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Separate Activation of the Cytoplasmic and Nuclear Calcium Pools in Maturing Starfish Oocytes

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The dynamics of the cytoplasmic and nuclear Ca^{2+} pools in starfish oocytes arrested at the prophase of the first meiotic division or after induction of meiosis by 1-methyladenine (1-MA) have been studied by confocal microscopy. A 70 kDa fluorescent Ca^{2+} indicator has been injected in either the cytoplasm or the nucleus, and shown to remain restricted to the compartment of injection. 1-MA induced a first Ca^{2+} transient in the cytosol, followed by a nuclear transient, and eventually by a second cytosolic transient. The latter failed to occur if the nuclear peak was suppressed. This required the nuclear injection of antagonists of the inositol 1,4,5-trisphosphate ($InsP_3$) and cyclic-ADPribose (cADPr) Ca^{2+} channels, showing that both channel types were active in the inner envelope membrane. The nuclear injection of the Ca^{2+} channel antagonists affected the process of meiosis reinitiation: in about one third of the injected oocytes no breakdown of the nuclear envelope (GVBD) was observed. In the others, even if GVBD eventually occurred, the intermixing of the nucleoplasm and cytoplasm was inhibited. © 1998 Academic Press

Key Words: **InsP3; cADPr; nucleus; maturation; starfish oocyte.**

The oocytes of several starfish species, including *Asterina pectinifera*, become arrested at the prophase of the first meiotic division, at which stage they contain a very large nucleus (germinal vesicle, 60 μm across). In 1969, Kanatani et al. (1) discovered that 1-methyladenine (1-MA), a hormone secreted by the follicle cells surrounding the oocytes, promoted the reinitiation of the meitotic cycle (maturation). 1-MA recognizes a plasma membrane receptor which interacts with a Pertussis-toxin sensitive heterotrimeric G-protein (2,3). The signalling cascade activated by 1-MA involves several kinases including the M-phase promoting factor (MPF) which induces the breakdown of the germinal vesicle (GVBD), entry into metaphase, and, eventually, the two meiotic divisions (4). The search for the second messenger that mediates the 1-MA signal has frequently focussed on Ca^{2+}, but the results have been controversial. While Moreau et al. (5) observed light emission in starfish oocytes injected with the Ca^{2+} sensitive photoprotein aequorin less than 2 seconds after the addition of 1-MA, others failed to detect changes in cell Ca^{2+}, or concluded that these changes were not necessary, since maturation could be elicited in situations where no calcium transient occurred (6), or in the presence of intracellular calcium chelators (7,8). More recent work in a number of laboratories, however, has shown that both inositol 1,4,5-trisphosphate ($InsP_3$) and ryanodine receptors can be stimulated to release endoplasmic reticulum (ER) Ca^{2+} during maturation (9–10), a finding that may be related to the observation that 1-MA induces structural changes in the ER (11). Recently, work in our laboratory has detected increases in both cytoplasmic and nuclear Ca^{2+} following the treatment with 1-MA. The clamping of Ca^{2+} in the latter compartment, by injecting BAPTA directly into the nucleus, was found to block the reinitiation of meiosis. This had been performed on oocytes injected with fura-2, and its changes in fluorescence were followed with a photomultiplier: we have thus decided to perform similar experiments using confocal microscopy, injecting the cytoplasm or the nucleus of prophase arrested starfish oocytes with the Ca^{2+} indicator Oregon Green BAPTA-1 coupled to 70 kDa dextran (OGBD). The results have documented separate changes in the cytoplasmic and nuclear Ca^{2+} pools induced by 1-MA. They have also shown that injection of the inhibitors of the $InsP_3$ and cyclic-

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Abbreviations: 1-Ma, 1-methyladenine; $InsP_3$, inositol 1,4,5-trisphosphate; cADPr , cyclic-ADPribose; MPF, M-phase promoting factor; GVBD, germinal vesicle breakdown; ER, endoplasmic reticulum; OGBD, Oregon Green BAPTA-1, dextran, 70 kDa; NSW, natural sea water.

