### Title

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# 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'*-tetra-acetic acid (BAPTA) buffer, via the tetra(acetoxymethyl) 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 chlortetracycline to visualize high concentrations of $Ca_{2+}$ sequestered in endomembrane organelles in mitotic cells of *Haemanthus*. The chlortetracycline 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-

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malian cells (Izant, 1983) showed that artificially elevated cytoplasmic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) can speed up anaphase, while lowered $[Ca^{2+}]_i$ can delay the metaphase–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 delays, respectively, to the onset of anaphase. These types of experimental findings suggested that $Ca^{2+}$ may play a functional role in mitotic progression and prompted the search for a "$Ca^{2+}$ trigger" for the metaphase-to-anaphase transition.

The first positive results were observed in fertilized sea urchin eggs (Poenie et al., 1985) which were shown to be capable of producing $Ca^{2+}$ transients correlated with various mitotic 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 investigations into the role of $Ca^{2+}$ signals in mitosis in cells of both plant and animal origin (Keith et al., 1985*a*,*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 mitotic $Ca^{2+}$ transients: (*a*) are the $Ca^{2+}$ transients reproducible 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. Furthermore, if $Ca^{2+}$ triggers were crucial to the progression of mitosis, then their abolition should severely reduce the probability of cells completing mitosis successfully.

In the present study we have made a comparison of the phenomenology of $Ca^{2+}$ transients at the metaphase–anaphase 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 metaphase into anaphase. With the aid of nitr-5, a chelator that releases $Ca^{2+}$ upon UV illumination (Adams et al., 1988), we were able to generate intracellular $Ca^{2+}$ pulses in mitotic cells by flash photolysis. The effects of the photoinduced $Ca^{2+}$ pulses on nuclear envelope breakdown (NEB),[1] entry into anaphase, and the timing of the metaphase-anaphase transition are presented below. Mechanistic implications of the results are discussed.

## 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 J. Feramisco, Cancer Center, University of California, San Diego) were grown in DME supplemented with 10% vol/vol FBS. Marsupial epithelial 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 cultured in the appropriate growth medium for 1–2 d before being used for experiments. All experimental observations were done at 33–37°C.

For imaging or photometric measurements with fura-2, the cells were incubated in air at 23°C for ~60 min with nominally 10–20 μM of the penta(acetoxymethyl) (AM) ester of fura-2 in the appropriate bicarbonate-free medium buffered with 20 mM Hepes. The nonionic surfactant Pluronic F-127 (BASF Wyandotte Corp., Wyandotte, WI) was used sparingly to aid dissolution of the difficultly-soluble AM esters into aqueous medium. (We prepared a 25% wt/wt solution of Pluronic F-127 in dry DMSO. For every 10 nmol AM ester used, 0.5–1 μl of the concentrated Pluronic stock was mixed in with the AM ester before the mixture was thoroughly dispersed into aqueous solution). For intracellular 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) buffering experiments, 1–10 μM BAPTA/AM was also present in the loading medium, in addition to fura-2/AM.

The AM ester of nitr-5, a photolabile chelator that releases $Ca^{2+}$ upon UV illumination, was used in experiments designed to study the effect of artificially induced $Ca^{2+}$ transients on the metaphase–anaphase transition in Swiss 3T3 fibroblasts. To monitor the rises in $[Ca^{2+}]_i$ resulting from UV flash photolysis of nitr-5, cells were also coloaded 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-9, 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 exposure.

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 fura-2/AM except that the loading medium contained 1–10 μM of the appropriate AM ester. The heavy metal chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (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, Berkeley, CA). All AM esters were dissolved in dry DMSO to make stock solutions of 1–10 mM concentration. Caged-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$] was purchased from Calbiochem-Behring Corp.

Control experiments using the heavy metal chelator, TPEN, were conducted using phophase Swiss 3T3 cells which were first incubated for 10–15 min in DME supplemented with 8% FBS and containing 30 μM 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 performed 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 monitoring of the progress of the mitotic cells in transmission mode using near-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 experiments involving nitr-5 photolysis and fluo-3 monitoring of $[Ca^{2+}]_i$, 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 photolysis, 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 MPI microspectrophotometer and photon-counted by a Thorn EMI 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 fluorescent $Ca^{2+}$ indicators occurs in some perinuclear regions as well as diffusely in the cell periphery. Whereas fluorescence from the center of the

1. *Abbreviations used in this paper*: AM, acetoxymethyl; BAPTA, 1,2-bis-(2-aminophenyl)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ins(1,4,5)$P_3$, *myo*-inositol 1,4,5-trisphosphate; NEB, nuclear envelope breakdown; TPEN, *N*,*N*,-*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine.

