On the Calcium Pulse during Nuclear Envelope Breakdown (NEB) in Sea Urchin Eggs

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The relationship between a calcium pulse and NEB was critically reviewed by Hepler in 1989 (1) and more briefly reviewed by Tombes *et al.* in 1992 (2). With regard to eggs, Hepler concluded that "the evidence . . . supports the occurrence of a Ca pulse preceding NEB. . . . However, there is much less agreement for the other phases of division . . ." We fully agree with Hepler but would add that the evidence has supported the need for (as well as the occurrence of) NEB pulses.

Particularly convincing is the report by Twigg *et al.* that injection of BAPTA calcium buffer into fertilized *Lytechinus* eggs at a final cytosolic concentration above about 2 m*M* regularly and permanently blocks NEB; while injection of CaEGTA at a final concentration of 25 m*M* so as to set free calcium at one micromolar regularly speeds NEB (3). This **indirect** evidence seems particularly cogent since this BAPTA effect is in quantitative agreement with shuttle buffer theory (4) as well as several other observations of inhibition by calcium buffer injections (4–7). Also consistent with a need for NEB pulses are reports that mitosis and NEB can be blocked by antibodies to intracellular calcium pumps (8–9), by antagonists to intracellular calcium channels (10) and by a specific peptide inhibitor to a Ca^{++}/calmodulin-dependent protein kinase (11).

On the other hand, **direct** evidence for NEB pulses has been less satisfactory. The first such report was primarily illustrated by a single recording showing six calcium peaks during the first cell cycle in *Lytechinus,* including one said to occur at NEB; however, as Hepler pointed out this particular egg was grossly abnormal and probably dispermic (12). The second group of such reports have only appeared in abstract form and seem impossible to critically assess (13). The third indicated that such pulses could only be seen regularly in *Lytechinus* eggs when they were activated by ammonia rather than fertilized (5). The latest reports that only 8 of 19 mouse eggs showed such pulses and is therefore cautious about their significance (2).

Here we briefly report observations of calcium pulses during first NEB in fertilized, monospermic *Lytechinus* eggs as observed via injection of the ultrasensitive h or f forms of the chemiluminescent protein, aequorin. [At cytosolic pCa's, but measured *in vitro,* these 'semi-synthetic' aequorins luminesce with about 30 to 60 times the intensity of natural aequorin (14).] In about 40 cases of aequorin-loaded, monospermic eggs that underwent normal first cleavage, all except one showed a striking calcium pulse that began within the minute that preceded NEB. The calcium level was observed to rise to a peak level of about 200 photons/s from a resting level of about 10 photons/s. These pulses usually rose to a peak within about 10 s and remained perceptible for about 30–60 s. Preliminary quantitation indicates that these pulses peak in the few micromolar range of free calcium while preliminary imaging of these pulses showed them to fill the whole egg.

However, to our considerable and continuing surprise, no detectible calcium pulses ever accompanied NEB during the second or later cell cycle in any of those two dozen eggs in which observations were continued beyond the first cell cycle. Subsequent tests for residual aequorin showed that an ample amount remained to have revealed later NEB pulses had they been present.

In four cases, we observed the course of free calcium in eggs that were visibly dispermic. In all of these cases, we again observed an extraordinarily large (about ten times usual) but otherwise typical pulse of aequorin light during NEBD. In three of these four dispermic eggs, the initial pulse was followed by a periodic series of pulses that continued until (delayed) cleavage occurred. The number of such additional pulses varied from 3 to 11, the pulse to pulse time was about 4 min and the amplitude tended to gradually decrease.

Finally, in two cases, we succeeded in observing the course of free calcium change in eggs that were immersed in calcium free seawater (containing 2 m*M* EGTA) right after fertilization. In both of these cases we observed an extraordinarily large (about ten times usual) but otherwise typical pulse of aequorin light during first NEB. Figure 1 illustrates one of these remarkable pulses. We suspect that giant pulses occur in calcium free seawater because much of the injected, ultrasensitive aequorin is normally destroyed by calcium entering the injection wound.

