# Analysis of the Calcium Transient at NEB During the First Cell Cycle in Dividing Sea Urchin Eggs

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Abstract. Accumulating evidence from several systems suggests that nuclear envelope breakdown (NEB) is triggered by an endogenous transient of free calcium. Using *h*- and *f*-semisynthetic aequorins as cytosolic calcium indicators, we have clearly and regularly visualized a single large, global calcium transient just before first NEB in normally developing, monospermic *Lytechinus* eggs. Although similar transients were not observed at NEB in subsequent cell cycles, microinjection of the calcium buffer BAPTA into one blastomere of the two-celled embryo resulted in the inhibition of NEB.

The NEB transient in the first cell cycle was some fivefold smaller than the one associated with egg activation. Our data suggest that this transient takes the form of a calcium wave that spreads inwards from the periphery of the egg toward the nucleus. We confirmed that these NEB transients did not require extracellular Ca2+. In polyspermic eggs, NEB-associated transients were fourfold larger than in monospermic eggs and were periodically repeated. Examination of the distribution of fluorescein-conjugated aequorins with a laser scanning confocal microscope indicated that aequorin both enters the nucleus and is evenly distributed within the cytosol of the egg. The use of *h*- and *f*-aequorins did not reveal any NEB transients during subsequent cell cycles, nor did we detect transients associated with other cell cycle events. However, a complex train of calcium transients in the form of both localized pulses and propagated waves was detected from embryos beginning at about the morula-to-blastula transition and continuing through to hatching.

# Introduction

The molecular basis of cell division appears to be remarkably similar in organisms as diverse as yeasts, sea urchins, and mammals (Murray and Hunt, 1993). However, the roles of key proteins such as cyclin B, p34, and cdc 25 are better understood than those of critical small molecules and ions such as Ca<sup>2+</sup>. The regulation of a large number of cellular processes by Ca<sup>2+</sup> suggests that it may also interact in cell cycle control. Indeed, evidence is accumulating that Ca2+ may regulate such events as pronuclear migration, pronuclear fusion, nuclear envelope breakdown (NEB), mitotic spindle formation, the transition between metaphase and anaphase, chromosome separation, and cytokinesis (Ciapa et al., 1994; Gillot and Whitaker, 1994; Fluck et al., 1991; Whitaker and Patel, 1990; Steinhardt and Alderton, 1988; Poenie and Steinhardt, 1987; Poenie et al., 1985). But clear experimental evidence in support of the contention that Ca<sup>2+</sup> transients directly regulate many of these events is still lacking (Hepler, 1994; 1992; 1989).

The argument for an association between a Ca<sup>2+</sup> transient and a cell cycle event is strongest in the case of NEB (Tombes *et al.*, 1992; Browne *et al.*, 1992; Whitaker and Patel, 1990; Poenie *et al.*, 1985). NEB and chromatin condensation can be induced in sea urchin eggs by microinjecting free Ca<sup>2+</sup> or IP<sub>3</sub> (Twigg *et al.*, 1988; Patel *et* 



Figure 1. Representative examples of calcium transients preceding NEB in Lytechinus variegatus eggs. Zero time indicates fertilization. INJ indicates the time of aequorin injection (in d, aequorin was injected before fertilization). Breaks in the record indicate DIC observations. Because the fertilized eggs were observed intermittently with DIC optics to detect nuclear envelope breakdown and cleavage, the times indicated for these events reflect when they were observed, rather than the precise time at which they occurred. NEI indicates the time closest to the transient at which the nuclear envelope was seen to be still intact; NEB, nuclear envelope breakdown, and CL, cleavage. (a) A monospermic egg cultured in ASW. (b) A monospermic egg cultured in Ca2+-free ASW. (c) A polyspermic egg cultured in ASW. Both polyspermic eggs and the monospermic controls in this experiment displayed first cleavage at around 100 min, rather than the 70 to 80 min often observed. (d) Comparison between the calcium transient at activation and that at NEB.

