The Activation Wave of Calcium in the Ascidian Egg and Its Role in Ooplasmic Segregation

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Abstract. We have studied egg activation and ooplasmic segregation in the ascidian *Phallusia mammillata* using an imaging system that let us simultaneously monitor egg morphology and calcium-dependent aequorin luminescence. After insemination, a wave of highly elevated free calcium crosses the egg with a peak velocity of 8–9 μm/s. A similar wave is seen in eggs fertilized in the absence of external calcium. Artificial activation via incubation with WGA also results in a calcium wave, albeit with different temporal and spatial characteristics than in sperm-activated eggs. In eggs in which movement of the sperm nucleus after entry is blocked with cytochalasin D, the sperm aster is formed at the site where the calcium wave had previously started. This indicates that the calcium wave starts where the sperm enters. In 70% of the eggs, the calcium wave starts in the animal hemisphere, which confirms previous observations that there is a preference for sperm to enter this part of the egg (Speksnijder, J. E., L. F. Jaffe, and C. Sardet. 1989. *Dev. Biol.* 133:180–184). About 30–40 s after the calcium wave starts, a slower (1.4 μm/s) wave of cortical contraction starts near the animal pole. It carries the subcortical cytoplasm to a contraction pole, which forms away from the side of sperm entry and up to 50° away from the vegetal pole. We propose that the point of sperm entry may affect the direction of ooplasmic segregation by causing it to tilt away from the vegetal pole, presumably via some action of the calcium wave.

Fertilization in ascidians, as in many other species, is accompanied by a transient increase in the concentration of free calcium ions in the egg cytoplasm (Gilkey et al., 1978; Eisen and Reynolds, 1984; Eisen et al., 1984; Busa and Nuccitelli, 1985; Miyazaki et al., 1986; Swann and Whitaker, 1986; Yoshimito et al., 1986; Kubota et al., 1987; Nuccitelli et al., 1988; Hafner et al., 1988; Speksnijder et al., 1989*a*). The release of intracellular calcium triggers a cortical contraction wave, meiosis, and a general reorganization of the egg (Steinhardt et al., 1974; Bevan et al., 1977; Jeffrey, 1982; Dale, 1988; Speksnijder et al., 1989*a*). This reorganization is characterized by two distinct phases of ooplasmic movements that define both the final distribution pattern of determinants and the bilateral symmetry of the embryo (e.g., Conklin, 1905; Whittaker, 1987; Bates and Jeffery, 1987, 1988; Sardet et al., 1989).

The factors that specify the timing and direction of ooplasmic segregation in ascidians are only partially understood. During the first phase of ooplasmic segregation, the subcortical myoplasm is moved vegetally by a contraction of the actin-rich cortex (Sawada, 1983; Sawada and Osanai, 1981, 1985; Jeffery and Meier, 1983; Sardet et al., 1989). Local application of the calcium ionophore A23187 causes this segregation to occur toward the site of application, which indicates that calcium might be involved in determining its direction (Jeffery, 1982). However, recent experiments by Bates and Jeffery (1988) suggest that the direction of the first phase is predetermined in the unfertilized egg and occurs along its animal–vegetal axis; a suggestion which is in accordance with the model proposed by Sawada (1983).

We decided to explore the role of calcium in defining patterns within the ascidian embryo by directly observing the spatial pattern of the increase in cytoplasmic calcium following fertilization. This paper complements our previous reports on the temporal aspects of intracellular calcium release in the egg of the ascidian *Phallusia mammillata*, in which we describe the occurrence of a large fertilization transient, which is immediately followed by a series of smaller, oscillatory calcium pulses (Speksnijder et al., 1989*a*). The present report is based on observations with an imaging system that allows one to simultaneously monitor the spatial distribution of free calcium in an individual egg cell and the morphology of that same egg with differential interference contrast (DIC) optics. We demonstrate that a wave of elevated calcium is initiated at the site of sperm entry, usually in the animal hemisphere of the egg. This calcium wave is followed by the wave of cortical contraction. Contrary to what we had assumed so far (Sardet et al., 1989), the axis of this contraction wave usually does not coincide with the animal–vegetal axis of the *Phallusia* egg.

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Materials and Methods

Biological Material

The European ascidian *Phallusia mammillata* was collected at Sète (Mediterranean Sea, France), and kept in aquaria at the Marine Biological Laboratory (Woods Hole, MA). Gametes were obtained by dissection. Since *P. mammillata* is self-fertile, care was taken that the oviduct and spermiduct of one animal were tapped separately. The dry sperm were stored at 4°C and the eggs were rinsed several times and subsequently dechorionated in a 0.1% solution of trypsin (Sigma Chemical Co., St. Louis, MO) in sea water for 1-2 h (Zalokar, 1979). Dechorionated eggs were rinsed in filtered sea water and kept in dishes coated with gelatin to prevent sticking and lysis of the eggs (Sardet et al., 1989). All manipulations were carried out at 18-21°C.

Microinjection of Aequorin

Dechorionated eggs were transferred to an injection chamber and pressure-injected with aequorin using the Hiramoto method (Hiramoto, 1962; Kiehart, 1982). The aequorin (iso-aequorin D; 4.1 mg/ml in 180 mM KCl, 100 μM EGTA, 30 mM BES [*N,N*-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid], pH 7.1) was a gift from Dr. Osamu Shimomura (Marine Biological Laboratory, Woods Hole). The eggs were injected with 15-30 pl of the aequorin solution (1-2% of the total egg volume). The aequorin was allowed to diffuse for at least 30 min before any luminescence measurements were made.

Activation of the Aequorin-injected Eggs

In most experiments, the injected eggs were inseminated by adding sperm preactivated with chorionated eggs (see Sardet et al., 1989). Unlike our previous study on the sperm entry point in *Phallusia* (Speksnijder et al., 1989*b*), in which we used very high sperm concentrations to ensure rapid and synchronous fertilization, we here used more dilute suspensions of sperm to prevent polyspermy and obtain good development. As a result, a delay of up to 30 min was sometimes found between insemination and fertilization. Eggs were also activated artificially by treatment with 50 μg/ml wheat germ agglutinin (WGA; Sigma Chemical Co.) in seawater.

The effects of removing external calcium on the pattern of the calcium-dependent aequorin signals was determined by washing the aequorin-injected eggs extensively in calcium-free seawater (Marine Biological Laboratory formula; 436.7 mM NaCl, 9.0 mM KCl, 22.9 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.1 mM $NaHCO_3$) to which 5 mM EGTA was added, preactivating sperm with chorionated eggs in natural seawater, and adding a small volume of concentrated sperm suspension to the injection chamber containing the injected egg in calcium-free seawater plus 5 mM EGTA.

Finally, in some experiments the movement of the sperm nucleus after entry was inhibited by blocking the cortical contraction that follows fertilization via treatment of the aequorin-injected eggs with 2 μg/ml cytochalasin D in seawater for 90-110 min, after which they were inseminated with preactivated sperm in the presence of cytochalasin D (see Speksnijder et al., 1989*b*). Control experiments were done to confirm that the sperm nucleus indeed does not move under these conditions by inseminating cytochalasin-treated eggs that were also preloaded with 10 μg/ml of the DNA-specific fluorescent dye H 33342 (Hinkley et al., 1987). In such eggs, the nucleus of the sperm that has entered the egg becomes fluorescent within seconds after fertilization (Speksnijder et al., 1989*b*), and any movements of the nucleus after fertilization can thus be observed in the living egg.

