A Localized Elevation of Cytosolic Free Calcium Is Associated with Cytokinesis in the Zebrafish Embryo

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Abstract. Cytokinesis, a key step in cell division, is known to be precisely regulated both in its timing and location. At present, the regulatory mechanism of cytokinesis is not well understood, although it has been suggested that calcium signaling may play an important role in this process. To test this notion, we introduced a sensitive fluorescent Ca^{2+} indicator into the zebrafish embryo and used confocal microscopy to measure the spatiotemporal variation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) during cell cleavage. It was evident that a localized elevation of $[Ca^{2+}]_i$ is closely associated with cytokinesis. First, we found that during cytokinesis, the level of free Ca^{2+} was elevated locally precisely at the cleavage site. Second, the rise of free Ca^{2+} was very rapid and occurred just preceding the initiation of furrow contraction. These observations strongly suggest that cytokinesis may be triggered by a calcium signal. In addition, we found that this cytokinesis-associated calcium signal arose mainly from internal stores of Ca^{2+} rather than from external free Ca^{2+}; it could be blocked by the antagonist of inositol trisphosphate ($InsP_3$) receptors. These findings suggest that the localized elevation of $[Ca^{2+}]_i$ is caused by the release of free Ca^{2+} from the endoplasmic reticulum through the $InsP_3$-regulated calcium channels.

INTRACELLULAR Ca^{2+} is an important second messenger known to be involved in many cell functions. For a long time, it has been suspected that calcium signaling may play an important role in regulating cytokinesis (for a review see Satterwhite and Pollard, 1992; Hepler, 1992). In fact, this view has been supported indirectly by a number of observations. For example, it was reported almost two decades ago that application of a calcium ionophore can accelerate furrow elongation in cleaving squid eggs (Arnold, 1975). Using a bioluminescence probe, aequorin, cyclic changes of intracellular calcium level have been observed in association with the cell cleavage cycle in echinoderm and medaka eggs (Yoshimoto et al., 1985). Elevation of free Ca^{2+} has also been detected during cell cleavage using a fluorescent dye, fura-2 (Poenie et al., 1985; Whitaker and Patel, 1990). Recently, Fluck et al. (1991) measured the aequorin signal in medaka eggs using a specially designed photon detector and was able to observe changes of the intracellular calcium level at the cleavage region. They suggested that a slow calcium wave accompanied cytokinesis in the medaka egg.

In spite of these efforts, however, the evidence that implicates calcium signaling in cytokinesis is still uncertain. First, there are conflicting reports about the direction and magnitude of the variation in the intracellular free calcium concentration ($[Ca^{2+}]_i$) during cytokinesis (Hepler, 1992). Depending on the method of measurements, some observed an elevation in $[Ca^{2+}]_i$ at cell cleavage (Fluck et al., 1991; Poenie et al., 1985; Whitaker and Patel, 1990), while others reported either a reduction (Yoshimoto et al., 1985; Grandin and Charbonneau, 1991) or no change (Rink and Tsien, 1980; Schantz, 1985; Hamaguchi and Mabuchi, 1978; Hepler and Callaham, 1987). Part of the difficulty may be that the change in $[Ca^{2+}]_i$ can be highly localized and thus may be difficult to detect in studies measuring total Ca^{2+}. Second, since most of the earlier work measured only the average $[Ca^{2+}]_i$ over the entire cell (Hamaguchi and Mabuchi, 1978; Poenie et al., 1985; Yoshimoto et al., 1985; Schantz, 1985; Hepler and Callaham, 1987; Whitaker and Patel, 1990; Grandin and Charbonneau, 1991), there was a lack of information on the spatial correlation between the calcium signal and the cellular response during cytokinesis. Third, in the recent study that intracellular distribution of Ca^{2+} was measured using aequorin (Fluck et al, 1991), there was insufficient spatial or temporal resolution to allow comparison of the timing of $[Ca^{2+}]_i$ change with the initiation of cell cleavage. It was not clear whether the elevation of free calcium was the cause or the result of cytokinesis.

To provide more detailed information on the role of calcium signaling in cytokinesis, we used a combination of newly developed imaging techniques to examine simultaneously the spatial and temporal distributions of free Ca^{2+} in zebrafish embryos during cell cleavage. Because of its

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optical transparency and its rapid cell division cycle, the zebrafish embryo is an ideal model system for studying calcium signaling in cell division. Our calcium-indicating probe is a fluorescent dye, Calcium Green-1, which was conjugated to 10 kD dextran (from Molecular Probes, Eugene, OR). The fluorescence signal was measured digitally using a laser-scanning confocal microscope. The major advantage of the confocal measurement is that we can examine the spatial distribution of the fluorescence signal within a single optical section of the cell, without interference from the out-of-focus regions. Also, using a time-series of digital recording, we can monitor the temporal variation of the Ca^{2+} distribution pattern second by second.

This study addresses two specific questions: (*1*) Is there a localized calcium signal which is closely associated with the onset of cytokinesis? (*2*) What are the sources of this calcium signal?

