DEVELOPMENTAL BIOLOGY 135, 182-190 (1989)

Free Calcium Pulses following Fertilization in the Ascidian Egg

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Accepted May 16, 1989

Using the calcium-specific, chemiluminescent photoprotein aequorin, we have measured changes in the concentration of free cytosolic calcium at fertilization in single eggs of the ascidians *Phallusia mammillata* and *Ciona intestinalis*. Shortly after insemination, the free calcium concentration rises within a minute from a resting level of about 90 n*M* in the unfertilized egg to a peak level of about 7 μM in *Phallusia* and about 10 μM in *Ciona*. The total duration time of this fertilization transient is 2-3 min. It is immediately followed by a series of 12 to 25 briefer calcium transients with peak levels of about 1-4 μM. These postfertilization pulses occur at regular intervals of 1-3 min during the completion of meiosis, and they stop as soon as the second polar body is formed at about 25 min. An interesting exception to this pattern was observed in eggs from *Ciona* that had been raised at lower temperatures during the winter months. Insemination in the absence of external calcium in *Phallusia* results in a pulse pattern very similar to the normal pattern. From this result we infer that the bulk (if not all) of the calcium required for both the fertilization pulse and the meiotic oscillations is released from internal sources.

INTRODUCTION

It is now well established that fertilization of a mature egg results in a large transient increase in the concentration of free calcium ions in the egg cytoplasm (for a review see Jaffe, 1985). This pulse of free calcium releases the egg's developmental block and results in the resumption of its development.

In a variety of species, such as the sea urchin, starfish, medaka, frog, and golden hamster, this pulse of free calcium starts at the point of sperm entry (or prick activation) and travels as a wave toward the opposite pole of the egg (Gilkey *et al.*, 1978; Eisen *et al.*, 1984; Eisen and Reynolds, 1984; Busa and Nuccitelli, 1985; Miyazaki *et al.*, 1986; Yoshimoto *et al.*, 1986; Swann and Whitaker, 1986; Tsien and Poenie, 1986; Kubota *et al.*, 1987; Hafner *et al.*, 1988; Nuccitelli *et al.*, 1988). The existence of such a wave had been suggested in some species by earlier observations on waves of cortical vesicle exocytosis and contraction waves at fertilization (see, e.g., Yamamoto, 1961). It now seems that all deuterostome eggs may show such a wave of free calcium at activation (Jaffe, 1985).

Eggs of ascidians have never been investigated for the presence of pulses and waves of free calcium at activation. There are indications, however, that calcium does play an important role in the activation of these eggs. Treatment with calcium ionophore results in egg activation, as assessed by egg deformation and polar body formation, or by the segregation of the pigmented, cortical myoplasm (Steinhardt *et al.*, 1974; Bevan *et al.*, 1977; Jeffery, 1982). Local application of the ionophore on the egg surface causes this segregation to occur toward the point of high calcium, suggesting that calcium might also be involved in determining the direction of ooplasmic segregation at egg activation (Jeffery, 1982). Finally, microinjection of inositol 1,4,5-trisphosphate which is thought to release calcium from intracellular stores, also leads to activation of the egg (Dale, 1988). Although there are no cortical granules and thus no wave of exocytosis during activation in the ascidian egg, the wave of contraction described for several species (Reverberi, 1971; Sawada and Osanai, 1981; Sardet *et al.*, 1986, 1989) might be indicative of a wave of free calcium at egg activation.

We have investigated whether a pulse or a wave of free cytoplasmic calcium is involved in the activation of the ascidian egg. In this first paper, we describe changes in calcium levels with time, using aequorin and a photon counting system. We found that indeed there is a large pulse of free calcium at fertilization in the eggs of *Phallusia mammillata* and *Ciona intestinalis*. Interestingly, this fertilization pulse is followed by a series of calcium oscillations, which usually occurs during the completion of meiosis. Preliminary reports on part of these findings have appeared elsewhere (Speksnijder *et al.*, 1986a,b).

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0012-1606/89 $3.00

MATERIALS AND METHODS

Biological Material

P. mammillata was obtained from Roscoff (Brittany, France) and from Sète (Mediterranean, France) and kept in aquaria at 15–22°C. *Phallusia* is self-fertile; therefore care was taken that the oviduct and spermiduct of one animal were tapped separately. The dry sperm were stored on ice and the eggs were dechorionated in a 0.1% solution of porcine pancreas trypsin (Sigma, St. Louis) in seawater for 1–2 hr (Zalokar, 1979). Dechorionated eggs were rinsed in filtered seawater and kept in dishes coated with gelatin to prevent sticking and lysis of the eggs. All experiments with *Phallusia* were carried out at 18–21°C.