Imaging of the Aequorin Luminescence

The injection chamber containing the aequorin-injected egg was mounted on the temperature-controlled stage of a Zeiss IM 35 inverted microscope. The aequorin light was collected with an Olympus 40×/NA 1.3 oil objective and projected onto the photocathode of an imaging photon detector (IPD; Instrument Technology Ltd., East Sussex, UK). This device consists of a microchannel plate intensifier with a resistive anode as the positional encoder (see Whitaker, 1985). The analogue signals provided by the tube are passed on to a signal processing unit, which calculates the x-y coordinates of each photon as it arrives and presents this information as both digital and analogue outputs. The digital output was fed and stored in a IBM PC/XT equipped with a parallel digital I/O board (Metrabyte Corp., Taunton, MA). The analogue signals for x and y were used for real-time display on an oscilloscope operating in a non-time-based mode, and the analogue output corresponding to the rate of events was fed into a chart recorder.

To correlate changes in egg morphology with changes in free cytosolic calcium, we devised a scheme to observe the image of the specimen in transmission-type illumination while simultaneously measuring and imaging the aequorin light output. This was done by taking advantage of the fact that Newvicon video cameras are highly sensitive to longer wavelengths of light, while aequorin emission occurs in the blue-green region of the spectrum (λ_{max} = 469 nm; Blinks, 1982). Light from a tungsten light source was prefiltered with a long-pass glass absorption filter (cut-off 650 nm). This red light was used to illuminate the specimen. A dichroic mirror was placed just below the objective to reflect the blueish aequorin light out the side of the microscope toward the IPD. The red illumination beam is transmitted by the dichroic mirror and thus enters the rest of the microscope optical train to be picked up by the red-sensitive Newvicon camera. Since not all of the red light is attenuated upon reflection by the dichroic mirror, the reflected beam was further filtered by two short-pass absorption filters. Although this cuts the aequorin signal by ~50%, the transmission type interference is reduced by about five orders of magnitude. This results in only a slight increase in the background to a signal of ~20 counts/s above the dark current of ~10 counts/s.

Finally, the aequorin image was reduced in size by passing it through a 75-mm optical doublet, and focussed on the photocathode of the IPD. By varying the distance between the IPD and the fixed lens, a variety of image sizes can be obtained. As described in a previous paper, small images are very useful for measuring very small signals, such as the resting glow of an unfertilized egg (Speksnijder et al., 1989*a*). Larger images are used to improve spatial resolution where count rate is not a limiting factor, as during the fertilization wave described in this paper.

Simultaneous imaging was obtained by displaying the analogue output of the IPD processor corresponding to the x-y coordinates on an oscilloscope. This real-time image of the photons as they are being registered by the IPD was monitored by a video camera synchronized with the transmission beam camera mounted on the microscope. The two resulting video signals were mixed and recorded in time-lapse mode on a Sony TVO 9000 Umatic recorder or on a GYYR (Anaheim, CA) VHS recorder.

Image Analysis

The digital output of the x and y coordinates of the registered photons was stored in an IBM PC/XT as a sequential file. This file was then used to reconstruct images of photons collected over any desired period of time. In addition, these data were used to analyze the direction and velocity of the calcium waves, and to generate profiles of luminescence intensity across the egg.

The temporal and spatial correlation between the aequorin luminescence and the morphological events accompanying activation were analyzed using the videotape records of the mixed video signals. An Image-1 image processing system (Universal Imaging Corp., Media, PA) was used for part of this analysis.

Results

The Calcium Wave at Fertilization

After the addition of preactivated sperm, the aequorin-injected *P. mammillata* egg shows an enormous increase in the calcium-mediated light output (Fig. 1 *a*). We have demonstrated previously that the peak levels of luminescence during the fertilization transient correspond to peak levels of calcium of ~7 μM (Speksnijder et al., 1989*a*). By using an imaging device, we have now determined that this increase starts locally, and propagates across the egg at a peak velocity of 8-9 μm/s (mean ± SEM = 8.5 ± 0.4; n = 3) (Figs. 2 *a* and 3). After the wave of elevated calcium has crossed the egg, the calcium level remains elevated in the entire egg and gradually decreases homogeneously to finally reach resting levels after ~3 min.

The relationship between the calcium wave and the morphological reorganization of the egg occurring at fertilization was investigated using an imaging system that allowed simultaneous observation of the aequorin luminescence and a DIC (differential interference contrast) image of the egg. For op-

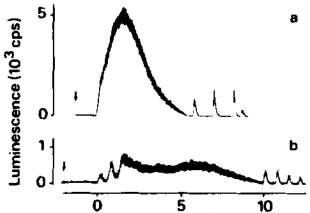


Figure 1. Photon emission rates from aequorin-injected *Phallusia* eggs. (*a*) Sperm activation, trace shows the 5,000 cps fertilization pulse as well as the first four post-activation pulses after the addition of sperm (*arrow*). (*b*) Activation after addition of WGA (*arrow*); the activation pulse is prolonged and has four separate peaks that are much lower than during sperm activation. The first four postactivation pulses are also shown.

timal analysis of these events, eggs were chosen that had a favorable orientation, i.e., with the animal–vegetal axis parallel to the microscope image plane. In the eggs of *P. mammillata*, the animal pole is defined by the location of the first meiotic spindle, which is right underneath the surface, and can be clearly visualized in DIC optics via a clear zone surrounding the spindle (Fig. 4). In this orientation, the processes of cortical contraction and ooplasmic segregation after fertilization can be clearly observed (see Sardet et al., 1989).

Simultaneous observation of both the calcium events and the DIC image of the egg shows that the calcium wave clearly precedes the wave of cortical contraction (Fig. 2 *a*). The first change of shape indicating the onset of the contraction wave can generally be detected shortly after the calcium wave has filled the entire egg, i.e., ~30–40 s after the calcium wave was seen to start.

In the majority of the eggs studied, the starting position of the calcium wave is located in the animal hemisphere. In only 5 out of 17 eggs analyzed (29%) did the wave start in the vegetal hemisphere; in the remaining 12 eggs (71%) the calcium wave started in the animal hemisphere. Similar percentages were obtained if only eggs that developed into tadpoles were considered ($n = 7$).

Calcium Waves in the Absence of External Calcium

We have reported that fertilization in the absence of external calcium results in a series of calcium pulses with amplitude and kinetics similar to those observed during normal fertilization (Speksnijder et al., 1989*a*). We have now compared their spatial characteristics. In the four eggs thus treated, the fertilization wave proceeded as in eggs fertilized in the presence of external calcium (Fig. 2 *c*). It started in the animal hemisphere in two eggs and in the equatorial region in the remaining two. This supports the notion that in the ascidian egg calcium is released from internal stores during activation and meiosis, and that apparently an influx of external calcium does not occur or is not required for the correct pattern, both in time and space, of the activation wave.

