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

ANDREW EISEN and GEORGE T. REYNOLDS
The Marine Biological Laboratory, Woods Hole Massachusetts 02543: The Children's Hospital of Philadelphia, Pennsylvania 19104; The Physics Department of Princeton, Princeton, NJ 08544

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 preceed 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*.

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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 sample was lyophylized, 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 chelator-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-aerquorin 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-methyladenine 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)[1] 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 ~⅓–½ s between exposures to accomodate 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 mid-way 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.

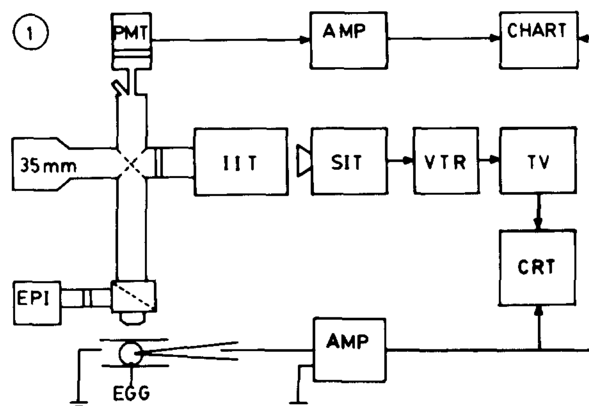

FIGURE 1 System schematic. The experimental system consists of a microscope with a DC-regulated Hg-arc epifluorescence (*EPI*) attachment, low noise/high gain EMI photomultiplier tube (*PMT*), image intensifier tube (*IIT*), Dage silicon intensified target (*SIT*) vidicon, and with various amplifiers (*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.

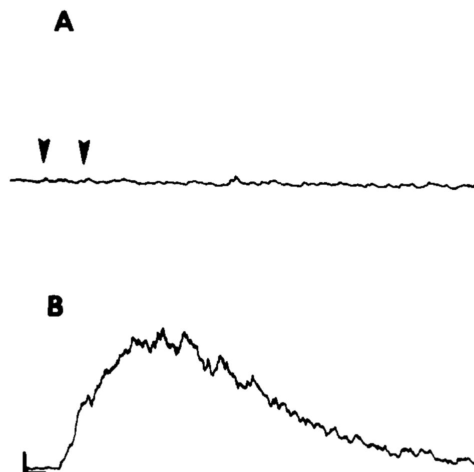

FIGURE 2 Photomultiplier records. The two traces show the measurement of the calcium-aequorin luminescence from a single aequorin injected *A. forbesi* oocyte in response to 1-methyl adenine (*A*) and, subsequently, to fertilization with sperm (*B*). The arrows in *A* indicate the insertion of the capillary 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