C. intestinalis was collected locally and kept in aquaria with running seawater at 12–18°C. Eggs and sperm were obtained by dissection and the dry sperm were stored on ice. Eggs were rinsed several times in filtered seawater, transferred to calcium/magnesium-free seawater (MBL formula; 461.9 m*M* NaCl, 10.7 m*M* KCl, 7.0 m*M* Na_2SO_4, 2.1 m*M* $NAHCO_3$) for 20–30 min to remove the follicle cells, and finally rinsed again in filtered seawater. During the fall, the eggs were kept at 18–21°C throughout all procedures; whereas during the winter all experiments were carried out at 14–15°C.

Microinjection of Aequorin

The dechorionated *Phallusia* eggs and defollicled *Ciona* eggs were transferred to an injection chamber and pressure-injected with aequorin as described by Hiramoto (1962) and Kiehart (1982). The calcium-specific photoprotein aequorin was generously supplied by Dr. Osamu Shimomura (Marine Biological Laboratory, Woods Hole, MA) as a solution of 4.1 mg/ml iso-aequorin D in 180 m*M* KCl, 100 μM EGTA (ethylene glycol bis(β-aminoethyl ether) *N,N,N'N'*-tetraacetic acid), 30 m*M* BES (*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, pH 7.1). We also used aequorin obtained from Dr. John Blinks (Mayo Foundation) as a 10 mg/ml solution in 150 m*M* KCl, 10 μM EGTA, 5 m*M* Hepes (*N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid, pH 7.5). The eggs were injected with 15–30 pl of the aequorin solution (1–2% of the total egg volume), after which the aequorin was allowed to diffuse for at least 30 min before any luminescence measurements were made.

Measurement of Aequorin Luminescence

In early experiments, aequorin-injected eggs were transferred from the injection chamber to a 1-ml polystyrene centrifuge tube (Fisher) filled with 200–250 μl filtered seawater. This tube was put in a brass holder and the entire assembly was pushed up against the window of a photomultiplier tube (Hamamatsu R464), equipped with a Pacific Precision Instruments photometer (Model 126) and amplifier/discriminator (Model AD126). A pool of mercury in the bottom of the brass holder envelops the bottom of the tube and serves to reflect the light emitted from the egg via a light pipe toward the window of the photo tube. The photomultiplier tube was operated with the cathode at −890 V and the anode near ground and cooled to −20°C to reduce the dark current to typically 1–3 counts/sec. The single photon pulses from the discriminator were lengthened via a pulse generator (Global, Model 4001) and counted in the 5-MHz clock-counter register of a Labmaster Data Acquisition Board (Scientific Solutions) located in one slot of an IBM PC/XT. The light output was displayed and stored as a function of time.

In most experiments, the aequorin luminescence was measured with an Imaging Photon Detector (IPD; Instrument Technology, Ltd., East-Sussex, UK) (Whitaker, 1985). For this purpose, the injection chamber containing the aequorin-injected egg was mounted onto the stage of a Zeiss IM 35 inverted microscope. The aequorin light was collected with an Olympus 40×/n.a. 1.3 oil objective and projected via a dichroic mirror, a reducing lens, and a shutter onto the photocathode of the IPD, which was cooled to −20°C. The anode signals were processed by the IPD-processor unit; the pulse output was displayed on a counter and the analog output corresponding to the rate of events was fed into a chart recorder.

In order to measure very small signals, such as the resting glow of an unfertilized egg, we increased the signal-to-noise ratio by focusing the full optical field on a region of only 2.5 mm in diameter. This is only 1/10 of the diameter of the full IPD photocathode; therefore the dark count in this small area is only 1/100 of the total (typically 10 counts/sec). This scheme allowed us to measure very small signals against a virtually zero noise level (only 0.1 count/sec). In order to record the signal from this small area only, a two-dimensional voltage window was constructed. Such a window was set typically around the 2.5-mm area on which the full optical field was focused. The resulting output was fed to a chart recorder.

First a measurement was made of the resting glow of an unfertilized aequorin-injected egg. Then the shutter was closed, sperm were added, and the shutter was opened again. The calcium-mediated light emission from the embryo was usually monitored for several hours, and the development of the embryo was checked afterward and followed for at least 24 hr.

The effects of removal of external calcium on fertilization and the kinetics and pattern of the calcium

pulses were determined by washing unfertilized aequorin-injected *Phallusia* eggs extensively in calcium-free seawater (MBL formula; 436.7 m*M* NaCl, 9.0 m*M* KCl, 22.9 m*M* $MgCl_2$, 25.5 m*M* $MgSO_4$, 2.1 m*M* $NaHCO_3$) to which 5 m*M* EGTA was added, preactivating sperm by incubation with chorionated eggs in natural seawater for 30 min (Sardet *et al.*, 1986, 1989), and adding a small volume of concentrated sperm suspension to the injection chamber containing the injected egg in calcium-free seawater plus 5 m*M* EGTA.

