Active Involvement of Ca\(^{2+}\) in Mitotic Progression of Swiss 3T3 Fibroblasts

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Abstract. Global Ca\(^{2+}\) transients have been observed to precede nuclear envelope breakdown and the onset of anaphase in Swiss 3T3 fibroblasts in 8% (vol/vol) FBS. The occurrence of these Ca\(^{2+}\) transients was dependent on intracellular stores. These Ca\(^{2+}\) transients could be (a) abolished by serum removal without halting mitosis, and (b) eliminated by increasing intracellular Ca\(^{2+}\) buffering capacity through loading the cells with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) buffer, via the tetra(acetoxyethyl) ester, without hindering the transition into anaphase. Microinjection of sufficient concentrations of BAPTA buffer could block nuclear envelope breakdown. Pulses of Ca\(^{2+}\) generated by flash photolysis of intracellularly trapped nitr-5, a "caged" Ca\(^{2+}\), could precipitate precocious nuclear envelope breakdown in prophase cells. In metaphase cells, photochemically generated Ca\(^{2+}\) pulses could cause changes in the appearance of the chromosomes, but the length of time required for cells to make the transition from metaphase to anaphase remained essentially unchanged regardless of whether a Ca\(^{2+}\) pulse was photoreleased during metaphase. The results from these photorelease experiments were not dependent on the presence of serum in the medium. Discharging intracellular Ca\(^{2+}\) stores with ionomycin in the presence of 1.8 mM extracellular Ca\(^{2+}\) doubled the time for cells to pass from late metaphase into anaphase, whereas severe Ca\(^{2+}\) deprivation by treatment with ionomycin in EGTA-containing medium halted mitosis. Our results collectively indicate that Ca\(^{2+}\) is actively involved in nuclear envelope breakdown, but Ca\(^{2+}\) signals are likely unnecessary for the metaphase–anaphase transition in Swiss 3T3 fibroblasts. Additional studies of intracellular Ca\(^{2+}\) concentrations in mitotic REF52 and PtK\(_1\) cells revealed that Ca\(^{2+}\) transients are not observed at all mitotic stages in all cells. The absence of observable global Ca\(^{2+}\) transients, where calcium buffers can block and pulses of Ca\(^{2+}\) can advance mitotic stages, may imply that the relevant Ca\(^{2+}\) movements are too local to be detected.

Three types of experimental findings have been invoked to support the notion that an elevation in intracellular Ca\(^{2+}\) concentration (possibly transient) may be the regulatory trigger for specific stages of mitosis: (a) Ca\(^{2+}\)-sequestering endomembrane organelles exist in close proximity to the mitotic apparatus; (b) disassembly of mitotic spindle microtubules is sensitive to Ca\(^{2+}\); and (c) anaphase could be hastened or delayed by microinjection of Ca\(^{2+}\)-EGTA buffers.

There have been numerous electron microscopic studies (see Hepler and Wolniak, 1984 for review) on a large variety of mitotic cells that revealed that there is an extensive, highly reticulated endomembrane system that intermeshes with the mitotic apparatus and which comes into intimate apposition with components of the mitotic apparatus. Serial reconstructions have shown that the mitotic membrane system is in fact continuous with the ER, which is outside the mitotic spindle. Because the ER is known to sequester Ca\(^{2+}\) (see Somlyo, 1984; Somlyo et al., 1985), extensions of the ER within the mitotic apparatus tantalized investigators into more direct measurements of Ca\(^{2+}\) sequestered within the mitotic endomembrane system. Wolniak et al., (1981) used chlorotetracycline to visualize high concentrations of Ca\(^{2+}\) sequestered in endomembrane organelles in mitotic cells of Haemanthus. The chlorotetracycline experiments revealed high levels of Ca\(^{2+}\) sequestered within the mitotic apparatus, indeed, very near the kinetochore microtubule bundles which connect the chromosomes to the poles of the spindle. Correspondingly, Keihart (1981) has demonstrated in echinoderm eggs that microtubules in the mitotic spindle display an in vivo sensitivity to Ca\(^{2+}\) levels in the physiological range and that an efficient Ca\(^{2+}\) sequestering system exists in the cytoplasm of mitotic cells.

Finally, direct manipulation of intracellular Ca\(^{2+}\) concentrations by microinjection of Ca\(^{2+}\)-EGTA buffers into mam-
malian cells (Izant, 1983) showed that artificially elevated cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) can speed up anaphase, while lowered [Ca\(^{2+}\)], can delay the meta-
phase-anaphase transition. We note however, that Keith (1987) reported that injection of Ca\(^{2+}\)-saturated calmodulin and CaCl\(_2\) in prometaphase PtK\(_2\) cells caused long and short de-
lays, respectively, to the onset of anaphase. These types of experimental findings suggested that Ca\(^{2+}\) may play a func-
tional role in mitotic progression and prompted the search for a “Ca\(^{2+}\) trigger” for the meta-
phase-to-anaphase transition.

The first positive results were observed in fertilized sea urchin eggs (Poenie et al., 1985) which were shown to be capa-
ble of producing Ca\(^{2+}\) transients correlated with various mito-
tic events, including nuclear envelope breakdown and the onset of anaphase. Subsequently, the same authors reported their observation of Ca\(^{2+}\) transients at the onset of anaphase in a mammalian cell line, PtK\(_1\) (Poenie et al., 1986). These observations generated much excitement amidst investiga-
tions into the role of Ca\(^{2+}\) signals in mitosis in cells of both plant and animal origin (Keith et al., 1985a,b; Ratan et al., 1987, 1988; Hepler and Callaham, 1987). Contemporaneous studies in the literature did not report close correlations between Ca\(^{2+}\) transients and mitotic events, however.

Two questions naturally arose after these reports of mito-
totic Ca\(^{2+}\) transients: (a) are the Ca\(^{2+}\) transients reproduc-
ible and universal? and (b) are the Ca\(^{2+}\) transients necessary for mitotic progression? Because mitosis is a fundamental cell biological process that appears to be highly conserved in all higher eukaryotes, one is justified in expecting that functional elements of the process would be observed with great regularity in mitotic cells from diverse sources. Fur-
thermore, if Ca\(^{2+}\) triggers were crucial to the progression of mitosis, then their abolition should severely reduce the prob-
ability of cells completing mitosis successfully.

In the present study we have made a comparison of the phenomenology of Ca\(^{2+}\) transients at the metaphase–ana-
phase transition in three different cell lines: Swiss 3T3, REF52, and PtK\(_1\). We have also conducted experiments to test the functional necessity of Ca\(^{2+}\) transients in the ability of Swiss 3T3 cells to enter prometaphase and to pass from met-
aphase into anaphase. With the aid of nitr-5, a chela-
table of Swiss 3T3 mouse fibroblasts. To monitor the rises in [Ca\(^{2+}\)]\(i\) resulting from UV flash photolysis of nitr-5, cells were also co-loaded with the AM ester of fluo-3, a fluorescent Ca\(^{2+}\) indicator that is excited at visible wavelengths. For experiments using nitr-5, loading of cells was always done in a dark room to guard against accidental photolysis of nitr-5.

The AM ester of nitr-5, a molecule that exhibits the same photochemistry as nitr-5 but lacking the Ca\(^{2+}\) binding site of the latter, was used to control for possible artifacts resulting from reactive photochemical intermediates or products generated by photolysis of nitr-5 intracellularly. Exhaustively UV-photolyzed nitr-5/AM was also used to control for the effects of UV expo-
sure.

In loading cells with nitr-5/AM, UV-photolyzed nitr-5/AM, nitr-9/AM, fluo-3/AM, or BAPTA/AM, the loading procedure was the same as that used for nitr-2/AM except that the loading medium contained 1-10 mM of the appropriate AM ester. The heavy metal chelator N,N,N',N'-tetakis(2-
pyridyldimethyl)ethylendiamine (TPEN), and the AM esters of fura-2, fluo-3, and BAPTA were purchased from Molecular Probes, Inc. (Eugene, OR). Nitr-5/AM was obtained from Calbiochem Behring Corp. (La Jolla, CA). Nitr-9 and the corresponding AM ester were synthesized by Dr. Stephen R. Adams in the Tsien laboratory (University of California, Berke-
ley, CA). All AM esters were dissolved in dry DMSO to make stock solu-
tions of 1-10 mM concentration. Caged-myo-inositol 1,4,5-trisphosphate [Ins\((4,5)P_3\)] was purchased from Calbiochem-Behring Corp.

