Calcium Transients during Early Development in Single Starfish (Asterias forbesi) Oocytes

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ABSTRACT Maturation and fertilization of the starfish oocyte are putative calcium-dependent events. We have investigated the spatial distribution and temporal dynamics of this calcium dependence in single oocytes of Asterias forbesi. We used the calcium photoprotein, aequorin, in conjunction with a microscope-photomultiplier and microscope-image intensifier. Surprisingly, in contrast to earlier work with Marasthenias glacialis, there is no detectable increase in intracellular-free calcium in the oocyte of A. forbesi in response to the maturation hormone 1-methyl adenine. During fertilization of the same, matured, A. forbesi oocyte there is a large increase in intracellular-free calcium. The calcium concentration increases to ~1 μM at the point of insemination and the region of elevated free calcium expands across the oocyte in ~20 s (17-19°C). After the entire oocyte reaches an elevated concentration of free calcium, the concentration decreases uniformly throughout the oocyte over the next several minutes.

Two processes during the early development of starfish oocytes have been associated with and are presumably dependent upon an increase in intracellular-free calcium. These are maturation of the oocyte in response to the hormone, 1-methyl adenine, and fertilization by sperm (2, 3, 10, 13, 14). With each there is reported or presumed to be a large increase in the concentration of free calcium from less than 0.01 to ~1 μM (14).

Of particular interest to many investigators is how these two processes can be kept physiologically separated. That is, the large calcium increase at maturation does not lead to the events of fertilization which are presumably calcium dependent (19). Likewise the large calcium increase at fertilization (measured in sea urchins and presumed to occur in the starfish) does not lead to the events of maturation. How are these processes kept separated? It is not simply a sequential change because fertilization of an oocyte can precede maturation of the oocyte (19). It has been speculated that the two processes could be physically separated within the cell (13, 14). It has also been argued that maturation may not be dependent upon an increase in the free calcium concentration (12, 15).

Image intensification microscopy in conjunction with microinjection of the luminescent, calcium-specific photoprotein, aequorin, provides an ideal way to examine the spatial distribution and the temporal dynamics of any calcium dependence that might exist (1, 18, 20, 21). This brief communication reports work in which single oocytes form the starfish Asterias forbesi were injected with a high photon-yielding acetylated aequorin (21), subsequently matured with 1-methyl adenine, and then fertilized with sperm. The calcium-aequorin luminescence was then measured with a microscope-photomultiplier or visualized with a microscope-image intensifier-SIT video system (16-18).

Surprisingly, and in contradistinction to previously reported work using aequorin-injected oocytes of the starfish Marasthenias glacialis, (14) we find no change in the intracellular-free calcium upon maturation of the aequorin-injected oocytes of A. forbesi in response to 1-methyl adenine. On the other hand, we find a large increase in the intracellular-free calcium upon fertilization of the same, matured oocyte. The region of elevated free calcium expands from the point of insemination across the oocyte until the entire oocyte is luminescing uniformly. The expansion occurs over ~20 s. The subsequent decay in the luminescence is uniform and occurs over several minutes (5).

MATERIALS AND METHODS

Gametes: Gonads were removed from the arms of ripe A. forbesi obtained at Woods Hole, MA, from mid June to mid July, 1983. The testes were placed in sealed dishes on ice until used. At that time a small amount of dry sperm was diluted ×1,000 with filtered sea water and used directly. The ovaries were removed to a dish of Ca- and Mg-free Marine Biological Laboratory artificial sea water. The extruded, follicle-free oocytes were collected with a Pasteur pipette and washed two times by settling and resuspension in filtered sea water. Room temperature was kept at 16-19°C for work with A. forbesi.
Aequorin Microinjection: Acetylated aequorin was the gift of Dr. O. Shimomura (21). The protein was initially in a dilute TRIS buffer at 1 mg/ml. The stock was lyophilized, resuspended in 10 mM HEPES, 0.2 mM EGTA, pH 7.0 at 10 mg/ml, and dialyzed on ice against this buffer to remove the TRIS. It has been our experience that microinjection of a HEPES-buffered solution is better tolerated by echinoderm eggs than solutions buffered by TRIS or PIPES (4). In addition, we find that inclusion of the EGTA prevents accidental discharge of the aequorin and improves the light output over the chlorinator-free preparations (4). Oocytes were immobilized, slightly compressed between parallel coverslips in a chamber containing filtered sea water. The cells were pressure injected with the aequorin solution with a volume equal to 3% of the cell volume. See the paper by Kiehart for details of the method (9).