Calcium Waves in Lectin-activated Eggs

Dechorionated unfertilized eggs can be activated by treatment with Con A or WGA. Typical postfertilization shape changes are induced, albeit more slowly, one and sometimes two polar bodies are extruded, and an aster is formed in the center of the egg. However, such eggs never cleave (Zalokar, 1980; Speksnijder, J. E., and C. Sardet, unpublished observations). We have investigated whether artificial activation with lectins is accompanied by an elevation of cytoplasmic free calcium, by treating aequorin-injected eggs with 50 μg/ml WGA, and measuring the calcium-mediated light output.

Several minutes after the addition of WGA, the luminescence levels gradually start to increase, and stay elevated for a longer period than during normal fertilization. The total duration time of the activation pulse was, respectively, 10 and 20 min in the two eggs studied. This activation pulse showed three to four peaks in both eggs, each next peak being higher than the previous one, and finally reached maximum peak levels of luminescence of ~200–600 counts/s (Fig.1 *b*).

The spatial distribution of the WGA-induced calcium signals was studied using the IPD. We observed that the three to four peaks within the activation pulse all start in the same region (not shown). This region might be determined by the number of occupied lectin binding sites per surface area, since in both eggs the calcium pulses started on the side facing the opening of the chamber, i.e., the side the lectin solution was applied from. The initial localized increase does not fill the whole egg; it is not until the final peak in the activation pulse that a sustained signal filling the entire egg is obtained (not shown). These results suggest that (*a*) WGA activates the egg by increasing intracellular levels of calcium; (*b*) a certain threshold of calcium needs to be reached before a calcium wave is initiated that travels the entire diameter of the egg.

The Calcium Wave in Relation to the Sperm Entry Point

As shown earlier in this paper, the calcium wave at fertilization starts in the animal hemisphere in 70% of the eggs analyzed. This number fits with our previous observation by independent means that in 70–80% of *P. mammillata* eggs, the fertilizing sperm enters the animal hemisphere (Speksnijder et al., 1989*b*). This suggests, that the calcium wave may be initiated at the site of sperm entry.

Unfortunately, determining the exact relationship between the sperm entry site and the starting position of the calcium wave in the ascidian egg is complicated, because it is extremely difficult to observe sperm entry directly in the living egg and simultaneously image aequorin luminescence. In addition, the sperm nucleus is quickly carried toward the vegetal pole during the cortical contraction wave that follows fertilization (Speksnijder et al., 1989*b*). Therefore we explored the relationship between the site of sperm entry and the starting position of the calcium wave in aequorin-injected eggs in which the cortical contraction was blocked with cytochalasin D (Zalokar, 1974; Sawada and Osanai, 1981; Sawada and Schatten, 1989; Speksnijder et al., 1989*b*). We monitored the calcium wave at fertilization in five such eggs and despite the interference with the action of actin filaments, all of these five eggs showed a normal wave of calcium at fertilization (Fig. 2 *b*). In addition, the location of the growing sperm aster, which is first clearly visible in differential interference contrast at ~10 min after fertilization (Sardet et al., 1989), coincided with the starting position of the calcium wave (Fig. 5).

To check whether the sperm indeed does not move after entry in cytochalasin-treated eggs and to confirm that the site of sperm aster formation is the site where the sperm entered,