A summary of the preliminary results of this work has been presented in the General Scientific Meeting of the Marine Biological Laboratory at Woods Hole (Meng and Chang, 1994) and at the Thirty-Fourth Annual Meeting of the American Society for Cell Biology.

Materials and Methods

Specimen Collection and Treatments

Zebrafish (*Brachydanio rerio*) obtained from North Carolina Biological Supplies, were maintained on a light/dark cycle as described by Westerfield (1994) to induce spawning. A single layer of marbles was laid at the bottom of the fish tank to trap the spawned eggs. Fertilized eggs were collected by suction 10–20 min after spawning. The eggs were washed three times, and then resuspended in the embryo medium (13.7 mM NaCl, 0.54 mM KCl, 0.025 mM Na_2HPO_4, 0.044 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$, pH 7.2) (Westerfield, 1994). In some experiments, eggs were dechorionated using a pronase treatment (Westerfield, 1994).

Calcium Probes, Calcium Buffers, and Chemical Reagents

The calcium-indicating dye used in this study, Calcium Green-1 conjugated to 10 kD dextran, was prepared from a stock solution (concentration 250 μM) dissolved in an injection buffer (10 mM Hepes, 3 mM $MgCl_2$, 30 mM sodium glutamate, 40 mM potassium phosphate and 80 mM sucrose, pH 7.2); this solution was stored in the dark at 4°C. In some experiments, rhodamine conjugated to 10 kD dextran was also added to the injection solution (2.5 mg/ml), so that the distribution of dye inside the embryo could be monitored. The calcium buffer BAPTA (1,2-bis(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid) was prepared as a 100-mM stock solution in injection buffer. Stock solutions of heparin (10 mg/ml) and ryanodine (2 mM) were also made in the injection buffer.

Unless specified otherwise, the embryos were bathed in the embryo medium. In the case when it was required to remove extracellular Ca^{2+}, the Ca-free buffer was prepared by substituting $CaCl_2$ in the embryo medium with 5 mM $MgCl_2$ plus 0.5 mM EGTA. When it was necessary to block the calcium channels at the plasma membrane, lanthanum chloride (0.5 mM) or nifedipine (0.2 mM) was added to the Ca-free buffer.

Dextran-conjugated Calcium Green-1, dextran-conjugated rhodamine, and BAPTA were purchased from Molecular Probes, Inc. Heparin, ryanodine, lanthanum chloride, nifedipine, and other reagents were obtained from Sigma Chem. Co. (St. Louis, MO).

Microinjection

The dye was microinjected into one-cell stage embryos at a final concentration of less than 4 μM. To immobilize the eggs for injection, eggs were inserted into the groove of an agar slab (1% agar). Under a dissecting microscope (Zeiss), the embryos were injected by pressurized gas using a PLI-100 pico-injector (Medical Systems Corp., Greenvale, NY). The micropipettes were pulled from 1.0-mm glass capillaries with filling fiber inside (World Precision Instruments, Inc., Sarasota, FL) using a Narishige PN-3 glass microelectrode puller. The injection volume was estimated by injecting a solution droplet into a bath of paraffin oil and measuring the droplet diameter using a micrometer attached to the dissecting microscope. The volume of injected solution was typically less than 5% of the cell volume.

Microscopy

The spatial distribution of the fluorescent signal emitted by Calcium Green-1 or rhodamine in the injected embryos was studied using a laser scanning confocal microscope. Two models were used: MRC-600 and MRC-1000 (from Bio-Rad Labs., Hercules, CA). Both microscopes were equipped with a krypton/argon mixed gas laser which was capable of providing three separate wavelengths for multi-channel measurement. The laser scanner was coupled to a compound microscope (Zeiss Axioskop) equipped with phase and epi-fluorescent optics. The objectives used were Zeiss Plan-Neofluar 20×/0.50 and 10×/0.30. To reduce photo-bleaching, minimum laser power was used in the measurements. Also, in order to monitor the temporal variation of the fluorescence signal, the controls on "gain" and "black level" were fixed throughout each z-series or time-series measurement.

The relationship between $[Ca^{2+}]_i$ and the measured fluorescence signal was calibrated using procedures similar to the in situ Ca^{2+} calibration method of Kao (1994). Briefly, at the end of each experiment, a high dosage of ionomycin (10–100 μM) was applied to the embryos in the presence of 2 mM of $CaCl_2$ to obtain the maximum fluorescence reading at saturated $[Ca^{2+}]_i$. The measured fluorescence signals were then converted into calcium concentrations using the calibration curves provided by Molecular Probes and equation (1) of Kao (1994).

Results

Injection of Calcium Probes Did Not Interfere with Normal Embryo Development

To determine the optimal concentration of calcium dye to be injected, two requirements must be considered. First, the signal-to-noise ratio must be high enough to detect the localized changes in $[Ca^{2+}]_i$ in the embryo. Second, the injected dye must not interfere with normal embryo development, otherwise the observed results could be artifacts caused by the injected dyes. We found that when the injected dye reached a final concentration of 2–4 μM, the fluorescent signal had a more than adequate signal-to-noise ratio. With this amount of injected dye, the development of the fish embryo was completely normal. Table I shows the results of seven experiments designed to examine this point. The percentage of zebrafish embryos injected with dye that successfully developed into normal fishes was virtually the same as that of the control (i.e., uninjected embryos). Furthermore, even when the injected embryos were extensively exposed to the laser of the confocal microscope, the success rate of hatching was not significantly altered. These results indicate that the combination of injecting the Calcium Green-1 dye and laser scanning during observation by the confocal microscope did not interfere with the normal development of the embryos.