*al.*, 1989a, Steinhardt and Alderton, 1988) or by photoactivating  $[Ca^{2+}]_i$  transients in nitr-5 loaded cells (Kao *et al.*, 1990). Furthermore, if the calcium buffers EGTA and BAPTA (Steinhardt and Alderton, 1988; Twigg *et al.*, 1988) or a precipitating antibody or a specific inhibitor to the multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinase (Baitinger *et al.*, 1990) are microinjected into fertilized and parthenogentically activated sea urchin eggs, NEB and further progress through mitosis are blocked. In addition, monospecific antibodies to intracellular calcium pumps (Silver, 1986; Hafner and Petzelt, 1987) and antagonists (ryanodine and 8-(diaminoethyl)octyl-3,4,5-trimethoxybenzoate) to intracellular  $Ca^{2+}$  channels (Silver, 1989) have also been shown to block NEB and the subsequent mitotic cycle.

Using fluorescent Ca2+ indicators such as Fura-2 or Ca<sup>2+</sup>-Green, several studies have presented evidence of fluctuations of [Ca<sup>2+</sup>], in the cell cycle of ammonia-activated and fertilized sea urchin eggs, including a transient that corresponded to the time of NEB (Ciapa et al., 1994; Whitaker and Patel, 1990; Patel et al., 1989b; Poenie et al., 1985). Similar signals have been reported in starfish (Stricker, 1995), cultured mammalian cells (Poenie et al., 1986; Tombes and Borisy, 1989; Kao et al., 1990), and fertilized mouse eggs (Tombes et al., 1992). Although attempts to correlate Ca<sup>2+</sup> transients with NEB have been unsuccessful in the eggs of Xenopus and zebrafish, failure to detect a transient in these systems might be attributed to the techniques used, rather than to the absence of a calcium signal (Rink et al., 1980; Busa and Nuccitelli, 1985; Reinhard et al., 1995).

Thus, while the weight of the evidence does indeed suggest that a  $Ca^{2+}$  transient precedes and regulates NEB, many questions remain. What initiates the transient? What form does the transient take (*i.e.*, is it a propagating wave)? Is the transient unique to the first cell cycle? What are the molecular targets of the transient?

We decided, therefore, to further investigate the NEB calcium transient in L. variegatus using new nontoxic, semisynthetic recombinant forms of the chemiluminescent calcium indicator aequorin, a complementary methodology to fluorescent calcium reporters (Miller et al., 1994; Shimomura et al., 1993, 1990). Using the highly sensitive h- and f-aequorins, we have consistently visualized a single global calcium transient just prior to NEB in the first cell cycle in monospermic Lytechinus eggs. In polyspermic eggs, significantly larger, repetitive Ca<sup>2+</sup> transients were observed. The NEB calcium transient is not dependent on external Ca2+, and preliminary data suggest that it takes the form of a wave that spreads from the periphery of the egg towards the nucleus. These data are interpreted in light of recent results relating to independent Ca2+ regulation in the nucleus (Himpens et al., 1994). A short preliminary report of some of the data has appeared previously (Browne et al., 1992).

#### Materials and Methods

# Preparation of gametes

Eggs and sperm of *Lytechinus variegatus* (supplied by the Duke Marine Laboratory) were obtained either by intracoelomic injection of 0.5 M KCl or by electrical stimulation (12-V DC). Eggs that had been washed sev-

#### NEB CALCIUM TRANSIENTS



**Figure 2.** Visualization of the NEB calcium transient shown in Figure 1a. The panels show successive 3-min periods of accumulated luminescent light beginning 35 min after fertilization. The resting level of  $[Ca^{2+}]_i$  within this egg is shown in panel a. A pulse of elevated calcium precedes NEB, fills the whole egg, and lasts for about 5 min (panels b–c). By panel d,  $[Ca^{2+}]_i$  has nearly returned to the resting level. The dashed boundaries indicate the locations of the nucleus (n) and the cell membrane.

eral times in artificial seawater (ASW—Marine Biological Laboratory, Woods Hole, MA) were fertilized by the addition of 10  $\mu$ l of concentrated sperm in ASW to 100 ml of egg suspension. After fertilization, the eggs were again washed in either ASW or calcium-free ASW containing 1 mM EGTA. Jelly coats were removed by vacuum filtration of the eggs through a 100- $\mu$ m nylon mesh.