Luminescence Detection: Single aequorin-injected oocytes were observed with either a Zeiss ×25/0.8 NA oil immersion or a Leitz ×50/1.0 NA water immersion objective in the apparatus depicted in Fig. 1. The apparatus has been described in detail by Reynolds (16–18). Using the native aequorin this apparatus can easily detect the calcium-aequorin luminescence from 0.1 μM free calcium (4). In our hands, under comparable conditions, the luminescence produced by the acetylated aequorin is 3–10 times greater than the luminescence from the native protein.

During each experiment a stock solution of 1-methyl adenine (1 mg/ml in filtered sea water) was added to the cell chamber through a capillary tube inserted into the chamber while the oocyte was under continuous observation. The final concentration of 1-methyl adenine was 150 μM. Maturation was confirmed by the observation of germinal vesicle breakdown. The diluted sperm suspension was similarly added to the chamber 15 min after the induction of maturation.

The light from the intracellular calcium-aequorin luminescence was directed to either (a) an EMI 6256SA photomultiplier (PMT)7 with a very low dark current of 12 pA when operated at −1,100 V at room temperature; or (b) an EMI four-stage, magnetically focused image intensifier tube (IIT). The output of the PMT was amplified by a Keithley electrometer and recorded on a Gould strip chart recorder. The output of the IIT was observed with a Dage silicon intensified target (SIT) video camera and recorded on a Sony time-lapse video recorder in real time. The sequence presented in Fig. 3 was photographed from the video monitor during playback of the real-time record. Exposure times were 1 s each with ~1/2 s between exposures to accommodate the shutter release and automatic advancement of the film.

The temporal dynamics can also be determined from the video record by measuring the brightness of the video image with a PMT. This was accomplished using an RCA 6655 PMT operated at −100 V, amplified by a Keithley electrometer, and recorded on a Gould strip chart recorder.

RESULTS

When 1-methyl adenine was added to the experimental preparation the oocytes, uninjected or injected with aequorin, underwent maturation. The germinal vesicle broke down in 9–10 min following the application of the hormone and was completely gone by ~15 min. Subsequently the meiotic divisions commenced and the polar bodies were extruded. Typically we added sperm to the preparation at 15 min after the addition of 1-methyl adenine. Both the uninjected and the injected eggs fertilized and elevated fertilization membranes. However the aequorin-injected eggs were arrested midway through the first mitotic division. The etiology of this developmental arrest was unknown. In this report we concentrated on the earliest events of development in the aequorin-injected oocyte as these occurred with identical timing and morphologic changes as seen in the uninjected controls. We have complete records (both maturation and fertilization) from six oocytes: PMT and IIT records from three each.

We found no change in the intracellular-free calcium that we could detect in response to 1-methyl adenine under continuous observation for as long as 5 min. This is in contrast to other investigators working with oocytes from another species of starfish, also injected with aequorin, who found a large (~21 μM) increase in intracellular-free calcium within 2 s of applying 1-methyl adenine (14).

1 Abbreviations used in this paper: IIT, image intensifier; PMT, photomultiplier.

Figure 1: System schematic. The experimental system consists of a microscope with a DC-regulated Hg-arc epifluorescence (EPI) attachment, low noise/high gain EMI photomultiplier tube (PMT), image intensifier tube (IIT), Dage silicon intensified target (SIT) vidicon, and with various amplifiers (AMP) and recording devices to obtain temporal and spatial records of the fluorescent or luminescent changes occurring within a single oocyte/egg. On occasions the membrane potential of the cell was also measured with a KCl-filled glass microelectrode.