A Localized Elevation of Intracellular Free Calcium Was Associated with Cell Cleavage

By using the confocal microscope, one can measure the distribution of free Ca^{2+} within an optical thin section of the embryo. Digital images of $[Ca^{2+}]_i$ can be obtained as a function of time. Fig. 1 shows the distribution of $[Ca^{2+}]_i$, as determined from the fluorescent signal of Calcium Green-1, in a zebrafish embryo undergoing the second cell cleavage.

Table I. Embryo Development after Microinjection of Calcium Green-1 Conjugated with 10 kD Dextran

Number of embryos	Injected with Ca Green-1	Exposed to laser light	Number of normal fish hatched	Normal development
				%
19	yes	no	16	84.2
5	yes	yes	4	80.0
13	no	no	12	92.3

Calcium Green-1 conjugated to 10 kD dextran was microinjected to a final concentration of 2–4 μM. Results are from seven experiments.

The level of free Ca^{2+} is shown here in pseudo-color. From this figure, it is apparent that the distribution of $[Ca^{2+}]_i$ was not uniform throughout the entire embryo. Elevation of free Ca^{2+} can be seen mainly in two local areas of the embryo: (*1*) at the cleavage furrow of the first cell cleavage, and (*2*) along the equators of the dividing cells, where the second cleavage furrow emerged. Thus, it appears that there is a close spatial correlation between elevated $[Ca^{2+}]_i$ and the cell cleavage furrow. In fact, one may see from Fig. 1 (*b* and *c*) that a localized elevation of $[Ca^{2+}]_i$ was detected just before the cleavage furrow began to appear.

Localized elevation of $[Ca^{2+}]_i$ at the cleavage furrow was detected in 91% of embryos that had been examined (n = 112). The pattern of $[Ca^{2+}]_i$ distribution as shown in Fig. 1 is typical among most of the embryos, although the magnitude of elevation in the Ca^{2+} level may vary significantly. Among those embryos whose actual $[Ca^{2+}]_i$ had been calibrated, the average resting level of intracellular free Ca^{2+} was estimated to be 164 (±42) nM (n = 43). At the initiation of cytokinesis, $[Ca^{2+}]_i$ was found to rise to ~605 (±380) nM (n = 43). The large variation in the peak $[Ca^{2+}]_i$ observed at the cleavage site could be partially related to the fact that the elevation of $[Ca^{2+}]_i$ may vary with the depth of the optical section in the measurement. We have also calculated the ratio of $[Ca^{2+}]_i$ elevation at the equator before and after the initiation of cytokinesis. On average, the local $[Ca^{2+}]_i$ was found to increase by 3.7 (±2.2)-fold (n = 43).

The reason that we failed to detect $[Ca^{2+}]_i$ changes in a small percentage of embryos is probably related to technical difficulties. For example, the cytoplasmic concentration of fluorescent probes could be significantly reduced when dyes were accidentally injected into the boundary between the cytoplasm and yolk. Under such a situation, it was difficult to observe the localized $[Ca^{2+}]_i$ changes due to the lack of sensitivity.

One alternative interpretation of the results shown in Fig. 1 is that the observed elevation of fluorescent signal could be an artifact caused by uneven distribution of the