# Microinjection into eggs

Fertilized eggs were microinjected with either *h*-aequorin, *f*-aequorin, or a fluorescein-conjugated aequorin, obtained from Dr. O. Shimomura of the Marine Biological Laboratory. The *h*- and *f*-aequorins are reported to have 16 and 20 times, respectively, the relative intensity (at pCa 7) of recombinant aequorin *in vitro* (Shimomura *et al.*, 1993). These aequorins were injected as a 1% solution in 50 mM KCl, 0.1 mM EDTA, 1 mM MOPS buffer, at pH 7.1. Fertilized eggs were microinjected in chambers consisting of a coverslip fragment mounted with beeswax to form a wedge on a 22-mm-square #0 coverslip. Microinjection was carried out by the method of Hiramoto (1962) and Kiehart (1982) but was modified for aequorin as outlined in detail by Miller *et al.* (1994). The injection volume was about 40 pl, less than 10% of the egg volume.

To compare the NEB calcium transient to that at activation, unfertilized eggs were loaded into gelatin-coated wedge chambers and microinjected with aequorin as described above. ASW containing active sperm was introduced into the outer part of the Hiramoto chamber. The time needed for sperm to swim to the eggs in the inner part of the chamber was more than sufficient to allow us to switch our imaging system to a photon-counting mode and thus monitor the initiation of the activation wave.

#### Luminescent imaging

The calcium-dependent luminescent light from *h*- and *f*-aequorin-loaded eggs was projected onto the photo-



Figure 3. Graphs in a and b illustrate data from two experiments that yielded the most light at NEB. Each egg profile was divided into concentric areas representing equal volumes of the egg. The times of maximal photon count rate (in photons/coordinate/second) were determined for each concentric area and plotted against the distance of the midpoint of each area from the center of the egg. A linear regression fit of each plot yielded the estimated speed of a wave peak starting at the periphery of the egg and converging on the center (or nucleus). The two plots yield wave velocities of 1.54 and 1.49  $\mu$ m/s.

cathode of an imaging photon detector (IPD) by a Zeiss Axiovert 100 TV microscope platform with an image port on the same axis as a Zeiss  $63 \times 1.25$ -NA Plan-Neofluor objective. The IPD system collects photons and directly digitizes the *x*-*y* coordinates and time of each photon in a sequential data file. Such files were later used to reconstruct images over any desired time interval (described in detail in Miller *et al.*, 1994). Photon collection was intermittently interrupted so we could observe the eggs directly with DIC (differential interference contrast) optics, and the DIC images were stored on videotape. The temporal and spatial correlation between the aequorin luminescence and morphological events was determined from analysis of computer files and videotaped records. After most experiments, the egg chamber was perfused with 1% Triton X-100 in ASW to lyse the eggs. The resultant luminescence enabled us to estimate the residual amount of photoactive aequorin. Embryos that were not lysed were continually imaged until hatching, thus highlighting the nontoxic nature of these new ultrasensitive aequorins.

# Fluorescent imaging

Fluorescein-conjugated aequorin was microinjected into fertilized eggs as described above. The eggs were examined by confocal fluorescence microscopy with a Zeiss LSM 410 Axiovert at the Zeiss Microscopy Facility at the Marine Biological Laboratory. Images were obtained with a  $40 \times 0.6$ -NA Achroplan lens and Zeiss LSM software. Other than an initial adjustment of brightness and contrast, these images were not processed further. Photographic records were stored on 35-mm film.

# Data analysis of calcium transient

With the aequorin method, one is essentially limited by the total amount of signal, rather than the signal-tonoise ratio. When the NEB signal was integrated over periods of a few minutes, this was not a problem. But to facilitate a more sophisticated analysis to determine the kinetics of the calcium rise and explore the possibilities of wave propagation, we had to generate an analysis routine suited to our limited signal. Moreover, our imaging equipment (Miller et al., 1994) is essentially two-dimensional and has no resolution in the z-axis. This is a serious limitation when observing a signal generated within a spherical volume. We devised an analysis routine in which each egg profile was divided into concentric areas representing equal volumes of the egg. Each concentric area was identified by the distance, in micrometers, between its midpoint and the center of the egg (or the nucleus, if the latter did not happen to be located in the center of the egg).

To determine whether the NEB signal took the form of a wave, the times of maximal photon count rate (in photons/coordinate/second) were determined for each concentric area and plotted against the distance of the midpoint of each area from the center of the egg. Such a plot should determine (1) whether a wave does indeed propagate, (2) the direction of propagation, and (3) the velocity of propagation. A linear regression fit of each plot yielded the estimated speed of a wave peak starting at the periphery of the egg and converging on the center (or nucleus).