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. 1 *a*. In mitotic Swiss 3T3 cells, resting $[Ca^{2+}]_i$ was typically 145 ± 42 nM ($n$ = 24); $[Ca^{2+}]_i$ at the peak of the transient associated with NEB was 630 ± 430 nm ($n$ = 11); and $[Ca^{2+}]_i$ 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 routinely to 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 $[Ca^{2+}]_i$ vs. time for an interphase Swiss 3T3 cell in DME supplemented with 8% FBS is shown in Fig. 1 *b*. The imaging experiments revealed that the transient rises in $[Ca^{2+}]_i$ were not localized to a subcellular region, but rather $[Ca^{2+}]_i$ 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 visual observation paralleled fura-2 monitoring of $[Ca^{2+}]_i$ 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 ± 3.0 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. 1 *a* 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 $[Ca^{2+}]_i$ 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.

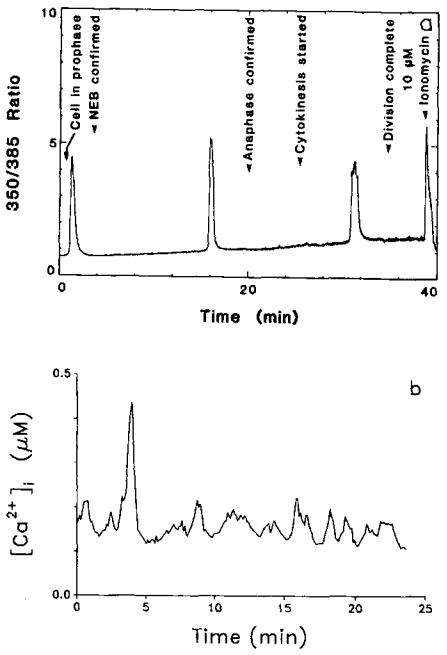



*Figure 1.* Intracellular $Ca^{2+}$ transient activity in mitotic and interphase Swiss 3T3 fibroblasts. (*a*) 350/385 excitation ratio vs. time trace for a mitotic Swiss 3T3 cell loaded with fura-2, the fluorescence was monitored with a photomultiplier. (*b*) $[Ca^{2+}]_i$ vs. time trace for an interphase Swiss 3T3 cell loaded with fura-2 and monitored in an imaging experiment. When calibrated to give $[Ca^{2+}]_i$, the NEB peak typically reached 630 ± 430 nM ($n$ = 11); the metaphase-anaphase peak, 620 ± 300 nM ($n$ = 13); baseline $[Ca^{2+}]_i$ was 140 ± 42 nM ($n$ = 24). The cells were loaded with fura-2 by incubation with the AM ester as described in Materials and Methods. During the experiments the cells were bathed in DME buffered with 20 mM Hepes at pH 7.4 and supplemented with 8% FBS.

### 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 $[Ca^{2+}]_i$ but should not affect the basal $[Ca^{2+}]_i$. Indeed, in our experiments, baseline $[Ca^{2+}]_i$ 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 I. At low intracellular concentrations of injected $Ca^{2+}$

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

| No. of cells injected | Average intracellular chelator concentration‡ | Time to NEB after injection |
|---|---|---|
| | $\mu M$ | *min* |
| 2 | 170 | 4.5 ± 1.8 |
| 11 | 420 | 37 ± 8 |
| 3 | 870 | >49 ± 6§ |

Time from early/mid-prophase to NEB in control cells (uninjected cells and cells injected with buffer containing no $Ca^{2+}$ chelator) = 6.4 ± 1.5 min ($n$ = 8). Time from early/mid-prophase to NEB in the presence of 30 $\mu$M 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.)

