Slow Calcium Waves Accompany Cytokinesis in Medaka Fish Eggs

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Abstract. Animal cells are cleaved by the formation and contraction of an extremely thin actomyosin band. In most cases this contractile band seems to form synchronously around the whole equator of the cleaving cell; however in giant cells it first forms near the mitotic apparatus and then slowly grows outwards over the cell. We studied the relationship of calcium to such contractile band growth using aequorin injected medaka fish eggs: we see two successive waves of faint luminescence moving along each of the first three cleavage furrows at ~0.5 μm/s. The first, narrower waves accompany furrow extension, while the second, broader ones, accompany the subsequent apposition or slow zipping together of the separating cells. If the first waves travel within the assembling contractile band, they would indicate local increases of free calcium to concentrations of about five to eight micromolar. This is the first report to visualize high free calcium within cleavage furrows. Moreover, this is also the first report to visualize slow (0.3–1.0 μm/s) as opposed to fast (10–100 μm/s) calcium waves. We suggest that these first waves are needed for furrow growth; that in part they further furrow growth by speeding actomyosin filament shortening, while such shortening in turn acts to mechanically release calcium and thus propagates these waves as well as furrow growth. We also suggest that the second waves act to induce the exocytosis which provides new furrow membrane.

THE uncertain relationship of free calcium patterns to cytokinesis has been critically reviewed by Mabuchi (1986), by Hepler (1989), as well as by Salmon (1989) and considered by several investigators at a recent symposium volume (Conrad and Schroeder, 1990). In our view, the most cogent evidence that a natural rise in calcium may favor furrow elongation as well as furrow initiation and apposition are Arnold's (1975) observations of the effects of A23187 on cleaving squid eggs. He reported that application of this calcium ionophore to cleaving eggs induces an immediate increase in the speed of furrow elongation as well as an immediate widening of the furrow. A minute or two later he saw a "relaxation" of the cleaving egg and a decrease in visibility of the furrow which probably indicated apposition of the separating cells. Longer term effects included an extension of the furrows beyond their normal extent. Moreover, if the ionophore is applied a few minutes before cleavage is scheduled, then the furrows appear prematurely. Also cogent is the more recent report of Conrad et al. (1987) that optimal concentrations of caffeine–well known to speed calcium release from the ER–substantially speeds the initiation of polar lobe furrows as well as cleavage furrows in *Ilyanassa* eggs.

We have pursued this question in the large (1,200-μm diam), hyaline medaka egg because calcium patterns within this system can be easily and reliably studied with aequorin (Gilkey et al. 1978). Fig. 1 diagrams the medaka egg's second cleavage. Each early cleavage furrow begins over (and is presumably initiated by) a centrally placed mitotic apparatus (Rappaport, 1990). It then extends laterally to the edge of the blastodisc, at about 0.6 μm/s (as well as inward towards the yolk membrane) so as to cleave a cell in 5 to 10 min. At this stage, the newly formed cells are rounded up and separated from each other by a broad and obvious groove. They then move up against each other so as to obliterate this groove. This process likewise starts centrally and moves laterally at ~0.6 μm/s. We call it "zipping".

Materials and Methods

To obtain gametes, gonads were removed from breeding medaka and placed in a balanced saline solution (BSS[1]: 111 mM NaCl; 5.37 mM KCl; 1.0 mM $CaCl_2$; 0.6 mM $MgSO_4$; 5 mM Hepes, pH 7.3). To prepare unfertilized eggs for microinjection, we transferred them through five successive washes of Ca^{2+}/Mg^{2+}-free BSS over a period of 1 h. Approximately 1.0 nl of a 0.62% solution of recombinant aequorin (Shimomura et al., 1990) in 100 mM KCl, 5 mM Hepes, and 0.05 mM EDTA was injected equatorially using micropipettes of ≤5-μm tip diameter. The injection technique used was Hiramoto's quantitative low-pressure method (Hiramoto, 1962). Details of the procedure used to introduce a micropipette into the thin peripheral cytoplasmic layer of the medaka egg were those described by Gilkey (1983).

