

Demonstration of Calcium Uptake and Release by Sea Urchin Egg Cortical Endoplasmic Reticulum

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Abstract. The calcium indicator dye fluo-3/AM was loaded into the ER of isolated cortices of unfertilized eggs of the sea urchin *Arbacia punctulata*. Development of the fluorescent signal took from 8 to 40 min and usually required 1 mM ATP. The signal decreased to a minimum level within 30 s after perfusion with 1 μ M InsP₃ and increased within 5 min when InsP₃ was replaced with 1 mM ATP. Also, the fluorescence signal was lowered rapidly by perfusion with 10 μ M

A23187 or 10 μ M ionomycin. These findings demonstrate that the cortical ER is a site of ATP-dependent calcium sequestration and InsP₃-induced calcium release. A light-induced wave of calcium release, traveling between 0.7 and 2.8 μ m/s (average speed 1.4 μ m/s, N = 8), was sometimes observed during time lapse recordings; it may therefore be possible to use the isolated cortex preparation to investigate the postfertilization calcium wave.

INTRACELLULAR free calcium rises near the sperm entry point in the eggs of many animals; a wave of elevated calcium then proceeds from this region by a release of calcium from internal stores (Gilkey et al., 1978; Jaffe, 1983; Hafner et al., 1988). The calcium wave is crucial in the activation of the egg (Whitaker and Steinhardt, 1982; Jaffe, 1983); for instance, its passage triggers the postfertilization wave of cortical granule fusion (Hamaguchi and Hiramoto, 1981).

In the sea urchin egg cortex preparation, the former interior of the egg surface is exposed and amenable to experimentation (Vacquier, 1975). This preparation retains some features of normal calcium regulation. Isolated cortices sequester ⁴⁵Ca in the presence of ATP, and release preloaded ⁴⁵Ca in response to inositol 1,4,5 trisphosphate (InsP₃)¹ (Oberdorf et al., 1986; Payan et al., 1986). However, an important issue that has not been resolved by the above isotope studies is the identity of the membrane compartments that sequester and release calcium. Several organelles are present on the sea urchin cortex, including cortical granules (Vacquier, 1975), pigmented and acidic vesicles (Sardet, 1984), and the ER (Sardet, 1984; Chandler, 1984).

Recently, a calsequestrin-like protein has been isolated from sea urchin eggs (Oberdorf et al., 1988), and an antibody to this protein labels the cortical ER (Henson et al., 1989). In striated muscle, calsequestrin is a low affinity, high capacity calcium binding protein; the presence of a similar calcium binding protein in the ER of sea urchin eggs suggests that the ER is capable of sequestering calcium, but direct evidence for calcium uptake and release from the ER is lacking.

We report here that the fluorescent calcium indicator dye, fluo-3 (Minta et al., 1989), when applied in its AM ester form, produces a signal from the cortical ER of cortices prepared from *Arbacia punctulata* eggs. Once present in the ER, the dye can be used to determine whether the ER sequesters or releases calcium.

Materials and Methods

Sea Urchin Eggs

Eggs were obtained from *Arbacia punctulata* by injection of 0.5 M KCl into the coelomic cavity. The eggs were dejellied by suspension for 1.5 min in EMC, a divalent cation-free sea water (Detering et al., 1975; NaCl, 29.34 g/liter, KCl, 0.745 g/liter, NaHCO₃, 0.21 g/liter, EGTA, 9.51 g/liter, pH 8.0) followed by centrifugation and resuspension in sea water. The eggs were then transferred onto polylysine-coated cover slips. After 1 min, the sea water was replaced with two or three changes of intracellular buffer and then a shearing force was applied to the eggs by pasteur pipet.

Intracellular Buffer

A modification of buffer X (a buffer based on the composition of squid axoplasm [Brady et al., 1984]) was used to simulate the internal conditions of the sea urchin eggs. The composition of the modified buffer is: 350 mM potassium aspartate, 130 mM taurine, 170 mM betaine, 20 mM Hepes, 3 mM MgCl₂, 10 mM EGTA, 3 mM CaCl₂, pH 7.2. This buffer preserves the network pattern of the cortical ER.