Control experiments using the heavy metal chelator, TPEN, were con-
ducted using phophate Swiss 3T3 cells which were first incubated for 10-15 min in DME supplemented with 8% FBS and containing 30 mM TPEN. The cells were then examined and scored as they underwent NEB while still bathed in the TPEN-containing medium.

Fura-2 fluorescence imaging and photometric measurements were per-
fomed essentially as described previously (Poenie et al., 1986), except that an infrared interference filter (760 nm) has been placed before the condenser in the microscope illuminator, and a second camera (5000 Series; Cohn, Inc., San Diego, CA) has been added to permit continuous monitor-
ing of progress of the mitotic cells in transmission with infrared light without interfering with the excitation of the Ca\(^{2+}\) indicators in the UV (in the case of fura-2) or the visible range (in the case of fluo-3). Although some photorelease experiments were performed using procedures and equipment previously described (Kao et al., 1989), for most of the ex-
periments involving nitr-5 photolysis and fluo-3 monitoring of [Ca\(^{2+}\)], we used a Zeiss IM35 inverted epifluorescence microscope equipped with UV optics and a filter wheel fitted with two interference filters at 180° from each other. To photolyze nitr-5, a 340-390-nm filter (Omega Optical, Inc., Brattleboro, VT) was transiently positioned in the light path for 50-1,000 ms. The UV exposure time was controlled by an IBM AT microcomputer and a stepping motor connected to the filter wheel. The light source, used for both photolysis of nitr-5 and excitation of fluo-3, was an Osram 75-W xenon (XBO/2) lamp, driven by a stabilized power supply. Before and after photol-
ysis, a 490-nm filter (20 nm bandpass) was in the light path, so that fluo-3 measurements could be made. A neutral density (1.0) filter was attached to the 490-nm interference filter to attenuate the exciting light. Fluorescence emitted by fluo-3 was collected through a 530-nm interference filter (40 nm bandpass) mounted in a Leitz MFI microspectrophotometer and photon-counted by a Thorn EM1 cooled photon counter. Fluorescence intensity readings were acquired at a rate of 2 Hz. All data including the time and duration of UV photolyses were stored by the computer for delayed analysis.

From imaging experiments, we know that compartmentation of fluores-
cent Ca\(^{2+}\) indicators occurs in some perinuclear regions as well as diffusely in the cell periphery. Whereas fluorescence from the center of the

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**Materials and Methods**

Swiss 3T3 mouse fibroblasts were cultured in DME supplemented with 8% FBS. Rat embryo fibroblasts of the cell line REF52 (a gift of I. Fer-
amisco, Cancer Center, University of California, San Diego) were grown in DME supplemented with 10% vol/vol FBS. Mouse lymphoid cells of the PtK\(_1\) line were cultured in Ham's F-12 nutrient mixture supplemented with 10% vol/vol FBS. To prepare cultures for experimental observations, cells were trypsinized, seeded onto 25-mm-diam glass coverslips and cul-

1. Abbreviations used in this paper: AM, acetoxymethyl; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; Ins\((4,5)P_3\), myo-inositol 1,4,5-trisphosphate; NEB, nuclear envelope breakdown; TPEN, N,N,N',N'-tetrakis(2-pyridyldimethyl)ethylendiamine.
cell rapidly dissipates when a dye-loaded cell is permeabilized with low levels of digitonin, compartmentalized dye fluorescence dissipates only slowly, requiring as long as tens of minutes in some cases. In our photomultiplier and imaging measurements, we took fluorescence data from the center of each cell examined to avoid giving emphasis to data from regions of the cell where compartmentation of the fluorescent indicators was noticeable.

Results

Swiss 3T3 Fibroblasts Show Ca\(^{2+}\) Transients during Mitosis and Interphase

When mitotic Swiss 3T3 cells loaded with fura-2 were examined in 20 mM Hepes-buffered DME supplemented with 8% FBS, they showed Ca\(^{2+}\) transients not only at the onset of anaphase but at the time of NEB and around cytokinesis/division as well. A typical trace from a photomultiplier experiment is shown in Fig. 1a. In mitotic Swiss 3T3 cells, resting [Ca\(^{2+}\)], was typically 145 ± 42 nM (n = 24); [Ca\(^{2+}\)], at the peak of the transient associated with NEB was 630 ± 430 nm (n = 11); and [Ca\(^{2+}\)], at the peak of the transient associated with the metaphase–anaphase transition was 620 ± 300 nM (n = 13).

The Swiss 3T3 cells proved remarkably insensitive to UV radiation during mitosis. This property enabled us to routinely apply the fura-2 imaging technique to study mitosis in Swiss 3T3 cells. When imaging Swiss 3T3 cells, we observed that Ca\(^{2+}\) transients were not exhibited by mitotic cells exclusively. Rather, interphase cells also showed Ca\(^{2+}\) transients at apparently random times. A trace of [Ca\(^{2+}\)], vs. time for an interphase Swiss 3T3 cell in DME supplemented with 8% FBS is shown in Fig. 1b. The imaging experiments revealed that the transient rises in [Ca\(^{2+}\)], were not localized to a subcellular region, but rather [Ca\(^{2+}\)], became elevated over the entire cell; i.e., the Ca\(^{2+}\) transients were "global" or "pancellular."

While the Ca\(^{2+}\) transient activity in interphase Swiss 3T3 cells appeared to be random; in mitotic cells, however, Ca\(^{2+}\) transients preceded and were always tightly correlated with the major mitotic events of NEB and the metaphase–anaphase transition. Experiments wherein continuous window observation paralleled fura-2 monitoring of [Ca\(^{2+}\)], revealed that NEB could be confirmed within 39 ± 18 s (n = 7) of the peak of the preceding Ca\(^{2+}\) transient, while entry into anaphase could be visually established within 7.4 ± 30 s (n = 5) of the peak of the preceding Ca\(^{2+}\) transient. The time elapsed between the peak of the Ca\(^{2+}\) transient and NEB was somewhat longer than the corresponding time for the metaphase–anaphase transition because our morphological marker for NEB, the spill-out of chromosomes into the cytoplasm after NEB, required more time to ascertain visually than the marker for entry into anaphase, namely poleward movement of aligned chromosomes.

In mitotic Swiss 3T3 cells, Ca\(^{2+}\) transients (often more than one) were also always observed around the time of cytokinesis and cell division, and sometimes even in the daughter cells immediately after division (see Figs. 1a and 3, e and f). These transients occurred within a rather wide window of time and we were unable to correlate them with specific cellular events.

When bathed in Hepes-buffered DME (pH 7.4) containing 8% FBS and 3–4 mM EGTA, mitotic Swiss 3T3 cells continued to undergo NEB and the metaphase–anaphase transition, with the corresponding Ca\(^{2+}\) transients. Peak [Ca\(^{2+}\)], values in EGTA-containing medium were 525 ± 85 nM (n = 3) at NEB, and 1,300 ± 260 nM (n = 3) at entry into anaphase. These results suggest that the source of Ca\(^{2+}\) for generating the transients observed in these mitotic cells is in large part intracellular.

Effect of Increasing Intracellular Ca\(^{2+}\) Buffering on Mitotic Progression

BAPTA Injections Can Block the Onset of NEB. To test for the necessity of Ca\(^{2+}\) in NEB, cells in early to middle prophase were injected with solutions containing 40–54 mM BAPTA (tetrapotassium salt), 2.7–11 mM fura-2, enough CaCl\(_2\) to give 55–75% Ca\(^{2+}\) saturation of the chelators, 1 mM MgCl\(_2\), and buffered at pH 7.1 with 9 mM Hepes. The amount of BAPTA and fura-2 injected in each case was estimated from the fluorescence intensity of fura-2 using a procedure previously described (Steinhardt and Alderton, 1988). Excess Ca\(^{2+}\) buffer should dampen out rapid changes in [Ca\(^{2+}\)], but should not affect the basal [Ca\(^{2+}\)]. Indeed, in our experiments, baseline [Ca\(^{2+}\)], in cells injected with Ca\(^{2+}\) buffer was 138 ± 17 nM, a value not significantly different from the 126 ± 10 nM found in uninjected cells.