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Comparison of the calcium transients at activation (Fert) and nuclear envelope breakdown

Egg #	Counts at Fert*	Counts at NEB*	Counts at lysis*	[Ca <sup>2+</sup> ] at Fert**	[Ca <sup>2+</sup> ] at NEB**	[Ca <sup>2+</sup> ] NEB [Ca <sup>2+</sup> ] Fert ** (%)
1	63.8	0.53	104.4	4.94	0.78	15.8
2	279.4	6.32	345.0	5.40	1.26	23.3
3	120.1	4.20	250.7	4.13	1.14	27.6
4	243.0	2.93	529.7	4.83	0.88	18.3
Avgs	176.6	3.50	307.5	5.07	1.06	21.3

\*Milliphotons/coordinate/second.

\*\*Assuming luminescence rises to the 2.6 power of  $[Ca^{2+}]$  and the resting levels before and after fertilization are 0.10  $\mu$ M and 0.25  $\mu$ M respectively.

#### Calibration of aequorin luminescence signal

Estimations of free  $[Ca^{2+}]_i$  levels during both activation and NEB transients were made on two assumptions: (1) the resting level of  $[Ca^{2+}]_i$  before activation is 0.10  $\mu M$ , whereas after activation, but prior to NEB, it increases to 0.25  $\mu M$  (Whitaker and Patel, 1990; Baitinger *et al.*, 1990; Poenie *et al.*, 1985; Steinhardt and Alderton, 1988); and (2) the luminescence of *h*- and *f*-aequorin varies with Ca<sup>2+</sup> concentration *in vivo* as it does *in vitro*. From plots #2 and #3 (*i.e.*, those for *h*- and *f*-aequorin) in figure 1 in Shimomura *et al.* (1993), we estimate that between pCa 7 and pCa 6.5 luminescence rises to the 2.6 power of  $[Ca^{2+}]$ .

#### Injection of BAPTA buffer during the second cell cycle

We used the microinjection technique described above to inject BAPTA (1,2-bis[o-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid) buffer into one of the two daughter blastomeres after the first cell division, but before the second NEB. From our previous experience with BAPTA-type shuttle buffers (Fluck et al., 1994; Miller et al., 1993; Speksnijder et al., 1989) and from data reporting the effects of BAPTA on NEB in the first cell cycle in L. pictus (Steinhardt and Alderton, 1988; Twigg et al., 1988), we estimated that a final cytosolic concentration of about 10 mM BAPTA should have a significant effect, if a calcium transient is involved in triggering second NEB. The injection solution contained 70 mMBAPTA; 80 mM KCL; 48 mM CaCl<sub>2</sub>; 3.4 mM MgCl<sub>2</sub>, and 8 mM HEPES, set to a pH of 7.0. Injection volumes were between 20 and 50 pl (i.e., less than 10% of the total blastomere volume).

#### Results

#### NEB calcium transient

Monospermic eggs in ASW. In 42 cases of f- or h-aequorin-loaded, monospermic L. variegatus eggs that developed in ASW, all but one showed a striking calcium transient that began soon before first NEB (Figs. 1a and 2). Throughout the experiments, photon collection was interrupted periodically to collect and store DIC images of eggs onto a video record. Examination of the video record showed that in every case the calcium transient immediately preceded or corresponded to NEB. In three experiments in which video images were acquired every 30 s, NEB was seen to start about a minute after the initiation of the calcium transient. The calcium rise occurred in about 10 s and reached  $1.9 \pm 0.3 \,\mu M$  (s.e.m.). After the NEB transient, the calcium levels usually fell back to near the pre-NEB resting level in about 5 min. As shown in Figure 2, the transient was global; *i.e.*, it eventually filled the whole egg.

In monospermic eggs injected with *h*- or *f*-aequorin, no transients were detected at NEB in subsequent cell cycles nor at any other phases of the first or subsequent cell cycles. These negative results were not due to exhaustion of the aequorin. Lysis of the eggs in Triton X-100 after each experiment resulted in photon emission, which indicated that the eggs still retained abundant active aequorin, with count rates often exceeding thousands of photons per second for several minutes.

Monospermic eggs in calcium-free ASW. In four cases, aequorin-injected eggs were cultured in calcium-free ASW containing 1 mM EGTA. These gave similar single, global transients at NEB (Fig. 1b) that peaked at 3.2  $\pm$  0.4  $\mu$ M (s.e.m.), a signal some twofold larger than was observed in monospermic aequorin-injected eggs at NEB.