Reagents

Fluo-3/AM (fluo-3 pentaacetoxymethyl ester) was obtained from Molecular Probes (Eugene, OR). It was kept as a 1 mM stock in DMSO. ATP (dipotassium salt) and A23187 were obtained from Sigma Chemical Co. (St. Louis, MO). InsP₃ and ionomycin were obtained from Calbiochem-Behring Corp. (San Diego, CA). ATP was stored as a stock solution of 100

1. Abbreviation used in this paper: InsP₃, inositol 1,4,5 trisphosphate.

mM at pH 6.5, InsP_3 was stored as a 200 and 100 μM stock solution in intracellular buffer, and A23187 and ionomycin were stored as a 1 mM stock in ethanol.

Loading Protocol

Cortices were exposed to 5 or 10 μM fluo-3/AM, 1 mM ATP in intracellular buffer for 8 to 40 min at room temperature. The cover slips were then washed with intracellular buffer and mounted on a silicon rubber chamber.

There was considerable variation in loading fluo-3. Variability was not seen among cortices prepared from eggs of the same animal, nor among the cortices on each cover slip. There was some variation among animals that had been collected together, and there was significant variation among different collections. One manifestation was in the ATP dependence for the development of the signal. In about two thirds of the collections, development of the signal was dependent on the presence of ATP. In some cases though, development of a fluorescent signal did not require ATP, and in other cases, a signal did not develop even after incubation in ATP for 45–60 min. In the first case, calcium may already be present in relatively high concentrations and in the second case, perhaps the enzyme responsible for cleaving fluo-3 AM is less active or absent. Another form of variability was in the time required to load fluo-3; this ranged from 8 to 40 min.

Fluo-3 loaded ER membranes often became vesiculated with time (see Fig. 6). It is not known what is the cause of this.

Microscopy, Video Recording, and Analysis

A Zeiss ICM 35 inverted microscope was used. For conventional fluorescence, a 100 W mercury arc lamp was used to provide illumination. A Zeiss planapo 63 \times NA 1.4 lens or a Nikon planapo 100 \times NA 1.4 lens was used. Photographs were taken with Kodak TMAX 3200 developed for 13 min in TMAX developer. Exposure times were usually 8 s.

For video observations, a 100 W halogen lamp connected to a voltage stabilized power supply was used. An electronically controlled shutter (Uniblitz model 100-2B shutter drive control; Vincent Assoc., Rochester, NY, modified by Atlantex and Zieler, Inc., Avon, MA) was used to reduce exposure to illumination. The eye piece of the microscope was replaced with a DAGE series 66 SIT camera mounted on an adapter tube. The black level and gain controls of the camera were set to manual control. An Image 1/AT image processing system (Universal Imaging, Media, PA) installed in a Dell system 310 personal computer was used for image processing; this system also controlled the shutter, a Panasonic 2028F optical memory disk recorder and time lapse sequencing. For analysis of recorded images, the video signal was sent through a FOR-A FA-210 digital time base corrector before being sent to the Image 1/AT image processor. For the time lapse sequences, a 4 or 8 frame average was used. For the experiment shown in Fig. 6, a 16 frame average was used.

In later experiments, a KS 1381 microchannel plate intensifier (Videoscope International, Ltd., Washington DC) coupled to a Hamamatsu C-2400-07 nuvicon video camera was used in place of the SIT camera. Using neutral density filters, the region of linear response to light was determined for given instrument settings; during experiments, the light source intensity was adjusted so as to produce fluorescent signals within this linear range. The image processor was used to quantitate the changes in fluorescence due to perfusion of InsP_3 and ionomycin. Images were collected during the perfusion experiment and stored on the hard disk of the computer. Afterwards, two rectangular areas were selected, one on the cortex and the other off of the cortex to serve as a background. For each time point, the average brightness of the background area was subtracted from that of the cortex to give a measure of the fluo-3 fluorescence from the ER.

Photographs of the monitor were taken on 35 mm film and were printed under identical conditions.

Perfusion

Cortices were prepared on large 24 \times 60 mm cover slips. Two parallel walls of high vacuum grease (Dow Corning Corp., Midland, MI) were laid down along the long dimension of the cover slip and a 22 \times 22 mm cover slip was placed on top to form a chamber with two open ends on the sides. Buffer to be perfused was put onto one side of this chamber and then drawn through rapidly by using filter paper triangles applied to the other side of the chamber.

