Intracellular Free Calcium and Mitosis in Mammalian Cells: Anaphase Onset Is Calcium Modulated, but Is not Triggered by a Brief Transient

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Abstract. Swiss 3T3 fibroblasts and LLC-PK epithelial cells in prometaphase or metaphase were either injected with fura-2 or loaded with the acetoxymethyl ester derivative of fura-2 (fura-2 AM) and monitored by microspectrofluorimetry. With both methods of loading, we observed two aspects of intracellular free calcium (Ca~) metabolism. (a) Most fibroblasts and epithelial cells exhibited a gradual rise from 75 nM in metaphase to 185 nM during cleavage, returning to baseline by early G1. (b) Mitotic Swiss 3T3 cells exhibited rapid transient Ca~ changes, similar to those previously reported [Poenie, M., J. Alderton, R. Y. Tsien, R. A. Steinhardt. 1985. Nature (Lond.). 315:147-149; Poenie, M., J. Alderton, R. Steinhardt, and R. Tsien. 1986. Science (Wash. DC). 233:886-889; Ratan, R., and M. L. Shelanski. 1988. J. Cell Biol. 107:993]. These Ca~ transients occurred repetitively, often beginning in metaphase and continuing long after daughter cell formation. Eliminating serum or calcium from the medium abolished the transients, but delayed neither the gradual Ca~ elevation nor anaphase onset.

Co-injection of EGTA or 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) with fura-2 in calcium-free medium, but not in calcium containing medium, blocked both anaphase and the sustained Ca~ elevation in almost all cases. Blocked cells were rescued by returning calcium to the medium, whereupon Ca~ slowly but steadily rose as the cell entered anaphase. Spindle microtubules persisted through the EGTA block. Depolymerization of spindle microtubules by nocodazole also reversibly blocked anaphase onset and the sustained Ca~ elevation, but did not block transients. This study has revealed the following: (a) anaphase in mammalian fibroblasts and epithelial cells is not triggered by brief calcium transients; (b) anaphase is a calcium-modulated event, usually accompanied by a sustained elevation of Ca~ above 50 nM; (c) the elevation of Ca~ is dependent upon an intact spindle; and (d) fibroblasts progress through mitosis by drawing upon either intracellular or extracellular sources of calcium.

The metaphase–anaphase transition is a critical point in the cell cycle. The abrupt nature of sister chromatid separation and spindle microtubule depolymerization suggest a regulatory mechanism that is tightly controlled, yet rapidly invoked.

A single transient rise in intracellular free calcium (Ca~) has been proposed to serve as the trigger of anaphase onset (Poenie et al., 1986). Such a trigger is plausible since intracellular calcium regulation is extensive and calcium can potentially interact with many components of the mitotic spindle. For example, ionized calcium affects the degree of polymerization of spindle microtubules both in vitro (Weisenberg, 1972; Weisenberg and Deery, 1981) and in vivo (Kiehart, 1981; Izant, 1983; Keith, 1987; Lee et al., 1987). The mitotic spindle is known to contain calcium transporting vesicles (Silver, 1986; Hafner and Petzelt, 1987; Petzelt et al., 1987), calmodulin (Andersen et al., 1987; Welsh et al., 1979; Zavortnik et al., 1983), and calpain II, a calcium-activated protease (Schollmeyer, 1988). Furthermore, sea urchin egg spindle microtubule depolymerization has been correlated with calmodulin-dependent phosphorylation of a mitotic apparatus protein (Dinsmore and Sloboda, 1988).

Direct measurements of Ca~ have used calcium-sensitive fluorescent, luminescent, or absorbing dyes to reveal both transient and gradual Ca~ changes during mitosis. Single, abrupt rises (transients) have been observed at anaphase onset in mammalian and sea urchin levels (Poenie et al., 1985, 1986). Less dramatic, but sustained increases in Ca~ have been observed during this same time period in mammalian and plant cells (Ratan et al., 1986; Hepler and Callaham, 1987), a result that questions an obligatory coupling of a transient with anaphase onset. Furthermore, in PtK epithelial cells, transients occur in interphase as well as in mitosis and do not statistically correlate with anaphase onset (Ratan et al., 1988). In separate experiments, but in the same cell types, attempts were made to artificially manipulate Ca~ with calcium chelators, channel blockers, ionophores or putative antagonists of membrane calcium pumps. When Ca~ was perturbed towards very low or very high levels,
anaphase onset was delayed (Izant, 1983; Ziegler et al., 1985; Keith, 1987; Lee et al., 1987), sometimes reversibly, after removal of the perturbation (Hepler, 1985; Wolniak and Bart, 1985a,b). At moderate levels, anaphase was prematurely induced (Izant, 1983; Chen and Wolniak, 1987). It was estimated that the delay in anaphase onset occurred at Ca$^{2+}$ levels below $10^{-8}$ M or above $10^{-7}$ M, and that precocious onset occurred between $10^{-7}$ and $10^{-8}$ M (Izant, 1983).