Calibration of the Aequorin Light Signal

Absolute calcium concentrations were estimated as described by Blinks (1982; pp. 29–31). In short, after the measurement of the light emission from the fertilized, aequorin-injected egg, the egg was lysed by exposure to a solution of 1.0% Triton X-100 in seawater. The total emitted light was measured and integrated over the period required for the full discharge. From this, the L_{max} (i.e., light emission under saturating calcium concentrations) was estimated and the fractional luminescence L/L_{max} was calculated. Finally, the absolute calcium concentration was estimated after correction for temperature and ionic conditions by using the calcium concentration–effect curves published by Blinks (1982) and by assuming that the free cytosolic potassium and magnesium concentrations in the *Phallusia* eggs are similar to those reported in the sea urchin egg and nerve and muscle cells of other marine organisms (e.g., Steinhardt *et al.*, 1971; Girard *et al.*, 1982; Alvarez-Leefmans *et al.*, 1987), i.e., about 200 and 1 m*M*, respectively. The amount of aequorin used up during the large fertilization pulse was calculated from the total light emitted during the fertilization pulse and the total light emitted during both the fertilization pulse and the full discharge during lysis of the egg. This ratio was found to range from 0.2 to 0.4, for which a correction was made in the calculation of the peak levels of calcium.

Control Experiments

The toxicity and stability of aequorin in the egg cytoplasm was tested by keeping injected *Ciona* eggs overnight before measuring the fertilization pulse. Similar peak levels of luminescence were found in these eggs when compared to the freshly dissected and injected ones.

The effects of aequorin injection were checked by monitoring the development of each injected egg. In *Phallusia*, 8/14 eggs developed in a tadpole larvae with a (sometimes reduced) tail and pigment spots and another 4 became arrested at gastrulation. Similar percentages were obtained in *Ciona* and in controls injected with carrier buffer only.

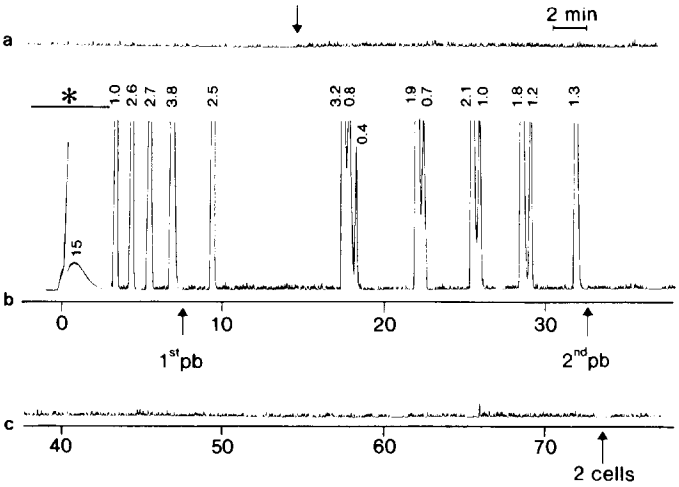

FIG. 1. Photon emission rates from *Phallusia* eggs. Trace a before arrow: An unloaded unfertilized control egg showing the 0.2 counts/sec background at maximum sensitivity using the voltage window. Trace a after arrow: An aequorin-loaded, unfertilized egg showing a 0.9 count/sec resting level above background. Trace b with asterix: The 15,000 counts/sec fertilization peak following insemination. The initial rise is shown at normal sensitivity, which is subsequently reduced to show the peak. Trace b after asterix: Return to maximum sensitivity (voltage window) to show the fine structure of the post-fertilization pulses as well as the resting level in between pulses. Peak levels in 10^3 counts/sec are indicated above each pulse. Trace c: Resting level after the completion of meiosis shown at maximum sensitivity. (Timing of the various events is slightly delayed due to the somewhat lower temperature of 18°C; cleavage in this egg occurred at about 70 min). Time in minutes after fertilization is indicated.

RESULTS

The Resting Level of Calcium in the Unfertilized Egg

The resting level of calcium in the unfertilized *Phallusia* egg was determined by averaging the luminescent signal over long periods of time (5–10 min) and comparing the signal of an aequorin-injected unfertilized egg with the background signal of an uninjected egg. The resting signal is rather small (about 1–6 counts/sec above an average total background of 12–13 counts/sec), but it can be clearly visualized (see Fig. 1) by reducing the size of the luminescent image on the IPD photocathode and using a window discriminator to monitor the count rate in the image area only as described under Materials and Methods (Fig. 1). From these measurements we calculated a resting level of calcium in the unfertilized egg of *Phallusia* of 90 ± 40 n*M* (mean ± SEM, n = 6; see Table 1).