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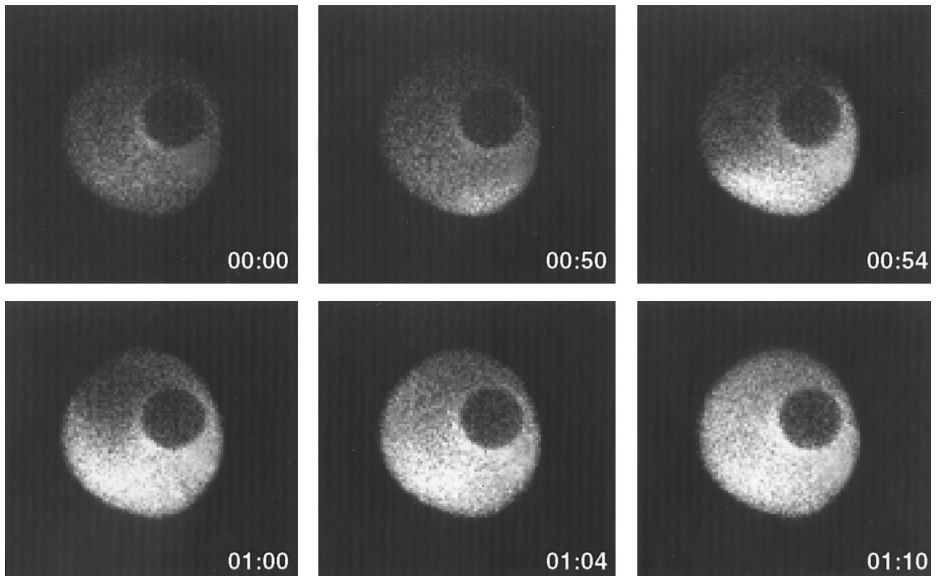

FIG. 1. Confocal scanning laser images of an oocytes in which OGBD has been injected into the cytoplasm. The dark nucleus is visible at the animal pole. 50 s after the addition of 1-MA an increase of cytosolic Ca^{2+} is detected at the site of action of 1-MA. The Ca^{2+} wave propagates in the central region of the oocyte and to the opposite pole in 20 s.

ADPribose (cADPr)-sensitive Ca^{2+} channels heparin and $8NH_2$cADPr directly into the nucleus blocked the GVBD induced by 1-MA in about one third of the oocytes.

MATERIALS AND METHODS

Preparation of gametes. *Asterina pectinifera* oocytes were collected from the Mutsu Bay near the Asamushi Marine Biological Station (Japan) during the breading season and kept in running sea water. Prophase-I arrested cells dissected from the ovaries in natural sea water (NSW) were washed several times in filtered sea water and kept in it for 30 min prior to use. Oocytes in which the GVBD spontaneously occurred during this period were discarded.

Microinjection procedures. The cytoplasm or the nucleus of the arrested oocytes were injected with a micropipette containing 5 mg/ml Oregon Green BAPTA-1 coupled to 70 kDa dextran (OGBD) (Molecular Probes, Eugene, OR) in 100 mM potassium aspartate, 10 mM Hepes, pH 7.0. The injections were performed with a micromanipulator (Leitz) and capillary pipettes according to the procedure of Hiramoto, (12). The injected volume was 1% of the oocyte and the final concentration was about 50 μg/ml in the injected compartment. When the Ca^{2+} channels were injected, their final concentration in the nucleus was 100-200 μg/ml heparin, and 30-100 μM of $8NH_2$cADPr, respectively.

Ca^{2+} measurements. The cytoplasmic or nuclear Ca^{2+} changes following hormonal treatment were assessed at 2 s intervals after the addition of 1-MA with an (LSM 410 Zeiss) inverted microscope on oocytes pre-injected in the cytoplasm or the nucleus, as indicated above. The changes of fluorescence were measured with a 20X objective and 488 nm excitation and 515 nm emission filters. The images were recorded on a Jaz, 1GB (Iomega) and analyzed using Metamorph software (Universal Imaging Corporation, West Chester, PA). The data were expressed as relative fluorescence intensity using a Microsoft Excel 97 program.