However, almost all of these experiments suffered from the lack of direct Ca$^{2+}$ determinations to confirm their effect. Without direct measurement of Ca$^{2+}$, it was not possible to demonstrate that the experimental agents acted in the expected manner, nor was it possible to exclude homeostatic regulation of Ca$^{2+}$ during the experiments. Although they clearly pointed to a role for Ca$^{2+}$ in the transition from metaphase to anaphase, these experiments did not resolve whether an abrupt transient or a sustained elevation was the significant variable.

We have attempted to determine whether Ca$^{2+}$ is the trigger for anaphase by combining the approaches of perturbation by calcium chelators with direct measurement of Ca$^{2+}$ by fura-2 fluorescence. We report that anaphase onset is not triggered by a brief Ca$^{2+}$ transient, but is nonetheless dependent upon Ca$^{2+}$ mobilization, which is usually manifested through a sustained Ca$^{2+}$ rise.

**Materials and Methods**

**Reagents**

Acetoxymethyl ester derivative of fura-2 (Fura-2 AM) and K$_2$fura-2 were obtained from Molecular Probes Inc. (Eugene, OR). K$_2$Fura-2 was dissolved in injection buffer to 10 mM and stored at ~20°C until use. Injection buffer was composed of 115 mM KCl, 20 mM NaCl, 1.2 mM MgCl$_2$, 10 mM NaH$_2$Pipes, pH 7.0. 50-μM aliquots of dry fura-2 AM were dissolved in 10 μl dry DMSO, 10 μl pluronic F-127 in DMSO and 8 μl FBS on the day of use according to published methods (Poenie et al., 1986). FBS was obtained from Hyclone Laboratories (Logan, UT).

Calcium-EGTA buffers were prepared by titrating solutions of CaCl$_2$ and K$_2$EGTA to their equivalency point by monitoring pH, as previously described (Moisescu and Pusch, 1975). Calibration curves were prepared by mixing stock solutions of 10 mM K$_2$EGTA and 10 mM Ca$_2$EGTA to achieve the desired final free calcium concentration, calculated from a computer program kindly supplied by Dr. Donner Babcock, Department of Biochemistry, University of Washington.

Cells were monitored in a modified UV fluorescence-free DME (Gibco Laboratories, Grand Island, NY) which lacked vitamins, NaHCO$_3$, and phenol red, but contained 135 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl$_2$, 25 mM dextrose, amino acids at standard dilution, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 1 mM Na$_2$PO$_4$, 20 mM NaH$_2$Pipes, pH 7.5, and either 1.36 mM CaCl$_2$ or 2 mM K$_2$EGTA. This was referred to as either FF-DME or Ca-free FF-DME.

**Cell Culture**

Swiss 3T3 primary cell lines were obtained from American Type Culture Collection (Rockville, MD) and cultured in DME with 10% FBS at 37°C with 7.5% CO$_2$. LLC-PK1 cells are a pig kidney epithelial cell line, also obtained from ATCC and cultured identically to 3T3 cells. Subconfluent cultures were subcultured at least every 4 days onto glass coverslips. We limited Swiss 3T3 cells to <30 passages before thawing a fresh aliquot. Cells on glass coverslips were monitored in a Sykes-Moore chamber within 3 d of subculturing. Coverslips were occasionally coated with 0.1% poly (l-lysine) to ensure attachment of cells to the coverslip, especially when cells were examined in calcium-free medium.

**Quantitative Fluorescence Microscopy**

An inverted microscope (Diaphot; Nikon Inc., Garden City, NY) equipped with UV transmitting optics, a UV-F 40×, 1.3 NA oil immersion objective, a 400-nm dichroic mirror, and a 420-nm barrier filter was used for fluorescence microscopy. We used fluorescent-type FF immersion oil (R. P. Cargille Laboratories, Inc., Cedar Grove, NJ). A Fluoroplaque III (Taconic Northern, Middlefield, WI) was used to cover a radiat ion from a 150-W xenon lamp that was split into and alternated between two monochromators set at 340 and 380 nm. Illumination was through the epifluorescence port and typically the intensity of the light beam was attenuated 100-1,000-fold with neutral density filters, since cells would not divide when exposed constantly to uninterrupted UV illumination, whether they had been loaded with fura-2 or not. Light filtering in both channels were decreased to the same extent with these filters. Whole cell fluorescence quantitation was by photomultiplier tube (PMT) synchronized with the alternating monochromators. To minimize background levels, we found it necessary not only to use fluorescence-free immersion oil and medium, but to turn off overhead room lights and drape the microscope in two layers of black photographic cloth. Typical signal/noise ratios were ≥20 at the start of an experiment.

Injections were performed with a micromanipulator (Narisige Scientific Laboratory, Greenwale, NY) with capillary pipettes drawn on a vertical pi-ette puller (David Kopf Instruments, Tujunga, CA) according to published methods (Kreis and Birchmeier, 1982). Each pipette was pressure driven from a 10 μl gas-tight Hamilton syringe (VWR). Fura-2 loadings or injections were both performed at room temperature (23 ± 1°C). Fura-2 needle concentrations for injections were between 0.25 and 0.5 μM, and the concentration of fura-2 AM used was 0.01 μM. After fura-2 was injected or loaded, fresh medium was placed in the chamber and then overlaid with mineral oil. Monitoring began at this time as cells were warmed to 32 ± 1°C at a rate of 1°C/min and treated as described. Temperature in the medium was determined by an electronic temperature probe (Bailley Instruments, Saddler Brook, NY).