The Calcium Pulse at Fertilization in Phallusia

Several minutes after the addition of preactivated sperm (see Sardet *et al.*, 1989), the aequorin-injected *Phallusia* egg shows an enormous increase in the cal-

cium-mediated light output (Figs. 1 and 2). In about 40–50 sec, the luminescence rises explosively to a peak level generally ranging from 2000 to 6000 counts/sec. Under optimal conditions, peak levels as high as 15,000 counts/sec can be measured (Fig. 1). This peak level is maintained for about 10–15 sec, after which the luminescent signal slowly returns to background levels. The total duration time of the fertilization pulse in *Phallusia* is about 2–3 min at 21°C (mean ± SEM = 2.8 ± 0.1, n = 10). From the peak level of luminescence during the fertilization pulse and the light signal obtained after lysis of the egg with Triton X-100 (Blinks, 1982), we calculated the peak level of free calcium during the fertilization pulse to be 6–7 μM (mean ± SEM = 6.7 ± 0.4, n = 4; see Table 1).

The Calcium Pulse at Fertilization in Ciona

As in *Phallusia*, the aequorin-injected *Ciona* egg shows a large increase in calcium-mediated luminescence after fertilization (Fig. 4). About 1 min after sperm addition, the luminescence rises rapidly within 30–50 secs to peak levels of 8000–40,000 counts/sec. These peak values are about fourfold higher than those measured for *Phallusia*. Yet the average total duration time of the fertilization pulse in both species is about the same, i.e., 2–3 min (2.8 ± 0.2, n = 8, for *Ciona* at 18–21°C, and 2.8 ± 0.1, n = 10, for *Phallusia* at 21°C). Moreover, the shape of the fertilization transient in both species is quite different: in *Ciona* one sees a very rapid, exponential rise to peak levels, which is immediately followed by an equally rapid fall to about 20% of the peak level, after which the luminescence levels gradually return to background. This slow falling phase lasts for about the final 60% of the total pulse time (Fig. 4). In contrast, the increase in luminescence in *Phallusia* is initially exponential and rapid, but the final rise to the peak level is much slower. The peak levels are maintained longer than those in *Ciona*, and the fall to background levels is slow and gradual (Fig. 2).

TABLE 1

FREE CYTOSOLIC CALCIUM CONCENTRATIONS BEFORE, DURING, AND AFTER FERTILIZATION IN EGGS OF THE ASCIDIANS *Phallusia mammillata* AND *Ciona intestinalis*

	Phallusia mammillata	*Ciona intestinalis*
Resting level (nM)	90 ± 40 (6)	—
Peak fertilization pulse (μM)	6.7 ± 0.4 (4)	10.8 ± 1.9 (5)
Peak postfertilization pulses (μM)	Max: 4.1 ± 0.4 (4) Min: 2.0 ± 0.4 (4)	Average: 2.4 ± 0.4 (6)

Note. Indicated are means ± SEM (n).

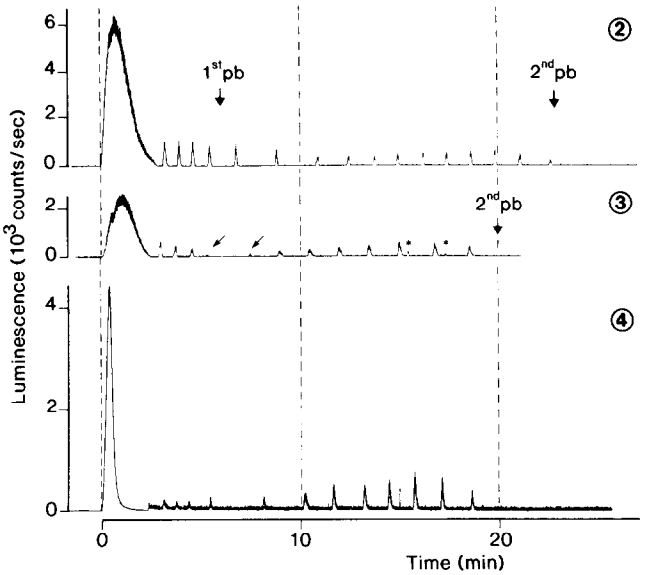

FIG. 2. Fertilization and postfertilization pulses in an aequorin-injected *Phallusia* egg. Note the sinusoidal change in the peak levels of the 16 subsequent postfertilization pulses (T = 21°C; cleavage started at 53 min).