*Polyspermic eggs in ASW.* In four cases, aequorinloaded eggs cultured in natural seawater proved to be polyspermic, as judged by observations of the number of asters and of subsequent cell division patterns. These appeared in four batches of eggs on four different days and most likely resulted from the fusion of two or more sperm with otherwise normal eggs. The NEB pulse



**Figure 4.** Acquorin distribution in the egg of *Lytechinus variegatus* microinjected with fluorescein-conjugated acquorin at about 20 min after fertilization. (a) Brightfield light micrograph of the egg indicating the position of the nucleus. (b) Corresponding confocal fluorescent image of a 10- $\mu$ m optical section in the equatorial region of the egg. Other than an initial adjustment for brightness and contrast, the images were not processed.

heights in these polyspermic eggs averaged  $7.6 \pm 1.0 \ \mu M$ (s.e.m.), a value around four times larger than the average in the monospermic eggs. Moreover, these polyspermic transients were always clearly repeated, decreasing in intensity over time. In the most remarkable case, the transient was repeated 14 times; in two others, 5–6 times; in the fourth, the three additional transients were smaller compared to the first one but still unmistakable (Fig. 1c). These repeated transients were not correlated with any observable structural change within the egg. Despite these large and repeated transients, cleavage began at about the same time in all of these eggs, as it did in aequorin-loaded, monospermic ones from the same batch. This confirms an earlier report in which polyspermy did not significantly delay cleavage (Timourian and Watchmaker, 1971). However, several of the subsequent cleavages in these polyspermic eggs eventually regressed, in part perhaps because of a combination of their polyspermy and their slight flattening in the Hiramoto/Kiehart chamber.

# Nature of the NEB calcium transient

Because the total amount of calcium-dependent light generated at first NEB was limited, the pattern of calcium release was examined only in those eggs that yielded the largest signals at NEB. Only 7 of the 47 eggs that were injected with aequorin had a photon emission per unit area per second that was large enough to permit an effective analysis of the pattern of photon emission. Figures 3a and b are examples of two of these. Five of the seven eggs analyzed gave similar results; however, the remaining two were not statistically significant. The two plots shown yield wave velocities of 1.54 and 1.49  $\mu$ m/s, with fits of 0.65 and 0.71, respectively, both statistically significant at the P < 0.01 level.

# Comparison of NEB and calcium transients associated with egg activation

In four experiments in which calcium transients at NEB were compared to those at fertilization, the  $[Ca^{2+}]_i$  peak at activation was about five times larger than at NEB (Table I). Eggs were imaged through first cleavage and lysed by the addition of 1% Triton X-100 in ASW. In all cases, the large luminescent signal resulting from the influx of extracellular  $[Ca^{2+}]$  (not shown) indicated that the smaller NEB signal was not due to a lack of active aequorin. Figure 1d shows a representative plot of  $[Ca^{2+}]_i$  against time of an egg injected with aequorin prior to fertilization.

# Distribution of aequorin in cytosol and nucleus

To determine the cytoplasmic and nuclear distribution of aequorin prior to NEB, fluorescein-conjugated aequorin was microinjected into fertilized eggs (Fig. 4a,b). Equatorial brightfield and fluorescent optical sections taken through a representative *L. variegatus* egg showed an intense fluorescent signal from the nucleus. This clearly indicates that aequorin ( $M_r \ge 20,000$  to 22,000) microinjected into the cytosol enters the nucleus. The increased intensity of the signal from the nucleus compared to that from the cytosol probably reflects the larger amount of aequorin in the nucleus due to an absence of inclusions. The cytosolic distribution of the aequorin appeared to be homogeneous, except for

Egg #	Time of 1st cleavage	Injection time	NEB in control blastomere	NEB in injected blastomere	Cleavage in control blastomere	Cleavage in injected blastomere
1	66	75	yes	no	116	no
2	65	71	yes	no	116	no
3	68	80	yes	no	115	no
4	66	84	yes	no	115	no
5	67	90	yes	no	115	no
6	67	74	yes	no	113	no
7	68	81	yes	no	113	no
8	68	84	yes	no	113	no

Inhibition of second nuclear envelope breakdown (NEB) by the microinjection of BAPTA into one blastomere at the two-cell stage

The second blastomere serves as a control. The final cytosolic concentration of buffer was 10 mM. Times are given in minutes after fertilization.

slightly more intense fluorescence in the cortex than in the endoplasm. There was no indication that cytosolic aequorin was compartmentalized.

the failure to detect any further calcium signals during cell cycles following the first mitotic cycle was not due to aequorin exhaustion.