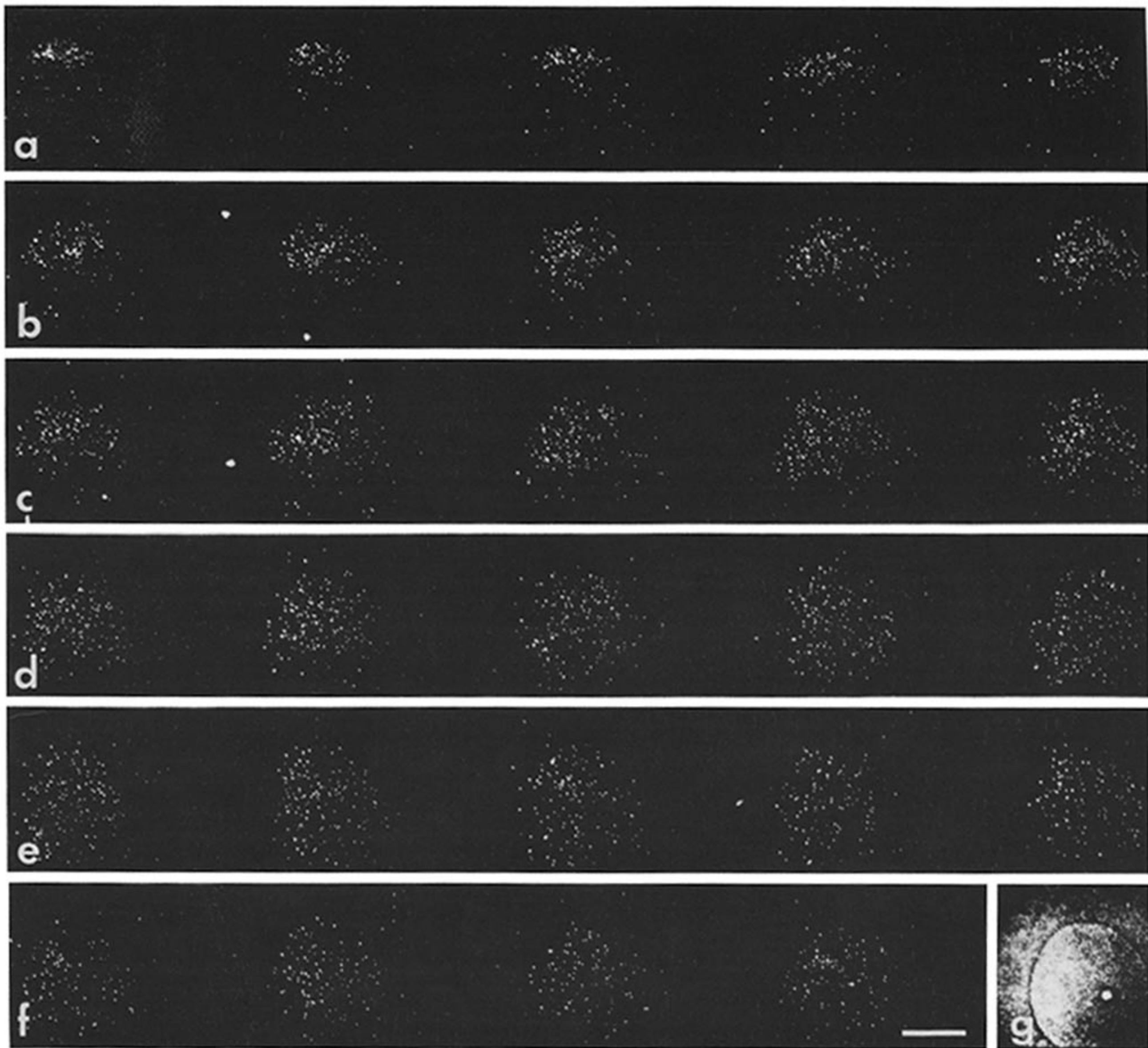

FIGURE 3 Image intensifier records. This figure presents photographs taken of the video monitor during playback of the real-time record of the calcium-aequorin luminescence during fertilization of a single, matured *A. forbesi* oocyte. Each spot is produced from a single photon originating from within 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 ⅓–½ 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

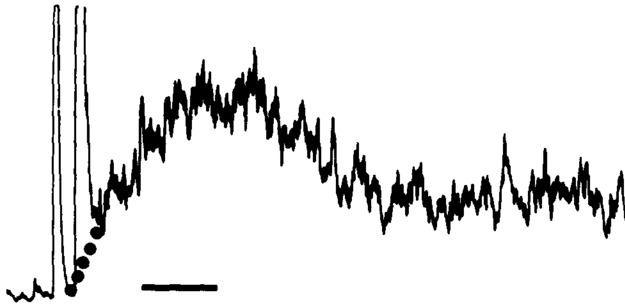

FIGURE 4 Video analysis. The brightness of the video image due to the calcium-aequoriun luminescence from the oocyte presented in Fig. 3 during fertilization is recorded (on an arbitrary scale) as a function of time. The spikes are video artifact and the dots extrapolate the signal to its origin. The record is similar in nature to Fig. 2*B*. The time from the onset to the peak of the signal is 21 s. This suggests that the full extent of the luminescence is attained in the first image of row *D* in Fig. 3. Bar, 12.5 s.