FIG. 3. Luminescence signals following fertilization of an aequorin-loaded *Phallusia* egg in the absence of external calcium. Pulse pattern as well as peak levels of luminescence are similar to those in eggs fertilized in the presence of external calcium (see Fig. 2). The small postfertilization pulses Nos. 4 and 5 (total number is 14) are indicated with arrows; the out of phase echo pulses are indicated with asterisks. After perfusion of the chamber with natural seawater at 25 min, the egg cleaved at 42 min after fertilization.

FIG. 4. Photon emission rates from an aequorin-injected *Ciona* egg showing the 43,500 counts/sec fertilization peak at about 10-fold reduced sensitivity (T = 21°C; onset cleavage at 45 min). This fertilization pulse is followed by 12 postfertilization pulses. Polar body formation in this egg was obscured by chorion and test cells, but shape changes in the control group indicated that the second polar body was formed at about 20 min.

Finally, from the peak levels of luminescence we calculated that in *Ciona* the level of free calcium at fertilization reaches a peak of 7–18 μM (mean ± SEM = 10.8 ± 1.9, n = 5), which is about twofold higher than the value measured for *Phallusia* (Table 1). This twofold difference in calcium levels with a corresponding fourfold difference in luminescence levels is explained by the nonlinear response of aequorin to calcium; i.e., aequorin luminescence changes with about the 2.5 power of calcium.

Periodic Calcium Oscillations during Meiosis in Phallusia

A series of about 12–25 smaller and briefer calcium pulses is observed in the fertilized *Phallusia* egg during meiosis. These pulses start as early as 8–24 sec after the fertilization pulse has ended and continue until about 22–25 min after fertilization (Fig. 2). The duration of

each individual pulse is usually 12–16 sec. The time period during which they occur corresponds remarkingly well with the time of the meiotic divisions; the first polar body is extruded at the animal pole at 2–3 min after the onset of the pulse series, whereas the second polar body is formed within a minute after the last calcium pulse in the series has occurred (Table 2).

The pattern of these calcium oscillations also shows a number of characteristic features. The intervals between the first three to five pulses gradually increase from 30–60 sec to 60–100 sec. Then follows a period of about 2–8 min during which either no pulses or just one or two pulses occur. During the final part of the pulse train, the interval between the pulses is initially 60–100 sec, which usually increases to about 110–130 sec for the final few pulses. Interestingly, in about 50% of the eggs we found that during this last part of the pulse series one to three "echos" are present which start within 30 sec after the start of the previous pulse (Figs. 1 and 3). The reason why we call these out-of-phase pulses "echos" is because we have now evidence that the starting position of these pulses is usually at the opposite pole of the starting position of the previous pulse (Speksnijder *et al.*, 1988; Speksnijder *et al.*, in preparation).

The amplitude of the pulses is of the order of 50–1000 counts/sec, from which we calculate that the peak levels of calcium during these meiotic oscillations vary from 1 to 3 μM for the smallest peak in each record to 3–5 μM for the largest peak (means ± SEM are respectively 2.0 ± 0.4, $n = 4$, and 4.1 ± 0.4, $n = 4$, see Table 1). Usually, the amplitudes of the pulses change in a sinusoidal fashion; i.e., the pulse height of subsequent pulses first increases, then decreases, increases again, and finally decreases at the end of the pulse train (Fig. 2). Moreover, in 9 of 12 eggs the amplitude of the pulses seems to fall into two discrete groups with a twofold difference in the mean value of the pulse height (data not shown).

TABLE 2
TIMING OF CALCIUM PULSES, MEIOTIC DIVISIONS, AND CYTOKINESIS FOLLOWING FERTILIZATION IN THE ASCIDIAN EGG

	Phallusia mammillata (21°C)[a]	*Ciona intestinalis* (18–21°C)[a]
End fertilization pulse	2.8 ± 0.1 (10)	2.8 ± 0.2 (8)
Start first postfertilization pulse	3.1 ± 0.2 (9)	3.5 ± 0.3 (7)
Formation first polar body	6.0 ± 0.1 (10)	5–7[b]
Start final postfertilization pulse	23.0 ± 0.9 (9)	25.5 ± 1.8 (6)
Formation second polar body	23.5 ± 0.5 (11)	20–25[b]
First cleavage	53.5 ± 1.2 (10)	55–60

Note. Times are expressed in minutes after the start of the fertilization pulse; indicated are means ± SEM (n).

[a] Temperature at which the experiments were carried out.

[b] The exact timing of the meiotic divisions is unknown, due to the presence of the chorion and the test cells which obscure the small polar bodies.