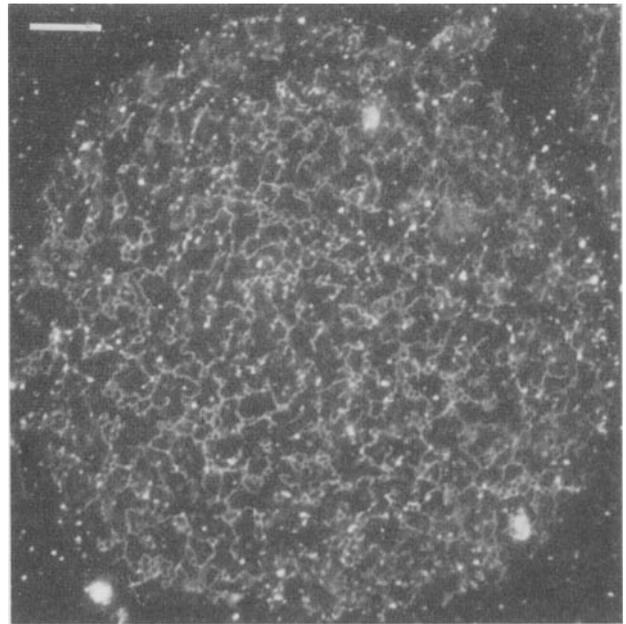


Figure 1. Fluorescent signal from a cortex incubated in 10 μM fluo-3/AM and 1 mM ATP for 20 min. The reticular network is the ER, indicating that fluo-3/AM has been cleaved by esterases in the ER and that calcium concentrations in these conditions are sufficiently high to produce a signal from fluo-3. The cortex was illuminated by a mercury arc lamp and photographed on 35 mm film. Bar, 10 μm .

Results

Fluo-3 Fluorescence in the Cortical ER

Cortices prepared from unfertilized eggs of the sea urchin *Arbacia punctulata* were loaded in 5–10 μM fluo-3/AM in an intracellular buffer with 1 mM ATP. Usually, after loading for 20 min, a reticular pattern was clearly visible by eye

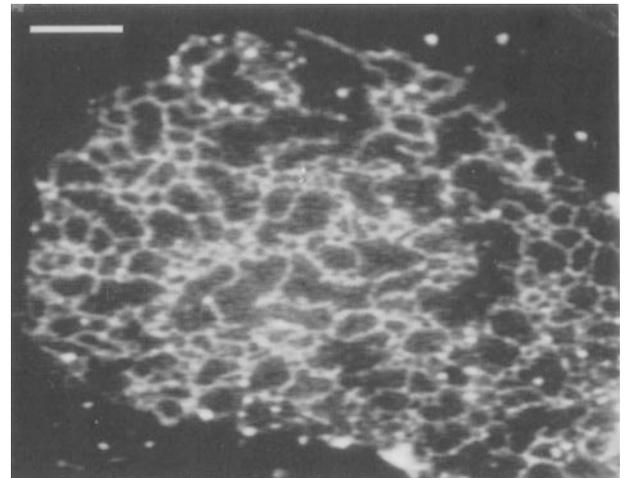


Figure 2. Fluorescent signal from a cortex in the same conditions as in Fig. 1. The cortex was illuminated by a mercury arc lamp and viewed by a SIT camera, with frame averaging by an image processor. Bar, 10 μm .