Fluorescence from fura-2 whether injected or loaded as the AM-ester steadily decreased intracellularly, independent of the change in the shape of the dividing cell. At 32°C, fluorescence was lost with an average half-life of 30 min, a rate similar to other reports (Malgaroli et al., 1987; Poenie et al., 1986). At 37°C, fura-2 had a similar half-life as at 32°C, but it was rapidly compartmentalized in small perinuclear vesicles in interphase cells, even when these cells were loaded at 23°C. Mitotic cells showed no apparent compartmentalization of the dye. We observed insignificant rates of dye bleaching in vitro under similar conditions of illumination, and so concluded that the fluorescence loss was because of active transport of the dye out of the cell, a conclusion also reached by others (Malgaroli et al., 1987).

Ca$^{2+}$ was calculated using a K$_2$ of 225 nM, a viscosity correction factor of 0.85, and the equation previously described (Poenie et al., 1986). The average of experimentally determined calcium-free and calcium-saturated fura-2 340/380 fluorescence ratios were 0.33 and 10, respectively.

Cells were monitored continuously for fura-2 fluorescence by PMT with data points typically integrated every 2.5 s. Every 8 min, data collection was interrupted and cells were examined and photographed using optics (Differential Interference Contrast; Nikon Inc., Garden City, NY) (Nomarski) (technical pan 2415 film; Eastman Kodak Co., Rochester, NY). Mitotic cells were distinguished from interphase cells by the presence of the mitotic spindle.

The approximate moment of anaphase onset was estimated from known kinetics of mitotic progression and the morphology of the cell at the beginning and end of each eight-min acquisition. Transit times were defined as the time between warming the cells to 32°C and anaphase onset, since we injected or loaded cells with fura-2 at temperatures that have been shown to drastically slow, if not arrest, mitotic progression (Rao and Engelberg, 1966; Rieder, 1981). Although we only monitored mitotic progression every 8 min, we felt that the phase of the mitotic cells (i.e., metaphase, early, or late anaphase) and the known rate of chromosome separation (~1 μm/min) permitted us to estimate transit times to within 5 min. Cells which had already been warmed to 32°C before injection were excluded from the transit time estimates shown in Tables I, II, and III, but may have been used to confirm general trends in Ca$^{2+}$.

**Immunofluorescence**

Cells were permeabilized, fixed and processed at room temperature for immunofluorescence microscopy, as described (Vandere et al., 1984). Briefly, coverslips were placed in 0.1% Triton X-100, 5 μg/ml taxol, PHEM buffer and then overlaid with fresh medium was placed in the chamber and then overlaid with mineral oil. Monitoring began at this time as cells were warmed to 32 ± 1°C at a rate of 1°C/min and treated as described. Temperature in the medium was determined by an electronic temperature probe (Bailley Instruments, Saddler Brook, NY).
Table I. Transit Times and Calcium Metabolism in Swiss 3T3 and LLC-PK Cells According to Method of Dye Introduction and Extracellular Calcium and Serum

| Condition                                      | No. cells monitored | No. cells entering anaphase | Transit time mean ± SD (range) | No. cells with sustained elevation | Base line (nM Ca) | Sustained elevation peak (nM Ca) | Difference (nM Ca) |
|------------------------------------------------|---------------------|-----------------------------|-------------------------------|-------------------------------------|------------------|----------------------------------|--------------------|
| **Swiss 3T3**                                  |                     |                             |                               |                                     |                  |                                  |                    |
| F2 injected in normal medium                   | 10                  | 10 (100%)                   | 16 ± 7 (10-29)                | 9 (90%)                             | 10 (100%)        | 65 ± 50                          | 160 ± 80           |
| F2AM loaded in normal medium                   | 9                   | 9 (100%)                    | 15 ± 6 (8-27)                 | 9 (100%)                            | 9 (100%)         | 90 ± 25                          | 210 ± 50           |
| F2 and F2AM in normal medium lacking calcium, containing EGTA | 5                   | 5 (100%)                    | 14 ± 4 (10-18)                | 1 (20%)                             | 4 (80%)          | 55 ± 35                          | 115 ± 55           |
| F2 and F2AM in normal medium low (<2%) in serum | 7                   | 7 (100%)                    | 21 ± 5 (15-27)                | 1 (15%)                             | 6 (85%)          | 105 ± 30                         | 195 ± 55           |
| **LLC-PK**                                     | 8                   | 8 (100%)                    | 19 ± 3 (16-25)                | 0 (0%)                              | 8 (100%)         | 75 ± 35                          | 165 ± 55           |

Normal medium was fluorescent-free, calcium containing DME, with 5–10% FBS. Fura-2(F2) needle concentration was 0.25–0.5 mM; fura-2 AM(F2AM) loading concentration was 0.01 mM; extracellular CaCl₂ concentration was 1.36 mM; and EGTA concentration was 2 mM. Transit times are between warming to 32°C and anaphase onset. Sustained elevations are defined as gradual rises in calcium from the baseline to the peak which exceed 1 nM/min.