Calcium Oscillations during Meiosis in Ciona

In *Ciona*, we observed that depending on the season and the temperature at which the experiment was performed, two different kinds of pulse patterns could be obtained. One series of 10 experiments was run during the fall: the animals were kept in aquaria at 18°C and the experiments were carried out at 18–21°C. Under these conditions, the pattern of calcium oscillations is very similar to that in *Phallusia*, i.e., a series of 12–22 pulses occurring at regular intervals, starting immediately after the fertilization pulse and ending around the time of second polar body formation (Fig. 4). The peak levels of calcium reached during these pulses are about 2–4 μM, which compares very well with the values obtained in *Phallusia* (Table 1).

However, during the winter, when we kept the animals at about 12°C and performed the experiments at 14–15°C, we found that in one aspect the pulse pattern was quite different. In all of the 14 eggs injected with aequorin and subsequently fertilized, the calcium oscillations did not stop at the time of second polar body formation, as they usually do in eggs developing at 21°C as described above. Instead, the oscillations occurred in two distinct groups; the first group of 3–6 pulses started immediately after the fertilization pulse and occurred over a period of several minutes. The second group of 7–12 pulses did not *start* (rather than end) until second polar body formation at about 25–29 minutes and continued for another 20–25 min. (Fig. 5; at this temperature cleavage occurs at about 70 min).

Fertilization in the Absence of External Calcium

In order to determine the source of the calcium responsible for the increase of the cytosolic calcium concentration during fertilization and meiosis, we inseminated eggs in the absence of external calcium. This experiment does not work in chorionated eggs of *Ciona*, perhaps because external calcium is required for the sperm to attach to and penetrate the chorion. On the other hand, dechorionated *Phallusia* eggs are readily fertilized in the absence of external calcium.

In a first series of experiments we examined the effect of removal of external calcium during fertilization and meiosis on development. For that purpose, large

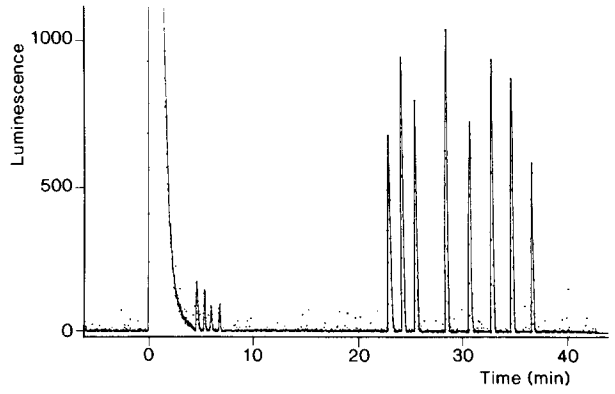

FIG. 5. Calcium-mediated aequorin luminescence (in counts/sec) following fertilization in *Ciona* at 15°C. At this temperature, a second series of postfertilization pulses starts around the time the control eggs form the second polar body (in this case at about 25 min after fertilization). The 32,000 counts/sec fertilization peak is not shown. In this experiment, the data were obtained with a photomultiplier tube and presented as individual data points every second rather than as a continuous chart recorder trace. For clarity, we connected the data points by a line leaving out the scattered noise. A realistic impression of the signal/noise ratio can be obtained from Figs. 2–4.

batches of several hundreds of eggs were rinsed in Ca-free seawater containing 5 m*M* EGTA and inseminated with a small volume of concentrated sperm that had been activated with chorionated eggs in seawater for 30 min (Sardet *et al.*, 1989). The EGTA present in the Ca-free seawater suffices to complex the calcium added to the medium with the sperm. Shortly after second polar body formation (i.e., the time at which the calcium oscillations normally stop), the eggs were transferred to natural seawater, and their development was followed. Interestingly, the percentage of embryos that develop into normal free-swimming tadpoles was very similar to that in nontreated control eggs of the same batch (76% in the treated embryos versus 74% in the controls). This shows that the ascidian egg is not dependent on external calcium for activation and the completion of meiosis.

In the second series of experiments we determined the effects of the absence of external calcium on the amplitude and the kinetics of the fertilization pulse and subsequent calcium oscillations in single aequorin-injected eggs. Figure 3 is an example of such an experiment; it shows that the timing and pattern of the pulses are similar to those in normal eggs. Although we have not quantified the peak calcium levels in these experiments, it appears that they fall within the same range as the controls, since the peak levels of luminescence are very similar (Table 3). This result suggests that most, if not all, of the calcium required for the fertilization pulse and subsequent calcium oscillations is released from internal stores.

