and was presented in green histogram. The exact Mean ± SD and the number of the eggs examined (*n*) were indicated above the corresponding bars of the histogram. The results indicating a significant difference from the values in the control were marked with special symbols: $^{\S}P < 0.05$, $^{*}P < 0.01$, $^{**}P < 0.0001$.

condition (100 nM LAT-A, 15 min), the eggs were hardly entered by spermatozoa (Fig. 4B).

The occurrence of the multiple Ca^{2+} peaks evoked by spermatozoa was not restricted to the eggs pretreated with actin-depolymerization drugs. We found that the eggs pretreated with JAS also produced 1.63 ± 1.10 Ca^{2+} peaks ($n = 24$), as opposed to 1.05 ± 0.21 peaks manifested by the control eggs ($n = 22$, $P < 0.05$). A similar trend was also observed in the PHAL-preinjected eggs (1.40 ± 0.56, $n = 30$) but the difference from the values in the DMSO-preinjected control eggs did not reach statistically significance (1.23 ± 0.51, $n = 26$; $P = 0.2026$). Thus, it appeared that the eggs pretreated with actin drugs were more permissive toward spermatozoa and let them trigger Ca^{2+} waves from multiple sites.

Although the global Ca^{2+} wave in the JAS-pretreated eggs was highly comparable to that of the control eggs in terms of its amplitude (Fig. 3A), the eggs treated in the same condition (12 μM, 20 min) was hardly entered by spermatozoa (Fig. 4B), whereas eggs displaying a single Ca^{2+} peak could occasionally incorporate multiple sperm (data not shown). Thus, an episode of Ca^{2+} wave at fertilization is neither necessary nor sufficient for individual sperm entry in sea urchin eggs, while the structural or functional status of the egg actin cytoskeleton was closely linked to the sperm

entry (Fig. 4). The universal failure at incorporating sperm displayed by the eggs pretreated with the actin drugs was not attributable to a potentially deleterious effect of the drugs on sperm motility because non-pretreated control eggs present in the same chamber were successfully fertilized amid the failing actin drug-pretreated eggs (data not shown).

The entry of supernumerary sperm in the eggs exhibiting normal elevation of the FE, together with the lack of sperm entry in the eggs with no FE elevation (Fig. 4A), implies that the formation of the FE might be of no practical service towards block to polyspermy [25]. Hence, monospermic fertilization in sea urchin eggs may be assured by a faster mechanism, as was first suggested by E.E. Just [20] and later postulated on the basis of a rapid depolarization of the egg membrane potential at fertilization [26]. While the exact mechanism of a fast block to polyspermy is still on the debate as was discussed in this issue [27] and elsewhere [28], the results of our study indicated that the exquisite coordination of the actin cytoskeleton is a prerequisite for proper sperm interaction (multiple Ca^{2+} peaks in LAT-A- and CYT-B-pretreated eggs) and incorporation, which might lead to regulation of a mechanism ensuring monospermic sperm entry (Fig. 4). In support of the idea that the actin cytoskeleton plays a dominating role in the control of monospermy independent of the FE, it was demonstrated that surf clam eggs, which do not substantially elevate the FE, became highly polyspermic at fertilization when they were pretreated with CYT-B [29].

Another important point in our study was that the JAS-pretreated eggs succeeded to elevate the FE in none of the 60 eggs examined (Fig. 4), although the same experimental condition (12 μM, 20 min) still produced a massive Ca^{2+} wave that is comparable with the control eggs (Fig. 3A). All other drugs manifested basically the same phenomena: a failure of triggering cortical granules exocytosis despite the fact that the cytosolic Ca^{2+} increase physically took place. Hence, it is evident that the increase of Ca^{2+} alone was not sufficient for the exocytosis of cortical granules at fertilization, but instead required an exquisite cooperation of the actin cytoskeleton, which corroborated our previous findings from starfish eggs [15–17,19].