§ These three cells did not undergo NEB during the period of observation.

buffer (170 $\mu$M), 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 $\mu$M), NEB occurred at 37 ± 8 min after injection, a delay of ~31 min relative to controls. At 870 $\mu$M intracellular $Ca^{2+}$ chelator, NEB was not observed after even 49 ± 6 min. These results suggest that addition of exogenous $Ca^{2+}$ buffer into prophase Swiss 3T3 cells can hinder NEB. Although NEB could be blocked by heavy $Ca^{2+}$ buffering, chromosome condensation could not be, as prophase chromosomes continued to condense in the presence of high intracellular concentrations of $Ca^{2+}$ chelator.

We note that while BAPTA was used as a $Ca^{2+}$ chelator, it also shows high binding affinity for transition metal ions such as $Fe^{2+}$ and $Zn^{2+}$, which are important for biological function. It was thus important to ascertain that the NEB-blocking effect of BAPTA was due to $Ca^{2+}$ 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 $Zn^{2+}$), but low affinities for the divalent cations $Mg^{2+}$ and $Ca^{2+}$ ($K_d$'s of 20 mM and 40 $\mu$M, respectively). TPEN is therefore capable of sequestering heavy metal ions without disturbing the $Ca^{2+}$ and $Mg^{2+}$ 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 $\mu$M 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 $Ca^{2+}$.

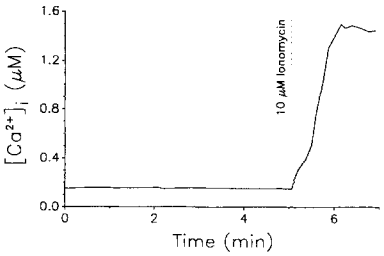



*Figure 2.* BAPTA loaded into cells via the AM ester can eliminate $Ca^{2+}$ transients. $[Ca^{2+}]_i$ vs. time profile for an interphase Swiss 3T3 cell that had been incubated for 60 min at room temperature with 10 $\mu$M each BAPTA/AM and fura-2/AM in Hepes-buffered DME supplemented with 8% FBS. The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

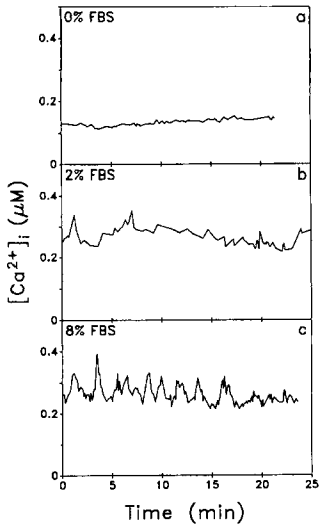
*Table II. Elapsed Time between Late Metaphase and Start of Chromosome Movement in BAPTA-loaded and Unloaded Swiss 3T3 Cells*

| BAPTA-loaded cells ($n$ = 14) | Control cells ($n$ = 14) |
|---|---|
| 4.1 ± 3.2 min | 3.7 ± 3.0 min |

Late metaphase is defined to be that stage during mitosis when ≥90% of the chromosomes have become aligned at the equatorial plate.

***BAPTA Buffering Can Abolish Global $Ca^{2+}$ Transients without Halting the Metaphase-Anaphase Transition in Swiss 3T3 Cells.*** To examine more specifically the requirement for global $Ca^{2+}$ 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 $Ca^{2+}$ 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 $Ca^{2+}$ homeostasis should titrate the newly formed BAPTA with $Ca^{2+}$ to maintain the resting $[Ca^{2+}]_i$ (Tsien et al., 1982). Because BAPTA has a $K_d$ for $Ca^{2+}$ of ~100 nM, at the resting $[Ca^{2+}]_i$ found in Swiss 3T3 cells (100–200 nM), 50–60% of the intracellular BAPTA is $Ca^{2+}$ bound, whereas 40–50% remains free to counteract any fast changes in $[Ca^{2+}]_i$. That intracellular BAPTA introduced via the AM ester was effective in buffering $[Ca^{2+}]_i$ is demonstrated in Fig. 2, which displays a $[Ca^{2+}]_i$ 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 $Ca^{2+}$ transients normally observed in abundance in interphase cells have been completely damped out. Addition of 10 $\mu$M ionomycin however, was effective in slowly bringing $[Ca^{2+}]_i$ in the BAPTA-buffered cell to elevated levels (Fig. 2).