DISCUSSION

Our main findings are: (1) The ascidian egg shows a large pulse of free cytosolic calcium at fertilization. During peak luminescence, the free calcium concentration reaches values of about 10 μM in *Ciona* and 7 μM in *Phallusia*, which is somewhat higher than the 1–3 μM reported for the sea urchin, frog, and golden hamster (Eisen *et al.*, 1984; Poenie *et al.*, 1985; Busa *et al.*, 1985; Swann and Whitaker, 1986; Miyazaki *et al.*, 1986; Hafner *et al.*, 1988; Nuccitelli *et al.*, 1988), but lower than the 30 μM estimated for medaka (Gilkey *et al.*, 1978). (2) This pulse is followed by a series of calcium transients with a period of several minutes and peak levels of about 1–4 μM; these calcium oscillations occur during a fixed time period which coincides with the completion of meiosis (however, an exception to this latter observation is found in *Ciona* eggs fertilized at lower temperatures during the winter months). (3) Removal of external calcium during fertilization and meiosis does not affect development nor the pulse pattern and amplitude, suggesting that the main source of calcium is internal. (4) About 50% of the aequorin-injected and fertilized eggs develop into tadpoles; this shows that the above described phenomena are natural and physiological ones.

Fertilization Pulse

Within a few minutes after sperm addition, the free cytosolic calcium concentration in the ascidian egg rises from a resting level of about 100 n*M* to peak levels of about 7 and 10 μM in *Phallusia* and *Ciona*, respectively. These values are based on an estimated cytosolic potassium and magnesium concentrations of 200 and 1 m*M*, respectively, and on the assumption that calcium is distributed homogeneously during peak luminescence. Our imaging data suggest that the latter is indeed the case

TABLE 3

PEAK LEVELS OF LUMINESCENCE FOLLOWING FERTILIZATION OF AEQUORIN-LOADED *Phallusia* EGGS IN THE PRESENCE OR ABSENCE OF EXTERNAL CALCIUM[a]

	Fertilization pulse (counts/sec)	Maximum postfertilization pulse (counts/sec)	Injected volume (pl)[b]
Ca-free seawater +5 m*M* EGTA	3020 ± 584 (5)	730 ± 110 (5)	28
Natural seawater (control)	3510 ± 375 (10)	533 ± 267 (12)	27

Note. Indicated are means ± SEM (*n*).

[a] Comparison based on experiments performed under identical measuring conditions.

[b] Injected volume of aequorin solution (same batch in all experiments).

(Speksnijder *et al.*, in preparation). However, it is very well possible that even higher free calcium levels are reached locally in the region of sperm–egg interaction at the time the calcium wave is initiated, as has been suggested for the sea urchin egg (Swann and Whitaker, 1986).

The explosive nature of the calcium transient during activation and the high calcium levels reached during this transient raise interesting questions as to the trigger and the mechanism of the increase in calcium. The fact that the initial rising phase is exponential suggests an autocatalytic phenomenon, which in other eggs has been proposed to involve either direct or indirect calcium-stimulated calcium release (Jaffe 1983, 1985; Busa *et al.*, 1985; Miyazaki *et al.*, 1986; Swann and Whitaker, 1986). The trigger of this autocatalytic process under natural conditions obviously is the sperm, but it is not clear yet *how* the sperm induces the chain reaction to start. It was first proposed that the sperm acts as a source of high calcium, which initiates the transient by locally introducing calcium at the trigger level (Schackmann *et al.*, 1978; Jaffe, 1983). More recently, evidence has been obtained that inositol 1,4,5-trisphosphate ($InsP_3$) may also be involved in the initiation of the calcium transient, since injection of $InsP_3$ at levels that will release calcium from isolated sea urchin cortices activates sea urchin eggs (Whitaker and Irvine, 1984; Turner *et al.*, 1986; Oberdorf *et al.*, 1986; Payan *et al.*, 1986). Injected $InsP_3$ will also activate ascidian eggs (Dale, 1988). The signal transduction pathway leading to the production of $InsP_3$ following fertilization in the sea urchin egg possibly involves a G-protein (Turner *et al.*, 1986, 1987). Finally, recent work of Swann and Whitaker (1986) on the sea urchin egg suggests that $InsP_3$ is also involved in the propagation of the fertilization wave by acting as a diffusible intermediate in calcium-stimulated calcium release.

Periodic Calcium Oscillations during Meiosis

The discovery of a series of 12–25 smaller calcium oscillations, which occurs between the end of the fertilization pulse and the second polar body formation—except in *Ciona* eggs that were fertilized at lower temperatures during the winter months—is another interesting aspect of our results. The peak levels of calcium reached during the oscillations are still fairly substantial (1–4 μM). Recently we have observed in *Phallusia* that during the same time period a series of pulsating surface and cytoplasmic movements occurs in the egg, with about the same period as the calcium oscillations (Sardet *et al.*, 1986, 1989). These calcium transients resemble in many ways the calcium pulses recently reported in hamster eggs (Miyazaki *et al.*, 1986; Miyazaki, 1988). However, the pulses in hamster eggs are dependent on external calcium (except for the first one or two), whereas the ones in ascidian eggs are not. In addition, the ascidian pulses are almost certainly natural developmental events, since they were observed in eggs which generally underwent several cleavages and frequently developed into swimming tadpoles. In contrast, the pulses in the hamster were observed in eggs that never cleaved.