In addressing general effects of the actin-binding drugs on the egg's Ca^{2+} response at fertilization, the amplitude of the cortical flash, which reflects the Ca^{2+} influx via voltage-gated Ca^{2+} channel, was selectively affected by the drugs stabilizing actin filaments (Fig. 3). On the other hand, the peak amplitude of the global Ca^{2+} wave was moderately repressed in the eggs pretreated with CYT-B and LAT-A by 9% and 26%, respectively, but was enhanced by 41% in PHAL-pretreated eggs. In contrast to PHAL, the JAS-pretreated eggs displayed virtually no significant changes in the peak amplitude of the Ca^{2+} waves in comparison with their control eggs (Fig. 3). These subtle differences between JAS and PHAL in their effects on Ca^{2+} signaling peak are difficult to explain since their exact influences on the actin filaments in specific subcellular loci around endoplasmic reticulum (ER) and in microvilli are largely unknown. Nonetheless, we found that both JAS and PHAL had a similar effect at the decay phase of the Ca^{2+} trajectories (Fig. 3). After being released from the stores, Ca^{2+} ions are to be buffered by Ca^{2+}-binding proteins or reabsorbed back to the intracellular stores, or even pumped out of the cell. Thus, after reaching the peak, the net Ca^{2+} mobilization is toward buffering or reuptake into the source, but we intriguingly found that the removal of the cytosolic Ca^{2+} came to an earlier saturation in the eggs pretreated with JAS and PHAL (Fig. 3). This phenomenon was not found in the eggs pretreated with actin-depolymerizing drugs, but was more evident in the eggs pretreated with PHAL (Fig. 3B) that displayed stronger hyperpolymerizing effects on the cortical actin (Fig. 1). The molecular mechanisms by which hyperpolymerization of cortical actin attenuated the Ca^{2+} buffering or reuptake in these eggs are only a matter of speculation. In view of the fact that ion channels and pumps on the plasma membrane and the ER are closely associated with the actin cytoskeleton, it is no surprise that the Ca^{2+} transport activities are modulated by the state of the local actin cytoskeleton that may alter the positioning or microenvironment of the Ca^{2+}-mobilizing channel components [30–34]. By the same token, it is conceivable that hyperpolymerization of cortical actin by PHAL and JAS might have negatively affected the Ca^{2+}-reuptake activities of the Ca^{2+}-ATPases on the ER or on the plasma membrane and thereby led to an elevated plateau of the residual Ca^{2+} level (Fig. 4). Indeed, it has been shown that treatment of hippocampal neurons with JAS synergizes thapsigargin that increases cytosolic Ca^{2+} by primarily inhibiting the Ca^{2+} uptake through the ER Ca^{2+}-ATPase [35]. Alternatively, the elevated plateau at the decaying phase of Ca^{2+} response might have happened because the monomeric actin pools in the cytoplasm were depleted due to the hyperpolymerization. Binding of Ca^{2+} by G-actin and its incorporation into actin filaments could serve as a supplemental mechanism to absorb Ca^{2+} and alleviate its local surge [19,36–39]. Thus, overstabilization of the actin filaments by PHAL and JAS might have repressed the Ca^{2+}-removal process by suppressing treadmilling. Nonetheless, it bears an emphasis that the functional mechanisms and the effects of the actin drugs are highly intricate, and that it is difficult to explain all our observations solely based on the direction of actin polymerization and depolymerization. Hence, the question of how the actin cytoskeleton affects the biological process of Ca^{2+} signaling and sperm entry awaits further investigation in various model systems.

Finally, we found that the time span for the Ca^{2+} wave to travel from the sperm interaction site to the antipode was significantly increased not only by CYT-B or LAT-A (see Results), but also by PHAL and JAS. The 'traverse time' in the PHAL-treated eggs was 20.3 ± 2.9 s ($n = 29$) as opposed to 16.8 ± 2.0 s of the control eggs ($n = 25$, $P < 0.0001$). Likewise, the traverse time in the JAS-pretreated eggs was much longer (25.6 ± 3.3 s, $n = 15$) than in their control eggs (19.8 ± 2.8 s, $n = 22$; $P < 0.0001$). Thus, the planar speed of the Ca^{2+} wave in the PHAL- and JAS-pretreated eggs at fertilization was also reduced by 17% and 23%, respectively, in comparison with their corresponding control eggs. Taken together, our results in sea urchin eggs again indicated that the actin cytoskeleton plays comprehensive roles at the early stages of egg activation by setting the tone for the excitability of the subplasmalemmal and cytoplasmic matrix and thereby contributes to sperm receptivity at fertilization.

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