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Intracellular Calcium Release at Fertilization in the Sea Urchin Egg

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Fertilization or ionophore activation of *Lytechinus pictus* eggs can be monitored after injection with the Ca-sensitive photoprotein aequorin to estimate calcium release during activation. We estimate the peak calcium transient to reach concentrations of 2.5–4.5 μM free calcium 45–60 sec after activation and to last 2–3 min, assuming equal Ca^{2+} release throughout the cytoplasm. Calcium is released from an intracellular store, since similar responses are obtained during fertilization at a wide range of external calcium concentrations or in zero-calcium seawater in ionophore activations. In another effort to estimate free calcium at fertilization, we isolated egg cortices, added back calcium quantitatively, and fixed for observation with a scanning electron microscope. In this way, we determined that the threshold for discharge of the cortical granules is between 9 and 18 μM Ca^{2+}. Therefore, the threshold for the *in vitro* cortical reaction is about five times the amount of free calcium, assuming equal distribution in the egg. This result suggests that transient calcium release is confined to the inner subsurface of the egg.

INTRODUCTION

It was recently demonstrated that the Lilly Ca^{2+} ionophore A23187 can activate sea urchin eggs (Steinhardt and Epel, 1974; Chambers *et al.*, 1974), as well as eggs of several other species widely separated phylogenetically (Steinhardt *et al.*, 1974). All these activations were shown to be independent of external calcium and led to the proposal that the release of intracellular ionic calcium may be the universal factor promoting activation of egg metabolism at fertilization (Steinhardt, *et al.*, 1974).

Since the pioneering experiments of Mazia (1937) who observed an increase in Ca^{2+} in the ultrafiltrates of homogenates of *Arbacia* eggs which had just been fertilized, several efforts have been made to confirm that calcium was released at fertilization. Nigon and Do (1963), Clothier and Timourian (1972), Steinhardt and Epel (1974), and Azarnia and Chambers (1976) used ^{45}Ca fluxes to demonstrate intracellular calcium release at fertilization in the sea urchin egg. However, the most direct demonstration of calcium release at fertilization was provided by experiments on the much larger egg of the medaka fish, in which it was possible to inject substantial amounts of the photoprotein aequorin and observe a flash at fertilization (Ridgway *et al.* 1976).

We report here on our success at using aequorin to study calcium release at fertilization in sea urchin eggs and on the details of the methods used with this more difficult material. We have also made quantitative estimates of the peak calcium transients at fertilization and ionophore activation and have compared these estimates to a bioassay method for free calcium using isolated cortices.

MATERIALS AND METHODS

Handling of gametes. The sheddings of *Lytechinus pictus* gametes were induced by intracoelomic injection of 0.5 *M* KCl. Jelly was removed by a 1-min exposure to acidified seawater (pH 5) and repeated

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washing with normal seawater. Sperm was added to dishes in the undiluted form as described below. Eggs were stuck down by the protamine method (Steinhardt *et al.*, 1971) to permit microinjection of the aequorin solution. The dish was placed in a water bath the temperature of which was maintained at 18 ± 0.5°C throughout these experiments.

Preparation and storage of aequorin. Dr. O. Shimomura kindly provided us with 1 mg of highly purified (better than 90%) aequorin, suspended in 10 m*M* EDTA (ethylenediamine tetraacetic acid) saturated with ammonioum sulfate. This sample was washed into a centrifuge tube with a solution of 10 m*M* K_2EDTA saturated with ammonium sulfate, was titrated to pH 7.1 with KOH, and was centrifuged for 15 min at 15,000 rpm and 2°C.

We used a microinjection solution consisting of 0.25 *M* K_2SO_4, 2 μM K_2EDTA and 10 m*M* potassium phosphate buffer at pH 7.5. This solution was designed to have minimal effects on the egg cytoplasm and electrical properties of the membrane, without using anions or other solutes likely to clog the injection pipet tip. The osmotic pressure is not very different from that of cytoplasm. The pH, although higher than estimates of egg cytoplasm (Johnson *et al.*, 1976), is the minimum pH at which aggregation of aequorin does not occur (Kohama *et al.*, 1971). The EDTA is needed to protect the aequorin from contamination during the preparative procedures. Upon injection into cells, the EDTA is diluted to less than 5×10^{-8} *M*, which should not interfere with either the calcium buffering of the cell or the calcium–aequorin reaction. The injection solution was cleared of traces of calcium by passage through a Chelex 100 column (potassium form, 200–400 mesh, bed volume 85 ml, equilibrated with 0.5 *M* potassium phosphate buffer to pH 7.5) and then was Millipore-filtered. The aequorin pellet (1 mg) was dissolved in 200 μl of this solution, yielding a concentration of 0.25 m*M* (assuming a molecular weight of 20,000; Blinks *et al.*, 1976). All vessels with which the aequorin or injection solution would come into contact were made of plastic and were thoroughly cleaned and rinsed with a solution of 20 μM K_2EDTA (made by diluting 20 m*M* K_2EDTA titrated to pH 7.5 with KOH) which had been Chelexed and Millipore-filtered.

The aequorin sample was freed of traces of ammonium sulfate and excess EDTA by passage through a microcolumn (2-mm i.d.) containing Sephadex G-25 fine beads (bed height 56 mm), topped by a 9-mm layer of Chelex 100 (potassium form, 200–400 mesh) which had been equilibrated with microinjection solution. This solution was also used to elute the aequorin, which was collected as 20-μl droplets in individual Teflon cups with tight-fitting tops and was stored at −80°C. After each droplet had been assayed, the most active droplet was transferred to a plastic petri dish and was stored at −10°C under light mineral oil which had been Chelexed and ultrafiltered.