Figure 2: Photomultiplier records. The two traces show the measurement of the calcium-aequorin luminescence from a single aequorin injected A. forbesi oocyte in response to 1-methyl adenine (A) and, subsequently, to fertilization with sperm (B). The arrows in A indicate the insertion of the capillary tube containing the hormone into the preparation and the completion of the hormone addition. Horizontal bar, 12.5 s; vertical bar, 25 pA.

However if the matured oocyte was then fertilized by sperm we detected a large luminescence signal resulting from the increase in intracellular-free calcium. The PMT record of the temporal changes in the luminescence from a single aequorin-injected oocyte is presented in Fig. 2. An IIT record of both the spatial and temporal changes in the luminescence from a single aequorin-injected oocyte at fertilization is presented in Fig. 3.

The signal at fertilization reached its peak in 15–25 s, maintained this peak value for 5–10 s, and then decayed over the next 100–300 s. Observations with the IIT showed that the luminescence was detected first at the site of insemination and that the region of luminescence spreads across the egg until the entire egg was luminescing. The subsequent decay appeared to be uniform throughout the egg.

The luminescence was relatively dim and was a photon-limited process. For this reason the IIT-SIT video records
FIGURE 3 Image intensifier records. This figure presents photographs taken of the video monitor during playback of the real-time record of the calcium-aequorin luminescence during fertilization of a single, matured *A. forbesi* oocyte. Each spot is produced from a single photon originating from within the aequorin-injected oocyte and spatially focused by the microscope objective. Across each row there are five sequential images integrating the video output for 1 s each. Approximately 1/3–1/2 s separates each image. The sequence is continuous through the figure. The final image (g) is of the fertilized oocyte taken through the system with very dim background illumination to locate the oocyte in the field of view. Bar, 50 μm. × 200.

appeared speckled and were not continuous in intensity. Each spot in Fig. 3 is the consequence of a single photon generated by the intracellular calcium-aequorin reaction. The end point of the expansion can be determined from a PMT analysis of the video image.

The brightness of the video image is proportional to the number of spots that comprises the image. With a PMT to measure the brightness of the image on the video monitor we have generated a dynamic record from the video record. The curve of intensity vs. time derived from the video monitor is similar to that directly obtained with a PMT. This is presented in Fig. 4. For a uniform, nongrowing, but merely expanding region of luminescence the time to reach the peak luminescence represents the propagation time. For the oocyte depicted in Fig. 3 such is the case and the propagation time obtained from Fig. 4 is 21 s.

The oocyte from which Fig. 3 was obtained is seen in brightfield micrographs in Fig. 5 before activation (a), after activation with 1-methyl adenine (b), and after fertilization (c). The oocyte is compressed to a slightly larger degree in b and c and therefore has a slightly larger diameter than in a.

DISCUSSION

The work described in this report was undertaken to resolve certain paradoxical findings with regard to the role of calcium during the early development of starfish oocytes. In particular we sought an explanation for how two processes with a putative calcium dependence, maturation, and fertilization, could be separately regulated within the same cell. The method that we chose to examine the putative calcium dependence relied upon a sensitive and specific luminescent indicator of the level of free calcium, aequorin, used in
Our studies were of single aequorin-injected oocytes, the fate of which could be precisely known. During maturation and confirm the heretofore untested assumptions about fertilization. Specifically, we find the full extent of the luminescence is attained in the first image of row D in Fig. 3. Bar, 12.5 s.

The results of this study differ significantly with the results of other studies during maturation and confirm and restrict the presumed calcium dependence of maturation with 1-methyl adenine, the fate of which could be precisely known.

The findings of this study seem to resolve the paradoxical and otherwise hard to explain separation of putative calcium-dependent events of early development in the starfish oocyte. They refute the purported calcium dependence of maturation and confirm and restrict the presumed calcium dependence to fertilization alone. These findings pose new challenges to uncover the effector of maturation with 1-methyl adenine, explain the function of oocyte calmodulin and the effects of calmodulin inhibitors on meiosis and fertilization.
and to explore further the role of calcium in the process of fertilization.

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