

# Long-lasting and Rapid Calcium Changes During Mitosis

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**Abstract.** A more complete understanding of calcium's role in cell division requires knowledge of the timing, magnitude, and duration of changes in cytoplasmic-free calcium,  $[Ca^{2+}]_i$ , associated with specific mitotic events. To define the temporal relationship of changes in  $[Ca^{2+}]_i$  to cellular and chromosomal movements, we have measured  $[Ca^{2+}]_i$  every 6–7 s in single-dividing Pt K2 cells using fura-2 and microspectrophotometry, coupling each calcium measurement with a bright-field observation. In the 12 min before discernable chromosome separation, 90% of metaphase cells show at least one transient of increased  $[Ca^{2+}]_i$ , 72% show their last transient within 5 min, and a peak of activity is seen at 3 min before chromosome separation. The mean  $[Ca^{2+}]_i$  of the metaphase transients is  $148 \pm 31$  nM (61 transients in 35 cells) with an average duration of  $21 \pm 14$  s. The timing of these increases makes it unlikely that these transient increases in  $[Ca^{2+}]_i$  are acting directly to trigger the start of anaphase. However, it is possible that a transient rise in calcium during late metaphase is part of a more complex progression

to anaphase. In addition to these transient changes, a gradual increase in  $[Ca^{2+}]_i$  was observed starting in late anaphase.

Within the 2 min surrounding cytokinesis onset, 82% of cells show a transient increase in  $[Ca^{2+}]_i$  to  $171 \pm 48$  nM (53 transients in 32 cells). The close temporal correlation of these changes with cleavage is consistent with a more direct role for calcium in this event, possibly by activating the contractile system.

To assess the specificity of these changes to the mitotic cycle, we examined calcium changes in interphase cells. Two-thirds of interphase cells show no transient increases in calcium with a mean  $[Ca^{2+}]_i$  of  $100 \pm 18$  nM ( $n = 12$ ). However, one-third demonstrate dramatic and repeated transient increases in  $[Ca^{2+}]_i$ . The mean peak  $[Ca^{2+}]_i$  of these transients is  $389 \pm 70$  nM with an average duration of 77 s. The necessity of any of these transient changes in calcium for the completion of mitotic or interphase activities remains under investigation.

THE rearrangements of organelles and the cytoskeleton required for the proper completion of cell division occur with great regularity and efficiency (1, 3, 10, 25). Such precision suggests the existence of signals which act spatially and temporally to orchestrate cell dynamics. A substantial body of biochemical, cell biological and pharmacological evidence supports a role for ionic calcium in mitotic events (8, 9, 28). Critical to this line of thinking is the direct demonstration of fluctuations in free cytosolic calcium that are correlated with the structural and biochemical changes of cell division. Several studies have examined calcium changes during mitosis, and these studies have begun to elucidate plausible temporal and spatial schemes for calcium action in the regulation of nuclear envelope breakdown, anaphase onset, vectorial kinetochore microtubule disassembly in anaphase, and cytokinesis (7, 13, 26, 27, 29).

Keith et al. (13) used digital image processing and the fluorescent calcium indicator, quin-2, to study mitotic endosperm of the African Blood Lily, *Haemanthus Katherinae Baker*. The fluorescent signal obtained allowed the generation of a temporal and spatial map of calcium in single cells

going through mitosis. This approach revealed the existence of local increases in calcium which occur in the chromosome-to-pole region throughout anaphase. The finding that calcium increases and remains elevated for several minutes during anaphase in plant cells was recently corroborated by studies of cellular ionic calcium using a nonfluorescent (metallochromic) class of calcium indicator, Arsenazo III, in dividing stamen hair cells of *Tradescantia* (7).

These studies were extended to mammalian Pt K2 cells as they progressed from metaphase to telophase (29). Consistent with the observations in plant endosperm, a gradient of calcium is observed between the chromosome-to-pole region and the spindle mid-zone in anaphase. Although the precise molecular target(s) of elevated calcium during anaphase in plant and mammalian cells remain unclear, several indirect lines of evidence suggest that calcium can act with spindle calmodulin to stimulate selective kinetochore disassembly and thus drive or facilitate chromosome separation (14, 15, 28, 31, 36, 37, 40).

In addition to these long-lived local changes in cytosolic calcium, more rapid transients in ionic calcium measured

over the whole cell have also been reported. Poenie et al. (26) continuously monitored fura-2 signals in echinoderm eggs and showed that, in addition to a large rise in cytoplasmic ionic calcium  $[Ca^{2+}]_i$  at fertilization, several smaller spikes (up to  $\sim 0.4 \mu M$ ) of calcium also occur during mitosis. They suggested that these transient changes may be correlated with pronuclear migration, streak stage, nuclear envelope breakdown, the metaphase-anaphase transition, and cytokinesis. However, these measurements were done without simultaneous morphological observation, making the correlation of calcium transients with specific events difficult. Poenie and coworkers (27) subsequently used digital image processing to show a transient rise (lasting  $\sim 20$  s) in calcium near the metaphase-anaphase transition. The authors concluded that a calcium pulse is required as a direct trigger for anaphase onset.