Detection of light emitted by aequorin. Light emitted by eggs or microsamples of aequorin solution was collected and transmitted to a photomultiplier tube by a fiber optics light guide (1/8 in. diameter, 1 ft long, N.A. 0.35, American Optical UVLGM-3-12) having a high transmittance to blue light. The light guide was usually mounted at the minimum distance from the source (4.5 mm) so that the light striking all of the surface of the light-guide target was within the acceptance angle of the light guide. This light guide transmitted about 60% of the incident light (manufacturer's calibration) to the photomultiplier tube. The preparation dish and light detection system were enclosed in a light-tight cage.

The aequorin luminescence was measured using an EMI 9789A photomultiplier tube, especially selected for low dark current (0.05 nA at highest anode voltage used). The dark current was maintained at

a low level by never exposing the photomultiplier tube cathode to bright light. This tube has a rated cathode sensitivity of 58 mA/W, and was calibrated by the manufacturer to have a gain of 4×10^7 at an anode voltage of 870 V. Thus, the tube has a rated overall sensitivity to a black body radiator of 2.32×10^{15} nA/W at the voltage used in most of our experiments. Using a constant light source, we measured the relative sensitivity of the tube as a function of anode voltage, in order to be able to compare responses obtained at different voltages.

The anode current was measured with an FET op amp (Burr Brown 3542) used as a current-to-voltage converter (1 mV/nA), with a time constant of 30 msec. This signal was recorded on a Vetter Model A FM Instrumentation tape recorder (3-db-bandwidth 0–700 Hz) and simultaneously was displayed on an oscilloscope and recorded on a chart recorder. Values for this paper were prepared by replaying tapes at a reduced speed and recording the signal on a Linear Instruments Model 255 chart recorder, with a recording time constant of 400 msec.

Microinjection procedures. Aequorin was injected into egg cells using specially-made micropipets. These were pulled from glass capillaries (1.2-mm o.d., 0.6-mm i.d.) made of low-calcium glass (Corning 7740), which were thoroughly precleaned with hot dilute nitric acid, acetone, glass double-distilled water, and finally with 20 μM K_2EDTA. Fairly blunt micropipets were pulled and then beveled by the technique of Werblin (1975), producing an elliptical tip opening of 1×3 μm while retaining a sharp 0.2-μm-diameter point (c.f. Chang *et al.*, 1974). After beveling, pipets were checked and cleaned by observing, under a compound microscope, the filling of the tip by capillarity with 20 μM K_2EDTA solution. This solution was blown out, and the electrodes were stored in a dust-free environment. Pipets were usually pulled and cleaned shortly before use.

Micropipets were filled by immersing the tip in a thawed aequorin droplet maintained at about 5°C on a cold plate (c.f. Rose and Loewenstein, 1976). The back of the electrode could be connected by a series of valves to either positive or negative pressure. Using a stereomicroscope, we sucked aequorin solution into the pipet until it clogged. The tip was unclogged by applying a brief pulse of positive pressure, and then the suction was reconnected, and filling was resumed. In this way, we were able to fill relatively sharp pipets with enough aequorin to inject as many as 25–50 eggs.

Eggs were filled with aequorin by pressure injection. The micropipet was held in a pressure-injection microelectrode holder which was connected through a three-way electronic interface valve (Clippard-Northeast Fluidics, Fluidamp 2013) to a regulated pressurized air tank. At rest, the pipet was connected to atmospheric pressure. Brief pulses (about 50 msec in duration) applied through a relay closed the interface valve and provided sharp pressure pulses, about 2.75 bar. The parameters of the pressure pulses were adjusted so that one pulse ejected a volume of aequorin solution approximately equal to 1% of the volume of an egg cell (egg volume about 700 pl; injection volume about 7 pl). The injection volume was estimated from the diameter (25 μm) of the droplet formed at the tip of the micropipet, when the pipet was held in air and a single pulse of pressure was delivered. This was the largest volume we could inject into egg cells 110 μm in diameter and still maintain electrical integrity of the cell membrane and obtain reasonably high yields of fertilization.

Certain precautions must be taken to prevent dilution and inactivation of the aequorin in the pipet tip while attempting to penetrate cells. The eggs are in normal seawater, the calcium concentration of which is high enough that pipets filled with aequorin emit a steady light, indicat-

ing the inactivation of aequorin in the pipet tip at a rate of 1–5 × 10^8 molecules/sec. An egg which is microinjected with such a pipet can be assayed for active aequorin by pumping it full of air until it bursts while light emission is monitored. Very little active aequorin is then found in the egg.

In order to prevent dilution and inactivation of aequorin in the pipet tip, pipets with a low tonic emission of light were selected. Moreover, we adopted a procedure in which pressure pulses were applied continuously, at about 0.5 Hz, to the pipet whenever it was immersed in seawater, and, in particular, while micromanipulating the pipet into position for penetrating the egg membrane. Under these circumstances, the resting level of light emission between pulses is very low, and each injection pulse leads to a transient light emission generating an anode charge of about 50 nC (range 40–80 nC), at an anode voltage of 600 V. For the volume of aequorin solution ejected by one pressure pulse (7 pl), this corresponds to an aequorin activity of 0.19 m*M*, which is very close to the assayed activity of aequorin (0.20 m*M*) in our injection solution before filling the micropipets (see Appendix for method of estimating aequorin activity). Apparently, this method of handling the micropipets is very effective in preserving the activity of aequorin in the tips of the pipets.

With continuous pulsing, we penetrate an egg and stop the pulses after a single-pulse volume of aequorin has been injected. At this instant, we observe a clear region in the cytoplasm just beyond the pipet tip corresponding to the injected fluid. This fluid disperses throughout the egg within about 10 sec. We have several reasons for believing that the injected aequorin remains inside the egg and active. (1) We can explode an egg up to 5 hr later and still observe a light emission [average anode charge, 48 ± 10 (SD) nC, 12 eggs] corresponding closely to the amount of light emitted by injecting the aequorin into seawater (about 50 nC). (2) There is no detectable resting emission from 50 eggs injected with aequorin, compared to the background noise of our detection system. (3) We believe the aequorin is dispersed throughout the cytoplasm, because penetration of the opposite pole of an egg from the injected end with a second micropipet leads to a small transient emission, lasting a few seconds until the membrane seals. Furthermore, removing an implanted pipet results in only a very small flash. These manipulations do not inactivate the aequorin in an egg, nor is there any evidence of prolonged leakage, except in eggs damaged by rough handling. (4) Once an aequorin pipet has penetrated an egg, no light is emitted by single-pulse (7 pl) injections. However, as soon as the pipet is withdrawn, single-pulse injections emit light just as before penetration (50-nC anode charge). Thus, the aequorin in the tip of the pipet is protected from inactivation by the low-calcium cytoplasm, and none of the aequorin injected is lost through the cell membrane.