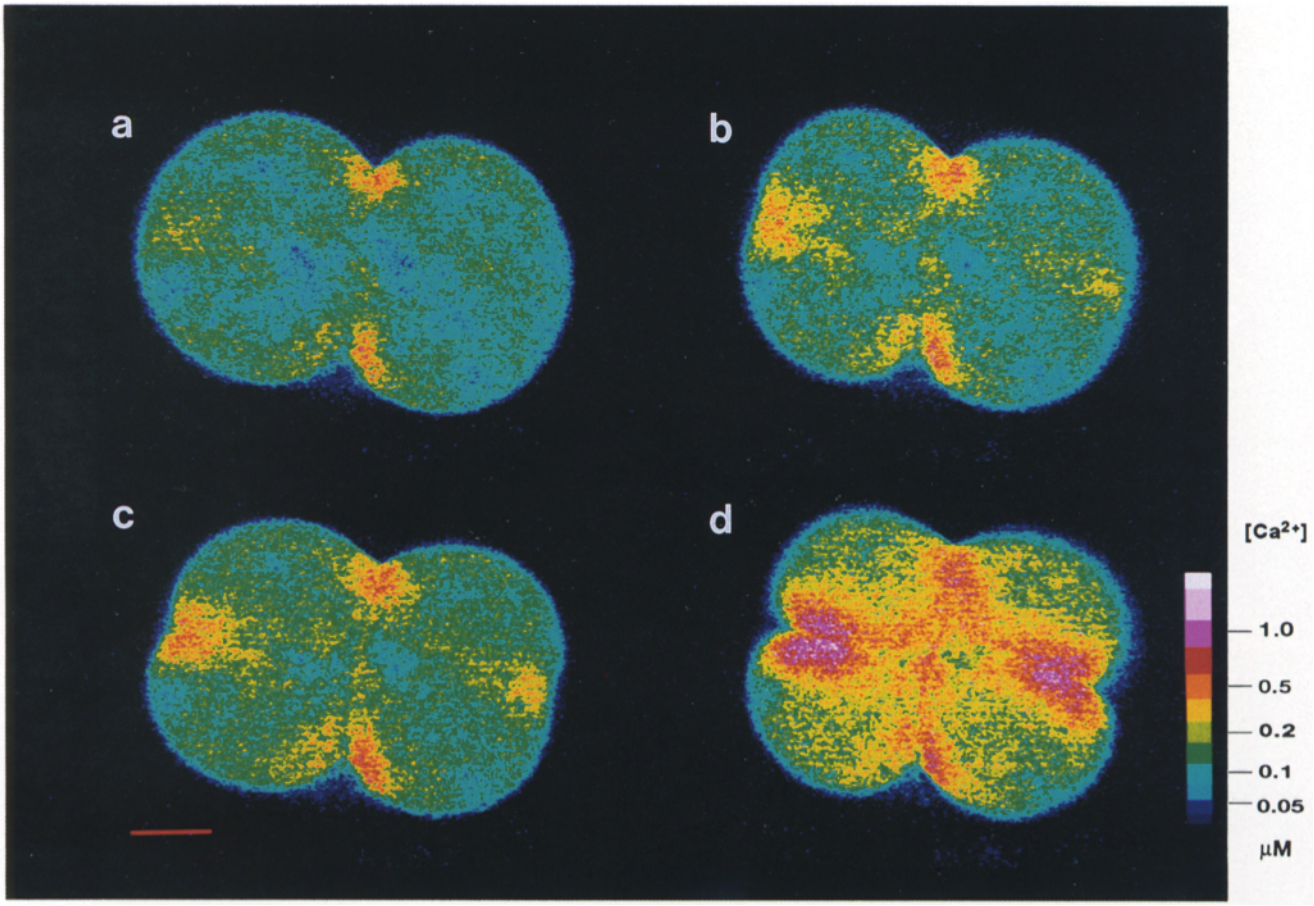

Figure 1. Spatial and temporal variation of $[Ca^{2+}]_i$ associated with the second cell division in a zebrafish embryo. The embryo was viewed from the animal pole at an optical section which was about 2-μm thick. Intracellular concentration of free Ca^{2+}, as determined from the intensity of fluorescence signal from Calcium Green-1, is displayed in pseudo-color. *a–d* represent a time series of calcium images obtained at different times (t). At the beginning of this time series, the embryo was at a 2-cell stage. t = 0 (*a*); 69 (*b*); 153 (*c*); and 365 (*d*) seconds. Bar, 100 μm.

calcium-indicating dyes that somehow aggregated at the cell cleavage furrow. To rule out this possibility, we co-injected two fluorescent dyes, rhodamine and Calcium Green-1, both of which were conjugated to 10 kD dextran, into the same embryo and recorded simultaneously the fluorescent images of the two dyes using two separate wavelength channels in the confocal microscope. Since the fluorescent signal of rhodamine is not sensitive to $[Ca^{2+}]_i$, it provides a direct measurement of the distribution of the injected dextran molecules. We found that the rhodamine signal shows no detectable spatial or temporal changes during cell cleavage (Meng and Chang, 1994), indicating that the dextran-conjugated dyes were evenly distributed throughout the process. Thus, the observed changes in the Calcium-Green signal should reflect mainly the variations in the distribution of Ca^{2+} in the embryo.

Dynamics of the Ca^{2+} Elevation in Association with Cytokinesis

How rapidly does the Ca^{2+} signal rise and how close are the timings between the $[Ca^{2+}]_i$ elevation and the onset of cytokinesis? Fig. 2 shows the results of a time-series measurement of the distribution of $[Ca^{2+}]_i$ in one fixed optical section of an embryo which was undergoing the third cell division. Ca^{2+} images were recorded at the rate of one image per second. Different panels represent sample images collected at different times. Hot spots of intense $[Ca^{2+}]_i$ elevation were found to appear before the initiation of cell cleavage. In fact, the hot spots developed very rapidly; the $[Ca^{2+}]_i$ took only a few seconds to rise from the resting level to the peak excited level (see panels *b*, *c*, and *d* in Fig. 2). The location of the hot spots (arrowhead in *b*) coincided exactly with the future sites of the cleavage furrow formation. Similar results were also observed in another embryonic cell which divided at a later time (see panels *f*, *g*, and *h* of Fig. 2).

