Local Perinuclear Calcium Signals Associated with Mitosis-Entry in Early Sea Urchin Embryos

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Abstract. Using calcium-sensitive dyes together with their dextran conjugates and confocal microscopy, we have looked for evidence of localized calcium signaling in the region of the nucleus before entry into mitosis, using the sea urchin egg first mitotic cell cycle as a model. Global calcium transients that appear to originate from the nuclear area are often observed just before nuclear envelope breakdown (NEB). In the absence of global increases in calcium, confocal microscopy using Calcium Green-1 dextran indicator dye revealed localized calcium transients in the perinuclear region.

We have also used a photoinactivatable calcium chelator, nitrophenyl EGTA (NP-EGTA), to test whether the chelator-induced block of mitosis entry can be reversed after inactivation of the chelator. Cells arrested before NEB by injection of NP-EGTA resume the cell cycle after flash photolysis of the chelator. Photolysis of chelator triggers calcium release. Treatment with caffeine to enhance calcium-induced calcium release increases the amplitude of NEB-associated calcium transients. These results indicate that calcium increases local to the nucleus are required to trigger entry into mitosis. Local calcium transients arise in the perinuclear region and can spread from this region into the cytoplasm. Thus, cell cycle calcium signals are generated by the perinuclear mitotic machinery in early sea urchin embryos.

INTRACELLULAR calcium is now known to perform a wide variety of functions as a second messenger. It is implicated in the control of exocytosis, protein synthesis, locomotion, and synaptic transmission (for reviews see Campbell, 1983; Berridge, 1993; Brostrom and Brostrom, 1990; Rasmussen and Rasmussen, 1990). It is increasingly clear that calcium signals can originate in specific cytoplasmic domains (Lechleiter et al., 1991; Rizzuto et al., 1993; Meyer et al., 1995; Petersen, 1995; Kasai, 1995). A body of evidence also points to a role for calcium ions as one of the mechanisms enabling passage through cell cycle control points (for reviews see Whitaker and Patel, 1990; Steinhardt, 1990; Lu and Means, 1993; Whitaker, 1995).

The evidence for a role of calcium ions during mitosis comes from observations that embrace many different aspects of cell cycle physiology. For example, many components of the intracellular calcium signaling system localize to the area of the nucleus just before mitosis entry and to the mitotic apparatus during mitosis (Silver et al., 1980; Keihart, 1981; Petzelt, 1979, 1984; Petzelt and Hafner, 1986; Wolniak et al., 1981; Paweletz and Firze, 1981; Hepler, 1980; Hepler and Wolniak, 1983, 1984). Preventing intracellular calcium increases with calcium chelators such as EGTA and BAPTA blocks both entry into, and exit from, mitosis (Steinhardt and Alderton, 1988; Twigg et al., 1988; Whitaker and Patel, 1990; Patel et al., 1989, 1990; Ohsumi and Anraku, 1983; Iida et al., 1990; Lindsay et al., 1995). Entry into mitosis can also be induced prematurely by microinjection of calcium during a defined period before mitosis entry (Steinhardt and Alderton, 1988; Twigg et al., 1988; Patel et al., 1989, 1990; Kao et al., 1990). These experiments suggest that calcium ions may be a necessary and sufficient stimulus for mitosis entry. There is also evidence that the cell cycle calcium release mechanism uses inositol trisphosphate as the calcium releasing agent (Han et al., 1992; Forer and Sillers, 1987; Wolniak, 1987; Ciapa et al., 1994). Finally, blocking the action of the calcium receptor calmodulin, or its target calcium/calmodulin kinase II also prevents entry into mitosis in various cell types, suggesting that calcium acts by causing calcium-dependent phosphorylation, possibly of certain cell cycle control proteins (Baitinger et al., 1990; for reviews see Rasmussen and Means, 1989; Lu and Means, 1993; Means, 1994; Gruver et al., 1992; Ohya and Anraku, 1992; Anraku et al., 1991; Whitaker, 1995).

Intracellular calcium transients can be detected before mitotic checkpoints in experiments on plant and animal cells in culture (Keith et al., 1985; Hepler and Callahan, 1987; Kao et al., 1990) as well as in oocytes and embryos (Poenie et al., 1985, 1986; Steinhardt and Alderton, 1988; Jones et al., 1995). However, sometimes they are appar-

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ently absent (Poenie et al., 1985; Tombes et al., 1992; Kao et al., 1990; for review see Whitaker and Patel, 1990; Hepler, 1992). So, while calcium transients show a good temporal correlation with mitosis when they are detected, mitosis has been observed to begin without a calcium transient being seen.

It has often been suggested, but not until now demonstrated, that calcium transients during mitosis are localized to specific regions of the cell, and therefore are often obscured when using whole-cell calcium measurement techniques (Whitaker and Patel, 1990; Kao et al., 1993; Kono et al., 1996). Indirect evidence strengthening this hypothesis comes from the observation that the putative intracellular calcium store, the endoplasmic reticulum, localizes to the nuclear area before mitosis in the sea urchin early embryo (Terasaki and Jaffe, 1991). Recently, evidence has emerged that points to a role for very local calcium increases in calcium signaling (Petersen, 1995), particularly in the region of the nucleus (Meyer et al., 1995). We used confocal microscopy to test the hypothesis that the mitosis entry calcium transient is fundamentally a local event. Our results show that when larger calcium transients are not detected during mitosis entry, localized calcium transients can be seen. We show that the small size and localization of these transients makes them difficult to detect using whole cell techniques. We therefore establish the importance of local nuclear calcium signals in the regulation of mitosis.