After microinjection, the eggs were transferred to BSS containing 0.2 mM Ca^{2+} for 30 min, transferred to BSS for 30 min, and then fertilized. All the eggs used in this study developed normally and hatched. Chorionated eggs were observed in their normal, blastodisc-down orientation with an IM-35 inverted microscope (Zeiss, Oberkochen, Germany), a Nikon Planapo 10/0.45 objective, and a 75-mm optical doublet, which together produced a 6× magnified image on the photocathode of an imaging photon detector or IPD (Imaging Technology Ltd., East Sussex, UK). This device

1. *Abbreviations used in this paper*: BSS, balanced saline solution; MLCK, myosin light chain kinase.

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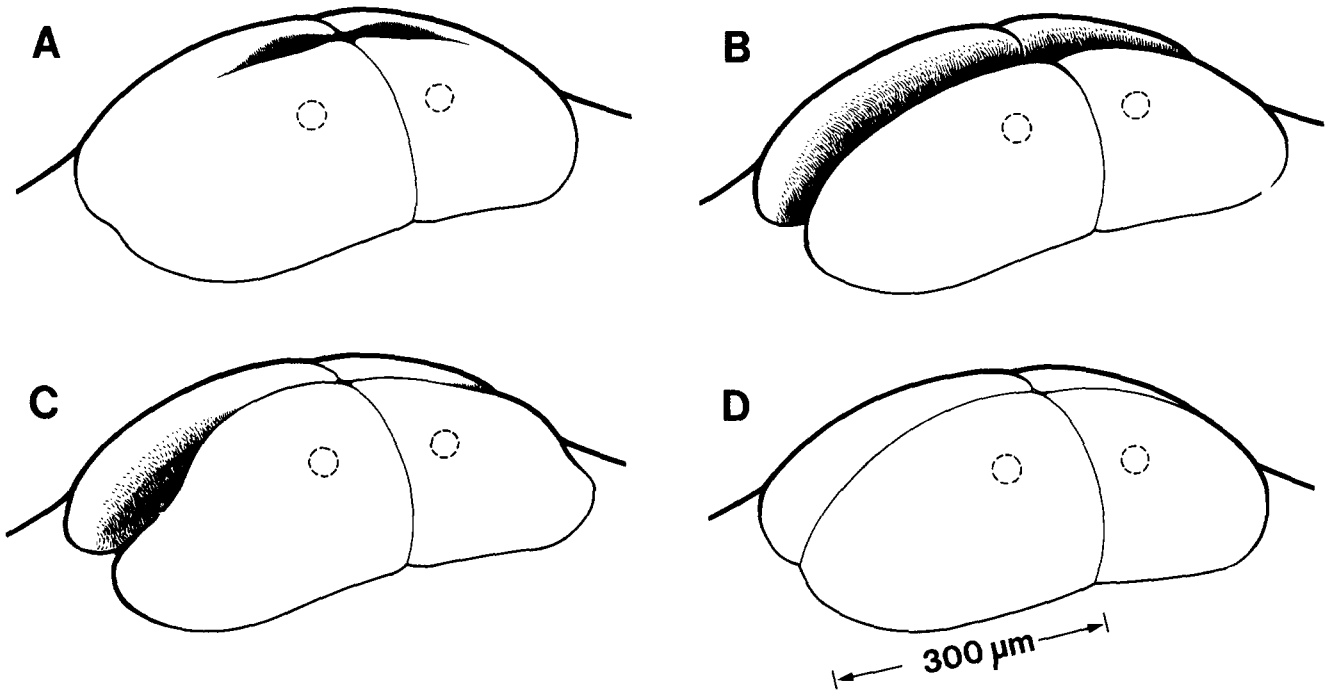

Figure 1. Diagram of the two structural waves which occur during the second cleavage of the medaka egg. *A* and *B* indicate the furrowing wave, *C* and *D*, the zipping wave.

consists of a microchannel plate intensifier with a resistive anode as the positional encoder (Speksnijder et al., 1990).

The raw data from the IPD system consists of a sequential record of photon positions and times, measured one at a time. This system will record up to around 100,000 photons per second. Since we never encountered more than about 30 photons per second, system saturation was never a problem. Images were generated by accumulating data over any desired interval and representing multiple photons per pixel with a color scale. Photon collection was briefly (2 s) interrupted at appropriate intervals to record brightfield images of the eggs. This allowed us to compare luminescent and bright-field images as the relatively slow process of cytokinesis proceeded. Moreover, we separately analyzed time lapse recordings of brightfield images of cleaving blastodiscs. This allowed us to compare the average velocities of structural waves and calcium waves.