Table II. Transit Times and Calcium Metabolism in Swiss 3T3 Cells Injected with Calcium Chelator in the Presence or Absence of Extracellular Calcium

| Condition                                      | No. cells monitored | No. cells entering anaphase | Transit time mean ± SD (range) | No. cells with sustained elevation | Base line (nM Ca) | Sustained elevation peak (nM Ca) | Difference (nM Ca) |
|------------------------------------------------|---------------------|-----------------------------|-------------------------------|-------------------------------------|------------------|----------------------------------|--------------------|
| (1) F2 with BAPTA or EGTA in normal medium     | 8                   | 8 (88%)                     | 29 ± 3 (8-74)                 | 8 (100%)                            | 50 ± 25          | 285 ± 85                         | 235 ± 90           |
| (2 a) F2 with BAPTA or EGTA in normal medium lacking calcium, containing EGTA | 18                  | 3 (17%)                     | 29 ± 3 (24-32)                | 0 (0%)                              | 30 ± 20          | 55 ± 20                          | 25 ± 25            |
| Other 15 cells blocked                         | 0                   | 0 (0%)                      | 20 ± 20                       | 0 (0%)                              | 50 ± 20          | 30 ± 20                          | 30 ± 20            |
| (2 b) Calcium added back to 9 of 15 cells blocked in 2a, after 30-70 min | 9                   | 8 (89%)                     | 77 ± 22 (52-120)*             | 9 (100%)                            | 50 ± 20          | 265 ± 140                       | 215 ± 140          |

Transit times are between warming to 32°C and anaphase onset, except in 2b, where the transit is between calcium addition and anaphase onset. Sustained elevations are defined as gradual rises in calcium from the baseline to the peak that exceed 1 nM/min. Normal medium was fluorescent-free, calcium containing DME, with 5–10% FBS. Needle concentrations were 0.25–0.5 mM for fura-2(F2), and 10 or 25 mM for EGTA and BAPTA. Extracellular CaCl₂ and EGTA concentrations were 1–2 mM and 2 mM, respectively. Calcium was added back to cells in EGTA by either exceeding the EGTA concentration with buffered CaCl₂ or replacing the entire medium.

Table III. Transit Times and Calcium Metabolism in Swiss 3T3 Cells Incubated with Nocodazole

| Condition                                      | Cells monitored | Cells entering anaphase | Transit time mean ± SD (range) | Cells with sustained elevation | Base line (nM Ca) | Sustained elevation peak (nM Ca) | Difference (nM Ca) |
|------------------------------------------------|-----------------|-------------------------|-------------------------------|-------------------------------|------------------|----------------------------------|--------------------|
| (1 a) F2 and F2AM in normal medium containing nocodazole | 8               | 0 (0%)                  | All 8 blocked                 | 0 (0%)                        | 115 ± 30         | 135 ± 30                         | 20 ± 40            |
| (1 b) Nocodazole removed from cells in 1a       | 8               | 8 (100%)                | 30 ± 7 (18-40)                | 8 (100%)                      | 135 ± 30         | 350 ± 105                        | 215 ± 110          |

Nocodazole was used between 10 and 40 ng/ml. Transit times are from nocodazole removal to anaphase onset. Sustained elevations are defined as gradual rises in calcium from the baseline to the peak that exceed 1 nM/min. Normal medium was fluorescent-free, calcium containing DME, with 5–10% FBS. Fura-2 needle loading concentration was 0.25–0.5 mM and fura-2 AM loading concentration was 0.01 mM.

Results

All prometaphase or metaphase Swiss 3T3 cells loaded or injected with fura-2 and monitored for fluorescence in medium containing both 1.4 mM CaCl₂ and 10% FBS, proceeded through mitosis with normal kinetics, entering anaphase in 16 ± 6 min after reaching 32°C (Table I). They exhibited two aspects of Ca metabolism, as shown for representative cells either loaded with 10 μM fura-2 AM (Fig. 1 A) or injected with 0.25 mM fura-2 (Fig. 1 B). One aspect was an
Figure 1. A, Prometaphase Swiss 3T3 cell, loaded with 10 µM fura-2 AM for 73 min at 22°C was warmed to 32°C at T = 0 min in calcium containing medium, 5% FBS. B, Metaphase Swiss 3T3 cell, injected with 0.25 mM fura-2 at 23°C, was warmed to 32°C at T = 0 min in calcium containing medium, with 10% FBS. Prometaphase (P), metaphase (M), anaphase (A), and the completion of cytokinesis (C) occurred as indicated. Nomarski images are listed in min after reaching 32°C and correspond to the cell in A, but represented the mitotic cycle of all control cells. Bar, 20 µm.

The baseline level from ~80 nM-185 nM (average of values in Table I). We did not consider this elevation to have begun until the initial rate exceeded 1 nM/min. It occurred in over 90% of cells that completed mitosis, but exhibited variability in onset kinetics, beginning in metaphase in two thirds of the cells and in anaphase in one third of the cells, typically peaking well after anaphase was complete. The elevated baseline did not recede to preanaphase levels until well after daughter cells had begun spreading out and nuclei had reformed. Because of loss of dye from the cells (see Materials and Methods), we seldom could observe the return of Ca to the original baseline levels (e.g., Figs. 5, 6, 8), even though it generally occurred when cells were monitored long enough (30 min to 2 h after anaphase onset).