Nitrophenyl-EGTA (NP-EGTA)[1] is a newly developed photoinactivatable calcium chelator (Ellis-Davis and Kaplan, 1994), which we have used to control the cytoplasmic calcium concentration during mitosis entry. We have found that the inhibition of mitosis entry is correlated with the ability of NP-EGTA to buffer intracellular calcium transients. These results show that entry into mitosis is completely dependent on increases in cytoplasmic calcium. These data themselves represent an advance on previous work because they show that the block of mitosis entry caused by buffering calcium can be reversed in individual cells, so demonstrating that this effect is not due to irreversible damage to early embryos as a consequence of calcium chelation.

Finally, we show that dextran-conjugation of calcium dyes prevents dye internalization and contributes to the enhanced detection frequency of calcium transients with these dyes during mitosis. Our results demonstrate unequivocally that hitherto undetected local intracellular calcium signals occur around the nucleus before mitosis entry, and that these signals tightly control entry into mitosis during the first cell cycle of the sea urchin embryo.

Materials and Methods

Sea Urchins

Lytechinus pictus (Marinus, Long Beach, CA) eggs were obtained by intracoelomic injection of 0.5 M KCl and collected in artificial sea water (ASW) (435 mM NaCl, 40 mM $MgCl_2$, 15 mM $MgSO_4$, 11 mM $CaCl_2$, 10 mM KCl, 2.5 mM $NaHCO_3$, 1 mM EDTA, pH 8.0). Eggs were washed, dejellied by passage through Nitex mesh (pore size 100 μm) four times, washed again in ASW, and resuspended in ASW a density of 5%. For microinjection, eggs were immobilized on glass slides precoated with 50 μg/ml poly-L-lysine (Sigma Chemical Co., Poole, Dorset, UK). Sperm were collected dry and stored at 4°C until further use. For caffeine experiments, caffeine (Sigma) was dissolved to 5 mM in ASW by heating to 70°C and then cooled to 16°C. Eggs were either fertilized in caffeine-ASW or perfused with caffeine-ASW after fertilization. All experiments were conducted at 16°C.

Confocal Microscopy

Calcium Green-1 and Calcium Green-1 dextran (Molecular Probes, Eugene, OR) were dissolved in injection buffer (10 mM in 0.5 M KCl, 20 mM Pipes, pH 6.7), and microinjected into eggs to give a cytoplasmic concentration of 10–20 μM, 30–40 min postfertilization. Confocal scans were started 45 min postfertilization, at an interval of 10 s per image on a confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). For single excitation scanning, a 488-nm line of an Argon ion laser was used. Images were then collected using a 530-nm long pass filter. For dual excitation, we used a method based on earlier work (Berger and Brownlee, 1993; McDougall and Sardet, 1995; Stricker, 1995). Calcium Green-1 dextran (20 μM) was coinjected with Tetramethylrhodamine dextran (1 μM, Molecular Probes). The dyes were excited with the 488-nm and 568-nm lines of an Argon ion laser. Emission intensities were separated by a 580-nm dichroic mirror. Calcium Green images were collected with a 530-nm band pass filter and Tetramethylrhodamine images using a 590-nm long pass filter. Images were processed using Leica analysis software. Calcium traces were calibrated according to the method of Gillot and Whitaker (1993): dye-injected eggs were fertilized and the mean unfertilized resting calcium concentration (200 nm) and mean peak calcium concentration during the fertilization transient (2 μM) found with fura-2 were used to calibrate the Calcium Green dextran/Rhodamine dextran ratio. The Rhodamine dextran signal was insensitive to this range of calcium concentration. Using a fluorescence enhancement (F_s/F_o) of threefold at saturating calcium for our optical system, we find a K_d of 2 μM for Calcium Green dextran within the egg.

Whole-Cell Fluorescence Measurement of Calcium

Whole-cell calcium measurement was performed in single eggs using Fura-2 pentapotassium salt and its 10,000 M_r dextran conjugate (Molecular Probes). The dyes were dissolved in injection buffer (10 mM in 0.5 M KCl, 20 mM Pipes, pH 6.7), and microinjected into eggs to give a cytoplasmic concentration of 10–20 μM. For Fura-2 and Fura-2-dextran experiments, the fluorescence from the egg was measured as described before (Swann and Whitaker, 1986). Permeabilization with digitonin was performed according to Crossley et al. (1991).

Flash Photolysis

NP-EGTA was synthesized using the method of Ellis-Davies and Kaplan (1994). It was dissolved in injection buffer (10 mM in 0.5 M KCl, 20 mM Pipes, pH 6.7) and microinjected into eggs to give a cytoplasmic concentration of 5.4 mM (0.6% injection, 0.9 M stock). Flash photolysis was performed on a Nikon microscope fitted with a Hi-Tech XF-10 flashgun (Hi-Tech, Salisbury, England). The flashgun delivers light through a 360-nm (10 nm FWHM, Glenn Spectra, Wembley, Middx., England) filter. Calcium measurements were simultaneously made by injection of 10 μM Calcium Green-1 dextran. Calcium Green dextran recordings were made with an XF22 filter set (Glenn Spectra, Watford, England). The calcium trace was recorded with an EMI photomultiplier attached to a PC installed with Thorn EMI software. Traces were calibrated for calcium according to Gillot and Whitaker (1993) as described above.

Reagents

All chemicals were of analytical grade purchased from BDH (Poole, UK) unless otherwise noted.