From these measurements, we estimate that we are injecting an egg with 1% of its volume of 0.19 m*M* aequorin, the final cytoplasmic concentration of which is about 2.0 μM and remains at this level throughout our experiments. We also estimate the total anode charge available in one injected egg as 50 nC at an anode voltage of 600 V or 2500 nC at 870 V.

Experimental procedures using injected eggs. Fertilization was accomplished by filling a 5-mm-long glass tube, 1.5-mm i.d., with a fresh suspension of sperm. The tube was lowered by remote control into the dish of eggs, about 1 cm from the eggs injected with aequorin. In parallel experiments we observed elevation of fertilization membranes to occur nearly synchronously (within 10 sec) among all eggs injected, which were clustered in an area of about 0.5 mm^2. Depending on the distance between eggs and sperm, elevation occurred 1–3 min after adding sperm. This

time could be reduced to 45 sec by continuously stirring the fluid in the dish.

The divalent cation ionophore A23187 (obtained from R. Hamill, Eli Lilly Co., Indianapolis) was administered by placing 20 μl of the ionophore, dissolved in DMSO (dimethyl sulfoxide) at a concentration of 5 m*M*, in the glass tube used for delivering sperm. The same remote control system was used with continuous stirring of the medium bathing the eggs. DMSO without ionophore had no effect on aequorin-filled eggs. The final concentrations in the dish were 40 μM A23187 and 0.8% DMSO.

Rapid changes of solution were accomplished by a perfusion system controlled from outside the light-tight cage. The perfusion rate was 0.5 ml/sec; the fluid in the petri dish containing the eggs was replaced every 5 sec. Changes in $CaCl_2$ were adjusted with NaCl to maintain osmotic balance in our artificial seawaters.

Preparation of isolated egg surfaces. Dejellied eggs were affixed to a polylysine-coated plastic petri dish (Mazia *et al.*, 1975) and were rinsed in seawater to remove the unattached eggs. These affixed eggs were then rinsed five times in a homogenization medium designed to mimic the internal ionic environment of the unfertilized egg minus all Ca^{2+} (0.45 *M* KCl, 50 m*M* Hepes buffer (*N*-2-hydroxyethylpiperazine *N'*-2-ethane-sulfonic acid), 1 m*M* EGTA [ethylene glycol bis (β-aminoethyl ether)-*N*,*N'*-tetraacetic acid], 1 m*M* $MgCl_2$, pH 6.7. The tops of the eggs were sheared off by a squirt of the homogenization buffer to reveal the inner surface of the bottom of the cell (Vacquier, 1975). The remaining monolayer of unfertilized egg surfaces was then observed with inverted phase optics in which the cortical granules appear as distinct spheres. Titration with buffers of known calcium concentrations was performed by thoroughly rinsing the petri dish five times with the Ca^{2+} buffer to replace the homogenization medium and to remove any residual unbuffered EGTA. The dish was then examined under inverted phase optics to determine the concentration of free Ca^{2+} which would discharge the cortical granules.

Scanning electron microscopy of isolated surfaces. Unfertilized egg surfaces were isolated as previously described and were rinsed in a Ca-EGTA buffer containing a fixed level of free Ca^{2+} (see Results and Table 1). Fixation was immediately performed in the Ca^{2+} buffer with 2.5% glutaraldehyde, pH 6.7, for 1 hr. The specimens were dehydrated in ethanol, infiltrated in Freon TC, and dried at the critical point in Freon 13. After carbon–platinum coating, the samples were examined in a field-emission scanning-electron microscope.

RESULTS

Fertilization

We could not detect the resting levels of calcium ion even if we injected as many as 30 eggs in the same field. However, with these multiple injections we could detect a response to fertilization. On the average, about half the eggs injected were invisibly injured by the pressure applied and did not elevate fertilization membranes. In five experiments, we filled 16–27 eggs without apparent injury and successfully fertilized from 5 to 17 eggs as judged by normal elevation of fertilization membranes. Figure 1 shows one of our best results and a

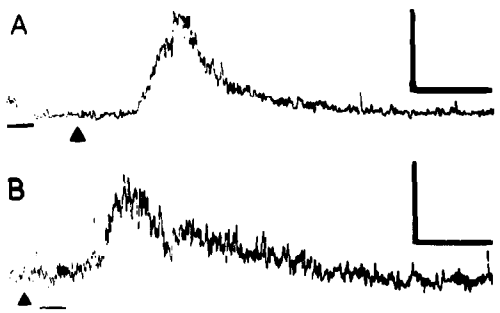

FIG. 1. Response to fertilization. Sperm addition at arrow. Time base is horizontal bar equal to 1 min. (A) Best aequorin emission: 25 eggs injected, 12 successfully fertilized. Vertical bar: 2 nA. (B) Typical aequorin emission: 27 eggs injected, 7 successfully fertilized. Vertical bar: 1 nA. In all figures, lower left horizontal bar is zero light level. All experiments conducted at 18°C.

typical result. In our best experiment, the peak current per egg was 0.21 nA (at a photomultiplier voltage of 870 V), and the average peak current per egg was 0.14 ± 0.07 nA. Our lowest peak current was 0.053 nA/egg. The latency of the response ranged from 45 to 105 sec and depended on the distance of the sperm delivery system and whether we provided mechanical mixing. Our shortest latencies of 45 and 60 sec were obtained with close delivery and vigorous mixing, and these experiments also gave high (0.21 nA) peak currents, indicating more synchronous fertilization. With short latencies, the duration of the detectable calcium transient was 140–150 sec. Since the resting level is below our level of detection we are limited to describing only the top shape of the response.