While the studies of Poenie and coworkers have highlighted the potential importance of rapid calcium changes, their fluorescence measurements (taken every 3 s) were paired with only a few transmitted-light images rendering the precise temporal relationship of fast calcium changes with the separation of the chromosomes problematic. Attribution of "triggering" functions to these changes requires, among other things, a precise correlation of the changes in calcium to the structural events of mitosis.

In an attempt to achieve this correlation, we have used a computer controlled microspectrofluorometric system. This apparatus has allowed us to assay free cytosolic calcium every 6–7 seconds for up to an hour in fura-2 loaded, dividing, and interphase Pt K2 cells and to generate a transmitted bright-field image for each calcium measurement. Under these conditions we fail to see a tight coupling between calcium transients and anaphase onset.

## Materials and Methods

### Cell Culture

Pt K2 rat kangaroo kidney epithelial cells were plated at 50–90% confluence in coverslip-bottom tissue culture dishes. Cells were maintained in DME with 16.7% (vol/vol) FCS, and penicillin/streptomycin in a humidified incubator at 37°C with a 9% CO<sub>2</sub>/91% air atmosphere. Experiments were performed 2–3 d after plating.

### Fura-2 Loading

Cells were incubated at 15–20°C with 20  $\mu M$  fura-2/AM (Molecular Probes, Eugene, OR), 0.025% Pluronic F-127 (BASF Wyandote, Wyandote, MI), and 3% FCS dissolved in incubation medium (150 mM NaCl; 5 mM KCl; 1 mM MgCl<sub>2</sub>; 1 mM CaCl<sub>2</sub>; 20 mM Hepes, 10 mM glucose; pH 7.4) for 2 to 2.5 h (27). Cells were then rinsed several times and placed in dye-free incubation medium for 1–2 h at 24°C to allow complete de-esterification of the ester. Before beginning the experiments, loaded cells were brought to 30–32°C by addition of incubation medium and placed on the microscope stage which had been prewarmed to 30–32°C using an air curtain incubator. Under these conditions, the cells were maintained at 30–32°C throughout the experiment. Two cells out of more than 100 were not included in the study because the temperature of the medium changed by more than one degree. To prevent evaporation of medium during the experiments, a coverslip was sealed with vacuum grease to the top of the 35-mm dish containing the loaded cells.

Visual inspection of cells excited at 360 nm (a Ca<sup>2+</sup>-independent excitation wavelength of fura-2) after completion of the loading protocol revealed that nearly 100% of the cells contain the dye and the majority (>90%) were

1. *Abbreviations used in this paper:*  $[Ca^{2+}]_i$ , concentration of intracellular (cytosolic) free calcium; AM, tetraacetoxymethyl ester of fura-2;  $I_{340}/I_{380}$ , ratio of intensity of fluorescence emission at 340 nm to that at 380 nm.

diffusely labeled without evidence of spotty or punctate fluorescence. Only diffusely labeled cells were selected for study. In agreement with the findings of Malgoroli et al. (18), when diffusely labeled cells were warmed to 30–32°C for calcium measurements, no compartmentalization of the dye (as seen by punctate fluorescence) was observed for periods up to at least 1 h.

Homogenization and centrifugation of loaded cells was performed as described (29) and revealed that 88% of the calcium-sensitive fluorescence is in the cytosol. Incubation of loaded cells at 30–32°C for periods of up to 60 min did not significantly affect the fraction of calcium-sensitive fluorescence in the cytosol. The emission and excitation spectra of the loaded cells after homogenization was not different from that of fura-2 free acid exogenously added to cell homogenates, indicating the dye is completely cleaved to its free acid form.

Finally, leakage of dye at 30–32°C never exceeded 15% per h. In all fura-2-loaded cells measured, the cell-associated fluorescence at the end of the experiment was at least 10 times greater than the average autofluorescence. Cells were not studied at 37°C because leakage of dye from cells incubated at this temperature was so rapid that measurements for periods greater than 5 min could not be obtained.

## Fluorescence Microscopy, $[Ca^{2+}]_i$ Measurements, and Quantitative Analysis

Identification of metaphase cells, microspectrofluorometric measurements of ratios of fura-2 fluorescence at 340 nm excitation and 380 nm excitation ( $I_{340}/I_{380}$ ) and conversion of  $I_{340}/I_{380}$  to calcium concentrations were carried out as described (12, 13, 16, 29) with the following exceptions. The 340-nm and 380-nm filters were paired with a 10% transmission quartz neutral density filter to reduce the level of incident UV illumination and thereby reduce phototoxicity. The 380-nm filter was paired with an additional 32.5% transmission filter to approximately equalize the intensities at the two wavelengths.

