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BRIEF NOTES

Periodic Calcium Waves Cross Ascidian Eggs after Fertilization

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Ascidian eggs respond to fertilization with one to two dozen periodic calcium pulses (J. E. Speksnijder, D. W. Corson, C. Sardet, and L. F. Jaffe, 1989a, *Dev. Biol.* **135**, 182-190). We examined the spatial pattern of these pulses and found that they are initiated in discrete regions from which they propagate as waves. The first few pulses start in the animal hemisphere, whereas the later ones are mostly initiated near the vegetal pole. Such vegetal waves are often followed by a contraction of the egg surface. Since these waves are attenuated as they spread, they repeatedly expose the vegetal pole region to more calcium. The mechanism of these repetitive calcium waves and their possible role in establishing pattern or completing meiosis is discussed.

INTRODUCTION

The mature egg cells of ascidians respond to fertilization with a series of calcium oscillations (Speksnijder *et al.*, 1989a). In the *Phallusia mammillata* egg, the first large (activating) pulse starts at the point of sperm entry and spreads across the egg as a wave (Speksnijder *et al.*, 1990). A similar wave of calcium at activation has been seen in the egg of the ascidian *Ciona intestinalis* (Brownlee and Dale, 1990). A series of 12 to 25 periodic postfertilization pulses starts shortly after the activating pulse. These pulses occur strictly in the period between fertilization and the completion of meiosis about 25 min later, they have free calcium peaks of about 1 to 4 μM—which is 15 to 60% of the fertilization pulse—and like the latter are independent of external calcium (Speksnijder *et al.*, 1989a).

The present study was undertaken to investigate the spatial pattern of these calcium pulses in relation to the developmental events that occur immediately following fertilization, such as meiosis, ooplasmic segregation, and axis determination (e.g., Sawada and Osanai, 1981; Bates and Jeffery, 1987; Sardet *et al.*, 1989). For this purpose we used an imaging system that allowed us to simultaneously monitor the intracellular calcium signals as well as the transmitted light image of the egg.

MATERIALS AND METHODS

Unfertilized *Phallusia mammillata* eggs were obtained and dechorionated as described previously (Sardet *et al.*, 1989), and pressure-injected with 15 to 30 pl (1-2% of the total egg volume) aequorin D (4.1 mg/ml) in 180 mM KCl, 100 μM EGTA (ethylene glycol bis (β-aminoethylether) (N,N,N',N'-tetraacetic acid), 30 mM BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, pH 7.1). Imaging of the aequorin luminescence and simultaneous generation of a transmitted light image of the egg, as well as data acquisition and analysis, were done as described before (Speksnijder *et al.*, 1990).

RESULTS AND DISCUSSION

Our results show that the postfertilization pulses are initiated in discrete regions of the zygote from which they spread as waves (Fig. 1). They travel at 11 to 20 μm/sec (mean ± SEM = 16 ± 2, n = 6), which is about twice the speed of the corresponding fertilization waves (8-9 μm/sec) (Speksnijder *et al.*, 1990).

The starting positions of these postfertilization waves display a distinct pattern. Analysis of 13 zygotes from five different egg clutches shows that a first series of 1 to 5 waves generally starts somewhat diffusely in the animal hemisphere or the equatorial zone, which corresponds to the region where the sperm usually enters (Speksnijder *et al.*, 1989b) and initiates the activation wave of calcium (Speksnijder *et al.*, 1990). The initiation site of the remaining 10 to 20 calcium waves is shifted to a region that includes the vegetal pole and the nearby contraction pole (Figs. 1a, 2). In addition, in about half of the eggs we analyzed, these vegetal calcium waves are often followed by an "echo" wave of smaller amplitude originating in the animal hemisphere and thus on the opposite side of the egg (Fig. 1b). These echo waves