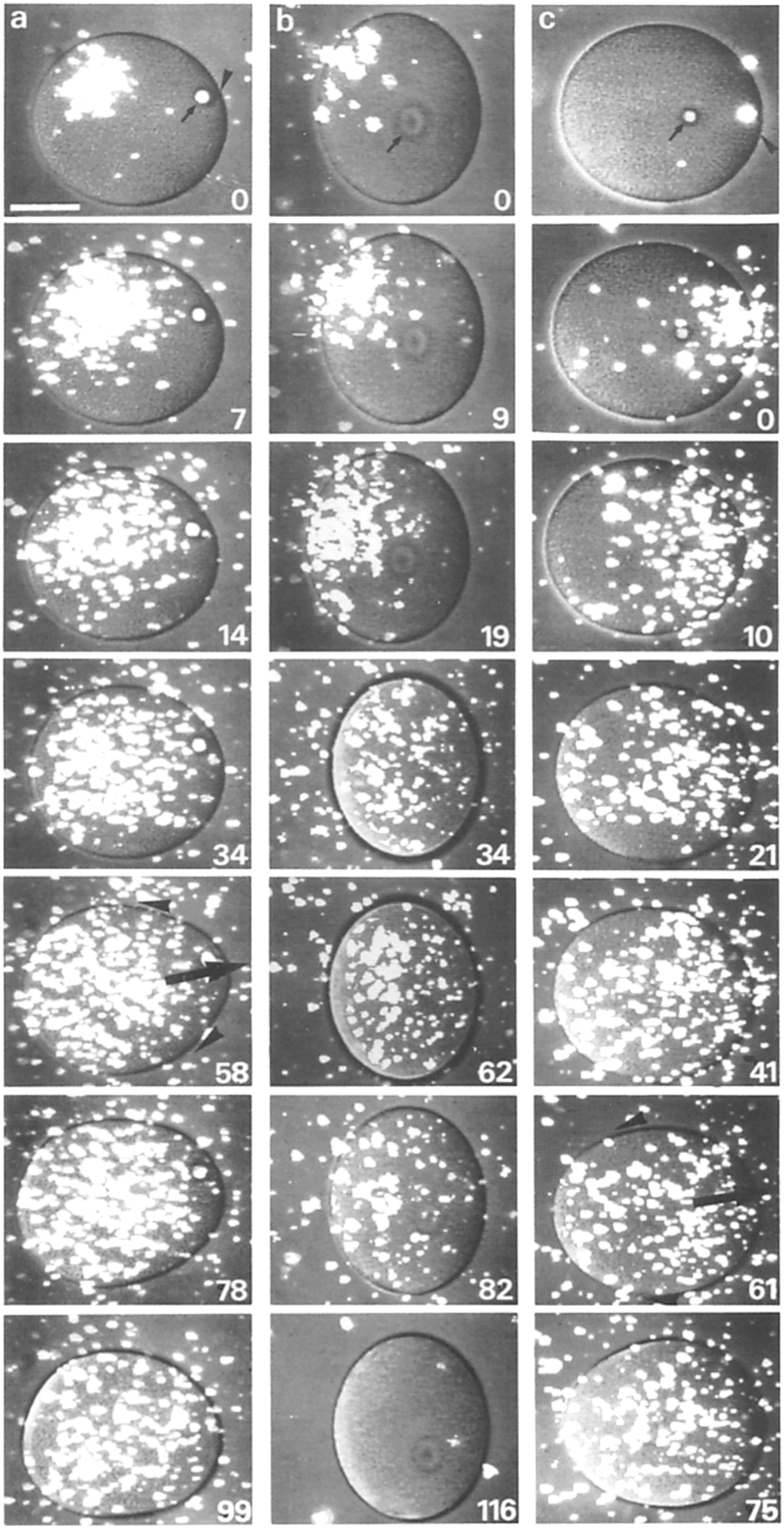


Figure 2. Overlay of the differential interference contrast (DIC) image and the calcium-dependent luminescent image of *Phallusia* eggs during activation. Indicated are times in seconds after the onset of the calcium wave. The arrowheads indicate the position of the meiotic spindle marking the animal pole; the arrows point to the small oil droplet that was coinjected with the aequorin. Bar, 50 μm. (*a*) Calcium wave at fertilization, which in this egg starts near the equator, and crosses in 30–40 s. It precedes the wave of cortical contraction, which starts between 34 and 58 s and travels along the surface in vegetal direction (*arrowheads*), thereby causing the internal cytoplasm to bulge out near the animal pole (*arrow*). (*b*) Fertilization wave in an egg pretreated with 2 μg/ml cytochalasin D for 2 h. The calcium wave progresses normally, but the wave of cortical contraction is blocked. The animal pole is located in the upper focal plane toward the objective and is therefore not visible in this series. The image is slightly elongated due to distortion by the video monitor. (*c*) Fertilization wave in the absence of external calcium, which in this egg starts close to the animal pole (*arrowhead*). The contraction wave has just started at 41 s, and is clearly visible at 61 s.

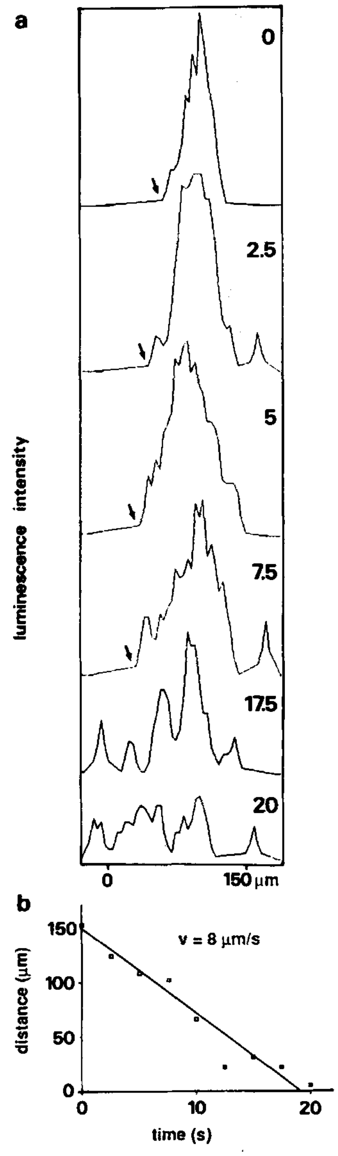


Figure 3. (*a*) Propagation of the calcium wave at fertilization shown by intensity distribution profiles of the aequorin luminescence taken parallel to the direction of the wave. Distributions of the light intensity (number of photons) were taken at 2.5 s intervals following the time (in seconds) shown at the right of each profile. Arrows indicate the position of the wave front. Data were obtained from the egg in Fig. 2 *a*. (*b*) Position of the wave front in the above intensity profiles as a function of time; the distance traveled by the wave front is corrected for the vertical component in the direction of the calcium wave (see Fig. 2 *a*). Curve fitting of the data from 0 to 10 s (*open squares*) reveals progression of the calcium wave at a velocity of 8 μm/s.

we inseminated and followed six cytochalasin-treated eggs that were also preloaded with the DNA-specific dye H 33342. In all of these eggs we could determine the position of the comma-shaped nucleus of the sperm that had entered within 1 min after fertilization. We followed its position during the 10-min period it takes to decondense and form an aster. No detectable movement of the sperm nucleus was observed during this period in these six different eggs (not shown). Thus we can be confident that the site where the sperm aster is formed is the site where the sperm entered the egg. This, together with the observation that the sperm aster is formed at the site where the calcium wave started, demonstrates that the calcium wave is initiated at the site of sperm entry in cytochalasin-treated eggs.

From this result we extrapolate that also in normal, untreated eggs the calcium wave starts at the point of sperm entry. This extrapolation is justified for two reasons: first, the physiological and temporal characteristics of the calcium wave are similar in cytochalasin-treated and normal eggs, which indicates that they are initiated and propagated by the same mechanism. Second, the fertilization waves of calcium in all species known to be activated by such a wave (i.e., medaka fish, sea urchin, starfish, golden hamster, and frog) are very similar to the ascidian one with respect to their characteristics: they do not require external calcium, and show similar propagation velocities. In addition, they are all shown to be initiated by the sperm at its entry point (Gilkey et al., 1978; Eisen et al., 1984; Busa and Nuccitelli, 1985; Miyazaki et al., 1986; Yoshimoto et al., 1986; Swann and Whitaker, 1986; Kubota et al., 1987; Hafner et al., 1988).

The Direction of the Calcium Wave and the Contraction Wave

Previous studies have suggested that the direction of the first phase of ooplasmic segregation and thus of the cortical contraction is toward the vegetal pole (Sawada, 1983, 1988; Bates and Jeffery, 1988). Our results confirm that independent of the starting position of the calcium wave, the cortical contraction indeed always occurs in the general direction of the vegetal pole. This is even true when the calcium wave starts in the vegetal hemisphere; a rare occurrence due to the preference of sperm to enter in the animal hemisphere (Speksnijder et al., 1989*b*). This suggests, that the direction of the calcium wave does not determine the direction of the contraction wave leading to the first phase of ooplasmic segregation.

However, careful examination of eggs that had completed the first segregation phase revealed that the position of the contraction pole and the myoplasmic cap is rarely directly opposite the first polar body (Fig. 6). This polar body is extruded at the animal pole and does not move from that position as confirmed by DIC optics on living eggs. In only one out of 13 eggs that were oriented properly for an analysis of this phenomenon, the contraction pole was at 180° to the animal pole. In the remaining 12 eggs, the contraction pole lay between 130 and 165° from the animal pole (Table I). Thus it was tilted 15 to 50° (mean ± SEM = 31 ± 4°) from the vegetal pole defined as the pole diametrically opposite to the meiotic site. In addition, the sperm aster was always found on the side of the egg along which the contraction wave had traveled over the longest distance (Fig. 6; Table I). We also observed that the closer sperm entry occurs toward the equator (at 90° from the animal pole), the further away the contraction pole is located from the vegetal pole (Fig. 7). However, this relationship was found only in eggs that developed into tadpoles, but not in those that became arrested at the gastrula stage.

From the observation that the sperm entry point, the position of the sperm aster and the contraction pole show a more or less fixed relationship, we infer that the site where sperm entry occurs along the animal–vegetal axis is of great relevance for the direction of the contraction wave. We suggest that the point of sperm entry may help determine the final location of the myoplasmic cap by shifting the direction of the contraction wave away from the vegetal pole, and that it may do so by way of the calcium wave.

Finally, the data in Table I also show that after entry, the sperm nucleus is carried toward the vegetal pole during the first phase of ooplasmic segregation. In the majority of eggs, sperm entry occurs in the animal hemisphere (Table I). Yet in those same eggs, the sperm aster is always found in the vegetal hemisphere at 10–15 min after fertilization. The sperm nucleus thus travels over an angular distance of 80–110° in vegetal direction after entry above the equator.