Results

Fig. 2 shows the two calcium waves during a representative second cleavage, while Fig. 3 shows computer-smoothed profiles taken from this "pixilated" data. Wave velocities were obtained from such smoothed curves. Nine eggs (including two in Ca^{2+}/Mg^{2+}-free media with 1 mM EGTA) all showed qualitatively similar results; five proved suitably orientated to yield the quantitative data shown in Table I. By comparing such luminescent light images with (intermittent) transmitted light images, we learned that each cleavage's first calcium wave starts within a minute or two of the start of furrowing and likewise ends within a minute or two of the end of the furrowing process; while each second calcium wave has this same relationship to the zipping process. A similar 1 to 2 min (and thus 30–60 μm) uncertainty held for the possible coincidence of calcium waves and furrowing or zipping during their progress. These conclusions are based upon the fact that we never clearly saw calcium waves precede or follow furrowing or zipping and the fact that it took 1 to 2 min to accumulate enough photons to clearly localize the front of an advancing calcium wave. The average velocity of both furrowing and zipping proved to be about 0.6 μm/s. This figure is within 20% of the corresponding calcium wave speeds (Table I). So we conclude that the calcium waves "accompany" the structural ones.

As the first calcium waves move outwards, they are seen to have a width of one or two pixels, which corresponds to 18 to 36 μm in our system. Structural data from many other cells indicate that the contractile bands in medaka eggs should be about 10- to 20-μm (or approximately one pixel) wide (Schroeder, 1990). This then suggests that the calcium waves form and spread within the extending contractile bands. Assuming this to be true, one can calculate the peak calcium level during a furrowing wave from the measurements of relative light intensity in blastodisc regions which do and do not include the furrow (the R_A's of Table I).

First we converted the measured ratios of light per unit area (R_A) to R_V's, i.e., ratios of light per active cell volume divided by light per resting cell volume. We did this via Equation 1:

$$R_v = R_A \cdot \frac{W_r}{W_a} \cdot \frac{t_r}{t_a} \qquad (1)$$

where W_a, W_r, t_a, and t_r are the widths and thicknesses of the active and resting regions. We took W_a and t_a to be 10 to 20 μm and 0.1 to 0.2 μm, since these cover the range of known contractile band dimensions in giant cells (Schroeder, 1990). W_r is 126 μm since this is the width of the 7-pixel-wide measurement box. We took t_r to be 90 μm since this is the blastodisc's thickness, since the depth of focus could be seen to exceed this value, and since the blastodiscs's depth is much smaller than its width.