# Effects of BAPTA injection on second NEB

The failure to visualize a  $Ca^{2+}$  transient in subsequent cell cycles raises a question as to its role in later NEBs. Calcium signals may actually be present, but of significantly lower intensity and not detectable by this method. Silver (1989) injected the calcium buffer BAPTA into one of two blastomeres of the two-celled sand dollar embryo, inhibiting NEB in the injected blastomere. In a similar procedure, injection of BAPTA buffer to a final cytosolic concentration of 10 m*M* into one cell of the two-celled sea urchin embryo blocked second NEB and subsequent cleavage in the injected blastomere (Table II). This suggests that, in spite of our failure to visualize a calcium signal with the aequorins and equipment at our disposal, a transient elevation of  $[Ca^{2+}]_i$  is required for the second NEB.

#### Post-morula to blastula calcium transients

Aequorin luminescence was monitored in six monospermic eggs developing in natural seawater for more than 17 h. Although no detectable light was seen above the resting level during the subsequent cell divisions, striking patterns of luminescence reappeared after about 5 h. Periodically thereafter, a train of strong signals was recorded until the embryos hatched and swam out of view of the detector. These signals were often localized to specific regions of the embryo and took the form of either waves or nonpropagating transients (Fig. 5). Because these data consist of luminescent signals but no video records, we were unable to precisely correlate these signals with specific developmental events. However, the presence and intensity of these transients confirms that

#### Discussion

We consistently observed a global Ca<sup>2+</sup> transient preceding NEB during the first cell cycle in monospermic *L. variegatus* eggs. This observation supports the gathering evidence that an endogenous calcium transient is needed to trigger at least the first NEB. In monospermic eggs, we found peak values of  $[Ca^{2+}]_i$  at first NEB of around  $1.9 \ \mu M$ . Assuming a pre-NEB cytosolic resting level of  $0.25 \ \mu M$ , this represents about an eightfold rise in  $[Ca^{2+}]_i$ . When comparing NEB  $[Ca^{2+}]_i$  peaks to those at egg activation, we found them to be around fivefold smaller. Our data are also consistent with reports that the source of this Ca<sup>2+</sup> appears to be intracellular stores, since no extracellular Ca<sup>2+</sup> is necessary for this signal (Poenie *et al.*, 1985).

In Table III we have summarized our data, as well as published data, reporting calcium transients at activation and first NEB in a variety of sea urchin species. In general, smaller transients have been reported when fluorescent calcium probes have been used. We suggest two explanations for this observation: (1) If present in sufficient quantity, these high-affinity calcium reporters may buffer  $[Ca^{2+}]_i$  levels in the same range as the resting level of  $[Ca^{2+}]_i$ . (2) They also tend to saturate below micromolar concentrations of Ca2+ in vivo (Diliberto et al., 1994). A combination of these effects would dampen both the activation and NEB calcium transients. The use of aequorins avoids these problems (Miller et al., 1994). Differences in experimental protocols, detection equipment, calibration procedures, and species examined may also contribute to the range of values displayed.

Our observation of larger NEB transients in polysper-



Figure 5. Calcium transients from a *Lytechinus variegatus* egg during the first 17 h of normal development. The first pulse corresponds to NEB in the first cell cycle. CL indicates first cleavage. The absence of any NEB transients, or indeed any cell-cycle-related transients, in subsequent cell cycles is apparent for several hours, even though the egg is developing normally. After 5 h, at about the morula-to-blastula transition, a few transients occur. This is followed, beginning about 10 h later, by a steady rise in  $[Ca^{2+}]_i$  over the whole embryo. This general rise appears to be due to a train of calcium transients that continue up to the time that the ciliated embryo swam out of the field of view of the detector. (b) Visualization of the data shown above. Each panel represents 20 min of accumulated light beginning 10 h after fertilization. Because a corresponding DIC record is lacking, these patterns of calcium release are difficult to correlate with developmentally significant events (see text). Clearly, however, the absence of signal following the first cell cycle is not due to the exhaustion of aequorin.

mic eggs is also consistent with what has been reported (Whitaker and Patel, 1990). Although this is the first report to link repetitive transients with polyspermy in sea urchin eggs, Stricker (1995) has reported numerous repetitive "secondary fertilization signals" in polyspermic starfish eggs. The observation of repetitive Ca<sup>2+</sup> pulses in