Results

Global and Local Calcium Transients during Mitosis Entry

Global Nuclear Envelope Breakdown Calcium Transients Detected Using Confocal Microscopy. In parthenogenetically

1. *Abbreviations used in this paper*: ASW, artificial sea water; NEB, nuclear envelope breakdown; NP-EGTA, nitrophenyl-EGTA.

activated eggs, a large calcium transient precedes nuclear envelope breakdown (NEB) in every case (Poenie et al., 1985; Patel et al., 1989). The time from the peak of the transient to NEB is 5 ± 2.1 min (mean and SEM, $n = 5$, from Patel et al., 1989). From these data, we should expect that 80% of NEB-associated transients will occur in the 5–10 min before NEB. We used a confocal microscope in conjunction with Calcium Green-1 and its 10,000 M_r dextran conjugate in order to look for NEB-associated calcium transients in eggs activated by fertilization. The dyes were injected 20–30 min before NEB. Calcium Green-1 revealed calcium transients 5–10 min before NEB that filled the egg cytoplasm in 75% of attempts (6/8 experiments). The results were similar with Calcium Green-1 dextran (7 global calcium transients out of 13 experiments, see Table I for statistics). The transients have a slow time course (2–3 min to peak) relative to the norm for calcium signals, as has previously been observed (Poenie et al., 1985; Ciapa et al., 1994). Global calcium transients were also observed in the absence of external calcium (in 5 out of 10 experiments). Furthermore, anaphase transients were also detected when the experiment was allowed to continue through this point of the cell cycle (see Fig. 1, *a.i* and *ii*). An example of a global NEB calcium transient is shown in Fig. 1 *b* (*i* and *ii*).

Division of these images by an image at basal resting calcium levels produces an image free from static dye distribution artefacts (see Gillot and Whitaker, 1993). The analysis suggests that calcium release initially occurs within the nuclear area, then spreads through the cytoplasm (see Fig. 1, *b.ii*). The initial localization of the calcium transient corresponds to the area of localization of the endoplasmic reticulum, the calcium store of the sea urchin egg (Terasaki and Sardet, 1991; Terasaki and Jaffe, 1991). These data suggest that calcium release in the nuclear region can diffuse or propagate through the cell cytoplasm.

In the experiments that did not display global calcium release during the interval before NEB, we examined the cells to look for localized increases in the nuclear region where the endoplasmic reticulum is concentrated.

NEB Calcium Transients Local to the Nucleus Are Detectable with Calcium Green-1 Dextran. Experiments in which global calcium transients were not visible before NEB were examined using global and localized measurement of pixel intensities to look for possible areas of calcium increase 5–10 min before NEB. With Calcium Green-1, we found no evidence of any change in calcium before NEB using this technique. However, localized changes in fluorescence (calcium increases) were found when the perinuclear region was analyzed in experiments with Calcium Green-1 dextran. A typical result from a local analysis of a Calcium Green-1 dextran cell cycle experiment is shown in Fig. 2 *a*. Here, very little change in calcium is observed when using an analysis that averages calcium increases over the whole cell. However, a local analysis in an area surrounding the nucleus produces an increase of the order of 100 nM over several min (see Fig. 2 *a*). Transients of this type were detectable in 40% of the experiments using Calcium Green-1 dextran. Out of a total of 13 experiments using the dextran dye, 12 were therefore found to have either global or localized NEB calcium transients (see Table I for statistics). Fig. 2 *b* shows images of a localized NEB calcium transient. There is a calcium increase within the nuclear region. Localized NEB calcium transients were also seen in the absence of external calcium in four of ten experiments. Out of a total of ten experiments in calcium-free sea water, therefore, nine showed either global or local calcium signals.

The ratio method used in the experiments of Figs. 1 and 2 *b* eliminates static dye distribution artefacts, but might give false information if dye distribution changed substantially from one image to the next. For example, this could occur as a consequence of organelle movement in the region of the nucleus. Any transient dye distribution artefacts can be eliminated by using a two-dye ratio method with calcium-sensitive and calcium-insensitive fluorophores coupled to dextran (Berger and Brownlee, 1993; McDougall and Sardet, 1995; Stricker, 1995). The two-dye method also enables visualization of localized calcium increases within the nuclear region (Fig. 2 *c*). These data indicate that the localized increases are due to the release of calcium, and not to artefacts due to the changes in dye distribution. They show that the underlying event before NEB involves local activation of a calcium domain in the region of the nucleus. Activation of the local domain can result in the spread of the local response into the cytoplasm.

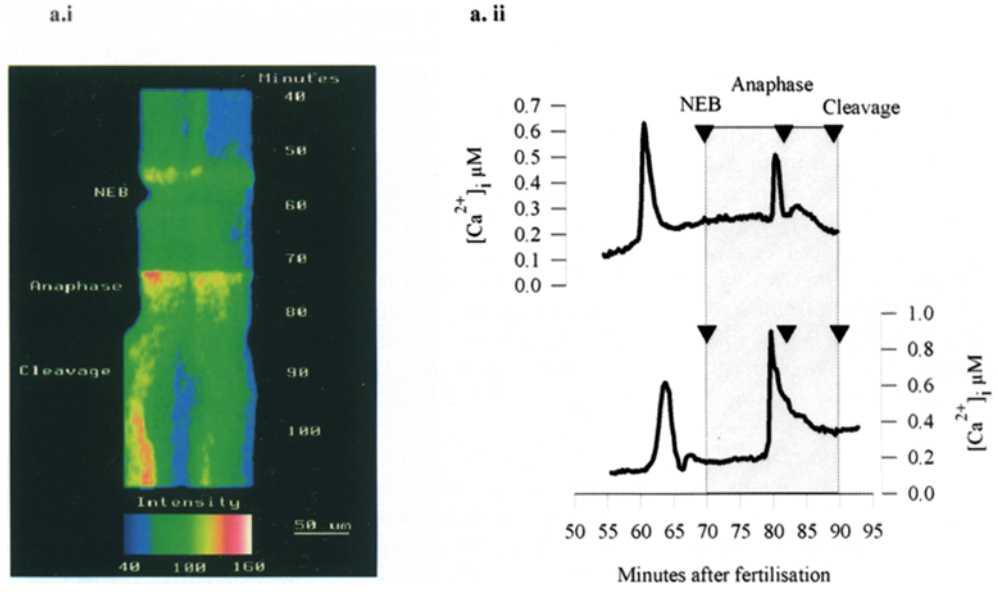
Cell Cycle Calcium Transients Can be Mimicked Using Caged Calcium