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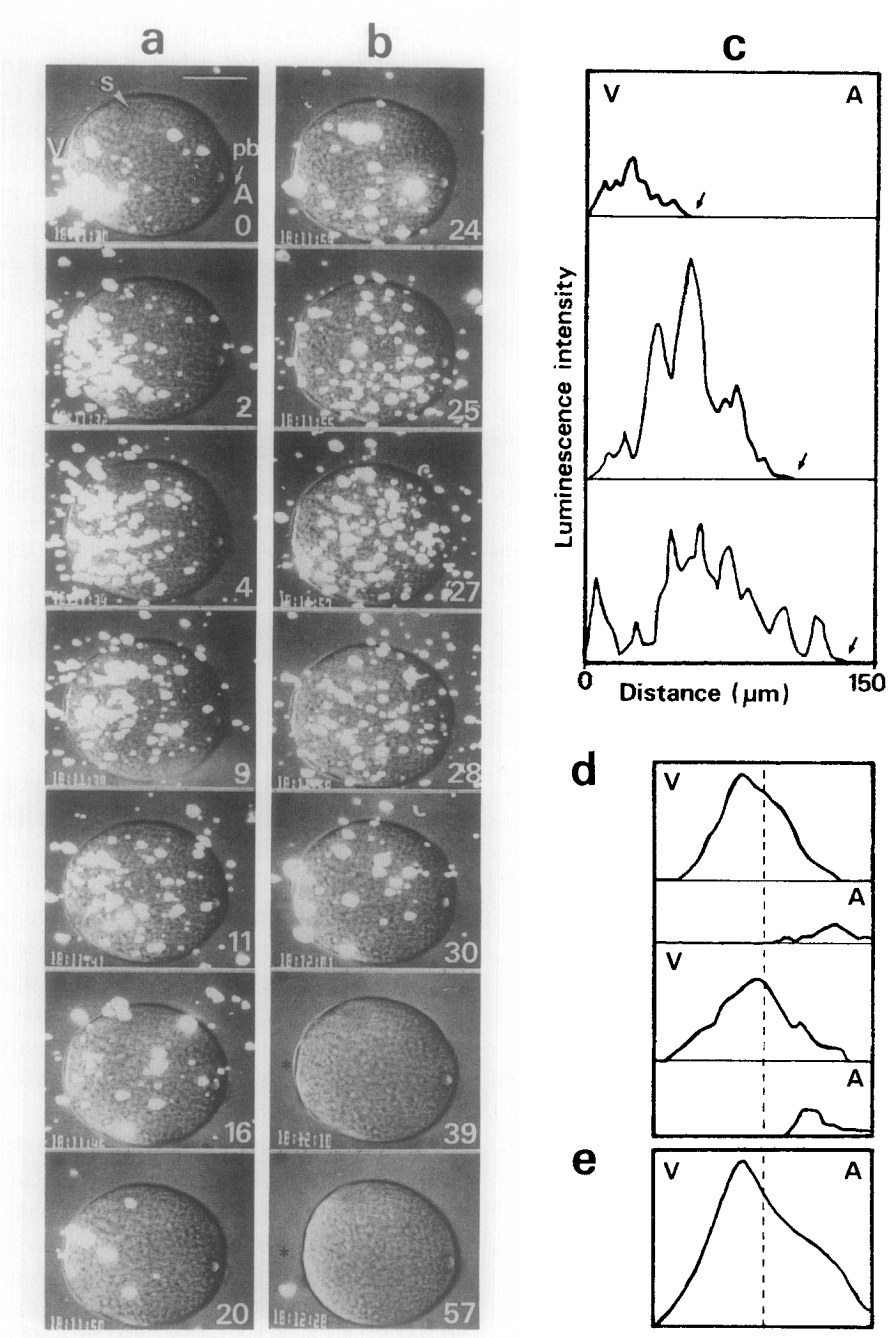

FIG. 1. Calcium waves through *Phallusia mammillata* eggs after fertilization. (a) Postfertilization wave of calcium starting near the vegetal pole at 18 min after fertilization. It spreads through the egg in less than 10 sec and is followed by (b) an echo wave starting in the animal hemisphere at 24 sec after the start of the previous wave (Waves 9 and 10 from Experiment 58). The egg responds to these calcium waves by a contraction of its surface, which travels from the vegetal pole in animal direction and is here visible as a flattening of the vegetal pole surface (see asterisk at t = 39 and 57 sec). Each image represents the sum of three video frames. pb, first polar body marking the animal pole (A); V, vegetal pole; S, sperm aster. Bar = 50 μm. (c) Luminescence intensity profiles summed over successive (2.5 sec) intervals along the propagation direction of Wave 7, Experiment 58, which starts near the vegetal (V) pole and spreads toward the animal (A) pole. The velocities of such waves were determined from successive wave fronts as indicated by arrows. In this particular wave the average velocity was calculated to be 14 μm/sec. (d) Luminescence intensity profiles summed over each of four successive waves in Experiment 57. The first of these, No. 16, starts vegetally (V) and because of its attenuated nature adds up to an asymmetric distribution of elevated calcium with its high point in the vegetal hemisphere. The second (echo) wave (No. 17) starts near the animal pole (A); the peak levels of luminescence reached during these echo waves are not as high but they also add up to an asymmetric calcium distribution profile. This pattern is repeated in the next 2 postfertilization waves (Nos. 18 and 19). (e) Intensity profile summed over all 25 postfertilization waves of this same Experiment 57 showing the asymmetric distribution of elevated calcium with its high point toward the vegetal pole (V).

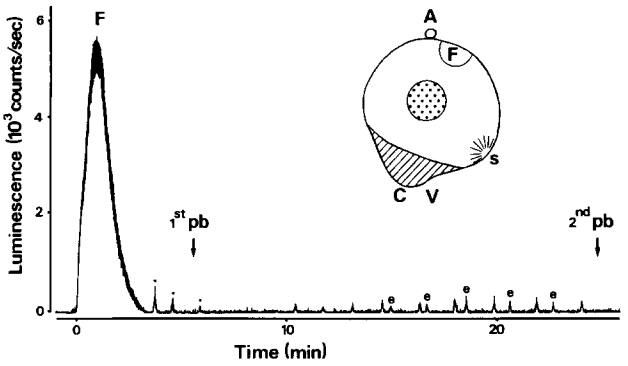

FIG. 2. Summary of the temporal and spatial characteristics of the periodic calcium waves in a fertilized *P. mammillata* egg. Trace shows the calcium-dependent photon emission rates after insemination of an aequorin-injected egg. The large fertilization pulse (F) is followed by a series of 17 postfertilization pulses, the last of which occurs shortly before the formation of the second polar body (2nd pb). During the last part of the pulse train, five out-of-phase pulses are present (Pulses 8, 10, 12, 14, and 16; data from Experiment 15).