The results of BAPTA injection experiments are listed in Table 1. At low intracellular concentrations of injected Ca\(^{2+}\)
buffer (170 µM), NEB was uninhibited (occurring in 4.5 ± 1.8 min as compared with 6.4 ± 1.5 min in control cells). At elevated levels of intracellular chelator (420 µM), NEB occurred at 37 ± 8 min after injection, a delay of ~31 min relative to controls. At 870 µM intracellular Ca²⁺ chelator, NEB was not observed after even 49 ± 6 min. These results suggest that addition of exogenous Ca²⁺ buffer into prophase Swiss 3T3 cells can hinder NEB. Although NEB could be blocked by heavy metal buffering, chromosome condensation could not be, as prophase chromosomes continued to condense in the presence of high intracellular concentrations of Ca²⁺ chelator.

We note that while BAPTA was used as a Ca²⁺ chelator, it also shows high binding affinity for transition metal ions such as Fe²⁺ and Zn²⁺, which are important for biological function. It was thus important to ascertain that the NEB-blocking effect of BAPTA was due to Ca²⁺ buffering and was not the result of chelation of essential heavy metal ions. To this end we have used a cell-permeant nonionic chelator, TPEN (Anderegg and Wenk, 1967; Arslan et al., 1985), which has very high affinities for heavy metal ions (e.g., $K_d = 0.26$ fM for Zn²⁺), but low affinities for the divalent cations Mg²⁺ and Ca²⁺ ($K_d$'s of 20 mM and 40 µM, respectively). TPEN is therefore capable of sequestering heavy metal ions without disturbing the Ca²⁺ and Mg²⁺ concentrations in the cell, and is thus the ideal control for possible artifacts arising from heavy metal binding by BAPTA. We have studied prophase Swiss 3T3 cells in medium containing 30 µM TPEN, which has previously been shown to be effective in sequestering intracellular heavy metals (Arslan et al., 1985). We found however, that prophase cells underwent NEB unhindered in the presence of TPEN. The mean time to reach NEB from early/midprophase was 5.8 ± 1.9 min, as compared with 6.4 ± 1.5 min in the absence of the heavy metal chelator (Table I). The results of the control experiments using TPEN show that the delay or blockage of NEB by BAPTA in prophase Swiss 3T3 cells was due, not to the ability of BAPTA to bind heavy metals, but rather to its ability to buffer Ca²⁺.

**BAPTA Buffering Can Abolish Global Ca²⁺ Transients without Halting the Metaphase–Anaphase Transition in Swiss 3T3 Cells.** To examine more specifically the requirement for global Ca²⁺ transients at the onset of anaphase in Swiss 3T3, we attempted to inject high doses of BAPTA into metaphase cells. However, Swiss 3T3 cells in metaphase were generally very rounded and poorly adherent, a fact that made it quite difficult to microinject reliably. As an alternative, we conducted a series of experiments in which we loaded cells with the Ca²⁺ chelator BAPTA by incubating the cells with BAPTA/AM, the cell-permeant ester. As BAPTA/AM is steadily hydrolyzed to BAPTA in a cell, the cellular mechanisms for Ca²⁺ homeostasis should titrate the newly formed BAPTA with Ca²⁺ to maintain the resting [Ca²⁺], (Tsien et al., 1982). Because BAPTA has a $K_d$ for Ca²⁺ of ~100 nM, at the resting [Ca²⁺], found in Swiss 3T3 cells (100-200 nM), 50-60% of the intracellular BAPTA is Ca²⁺ bound, whereas 40-50% remains free to counteract any fast changes in [Ca²⁺]. That intracellular BAPTA introduced via the AM ester was effective in buffering [Ca²⁺], is demonstrated in Fig. 2, which displays a [Ca²⁺], vs. time trace for an interphase cell loaded with BAPTA and fura-2 by incubation with the respective AM esters. If one compares Fig. 2 with Fig. 1 b, one sees that the random Ca²⁺ transients normally observed in abundance in interphase cells have been completely damped out. Addition of 10 µM ionomycin however, was effective in slowly bringing [Ca²⁺], in the BAPTA-buffered cell to elevated levels (Fig. 2).

We have incubated Swiss 3T3 cells with 1-10 µM BAPTA/AM and 10 µM fura-2/AM and then imaged the cells in DME containing 8% serum. The buffering afforded by the intracellular BAPTA completely eliminated the Ca²⁺ transients normally observed in mitotic Swiss 3T3 cells in

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**Table I. Blocking NEB by Injection of BAPTA Buffer into Early/Midprophase* Swiss 3T3 Cells**

<table>
<thead>
<tr>
<th>No. of cells injected</th>
<th>Average intracellular chelator concentration µM</th>
<th>Time to NEB after injection min</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>170</td>
<td>4.5 ± 1.8</td>
</tr>
<tr>
<td>11</td>
<td>420</td>
<td>37 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>870</td>
<td>&gt;49 ± 6</td>
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| Time from early/mid-prophase to NEB in control cells (uninjected cells and cells injected with buffer containing no Ca²⁺ chelator) = 6.4 ± 1.5 min (n = 8). Time from early/mid-prophase to NEB in the presence of 30 µM TPEN = 5.8 ± 1.9 min (n = 23). Early/mid-prophase is defined to be that stage during mitosis when chromatin condensation has progressed sufficiently to permit observation of threadlike structures in the nucleus. Intracellular chelator concentration = [BAPTA] + [fura-2]. (Cells were injected with a buffer containing BAPTA as well as fura-2. See Materials and Methods section for details of buffer composition.)

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**Figure 2. BAPTA loaded into cells via the AM ester can eliminate Ca²⁺ transients. [Ca²⁺], vs. time profile for an interphase Swiss 3T3 cell that had been incubated for 60 min at room temperature with 10 µM each BAPTA/AM and fura-2/AM in Hepes-buffered DME supplemented with 8% FBS. The experiment was carried out in the presence of 8% FBS, in Hepes-buffered DME, at 35°C. The random interphase Ca²⁺ transients normally observed (see Fig. 1 b) have been eliminated by the buffering effect of BAPTA, although addition of 10 µM ionomycin was effective in raising [Ca²⁺].**

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**Table II. Elapsed Time between Late Metaphase and Start of Chromosome Movement in BAPTA-loaded and Unloaded Swiss 3T3 Cells**

<table>
<thead>
<tr>
<th>BAPTA-loaded cells (n = 14)</th>
<th>Control cells (n = 14)</th>
</tr>
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<tbody>
<tr>
<td>4.1 ± 3.2 min</td>
<td>3.7 ± 3.0 min</td>
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Late metaphase is defined to be that stage during mitosis when ≥90% of the chromosomes have become aligned at the equatorial plate.
the presence of 8% serum. However, the cells all progressed successfully from metaphase through the end of mitosis in the absence of global intracellular Ca\textsuperscript{2+} transients. It is apparent from the data shown in Table II that BAPTA loading at 1-10 \mu M in the medium, while abolishing global Ca\textsuperscript{2+} transients, had no significant effect on the progression of mitosis, as judged by the mean time of passage from metaphase to anaphase.\footnote{2}

2. It seems surprising at first that incubating cells with 1-10 \mu M BAPTA/AM could introduce sufficient BAPTA into the cells to block Ca\textsuperscript{2+} transients, whereas incubation with 10 \mu M fura-2/AM did not apparently lead to drastically increased intracellular Ca\textsuperscript{2+} buffering. The reason for this apparent disparity is to be found in the true aqueous solubilities of the two AM esters. The true aqueous concentration of BAPTA/AM, 10 \mu M fura-2/AM only represents the total amount of compound placed into the incubation vessel and in no way reflects the true solution concentration of fura-2/AM. (Most of the fura-2/AM in the incubation medium is probably in the form of colloidal precipitates or aggregates). We believe that the large difference in the solubilities of BAPTA/AM and fura-2/AM leads to very different availabilities of the two compounds to cells, and consequently, very different intracellular concentrations of the two chelators.

Figure 3. Ca\textsuperscript{2+} transient activity in interphase and mitotic Swiss 3T3 fibroblasts are serum dependent. [Ca\textsuperscript{2+}], vs. time traces for interphase cells bathed in Hepes-buffered DME supplemented with (a) 0% FBS, (b) 2% FBS, and (c) 8% FBS. Also shown are [Ca\textsuperscript{2+}], vs. time traces for mitotic cells in (d) 0% FBS, (e) 2% FBS, and (f) 8% FBS. The times at which mitotic events were observed are marked by dotted lines and annotations in d through f. All data were from fura-2 imaging experiments.