The resting level of calcium in the medaka egg cytosol is

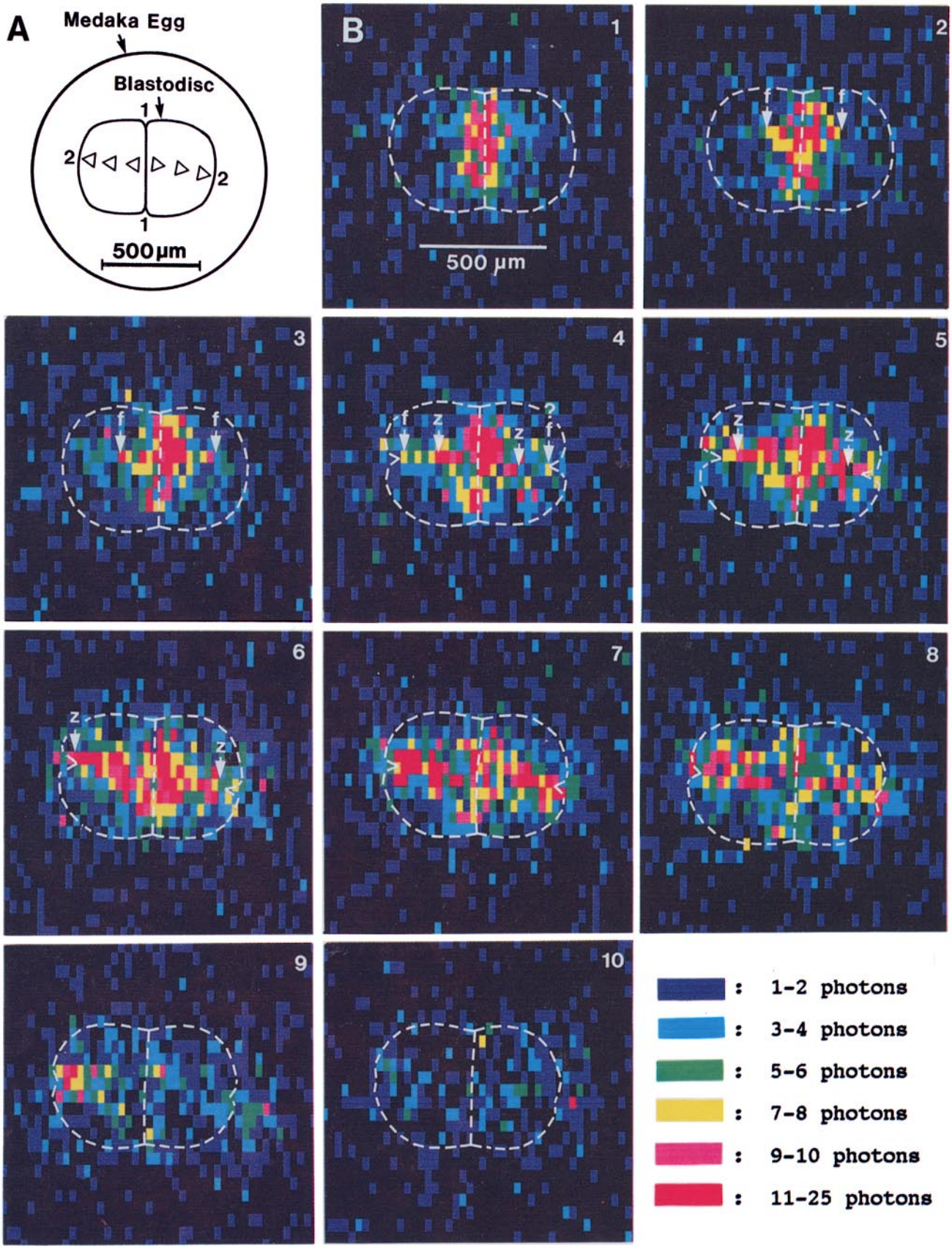

Figure 2. (*A*) Calcium waves during the second cleavages of medaka egg #9. The second cleavage furrows arose near the middle of the first furrow (whose ends are marked "1") and grew in the direction of the arrowheads, towards the edges of the blastodisc (marked "2"). (*B*) In *1–10*, the dashed lines approximate the position of the first cleavage furrow and the perimeter of the blastodisc; this is only an approximation because the blastodisc moves slightly as it undergoes cleavage. As the second furrows formed, the ends of the blastodisc became slightly indented as first shown in *4*. This particular blastodisc was tilted out of the optical plane, and we chose to focus on its left half. Each panel shows six minutes of accumulated light, and each successive panel started one minute later. The luminescent wave fronts accompanying furrowing (*f*) and zipping (*z*) are marked. An analysis of these images is shown in Fig. 3.