As observed earlier in Fig. 1, the localized elevation of $[Ca^{2+}]_i$ seems to appear first before the membrane started to curve inward to form the cleavage furrow. With the results of the time-series measurement as shown in Fig. 2, it is possible to determine the time lapse between the rise of the local Ca^{2+} signal and the onset of cytokinesis in the dividing cell. Fig. 3 shows the variation of the Calcium Green-1 fluorescent signal as a function of time at the location where the cell cleavage took place. It was found that a rapid increase of local $[Ca^{2+}]_i$ can be detected about 20 s before the cleavage furrow starts to form. This time lag was found to vary from embryo to embryo, but the elevation of $[Ca^{2+}]_i$ was always preceding the furrow contraction. Among embryos that had been examined in time-series

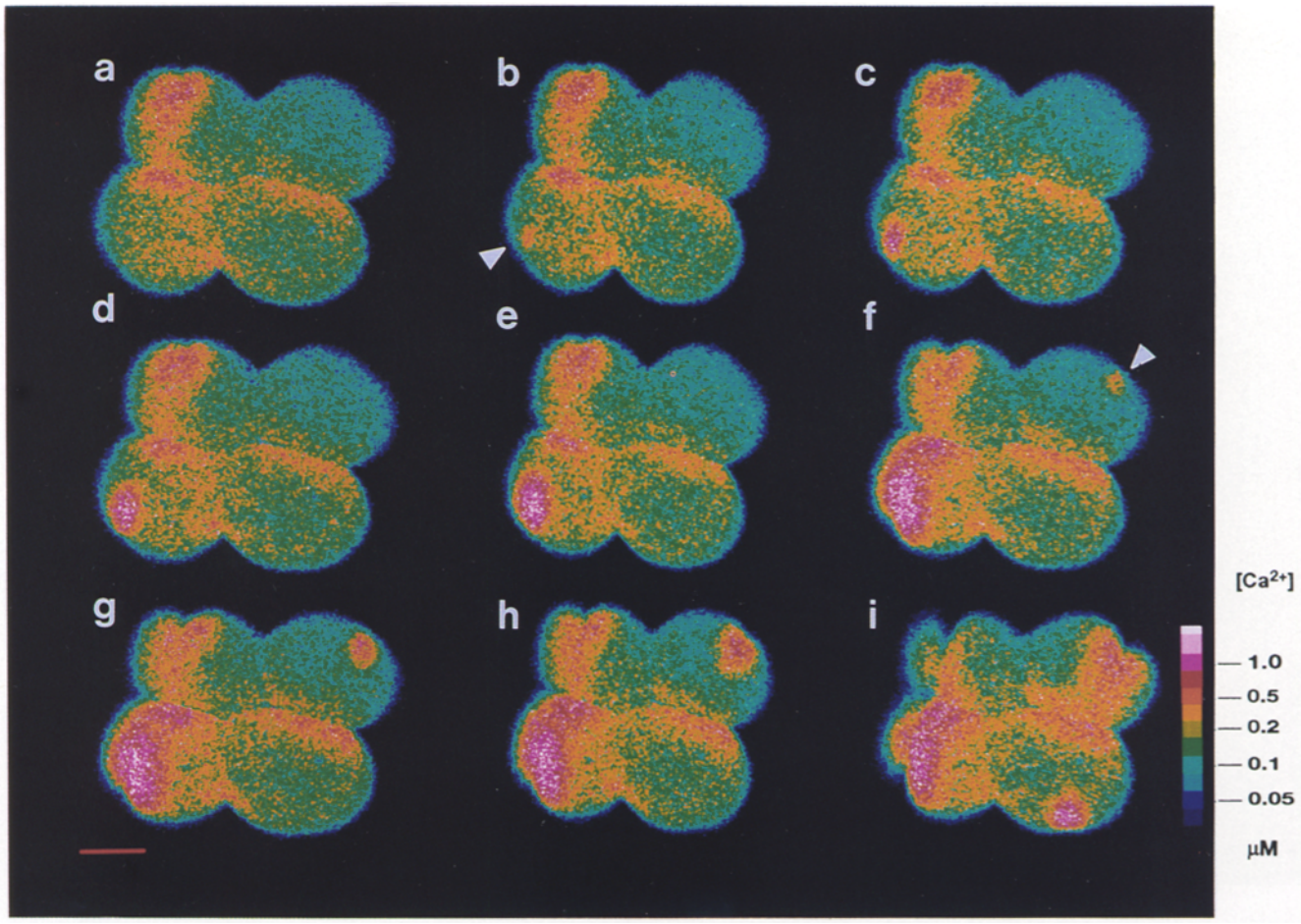

Figure 2. Time series of $[Ca^{2+}]_i$ variation during the third cell division. The zebrafish embryo was viewed from the animal pole. The level of free Ca^{2+} within one optical section of a 4-cell stage embryo was determined from the fluorescence signal of Calcium Green-1 and displayed here in pseudo-color. Different panels represent images obtained at different times (t); $t = 0$ (*a*), 1 (*b*), 2 (*c*), 3 (*d*), 6 (*e*), 50 (*f*), 51 (*g*), 54 (*h*), and 116 (*i*) seconds. Arrowheads mark the position where the localized Ca^{2+} signal arose. Bar, 100 μm.

measurements while they were undergoing the third cell cleavage, the average time lag between the rise of the Ca^{2+} signal and the initiation of furrow contraction was 30.1 (±14.2) s (n = 29).