Figure 4. UV flash photolysis of intracellularly trapped nitro-5 rapidly elevated [Ca\textsuperscript{2+}], as monitored by fluo-3 indicator. (a) Fluor-3 fluorescence intensity vs. time trace for a middle metaphase Swiss 3T3 cell co-loaded with nitro-5 and fluo-3 by incubation at room temperature in Hepes-buffered DME supplemented with 4% FBS containing 1.0 \mu M nitro-5/AM for 40 min, after which 0.5 \mu M fluo-3/AM was added to the loading medium and the cells incubated for a further 20 min. The experiment was performed at 35°C in Hepes-buffered DME supplemented with 8% FBS. At the time indicated by the first arrowhead, a 300-ms flash of UV was used to photolyze the intracellular nitro-5, resulting in a sharp rise in [Ca\textsuperscript{2+}], which was reflected by an increase in the fluo-3 fluorescence intensity. The second arrowhead marks the time when changes in the appearance of the chromosomes were seen to have occurred. (b) Fluor-3 intensity vs. time trace for a metaphase cell incubated for 60 min at room temperature with 1 \mu M nitro-5/AM and 2 \mu M fluo-3/AM in Hepes-buffered DME supplemented with 8% FBS. The Ca\textsuperscript{2+}-dependent fluorescence intensity of intracellular fluo-3 first increased sharply in response to Ca\textsuperscript{2+} influx caused by digitonin permeabilization, and then dropped rapidly as soluble cytosolic contents escaped through the permeabilized plasma membrane. Flashing the permeabilized cell with UV for 1 s did not elicit any changes in the residual fluorescence.

The Occurrence of Ca\textsuperscript{2+} Transients in Mitotic and Interphase Swiss 3T3 Cells Is Contingent on the Presence of Serum in the Culture Medium

That moderate levels of BAPTA buffering could abolish global Ca\textsuperscript{2+} transients without halting mitosis suggested that perhaps global rises in [Ca\textsuperscript{2+}], may not be necessary in the progression of mitosis. We thus asked if conditions exist under which a cell could successfully pass through mitosis in the absence of endogenous [Ca\textsuperscript{2+}], rises. Indeed, we found that the Ca\textsuperscript{2+} transient activity in mitotic as well as interphase Swiss 3T3 cells is strongly dependent on the level of serum supplementation in the culture medium.

Interphase Ca\textsuperscript{2+} Transients in Swiss 3T3 Are Serum Dependent. The random Ca\textsuperscript{2+} transient activity in interphase cells is dependent on the serum concentration in the experimental medium, as illustrated in Fig. 3, a-c. With no serum in the medium, the interphase cells were silent (Fig. 3 a). At low levels of serum (e.g., 2% vol/vol), the interphase cells showed a moderate level of transient activity (Fig.
Figure 5. Time course of NEB in Swiss 3T3 prophase cells with and without photoreleased Ca\(^{2+}\) pulse. Cells were bathed in Hepes-buffered DME (pH 7.4) containing 8% FBS. Panels a–e show the course of NEB in a prophase Swiss 3T3 cell loaded with nitr-5 and flashed with UV; the time of the UV flash is taken to be \(t = 0\) s. (a) Mid-prophase cell 15 s before Ca\(^{2+}\) photorelease. The partially condensed chromosomes were confined within the nuclear envelope, which formed a smooth, well-defined boundary. (b) 15 s after photorelease of Ca\(^{2+}\), the smooth outline of the nuclear envelope has begun to disappear and the boundary around the chromosomes has become uneven. (c) 45 s after photorelease, NEB was clearly complete, with no remnant of the nuclear envelope visible under Nomarski optics. (d) 140 s after photorelease. After photoinduced NEB, the chromosomes did not immediately begin to organize on the spindle as after normal NEB (see h–i), but rather remained randomly distributed around the center of the cell. (e) At 220 s after photorelease, the chromosomes finally appeared to organize, as evidenced by clustering towards the center of the cell as normally observed shortly after NEB (see h and i). Arrows mark where the nuclear envelope is most readily discernible.

(f–i) show the course of NEB in a cell which was neither loaded with nitr-5 nor irradiated with UV light; time \(t = 0\) is defined to be the time of the first micrograph. (f) When observation began, the cell was in early prophase. The nuclear envelope was visible as a sharply defined oval boundary. (g) 180 s into the observation, NEB was under way, and most of the nuclear envelope was no longer discernible. (h) At 240 s, chromosomes began to cluster toward the center of the cell. (i) At 300 s, chromosomes have formed the roughly radial arrangement usually observed in prometaphase. Arrows mark where the nuclear envelope is most readily discernible. Bar, 10 \(\mu\)m.

3 b). The probability for the appearance of Ca\(^{2+}\) spikes increased significantly when the cells were bathed in DME containing 8% serum (Fig. 3 c).

Mitotic Ca\(^{2+}\) Transients in Swiss 3T3 Are Also Serum Dependent. Examination of mitotic cells under comparable conditions yielded parallel results (Fig. 3, d–f). In serum-free medium, a mitotic Swiss 3T3 cell could enter anaphase and subsequently complete division without any Ca\(^{2+}\) spikes (Fig. 3 d). With moderate increases in serum concentration (e.g., 2% vol/vol), the Ca\(^{2+}\) peaks loosely associated with
cytokinesis/division appeared (Fig. 3 e). Finally, with 8% serum supplementation, the metaphase-anaphase Ca$^{2+}$ spike could also be observed (Fig. 3 f). Similarly, the Ca$^{2+}$ transient associated with NEB could be observed in the presence of 8% FBS but not when serum was removed from the bathing medium (data not shown). The only difference between NEB and the metaphase–anaphase transition was that in the absence of serum, the [Ca$^{2+}$], baseline was somewhat noisier for NEB than in experiments where the metaphase–anaphase transition was monitored. Our failure to record Ca$^{2+}$ transients in mitotic cells bathed in serum-free medium was not the result of the fura-2 indicator having somehow been rendered unresponsive, as ionomycin treatment after an experiment always elicited a sharp rise in [Ca$^{2+}$], (not shown). Finally, we observed no significant difference in the timing of mitotic events between cells bathed in 8% FBS and cells bathed in serum-free medium.

The above experiments demonstrate that whereas Swiss 3T3 cells show global Ca$^{2+}$ transients correlated with NEB and entry into anaphase in the presence of 8% serum, at diminished levels of serum supplementation, a cell could show no Ca$^{2+}$ transients but nevertheless complete mitosis successfully. In a recent study of mitosis in Swiss 3T3 cells, Tombes and Borisy (1989) suggested that a gradual rise, or ramp, in [Ca$^{2+}$], might be important for progression of mitosis. In view of this proposal, it is worth noting that in our cells the resting [Ca$^{2+}$], in mitotic Swiss 3T3 cells appeared to increase with time. This Ca$^{2+}$ ramp was rather noticeable in the presence of serum (see Fig. 3, e–f) but essentially nonexistent in the absence of serum (Fig. 3 d). Because mitosis could readily proceed in the absence of serum, we infer that Ca$^{2+}$ ramps may in fact not be necessary for mitotic progression.

**The Effect of Artificially Generated Ca$^{2+}$ Transients on Mitotic Progression in Swiss 3T3 Cells**

Because global Ca$^{2+}$ transients in mitotic Swiss 3T3 cells are correlated with NEB and the transition into anaphase, and yet cells could also successfully complete mitosis in the

| Table III. Elapsed Time between Midprophase and NEB |
|---------------------------------|------------------|
| Cells loaded with nitr-5 and flashed with UV (n = 7) | Control cells* flashed with UV (n = 15) |
| 29 ± 14 s | 5.3 ± 2.3 min |
| 0.5 ± 0.2 min | |

* Controls included cells not loaded with nitr-5/AM and cells loaded with either nitr-9/AM or exhaustively photolyzed (and thus inactive) nitr-5/AM.
absence of these transients, it was natural to ask whether 
transient rises in Ca\(^{2+}\) were actively involved in the mitotic 
events. We thus performed experiments where we observed 
a mitotic cell's response to a Ca\(^{2+}\) pulse that was artificially 
generated using nitr-5. Nitr-5 is a photolabile Ca\(^{2+}\) chelator 
whose fluorescence quantum yield increased ~40-fold upon binding Ca\(^{2+}\). Fig. 4 a displays 
a plot of the fluo-3 fluorescence intensity from a mitotic 
Swiss 3T3 cell that had been coloaded with fluo-3/AM and 
nitr-5. Nitr-5 is a photolabile Ca\(^{2+}\) chelator 
that is excitable by visible light, fluo-3 (Minta et al., 
1989; Kao et al., 1989), whose fluorescence quantum yield 
is increased by UV flash photolysis of nitr-5 can very rapidly raise [Ca\(^{2+}\)]. Whereas 
one to several hundred nanomolar above resting level. 