It should be noted that there may be systematic differences between the behavior of fura-2 in the aqueous buffers used for our calibrations vs. the cytoplasm. We and others have not been able to fully collapse  $[Ca]$  gradients across the plasma membrane while retaining the dye in cells. Thus, appropriate in-cell calibration curves were not obtained. Other workers (27) have attempted to correct for the effects of viscosity on the spectroscopic properties of the dye. Such corrections would increase both the basal and peak  $[Ca^{2+}]_i$  values reported here. The correction methods used by Poenie et al. (27) raise basal  $[Ca^{2+}]_i$  by  $\sim 30$ –35 nm. We have not applied them since the appropriate correction factors are uncertain.

To detect fast changes in calcium and their relation to mitotic events, we assembled a computer-controlled system which gives greater temporal resolution of calcium signals, allows correlation of calcium levels with morphology, and stores and displays a digital readout of  $I_{340}/I_{380}$  as a function of time.

The microscope spectrofluorometer is based on a Leitz Diavert inverted fluorescence microscope. The fluorescence illuminator is a 100 W Hg lamp. A six position filter wheel with a stepping motor is used to change illumination filters. Approximately 0.25 s was required to change filters. Fluorescence illumination and bright-field illumination are turned on and off by high speed shutters (Uniblitz, Rochester, NY). The filter cube for epifluorescence illumination was a Leitz A filter block with the excitation filter removed. This provides a dichroic beam splitter centered at 400 nm and a 430-nm long pass filter on the emission side. The objective used was a 40 $\times$  Nikon UV fluor, which passes 340 nm light. All optical components on the illumination light path were quartz or fused silica to maximize transmission of 340 nm light. The illumination area was adjusted by an iris diaphragm to include a circle slightly larger than the cell being observed. Since all the cells were plated at low density, only one cell contributed to the fluorescence intensity. Cells which moved outside of the measurement circle were excluded from the analysis.

Fluorescence intensities were measured with an EMI 9558 B (S20 cathode) photomultiplier housed in a Leitz MPV photometer head. The photomultiplier was operated at 800 V. Intensities were measured by integrating the photometer current for 0.5 s. The photometer head contains a variable aperture diaphragm; which was adjusted for each cell to match the illumination area. The positions of the illumination and measurement fields were carefully aligned for each cell. The system is commercially available (Kramer Scientific, Yonkers, NY).

Using this apparatus, one  $I_{340}/I_{380}$  measurement was acquired in 2.7 s with 0.8 s between each wavelength. Each measurement bleached less than 0.05% of the fura-2. Immediately after each  $I_{340}/I_{380}$  measurement, trans-

mitted bright-field illumination with a 546-nm bandpass filter permitted a 3-s observation of cell morphology and chromosome location. The complete sequence of fluorescence and bright-field measurements, which lasts  $\sim 6.6$  s, was recorded on videotape. Measurements were continued until the particular cell being examined had completed telophase. In some cells, this was for periods as long as an hour. Ionomycin ( $2 \mu\text{M}$ ) was added at the end of the data acquisition period to verify the calcium sensitivity of intracellular fura-2. All cells measured showed increases in  $[\text{Ca}^{2+}]_i$  greater than  $1 \mu\text{M}$  after ionomycin treatment.

Various sources of noise and interfering signals have been analyzed. Under the conditions of our measurements, the standard deviation in the intensity of a fluorescent standard (comparable in brightness to the cells) was always less than 2% of the average intensity. Most of this variation is due to fluctuations in lamp intensity; lamps were changed when the variation exceeded 2%. The SD in the photometric dark current was typically less than 0.1% of the fluorescence intensity of a fura-2 loaded cell. Measurements of  $[\text{Ca}^{2+}]_i$  in individual cells are highly reproducible. In stable interphase cells, the SD of repeated  $[\text{Ca}^{2+}]_i$  measurements was 5 nM. Similar reproducibility of basal  $[\text{Ca}^{2+}]_i$  values has been found in macrophages (17). Since the photomultiplier measures light from the whole cell, motion of the cell does not significantly affect intensity measurements.

A transient rise in calcium was defined as one which met all of the following criteria: (a) A  $[\text{Ca}^{2+}]_i$  maximum to which there has been a consistent increase and a consistent decrease in at least two consecutive readings as reflected by reciprocal changes in the fluorescence with excitation at 340 nm and 380 nm. (b) A  $[\text{Ca}^{2+}]_i$  maximum that was two SDs from the mean  $[\text{Ca}^{2+}]_i$  for that particular record. The mean and SD were determined from all points in the record. (c) A  $[\text{Ca}^{2+}]_i$  maximum that is greater than the  $[\text{Ca}^{2+}]_i$  at the local (adjacent) minima by at least 30 nM.

The definition of transients was complicated by the slow increase in resting calcium that most cells showed from metaphase to anaphase. Therefore, before transients were defined, a corrected baseline was generated for each record. This was done by first determining an appropriate polynomial curve fit for each individual record by the method of least squares. The corrected baseline was then obtained by subtracting the fitted curve from the actual record on a point by point basis. The mean and SD of the points on the corrected baseline were calculated and transients defined according to the criteria above. The duration of each transient was defined as the time that elapsed from one half maximum  $[\text{Ca}^{2+}]_i$  on the upward portion of the peak to one half maximum  $[\text{Ca}^{2+}]_i$  on the downward portion of the peak.