#### Table III

Species	Reporter	Activation $(Ca^{2+} \mu M)$	First NEB $(Ca^{2+} \mu M)$	Reference
L. variegatus	h-aequorin, f-aequorin	5.07	1.9	This paper
L. pictus	Fura-2, dextran	ND	0.41	Ciapa et al., 1994
L. pictus	Ca <sup>2+</sup> -green, dextran	$2.0^{2}$	ND	Gillot and Whitaker, 1994
L. pictus	Fura-2	1.53	ND	Galione et al., 1993
L. pictus	Fura-2	1.0-5.0	0.35	Whitaker and Patel, 1990
L. pictus	Fura-2	ND	0.566	Steinhardt and Alderton, 1988
L. pictus	Fura-2	2.5	ND	Hafner et al., 1988
L. pictus	natural aequorin	1.3-8.0	ND	Swann and Whitaker, 1986
L. pictus	Fura-2	1.95	0.404	Poenie et al., 1985
L. pictus, A. punctulata	natural aequorin	1.0	ND	Eisen et al., 1984
L. pictus	natural aequorin	2.5-4.5	ND	Steinhardt et al., 1977

Comparison of our results with published data reporting calcium transients at activation and nuclear envelope breakdown (NEB) in a variety of sea urchin species in the genus Lytechinus and one species of Arbacia

ND = No data.

<sup>1</sup> Authors report a mean transient increase of 0.85  $\mu M$  from what appears to be a resting level of between 0.2 and 0.3  $\mu M$ .

<sup>2</sup> From figure 1d.

<sup>3</sup> From figure 4c.

polyspermic eggs might provide a clue to the mechanism that initiates and propagates transients in monospermic eggs. It has been suggested that periodic calcium transients are generally triggered by repetitive overloading of the lumen of the endoplasmic reticulum with calcium (Jaffe, 1993), and that centrosomes organize calciumrich endoplasmic reticulum (Wolniak et al., 1980; Henson et al., 1990; Terasaki and Jaffe, 1991). Injected centrosomes have also been reported to induce parthenogenesis of Xenopus oocytes (Schiebel and Bornens, 1995). Recent evidence also suggests that, in the embryonic cell, a sperm-derived factor plays a role in the generation of cell-cycle-related calcium transients, including the one at NEB (Carroll et al., 1994; Kono et al., 1995; Osawa et al., 1994). Together, these point to the possibility that some additional factors introduced by extra sperm somehow contribute to triggering these repeated calcium transients.

The NEB transient during the first cell cycle quickly fills the whole egg. Our working hypothesis was that some sort of calcium-wave propagation was involved. In none of the 42 examples we examined, however, did we see an NEB transient that crossed an egg from pole to pole as a wave. This suggested that if the transient was propagating as a wave, it had to be traveling from the outside towards the nucleus, or *vice versa*. Our analysis was designed to explore these possibilities.

Although our analysis was circumscribed by limited signal and a lack of resolution in the z-axis, the data indicate that the NEB transient takes the form of a calcium wave that begins in the outer regions of the egg and spreads inward toward the central nuclear region with a velocity of about  $1.5 \,\mu$ m/s. We have not as yet explored possible propagation mechanisms of this wave. A suggestion might be calcium-induced calcium release (CICR). The wave velocity determined by our analysis is slow compared to some reports of waves propagated by CICR (Jaffe, 1993). It represents, however, an average velocity from the periphery to the center. A situation might exist in which the wave moves at different velocities in different regions of the egg. This phenomenon has been reported during activation in sea urchin eggs, when the wave moves faster in the cortex than it does in the central region of the egg (Mohri and Hamaguchi, 1991; Shen and Buck, 1993).

The best immediate hope for further exploring the nature of this wave would appear to lie in fluorescent confocal microscopy. Using this technique, Gillot and Whitaker (1994) recently reported two additional  $Ca^{2+}$  transients that follow the activation transient in *L. pictus:* the first coincided with the beginning of pronuclear migration; the second with pronuclear fusion. This report did not, however, continue to include a description of the transient at NEB. Stricker (1995), using ratio imaging and confocal microscopy, reported postfertilization waves in starfish embryos, but he was unable to demonstrate that these signals took the form of a wave.