The Effect of Photoinactivatable Calcium Chelators on the Cell Cycle. It has previously been shown that preventing increases in intracellular calcium with calcium buffers also efficiently blocks entry into mitosis in many cell types (see

Table I. Parameters of Confocal NEB Calcium Transients

	Detection frequency	NEB	Calcium transient peak-NEB	Transient duration	Basal calcium	Rise in calcium
		min	*min*	*min*	*nM*	*nM*
Calcium Green-1 (Global transients)	75% (6/8)	76 ± 4	7 ± 2	8 ± 2	188 ± 0.3	255 ± 59
Calcium Green-1 dextran (Global transients)	53% (7/13)	71 ± 3	3 ± 1	6 ± 1	191 ± 4	313 ± 108
Calcium Green-1 dextran (localized transients)	40% (5/13)	77 ± 4	2 ± 1	4 ± 1	199 ± 6	44 ± 8

Calcium-sensitive dyes were injected to a final concentration of 10 μM, 30–40 min postfertilization. Individual eggs were then scanned using confocal microscopy, at 10-s intervals. Pixelintensities were analyzed over a defined area of the images and traces were formed from those using Leica software. Traces were calibrated for calcium using the methods of Gillot and Whitaker (1993). Cell cycle events were scored using bright-field microscopy.



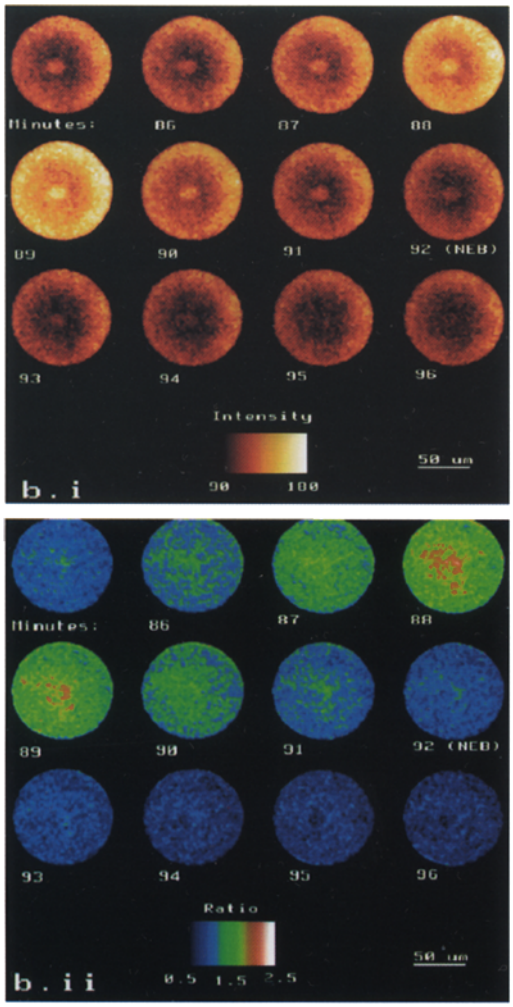


Figure 1. Global mitotic cell cycle calcium transients. (*a*) *i.* The figure is a stacked series of images taken at 10-s intervals throughout mitosis, equivalent to taking a line-scan on the confocal microscope through the centre of the egg every 10 s. Cells were injected with Calcium Green-1 dextran, 30–40 min post-fertilization. The cell was then washed into Ca^{2+}-free ASW. The image is presented as raw data, in false color (refer to color bar). (*ii*) These traces are examples of analyses of confocal calcium recordings obtained from cells producing global calcium transients during mitosis. Leica software was used to analyze pixel intensities, and the intensities were then calibrated according to Gillot and Whitaker (1993). Cleavage transients were present in these experiments, but could not be quantified satisfactorily because the forming daughter cells moved out of the field of view during cleavage. Cell cycle times can vary up to 25% from one egg batch to another, though the relative timing of cell cycle landmarks is consistent. NEB has been normalized in these traces to 70 min to aid comparison by applying the factor $t_{NEB}/70$, where t_{NEB} is the time to NEB in a given experiment. The mitotic period is shaded. (*b*) (*i*) Raw intensity image from a cell injected with Calcium Green-1. The Ca^{2+} increase occurs between 87 and 90 min after fertilization. The nucleus is visible in the center of the image. After NEB, dye diffuses away from the nuclear area. (*ii*) Ratio image of the same experiment. The ratio image was obtained by dividing each image pixel-by-pixel by the first image shown; this results in an image that reports Ca^{2+} and is free from static dye distribution artefacts. The Ca^{2+} increase is global although somewhat concentrated in the nuclear area and the image of the nucleus is now absent, indicating that nuclear Ca^{2+} and cytoplasmic Ca^{2+} are comparable (see Gillot and Whitaker, 1993). The image is presented in false color.