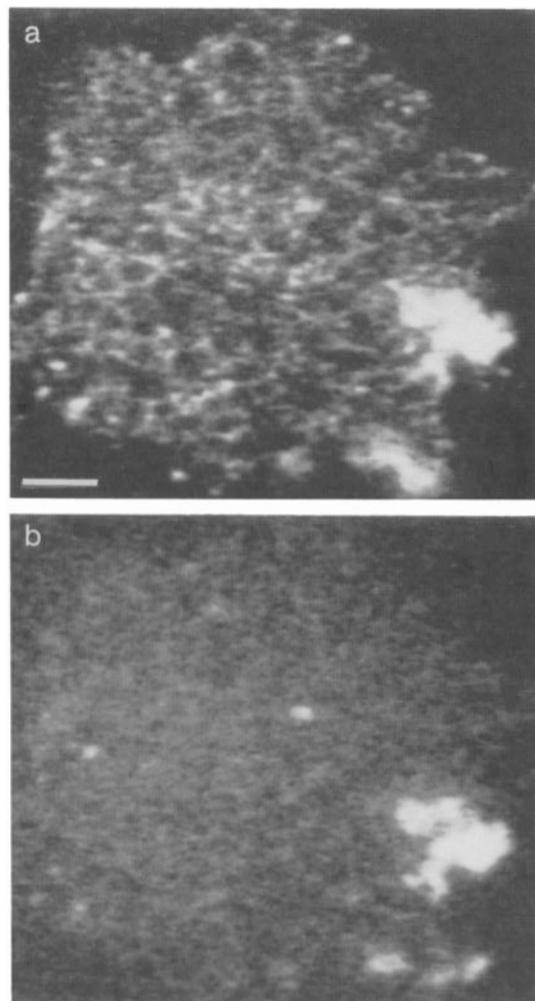


Figure 3. Perfusion with 10 μM calcium ionophore A23187. The cortex was loaded with fluo-3/AM and illuminated with a halogen lamp. The fluorescence was imaged by a SIT camera with frame averaging by an image processor. Images were obtained every 7.4 s. (a) The frame taken just before beginning perfusion. (b) The frame taken 52 s after beginning perfusion; the fluorescence signal has been reduced to background levels. Most of the reduction in fluorescence occurred by 30 s. Bar, 10 μm .

in the fluorescence microscope using illumination from a mercury arc lamp. The fluorescence was bright enough that an image could be photographed by high speed film (Fig. 1). The reticular pattern on the cortices is identical to the cortical ER as localized by an antibody to calsequestrin (Henson et al., 1989) and by a fluorescent dye staining procedure (Terasaki et al., 1988). The fluo-3 fluorescence pattern indicates that esterase activity that can cleave fluo-3/AM is present in the ER of these eggs. Other species of sea urchin (*Strongylocentrotus purpuratus*, *Strongylocentrotus droebachiensis*, *Lytechinus pictus*, and *Arbacia lixula*) did not show specific staining of the network pattern with fluo-3/AM.

Previous work with ^{45}Ca demonstrated that sea urchin cortices sequester calcium in similar conditions (Oberdorf et al., 1986; Payan et al., 1986). It seems very likely that

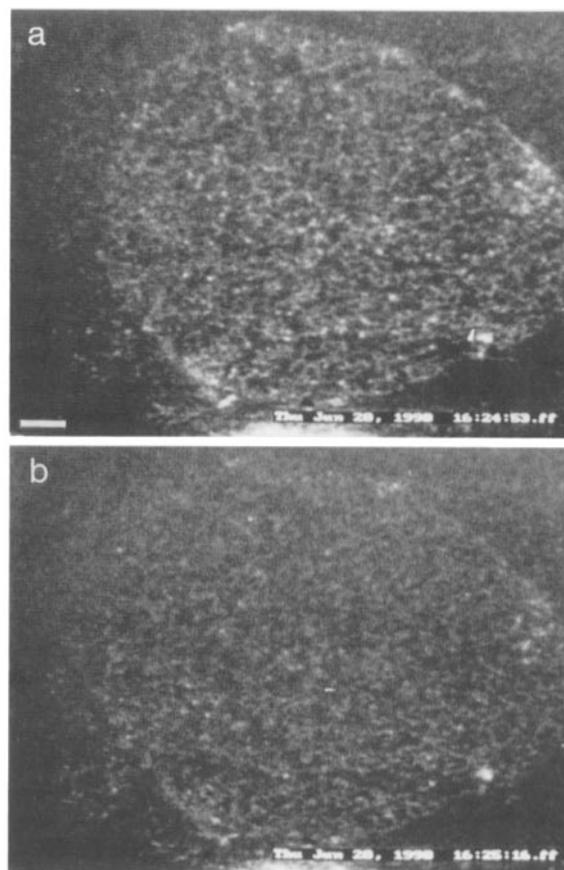


Figure 4. Perfusion with 1 μM InsP_3 . Images were obtained every 3.8 s during this sequence. (A) The frame taken just before beginning perfusion. (b) The frame taken 23 s after perfusion began; the fluorescence signal has been reduced significantly, indicating that InsP_3 has caused calcium release from the ER. Bar, 10 μm .

the ATP-dependent fluo-3 fluorescence is due to calcium pumping into the ER.