Events that are excitable by visible light, fluo-3 (Minta et al., 
1989; Kao et al., 1989), whose fluorescence quantum yield 
is increased by UV flash photolysis of nitr-5 can very rapidly raise [Ca\(^{2+}\)]. Whereas 
the magnitude of the increase in [Ca\(^{2+}\)], was a function of 
the extent of loading as well as the duration of UV photoly-
sis, separate calibrations showed that in our experiments, af-
other flash photolysis of nitr-5, [Ca\(^{2+}\)] typically increased from 
one to several hundred nanomolar above resting level.

Fibroblasts loaded with fluo-3 via the AM ester have been 
shown to take up some of the dye into noncytosolic compart-
ments (Kao et al., 1989). To have complete confidence in 
results derived from experiments using fluo-3 and nitr-5, it 
was important to show that the Ca\(^{2+}\) photorelease we ob-
served in fact took place in the cytosol, rather than in subcel-
lular compartiments. Fig. 4 b shows the fluorescence inten-
sity vs time trace for a cell loaded with fluo-3/AM and 
nitr-5/AM. The trace clearly demonstrates that UV flash 
photolysis of nitr-5 can very rapidly raise [Ca\(^{2+}\)]. Whereas 
the magnitudes of the increase in [Ca\(^{2+}\)], was a function of 
the extent of loading as well as the duration of UV photoly-
sis, separate calibrations showed that in our experiments, af-
other flash photolysis of nitr-5, [Ca\(^{2+}\)] typically increased from 
one to several hundred nanomolar above resting level.

Note that although the disassembly of the nuclear envelope 
could be drastically accelerated by the pulse of photore-
leased Ca\(^{2+}\), another process, chromosome condensation, 
which normally occurs during prophase, did not appear to 
be affected in so dramatic or obvious a way. A prophase cell, 
in response to the photoreleased Ca\(^{2+}\) pulse will undergo 
NEB, but the partially condensed chromosomes, now in the 
cytosplasm, will continue to condense before congression at 
the metaphase plate.

**Photoreleased Ca\(^{2+}\) Induces Changes in the Appearance of Metaphase Chromosomes But Does Not Cause the Onset of Anaphase.** Our microscopic observations of mitotic 
Swiss 3T3 cells revealed that immediately after photore-
lease of Ca\(^{2+}\) during metaphase, subtle morphological 
changes in the aligned chromosomes took place. Under 
Nomarski optics, these changes may be variously characterized 
as a sudden manifestation of granular texture, or a criss-
cross pattern, or a more distinctly threadlike appearance in 
the chromosomes at the metaphase plate. We have examined 
the effect of photoreleased Ca\(^{2+}\) pulses on the timing and 
appearance of these morphological changes as well as the 
effect on the metaphase–anaphase transition time. Because 
the photochemically induced morphology changes in the metaphase 
chromosomes were rather subtle, it was important 
to ascertain that these changes were reproducible and 
not the result of biased expectations on the part of the 
experimenters. To address these concerns, we have performed 
double-blind experiments wherein metaphase Swiss 3T3 
cells were loaded with mixtures (prepared and number-
Controls included cells not loaded with nitr compound as well as cells loaded with nitr-9/AM or exhaustively prephotolyzed (and thus inactive) nitr-5/AM.

Cells loaded 12.0 ± 7.0 s
with nitr-5 0.20 ± 0.12 min
Control cells — —

For late metaphase cells: nitr-5/AM-loaded, n = 18; control, n = 28.

Control cells — 6.7 ± 3.0 min

Middle/metaphase is defined as that stage at which >175% of the chromosomes are aligned.

Late metaphase is defined to be the stage at which >90% of the chromosomes are aligned.

Table IV. Effect of Photoreleased Ca²⁺ on the Timing of the Metaphase–Anaphase Transition

<table>
<thead>
<tr>
<th></th>
<th>Middle/metaphase</th>
<th>Late metaphase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From flash</td>
<td>From appearance</td>
</tr>
<tr>
<td></td>
<td>To appearance</td>
<td>change to poleward</td>
</tr>
<tr>
<td>Cells loaded</td>
<td>change</td>
<td>movement</td>
</tr>
<tr>
<td>with nitr-5</td>
<td>12.0 ± 7.0 s</td>
<td>4.9 ± 2.0 min</td>
</tr>
<tr>
<td>Control cells</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* For middle/metaphase cells: nitr-5/AM-loaded, n = 24; control, n = 24.
For late metaphase cells: nitr-5/AM-loaded, n = 18; control, n = 28.

To appearance of metaphase cells being examined prevented the second experimenter from making morphological observations; these two experiments were thus judged inconclusive. The results of the double-blind as well as non–double-blind experiments were combined and are presented in Table IV and further described below. In 24 middle and mid-late metaphase cells loaded with nitr-5/AM and bathed in Hepes-buffered DME containing 8% FBS, changes in chromosome appearance occurred within 12 ± 70 s after the UV flash. In contrast, in 24 control middle and mid-late metaphase cells that were not loaded with nitr compound, or were loaded with either nitr-9/AM or exhaustively UV-irradiated (inactive) nitr-5/AM, no corresponding changes in chromosome appearance were observed, but anaphase started 6.7 ± 3.0 min after the flash. The situation was similar for cells in late metaphase. In 18 late metaphase cells loaded with nitr-5/AM, changes in chromosome appearance occurred 10.8 ± 5.1 s after the UV flash, while in 26 control cells, no corresponding changes in chromosome appearance were observed, but the cells entered anaphase 3.9 ± 2.0 min after the UV flash. Thus a sharp rise in [Ca²⁺], during metaphase can cause changes in the appearance of chromosomes. This result is not dependent on the presence of FBS in the bathing medium. Essentially identical results were obtained when the experiments were repeated with metaphase cells loaded with nitr-5 but in the absence of FBS: changes in chromosome appearance occurred within 7 ± 2 s (n = 7) after the UV flash.

A significant feature of the timing of the metaphase–anaphase transition is common to the group loaded with nitr-5 and the control group. In the nitr-5/AM-loaded middle and mid-late metaphase cells, although changes in chromosome appearance occurred almost immediately (0.20 ± 0.12 min, Table IV) after the photoinduced Ca²⁺ jumps, poleward migration of the chromatids did not start until 5.1 ± 2.1 min after photorelease of Ca²⁺. In the control cells, where the UV flash produced no Ca²⁺ jump and no change in the appearance of the chromosomes, poleward movement of the separated chromatids took place in 6.7 ± 3.0 min. Exactly analogous results were obtained for late metaphase cells (Table IV). These results show that while artificially induced Ca²⁺ transients could elicit changes in chromosome appearance, they did not hasten the transition from metaphase to anaphase, since the total time it took for a cell to progress from metaphase to the start of poleward chromosome movement is conserved (e.g., 3.1 ± 2.0 min in the nitr-5/AM-loaded middle and late metaphase cells, and 3.9 ± 1.8 min in the controls; see Table IV).

The Effect of Ca²⁺ Deprivation and of Discharging Intracellular Ca²⁺ Stores on Mitosis in Swiss 3T3 Cells

Ca²⁺ Deprivation by Treatment with Ionomycin in EGTA-containing Medium Halts Mitosis in Swiss 3T3 Cells. We have performed experiments wherein mitotic Swiss 3T3 cells were placed in DME containing sufficient EGTA to keep the extracellular [Ca²⁺] at <60 nM. Under these conditions, cells could be observed entering and completing mitosis for >1 h after being transferred into EGTA medium (data not shown). In other experiments, the intracellular Ca²⁺ stores of cells bathed in EGTA-containing medium were then discharged by treatment with one or two 0.5–10 μM doses of the Ca²⁺ ionophore ionomycin. Ionomycin was very effective at depleting the cells of internal Ca²⁺ stores, as shown in Fig. 6 a, where a metaphase cell bathed in EGTA-containing DME was first treated with 0.5 μM ionomycin, which caused a rise in [Ca²⁺], due to discharge of intracellular stores, before all the Ca²⁺ was bled into the bathing medium through the facilitation by ionomycin. A second treatment with 0.5 μM ionomycin at 7 min after the first dose produced no further change in [Ca²⁺], suggesting that intracellular Ca²⁺ stores have been depleted.