Introduction). Although these experiments strongly suggest the link between intracellular calcium release and mitosis entry, we wanted to show that this effect was due only to calcium buffering rather than an unknown toxic effect of calcium chelators. The development of new photoinactivatable calcium chelators, the latest of which is nitrophenyl-EGTA (NP-EGTA) has enabled this type of experiment to be undertaken (for review see Ellis-Davies and Kaplan, 1994; Zucker, 1994). The advantage of NP-EGTA over earlier photoinactivatable calcium buffers is

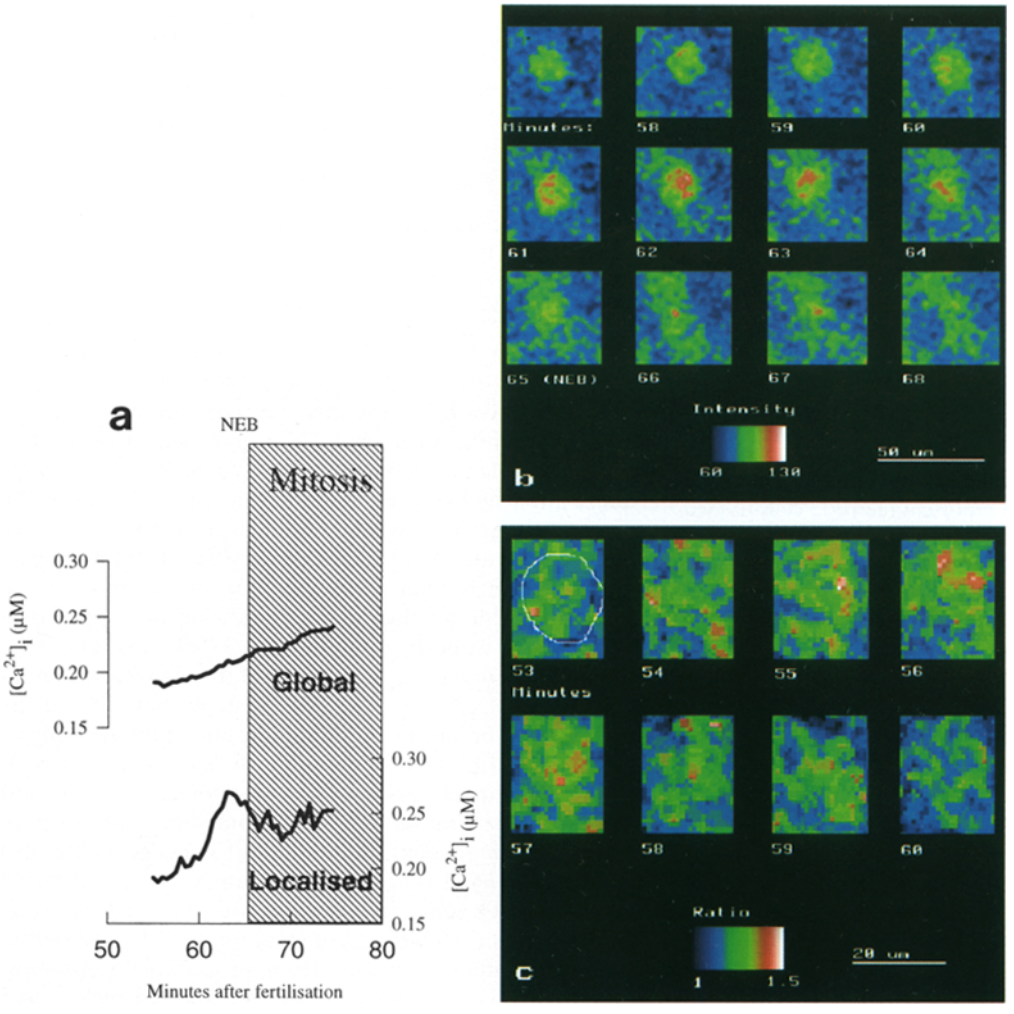


Figure 2. Localization of NEB Ca^{2+} transients. (*a*) A typical example of an experiment where a global analysis revealed no calcium transients at the time of entry into mitosis. If we then analyse an area over the nucleus, roughly 10% the total surface area of the confocal section, a local calcium transient is revealed. (*Top trace*) global analysis; (*Bottom trace*) local analysis. (*b*) This is a series of images of the nuclear area from an egg injected with Calcium Green-1 dextran, 30–40 min postfertilization. A false color look-up table is used to aid visualization of the data. A calcium transient localized to the nuclear area can be seen 61–65 min postfertilization, immediately before the nuclear envelope breaks down. (*c*) Ratiometric imaging of a localized NEB calcium transient. These images are a series of ratioed images just before NEB, recorded using Calcium Green-1 dextran and Tetramethylrhodamine dextran (see Materials and Methods). Images are magnified to show the nuclear area. The position of the nucleus itself is indicated by the white circle. A transient increase in calcium can be seen in the region of the nucleus. In this experiment, the embryo underwent NEB 63 min after fertilization. Images are plotted in false color.

its high affinity and specificity for calcium, which is completely abolished by photolysis (see Ellis-Davis and Kaplan, 1994). We had previously determined that 5.4 mM NP-EGTA is sufficient to block NEB in the sea urchin embryo, when injected 20–30 min before this event (not shown). Entry into mitosis was blocked in 88 ± 2.4% of cells with this concentration in the present experiments (mean ± SEM, $n = 6$, see Fig. 3).

After the light flash, cells entered mitosis (scored by NEB) within 10 min of photolysis (see Fig. 3). The fact that cells enter mitosis within 10 min after flashing is interesting because NP-EGTA was injected 20–30 min before NEB. NP-EGTA does not therefore appear to block the cell cycle immediately on injection, but seems to allow cells to progress until they arrest at a calcium-dependent step, suggesting that the NP-EGTA induced block is specifically due to its capacity to buffer calcium. Arrested cells also proceed to cleave after flashing, whereas unflashed cells remain arrested before NEB. This shows that increases in intracellular calcium are a fundamental component of cell cycle regulation at mitosis entry.