The calcium ionophores A23187 and ionomycin (both 10 μM) rapidly abolished the fluo-3 fluorescence signal from the ER (within 1 min). InsP_3 (1 μM) also caused the fluorescence to decrease rapidly, though not as completely as did the ionophores. When cortices were washed free of InsP_3 and incubated in ATP (1 mM), the fluorescence returned to a bright level. This indicates that calcium was pumped back into the ER.

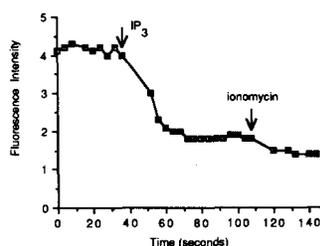


Figure 5. Quantitative measurements of fluo-3 fluorescence during perfusion with InsP_3 . A large rectangular area covering most of the area of the cortex of Fig. 4 was analyzed by an image processor. The average brightness from an area outside of the cortex was subtracted from the average brightness on the cortex to give a measure of fluo-3 fluorescence from the ER. Perfusion with InsP_3 causes a significant decrease in fluorescence; ionomycin causes an additional small decrease in fluorescence.

Fluorescence Imaging by Low Light Level Video Microscopy

A SIT camera coupled with an image processor was used to image the fluorescence. A clear image of the network could be obtained by frame averaging the image of a cortex (Fig. 2). For perfusion experiments, light levels and exposure time were reduced to a minimum in order to lower the rate of bleaching and photodynamic damage. In so doing, the image quality was sacrificed so that the network pattern was barely discernible; otherwise the fluorescence bleached too rapidly for the following studies. Perfusion of the ionophore A23187 caused a large decrease in fluorescence; most of the decrease occurred within 30 s and was complete by 50 s (Fig. 3). Due to the perfusion method used, the response time to ionophore may be significantly faster.

In later experiments, quantitative data on the fluorescence changes after perfusion with 1 μ M InsP₃ were obtained (Fig. 4). The average brightness of a large area on the cortex was drastically reduced by \sim 30 s after the beginning of the perfusion (Fig. 5). Again, due to the perfusion method used, the response to InsP₃ may be significantly faster than recorded here. Ionomycin caused a small additional decrease in fluorescence. Both InsP₃ and the ionophores seemed to cause a uniform fluorescence decrease on the cortex. When images were obtained every few seconds, there was little increase in fluorescence in conditions which calcium sequestration was shown earlier to occur. Perhaps calcium sequestration is highly sensitive to photodynamic damage. Calcium resequestration after InsP₃-induced release was demonstrated by reducing the number of images taken to three: after loading of fluo-3, 1 min after perfusion of InsP₃, and 5 min after perfusion of ATP (Fig. 6).

Spontaneous Propagated Decreases in Fluo-3 Fluorescence

The fluo-3 signal from the cortices was stable enough to record many images per cortex (in these experiments, 50–85 images were taken). Sometimes, during the course of imaging a cortex, a wave of calcium release occurred, in the absence of ATP (Fig. 7; frames taken every 6.3 s). This phenomenon begins with a small circular region of diminished fluorescence, which expands in succeeding frames. Ten instances of this type of release were observed on three separate days. On these days, an approximately equal number of cortices did not exhibit waves within the 5–10 min that they could be observed before becoming bleached. On four other days, the waves could not be observed on any cortex in spite of observations of several cortices on each day. The cause of this variation is not known. Of the eight instances that could be clearly measured, the velocities ranged between 0.7 and 2.8 μ m/s with an average of 1.4 μ m/s. The average is three to four times slower than the postfertilization wave in intact echinoderm eggs, which travels at \sim 5 μ m/s (Eisen et al., 1984; Hafner et al., 1988; Hamaguchi and Hamaguchi, 1990; Gillot et al., 1990). In two instances, the wave did not travel over the whole cortex but instead stopped at a distinct boundary within the periphery of the cortex. In two other instances, the wave spread to a boundary in the cortex and traveled around that boundary to the rest of the cortex. In several instances, short segments of ER tubules remained brightly stained after the release was complete. The