The Ca²⁺ deprivation studies were performed both in the presence and absence of 8% dialyzed fetal bovine serum, though the results obtained under these two conditions were

Kao et al. Ca²⁺ in Mitosis of Swiss 3T3 Fibroblasts

191
Figure 6. Ionomycin is effective in dissipating intracellular Ca\(^{2+}\) stores. (a) A metaphase Swiss 3T3 cell loaded with fura-2 was placed into DME containing 4 mM EGTA and treated with 0.5 \(\mu\)M ionomycin two times, at \(~7\) min apart. (b) A metaphase Swiss 3T3 cell loaded with fura-2 and bathed in regular DME was given two treatments with 2.5 \(\mu\)M ionomycin at \(~7\) min apart. In both cases, the first ionomycin treatment discharged intracellular stores, as evidenced by the sharp but transient rises in [Ca\(^{2+}\)]. The stores apparently remained discharged in the continued presence of ionomycin, as a second treatment with ionomycin did not produce corresponding sharp rises in [Ca\(^{2+}\)]. (c) A Swiss 3T3 cell injected at room temperature in Hepes-buffered DME with a solution containing 2.55 \(\mu\)M caged Ins(1,4,5)P\(_3\) and 10 mM fluo-3, pentapotassium salt. The cell was allowed to recover from injection for \(~25\) min before the experiment was begun at 33°C. A 0.5-s UV flash photoreleased sufficient Ins(1,4,5)P\(_3\) to trigger release of Ca\(^{2+}\) from intracellular stores, resulting in a sharp but transient increase in the fluo-3 fluorescence. Application of 0.5 \(\mu\)M ionomycin produced a large, more protracted rise in [Ca\(^{2+}\)], similar to that seen in b. (d) Fluorescence intensity vs. time profile for a cell prepared in precisely the same way as described for c. During the experiment, the cell was first treated with 0.5 \(\mu\)M ionomycin, which produced a large rise in [Ca\(^{2+}\)]. When [Ca\(^{2+}\)], had recovered to baseline levels, the cell was pulsed for 0.5 s with UV to release Ins(1,4,5)P\(_3\), which did not lead to a rise in [Ca\(^{2+}\)], of the ionomycin-treated cell, consistent with internal Ca\(^{2+}\) stores having been dissipated by ionomycin. A second application of ionomycin again produced no sharp rise in [Ca\(^{2+}\)], but rather a gradual approach to a slightly higher [Ca\(^{2+}\)], as was seen in b.

Table V. Late Metaphase to early Anaphase Transit Times in Swiss 3T3 Cells Treated with Ionomycin

<table>
<thead>
<tr>
<th>Ionomycin/ EGTA(^*)</th>
<th>Ionomycin/ Ca(^{2+})</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells undergone metaphase–anaphase transition</td>
<td>1/17</td>
<td>13/13</td>
</tr>
<tr>
<td>Time between metaphase and start of poleward movement (min)</td>
<td>13</td>
<td>7.8 ± 3.8</td>
</tr>
</tbody>
</table>

\(^*\) 10 cells in DME/0% FBS/4mM EGTA treated with 0.5 \(\mu\)M ionomycin, and 7 cells in DME/8% FBS/5mM EGTA treated with 10 \(\mu\)M ionomycin.

\(^\dagger\) Cells in DME/0% FBS treated with 2.5 \(\mu\)M ionomycin.

Discharging Intracellular Stores by Ionomycin Treatment in the Presence of 1.8 mM Extracellular Ca\(^{2+}\) Slows Mitosis in Swiss 3T3 Cells. Fig. 6 b shows the [Ca\(^{2+}\)] vs. time profile for a metaphase Swiss 3T3 cell bathed in unsupplemented DME in which the Ca\(^{2+}\) concentration was the normal 1.8 mM, and subjected to two treatments with 2.5 \(\mu\)M ionomycin. The first dose of ionomycin produced a large rise in [Ca\(^{2+}\)], which was the combined result of both discharging internal Ca\(^{2+}\) stores as well as ionomycin-facilitated Ca\(^{2+}\) entry from the Ca\(^{2+}\)-rich external medium. The cell subsequently restored [Ca\(^{2+}\)], to a plateau value some-

essentially identical (results summarized in Table V). The majority (97\%, 16 out of 17 cells) of mitotic Swiss 3T3 cells whose intracellular Ca\(^{2+}\) stores have been discharged by ionomycin treatment in the presence of external EGTA were halted at various stages in mitosis (no apparent morphological changes after at least 30 min). A single cell (6\%, 1 out of 17 cells) did enter anaphase and proceed through cytokinesis, although the progress of this cell was extremely slow when compared with untreated cells in normal medium (13 min between late metaphase and the start of poleward chromosome movement, as compared with 3.7 ± 3.0 min in untreated controls). This gross slowing or halting of mitosis was due to Ca\(^{2+}\) deprivation and not due to ionomycin toxicity, since ionomycin treatment of mitotic Swiss 3T3 cells in normal DME medium (Ca\(^{2+}\) concentration = 1.8 mM) only roughly doubled the transition time without stopping any of the cells from completing mitosis (see next section). These Ca\(^{2+}\) deprivation experiments suggest that mitotic Swiss 3T3 fibroblasts also have a threshold requirement for Ca\(^{2+}\), below which mitosis cannot progress.
what higher than the preionomycin resting level. A second 2.5 µM dose of ionomycin given 7 min after the first produced no corresponding sharp rise in [Ca\(^{2+}\)], suggestive of the discharged state of the internal Ca\(^{2+}\) stores. There was, however, a slow approach to a new, somewhat higher [Ca\(^{2+}\)], as the newly introduced ionomycin contributed additively to Ca\(^{2+}\) entry from the external medium.

It is important to verify that ionomycin treatment can in fact dissipate intracellular Ca\(^{2+}\) stores even when the cell is bathed in normal DME, which contains 1.8 mM Ca\(^{2+}\). We first showed that Ins(1,4,5)P\(_3\), when photoreleased in the cytoplasm, could, as expected, trigger release of Ca\(^{2+}\) from intracellular stores into the cytosol. Fig. 6 c shows the fluorescence intensity vs. time trace for a cell that had been pre-injected with a buffer containing fluo-3 and caged Ins(1,4,5)P\(_3\). A UV flash of 0.5 s duration liberated biologically active Ins(1,4,5)P\(_3\), which in turn triggered Ca\(^{2+}\) release from intracellular stores into the cytosol, as reflected by the sharp transient increase in fluo-3 fluorescence. Subsequent application of 0.5 µM ionomycin caused a large, transient elevation in [Ca\(^{2+}\)], similar to that shown in Fig. 6 b. Fig. 6 d also shows a fluorescence intensity vs. time profile for a cell pre-injected with fluo-3 and caged Ins(1,4,5)P\(_3\). In contrast to Fig. 6 c, however, the cell was treated with 0.5 µM ionomycin before UV flash photolysis. It can be seen from Fig. 6 d that photorelease of Ins(1,4,5)P\(_3\) in the ionomycin-treated cell elicited no increase in [Ca\(^{2+}\)], at all, a fact consistent with the Ca\(^{2+}\) content of the internal stores being greatly diminished. A second application of 0.5 µM ionomycin, just as in Fig. 6 b, caused no sharp rise in [Ca\(^{2+}\)], but rather only a slow approach to a slightly higher [Ca\(^{2+}\)] level.

The experiments illustrated in Fig. 6, b–d together demonstrate that ionomycin was effective in dissipating intracellular Ca\(^{2+}\) stores in the presence of 1.8 mM extracellular Ca\(^{2+}\). Under these conditions, we observed that metaphase Swiss 3T3 cells took about twice as long as untreated controls to enter anaphase (7.8 ± 3.8 min in ionomycin-discharged cells, as compared with 3.7 ± 3.0 min in controls; Table V).

This set of experiments suggests that the degree of depletion of the intracellular Ca\(^{2+}\) stores may have a direct effect on the mitotic apparatus, at least at the metaphase–anaphase transition in Swiss 3T3 cells.