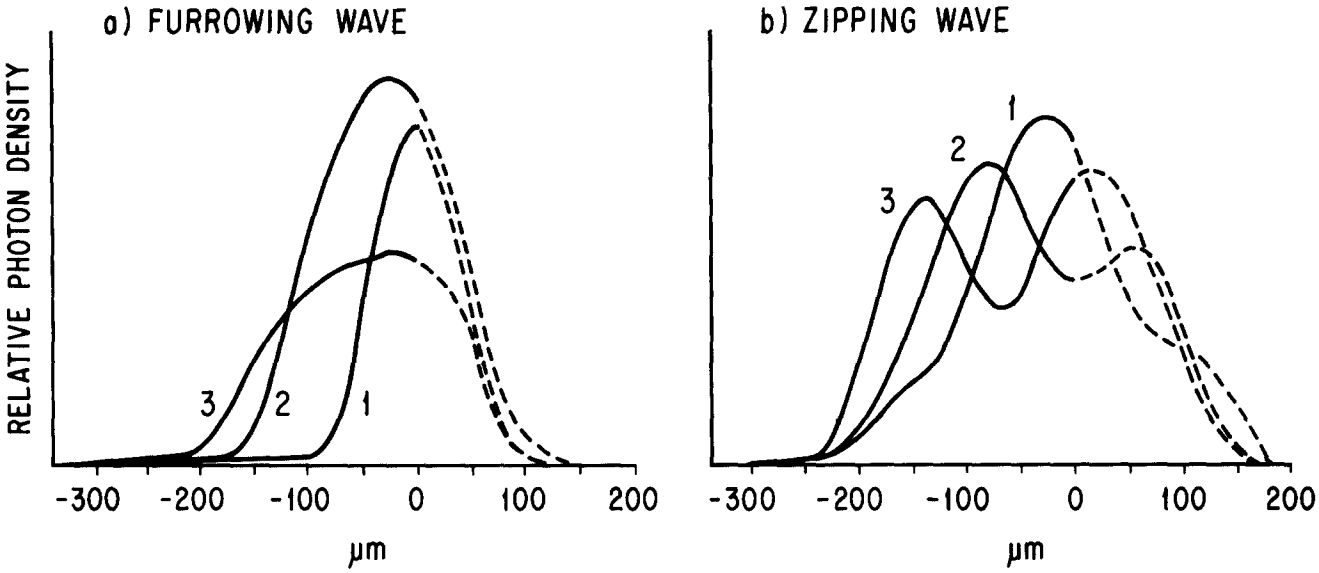

Figure 3. Analysis of the luminescence along the second cleavage furrows shown in Fig. 2. The profiles show photon densities along the forming or formed furrows and were measured within rectangles just wide enough to encompass the waves involved. They show data smoothed with a linear convolution filter so as to better reveal the wave fronts. Each profile shows photon intensity along the furrow minus average photon intensity near the furrow and came from the photons collected for one minute. Successive profiles in both the furrowing and the zipping series were collected during successive minutes. The point "O" represents the point along the first furrow at which the two furrows arose during the second cleavage; the solid lines represent the left-hand furrow in Fig. 2 and the dashed lines the right-hand furrow. As noted in the legend to Fig. 2, the left-hand furrow was in better focus and gave clearer results. The velocity of the waves were estimated by measuring the distance between the two wave fronts at successive intervals, computing a velocity, and correcting for scale.

reported to be about 0.14 μM (Schantz, 1985). From this value, from R_V values and from a graph of aequorin luminescence vs calcium (Blinks, 1982), one can then estimate the peak calcium level in the contractile band region during furrowing to be about 5 to 8 μM.

In contrast to the first or furrowing waves, the second or zipping waves are about 90–130-μm wide. As expected, these broad waves clearly progress to the edge of the blastodisc. Moreover, these "zipping" waves often persist for 10 to 20 min peripherally. This relatively long persistence was the only feature of the calcium waves to be suppressed by use of a calcium/magnesium-free medium.

Discussion

The First Calcium Waves and Furrow Elongation

We have seen calcium waves accompany furrow elongation in each of the early cleavages of medaka eggs. These may be usefully compared with the calcium wave which immediately follows fertilization and serves to initiate the development of medaka eggs (Gilkey et al., 1978). Like the fertilization wave, these first furrowing waves seem to peak at rather high calcium levels. Our present estimate (which is discussed below) is that they peak at a local free calcium con-

Table I. Quantitative Data from Individual Cleavages

Egg and cleavage numbers	Wave speeds (μm/s)		Relative intensities, R_A	
	Furrowing	Zipping	Furrowing	Zipping
5-1	0.54	0.18	–	–
5-2	–	0.42	1.2	1.8
6-1	–	–	3.6	4.6
6-2	0.41	0.59	–	4.5
7-1	–	0.52	–	–
7-2	0.63	0.55	–	–
8-1	–	0.47	–	3.5
8-2	0.42	0.57	3.7	3.1
9-2	0.50	0.21	2.4	(14.3)
9-3	0.39	0.40	2.7	4.3
Mean ±SEM	0.48 ± 0.04	0.47 ± 0.04	2.7 ± 0.5	3.6 ± 0.4

Wave speeds are speeds of the advancing front taken from profiles similar to those in Fig. 3. Relative intensities are ratios of photons within a 7-pixel-wide box encompassing the wave peak to such a box over a nearby, inactive region of the blastodisc. Egg #8 was bathed in Ca^{2+}/Mg^{2+}-free medium containing 1 mM EGTA. The anomalous value in zipping wave 9-2 was ignored in calculating the mean.

centration of 5 to 8 μM. This is comparable to the 30 μM level estimated for the fertilization wave. However, unlike the fertilization wave—which traverses the whole cytoplasm—they travel through a very narrow (and presumably even shallower) part of the cytoplasm. The first furrowing waves have a width less than our 20 μm lateral resolving power and may well have a depth comparable to the 0.1 to 0.2-μm depth of the contractile bands. Moreover, the furrowing waves are far slower than the fertilization one. While the latter traverses the activating egg at ~10 μm/s, the furrowing waves traverse the dividing blastodisc at about 0.5 μm/s and thus about twenty times slower.