To determine general patterns of calcium fluctuations in relation to morphologically definable events, transients from all cells were plotted as a function of time using chromosome separation or cytokinesis onset as "zero time" in separate analyses. Chromosome separation was defined as that point when the chromosomes are first seen to be clearly separated. Cytokinesis onset is defined as that point when the cell can first be seen to be constricting at its waist. To assess the role of calcium as a trigger for anaphase, a rough approximation of when anaphase onset occurred was made. This was determined by digitizing bright-field images of cells in anaphase and plotting the distance the sister chromosomes had separated (measured approximately from kinetochores in each half spindle) as a function of time. Assuming a linear rate of chromosome migration to separate poles one can extrapolate back to the distance between the kinetochores at metaphase to obtain an estimate of the time of anaphase onset.

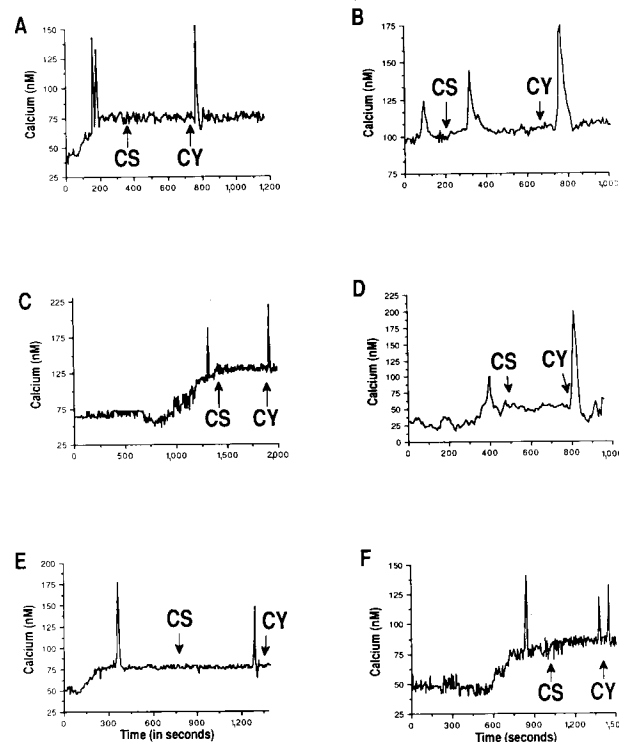
## Results

Rapid changes in free cytosolic calcium during mitosis were examined here by fura-2 microspectrofluorometry in the rat kangaroo epithelial cell line, Pt K2. These cells are well-suited for studies of mitosis as they remain relatively flat while they divide. Fura-2-loaded Pt K2 cells in prometaphase or metaphase were located and analyzed until cytokinesis was complete. Under our loading and recording protocol, 97% of these cells ( $n > 100$ ) progressed from metaphase to telophase with normal cytology (as judged by bright-field) and rate (time from chromosome separation to cytokinesis onset,  $6.2 \pm 2.7$  min in fura-2-loaded cells;  $5.8 \pm 2$  min for control cells). Comparison of cells at  $32^\circ\text{C}$  and  $37^\circ\text{C}$  shows a prolongation of metaphase  $\sim 30$  to  $40\%$  at  $32^\circ\text{C}$  while anaphase duration does not differ significantly.

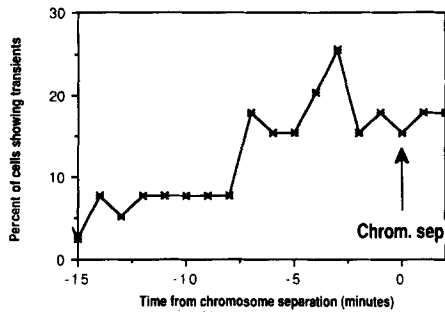
## $[\text{Ca}^{2+}]_i$ Transients Correlated with the Metaphase-Anaphase Transition

Calibrated  $[\text{Ca}^{2+}]_i$  measurements were made in 39 cells as they progressed from metaphase to telophase. Under our experimental conditions, metaphase lasts 35–40 min. 90% of metaphase cells showed at least one transient rise in  $[\text{Ca}^{2+}]_i$  within 12 min of chromosome separation with a mean duration of  $21 \pm 14$  s ( $\pm\text{SD}$ ). 52% of these cells showed more than one transient before chromosome separation (e.g., Fig. 1A). The mean calcium concentration at the peak of the transients was  $148 \pm 31$  nM (mean  $\pm$  SD, 61 transients in 35 cells), a value which is 82% over the mean metaphase baseline calcium for these cells ( $83 \pm 24$  nM,  $\pm$  SD). The range of peak  $[\text{Ca}^{2+}]_i$  during these transients was 103–250 nM. In our system, an increase from 83 nM to 148 nM corresponds to approximately a 45% increase in the 340 nm intensity and a 24% decrease in the 380 nm intensity.