We made numerous attempts to observe an emission of light from a single injected egg following fertilization. Only once did we detect any response, and this appeared as a miniscule increase in the photon noise which remained detectable for about 1 min. The response was undetectable when plotted on a chart recorder at low speed. We feel that it might be possible to measure the response from one egg using a photomultiplier tube in a pulse-counting configuration, but, with our present techniques, the response from a single egg was too small and unreliable to warrant further study.

Ionophore Activation

Figure 2 shows two activations by calcium ionophore, one in seawater and one in zero-calcium 2 m*M* EGTA seawater. With ionophore, the peak current is similar (0.13 nA/egg), and the duration of detectable light emission is longer (240–300 sec) than that following fertilization.

The ionophore result in zero calcium suggests that external Ca^{2+} does not play a significant role in elevation of internal free Ca^{2+} ion. This has been confirmed in the following experiments in which we quickly increased or decreased external Ca^{2+} a few orders of magnitude during fertilization without significantly altering the response.

Fertilization during Changes in External Calcium

Rapid changes of solution had only minor effects on the fertilization reaction. Figure 3 shows a number of solution changes and the corresponding levels of aequorin emission.

The process of perfusion itself occasionally produced a small brief change of light, recorded as seen in Fig. 3A in which we changed the seawater in the petri dish to identical seawater from our perfusion bottle. But, decreasing the external Ca^{2+} from 11 to 0 m*M* plus 2 m*M* EGTA had no effect either on unfertilized eggs or on the emission recorded during fertilization (Fig. $3B_1$, B_2).

We could obtain momentary increases in aequorin emission by rapid changes from very low levels of Ca^{2+} (1 m*M* or less) to very high levels (up to 110 m*M*) in both unfertilized eggs and during emission at fertilization (Fig. $3C_1$, C_2). However, these increases were very limited in duration and did not change the basic characteristic response at fertilization. These momentary increases are interpreted by us as a

FIG. 2. Response to ionophore activation. Addition of 40 μM A23187 at arrow. Vertical bar: 1 nA. Horizontal bar: 1 min. (A) Normal seawater containing 11 m*M* $CaCl_2$; out of 27 eggs injected, 6 successfully elevated fertilization membranes. (B) Zero-calcium seawater (0-Ca SW) with 2 m*M* EGTA added. Twenty-four eggs injected; fertilization membranes are not stable in zero-calcium seawater and could not be scored.

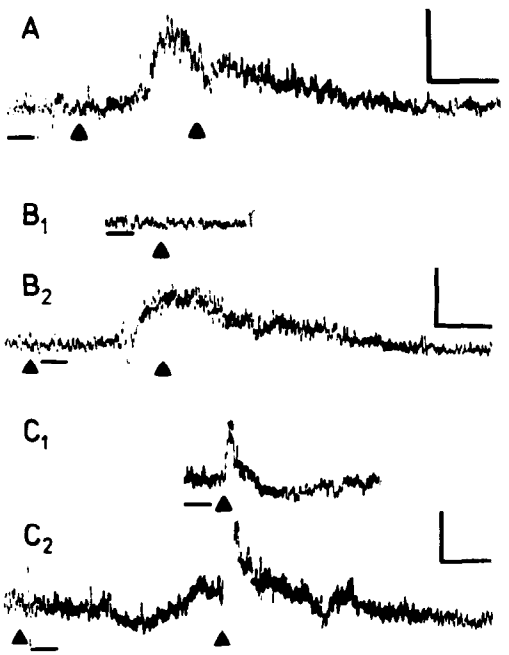

FIG. 3. Responses to fertilization and changes in Ca^{2+} concentration. Horizontal bar: 1 min; vertical bar: 1 nA. Unless otherwise noted, sperm addition at first arrow. (A) Artificial seawater (ASW) containing 460 m*M* NaCl, 55 m*M* $MgCl_2$, 11 m*M* $CaCl_2$, 10 m*M* KCl, and 5 m*M* $NaHCO_3$, pH 8, changed at second arrow to identical solution during successful fertilization of 7 eggs out of 27 injected. (B_1) ASW changed to 0-Ca SW with 2 m*M* EGTA at arrow while monitoring 24 injected unfertilized eggs. (B_2) ASW changed to 0-Ca SW containing 2 m*M* EGTA during the successful fertilization of 17 eggs out of 22 injected. (C_1) ASW changed to 55 m*M* Ca SW at arrow while monitoring 16 injected unfertilized eggs. (C_2) ASW changed to 55 m*M* Ca SW during fertilization of 31 injected eggs. Fertilization was not scored. Peak current at second arrow was 9 nA.

loss of membrane permeability control in abnormally low calcium, but do not affect our basic conclusion that external calcium does not participate in the fertilization reaction under physiologically normal conditions.

To test this conclusion further, we fertilized aequorin-injected eggs at different levels of calcium without changing solutions during the experiment. At 1 m*M* Ca^{2+} the peak current per egg was 0.09 nA; at 5.5 m*M* Ca^{2+}, it was 0.1; and at 55 m*M* Ca^{2+}, it was also 0.1. These values are not significantly different from the average 0.14 nA obtained from fertilizations in normal seawater with 11 m*M* Ca^{2+}.