NP-EGTA and Calcium Release. Apart from inactivating the buffering capacity of NP-EGTA, flash photolysis should also release the calcium bound to NP-EGTA. To measure the calcium transients produced by flash photolysis of NP-EGTA, we coinjected NP-EGTA with 10 μM Calcium Green-1 dextran. We then measured calcium levels as cells were flashed to inactivate the NP-EGTA Fig. 4. In eight cells, calcium rose from 77 ± 12.4 nM to 155 ± 29.2 nM (mean ± SEM). This indicates that, as expected, NP-EGTA chelates a detectable amount of calcium before NEB.

Caffeine Enhances Local Calcium Signals. Our experiments with confocal calcium imaging indicate that local release in small calcium domains will not always be detectable using whole-cell fluorescence measurement in monospermic, fertilized embryos (see Whitaker and Patel, 1990; Table II). Caffeine is an agent known to enhance sensitivity to

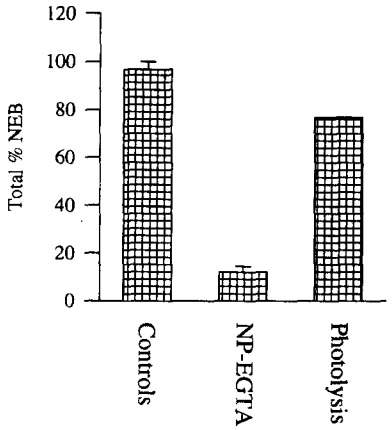


Figure 3. Effect of nitrophenyl-EGTA on NEB. Nitrophenyl-EGTA was injected to a final concentration of 5.4 mM, 20–30 min before nuclear envelope breakdown. Controls were injected with vehicle (0.5 M KCl). NEB was scored by bright field microscopy. The data show the extent of NEB at the end of the experiment (mean of six experiments injecting at least 20 embryos per experiment, SEM as bars). After the controls had divided (usually 120 min postfertilization), one or two NP-EGTA arrested embryos were flashed to inactivate NP-EGTA. Photolysis shows the result of flashing. These data represent the 10/13 cells flashed (in the six experiments) that succesfully completed NEB after the flash. These data are therefore plotted as a percentage, without error bars.

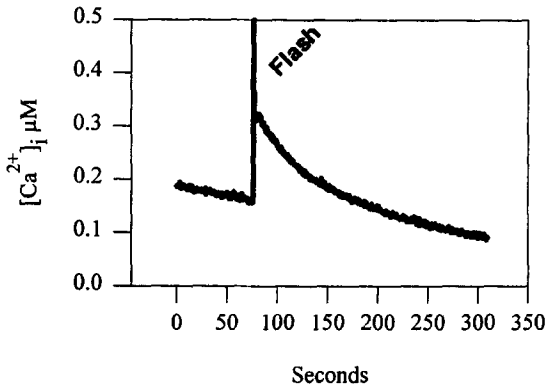


Figure 4. Calcium transients measured during flash photolysis. Example of a typical rise in calcium after flash photolysis of an egg arrested before NEB by NP-EGTA. Traces are calibrated according to Gillot and Whitaker (1993).

CICR (Endo et al., 1970; Fleischer and Inui, 1989; for review see Endo, 1977). Addition of caffeine to embryos increases the frequency of detection of NEB calcium transients (see Fig. 5 *a*, Table II for statistics). These data are consistent with and confirm the hypothesis that small, local calcium transients, below the limit of resolution using whole cell measurement, are always present and can be enhanced when caffeine is applied to sensitize CICR.

Resolving Local Calcium Signaling Domains Can Be Compromised by Dye Internalization

There are other reasons why events in local calcium signaling domains may be difficult to resolve. We were concerned that the sensitivity of the injected calcium-sensitive dyes might decrease as the experiment progressed, preventing detection of localized calcium transients. A possible cause is the removal of the dye from the cytoplasm into intracellular organelles (Connor, 1993). There, it is no longer sensitive to cytoplasmic calcium, but may also contribute a significant unchanging signal due to high intraorganelle calcium concentration. We tested for dye sequestration by using digitonin to make large pores in the plasma membrane, which will release cytoplasmic dye (Crossley et al., 1991). The fluorescence signal after permeabilization is then divided by the initial fluorescence signal to obtain a value of the proportion of trapped dye. We used the isobestic point of Fura-2 (360 nm) for excitation, which enables measurement of dye concentration independent of calcium. The results show that 38% of the dye injected before fertilization is retained by the embryo by 60 min after fertilization (Fig. 5 *b.i*). Dextran-conjugated dye is much less heavily sequestered (Fig. 5 *b.i* and *ii*). Internalization of the pentapotassium salt form of Fura-2 is independent of the time of injection of the dye (see Fig. 5 *b.ii*). It is not feasible to analyze internalization of Calcium Green-1 in a similar way due to lack of an isobestic point. However, confocal images of the distribution of Calcium Green-1 in cells approaching mitosis show that this dye is also internalized during the cell cycle (Fig. 5 *b.iii*). The lack of internalization of Fura-2 dextran through the cell cycle appears to account for the increased sensitivity of this dye, enabling better resolution of local calcium signaling events than the unconjugated form (Fig. 5 *c* and Table II for statistics).