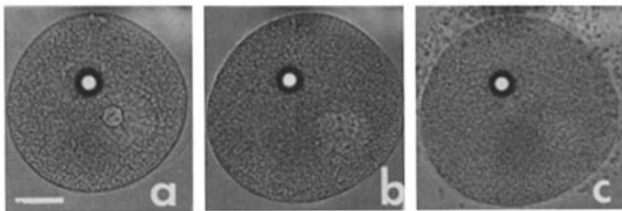

FIGURE 5 Oocyte morphology. The oocyte from which the video record in Fig. 3 was produced is seen in these bright field micrographs. The oocyte after microinjection and before maturation is seen in *A*. The germinal vesicle is intact. The oil droplet accompanied the microinjection of the aequorin by the Hiramoto method. The oocyte after the application of 1-methyl adenine and the dissolution of the germinal vesicle is seen in *B*. The oocyte after fertilization is seen in *C*. Bar, 50 μm. × 140.

conjunction with image intensification microscopy. In addition our studies were of single aequorin-injected *A. forbesi* oocytes, the fate of which could be precisely known.

The results of this study differ significantly with the results of other studies during maturation and confirm the heretofore untested assumptions about fertilization. Specifically, we find in single oocytes from *A. forbesi* that there is no change in the level of free calcium during maturation induced by the natural hormone 1-methyl adenine but there is a large increase in the level of free calcium as a consequence of fertilization by sperm.

Several important methodological differences exist between this study and others with regard to the findings during maturation. In this study, where no calcium transient was detected during maturation, oocytes from *A. forbesi* were used. In other studies, where a large calcium transient was detected during maturation, oocytes from *M. glacialis* were used (14). Species differences alone would be a remarkable cause for the discrepancy as 1-methyl adenine has been found to be a universal maturation hormone for starfish oocytes. It seems unlikely that there could be so radical a difference as no calcium transient vs. a calcium transient for the mechanism of such a universal hormone. Nonetheless each group of investigators should consider investigating oocytes from the other species. In addition to species differences, technical factors should also be considered as the source of these differing observations. Here two points of view should be considered: could we have failed to detect a change in the level of free calcium during maturation or could the previously detected changes have been artifactual?

It seems unlikely that we could have failed to detect a rise in the intracellular-free calcium at maturation if, as it has been reported, the level is 1 μM. This is at least one order of magnitude greater than our level of sensitivity using the microscope-PMT (4). Furthermore a smaller rise in the level of free calcium or a restricted region of elevated free calcium within the oocyte would not escape detection of the microscope-IIT-SIT video system. This is by virtue of the high signal-to-noise characteristics of this effectively photon-counting area detector.

On the other hand we have some reservations with technical aspects of the studies in which an increase in the free calcium at maturation has been reported (2, 14). Either the increase has been detected from a population of aequorin-injected oocytes, from a single oocyte that is simultaneously impaled with a micropipette, or an isolated cell cortex. In the first situation the origin of the signal is unknown and a single injured oocyte could yield a large luminescent response. In the second situation the fragile oocyte is at risk for continued mechanical injury especially during the addition of the activating hormone. If in the second situation the oocyte were under observation with a microscope-IIT video system it might show a localized response at the site of impalement. The third situation is far from physiologic.

We are convinced that the absent response during maturation of the *A. forbesi* oocyte is meaningful. This is because this same oocyte can be fertilized and that a change in the level of intracellular-free calcium is detected. Furthermore, this response at fertilization in the starfish oocyte is very similar to the response at fertilization which has been seen in a number of other echinoderm eggs—principally those from sea urchins (4, 6, 8). As with the sea urchin egg, fertilization of the starfish oocyte results in an increase in the free calcium to ~1 μM beginning at the site of insemination which then expands across the oocyte (6).

There are some subtle differences between the calcium transient which occurs at fertilization in the starfish oocyte and the sea urchin egg. In the starfish oocyte the region of elevated free calcium expands slowly. The calcium-aequorin luminescence reaches its peak value when the entire oocyte attains a uniform elevated level of free calcium. The sea urchin egg expands quickly and then the calcium-aequorin luminescence increases to its peak value (6, 8). Subsequently and similarly to the sea urchin egg the level of free calcium in the starfish oocyte decays uniformly throughout the oocyte. The expansion of the region of elevated-free calcium and its subsequent decay at fertilization in both the starfish oocyte and the sea urchin egg differ appreciably from the response seen in the egg of the freshwater fish, medaka (7). Thus, while the release of intracellular free calcium seems to be a common feature of fertilization, the fine details of that release are variable.

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 (11, 12),

and to explore further the role of calcium in the process of fertilization.

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