Calibration of the Aequorin Reaction

We would like to be able to estimate the level of free calcium attained in eggs from the level of anode current recorded during membrane elevation. Our approach to this problem was to inject aequorin into drops of solutions (under mineral oil) containing fixed amounts of free calcium. For this purpose, we used a 10 m*M* EGTA buffer system, in which the concentration of Ca^{2+} was varied between 1 and 30 μM. The Ca^{2+}-aequorin reaction is influenced by Mg^{2+}, pH, and ionic strength (Blinks *et al.*, 1976). Therefore, we used a buffer solution with 10 m*M* $MgCl_2$, 50 m*M* Na-Hepes at pH 6.7, and 0.45 *M* KCl, in which the Mg^{2+} concentration, pH, and ionic strength were similar to the corresponding levels in sea urchin cytoplasm (Johnson *et al.*, 1976; Steinhardt and Epel, 1974).

In order to obtain desired levels of free calcium and magnesium, the following system of equations was solved simultaneously for the total calcium and magnesium concentrations to be added to a total EGTA concentration of 10 m*M*:

$$[\text{Ca-EGTA}] = [Ca^{2+}]\,[\text{EGTA}^{2-}]\,K_1,$$
$$[\text{Mg-EGTA}] = [Mg^{2+}]\,[\text{EGTA}^{2-}]\,K_2,$$
$$[\text{Ca-EGTA}] + [\text{Mg-EGTA}] + [\text{EGTA}^{2-}] = [\text{EGTA}_{\text{total}}],$$
$$[Ca^{2+}] + [\text{Ca-EGTA}] = [\text{Ca}_{\text{total}}],$$
$$[Mg^{2+}] + [\text{Mg-EGTA}] = [\text{Mg}_{\text{total}}],$$

where K_1 and K_2 are the apparent binding constants of EGTA with calcium and magnesium (Portzehl *et al.*, 1964). At pH 6.7, we calculated K_1 to be 6.1×10^5 and K_2 as 18.4, using $10^{10.7}$ as the association constant for EGTA with calcium (Ogawa, 1968).

The correct estimation of free Ca^{2+} present in solutions of Ca-EGTA buffers requires that the association constants for reactions of EGTA with Ca^{2+}, Mg^{2+}, and H^+ be known accurately. We have reported here the actual composition of each

solution used so that, if new constants are obtained at some future date, our data can then be recalculated (Table 1).

With droplets equal in volume to 10 eggs and injected with the amount of aequorin we would normally inject into 10 eggs, we plotted the current vs free Ca^{2+} for the range of 1–30 μM. During each injection of aequorin in these calibrations, the aequorin micropipet is quickly removed from the field to erase emission from the tip due to possible mixing after contact. This relation was found to have a slope of 2.24 ± 0.5 (SE), N = 8 points. The second power relationship of aequorin emission to Ca^{2+} concentration conforms to that previously found (Blinks *et al.*, 1976). This plot allows us to compare aequorin emission reactions on a per-egg basis during various activation procedures (Fig. 4).

When calibrated in this manner, which assumes equal distribution of free calcium in the cytoplasm, the peak currents of 0.053–0.21 nA/egg correspond to 2.5–4.5 × 10^{-6} M Ca^{2+} during fertilization.

These calibrations also assume that all the aequorin is accessible to the changes in calcium ion. To test this assumption, we poisoned aequorin-injected eggs with *n*-ethyl maleimide (NEM) and monitored the tonic emission as calcium was released. Figure 5 shows this experiment in which light corresponding to 2000 nC (anode voltage, 870 V) was emitted from 7 eggs over a 1 hr period. When the response had died down, we blew up each egg in turn with air injections while monitoring light output. No further emission was recorded, indicating that the NEM-induced Ca^{2+} release had exhausted all of the injected aequorin. Apparently, none of the injected aequorin was isolated from or prevented from reacting with internal Ca^{2+}.

TABLE 1

DIVALENT CATION BUFFER COMPOSITION OF CALIBRATION SOLUTIONS[a]

Solution No.	Total Ca (mM)	Total Mg (mM)	Total EGTA (mM)	Free Ca (μM)	Free Mg (mM)
1	3.40	11.03	10	1	10
2	6.07	10.61	10	3	10
3	7.56	10.38	10	6	10
4	8.38	10.25	10	10	10
5	9.42	10.09	10	30	10

[a] See text for details.

Bioassay of Peak Calcium Transients

In another effort to estimate free Ca^{2+} at fertilization, we isolated cortices, added back calcium quantitatively with calibrated solutions prepared in the manner described above, and fixed for observation

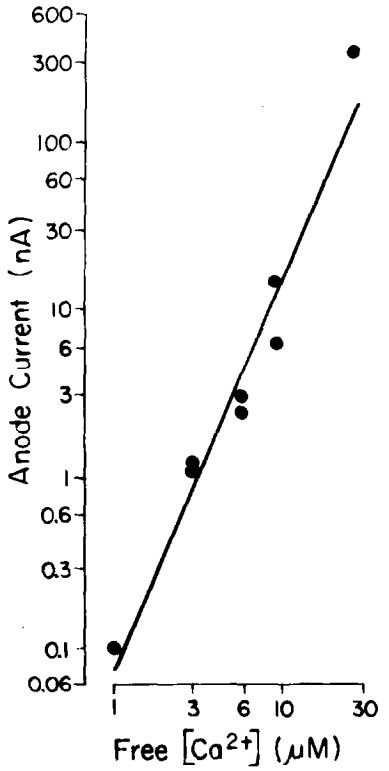

FIG. 4. Calibration of aequorin emission versus free Ca^{2+} on the basis of 10 injected eggs (see text). One nanoampere corresponds to 4 × 10^{-5} part injected aequorin exhausted per second. Anode voltage: 870 V. Temperature is 18°C.

FIG. 5. Aequorin emission from seven injected eggs poisoned with 1 mM NEM added at arrow. Blank spot in record is where tape reels were changed. Horizontal bar: 10 min. Vertical bar: 1 nA.

with a scanning electron microscope. In this way, we determined that the threshold for the discharge of the cortical granules is between 9 and 18 $\times 10^{-6}$ *M* Ca^{2+} (Fig. 6). Therefore, the threshold for the *in vitro* cortical reaction is about five times the amount of free Ca^{2+}, assuming equal distribution in the egg. This result suggests that Ca^{2+} release is localized in the cortical layer and that the highest Ca^{2+} levels are confined to the subsurface of the egg.