Indeed, the calcium waves through cleaving medaka eggs move far more slowly than any hitherto visualized calcium waves. Such waves have been seen in more than two dozen different eggs and somatic cells so far and their speeds range from 10 to 100 μm/s (Jaffe, 1991). For this and other reasons we suggest that medaka furrow waves will prove to be the first visualized member of a new class of slow, as opposed to fast, calcium waves. Specifically, we repeat an earlier suggestion that slow calcium waves will prove to accompany all slow surface contractile waves (Jaffe, 1985). The latter are a small but well documented class of 0.3 to 1.0 μm/s waves which traverse a variety of eggs during normal development (Schroeder, 1975; Sawai, 1985; Wolf, 1985; Cheer et al., 1987). There is even a hint of a comparable wave in asymmetrically cleaving PtK2 cells (Sanger et al., 1989; see Fig. 7). Of particular interest are the surface contraction waves seen in several amphibian eggs. These spread out radially rather than linearly and are of two different types. Hara's "post fertilization waves" start at the sperm entry point and have been studied only in *Xenopus* (Hara et al. 1977); while the better studied "pre-cleavage" waves start at the animal pole and have been reported in several amphibian eggs (Hara, 1971; Yoneda et al., 1982; Sawai, 1982; Sawai and Yomota, 1990). Cheer et al. (1987) have discussed whether these slow surface contraction waves in amphibian eggs are indeed calcium waves; it would be of great interest to know if further technical advances show them to be such waves or not.

We have estimated the peak local calcium level in the first furrowing wave to be ~5–8 μM on the assumption that the depth of the high calcium region is comparable to that of the contractile band. This is a plausible but uncertain assumption. Nevertheless, because aequorin luminescence varies with the first power of the aequorin concentration (and thus the depth of the high calcium source) but nearly the third power of the calcium concentration, for this reason, the inferred calcium concentration is surprisingly insensitive to the depth which is assumed. Even if one makes the assumption that the high calcium region is ten times deeper than the contractile band, the inferred calcium concentration is ~3 μM. So the high peak calcium (as well as the many minute duration) of the first calcium waves immediately suggest that they are more than an epiphenomenon, that they act back to speed and control growth of the contractile band. Such feedback is further supported by the three reports discussed in the Introduction. Furthermore, we ourselves have observed that injections of 5,5′-dibromo-BAPTA, a calcium buffer with a k_D of ~5 μM, will block cytokinesis in medaka eggs at a final concentration of ~1 mM or more (Fluck, R., A. Miller, Y. Kühtreiber, and L. Jaffe, manuscript in preparation). This is just the inhibiting concentration which is theoretically expected for such a buffer if it acts by inhibiting formation of a high calcium zone in the 3 to 10 μM range (Speksnijder et al., 1989). Altogether, the evidence indicates that the first calcium waves act back to speed furrow elongation.

The high intensity and long duration of these waves also suggests that they control furrow growth through more than one molecular target. Moreover, the obvious analogy to smooth muscle contraction suggests that one key molecular target is myosin light chain kinase (MLCK). Two main arguments support this idea. First, there is good reason to believe that MLCK is naturally activated by calcium in the same high 1 to 10 μM range (Cassidy et al., 1981; Nishimura et al., 1990 [Fig. 3, control curve]; Means et al., 1982). Second, activation of MLCK would be expected to favor growth of the contractile band and thus of the cleavage furrow. As Mabuchi has emphasized, activated MLCK acts to favor the assembly of both nonmuscle as well as smooth muscle myosins into stable filaments; such myosin filaments in turn can organize actin filaments (Mabuchi, 1986; Mabuchi and Takano-Ohmuro, 1990). Moreover, activated MLCK would be expected to favor actomyosin contraction and in this way extend contractile bands by tightening and aligning a preexisting network of cortical actomyosin filaments (Bray and White, 1988; Cao and Wang, 1990).