**Comparison of REF52 and PtK\(_1\), with Swiss 3T3 Shows That Global Ca\(^{2+}\) Transients at All Mitotic Stages Are Not a Universal Phenomenon**

In extending the investigations of [Ca\(^{2+}\)], transients associated with the metaphase–anaphase transition, we have studied two additional cell lines, REF52 and PtK\(_1\). Whereas PtK\(_1\) cells are often favored for microscopic observations owing to their having only a few large, well-defined chromosomes, their use in fura-2 imaging studies has been difficult for two reasons. Firstly, PtK\(_1\) cells are difficult to load with fura-2/AM. Secondly, mitotic PtK\(_1\) cells are highly sensitive to UV damage; the amount of UV light required for acceptable signal-to-noise in imaging measurements often resulted in nondisjunction, anaphase chromosome “bridges,” or in mitosis being arrested altogether. To ensure minimal damage from the UV light used to excite the intracellularly trapped fura-2, we largelyeschewed the imaging technique and made photometric measurements using a photomultiplier, whose sensitivity allowed us to reduce markedly the amount of UV light delivered to the cells during an experiment. The reduced UV exposure permitted most of the cells observed to complete mitosis successfully without obvious morphological irregularities. Under these improved experimental conditions we ascertained that only ~14% (3 out of 21) of the PtK\(_1\) cells and ~37% (6 out of 16) of the REF52 cells that successfully completed mitosis showed a transient rise in [Ca\(^{2+}\)], at the onset of anaphase (Table VI). In those cells where a [Ca\(^{2+}\)] spike was recorded, the transient had a shape and duration very similar to that described previously for mitotic PtK\(_1\) cells (Poenie et al., 1986). The percentage we have found of metaphase PtK\(_1\) cells displaying Ca\(^{2+}\) transients at the onset of anaphase is lower than that previously reported for the same cell line (Poenie et al., 1986). In this study, we were able to monitor simultaneously [Ca\(^{2+}\)]; as well as morphology changes in a mitotic cell, whereas this was not possible in the earlier study. We suspect that the inability to correlate [Ca\(^{2+}\)] continuously with morphology might have somewhat biased the statistics reported in the earlier work. Imperfect correlation of Ca\(^{2+}\) transients and initiation of anaphase has also been reported for the PtK\(_1\) cell line (Ratan et al., 1988). We have relatively little data concerning NEB in these two cell types. However, in all the prophase cells we have examined (3 PtK\(_1\) and 3 REF52 cells), we have recorded Ca\(^{2+}\) transients immediately preceding NEB.

We have considered two possible experimental reasons for our inability in the majority of PtK\(_1\), and REF52 cells to observe a Ca\(^{2+}\) transient correlated with the metaphase–anaphase transition. The first possibility is that in the great majority of cells examined, the Ca\(^{2+}\) spikes were of such short duration as to escape detection given the time resolution of our photomultiplier measurements. We thus reduced our data acquisition time from the normal 1 s per point to 100–200 ms per point, which is our instrumental limit. Even at these much faster data acquisition rates, we failed to observe a Ca\(^{2+}\) transient in those cells undergoing the metaphase–anaphase transition. The second possible explanation is that the [Ca\(^{2+}\)], rise accompanying the onset of anaphase was restricted to such a small, localized volume of the mitotic cell that a photomultiplier measurement, which effectively reads the whole-cell average [Ca\(^{2+}\)], would not have recorded a significant increase above the apparent intracellular resting Ca\(^{2+}\) level. We investigated this second possibility with fura-2 fluorescence imaging techniques. Within the spatial and temporal resolution of our imaging measurements (~0.1 µm\(^2\)/pixel and ~3–5 s/image, respectively), we were again unable to observe any Ca\(^{2+}\) transients, localized or global, in the majority of mitotic REF52 and PtK\(_1\) cells. The observed statistical norm in REF52 as well as PtK\(_1\) is for a mitotic cell to pass from metaphase to anaphase immediately.
phase generally without an attendant global transient rise in [Ca$^{2+}$]. The totality of our results in REF52 and PtK$_1$ shows that global Ca$^{2+}$ transients at some stages of mitosis are not a universal phenomenon.

**Discussion**

We have established in this work the following experimental results regarding the role of Ca$^{2+}$ in mitotic progression:

(a) Global (pan cellular) Ca$^{2+}$ transients could be observed reproducibly in mitotic as well as interphase Swiss 3T3 fibroblasts in the presence of 8% FBS. But whereas the occurrence of interphase Ca$^{2+}$ transients was apparently random and unpredictable, transients in mitotic cells preceded and were tightly correlated with NEB and the transition from metaphase to anaphase; i.e., these mitotic events appeared to be entrained to the endogenous Ca$^{2+}$ pulses. We have also determined that the source of Ca$^{2+}$ for generating the Ca$^{2+}$ transients in mitotic cells is, in large part, intracellular.

(b) Global Ca$^{2+}$ transients in interphase and mitotic cells could be abolished by serum removal, though mitosis could still proceed in the absence of serum-dependent Ca$^{2+}$ transients. The Ca$^{2+}$ transients could also be quelled by loading cells with BAPTA via the AM ester. Mitotic Swiss 3T3 cells loaded with BAPTA/AM could still undergo the metaphase–anaphase transition.

(c) Increased levels of intracellular Ca$^{2+}$ buffering achieved through microinjection of BAPTA buffer could delay or block NEB in prophase Swiss 3T3 cells.

(d) Artificially generated global Ca$^{2+}$ pulses could precipitate precocious NEB in prophase Swiss 3T3 cells, after which the partially condensed prophase chromosomes continue to condense before aligning at the metaphase plate. In metaphase cells however, artificial Ca$^{2+}$ pulses caused changes in the appearance of metaphase chromosomes but not significantly alter the time it took cells to make the transition from metaphase to anaphase. These results were obtained regardless of the presence of FBS in the bathing medium. The total time elapsed from middle/late metaphase to the start of poleward movement was conserved, irrespective of whether an intracellular Ca$^{2+}$ pulse was generated photochemically.

(e) Severe Ca$^{2+}$ deprivation by treatment with ionomycin in EGTA-containing medium could halt mitosis, whereas discharging the intracellular Ca$^{2+}$ stores with ionomycin in normal medium (1.8 mM extracellular Ca$^{2+}$) only lengthened the time for the metaphase–anaphase transition.

(f) Observations of [Ca$^{2+}$], in two additional cell lines, REF52 and PtK$_1$, revealed that observable global Ca$^{2+}$ transients could be recorded only in a relatively small percentage of mitotic cells at the onset of anaphase, although they were always associated with NEB in the few cases studied.

That global Ca$^{2+}$ transients, when they occur at all, are tightly correlated with mitotic events does not necessarily imply a causal relationship between the two. Experiments wherein we actively manipulate [Ca$^{2+}$] in mitotic cells are better suited for assessing the role of Ca$^{2+}$ in mitotic progression. That artificial Ca$^{2+}$ transients delivered photochemically could cause NEB in prophase Swiss 3T3 cells suggests that Ca$^{2+}$ can be actively involved in the NEB process. This view is strengthened by the observation that BAPTA buffering could block NEB in Swiss 3T3 cells. Our findings in Swiss 3T3 fibroblasts, a mammalian system, are in agreement with the report of Steinhardt and Alderton (1988), who showed that injection of the Ca$^{2+}$ chelators EGTA and BAPTA into sea urchin embryos delayed NEB, whereas injection of Ca$^{2+}$ buffers with high free Ca$^{2+}$ concentrations induced precocious NEB. Similarly, Whitaker and colleagues (Twigg et al., 1988) found that injection of InsP$_3$ in early sea urchin embryos. The same workers also found that the InsP$_3$ effect could be blocked by the intracellular presence of EGTA, suggesting that the principal effect of InsP$_3$ was to generate a rise in [Ca$^{2+}$], which promoted NEB. The results from our BAPTA blocking and Ca$^{2+}$ photorelease experiments show that NEB in mammalian cells is likewise a Ca$^{2+}$-sensitive process. Recent studies have revealed that microinjection of antibodies against Ca/CaM type II kinase, or of inhibitory regulatory peptides of the same kinase, into embryos of the sea urchin _L. pictus_ can block NEB (Baitinger, C., J. Alderton, M. Poenie, H. Shulman, and R. Steinhardt, manuscript in preparation). Ca/CaM kinase II might therefore be the Ca$^{2+}$-dependent control element which regulates NEB and entry into prometaphase.