DISCUSSION

The chief biological importance of these observations lies in the confirmation by the most specific means possible that calcium release is one of the first consequences of fertilization. It is especially interesting that we are also able to confirm by these direct methods that calcium ionophore A23187 releases equivalent amounts of calcium. Exposure of sea urchin eggs to micromolar amounts of this ionophore initiates in the normal fashion: the cortical reaction with the elevation of the fertilization membrane, the increase in potassium conductance, the respiratory burst, and the increases in protein and DNA synthesis (Steinhardt and Epel, 1974). Now, with this unequivocal demonstration of normal Ca^{2+} release with ionophore, the calcium hypothesis of activation is greatly strengthened.

The second finding is that we can confirm that the calcium is released from an intracellular store both by normal fertilization and by treatment with the calcium ionophore. There has been one brief report that washing sea urchin eggs with 0 Ca^{2+} seawater containing 3 m*M* EGTA prevents ionophore activation of the cortical reaction (Ivy and Rebhun, 1976). However, this effect requires such extensive washing that 50% of the eggs lyse, and the authors of this report feel that the EGTA is removing intracellular calcium under their conditions (Rebhun, personal communication).

The third finding is the quantitative estimate of 2.5–4.5 μM for peak free Ca^{2+} during fertilization by the aequorin method, assuming equal distribution in the cytoplasm. This value is two to three orders of magnitude lower than the necessarily crude estimates from homogenates (Mazia, 1937; Steinhardt and Epel, 1974). In our bioassay, disruption of cortical granules *in vitro*, the threshold is between 9 and 18 μM, roughly five times the amount assuming equal distribution in the cytoplasm. We, at least tentatively, conclude that this difference indicates that the transient Ca^{2+} release is confined to the cortical layer and, because of the second power relationship of Ca^{2+} concentration to aequorin emission, we estimate the release space to be, at most, 4% of the volume of the egg. Thus, our interpretation of this data leads us to propose that the bulk of the cytoplasm never has a Ca^{2+} concentration as high as 1 μM even during activation. This upper limit for free Ca^{2+} could prove to be important in designing conditions for testing biochemical steps in *in vitro* models of metabolic activation. We are especially pleased to be able to offer this quantitative estimate for sea urchin eggs, since, so far, they are the best material for biochemical studies of fertilization (see Epel, 1975, for review).

APPENDIX

R. ZUCKER

Assay of Aequorin Activity

In order to satisfy any doubts about the efficiency and adequacy of our procedures for storing, handling, and desalting the aequorin, we felt it was necessary to be able to assay quantitatively its activity. In the absence of an appropriate liquid light standard, we found it necessary to rely on the manufacturer's calibration of our photomultiplier tube, performed at our request.

The effective concentration of active aequorin in microinjection solution may be estimated by adding a known volume, *V*, of the solution (0.1 ± 0.01 μl, using a Hamilton syringe plus Chaney adaptor) to an excess (10 μl) of 1 m*M* $CaCl_2$, while monitoring the light emitted with the photomultiplier tube. The anode current $I(t)$ rises rapidly to a peak and de-