Examination of individual records revealed no fixed temporal relationship of transient changes in  $[\text{Ca}^{2+}]_i$  to chromosome separation (Fig. 1, A–F). Therefore, the percent of cells showing transients was plotted as a function of time before chromosome separation, and all transients from all spik-



**Figure 1.** (A–F) Time courses of  $[\text{Ca}^{2+}]_i$  in several typical dividing Pt K2 cells as they progress from metaphase to telophase. Cells were loaded with fura-2/AM and placed on the microscope stage which had been equilibrated at  $30$ – $32^\circ\text{C}$  using an air curtain incubator as described in Materials and Methods. Cells at a random point in prometaphase or metaphase were selected by bright-field observation and measurements of  $[\text{Ca}^{2+}]_i$  were made every 6–7 s until telophase was complete. Each  $[\text{Ca}^{2+}]_i$  measurement was accompanied by 3 s of bright-field observation to determine chromosome location. CS represents the time when the chromosomes can first be seen to be clearly separated. CY represents the point when the cell can first be seen to be constricting at its waist (the onset of cytokinesis).

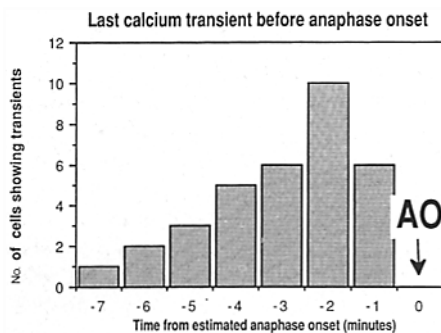


**Figure 2.** The percentage of Pt K2 cells showing transient increases in  $[Ca^{2+}]_i$  in each minute before chromosome separation. Transients in each cell were defined as described in Materials and Methods and were plotted as a function of time before chromosome separation (designated as  $t = 0$ ). For each minute before chromosome separation, the number of cells showing transients was counted and divided by the total number of cells examined ( $n = 39$ ).

ing cells were included in the analysis (Fig. 2). The graph reveals that although there is a peak of spiking activity at 3 min before chromosome separation, the increase in activity begins as early as 8 min before the daughter chromosomes have clearly moved apart.

To assess the role of such transient changes in the triggering of anaphase onset, the last time a cell underwent a transient increase in calcium before anaphase onset was plotted as a function of time before the estimated onset of anaphase (anaphase onset was estimated to be  $79 \pm 39$  s or  $\sim 1$  min before chromosome separation) (Fig. 3). Again, the graph indicates that although there is a peak number of transients (25%) 2 min before the start of anaphase, the increase in spiking activity begins as early as 6 to 7 min before anaphase has begun. In sum, 82% of the cells show their last transient increase in  $[Ca^{2+}]_i$  within the 8 min before anaphase onset.

In several cells that showed a transient before chromosome separation and in several cells that did not, a transient increase in calcium was observed during anaphase after the chromosomes had clearly separated (e.g., Fig. 1 B). There



**Figure 3.** The number of Pt K2 cells showing their last transient increase in calcium before anaphase as a function of time before the estimated onset of anaphase. Transient increases in  $[Ca^{2+}]_i$  in each cell were defined as described in Materials and Methods, and the last one occurring before anaphase onset was plotted as a function of minutes before anaphase onset. Anaphase onset was estimated to occur 1 min before observable chromosome separation. The number of transients occurring at each minute prior to the start of anaphase was counted and plotted.

appeared to be no fixed time interval between the transients in metaphase and anaphase when both occurred, and the magnitude and duration of the transients in anaphase are similar to those found in metaphase.

### Transient Changes in $[Ca^{2+}]_i$ at Cytokinesis

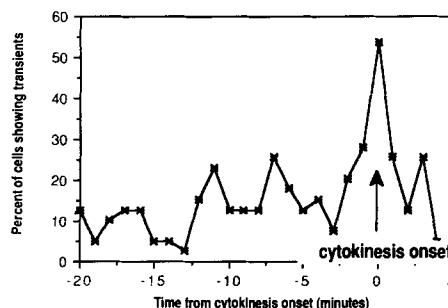
Transient fluctuations in calcium were also found during telophase and appear to be tightly correlated with the onset of cytokinesis (e.g., Fig. 1, A–F). 82% of the cells showed at least one transient within 2 min of cytokinesis onset. The mean  $[Ca^{2+}]_i$  at the peak of the transients was  $171 \text{ nM} \pm 70 \text{ nM}$  ( $\pm$ SD, 53 transients in 32 cells) with a mean duration of  $23 \pm 14$  s. The mean resting  $[Ca^{2+}]_i$  for anaphase and telophase cells is  $101 \pm 31 \text{ nM}$ , and the peak  $[Ca^{2+}]_i$  of the transients ranged from 103–324 nM. Using cytokinesis onset as “zero time”, a plot of the percent of cells showing transients as a function of time from cytokinesis onset demonstrates that the majority of cells (55%) show a transient increase in calcium at the onset of furrowing (Fig. 4).