Finally, this raises the question of whether contracting bands in turn pull on stretch-sensitive channels ahead of the wave so as to release calcium and thus propagate the wave. Zotin (1964) reported a rapid and persistent wrinkling reaction in fertilized sturgeon eggs in response to prodding with a blunt glass needle. This reaction clearly results from a localized contractile response. Like furrow elongation (Sawai and Yamota, 1990) such wrinkling can be blocked with colchicine. It suggests the presence of stretch-sensitive calcium channels which are attached to cytoskeletal cables. Moreover, cogent arguments against the three formal alternatives to mechanical propagation are available.

One such alternative is that furrow elongation is not a propagated, epigenetic process at all but growth along a preexisting preprogrammed track. This idea seems to be ruled out by several observations on giant amphibian eggs. For example, their first cleavage plane can be rotated up to 90° simply by deforming them before metaphase (Sawai and Yamota, 1990). A second alternative is growth of the contractile band by terminal addition of actin monomers in the manner of microtubule or microfilament growth. However, actin filaments in the contractile band lack the simple polarity needed for such quasicrystalline growth. A third alternative is that the slow calcium waves, like fast ones, are propagated by calcium-induced calcium release or by some related reaction–diffusion process. Such mechanisms seem most unlikely when one considers just how slow the pertinent calcium release reaction would have to be to explain slow calcium waves. The velocity of such waves is given by the Luther equation, a basic equation for reaction/diffusion waves (Showalter and Tyson, 1987).

$$V = \alpha \sqrt{\frac{D}{t_r}} \tag{2}$$

where V is wave velocity, D is the diffusion constant of the autocatalytic species, (here that of free cytosolic cal-

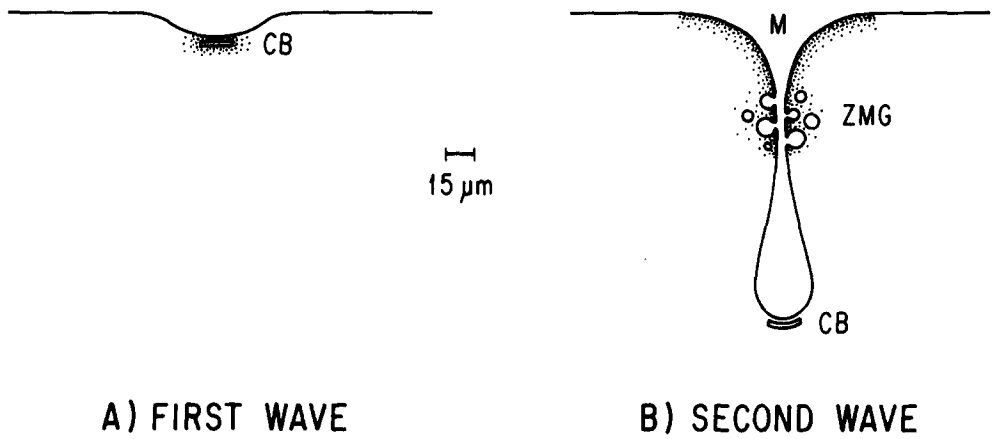

Figure 4. Diagram of high calcium regions (*stippled*) which underlie the two calcium waves in the cleaving medaka egg. It is mainly based upon a study of cleaving *Xenopus* eggs by Byers and Armstrong (1986). The somewhat different furrow structure described by Singal and Sanders (1974, see Fig. 8) probably reflects a much later stage in cleavage. *CB*, contractile band; *ZMG*, zone of membrane growth; M, furrow's mouth. For the sake of clarity, the thicknesses of the contractile bands and of the calcium-rich regions have been much exaggerated.

cium ions, which is known to be ~600 $\mu m^2/s$ [Donahue and Abercrombie, 1987; Speksnijder et al., 1989]), t_r is the reaction time or time taken for Ca^{2+} to rise e-fold, and α is a factor which largely depends on the exact (and in this case unknown) kinetics of the autocatalytic reaction. However, α quite generally lies between 0.5 and 2.0 for chemical waves in vitro and proves to be ~1.0 for fast intracellular calcium waves (Jaffe, In press). So we can safely assume that α is about the same for both fast and slow calcium waves. Application of the Luther equation, then, indicates that t_r (found to be 2.5 to 5 s for the first [11 to 16 μm/s] wave through fertilizing medaka eggs) would have to rise to a half an hour to explain 0.5 μ/s slow waves. It is hard to imagine how that could work.