This is the first report of calcium pulses clearly and regularly

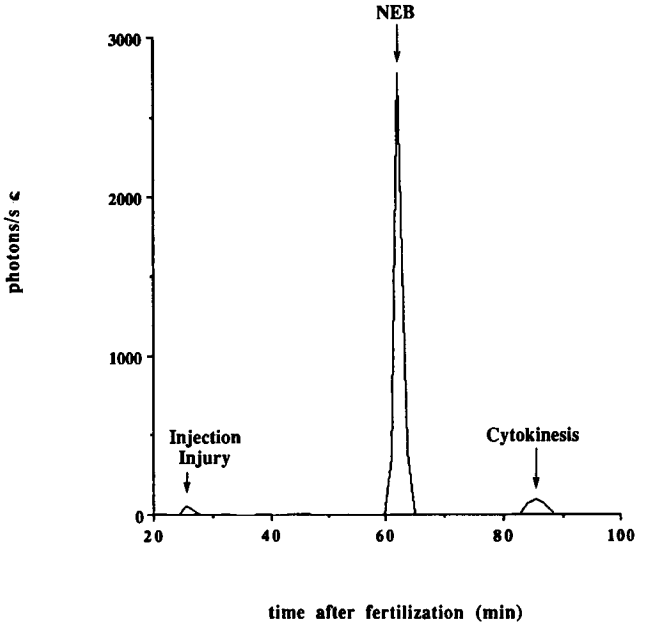

Figure 1. *NEB calcium signal from a* Lytechinus pictus *egg that was injected with f-aequorin about 20 min after fertilization and allowed to develop in calcium free artificial seawater. The resting level of about 10 photons/s was too small to appear on this linear graph. Similar NEB signals of about one tenth this height are regularly seen in* Lytechinus *eggs developing in natural seawater (see text). The smaller second signal accompanied cytokinesis. Such signals have only been seen occasionally.*

associated with NEB in fertilized cleaving eggs. It is also the first report that such pulses are absent during subsequent NEB's, do not require external calcium, and may be periodically repeated in dispermic eggs. The further use of ultrasensitive aequorins should make it possible to better characterize NEB calcium pulses and better study their function. In particular, it should now be possible to test the prediction that such pulses initially spread through the egg in the form of fast calcium waves (15).

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Calcium Buffer Injections Block Ooplasmic Segregation in *Oryzias latipes* (Medaka) Eggs

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We used the calcium buffer, 5,5'-dibromo-BAPTA, to investigate the possible role of Ca^{2+} in ooplasmic segregation in the medaka fish egg. The unfertilized medaka egg consists of two compartments: a large (~1 mm in diameter) central yolk vacuole and a thin (~30 μm thick) peripheral layer of ooplasm; a unit membrane (the yolk membrane) separates the two compartments. Following activation of the egg, the bulk of the ooplasm and its inclusions move toward the animal pole and form a blastodisc there, while oil droplets and other, smaller inclusions move toward the vegetal pole (1). At 25°C, segregation is complete within about 70 min.

Throughout the period of segregation, zones of elevated cytosolic [Ca^{2+}] are present at both the animal and vegetal poles of the medaka egg (2). To determine whether these zones are required for ooplasmic segregation, we injected dibromo-BAPTA (final concentration = 0.5–7.0 m*M*) into the ooplasm near the vegetal pole of the egg or near its equator (midpoint along the animal-vegetal axis) within 10 min after fertilization, using methods described previously (2). Our idea was to use this relatively weak calcium buffer (K_D = 1.5 μM) as a shuttle buffer to suppress or reverse the formation of needed high calcium zones in the micromolar range (3). Eggs were co-injected with sufficient Ca^{2+} to give free [Ca^{2+}] in the injectate of 0.14 μM, 0.2 μM, or 0.3 μM and thus in the range of natural resting levels (4). Injections of such buffer/Ca^{2+} mixtures, set at the resting level of [Ca^{2+}], cannot act by shifting [Ca^{2+}] away from the resting level (3). To estimate the time required for the buffer to diffuse around the egg from the site of injection, we monitored the spread of fluorescein injected near the equator of the egg. We found that it reached the poles of the egg within 10 min of injection and the antipode of the injection site within 20 min.

Low concentrations of the buffer (≤2.0 m*M*) had little or no effect on the eggs, while higher concentrations of the buffer (≥2.6 m*M*) inhibited ooplasmic segregation and blocked cleavage at all three levels of [Ca^{2+}] (Table I). The fact that the results were independent of pCa in this range confirms that the injectate was acting as a shuttle buffer. The slightly higher concentration of dibromo-BAPTA required to inhibit segregation and cleavage in the medaka egg *vs.* fucoid egg development may be due to a slightly higher calcium flux in the medaka egg.

The immediate reaction of the eggs to the injectate was an apparent expansion of the yolk membrane near the injection site, which caused the yolk vacuole to bulge into the ooplasm locally. At lower concentrations of buffer (<4 m*M*), this bulge usually subsided within 10 min. Sometimes, however, the yolk membrane over the bulge lysed; this was the predominant outcome at ≥4.0 m*M* buffer. This effect on the yolk membrane may have been caused by the initially high concentration of buffer near the injection site. Another consistent local response to the injection was the movement of nearby oil droplets away from the injection site and toward the top of the egg; this movement was over within 15 min.

Except for this early movement of oil droplets, segregation of ooplasm and oil droplets was strongly inhibited by ≥2.6 m*M* dibromo-BAPTA. The blastodisc formed slowly, and its final size was smaller than in control eggs; this effect was more pronounced in eggs in which we injected buffer near the equator. The effect of buffer injection on oil droplet movement also varied with the site of injection. When buffer was injected near the equator, oil droplet movement was strongly inhibited all along the animal-vegetal axis, but when it was injected near the vegetal pole, oil droplets moved away from both poles and formed a