RESULTS AND DISCUSSION

In a first series of experiments OGBD was microinjected into the cytoplasm of prophase arrested oocytes, i.e. at a stage when the nucleus was well visible at the animal pole of the cell. The large size of the dextran molecule (70 kDa) slowed the diffusion of the Ca^{2+} indicator, which remained restricted to the region of the cytoplasm where the injection was performed. Figure 1 shows the dark nucleus embedded in the fluorescent cytoplasm. Only 50 s after the addition of 1-MA into the oocyte an increase of fluorescence could be detected at a site which presumably was the selective point of interaction of the hormone. The Ca^{2+} increase wave then spread, and 4 s later it had diffused to the central region of the cell and then gradually to the opposite pole. Interestingly, the diffusion of the Ca^{2+} wave during maturation was significantly faster (20 s) than that of the Ca^{2+} wave at fertilization (80 s). The peak amplitude and the time of propagation of the Ca^{2+} wave induced by 1-MA were calculated as relative fluorescence intensity measured in the central region of the cell. The graph in Figure 2 shows the changes 1 min after the addition of the hormone. A first cytoplasmic Ca^{2+} transient reached a relative fluorescence of about 1.6 (see the graph). After an abortive decay, a second, damped transient ensued leaving in the end a somewhat more elevated deadline than at the start of the experiment. The response differed somewhat from that observed in a previous report from our laboratory (13) in which the hormone triggered a sharp cytoplasmic transient which decayed relatively rapidly and more completely, before the onset of the second

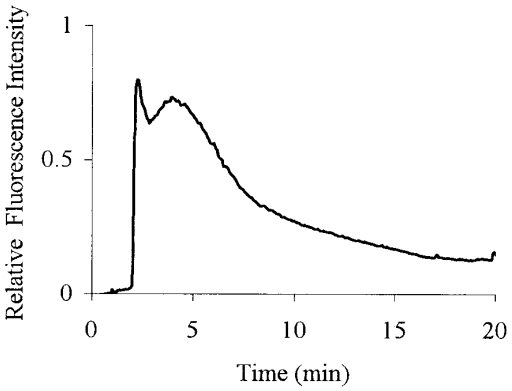

FIG. 2. Ca^{2+} release in starfish oocytes injected with OGBD into the cytoplasm. The data on the Ca^{2+} changes recorded with the confocal microscope were calculated as relative fluorescence intensity. Two Ca^{2+} elevation are detected following the hormonal stimulation.

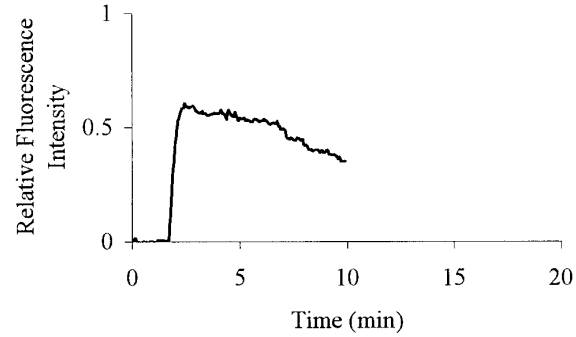

FIG. 4. Relative fluorescence intensity of the nuclear Ca^{2+} increase induced by the 1-MA, showing a delay in its decay.

cytosolic transient. The differences in kinetics may have been due to the large size of the fluorescent dye used in the present work, since the cytosolic injection of OGBD 10 kDa induced the same kinetics of the Ca^{2+} transients which had been previously observed using fura-2 (data not shown) (13). The confocal microscopy observations thus confirm the previous observation (13–16) that an increase in the nuclear Ca^{2+} pool always followed the first cytosolic Ca^{2+} transient. Figure 3, which shows the fluorescence in the nucleus after OGBD was injected into it, clearly shows that the dye remained exclusively localized in the nucleus, i.e. it failed to diffuse to the cytoplasm. In the oocyte used for the experiment, the Ca^{2+} increase in the nucleus became evident 70 s after the addition of 1-MA, reaching a peak in a few seconds and remaining elevated for the 10 minutes during which the images were recorded. Figure 4 graphically confirms that the increase in nuclear Ca^{2+} indeed failed to decay. Again, the failure to decay was at variance with the results of the earlier experiments (13). The reasons for the difference are not completely understood and may be manifold, but the explanation offered above appears plausible.

Ca^{2+} transients in the cytosol of prophase arrested starfish oocytes have been elicited by $InsP_3$, ryanodine, and cADPR (9,10,17). $InsP_3$ and cADPr-sensitive Ca^{2+} channels/stores have also been documented in the inner nuclear membrane of these oocytes. Nuclear microinjection experiments have shown that these channels could be induced to liberate Ca^{2+} stored in the envelope by their agonists (18). Since both channels became activated in response to 1-MA, they evidently are the end target of the signalling pathway initiated by the hormone (18). A previous contribution from our laboratory had found that the two cytoplasmic and the nuclear Ca^{2+} transients were causally related (13), since the blockade of the nuclear Ca^{2+} increase by clamping Ca^{2+} in the nucleus to very low concentrations abolished the second cytoplasmic transient, leaving the first unaffected. In this report, the matter has been re-investigated by co-injecting heparin (final concentration after the injection of 100-200 μg/ml) and $8NH_2cADPr$ (final concentration, 30-100 μM) into the nucleus. Figure 5 shows that the first cytoplasmic transient after the 1-MA challenge still occurred, albeit with a peak amplitude smaller than that in the control. By contrast, in confirmation of the earlier results mentioned above, the second cytoplasmic transient was completely suppressed, as was, of course, the nuclear transient. This clearly indicates that a relationship exists between the nuclear Ca^{2+} increase and the second cytoplasmic transient. In addition, the nuclear coinjection of the two antagonists reduced the baseline level of Ca^{2+} after the first cytoplasmic transient to the