The second aspect of mitotic Ca changes involved dramatic, repetitive increases or "transients" from the baseline level to an average of 360 nM ranging between 250 and 700 nM. The transients were repetitive, but typically not highly regular, regardless of the method used to introduce fura-2. In instances where transients were sufficiently regular (e.g., Fig. 1), their period was 190 ± 75 s (n = 104 transients from 10 cells) and their duration at half maximal amplitude was 42 ± 22 s (n = 85 transients from 10 cells). They began anytime from early metaphase to late anaphase, with only 30% beginning within 5 min of anaphase onset (not shown).

Transients normally continued through telophase and into early G1, but, if monitored long enough, subsided as the baseline returned to preanaphase levels. Mitotic cells never exhibited only one or two transients; when transients occurred, they happened anywhere between 5 and 40 times, averaging 18 ± 10 (n = 22). This number was an underestimate, since monitoring frequently ended before transients had begun to diminish. Both the sustained rise and the transients were observed regardless of whether monitoring had begun at any time between prophase and anaphase.

**Transients are not Essential for Mitotic Progression**

Single transients had previously been suggested to represent the trigger for anaphase onset (Poenie et al., 1986). Multiple transients were perplexing since they occurred throughout the mitotic interval and did not correlate precisely with anaphase onset. Nevertheless, we sought to test the possibility that repetitive Ca transients were responsible for initiating anaphase onset by exploring conditions that might affect Ca activity. When calcium was removed from or serum was decreased in the medium or both, the Ca transients were lost, yet the kinetics of mitosis were unaltered and the baseline Ca elevation persisted (Figs. 2, A and B; Table I). Although variable between batches of serum, at least 2-5% FBS was required to observe the repetitive transients. In one example, transients were eliminated by removing calcium from the medium and restored when calcium was returned to the medium, without preventing the baseline elevation (Fig. 2 C).

We also observed Ca transients in some interphase cells (Fig. 3 A), but did not pursue these observations exhaustively. The apparent rise in the baseline of the interphase cell
Figure 2. A, metaphase Swiss 3T3 cell, loaded with 10 μM fura-2 AM for 120 min at 23°C was warmed to 32°C at T = 0 min in calcium containing medium, lacking FBS. Cell lacks Ca, transients, but exhibits slow elevation and divides within the normal range. B, metaphase Swiss 3T3 cell, loaded with 10 μM fura-2 AM for 60 min at 23°C was warmed to 32°C at T = 0 min in calcium-free medium, 5% FBS. Again, cell lacks Ca, transients, but exhibits slow elevation and enters anaphase without delay. C, prometaphase Swiss 3T3 cell loaded with 10 μM fura-2 AM for 63 min at 22°C, was warmed to 32°C at T = 0 min in 10% FBS containing, calcium-free medium. Calcium was returned to medium at T = 34 min, removed at T = 53 min, and returned again at T = 79 min. Monitoring of this cell began in prophase; thus, its mitotic kinetics were extended. Prometaphase (P), metaphase (M), anaphase (A), and the completion of cytokinesis (C) occurred as indicated.

Figure 3. A, Interphase Swiss 3T3 cell injected with 0.25 mM fura-2 at 24°C was warmed to 32°C in calcium containing medium, 5% FBS at T = 0 min. Nomarski image was taken at T = 40 min. B, Interphase Swiss 3T3 cell loaded with 10 μM fura-2 AM at 24°C was warmed to 32°C in calcium containing medium, 10% FBS at T = 0 min. Bar, 20 μm.

in Fig. 3 A was not typical; a cell that lacked transients, but had a more typical flat baseline, is shown in Fig. 3 B. These two cells are illustrative of the diversity of interphase patterns in mammalian cells, as previously reported (Ratan et al., 1988). Interphase transients occurred unpredictably; their period (82 ± 42 s; n = 17 periods) was shorter than in mitotic cells, but they reached similar amplitudes.

To assay the generality of the transient activity, we examined Ca, in the LLC-PK pig kidney epithelial cell line. These cells remain flat during mitosis and have been used in previous studies of microtubule dynamics and mitotic phosphoproteins at anaphase onset (Gorbsky et al., 1987; Vandre et al., 1984). Although we observed an average gradual Ca, elevation from 75 to 165 nM beginning at metaphase or anaphase in all LLC-PK cells monitored, peaking after cell division, similar to that seen in Swiss 3T3 cells, we never saw Ca, transients during the entire prometaphase to interphase period, regardless of the method of fura-2 introduction or content of the extracellular medium (Table I; Fig. 4). The LLC-PK trace shows a typical gradual elevation of Ca, beginning just after anaphase onset; the slight dip in the trace just before anaphase onset is atypical.

Since the Ca, transients could be eliminated from fibroblasts by simple changes in the extracellular medium such as calcium removal or serum depletion and since they were not present in epithelial cells during mitosis, we conclude that transients are not essential for mitotic progression in mammalian cells and thus do not trigger specific mitotic events.