The results of experiments wherein Ca$^{2+}$ was photoreleased in metaphase cells are interesting for two reasons. First and most significantly, in contrast to what we found at NEB, photochemically generated Ca$^{2+}$ pulses did not cause precocious entry into anaphase. In fact, the time it took for metaphase cells to enter anaphase was conserved, i.e., the time required for the metaphase–anaphase transition remained essentially unchanged regardless of whether a Ca$^{2+}$ pulse was artificially generated during metaphase. Secondly, we showed, through the double-blind experiments with nitr-5, nitr-9, and inactivated nitr-5, that changes in the appearance of metaphase chromosomes could be reliably elicited by photochemically generated Ca$^{2+}$ pulses. Unfortunately, whereas these changes were reproducible, we have no knowledge of the molecular processes underlying these changes. Thus, in the absence of further cytological or ultrastructural information, our observations of Ca$^{2+}$-dependent changes in the appearance of metaphase chromosomes must remain as a curiosity at the phenomenological level.

We now summarize our findings on the metaphase–anaphase transition. That BAPTA buffering could eliminate Ca$^{2+}$ transients without hindering the metaphase–anaphase transition and that artificial Ca$^{2+}$ pulses did not significantly alter the time required for cells to pass from metaphase into anaphase argue for lack of an active role for Ca$^{2+}$ signals in the metaphase–anaphase transition.

There is in the literature some evidence that anaphase may be dependent on a Ca/CaM dependent kinase associated with the mitotic apparatus (work on mitotic apparatus isolated from fertilized eggs of _L. pictus_; Dinsmore and Sloboda, 1988). The kinase phosphorylated an endogenous spindle-associated 62-kD protein. Phosphorylation of the 62-kD protein facilitated depolymerization of the kinetochore microtubules in vitro and monitoring of the 62-kD proteins in vivo showed that the level of phosphorylation was cyclical in time and reached its maximum at the metaphase–anaphase transition (Dinsmore and Sloboda, 1988). The exact function and location of the 62-kD protein are still unknown at present. In view of these published results, it is possible that
even though the metaphase–anaphase transition does not appear to require a Ca$^{2+}$ signal, processes subsequent to the transition may require the participation of Ca$^{2+}$.

Having established that Ca$^{2+}$ can promote progression of NEB in Swiss 3T3 cells, we need to consider whether the role of Ca$^{2+}$ is essential or auxiliary. An auxiliary role would have Ca$^{2+}$ augment other cellular signals, which in the absence of the Ca$^{2+}$ signal could still effect mitotic progression (provided threshold Ca$^{2+}$ requirements for overall well-being of the cell are met). Thus if Ca$^{2+}$ had an auxiliary function in a particular mitotic event, elevation of [Ca$^{2+}$], may promote the event but absence of the Ca$^{2+}$ signal should not block the event. By this criterion, Ca$^{2+}$ has an essential rather than auxiliary role in NEB, because increases in intracellular Ca$^{2+}$ buffering capacity by injection of BAPTA could block NEB. The case of the metaphase–anaphase transition is rather different. That entry into anaphase could be blocked by ionomycin treatment in EGTA medium may merely reflect the cell’s requirement for a certain threshold [Ca$^{2+}$], to maintain proper overall cellular function. In fact, our results tend to establish the lack of a need for Ca$^{2+}$ signals at the metaphase–anaphase transition. Therefore, the possibility remains that Ca$^{2+}$ signals are not essential for entry into anaphase. If Ca$^{2+}$ signals were not essential in a particular mitotic event, as might be the case in entry into anaphase, then it would not be surprising that conditions could be found (e.g., 0% FBS, or BAPTA buffering) under which the event could occur in the absence of observable Ca$^{2+}$ signals. In NEB, however, where we have surmised the necessity of Ca$^{2+}$ signals, the fact that the process could take place in the absence of observable Ca$^{2+}$ signals (in 0% FBS) is more puzzling, and may have mechanistic implications which will be discussed below.

Since pancellular Ca$^{2+}$ transients can either be suppressed by BAPTA buffering without halting the metaphase–anaphase transition or abolished by serum removal without blocking mitosis in Swiss 3T3 cells, these global rises in [Ca$^{2+}$], appear not to be essential. The fact that global rises in [Ca$^{2+}$], are not necessary for mitotic progression and the observation that some mitotic processes (e.g., NEB) require Ca$^{2+}$ signals together lead one to suggest that the cell must have a mechanism whereby cytosolic Ca$^{2+}$ concentration can be raised locally, near the Ca$^{2+}$-sensitive mitotic machinery, without causing a global rise in [Ca$^{2+}$], at the same time. In such a scheme, endomembrane-enclosed Ca$^{2+}$ stores must release Ca$^{2+}$ site specifically to parts of the mitotic apparatus that are Ca$^{2+}$-sensitive. These localized releases would be expected to create small subcellular regions of elevated [Ca$^{2+}$], but as the released Ca$^{2+}$ is diffusionally diluted into the bulk cytoplasm, the global [Ca$^{2+}$] would remain essentially unchanged (for indirect evidence of similar localized phenomena at the plasma membrane, see Marty and Neher, 1985; Foskett et al., 1988). Our observation that NEB could be blocked by high intracellular concentrations of BAPTA is consistent with this proposal: The presence of an exogenous buffer is not expected to change the steady-state resting [Ca$^{2+}$], it is, however, expected to abolish or reduce Ca$^{2+}$ concentration gradients across different regions of the cytoplasm. This type of hypothesis has been advanced in studies of Fucus egg development (Speksnijder et al., 1989). If localized rises in [Ca$^{2+}$] were required, extra buffering due to BAPTA would reduce the competence of the Ca$^{2+}$-sensitive machinery, thus leading to blockage or delay of the relevant mitotic events, which was what we indeed observed for NEB. Furthermore, since Swiss 3T3 cells bathed in EGTA medium (but not treated with ionomycin) could successfully complete mitosis, one may deduce that the immediate source of the Ca$^{2+}$ required for mitosis resides in the intracellular Ca$^{2+}$ stores. It is thus reasonable to expect that if the internal stores were discharged, mitosis would be impeded. Indeed, when intracellular Ca$^{2+}$ stores were dissipated by treatment with ionomycin in normal DME, the transition time from metaphase to anaphase more than doubled (Table V). These results, which are in accord with the reports of mitotic HeLa cells treated with the Ca$^{2+}$ ionophore A23187 (Ziegler et al., 1985), lend support to the view that intracellular stores deliver Ca$^{2+}$ locally to the Ca$^{2+}$-sensitive components of the mitotic apparatus. We wish to point out however, that while we have demonstrated that ionomycin, a Ca$^{2+}$ ionophore, could dissipate intracellular Ca$^{2+}$ stores, prolonged treatment with Ca$^{2+}$ ionophores could have pleiotropic effects. Experimental results derived from prolonged treatments with ionophores should thus be interpreted with caution.

Finally, our studies of the rat embryo fibroblast line, REF52, and the marsupial epithelial line, PtK1, showed that global Ca$^{2+}$ transients were not always observed during all mitotic stages in these cells. Our findings on PtK1 cells are not inconsistent with results on PtK1 cells reported by Ratan et al. (1988), who found Ca$^{2+}$ transients in a much larger percentage of the mitotic PtK1 cells examined. These authors found only poor temporal correlation between the observed Ca$^{2+}$ transients and the start of anaphase and rejected the hypothesis of Ca$^{2+}$ transients as triggers for anaphase onset, a conclusion in accord with our understanding of the metaphase–anaphase transition in Swiss 3T3 cells. Our own finding, as well as the findings of others of non-universality of global Ca$^{2+}$ transients during mitosis, lead us to propose that localized delivery of Ca$^{2+}$ from internal stores might be the means for mobilizing Ca$^{2+}$ during mitosis.

In conclusion, although Ca$^{2+}$ transients are not always observed at all mitotic stages in all cells, our results in Swiss 3T3 fibroblasts provide strong evidence for the active involvement of Ca$^{2+}$ at nuclear envelope breakdown. At the metaphase–anaphase transition, however, Ca$^{2+}$ signals appear not to be necessary.

We are grateful to Dr. Stephen Adams for synthesizing nitr-9 and its AM ester, and we wish to thank Dr. Zac Cande for helpful discussions.

This work was supported by National Institutes of Health grants GM-39374, GM-31004, and CA-09641.

Received for publication 11 August 1989 and in revised form 20 March 1990.

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