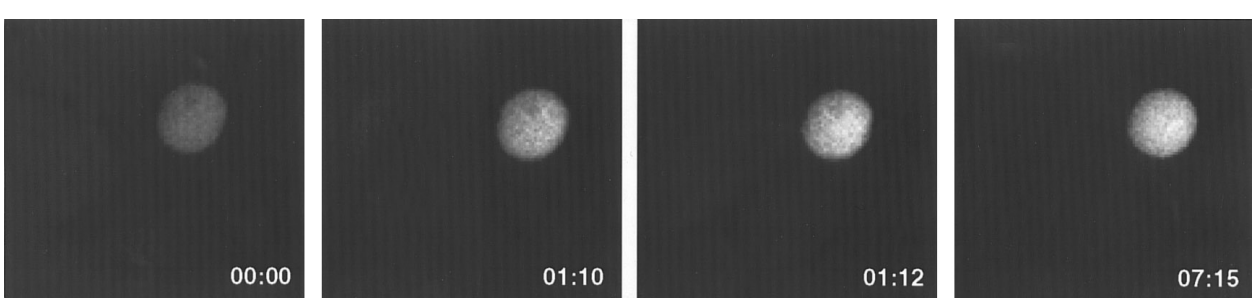

FIG. 3. Confocal images of the changes in the nuclear Ca^{2+} following the 1-MA treatment. A rapid increase in the nuclear Ca^{2+} is observed which remains elevated for the entire time (10 min) necessary to record the images.

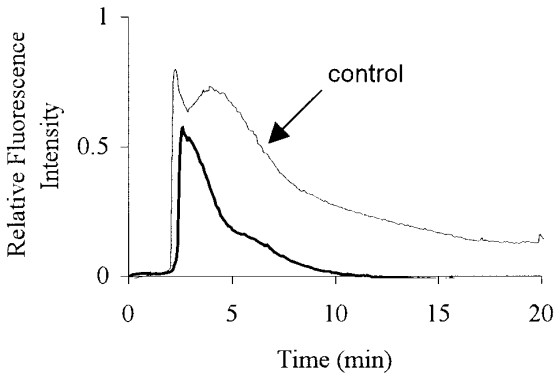

FIG. 5. Ca^{2+} release in starfish oocytes injected with heparin and $8NH_2cADPr$ into the nucleus. The addition of the 1-MA induced an inhibition of the second cytoplasmic Ca^{2+} peak.

value observed before the treatment with 1-MA, whereas (see above) in the controls the second cytoplasmic transient decayed to a value higher than the resting baseline level.

Thus, $InsP_3$ and cADPr-sensitive Ca^{2+} channels apparently become activated within the nucleus during the hormonal stimulation, and are essential to the production of the second cytoplasmic peak prior to the reinitiation of meiosis. It was logical at this point to explore whether the injection of the Ca^{2+} antagonists into the nucleus influenced (i.e., abolished or delayed) the GVBD induced by 1-MA. Therefore, the two antagonists were injected into the nucleus of 44 oocytes. 60 min after the treatment with 1-MA, i.e. at a time when GVBD had occurred in all the controls, 13 oocytes still had an intact nucleus at the animal pole. In the remainder of the injected cells the nuclear membranes were no longer visible at this time, but the nucleoplasm still remained located in the same area of the cell. Somehow, then, the intermixing between the nucleoplasm and the cytoplasm had failed to occur. The finding that the blockade of the two nuclear Ca^{2+} channels only inhibited the full completion of GVBD in a fraction of the oocytes may suggest that additional Ca^{2+} signalling pathways (nuclear or otherwise) could be involved in the signal transduction cascade which eventually induces the dissolution of the nucleus.

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