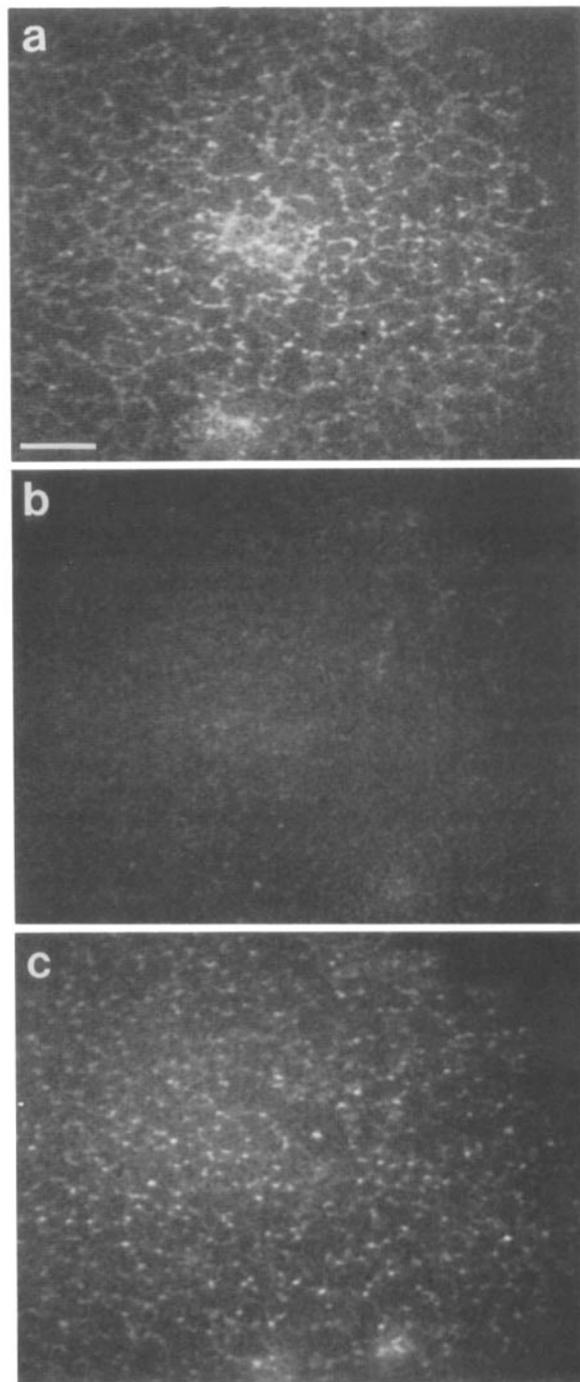


Figure 6. Calcium sequestration after InsP₃-induced calcium release. A cortex was imaged three times during this experiment: (a) after the ER was loaded with fluo-3/AM; (b) 1 min after perfusion with 1 μ M InsP₃; and (c) 5 min after subsequent perfusion with 1 mM ATP, showing resequestration of calcium into the ER. The ER appears to be mostly vesiculated, but in other experiments the ER was continuous after reloading. The average brightness of a large rectangular area on the cortex and of a smaller area outside the cortex was determined using an image processor. The average brightness of the cortex fluorescence minus background was 3.45 (a), 2.58 (b), and 3.23 (c).