The starting positions of the calcium waves are illustrated in the schematic drawing of a zygote at about 10 min after fertilization. The fertilization wave starts at the site of sperm entry (F) near the animal pole (A) and triggers a wave of cortical contraction that segregates the mitochondria-rich subcortical myoplasm (///) to a contraction pole (C), which is at some distance from the vegetal pole (V) (see Speksnijder *et al.*, 1990). The first 3 postfertilization waves start in the lower part of the animal hemisphere (area with asterisks), whereas the following ones start in the contraction/vegetal pole area, where the myoplasm (///) is then concentrated. The out-of-phase pulses (which occur in about half of the eggs) start opposite the contraction/vegetal pole in the animal hemisphere (area with asterisks) and behave as echos (e). S, sperm aster after its vegetal migration (see Speksnijder *et al.*, 1989b).

correspond to the out-of-phase pulses that we described earlier (Speksnijder *et al.*, 1989a), and which start within 30 sec (instead of the usual 60- to 100-sec period) after the previous pulse (Fig. 1). Except for these echos, all of the later waves are initiated at a discrete site in the contraction/vegetal pole area (Speksnijder *et al.*, 1990), where the mitochondria-rich myoplasm is concentrated. Finally, these later waves are frequently followed by a contraction of the egg surface starting near the vegetal pole and a movement of the internal cytoplasm toward the animal pole (Sardet *et al.*, 1989) (Fig. 1b), which suggests that they trigger contraction-relaxation cycles of the cortical actin network. These cortical contractions generally start within 1 min after the start of the vegetal calcium wave (Fig. 1b) and occur also in the absence of an echo wave.

The localization of the main wave initiation site in the vegetal pole/contraction pole area does not occur in eggs treated with 2 μg/ml cytochalasin D for 1–2 hr (observations of five eggs from three different clutches). Rather, the waves tend to start near the site of sperm entry (data not shown). This site can be readily identified at the time of fertilization as the region in which the first, activating wave of calcium starts and was marked as such on video records. Cytochalasin treatment is known to block the actin-mediated segregation of the cortical mitochondria-rich myoplasm to the vegetal pole (Sawada and Osanai, 1981; Speksnijder *et al.*, 1989b), which suggests that the segregation of some component of the egg's calcium regulating system is responsible for the localization of the main wave initiation site in this area. Our observation that the starting positions of these waves—like their timing and magnitude (Speksnijder *et al.*, 1989a)—are not affected by the absence of external calcium (observations on five eggs from two different clutches; data not shown), further suggests that this component does not operate via calcium influx across the plasma membrane.

Finally, integrations of the waves originating from the vegetal area show that they repeatedly expose this region to higher calcium levels. The smaller echo waves similarly expose the animal hemisphere to more calcium (Figs. 1d, 1e). Apparently these postfertilization waves are somewhat attenuated as they spread out from their starting points.

So far, the only comparable report of postfertilization waves of calcium concerns zona-free hamster eggs (Miyazaki *et al.*, 1986). As in *P. mammillata*, the first few postfertilization pulses spread like waves which start near the fertilizing sperm and move twice as fast as the fertilization wave. Later pulses were not seen as waves, but their nature may have been obscured by limitations of this preparation, e.g., their failure to divide and continue development. In contrast, aequorin-injected ascidian eggs regularly develop into normal tadpoles (Speksnijder *et al.*, 1989a, 1990).

The calcium released during fertilization waves in various eggs on the deuterostome line is generally considered to come from the endoplasmic reticulum (ER) (Jaffe, 1985; Eisen and Reynolds, 1985; Busa *et al.*, 1985). In *P. mammillata*, this view is strengthened by recent evidence that the mature egg has an extensive cortical ER which contains about 40–50 m*M* net calcium (Gualtieri and Sardet, 1989). This approaches the amount of calcium in skeletal muscle ER (Somlyo *et al.*, 1981). Since the postfertilization waves are unaffected by the absence of external calcium, it is likely that they are also supported by endogenous calcium released from the ER.

The fact that the larger, vegetally initiated waves are often attenuated and thus expose the vegetal end to more calcium seems comparable to earlier indications of high vegetal calcium in medaka fish eggs (Jaffe, 1986) and may prove developmentally significant. Among the early developmental events that these waves may affect are the subsequent completion of meiosis, movement of

most of the myoplasm to the future posterior pole (Sardet *et al.*, 1989), and localization of factors near the vegetal pole that determine the site of gastrulation and thus the dorsal side of the embryo (Bates and Jeffery, 1987).

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