Blocking Calcium Elevation Reversibly Blocks Anaphase Onset

To test the requirement of the baseline Ca, elevation for mitotic progression, we co-injected Swiss 3T3 cells with fura-2 and one of two calcium chelators, EGTA or (1,2-bis-[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid) BAPTA, in an attempt to block anaphase onset. As previously described for PtK, epithelial cells (Izant, 1983), LLC-PK epithelial cells did not withstand injections of calcium-free EGTA, even in calcium containing medium, as assayed by the rapid loss (within 30 s) of fluorescent dye and cytoplasmic components into the medium. However, injections into Swiss 3T3 cells were much more successful. It is not clear why Swiss 3T3 cells, but not epithelial cells, can reseal their membranes and retain injected chelators, but this trait made possible the following experiments directed at blocking Ca, elevations.
Figure 4. Metaphase LLC-PK cell injected with 0.50 mM fura-2 at 24°C was warmed to 32°C in calcium containing medium, 10% FBS at T = 0 min. Metaphase (M), anaphase (A), and the completion of cytokinesis (C) occurred as indicated. Nomarski images are listed in min after reaching 32°C. The slight dip in Ca~ at anaphase is atypical. Bar, 20 μm.

As already shown (Table I), mitosis occurred with normal kinetics when calcium was removed from the extracellular medium despite the elimination of Ca~ transients. Therefore, the putative calcium requirement does not derive exclusively from extracellular sources. When either 10 mM EGTA or BAPTA in injection buffer was injected into the cell and monitored in calcium containing medium, Ca~ levels were initially low (50 nM), but after a short delay, rose above 100 nM after the cell entered anaphase (Table II; Fig. 5). Anaphase onset failed in one of eight cells treated in this manner, but occurred normally in the rest, although taking twice as long (on average) as untreated cells and lacking any Ca~ transients. This result made it clear that cells can homeostase Ca~ despite the injection of high concentrations of calcium chelators; i.e., an imposed intracellular calcium clamp can be overcome by extracellular calcium.

However, when both sources of calcium were removed, by injecting at least 10 mM EGTA or BAPTA and maintaining the cell in a calcium-free medium, the cell remained in whichever mitotic phase (prometaphase or metaphase) that it was in at the time of injection. Although the loss of fura-2 from cells placed an upper limit on the time that cells could be monitored, we can state that the block lasted at least four times (>70 min) the transit time of control cells (16 min). Ca~ was initially reduced to 20 nM, and rose very slowly to 50 nM over the course of 1 h as the cells remained in the block (Table II; Fig. 6). In cells treated in this manner, this rise did not exceed 1 nM/min (data not shown). The block was reversible in that mitosis resumed when calcium was added to 1–2 mM in the extracellular medium by either exceeding the EGTA concentration in the medium with buffered CaCl2 or replacing the calcium free medium with calcium containing medium (Table II; Fig. 6). The latter approach was risky since, in the absence of calcium, the cells rounded up (Fig. 6), lost their grip on the coverslip and were occasionally washed away with the change of medium. The chromosomes tended to clump during calcium deprivation, but the microtubules of the mitotic spindle appeared normal (Fig. 7). After calcium addition, Ca~ elevation accelerated immediately to 5 nM/min, but it took much longer for the chromatids to regain their normal morphology and separate (Fig. 6). At the time of anaphase onset in these rescued cells, Ca~ had exceeded the levels seen in control mitotic cells, eventually reaching a higher peak value (Table II). Ca~ returned towards baseline levels after division (Fig. 6). In one third of the cells that were rescued from the metaphase block, anaphase occurred, but cytokinesis did not, resulting in a binucleated cell. This suggested that cytokinesis was even more sensitive to extended periods of calcium-deprivation than anaphase, when cells were blocked in metaphase.

3 of 18 cells treated in such a manner were anomalous in that anaphase occurred after a delay, but before readdition of calcium (Table II). Since chelator was always introduced by co-injection with fura-2, integrating fura-2 levels between both excitation channels allowed us to estimate that these three anaphase-delayed cells had no less chelator than cells...
Figure 6. Prometaphase Swiss 3T3 cell injected with 10 mM BAPTA/0.25 mM fura-2 at 24°C was warmed to 32°C at T = 0 min in calcium free medium, 10% FBS. Calcium was restored to medium at T = 67 min. Prometaphase (P), metaphase (M), anaphase (A) and the completion of cytokinesis (C) occurred as indicated. Nomarski images are listed in min after reaching 32°C. Bar, 20 µm.

that were blocked. Furthermore, like blocked cells, the initial rate of their Ca2+ rise did not exceed 1 nM/min.

Although blocked cells could be rescued, the transit times in these cells (from calcium addition to anaphase onset) were much longer than a normal transit time (73 min, as opposed to 16 min). This delay was, in part, because of overcoming intracellular chelator, as illustrated in the two-fold delay in kinetics in cells injected with chelator in calcium containing medium. However, the delay was more complex since in rescued cells, the Ca2+ level at anaphase was higher than the Ca2+ level in normal cells at anaphase onset. Variability in these rescue times was probably because of a number of factors such as injection volumes, endogenous calcium stores, and/or calcium influx during injection. Also, the duration of the block varied between 20 and 66 min and probably affected the recovery time.