It is not yet clear what the developmental significance of these postfertilization pulses is, but it is interesting to compare them with the agonist-induced calcium pulses reported in rat hepatocytes (Woods *et al.*, 1986, 1987) and recently also in human endothelial cells (Jacob *et al.*, 1988). Woods *et al.* (1987) suggest that the *frequency* rather than the amplitude of these calcium pulses is the principal determinant of the amplitude of the cellular response to calcium-mobilizing agonists. Such a frequency-encoded control mechanism of the cellular response to agonists might very well be a more general process, occurring in various types of cells with oscillating phenomena (see also Berridge, 1987; Berridge *et al.*, 1988; Jacob *et al.*, 1988).

Finally, our experiments demonstrate that the bulk of the calcium required for the large fertilization transient and subsequent meiotic oscillations is released from internal sources. This adds the ascidians to the group of deuterostomes such as the sea urchin, the medaka fish, and the frog in which it has been demonstrated that the source of calcium required for egg activation is internal and may consist of the cortical endoplasmic reticulum (Gilkey *et al.*, 1978; Charbonneau and Grey, 1984; Busa *et al.*, 1985; Eisen and Reynolds, 1985). We know that the *Phallusia* egg does possess a very elaborate network of endoplasmic reticulum in the cortex, which contains most of the sequestered calcium (Sardet *et al.*, 1988; Gualtieri and Sardet, 1989). However, it remains to be investigated whether this cortical endoplasmic reticulum is in fact the calcium store from which calcium is released during fertilization. It has been postulated that nonmuscle cells may possess $InsP_3$-sensitive calcium stores other than the endoplasmic reticulum (Volpe *et al.*, 1988). Finally, it is not clear whether the ascidian egg uses the same calcium store for both the fertilization and postfertilization pulses or whether it may possess two functionally separate calcium pools, as has been suggested for the *Xenopus* egg (Busa *et al.*, 1985). One thing is evident however, that apparently the kinetics of calcium release and sequestering during the fertilization pulse is species specific, as demonstrated by the differences between *Ciona* and *Phallusia* presented in this paper, i.e., the faster rise to peak levels of luminescence in *Ciona* and the twofold

higher levels of calcium reached during peak luminescence.

One matter still requires some discussion; i.e., the two different types of pulse patterns found in *Ciona*. At 18–21°C, the pattern of calcium oscillations is very similar to the pattern in *Phallusia* eggs at the same temperature. During the wintertime, however, the animals had to be kept at a lower temperature to prevent temperature shock and subsequently the experiments had to be carried out at a lower temperature to ensure normal development of the embryos. Under these conditions, the timing of the pulse pattern was very different from that observed at 21°C; rather than one almost continuous series of oscillations up to the time of second polar body formation, we see two series of pulses, with the second one starting at about the time the second polar body is formed. Yet development of these embryos was good. A similar seasonal difference has been observed in *Ciona* eggs with respect to the occurrence of spontaneous action potentials (Dale *et al.*, 1983). It is possible that the observed differences originate from season-dependent differences in the process of oocyte maturation.

In this report, we have described the magnitude and time course of the calcium transients following fertilization in the ascidian egg. Obviously, our research is now focused on resolving the spatial aspects of the calcium transients. Preliminary experiments with an imaging photon detector have already demonstrated that the calcium pulse at activation is in fact a wave of free calcium (Speksnijder *et al.*, 1986a; 1988). Using imaging techniques, we should be able to answer some important questions, such as (1) where does the fertilization wave start with respect to the polarity of the egg and the site of sperm entry—which we have recently determined to be preferentially in the animal hemisphere rather than near the vegetal pole, as previously thought (Speksnijder *et al.*, 1989); (2) are the meiotic oscillations localized, and if so, do they also travel as waves; and (3) what triggers these meiotic oscillations and what is their function?

The authors thank Dr. Osamu Shimomura for his generous gift of aequorin D, Philip Williams for designing the optical system and voltage window, Allen Shipley for technical assistance, and Miriam van Hattum for typing the manuscript. This work was supported by NIH Grant HD 18818 to L.F.J. and a NATO grant to C.S., J.E.S., and L.F.J.

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