Table II. Fura-2, Fura-2 Dextran and the NEB Calcium Transient

	Detection frequency	NEB	Calcium transient peak-NEB	Transient duration	Basal calcium	Rise in calcium
		min	*min*	*min*	*nM*	*nM*
Fura-2 injected before fertilization	17% (5/29)	71 ± 2	3 ± 1	5 ± 1	344 ± 50	313 ± 69
5 mM Caffeine (Fura-2 injected before fertilization)	63% (10/16)	73 ± 6	4 ± 1	6 ± 1	160 ± 26	360 ± 85
Fura-2 injected 40 min. post fertilization	30% (3/10)	74 ± 8	4 ± 1	7 ± 1	117 ± 9	77 ± 8
Fura-2-dextran injected 40 min. post fertilization	77% (23/30)	61 ± 1	3 ± 1	4 ± 0.4	184 ± 11	85 ± 8

Fura-2 and its dextran conjugate were injected according to the protocols listed in the table. The calcium trace was then recorded by measuring calcium at 4-s intervals using whole-cell fluorescence microscopy. Detection frequencies represent successful observation of the NEB calcium transient over background fluorescence. Cell cycle events were scored using bright-field microscopy. Calibration of the Fura-2 was performed according to Poenie et al. (1985).

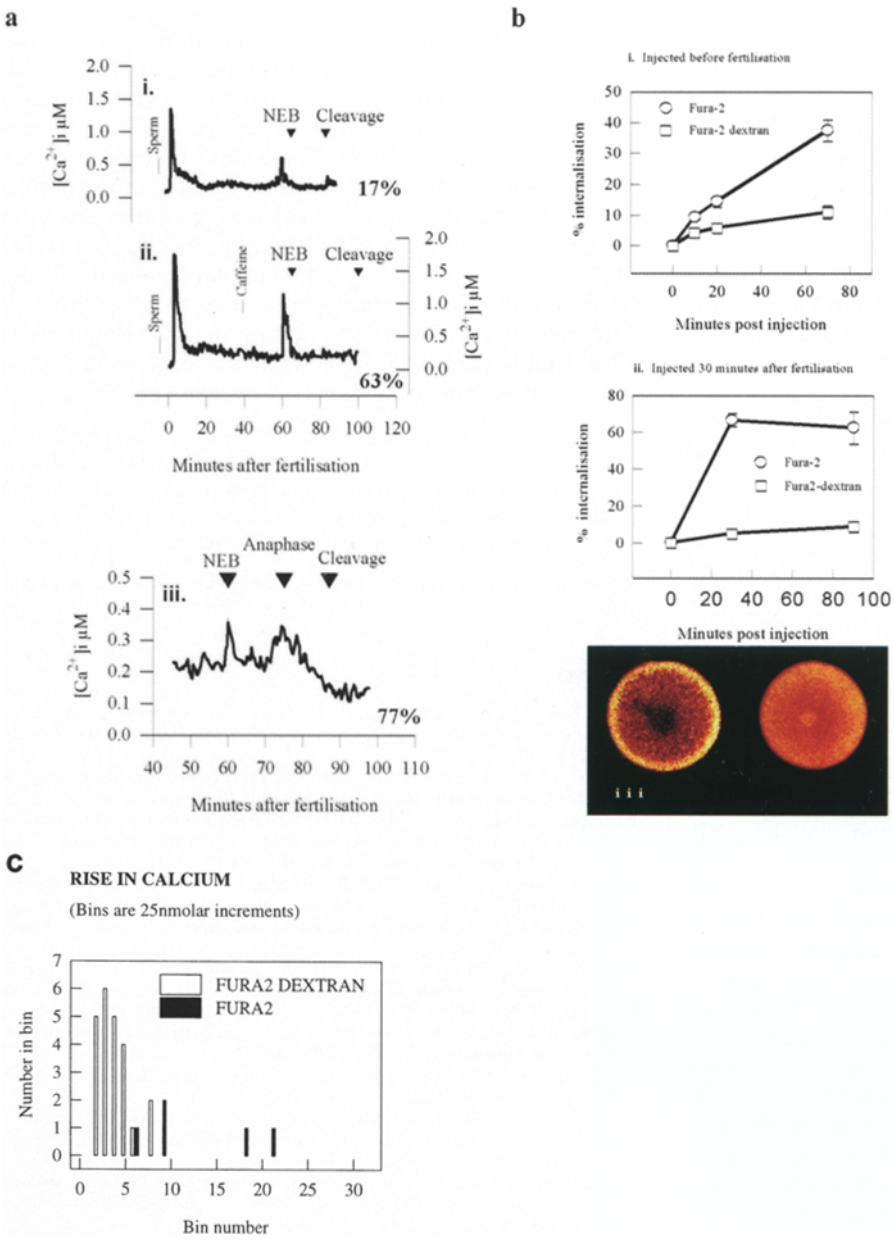


Figure 5. Effect of dextran-conjugation on measurement of cell cycle calcium transients. (*a*) Cell cycle calcium transients detected with Fura-2 pentapotassium salt and its dextran conjugate. (*i*) Example of an NEB-associated Ca^{2+} transient in monospermic eggs injected with Fura-2 pentapotassium salt. The dye was injected to 10 μM before fertilization. Percentage refers to detection frequency of the NEB calcium transient. (*ii*) Example of an NEB-associated Ca^{2+} transient in embryos treated with 5 mM caffeine in sea water. Fura-2 pentapotassium salt was injected to 10 μM before fertilization. Transients of this type were observed in 63% of experiments, and were independent of time of addition of caffeine. Note that addition of caffeine does not lead to an immediate calcium release, but that NEB-associated Ca^{2+} release is potentiated. Percentage refers to detection frequency of the NEB calcium transient. See Table II for statistics. (*iii*) Embryos were microinjected with Fura-2 dextran to a final concentration of 10 μM 30–40 min after fertilization. We found Ca^{2+} transients at anaphase onset and cleavage in addition to the NEB-associated transients, but the main aim of the figure is to illustrate our results vis-à-vis the NEB-associated transient. Percentage again refers to detection frequency of the NEB calcium transient. (*b*) Internalization of calcium-sensitive dyes and their 10,000 M_r dextran conjugates through the cell cycle. Each experiment represents mean ± SEM (shown unless smaller than symbols) of five separate determinations. Different eggs were used at each time point. In *i*, dyes (10 μM) were injected before fertilization. Eggs were immediately fertilized and permeabilized at the points shown. (*Circles*) Fura-2 pentapotassium salt. (*Squares*) Fura-2 dextran. In *ii*, 10 μM of each dye was injected 30 min postfertilization. (*Circles*) Fura-2 pentapotassium salt. (*Squares*) Fura-2 dextran. *iii* shows confocal images of internalization of Calcium Green-1. In the left image, 10 μM Calcium Green-1 was injected before fertilization and the image taken 50 min after fertilization (50 min also after injection of the dye). The dye has an uneven distribution, concentrated around the periphery and excluded from the nuclear area. We interpret this distribution as indicating dye internalization. This image is representative of the dye distribution in 10 experiments. The image on the right represents an experiment in which 10 μM Calcium Green-1 was injected 40 min after fertilization and scanned at 50 min (10 min therefore after injection). The distribution of the dye is much more even. The higher dye intensity in the nucleus is due to the higher water space of the nucleus with respect to the cytoplasm (Gillot and Whitaker, 1994). (*c*) Distribution of Ca^{2+} transient amplitudes above resting Ca^{2+} in experiments using either Fura-2 or Fura-2 dextran. The Ca^{2+} transients detected by Fura-2 dextran are substantially smaller than those measured with Fura-2, suggesting that dextran conjugation increases the detection efficiency of the dye.

The detection frequency of NEB calcium transients is lower using Fura-2 dextran with whole-cell fluorescence measurement, than using Calcium Green-1 dextran with confocal microscopy, confirming that whole-cell recording is not sensitive enough to detect highly localized calcium transients above the background fluorescence. This inference is consistent with our finding that localized calcium transients are resolved by regional analysis of confocal data (refer to Fig. 3 *a*). Two factors therefore contribute to the detection frequency of localized cell cycle calcium transients. First, the degree of internalization of the dye, and second, the ability of the measurement system to resolve small calcium increases above the unchanging fluorescence from the remainder of the cell. Our data provide a plausible reason for the differences between reports documenting cell cycle calcium transients in different cell types.