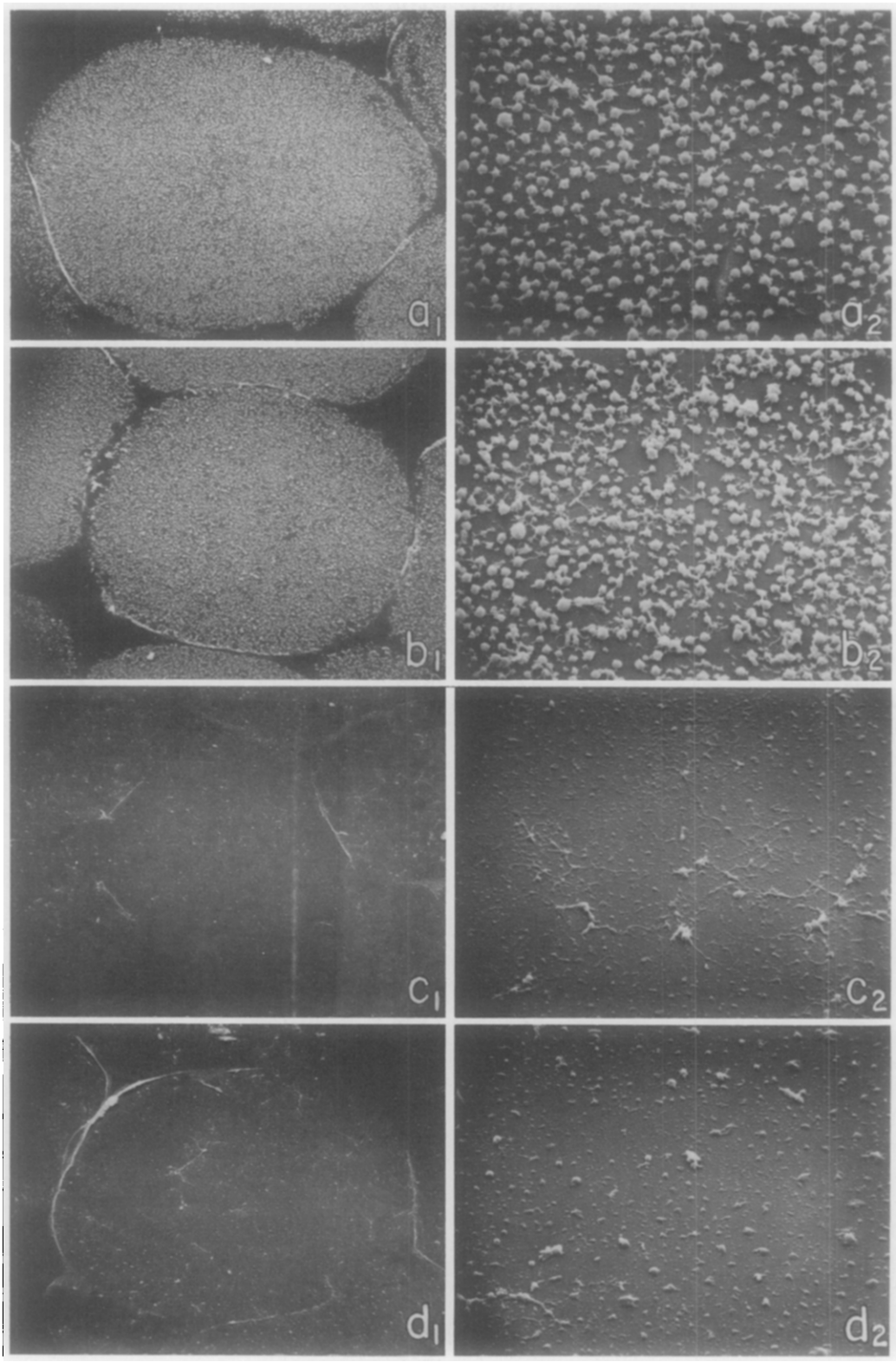

FIGURE 6.

clines exponentially with a time constant of 2.5–3 sec. The total anode charge (in nC) was computed as $Q = \int I(t)dt$. Based on this, the aequorin activity was calculated from the following relationship:

$$M = \frac{\bar{\lambda}Q}{EAVhcK(G/4\pi)} \cdot \frac{\int B(\lambda)S(\lambda)d\lambda \int A(\lambda)d\lambda}{\int A(\lambda)S(\lambda)T(\lambda)d\lambda \int B(\lambda)d\lambda}, \quad [1]$$

where M = molar concentration of active aequorin solution; $\bar{\lambda}$ = mean wavelength of the emission spectrum of aequorin, 4.74×10^{-7} m (Baker *et al.*, 1971); E = quantum efficiency of the calcium-aequorin reaction, 0.17 at 18°C, extrapolated from Shimomura and Johnson (1970) after correction for revised molecular weight (Blinks *et al.*, 1976); A = Avogadro's number, 6.025×10^{-23}; V = volume of aequorin solution in liters, usually 10^{-7} liter; h = Planck's constant, 6.625×10^{-35} J-sec; c = velocity of light, 3×10^{8} m/sec; K = calibration constant of photomultiplier tube for black body radiator at 2754°K at the anode voltage used, e.g., 5.3×10^{11} nA/W at 400 V; G = solid angle subtended by light guide from emission source, typically 0.03 sr; $A(\lambda)$ = relative emission spectrum of aequorin, from Shimomura and Johnson (1970); $S(\lambda)$ = relative spectral sensitivity of photomultiplier tube (from manufacturer); $B(\lambda)$ = relative emission spectrum of a black body at 2754°K (RCA, 1970); $T(\lambda)$ = absolute transmission spectrum for light guide (from manufacturer).

Equation [1] was derived as follows (cf. Baker *et al.*, 1971).

The anode current (I) resulting from a calcium-aequorin reaction is

$$I = (G/4\pi)\int A(\lambda)S(\lambda)T(\lambda)d\lambda. \quad [2]$$

The photomultiplier was calibrated by the manufacturer by measuring the anode current (I_s) resulting from exposure to a standard black body radiator at 2754°K. This current was

$$I_s = (G'/4\pi)\int B(\lambda)S(\lambda)d\lambda, \quad [3]$$

where G' was the solid angle subtended by the photomultiplier tube cathode from the black body radiator. The manufacturer provides a calibration factor (K) which is the ratio of the anode current to the power from the black body incident on the photomultiplier tube,

$$K = I_s/[(G'/4\pi)\int B(\lambda)d\lambda]. \quad [4]$$

From Eq. [2], [3], and [4], we have

$$\frac{I}{I_s} = \frac{(G/4\pi)\int A(\lambda)S(\lambda)T(\lambda)d\lambda}{(G'/4\pi)\int B(\lambda)S(\lambda)d\lambda} = \frac{I}{K(G'/4\pi)\int B(\lambda)d\lambda}, \quad [5]$$

or

$$\frac{I\int B(\lambda)S(\lambda)d\lambda}{K(G/4\pi)\int B(\lambda)d\lambda \int A(\lambda)S(\lambda)T(\lambda)d\lambda} = 1.$$

The total power (P) emitted by the calcium-aequorin reaction is

$$P = \int A(\lambda)d\lambda, \quad [6]$$

and, from Eq. [5] and [6], we have

$$P = \frac{I\int B(\lambda)S(\lambda)d\lambda \int A(\lambda)d\lambda}{K(G/4\pi)\int A(\lambda)S(\lambda)T(\lambda)d\lambda \int B(\lambda)d\lambda}. \quad [7]$$

In the derivation up to this point, $A(\lambda)$, $B(\lambda)$, and $S(\lambda)$ are absolute emission and sensitivity spectra. However, since these functions appear in both the numerator and denominator of Eq. [7], [9], and [1], relative spectral functions are sufficient. The rate of photon emission (R) is related to the power by:

$$R = P\bar{\lambda}/hc. \quad [8]$$

The number (N) of aequorin molecules reacting per second is $N = R/E$, where E is the quantum efficiency of the calcium-aequorin reaction. The rate of exhaustion of aequorin (dM/dt, in moles per liter per second) in a volume (V) of solution is $dM/dt = N/AV = R/EAV$, and

$$\frac{dM}{dt} = \frac{I\bar{\lambda}}{EAVhck(G/4\pi)} \cdot \frac{\int B(\lambda)S(\lambda)d\lambda \int A(\lambda)d\lambda}{\int A(\lambda)S(\lambda)T(\lambda)d\lambda \int B(\lambda)d\lambda}. \quad [9]$$

FIG. 6. Scanning electron micrograph of isolated inner surfaces before and after discharge with known concentrations of Ca^{2+}. (a_1) Inner surface in homogenization buffer at less than 75 nM Ca^{2+}. 700 ×. (a_2) Higher magnification of a_1 at 5000 × with individual 1.1-μm cortical granules intact. (b_1) Inner surface after washing and fixing in a Ca-EGTA buffer with 9 μM free Ca^{2+}, which was subthreshold for cortical granule discharge. (b_2) Higher magnification of b_1. 5000 ×. (c_1) Discharge of the cortical granules rapidly occurs in higher Ca^{2+} concentrations. Here, free Ca^{2+} = 18 μM. 700 ×. (c_2) Higher magnification of c_1. 5000 ×. (d_1) The image of surfaces exposed to even higher Ca^{2+} concentrations does not differ much from those at threshold concentrations. Free Ca^{2+} = 27.5 μM. 700 ×. (d_2) Higher magnification of d_1. 5000 ×.