Cytokinesis Was Blocked by Injecting Calcium Buffer in a Dosage-dependent Manner

To further evaluate the importance of the calcium signal in regulating cytokinesis, we examined whether cytokinesis in the zebrafish embryo can be blocked by abolishing the $[Ca^{2+}]_i$ elevation. The intracellular level of free Ca^{2+} can be suppressed by injecting a Ca^{2+} buffer, BAPTA, into the zebrafish embryo. With increasing concentrations of BAPTA injected, a larger proportion of injected embryos failed to enter cytokinesis (see Fig. 4). When the final concentration of BAPTA reached about 5 mM, cell cleavages in all injected embryos were blocked. Also, no localized elevation of intracellular Ca^{2+} could be observed in these embryos (data not shown). These results indicate that an elevated Ca^{2+} level is essential in triggering cytokinesis.

Source of the Calcium Signal in Association with Cytokinesis

An important question remains; that is, what is the source of the elevated free Ca^{2+}? One possibility is that Ca^{2+} ions stored in the ER system may be released through the inositol trisphosphate ($InsP_3$)[1] receptors either by direct action of $InsP_3$ or by a calcium-induced calcium release (CICR) mechanism (Thomas et al., 1992; Berridge, 1993; Berridge and Dupont, 1994; Amundson and Clapham, 1993; Ciapa et al., 1994; Girard and Clapham, 1993; Han et al., 1992; Stith et al., 1993). To examine the functional contribution of this pathway, we injected an $InsP_3$ receptor antagonist, heparin, into the embryo and studied its effects on cell cleavage and on the associated Ca^{2+} elevation. Fig. 5 shows the distribution of $[Ca^{2+}]_i$ in a zebrafish embryo in which heparin (in a final concentration of 100 μg/ml) was previously injected into one of the embryonic cells (marked by arrowhead) at the two-cell stage. The uninjected cell (on the right) was found to undergo a new round of cell cleavage, and displayed a localized elevation of intracellular Ca^{2+}, while the injected cell (on the left) failed to undergo cytokinesis, and no localized $[Ca^{2+}]_i$ elevation was found (Fig. 5). These results suggest that a major fraction of the cytokinesis-associated calcium signal must be released from internal stores of Ca^{2+} (such as ER) through the $InsP_3$-dependent calcium channel.

1. *Abbreviations used in this paper*: $InsP_3$, inositol trisphosphate, MLC, light chain of myosin II; MLCK, myosin light chain kinase; RYR, ryanodine receptor.

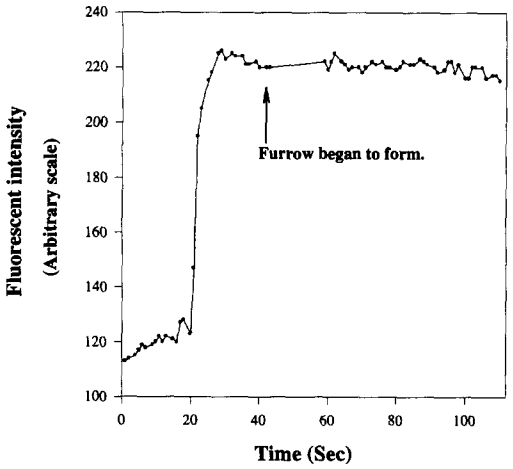

Figure 3. Temporal change of free Ca^{2+} level at the site of cleavage furrow formation during the third cell division, based on data from the same time-series measurement as presented in Fig. 2.

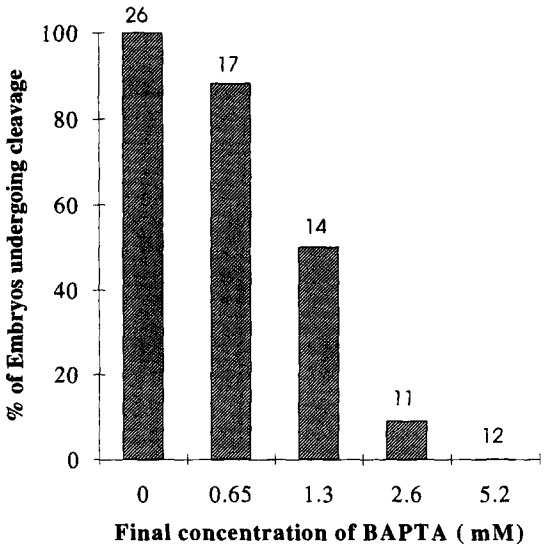

Figure 4. Effects of the calcium chelator, BAPTA, on cell cleavage. Percentage of embryos undergoing cell cleavage is plotted as a function of the amount of BAPTA injected into the embryo. The number above each bar represents the sample size.