Altogether then, we are led to propose that the first calcium waves are propagated by a traveling cycle in which a local release of calcium induces both assembly and initial contraction of the actomyosin band and that this contraction in turn releases calcium ahead of the growing band by pulling on distant stretch-sensitive calcium channels. These channels might lie in the plasma membrane and be comparable to those in muscle cells (Franco and Lansman, 1990) or they might lie in the ER. Since egg #8 exhibited calcium waves of unchanged speed and strength in calcium-free, EGTA-buffered media, such channels should be partly or wholly in the ER.

The Second Calcium Wave and Furrow Apposition

The second waves of luminescence are three to seven times wider than the first ones, far longer lasting, slightly more intense and altogether more obvious than the first ones. Nevertheless, they occur at a much later stage in cytokinesis, when the underlying furrow is far deeper and more complex and the location of the underlying high calcium region relatively uncertain. Fig. 4 compares schematic cross-sections of the probable early and late furrow structures. It is based upon the better known structure of cleavage furrows in *Xenopus* eggs (Byers and Armstrong, 1986) but is consistent with our limited knowledge of such furrows in fish eggs (Thomas, 1968). In this scheme the ingressing furrow is divided into three zones or domains: at the bottom is the leading edge, which is being driven deeper by the shrinking contractile band; at the top is its mouth; in between are its lateral walls. The latter includes a zone of membrane growth which is fed by exocytosis. This zone seems to lie just under the furrow's mouth (Byers and Armstrong, 1986). While the first waves of luminescence seem likely to come from a high calcium region within the assembling contractile band, the second ones almost certainly do not come from the shrinking band deep within the furrow groove. On the contrary, good biochemical evidence indicates that this deep shrinking band lies within a region of very low calcium well below the general cytosolic level. This evidence comes from observations on the later stages of contractile ring shortening in three different preparations, including permeabilized PtK_1 cells and sea urchin eggs (Cande, 1980; Yoshimoto and Hiramoto, 1985) as well as isolated newt egg furrows (Mabuchi et al., 1988). All three actively shorten in vitro and do so most quickly at very low calcium levels. The newt furrow preparation, for example, shortens fastest at 0.01 to 0.1 μM calcium.

The source of the second calcium wave is likely to include the zone of membrane growth, since exocytosis is generally driven by a rise in calcium. However, it may also include the furrow's mouth since it seems difficult to account for the 100-μm width of the second wave by calcium within the cortex of the growth zone alone. Furthermore, Kline et al. (1983), have measured substantial steady electrical currents entering the old, pigmented membrane in the mouth of the cleavage furrows of *Xenopus* eggs. Sharp reductions of these inward currents by calcium channel blockers suggests that they include a calcium component as large at 1 pM/cm^2 s. Such a current could easily maintain a high, subsurface calcium region in the furrow's mouth. All things considered, the calcium levels which underlie the second waves of luminescence should prove comparable to those which underlie the first ones.

We know of no evidence suggesting that the second wave is actively propagated along the zipping furrow. Thus, we would make the simpler assumption that it is not truly propagated at all. Rather it is a "phase wave" (Tyson and Keener, 1988, see page 328), the (longitudinal) spread of which represents a sequential recurrence of the first wave. However, the far greater width and duration of the second wave does require some mechanism(s) for its lateral spread and persistence. In part this may be mediated by a lateral

component of the tension field generated by band contraction. In part it may also be mediated by the exocytotic insertion of new furrow membrane rich in calcium channels together with the subsequent diffusion (or even electrophoresis) of these channels into old membrane at the furrow's mouth.

Finally we should consider the possible function of the second calcium waves. One may be to induce the exocytosis which supports the growth of the furrow membrane during cleavage (Fig. 4). This new surface is known to be far more adhesive than the original one (Bozhkova et al., 1983; Roberson et al., 1980); this adhesiveness may help effect or at least maintain apposition of the cleaving cells. Another may be to somehow relax cortical tensions and in this way allow apposition or zipping to proceed.

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