### Slow Changes in $[Ca^{2+}]_i$ From Metaphase to Anaphase

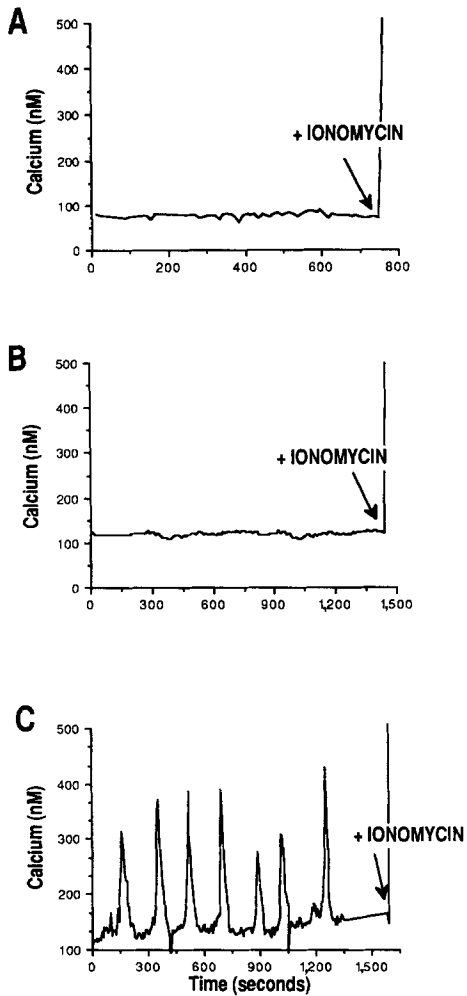
Continuous monitoring of  $[Ca^{2+}]_i$  confirms previous observations from our laboratory that resting calcium increases in most cells from metaphase to anaphase (29). The mean minimum  $[Ca^{2+}]_i$  during metaphase is  $53 \pm 19 \text{ nM}$  ( $\pm$ SD) with a mean baseline  $[Ca^{2+}]_i$  throughout metaphase of  $83 \pm 31 \text{ nM}$  ( $\pm$ SD), significantly different from mean baseline  $[Ca^{2+}]_i$  during anaphase ( $101 \pm 30 \text{ nM}$ ,  $n = 39$ , paired  $t$ -test,  $p < .0005$ ). The slow increase in  $[Ca^{2+}]_i$  occurs gradually (over several minutes) and begins variably during late metaphase (e.g., Fig. 1, A, C, D, and F). In one cell the rise began 10 min before chromosome separation (Fig. 1 E). There appears to be no fixed relationship of the slow rise in resting  $[Ca^{2+}]_i$  to rapid fluctuations occurring during the same period.

### Interphase Pt K2 Cells Show Oscillating $[Ca^{2+}]_i$

To assess the specificity of the fast and slow changes for mitosis, we obtained continuous calcium recordings in interphase cells. Most interphase cells showed stable baselines with no detectable changes in calcium (Fig. 5, A and B). However,



**Figure 4.** The percentage of Pt K2 cells showing transient increases in  $[Ca^{2+}]_i$  near the onset of cytokinesis. Transients in each cell were defined as described in Materials and Methods and were plotted as a function of minutes before cytokinesis onset (designated as  $t = 0$ ). For each minute before and after the start of cleavage, the number of cells showing transients was counted and divided by the total number of cells examined ( $n = 39$ ).



**Figure 5.** (A and B) Time course of  $[Ca^{2+}]_i$  changes in typical interphase Pt K2 cells showing no transient increases in  $[Ca^{2+}]_i$ . Cells were loaded with fura-2 and placed on the microscope stage which had been equilibrated at 30–32°C using an air curtain incubator as described in Materials and Methods. Cells at a random point in interphase were selected by bright-field observation and measurements of  $[Ca^{2+}]_i$  were made every 6–7 s for periods up to 3,000 s. Each  $[Ca^{2+}]_i$  measurement was accompanied by a 3-s bright-field observation. Ionomycin addition demonstrates the calcium sensitivity of fura-2 in these quiescent interphase cells. (C) Time course of  $[Ca^{2+}]_i$  in a typical oscillating interphase Pt K2 cell.

approximately one-third of the interphase cells showed dramatic brief fluctuations in calcium which could not be correlated with any morphological change. The mean  $[Ca^{2+}]_i$  at the peak in calcium was  $389 \pm 33$  nM (19 transients in 6 cells, range 150 nM to 700 nM) with a mean duration of  $77 \pm 12$  s. In several cells, the transients were oscillatory in nature, demonstrating remarkably regular periodicities (200–300 s) (Fig. 5 C). In over 100 mitotic cells examined, we saw no similar periodic oscillations in calcium.

## Discussion

Previous studies have investigated calcium changes during mitosis in mammalian cells. These studies have provided im-

portant information regarding long lasting ( $\approx 20$  min) local increases in  $[Ca^{2+}]_i$  (29) or more short-lived ( $\approx 20$  s) whole cell increases in  $[Ca^{2+}]_i$  (27) during the mitotic cycle, but they have failed to characterize precisely the relationship of fluctuations in calcium to mitotic events. In the present study, each calcium measurement (taken every 6.6 s) has been paired with a bright-field observation to define the temporal relationship of these changes in  $[Ca^{2+}]_i$  to the cellular and chromosomal movements characteristic of normal cell division.