In two cells injected with EGTA in calcium-free medium that were thought to be in late metaphase, (i.e., the chromosomes were in a tight conformation at the equator) Ca2+ in these cells was initially as low as in cells that were blocked, but these cells entered anaphase within 10 min of warm-up. These cells then passed through anaphase and cytokinesis with normal kinetics. It was not clear whether the cells were

Figure 7. Antitubulin-stained metaphase Swiss 3T3 cell injected with 10 mM EGTA/0.25 mM fura-2 in calcium free medium. Cell was monitored before fixation and exhibited a Ca2+ trace and morphology similar to the cell in Fig. 6. It was permeabilized and fixed 31 min after reaching 32°C. Cell was stained with a rabbit polyclonal antibody against the tyrosinated-tubulin peptide (B); its phase image is also shown (A). Bar, 20 µm.
already in early anaphase when injected or in late metaphase. Because of this potential ambiguity, we did not include these cells in the tabulations and attempted to restrict injections to those cells that were clearly in either prometaphase or early metaphase.

**Nocodazole Treatment and Calcium Metabolism**

Clamping Ca\textsuperscript{2+} at a low level blocked mitotic progression, but did not indicate how mitotic progression was coupled to Ca\textsuperscript{2+} elevation. To address this question, we briefly blocked mitotic progression with the microtubule depolymerizing drug nocodazole and examined whether Ca\textsuperscript{2+} transients and/or the baseline elevation occurred. 10–40 ng/ml nocodazole (33–133 nM) caused rapid loss of the clear zone around the spindle and the chromosomes drifted away from their tight metaphase configuration (Fig. 8). We interpreted this as the expected depolymerization of spindle microtubules. Before release from nocodazole, calcium transients were observed, but the baseline remained low, rising only slightly from 115 to 135 nM over 30 min (Fig. 8; Table III) at an average rate of 0.6 nM/min. After removing nocodazole from the medium, the spindle reformed, as judged by the return of the chromosomes to the metaphase plate and the clear zone surrounding the chromosomes. The repetitive calcium transients continued, the baseline calcium rise accelerated and anaphase onset occurred after a standard time period (Fig. 8). However, as in chelator blocked and released cells, the level of Ca\textsuperscript{2+} at anaphase onset was much higher than levels in control cells (Fig. 8) and continued to rise to peak levels that were twice as high as normal cells (Table III). As in control cells examined for extended times, the baseline calcium returned towards preanaphase levels once the cell had completed division. We have interpreted these results as a specific effect of nocodazole on spindle microtubules and not on other microtubule-associated organelles because of the rapidity of both onset and reversal of the effect on Ca\textsuperscript{2+} metabolism and spindle structure and the fact that most cellular microtubules are devoted to the mitotic spindle during mitosis.

We conclude that transients and mitotic progression are uncoupled events, but that there is a stronger association between the sustained baseline elevation and the formation of the spindle. Taken together, the chelator and nocodazole blocking experiments suggest an interdependence between mitotic progression and mobilization of Ca\textsuperscript{2+}.

**Discussion**

We have used two mammalian cell types to test the hypothesis that changes in Ca\textsuperscript{2+} trigger anaphase onset. By using ratiometric microfluorimetry with calcium-specific dyes, introduced by both injection and AM ester permeation, we recorded transient and sustained increases in Ca\textsuperscript{2+} during mitosis. We monitored cells continuously, documented their morphology intermittently and perturbed Ca\textsuperscript{2+} metabolism with calcium chelators and microtubule disrupting agents.

Our results reject the hypothesis that a calcium transient is the trigger for anaphase in mammalian cells. Calcium tran-
sients did not regularly occur at the metaphase/anaphase transition as would be expected for a triggering event. They generally occurred repetitively, not individually, throughout the mitotic interval and occasionally in interphase. They were not universally observed; LLC-PK epithelial cells lacked them and they could be eliminated from Swiss 3T3 cells, where they were normally observed, by calcium or serum deprivation, without altering the kinetics of mitotic progression. Finally, transients occurred in cells blocked in mitosis by the microtubule depolymerizing drug, nocodazole. All of these results point to the conclusion that calcium transients are essentially an independent phenomenon displayed in some cell types for as yet unknown reasons and not a universal requirement for or trigger of mitosis.

In contrast, a more gradual elevation and subsequent decline of Ca, was strongly correlated with mitotic progression. Elevations occurred in 92% of all mitotic cells examined, always began in either metaphase or anaphase and then declined in early interphase. Both epithelial and fibroblast cells exhibited this behavior in normal medium as well as in calcium or serum deprived medium. Furthermore, interphase cells and cells blocked in mitosis with nocodazole failed to display elevations and most mitotic cells in which the elevation was prevented by a calcium chelator clamp failed to enter anaphase. From these results, we conclude that the mobilization of Ca, is required for the metaphase/anaphase transition and that the Ca, elevation, which usually accompanies anaphase is dependent upon the formation of the mitotic spindle.

However, our correlation of sustained Ca, rises with anaphase onset is not without exceptions. Even though 83% of all cells in which all sources of calcium were removed were blocked in metaphase until their Ca, rises were restored, 3 of 18 cells treated as such entered anaphase despite lacking Ca, rises. We interpret these exceptional cells as indicating that there may be no strict temporal linkage between anaphase onset and the onset of the Ca, rise. For example, in cells rescued from nocodazole or calcium chelator blockage, Ca, begins to rise immediately upon release from the block, but well before anaphase onset. Furthermore, the onset of the sustained elevation in unperturbed cells can occur anytime from midmetaphase to early anaphase. In other words, Ca, mobilization may be one of many requirements that must be met for anaphase to occur, but in these three cells, calcium may have already exerted its influence on the developing spindle before chelator injection. We could not distinguish these three cells from those that were truly blocked by any of our experimental parameters.