The initial concentration of active aequorin is obtained by integrating Eq. [9]. Since only $I(t)$ is a function of time and $Q = \int I(t)dt$, Eq. [1] follows directly from Eq. [9]. For our photomultiplier tube and light guide, the right-hand factor in Eq. [1] and [9] had a value of 1.63.

Equation [1] indicated that our most active aequorin sample had an activity of 0.20 m*M*. This compares well with the concentration of aequorin before desalting on the Sephadex column, namely 0.25 m*M*. This indicates a small (20%) loss of aequorin activity caused by our preparation and storage procedures, due largely to dilution in the Sephadex column.

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REFERENCES

AZARNIA, R., and CHAMBERS, E. L. (1976). The role of divalent cations in activation of the sea urchin egg. 1. Effect of fertilization on divalent ion content. *J. Exp. Zool.* **198**, 65–78.

BAKER, P. F., HODGKIN, A. L., and RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.* **218**, 709–755.

BLINKS, J. R., PRENDERGAST, F. G., and ALLEN, D. G. (1976). Photoproteins as biological calcium indicators. *Pharmacol. Rev.* **28**, 1–93.

CHAMBERS, E. L., PRESSMAN, B. C., and ROSE, B. (1974). The activation of sea urchin eggs by the divalent ionophores A23187 and X-537A. *Biochem. Biophys. Res. Commun.* **60**, 126–132.

CHANG, J. J., GELPERIN, A., and JOHNSON, F. H. (1974). Intracellularly injected aequorin detects transmembrane calcium flux during action potentials in an identified neuron from the terrestrial slug, *Limax maximus*. *Brain Res.* **77**, 431–442.

CLOTHIER, G., and TIMOURIAN, H. (1972). Calcium uptake and release by dividing sea urchin eggs. *Exp. Cell Res.* **75**, 105–110.

EPEL, D. (1975). The program of and mechanisms of fertilization in echinoderm egg. *Amer. Zool.* **15**, 507–522.

IVY, N., and REBHUN, L. I. (1976). Calcium dependent activation of sea urchin eggs by sulfhydryl agents. *J. Cell Biol.* **70**, 202a.

JOHNSON, J. D., EPEL, D., and PAUL, M. (1976). Intracellular pH and activation of sea urchin eggs after fertilization. *Nature (London)* **262**, 661–664.

KOHAMA, Y., SHIMOMURA, O., and JOHNSON, F. H. (1971). Molecular weight of the photoprotein aequorin. *Biochemistry* **10**, 4149–4152.

MAZIA, D. (1937). The release of calcium in *Arbacia* eggs upon fertilization. *J. Cell. Comp. Physiol.* **10**, 291–304.

MAZIA, D., SCHATTEN, G., and SALE, W. (1975). Adhesion of cells to surfaces coated with polylysine. *J. Cell Biol.* **66**, 198–200.

NIGON, V., and DO, F. (1963). Les mouvements du calcium dans l'oeuf d'*Arbacia lixula* au moment de la fécondation. *C.R.H. Acad. Sci.* **257**, 2178–2180.

OGAWA, Y. (1968). The apparent binding constant of glycoletherdiaminetetraacetic acid for calcium at neutral pH. *J. Biochem.* **64**, 255–257.

PORTZEHL, H., CALDWELL, P. C., and RUEGG, J. C. (1964). The dependence of contraction and relaxation of muscle fibers from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. Biophys. Acta* **79**, 581–591.

RCA (1970). "Photomultiplier Manual." RCA Electronic Components, Harrison, N. J.

RIDGWAY, E. B., GILKEY, J. C., and JAFFEE, L. F. (1976). Free calcium increases explosively in activating medaka eggs. *J. Cell Biol.* **70**, 227a.

ROSE, B., and LOEWENSTEIN, W. R. (1976). Permeability of a cell junction and the local cytoplasmic free ionized calcium concentration: A study with aequorin. *J. Membrane Biol.* **28**, 87–119.

SHIMOMURA, O., and JOHNSON, F. H. (1970). Calcium binding, quantum yield, and emitting molecule in aequorin bioluminescence. *Nature (London)* **227**, 1356–1357.

STEINHARDT, R. A., LUNDIN, L., and MAZIA, D. (1971). Bioelectric responses of the echinoderm egg to fertilization. *Proc. Nat. Acad. Sci. USA* **68**, 2426–2430.

STEINHARDT, R. A., and EPEL, D. (1974). Activation of sea urchin eggs by calcium ionophore. *Proc. Nat. Acad. Sci. USA* **71**, 1915–1919.

STEINHARDT, R. A., EPEL, D., CARROLL, E. J., JR., and YANAGIMACHI, R. (1974). Is calcium ionophore a universal activator for unfertilized eggs? *Nature (London)* **252**, 41–43.

VACQUIER, V. D. (1975). The isolation of intact cortical granules from sea urchin eggs: Calcium ions trigger granule discharge. *Develop. Biol.* **43**, 62–74.

WERBLIN, F. S. (1975). Regenerative hyperpolarization in rods. *J. Physiol.* **244**, 53–81.