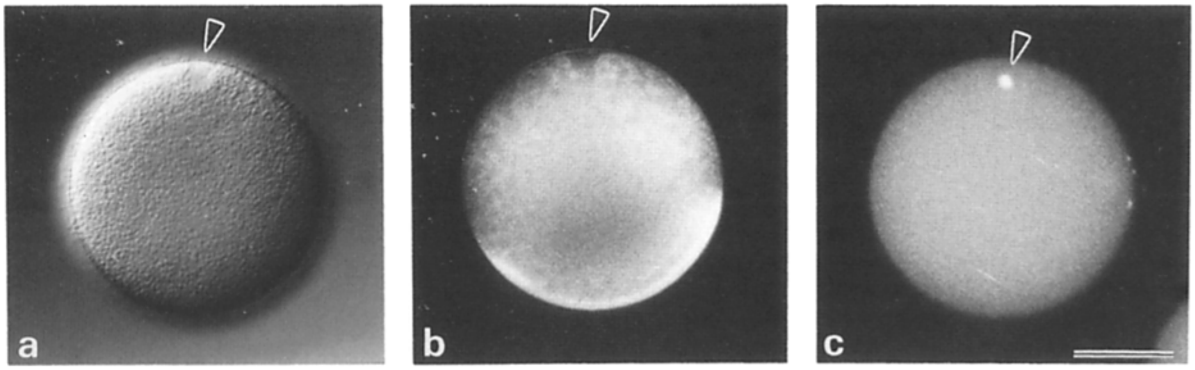


Figure 4. Visualization of the animal pole (*arrowheads*) in the unfertilized egg of *Phallusia*. Bar, 50 μm. (*a*) Differential interference contrast image showing the clear zone surrounding the meiotic spindle. (*b*) Egg stained with the fluorescent mitochondria-specific dye diOC_2, showing the mitochondria-rich subcortical myoplasm that is absent in the animal hemisphere. (*c*) Fluorescence image after labeling with the DNA-specific dye H33342 showing the female chromosomes that mark the animal pole.

These data confirm our previous observations with the DNA-specific dye H33342, that the sperm travels toward the vegetal pole within minutes after fertilization (Speksnijder et al., 1989*b*).

Development of Aequorin-injected Eggs

As reported in our previous paper (Speksnijder et al., 1989*a*), about half of the aequorin-injected fertilized eggs develop into tadpoles. In the particular experiments described in this paper, 2 out of 17 eggs showed an abnormal first or second cleavage, 8 eggs cleaved normally but failed to gastrulate, and finally 7 eggs (41%) developed into tadpoles. One such tadpole is shown in Fig. 9. No differences in the starting position of the calcium wave, nor in the progression of this wave or in the direction of the contraction wave were observed between the group of eggs that did develop into tadpoles and the group that did not. From this we infer that the early developmental events we studied proceeded normally. Furthermore, considering that in general further development of early zygotes used for the measurement of intracellular calcium levels is not documented or poor, we feel confident that the calcium patterns we observed represent natural and physiological phenomena.

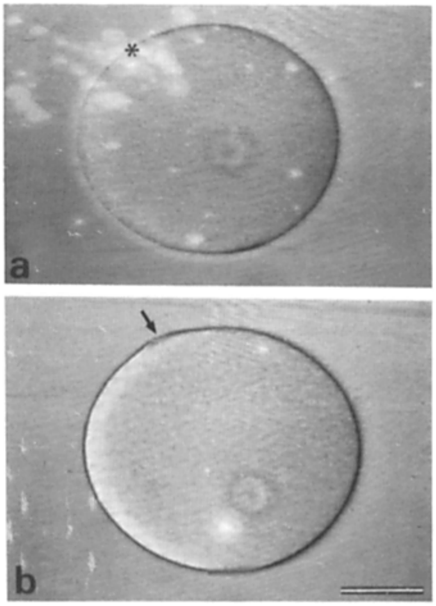


Figure 5. Relationship between the starting position of the calcium wave and the sperm entry point in a *Phallusia* egg pretreated with cytochalasin to block movement of the sperm nucleus following fertilization (same egg as in Fig. 2 *b*). Bar, 50 μm. (*a*) Onset of the calcium wave near the equator of the egg (*asterisk*); the animal pole is in the upper focal plane and therefore not visible. (*b*) The sperm aster is first clearly detected at 9 min following fertilization and is located near the starting position of the calcium wave. It slightly bulges the egg surface (*arrow*).

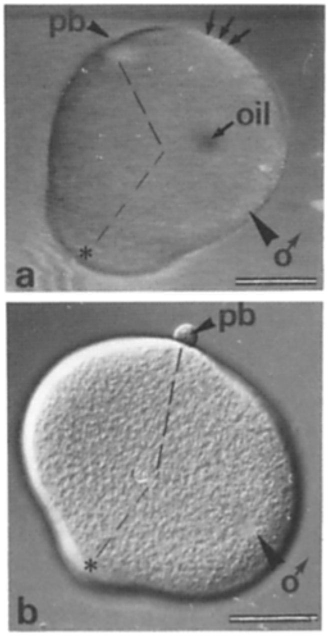
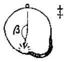


Figure 6. Position of the contraction pole and the sperm aster relative to the animal pole following the first phase of ooplasmic segregation. Bar, 50 μm. (*a*) Picture taken from a videorecord of an aequorin experiment: The triple arrows indicate the starting position of the fertilization wave of calcium at ~50° from the animal pole as observed ~9 min earlier; the sperm aster (♂) is now located in the vegetal hemisphere on the same side of the egg. The contraction pole (*asterisk*), around which the myoplasm is centered, is not directly opposite the polar body (*pb*) marking the animal pole, but is at ~120° from the latter. The oil droplet which was coinjected with the aequorin is visible. (*b*) Egg observed with differential interference contrast (DIC) optics, showing its contraction pole at ~150° from the animal pole.

Table I. Relationship between the Animal Pole, the Sperm Entry Point, and the Contraction Pole in Eggs of Phallusia mammillata

Exp No.	*	‡	§	Development‖
36	20 ± 20	?	160	+++
38	25	125	165	++
48	50	140	130	++
52	70 ± 20	160	130	+++
41	165 ± 15	180	165	++
42	180 ± 20	150	165	++
37	?	195	165¶	+++
58	10	120	140	+
39	20	100	145	+
44	60	220	140¶	+
46	65	150	155	+
50	165 ± 15	160	130	+
43	?	180	180¶	+

* The angular distance α (in degrees) between the meiotic site marking the animal pole, and the site of sperm entry identified by the starting position of the calcium wave.

‡ The angular distance β between the polar body marking the animal pole and the position of the sperm aster at 10–15 min following fertilization.

§ The angular distance γ between the animal pole and the contraction pole at 10–15 min after fertilization.

‖ Development at 15–18 h after fertilization: +++, normal tadpole; ++, slightly abnormal larva (e.g., short or crooked tail); +, embryo arrested at the gastrula stage.