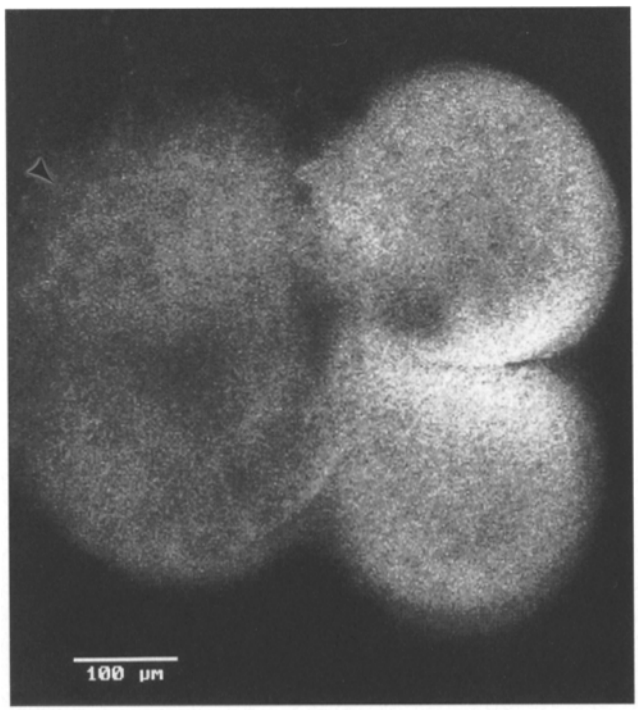

Figure 5. Ca^{2+} image of an embryo which was injected with heparin at the 2-cell stage. The free Ca^{2+} level is indicated by the intensity of the fluorescence signal. The injected cell (*marked by arrowhead*) did not divide; while the uninjected cell (*on the right*) underwent the second cell cleavage. Localized elevation of $[Ca^{2+}]_i$ was observed only in the uninjected cell.

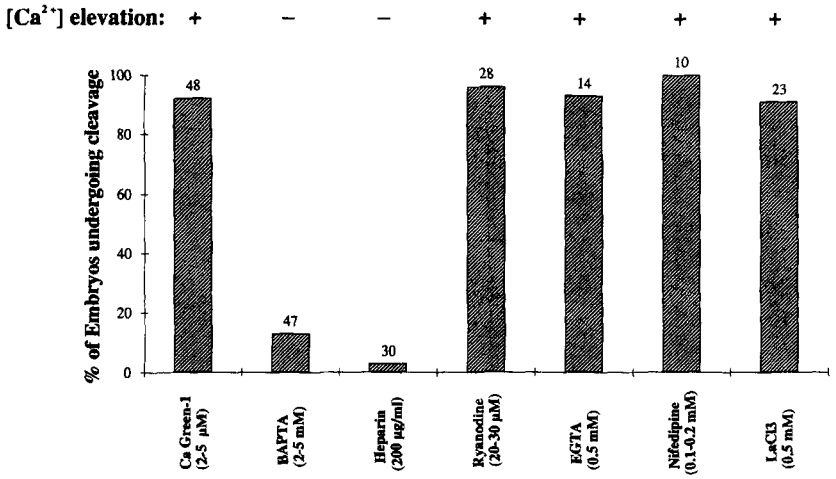

Figure 6. A summary of the effects of various treatments on the zebrafish embryos. All embryos were injected with dextran-conjugated Calcium Green-1. Thus, the first column represents the control. BAPTA, heparin, and ryanodine were introduced into the embryos by injection. EGTA, nifedipine, and $LaCl_3$ were applied externally in a Ca-free medium. The number on top of each bar represents the sample size. The "+" sign means that a localized $[Ca^{2+}]_i$ elevation was observed in the embryo; while a "−" means that the $[Ca^{2+}]_i$ elevation was abolished by the specific drug treatment.

Using a similar pharmacological approach, we examined the contribution of other Ca^{2+} sources to the $[Ca^{2+}]_i$ elevation. The results are summarized in Fig. 6. One possible pathway for internal Ca^{2+} release is through the ryanodine receptor (RYR) (Berridge, 1993; Amundson and Clapham, 1993; Ehrlich et al., 1994). RYR is known to exist in embryos of some species, such as sea urchin (McPherson et al., 1992), but not in others, such as *Xenopus* (Parys et al., 1992). Whether RYR is present in the zebrafish embryo has not yet been documented. We injected a high dosage of ryanodine (final concentration of 20–30 μM) into the zebrafish embryos, but observed no effects on either cell cleavage or its associated $[Ca^{2+}]_i$ elevation (Fig. 6). This result suggests that the calcium signal involved in cytokinesis was not processed through the RYR pathway.

Finally, we examined the contribution of external Ca^{2+} ions to the Ca^{2+} signal associated with cytokinesis by subjecting the embryos to a number of treatments that selectively blocked Ca^{2+} entry from the external medium. These treatments included removal of Ca^{2+} from the external medium, adding a chelator, EGTA, and additional application of high dosages of Ca^{2+} channel blockers such as La^{3+} or nifedipine (Hille, 1992; Girard and Clapham, 1993). All of these treatments were found to have no discernible effects on either cell cleavage or its associated $[Ca^{2+}]_i$ elevation (Fig. 6). Apparently, the cytokinesis-associated Ca^{2+} signal did not depend on calcium flux contributed from the extracellular Ca^{2+} pool.