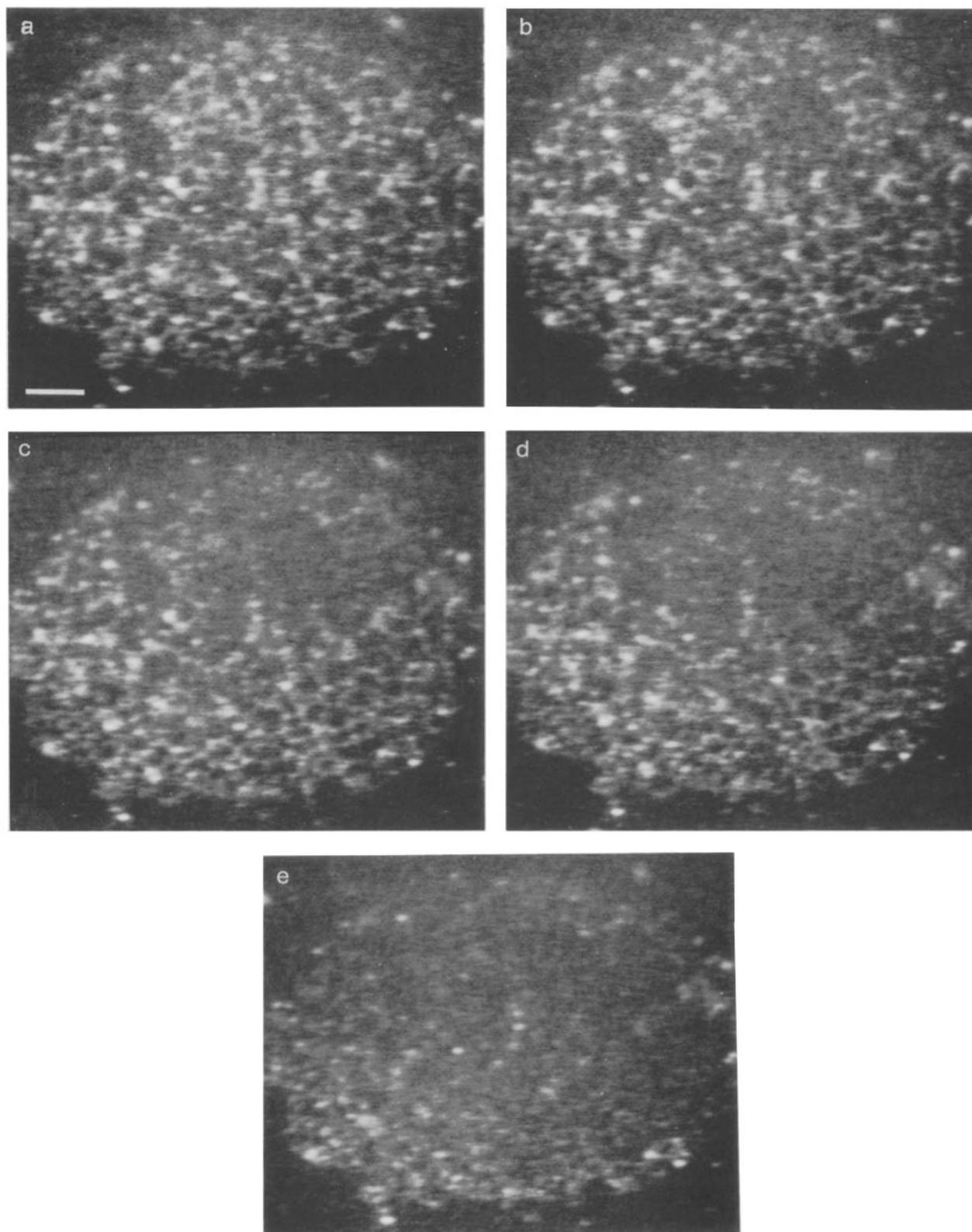


Figure 7. A spontaneous wave in the fluorescence signal from the cortical preparation. The fluo-3 loaded cortex was imaged as in Figs. 3 and 4, with images taken every 6.3 s. (a) 12th image of the sequence showing a uniformly labeled cortex. (b–e) Consecutive images (13–16) of the sequence showing a local diminution in the fluorescence signal near the top of the cortex that progressively spreads throughout the cortex in the succeeding frames. Bar, 10 μm .

cortical ER has occasional breaks in the network which form rifts of discontinuity (see Figs. 1 and 2); the indirect path sometimes taken by the wave suggests that the calcium release occurs only along continuous membrane compartments.

After observing a wave on a particular cortex, the cortices on the rest of the cover slip still had bright signals from their ER. Also, waves typically occurred after 2–4 min of observation. This suggests that the waves are triggered by the effects of accumulated exposure to illumination. When observed by transmitted light, the cortical granules were still present after passage of a wave. Since the buffer contains 10 mM EGTA, calcium released from the ER probably becomes bound to EGTA before it can trigger cortical granule fusion.