¶ Eggs in which the sperm aster was located at the contraction pole.

Note: Not included in this table are eggs that were dispermic (three cases), cleaved abnormally (two cases), or were oriented such that analysis of the angles was not possible (two cases).

Discussion

We have previously described the temporal aspects of the increase in cytosolic calcium in the egg of *P. mammillata* upon fertilization, and considered the physiological mechanisms by which the sperm induces this increase (Speksnijder et al., 1989*a*). In this report, we have documented the spatial pattern of the calcium signals that follow fertilization in the ascidian egg. These results, together with our previous work on the sperm entry point and the various movements that follow fertilization (Speksnijder et al., 1989*b*; Sardet et al., 1989), provide an integrated picture of the events that take place when a *P. mammillata* egg is fertilized (see Fig. 9).

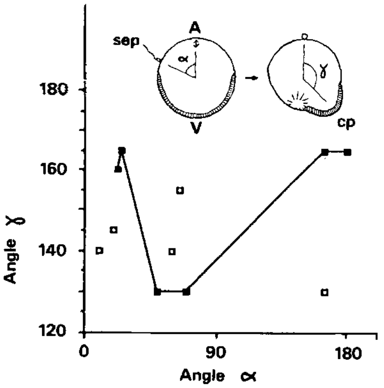


Figure 7. Relationship between the angular distance α between the animal pole and the sperm entry point (*sep*), and the angular distance γ between the animal pole and the contraction pole (*cp*) (see diagram at top of figure). The plot shows that in the eggs that developed into tadpoles (*filled squares*), the angle γ is smallest (and thus the angular distance between the contraction pole and the vegetal pole largest) when sperm entry occurs near the equator (α ∼90°). See text and Table I for further explanation.

The Calcium Wave at Fertilization and its Relationship to the First Phase of Ooplasmic Segregation

The first detectable event after insemination is a local elevation of the free cytosolic calcium concentration, which spreads to the opposite pole of the egg at a speed of 8–9 μm/s (at 18–21°C). This value corresponds to the speeds reported for the calcium wave in a wide variety of eggs (Jaffe, 1985; Busa and Nuccitelli, 1985; Swann and Whitaker, 1986; Kubota et al., 1987; Miyazaki et al., 1986). In all closely observed deuterostome eggs other than those of ascidians, fertilization induces a wave of cortical granule exocytosis, which is used as an indication of the propagation of a calcium wave even without direct measurement. Our finding of a calcium wave even in ascidians, which lack an exocytotic response to fertilization, strengthens the evidence that a calcium wave is a universal and highly conserved mechanism for activating deuterostome eggs (Jaffe, 1983).

It is not until 30–40 s after the onset of the fertilization wave that changes in egg morphology occur. A bulging at the animal pole is the first manifestation of the wave of cortical contraction, which travels to the vegetal hemisphere with a surface velocity of 1.4 μm/s (Sardet et al., 1989), and accompanies the segregation of the subcortical myoplasm. It is therefore clear that the contraction wave does not correspond directly to the calcium wave in time nor velocity. However, it seems likely that the elevation in calcium is the trigger of the contraction wave, since the latter can be induced by treatment with calcium ionophore (Bevan et al., 1977; Dale, 1988), as well as by injection of the calcium-releasing second messenger inositol 1,4,5-trisphosphate ($InsP_3$) (Dale, 1988; Speksnijder, J. E., unpublished observations). The 30–40-s delay between the rise in calcium and the contraction wave suggests that a complex machinery needs to be assembled or activated by calcium before contraction of the cortical actin network takes place.

Our cytochalasin experiments show that the calcium wave is initiated at the site of sperm entry. In nontreated eggs, the calcium wave usually starts in the animal hemisphere, which corresponds to the preference for sperm entry in that area (Speksnijder et al., 1988*b*). However, even in the eggs in which the calcium wave is initiated in the vegetal hemisphere, the contraction wave starts near the animal pole and travels in a generally vegetal direction. Thus, as suggested before, the location of the myoplasmic cap appears to be mainly determined by the animal–vegetal axis of the egg (Sawada, 1988; Bates and Jeffery, 1988).

However, careful observations on the position of sperm entry, sperm aster, and contraction pole suggest that the direction of contraction is determined by the animal–vegetal axis only to a certain extent, since we found that the myoplasmic cap is almost never located right at the vegetal pole, but can be as much as 50° away from the latter. In retrospect, we had already observed this phenomenon in a previous paper (Sardet et al., 1989; see Fig. 1, *A–H*), but at that time we did not appreciate its significance. Furthermore, upon rereading the literature we realized that Zalokar had already documented this phenomenon in *Phallusia* (Zalokar, 1974;

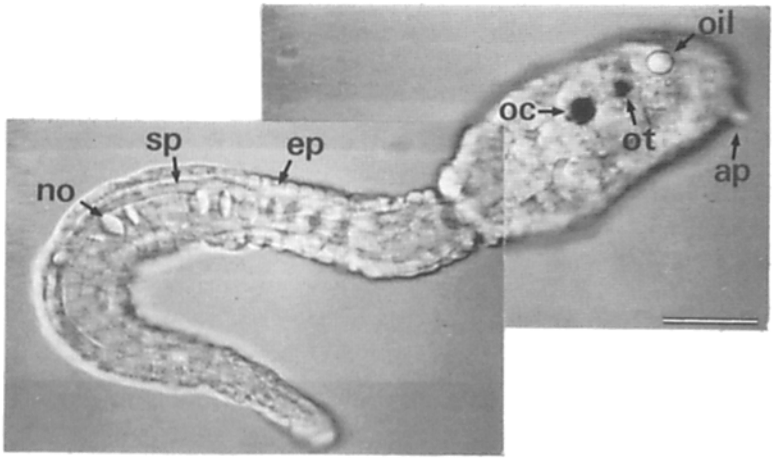


Figure 8. 15-h-old larva raised after fertilization of an aequorin-injected egg in the injection chamber. Note the oil droplet that was coinjected with the aequorin in its head. *ap*, adhesive papillae; *ep*, epidermis; *no*, notochord; *oc*, ocellus; *ot*, otolith; *sp*, spinal cord. Bar, 50 μm.

Fig. 1) without further commenting on it, and that Dalcq had described this phenomenon in the egg of the ascidian *Ascidiella*, although his finding was not clearly expressed (Dalcq, 1938; p. 109) and minimally documented (Dalcq and Vandebroek, 1937; Fig. 1). This asymmetry could already be present in the unfertilized egg, as was claimed for *Ascidiella* (Dalcq, 1938), and become subsequently reinforced after fertilization. However, we have not found evidence for such asymmetry in the unfertilized egg of *Phallusia*. Rather, the distinct relationship between the position of the sperm aster and the contraction pole (Table I) suggests that the sperm is involved in determining the direction of the first phase of ooplasmic segregation. We feel that the point where sperm entry occurs along the animal–vegetal axis might determine the angular distance over which the contraction pole is shifted away from the vegetal pole.