The amplitude of the sustained rise was also variable, ranging from only 60 nM in cells studied in the absence of extracellular calcium to over 200 nM in cells rescued from either calcium deprivation or nocodazole blockage. The overshoot of Ca, in rescued cells may be an exaggerated response by cells whose calcium channels have become hyperactivated in a metaphase-prolonged state, but this variability made it difficult to estimate whether a threshold of Ca, existed. The probable role that calcium plays in cytokinesis (Baker and Warner, 1972) may be the reason why the sustained Ca, elevation in normal cells does not peak until late cytokinesis.

Extended metaphase kinetics in cells released from the metaphase block may be because of the reinitiation of a normal, not necessarily sequential pathway that assesses whether conditions are permissive for mitosis. Part of this pathway appears to require an increase in calcium metabolism, usually manifested as a rise in Ca, whose kinetics and amplitude are variable.

Swiss 3T3 cells can obtain the needed calcium for this mitotic transition from either intracellular stores or from the medium. The intracellular calcium stores in these cells appear vast since a high concentration of chelator (~10⁻³ M final intracellular concentration) was required to block the normal level of Ca, (~10⁻⁷ M) from rising and leading to anaphase. This is consistent with the estimate that ionized calcium constitutes <0.1% of total cellular calcium (Carafoli, 1987). The existence of an optional extracellular source for calcium suggests that during metaphase, the mechanism exists to open calcium channels either on the plasma membrane or on endomembranes to effect a sustained Ca, elevation. The Ca, transients observed here, on the other hand, appear to be limited to calcium ion influx across the plasma membrane, implying a different mechanism or channel class than that used for the sustained elevations. That possibility is supported by the existence of L and T calcium channel types in the plasma membrane of Swiss 3T3 cells, the former of which is open for prolonged time periods and the latter only transiently (Chen, 1988). In plants, where only sustained elevations have been seen during mitosis, extracellular calcium appears to provide the sole source for the metaphase–anaphase transition (Hepler, 1985; Wolniak and Bart, 1985a,b; Chen and Wolniak, 1987).

The role of Ca, transients during mitosis or interphase in fibroblasts is unclear. Other nonexcitable cells exhibit transient elevations of Ca, in response to agonists, but unlike action potentials in excitable cells, these changes have not been attributed to a particular locus of action (Kruskal and Maxfield, 1987; Woods et al., 1986; Jacob et al., 1988; Berridge and Galione, 1988). Nevertheless, the serum dependency of mitotic transients may prove interesting, since the transient elevation of Ca, in Swiss 3T3 cells can be induced by growth factors (Pandiella et al., 1987) and since receptor-mediated channel activity is not completely understood (Rink, 1988). A model exists, however, that attempts to relate temporally transient Ca, elevations to long-term cellular responses (Alkon and Rasmussen, 1988).

Our results suggest that the mechanism that leads to sustained Ca, elevations, but not Ca, transients, is linked to the integrity of spindle microtubules. This spindle dependency has also been observed with other molecules that may be involved in mitotic regulation. Cyclin, a protein that can induce entry into meiosis and mitosis and whose level normally decreases abruptly at anaphase onset, can be sustained by nocodazole treatment (Murray and Kirschner, 1989). Similarly, the dephosphorylation of spindle phosphoproteins that normally occurs at anaphase onset can be delayed by nocodazole treatment (Vandre, manuscript in revision). Thus, Ca, elevation, cyclin destruction, and spindle protein dephosphorylation are not determined by a temporal program beginning in early mitosis, but await a signal from an intact spindle. It is not known whether Ca, controls or is controlled by cyclin degradation or spindle protein dephosphorylation, but this potential interaction could be mediated through several potential targets including spindle microtubules and kinetochores. Our results suggest that the pathways

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that govern mitosis are less likely to rely upon a simple Ca\textsubscript{i} transient than on a more complex interdependency between Ca\textsubscript{i} mobilization and other mitotic regulatory components.

We are very grateful to Rick Steinhardt who suggested the use of the Swiss 3T3 cell line and to Roger Tsien, Gerry Schatten and Gordy Hering for their helpful suggestions. Chloé Bulinski provided the antityrosinated tubulin antibody. Cody Martin, Leanne Olds, and Steve Limbach shared their expert graphic arts talents, and Donner Babcock provided the computer program for calculating free calcium levels from calcium-EGTA buffers. We are grateful to Tracor Northern for supplying the spectrofluorometric equipment used in this work.

This work was supported by postdoctoral grant PF-2989 from the American Cancer Society (to R. M. Tombes), by National Institutes of Health grant GM25062 (to G. G. Borisy), and by the Integrated Microscopy Resource for Biomedical Research which is funded by an NIH Biomedical Research Technology grant RR-570.

Received for publication 15 November 1988 and in revised form 7 April 1989.

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