Discussion

The identity of membrane organelle(s) that are the calcium stores has been a subject of controversy. Microsomal fractions sequester and release calcium; for instance, InsP_3 induces calcium release from microsomal fractions of sea urchin eggs (Clapper and Lee, 1985). It is very difficult though, to show that microsome preparations contain only ER membranes. An antibody to rabbit fast twitch skeletal muscle calsequestrin labels vesicular compartments in HL-60 cells and neutrophils that do not appear to be part of the ER (Volpe et al., 1988; Hashimoto et al., 1989). These workers isolated a membrane fraction that did not contain some ER markers but which released calcium in response to InsP_3 , leading to the proposal of a new organelle, the "calciosome" (Volpe et al., 1988). Recent work provides strong evidence that the ER is the InsP_3 -sensitive calcium store. An antibody to the InsP_3 receptor of cerebellar tissue labels stacks of ER cisternae, labels less strongly parts of the rough ER, and apparently does not label other organelles in Purkinje cells of the cerebellum (Sato et al., 1990; Otsu et al., 1990). However, not all of the receptors in the ER recognized by the antibody are necessarily active; they may be less active because of phosphorylation (Suppatapone et al., 1988) or interaction with allosteric regulators (Ferris et al., 1990). In addition, the InsP_3 receptor is presumably synthesized and assembled in the ER, so some of the receptors may not be functional yet.

Here, we present direct evidence that the sea urchin egg cortical ER is a site of ATP-dependent calcium sequestration and InsP_3 -induced calcium release. An interconnected network of ER adjacent to the sea urchin egg plasma membrane has been observed by EM, immunofluorescence and dye staining methods (Sardet, 1984; Chandler, 1984; Henson et al., 1989; Terasaki et al., 1991; Terasaki and Jaffe, 1991); we have used the calcium indicator dye fluo-3 to detect calcium uptake and release from these membranes. Fluo-3 is usually introduced into the cytosol of whole cells as the AM ester, which permeates the plasma membrane (Minta et al., 1989). Cytosolic esterases then cleave the thioester bond, generating a charged product that becomes trapped and concentrated in the cytosol. Very probably, the ER of *Arbacia punctulata* eggs contains an esterase capable of cleaving fluo-3/AM, enabling fluo-3 to accumulate in the ER in this open preparation. Since fluo-3 did not generate a signal from the other organelles on the cortex, our data cannot be used to investigate calcium regulation by the other organelles.

Development of the fluo-3 signal from the ER usually required 8–40 min incubation in the presence of ATP. Most of the signal was eliminated within 30 s after the beginning of perfusion of A23187. The signal also decreased within 30 s after perfusion of InsP_3 . This is consistent with previous observations of the release of preloaded ^{45}Ca induced by InsP_3 (Oberdorf et al., 1986; Payan et al., 1986). The InsP_3 -induced decrease in fluo-3 signal recovered over a period of 5 min when InsP_3 was replaced with ATP. These fluorescence changes demonstrate that the cortical ER is a site of ATP-dependent calcium sequestration and InsP_3 -induced calcium release. Since the cortical ER is RER (Sardet, 1984; Terasaki, M., unpublished results), the results indicate that this part of the egg ER is capable of supporting protein synthesis as well as calcium sequestration and release.

Although fluo-3 can be used to detect calcium release and uptake into the ER, development of a dye with lower affinity for calcium might be more desirable for investigations of calcium regulation by the ER. Fluo-3 has a much greater affinity for calcium than the calsequestrin-like protein in the ER ($K_d = \sim 1$ mM; B. Kammer, unpublished results), so depending on the actual concentrations of fluo-3, total calcium, calsequestrin-like protein, and other calcium binding proteins, fluo-3 may significantly alter the normal balance of free and bound calcium in the ER.

We also observed an apparently propagated release of calcium from the ER; the velocity was three to four times slower than that of the postfertilization Ca wave in sea urchins. Since other cortices on the cover slip were still labeled, the calcium release is very probably due to illumination by light. Our interpretation is that free radicals produced by photodynamic processes trigger calcium release in one area, after which calcium release is propagated through the entire network. If the waves we have observed are indeed related to the wave in the intact egg, then the isolated cortex possesses all of the components required for propagated release of calcium. Since many cytosolic components have been washed away during preparation of the cortices, the ER and plasma membrane, or the ER by itself may be sufficient for propagation of the calcium wave. Since the wave sometimes stopped or traveled around distinct boundaries on the cortex, propagated calcium release may only occur along continuous membranes. Further investigation requires a means for experimentally initiating this phenomenon.

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