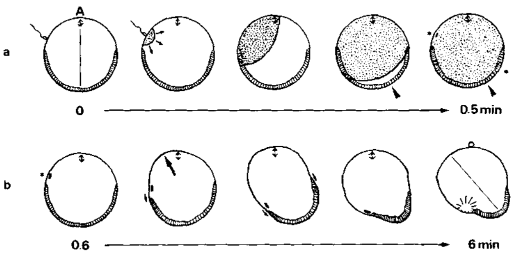


Figure 9. Summary of the events that follow fertilization in the ascidian egg. (*a*) After the addition of sperm to an aequorin-injected unfertilized egg, a large pulse of free calcium is observed. This increase starts locally at the point of sperm entry, which occurs preferentially in the animal hemisphere. The increase of calcium spreads across the egg as a wave in ~30 s with a peak velocity of 8–9 μm/s. The diagram illustrates our model in which the calcium wave lags in the vegetal hemisphere, and therefore ends at a point between the vegetal pole and the antipode to the starting position (*asterisk*). If a residue of calcium were left at this endpoint (*arrowhead*), it could provide a focus for the wave of cortical contraction that segregates the subcortical myoplasm in vegetal direction. (*b*) The wave of cortical contraction starts shortly after the calcium wave has filled the egg and is first visible as a bulge near the animal pole (*arrow*). This wave is driven by the contraction of a cortical actin network, which, like the subcortical myoplasm (*striped area*), is more sparse or absent from the animal pole region. The wave ends in a contraction pole, around which the myoplasm is centered, and which is usually not located directly opposite the polar body marking the animal pole. The contraction of this cortical actin network is triggered by the wave of elevated free calcium. This diagram illustrates the possibility that this cortical network contracts asymmetrically, simply because the contraction is likely to start first near the site of sperm entry (*asterisk*). As a result, the contraction pole will be located away from the side of sperm entry and away from the vegetal pole. Thus a second axis of asymmetry is established (*line*). See text for further explanation.

To explain how the position of sperm entry may affect the direction of segregation, we suggest that there might be two mechanisms at work, which are not mutually exclusive (see Fig. 9). The first mechanism is based on observations on activation in the medaka fish egg. When this egg is prick-activated at its equator, the resulting exocytotic wave proceeds more quickly near the animal pole than near the vegetal one, with the result that it ends at a point halfway between the pole diametrically opposite the pricking site and the vegetal pole (Yamamoto, 1961). More recently Yoshimoto et al. (1986) have confirmed these results by activating a medaka egg at its equator via microinjection of calcium and observing the resulting calcium wave directly with aequorin. If a similar reduction of calcium wave speed occurs in the egg of *Phallusia*, then the end point of the calcium wave would be located in the vegetal hemisphere even if the calcium wave is initiated near the equator (Fig. 9 *a*). Therefore we suggest that the contraction pole forms at the terminus of the activating wave and the reason it would form there is because unattenuated calcium waves are thought to leave a residue of high calcium. Even though this slowing down of the calcium wave in the vegetal half and the proposed residue are not obvious in the *Phallusia* egg, which could be due to its much smaller size (140 μm) as compared with the medaka egg (1.2 mm), this model is consistent with the observation of Jeffery (1982), that if calcium ionophore gradients are applied to ascidian (*Boltenia*) eggs, the myoplasm tends to aggregate toward the high ionophore end. It therefore seems that the contraction pole can be induced to form in all or most regions of the egg if cytosolic calcium is raised there. This in turn suggests that indeed a rise in cytosolic calcium naturally occurs in the forming contraction pole and is a major factor in determining its final location.

The second mechanism that could contribute to an asymmetric position of the contraction pole involves an asymmetric contraction of the cortical actin network, which is initiated at the starting position of the calcium wave (Fig. 9 *b*). The actin network has been described as a basket with its opening toward the animal pole (Sawada, 1983, 1988), a situation also found to be true in unfertilized eggs of *Phallusia* (Sardet, C., and J. E. Speksnijder, unpublished observations). Contraction of this basket results in its movement toward the vegetal pole, and as a result, in a bulging of the centrally located cytoplasm toward the animal pole. Assuming that the contraction is triggered by the elevation of free calcium, then it would start first at the side of the egg where the sperm enters and where the calcium wave is initiated, and last at the side opposite the sperm entry point. In those eggs in which sperm entry does not occur exactly on either the animal or vegetal pole, the contraction of the actin network would thus be asymmetric, and the myoplasm would be segregated to a site near but not right at the vegetal pole. If this model were true, one would expect the contraction pole to be furthest away from the vegetal pole when sperm entry occurs near the equator. We found this to be the case in the aequorin-injected eggs that developed into tadpoles.

It is clear that further research is required to elucidate the mechanism by which the point of sperm entry and the calcium wave affect the direction of ooplasmic segregation. In this light, it is important to note that neither of the above described mechanisms by itself can explain all the observed phenomena. For example, the first model fails to explain why sperm entry at or near the vegetal pole, even though rare, still results in segregation of the myoplasm toward the vegetal hemisphere (Table I; Exps. 41 and 42). Yet it would account for the observation that ooplasmic segregation tends to be directed toward the high point of an artificial calcium gradient. On the other hand, the latter observation would be difficult to explain by the mechanism proposed in the second model. Therefore, both of the proposed mechanisms might be involved in the observed shift in the direction of contraction.

As yet it is unclear whether such a shift is a general phenomenon occurring in eggs of all ascidians. Interestingly, the only other ascidian in which the formation of a contraction pole away from the vegetal pole has been described is *Ascidiella*, a species which is considered to be closely related to *Phallusia*. To our knowledge, such a shift has not been observed in the more distant species, such as *Styela*, *Molgula*, and *Ciona*. It seems unlikely that this phenomenon is the result of dechorionation of the egg, since ooplasmic segregation in the denuded egg of *Ciona* has been investigated extensively by Sawada and co-workers, and yet they have not described or otherwise documented a similar shift in this species (Sawada and Osanai, 1981, 1985; Sawada, 1988).

From our observations we infer that in the egg of *Phallusia mammillata*, the point of sperm entry helps determine the direction of ooplasmic segregation, and we suggest that it may do so via some action of the calcium wave. The relevance of this phenomenon for early embryonic development should be sought in the establishment of a second axis of developmental asymmetry, which runs through the contraction pole around which the myoplasm is centered, and its antipode. This axis might well correspond to the dorsal/ventral axis, since recent work on the egg of the ascidian *Styela* has demonstrated that axial determinants are cosegregated with the myoplasm during the first phase of ooplasmic segregation (Bates and Jeffery, 1987). These factors determine the future site of gastrulation and thus the dorsal side of the embryo. This asymmetry is subsequently reinforced during the second phase of ooplasmic segregation, when the larger part of the myoplasm becomes located at the future posterior side of the embryo.

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References

Bates, W. R., and W. R. Jeffery. 1987. Localization of axial determinants in the vegetal pole region of ascidian eggs. *Dev. Biol.* 124:65–76.

Bates, W. R., and W. R. Jeffery. 1988. Polarization of ooplasmic segregation and dorsal-ventral axis determination in ascidian embryos. *Dev. Biol.* 130:98–130.