The accuracy and physiological relevance of nearly continuous measurements of calcium with fura-2 has been ensured by performing the experiments under the following conditions. We have used a loading protocol for the permeant ester of fura-2 in which it is completely cleaved to its cytoplasmically trapped, calcium sensitive form. We have carried out calcium measurements at 30–32°C, a temperature which retards compartmentalization of the dye into subcellular organelles and that slows leakage of the dye from the cell. We have taken advantage of the greater sensitivity of fluorescence microspectrophotometry, as compared with the image intensifier cameras we have used for studies of mitosis in the past, to attenuate the incident UV excitation and avoid phototoxicity that results from repeated measurements over a long period of time. Finally, we have shown that repetitive measurements of fura-2 loaded Pt K2 cells under our experimental conditions significantly affect neither their mitotic transit time nor their morphology during mitosis.

### The Metaphase–Anaphase Transition

A number of events during the mitotic cycle have been proposed to be under the control of ionic calcium, but the mitotic process which has received the most attention in this regard has been the metaphase–anaphase transition, the period of mitosis when incipient daughter chromosomes, aligned at the metaphase plate, abruptly separate and are rapidly displaced to opposite poles. Several lines of evidence have supported the notion that chromosome separation is triggered by a sudden change in spindle calcium concentration (11, 15, 27, 31). However, the results presented here, showing that cells vary considerably in the time at which they undergo a transient increase in calcium for the last time before anaphase onset, argue against the hypothesis that a transient rise in calcium is the proximate “trigger” for the start of anaphase (Fig. 1, A–F). Examination of the spiking behavior of a population of cells shows that the percentage of cells showing transients begins to increase as early as 8 min before the chromosomes are separated with a maximum of 25% at 3 min, further evidence against an immediate triggering role for calcium (Fig. 2). Nevertheless, a plot of the last calcium transient in each cell before the onset of anaphase as a function of time before this event (Fig. 3) indicates the large majority of cells (82%) spike within the 8-min window before the start of anaphase or approximately the last 20% of the 35–40 min of metaphase. Such a pattern indicates that although a transient change in calcium is not likely to act as an immediate trigger, it may activate an intermediate step or a more complex series of events required for anaphase onset or anaphase progression. This view is supported by several studies that have manipulated  $[Ca^{2+}]_i$  internally and externally during metaphase and shown a slowing or inhibition of the transition to anaphase (6, 11, 38, 39).

Although the targets for calcium changes in metaphase are unknown, putative calcium-stimulated activities in the mitotic apparatus are numerous. Microtubules, actin filaments, and myosin possess well documented calcium-regulated activities. Stimulation of such activities could result in alterations in polymerization state, ATPase activity, or in homologous or heterologous interactions of filaments. These changes could be mediated by calcium-induced modifications of microtubule-associated proteins or actin-binding proteins in the spindle. Most notable among these is calmodulin, which has been colocalized with kinetochore microtubules in the mitotic apparatus (36, 37, 40). Recently, calcium activation of a  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase has been shown to result in autophosphorylation of the enzyme, thereby converting it to a calcium-independent form (38). Such a mechanism would allow a transient change in calcium during metaphase to effect calmodulin-mediated activities later in the mitotic cycle (e.g., anaphase onset or anaphase progression).

Calcium could also serve to facilitate progression to anaphase by degrading a cytostatic factor-like activity in metaphase. Cytostatic factor (CSF) is found in a variety of tissues and has been shown to arrest meiotic and mitotic cells in metaphase (19, 20). This factor has been demonstrated to be labile to calcium ions *in vitro*, but the precise mechanism of calcium-stimulated degradation of CSF is not well understood. Under such a scheme, degradation of cytostatic factor by calcium ions would be required before anaphase can take place. In fact, an analogous group of proteins, called cyclins, have been shown to arrest oocytes in meiotic metaphase and to be degraded with great regularity 5–6 min before the metaphase–anaphase transition (4, 34). The cyclin proteins must then be resynthesized before subsequent mitoses will occur. The relation of such factors to mitotic mechanisms may explain why *in vitro* models of mitosis have been difficult to develop.

Several laboratories have examined rapid calcium changes during the metaphase–anaphase transition using different dyes and a variety of cell types. We feel that despite disagreements in interpretation, the data can be reconciled. The results of Poenie et al. (27) in Pt K1 cells demonstrate a transient rise in calcium near the metaphase–anaphase transition. The authors concluded that this calcium pulse acts as an immediate trigger for anaphase, but due to the small number of cells studied and limitations in the temporal resolution of morphological observations, they could not document precisely the temporal relation of transient increases in  $[\text{Ca}^{2+}]_i$  to chromosomal movements. Additionally, despite the differences in the magnitude of the peak of the transient increases in calcium that are observed in Ptk1 cells (0.5–0.8  $\mu\text{M}$ ) and PtK2 cells (0.1–0.25  $\mu\text{M}$ ), the transients we observe here during the metaphase–anaphase transition in Pt K2 cells are similar in duration ( $\approx 20$  s) to those seen by Poenie et al. in Pt K1 cells. Part of the difference in magnitude is due to the use of a correction for microviscosity effects in the Pt K1 studies (27). This correction increases the values obtained for basal  $[\text{Ca}^{2+}]_i$  and for  $[\text{Ca}^{2+}]_i$  during transients.