Discussion

Evidence of a Localized Calcium Signal That Is Spatially and Temporally Correlated with the Onset of Cytokinesis

Results of this work demonstrate that in association with cytokinesis there is an increase in the intracellular concentration of Ca^{2+}. This increase is not uniform over the entire cell, but only at specific regions where the cell cleavage furrow forms. In other words, there is a close spatial correlation between the calcium elevation and the site of cellular action during cytokinesis.

Furthermore, the elevation of free Ca^{2+} occurred not only in the "right" place, but also at the "right" time. As can be seen in Figs. 2 and 3, the emergence of the Ca^{2+} hot spot actually preceded the contraction of the cleavage furrow by almost twenty seconds. Demonstrating such a sequence of timing is important; if the elevation of $[Ca^{2+}]_i$ is the signal that triggers cytokinesis, the signal ought to arise slightly ahead of the triggered action. Had the $[Ca^{2+}]_i$ elevation appeared after the cleavage began, it would be less convincing to regard the calcium elevation as a regulating signal for cytokinesis. In that situation, the calcium elevation would seem to be a by-product of the cytokinesis process.

This calcium signaling hypothesis has another attractive feature; that is, it provides a natural explanation for cell cleavage furrow advancement. We know that during cytokinesis, the cell cleavage furrow of an embryonic cell does not contract simultaneously around its entire equator; instead, the cleavage usually starts from the top (toward the animal pole) and then spreads along the sides. At present, it is not clear how the cleavage furrow spreads. If the cleavage furrow contraction is triggered by a localized calcium signal, one may speculate that the advancement of the cleavage furrow could be simply controlled by a self-propagating calcium wave. Currently, we are undertaking a series of 4-dimensional confocal measurements (i.e., time-dependent z-series measurements) of $[Ca^{2+}]_i$ in zebrafish embryos. The preliminary results (data not shown) appear to be consistent with this speculation.

Characteristics of the Cytokinesis-associated Calcium Signal

With the resolution obtained in our Ca^{2+} images, we can examine the properties of the cytokinesis-associated calcium signal in detail. Previously, it was not clear about the depth of the calcium signal that is associated with cell cleavage. Fluck et al. (1991) proposed that the calcium signal spreads mainly along the surface of the cleavage furrow. The thickness of the elevated Ca^{2+} region was thought to be 0.1–0.2 μm, a depth comparable to that of the contractile band. Our results, as shown in Figs. 1 and 2, indicate that the calcium signal is much deeper, i.e., on the order of tens of microns.

This study also provides a more direct estimate of the concentration of Ca^{2+} at the peak of the calcium elevation. In the previous study of medaka eggs, the elevated calcium concentration was estimated to be 5–8 μM, based on certain assumptions about the thickness of the calcium signal

(Fluck et al., 1991). In our confocal measurements, $[Ca^{2+}]_i$ was determined directly from the fluorescence signal of Calcium Green-1; there was no assumption about the spatial distribution of the calcium signal. Our results indicate that, on the average, the local $[Ca^{2+}]_i$ at the peak of the calcium signal is on the order of 1 μM; this value is slightly smaller than the previous estimate (Fluck et al., 1991).

Signal Transduction Pathway between a Localized Elevation of $[Ca^{2+}]_i$ and the Initiation of Cleavage Furrow Contraction

Results of this work provide strong evidence that cytokinesis is controlled by a calcium signal. The remaining question is how a localized elevation of free Ca^{2+} can trigger the formation of a cleavage furrow. The answer could be that Ca^{2+} ions activate certain kinase(s) which in turn modify the properties of the cytoskeletons. Since cell cleavage depends on the contraction of a submembrane contractile band composed mainly of actin and myosin II (Mittal et al., 1987; Cao and Wang, 1990; Sanger et al., 1989), an elevated calcium concentration could trigger the actomyosin contraction by a mechanism analogous to that in smooth muscle (Satterwhite and Pollard, 1992). It is well known that elevated free calcium can activate the calcium/calmodulin–dependent enzyme of myosin light chain kinase (MLCK). It has been suggested that, once activated, MLCK can promote the growth of the contractile band by inducing aggregation of actin and myosin filaments (Mabuchi and Takano-Ohmuro, 1990; Cao and Wang, 1990). In fact, recent evidence has indicated that the actomyosin contraction at cytokinesis is regulated through phosphorylation of different sites of the regulatory light chain of myosin II (MLC) (Satterwhite et al., 1992; Yamakita et al., 1994). In the early mitotic phase, MLC was found to be phosphorylated at Ser-1 and Ser-2, mainly through the action of the cdc2 kinase. At the onset of cytokinesis, Ser-1 and Ser-2 were found to be dephosphorylated, while another site, Ser-19, was phosphorylated by the activated MLCK (Yamakita et al., 1994). Interestingly, phosphorylation of Ser-19 is known to be connected with the initiation of contraction in smooth muscle (Sellers, 1990). Thus, an elevated $[Ca^{2+}]_i$ can play an important role in regulating cytokinesis by activating the target enzyme MLCK which in turn phosphorylates the Ser-19 site of MLC and triggers the actomyosin contraction in the contractile band.

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