Discussion

The major novel finding here is the demonstration that calcium transients can be localized to the nuclear area before mitosis entry in early sea urchin embryos. The fact that local pre-NEB calcium transients can be resolved strongly suggests that calcium is a major second messenger during mitosis. There is an increasing awareness that cells may possess functional calcium signaling domains (Rizzuto et al., 1993; Petersen, 1995; Meyer et al., 1995). Aside from the well known calcium microdomains close to the plasma membrane, local calcium signals have not until now been linked to the control of specific cellular events. Furthermore, our observations show that an initially local calcium signaling event may spread and generate a more global calcium response. It is noteworthy that both local and global pre-NEB calcium signals are both smaller and slower than the fertilization calcium signal, and, indeed, than calcium signals seen in mammalian somatic cells in response to agonists (e.g., Kasai, 1995). Nonetheless, they appear to be generated by inositol trisphosphate via the inositol trisphosphate receptor (Ciapa et al., 1994). These observations provide an explanation of why several studies have reported calcium transients correlated with NEB in only a proportion of experiments. The data show that both the type of detection system used and the method of dye loading are important considerations when resolving localized calcium signals.

We used a photoinactivatable calcium chelator, NP-EGTA, to test whether the block to mitosis entry caused by injection of calcium buffers can be reversed. The experiments show that the presence of NP-EGTA does not affect the cell other than at the level of calcium signaling. The experiments therefore demonstrate a link between calcium release and NEB because they provide an internal control for the effect of calcium buffering on the viability of the cell. The fact that cells remain competent to enter mitosis despite the presence of NP-EGTA shows that calcium chelation by NP-EGTA prevents the transmission of the cell cycle calcium signal, but has no less-specific effects on cell cycle progression. These results demonstrate that local increases in intracellular calcium are a prerequisite for NEB in the sea urchin embryo.

Our finding that caffeine treatment enhances the NEB calcium transient suggests the idea that the locus of initiation of the signal is a local perinuclear increase in $[Ca^{2+}]_i$ that may propagate via CICR. However, we have no direct evidence at present that the spread of the calcium signal from nucleus to cytoplasm involves a regenerative mechanism.

We find that minimizing dye internalization of calcium-sensitive dyes by dextran conjugation and injection of the indicator dye just before the stage of interest both enhance detection of the NEB calcium transient. These sets of experiments are consistent with our finding using confocal imaging: that local NEB-associated calcium transients always occur just before NEB, but that resolving these transients depends critically on the methods used. It is hard to conceive of any other explanation. The observation that highly sensitive aequorins also detect NEB calcium transients with high frequency supports this hypothesis (Browne et al., 1992). Aequorin has a highly nonlinear response to $[Ca^{2+}]_i$ (Allen et al., 1977), so it is better able to resolve local calcium increases in whole cell records. There are no reports of aequorin imaging applied to calcium transients before mitosis, though calcium transients in the cleavage furrow have been reported using this techniques (Fluck et al., 1991). These data then provide the first evidence of localized calcium signals before mitosis entry. The idea that localized calcium release events form the basis of calcium signaling is gaining ground (Cannell et al., 1994; Yao et al., 1995). The fact that localized calcium transients during anaphase have also been reported in PtK_2 cells and *Haemanthus* endosperm cells (Ratan et al., 1986; Keith et al., 1985) suggests that local calcium release during mitosis may be a universal mechanism.

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