Bevan, S. J., D. S. O'Dell, and G. Ortolani. 1977. Experimental activation of ascidian eggs. *Cell Diff.* 6:313–318.

Blinks, J. R. 1982. The use of photoproteins as calcium indicators in cellular physiology. *Tech. Cell. Physiol.* P126:1–38.

Busa, W. B., and R. Nuccitelli. 1985. An elevated free cytosolic calcium wave follows fertilization in eggs of the frog, *Xenopus laevis. J. Cell Biol.* 100: 1325–1329.

Conklin, E. G. 1905. The organization and cell-lineage of the ascidian egg. *J. Acad. Natl. Sci. (Phila.).* 13:1–126.

Dalcq, A. M. 1938. Form and Causality in Early Development. Cambridge University Press, Cambridge, UK. p 108.

Dalcq, A., and G. Vandebroek. 1937. On the significance of the polar spot in ripe unfertilized and in fertilized ascidian eggs. *Biol. Bull.* 72:311–318.

Dale, B. 1988. Primary and secondary messengers in the activation of ascidian eggs. *Exp. Cell Res.* 177:205–211.

Eisen, A., and G. T. Reynolds. 1984. Calcium transients during early development in single starfish (*Asterias forbesi*) oocytes. *J. Cell Biol.* 99:1878–1882.

Eisen, A., D. P. Kiehart, S. J. Wieland, and G. T. Reynolds. 1984. Temporal sequence and spatial distribution of early events of fertilization in single sea urchin eggs. *J. Cell Biol.* 99:1647–1654.

Gilkey, J. C., L. F. Jaffe, E. B. Ridgway, and G. T. Reynolds. 1978. A free calcium wave traverses the activating egg of the medaka, *Oryzia latipes. J. Cell Biol.* 76:448–466.

Hafner, M., C. Petzelt, R. Nobiling, J. B. Pawley, D. Kramp, and G. Schatten. 1988. Wave of free calcium at fertilization in the sea urchin egg visualized with fura-2. *Cell Motil. Cytoskel.* 9:271–277.

Hinkley, R. E., R. N. Edelstein, and P. Ivonnet. 1977. Selective identification of sperm fused with the surface of echinoderm eggs by DNA-specific bisbenzimid (Hoechst) fluorochromes. *Dev. Growth Differ.* 29:211–220.

Hiramoto, Y. 1962. Microinjection of the live spermatozoa into sea urchin eggs. *Exp. Cell Res.* 27:416–426.

Jaffe, L. F. 1983. Sources of calcium in egg activation: a review and hypothesis. *Dev. Biol.* 99:265–276.

Jaffe, L. F. 1985. The role of calcium explosions, waves and pulses in activating eggs. *In* Biology of Fertilization. C. B. Metz and A. Monroy, editors. Vol. 3. pp. 127–165. Academic Press, San Diego, CA.

Jeffery, W. R. 1982. Calcium ionophore polarizes ooplasmic segregation in ascidian eggs. *Science (Wash. DC).* 216:545–547.

Jeffery, W. R., and S. Meier. 1983. A yellow crescent cytoskeletal domain in ascidian eggs and its role in early development. *Dev. Biol.* 96:125–143.

Kiehart, D. P. 1982. Microinjection of echinoderm eggs: apparatus and procedures. *Methods Cell Biol.* 25:13–31.

Kubota, H. Y., Y. Yoshimoto, M. Yoneda, and Y. Hiramoto. 1987. Free calcium wave upon activation in *Xenopus* eggs. *Dev. Biol.* 119:129–136.

Miyazaki, S.-I., N. Hashimoto, Y. Yoshimoto, T. Kishimoto, Y. Igusa, and Y. Hiramoto. 1986. Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. *Dev. Biol.* 118:259–267.

Nuccitelli, R., D. Kline, W. B. Busa, R. Talevi, and C. Campanella. 1988. A highly localized activation current yet widespread intracellular calcium increase in the egg of the frog, *Discoglossus pictus. Dev. Biol.* 130:120–132.

Sardet, C., J. E. Speksnijder, I. Inoué, and L. F. Jaffe. 1989. Fertilization and ooplasmic movements in the ascidian egg. *Development.* 105:237–249.

Sawada, T. 1983. How ooplasm segregates bipolarly in ascidian eggs. *Biol. Bull. Mar. Biol. Station, Ashamushi, Tohoku Univ.* 17:123-132.

Sawada, T. 1988. The mechanism of ooplasmic segregation in the ascidian egg. *Zool. Sci.* 5:667-675.

Sawada, T., and K. Osanai. 1981. The cortical contraction related to the ooplasmic segregation in *Ciona intestinalis* eggs. *Roux's Arch. Dev. Biol.* 190: 208-214.

Sawada, T., and K. Osanai. 1985. Distribution of actin filaments in fertilized egg of the ascidian *Ciona intestinalis*. *Dev. Biol.* 111:260-265.

Sawada, T., and G. Schatten. Effects of cytoskeletal inhibitors on ooplasmic segregation and microtubule organization during fertilization and early development in the ascidian *Molgula occidentalis*. *Dev. Biol.* 132:331-342.

Speksnijder, J. E., D. W. Corson, C. Sardet, and L. F. Jaffe. 1989*a*. Free calcium pulses following fertilization in the ascidian egg. *Dev. Biol.* 135:182-190.

Speksnijder, J. E., L. F. Jaffe, and C. Sardet. 1989*b*. Polarity of sperm entry in the ascidian egg. *Dev. Biol.* 133:180-184.

Steinhardt, R. A., D. Epel, E. J. Carrol, and R. Yanimachi. 1974. Is calcium ionophore a universal activator for unfertilised eggs? *Nature (Lond.).* 252:41.

Swann, K., and M. J. Whitaker. 1986. The part played by inositol trisphosphate and calcium in the propagation of the fertilization wave in sea urchin eggs. *J. Cell Biol.* 103:2333-2342.

Whitaker, M. J. 1985. An imaging photon detector for the measurement of low-intensity luminescence. *J. Physiol. (Lond.).* 365:5P.

Whittaker, J. R. 1987. Cell lineages and determinants of cell fate in development. *Am. Zool.* 27:607-622.

Yamamoto, T.-O. 1961. Physiology of fertilization in fish eggs. *Int. Rev. Cytol.* 12:361-405.

Yoshimoto, Y., T. Iwamatsu, K. Hirano, and Y. Hiramoto. 1986. The wave of free calcium upon fertilization in medaka and sand dollar eggs. *Dev. Growth Differ.* 28:583-596.

Zalokar, M. 1974. Effect of colchicin and cytochalasin B on ooplasmic segregation of ascidian eggs. *Wilhelm Roux's Arch. Dev. Biol.* 175:243-248.

Zalokar, M. 1979. Effects of cell surface binding on development of ascidian egg. *Wilhelm Roux's Arch. Dev. Biol.* 187:35-47.

Zalokar, M. 1980. Activation of ascidian eggs with lectins. *Dev. Biol.* 79:232-237.