In contrast to these data in mammalian cells, Hepler and Callaham failed to observe a change in calcium during metaphase with continuous monitoring of Arsenazo III signals in *Tradescantia*, a plant cell line (7). Although species differences cannot be excluded, the authors point out that the indicator they used (Arsenazo-III) is not sensitive to transient in-

creases in calcium concentrations below 0.3  $\mu\text{M}$ , the range of calcium changes observed here in dividing Pt K2 cells. In fact, the observation by Hepler and Callaham that metaphase transit is slowed in the presence of Arsenazo-III, presumably due to the calcium buffering capacity of the indicator, supports a role for calcium in the metaphase–anaphase transition in *Tradescantia*. Earlier studies from our laboratory have also failed to document a rapid 20–30 s rise in calcium during metaphase. However, in these studies we were sampling fura-2 and quin-2 signals only once or twice during each stage of mitosis and could have easily missed such an increase (12, 13, 29).

### Cytokinesis

Assembly and contraction of the contractile ring for cytokinesis begins during late anaphase and can be recognized by furrowing in the plane of the former metaphase plate. We find that within 2 min of the first signs of cytokinesis, a pulse of calcium occurs in over 82% of the cells observed (Fig. 4). The magnitude and duration of the transient changes in calcium are similar to those found during metaphase, and a number of cells are found to spike more than once during cleavage (e.g., Fig. 1 F). Several laboratories have suggested a role for calcium in the formation of the contractile ring based on manipulations of  $[\text{Ca}^{2+}]_i$  using injected calcium buffers and by addition of the ionophore A23187 (2, 32). Attempts to characterize or refute calcium's involvement in cytokinesis by direct measurements of free calcium have been inconclusive (2, 5, 30), with the exception of one study by Poenie et al. (26) which showed an increase in calcium associated with cleavage in echinoderm eggs. The findings of the present study extend the results of Poenie and coworkers to mammalian cells and place the calcium rise in the majority of cells right at the onset of cytokinesis. These data suggest that further examination of calcium's role in cytokinesis is in order.

### Interphase PT K2 Cells

To address the specificity of the transient increases in calcium observed during division to the mitotic process, we investigated calcium changes in interphase cells and have discovered striking differences between two groups. While most interphase cells showed stable baselines with none of the transient changes characteristic of the mitotic cells, one-third showed apparently spontaneous and sometimes periodic increases in calcium. The period of these oscillations was  $\sim 200$ –300 s. No visible morphologic changes could be correlated with the calcium oscillations, but the occurrence of subtle changes, below the resolution of bright-field optics, cannot be ruled out. Oscillations in calcium have been documented before in fibroblasts (22, 24), and macrophages (17), and are thought to be correlated with phagocytosis (21), pinocytosis (35), and chemotaxis (23), activities which are suppressed during mitosis. In over 100 cells observed we have never seen periodic oscillations in calcium to the levels observed in interphase cells. It is possible that the spiking is characteristic of another stage of the cell cycle such as G1. Such stage specificity could explain the fact that only about one-third of the cells show spiking activity in our unsynchronized cultures. Much work is needed to define the precise mechanism by which calcium oscillations are generated.

Our results suggest that whatever the mechanism for generating large, periodic transient increases in calcium in interphase, such a mechanism is inhibited or deficient during mitosis.

## Conclusion

In the present study, we have defined the temporal relationship of slow and fast calcium changes to morphologically definable mitotic events in Pt K2 cells from metaphase to telophase. In most cells, there is a slow increase in  $[Ca^{2+}]_i$  during late metaphase, and one or more transient increases in  $[Ca^{2+}]_i$  are seen during the last 20% of metaphase. The timing of these transients indicates that they are not an immediate trigger for anaphase onset. A much closer temporal correlation is observed between transient  $[Ca^{2+}]_i$  increases and cytokinesis. The data present here and elsewhere (7, 13, 26, 27, 29) indicate where and when the calcium is elevated during mitosis. The precise role for these calcium changes remains to be demonstrated. Controlled manipulation of  $[Ca^{2+}]_i$  in living cells either by intracellular buffering or by release of calcium into the cytosol will aid in establishing the requirement for  $[Ca^{2+}]_i$  changes.

We would like to acknowledge helpful discussions with Benjamin Kruskal, Dr. Charles Keith, and Oliver Priceman.

This work was funded by GM-34770 to F. R. Maxfield and NS-15076 to M. L. Shelanski from the National Institutes of Health. R. R. Ratan was supported by grant No. GM-07308 from the Medical Scientist Training Program of the National Institutes of Health.

Received for publication 3 February 1988, and in revised form 2 May 1988.

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