The location of initiation and the direction of the wave suggest that the first cell cycle may be triggered by a cortical biological clock, such as the one described by Hara *et al.* (1980). The latter had the same periodicity as the cell division cycle in *Xenopus* embryos and functioned in the absence of any nuclear material. We suggest that, at least for the first cell cycle, there may be an analogous situation in the sea urchin egg.

We failed to visualize any NEB calcium transients in later cell cycles, but we could consistently block second NEB with the calcium buffer BAPTA. A plausible explanation might be that after the first cell cycle the NEB calcium transients are restricted to the nuclear or perinuclear region rather than the whole cytosol. The nucleus clearly possesses all the component parts (endomembranes, sequestered calcium, receptors, channels, pumps, etc.) to transduce a localized calcium signal (Minamikawa et al., 1995; Himpens et al., 1994). This idea of a progression from global to localized transients is supported by a study from the sand dollar Echinaracnius parma, in which a calcium transient was reported to precede second NEB by around 6 min, to last about 40 s, and to be restricted to the perinuclear region (Silver, 1994; Silver et al., 1994). Our failure to detect localized transients in subsequent cell cycles of L. variegatus might be explainable as follows: if these signals were as intense as the first global transient we recorded, but were restricted to a volume  $10 \,\mu m$  in diameter, they would result in only about 0.1% of the luminescent emission seen from the whole egg during first NEB.

The first cell cycle may be unique, and signal transduction pathways might be different in subsequent cycles. An understanding of calcium signaling in subsequent cycles is limited by the fact that most data have been collected from, and are relevant to, only the first cell cycle. In mouse embryos the ability to induce global  $Ca^{2+}$  transients is a unique property associated with the early NEBs and is lost in subsequent cell cycles (Kono *et al.*, 1995). Sea urchin embryos might exhibit a similar condition in which NEB is preceded by a global calcium transient in the first cell cycle but is lost in subsequent cell cycles. However, in somatic cells such as Swiss 3T3 fibroblasts, global rather than local  $Ca^{2+}$  transients are seen to precede NEB (Kao *et al.*, 1990).

The trigger for Ca<sup>2+</sup> release, the targets involved, and thus the overall mechanism by which Ca<sup>2+</sup> might regulate NEB are currently under investigation. An IP<sub>3</sub> receptor has been shown to be present in the nuclear envelopes of several cell types (Bachs *et al.*, 1992; Matter *et al.*, 1993), providing a mechanism for the initial localization of Ca<sup>2+</sup> fluxes at the nuclear envelope. Sea urchin eggs have been shown to possess IP<sub>3</sub>-sensitive and -insensitive calcium stores (Clapper and Lee, 1985; Turner *et al.*, 1986; Oberdorf *et al.*, 1986; Swann and Whitaker, 1986), as well as a calcium-induced calcium release mechanism mediated by a ryanodine receptor (Buck *et al.*, 1992; Fujiwara *et al.*, 1990; Galione *et al.*, 1991, 1993; Gerasimenko *et al.*, 1995; Lee *et al.*, 1993; McPherson *et al.*, 1992; Rakow and Shen, 1990). In addition, IP<sub>3</sub> and the calcium-binding proteins calsequestrin, calreticulin, and calsequestrin-like protein have been localized throughout the cytoplasm of sea urchin eggs (Parys *et al.*, 1994; Lebeche and Kaminer, 1992; Henson *et al.*, 1990). In *Xenopus*, overexpression of calreticulin inhibits the occurrence and frequency of repetitive Ca<sup>2+</sup> wave activity (Camacho and Lechleiter, 1995). The calcium transient, be it global or localized, could function through the multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinase that has been identified in sea urchin eggs and shown to be necessary for NEB (Baitinger *et al.*, 1990).

In prophase-arrested oocytes of both mouse and starfish, NEB during germinal vesicle breakdown does not need to be preceded by a calcium transient (Tombes *et al.*, 1992; Witchel and Steinhardt, 1990). Thus, it is premature to draw any broad conclusions as to the absolute requirement for a pre-NEB calcium trigger or the distinction between global or localized transients. Tombes *et al.* (1992) suggest that because germinal vesicle breakdown appears to be  $Ca^{2+}$ -independent, whereas NEB is  $Ca^{2+}$ dependent, there may be regulation by more than one signaling pathway. This would not be surprising, as it is becoming obvious that many important signaling pathways associated with key events in early development have redundant mechanisms (Galione *et al.*, 1993).

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