We have incubated Swiss 3T3 cells with 1–10 $\mu$M BAPTA/AM and 10 $\mu$M fura-2/AM and then imaged the cells in DME containing 8% serum. The buffering afforded by the intracellular BAPTA completely eliminated the $Ca^{2+}$ transients normally observed in mitotic Swiss 3T3 cells in



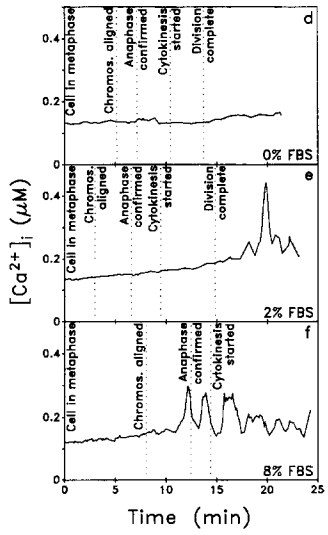




*Figure 3.* $Ca^{2+}$ transient activity in interphase and mitotic Swiss 3T3 fibroblasts are serum dependent. $[Ca^{2+}]_i$ vs. time traces for interphase cells bathed in Hepes-buffered DME supplemented with (*a*) 0% FBS, (*b*) 2% FBS, and (*c*) 8% FBS. Also shown are $[Ca^{2+}]_i$ vs. time traces for 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.

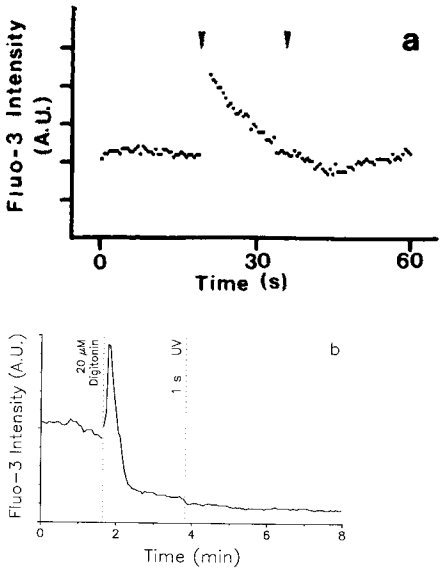



*Figure 4.* UV flash photolysis of intracellularly trapped nitr-5 rapidly elevated $[Ca^{2+}]_i$ as monitored by fluo-3 indicator. (*a*) Fluo-3 fluorescence intensity vs. time trace for a middle metaphase Swiss 3T3 cell co-loaded with nitr-5 and fluo-3 by incubation at room temperature in Hepes-buffered DME supplemented with 4% FBS and containing 1.0 $\mu$M nitr-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 nitr-5, resulting in a sharp rise in $[Ca^{2+}]_i$, 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*) Fluo-3 intensity vs. time trace for a metaphase cell incubated for 60 min at room temperature with 1 $\mu$M nitr-5/AM and 2 $\mu$M fluo-3/AM in Hepes-buffered DMA supplemented with 8% FBS. The $Ca^{2+}$-dependent fluorescence intensity of intracellular fluo-3 first increased sharply in response to $Ca^{2+}$ 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 presence of 8% serum. However, the cells all progressed successfully from metaphase through the end of mitosis in the absence of global intracellular $Ca^{2+}$ 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 $Ca^{2+}$ transients, had no significant effect on the progression of mitosis, as judged by the mean time of passage from metaphase to anaphase.[2]

## *The Occurrence of $Ca^{2+}$ 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 $Ca^{2+}$ transients without halting mitosis suggested that perhaps global rises in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ rises. Indeed, we found that the $Ca^{2+}$ 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 $Ca^{2+}$ Transients in Swiss 3T3 Are Serum Dependent.*** The random $Ca^{2+}$ 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.

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 $Ca^{2+}$ transients, whereas incubation with 10 $\mu$M fura-2/AM did not apparently lead to drastically increased intracellular $Ca^{2+}$ buffering. The reason for this apparent disparity is to be found in the true aqueous solubilities of the two AM esters. At 25°C, BAPTA/AM is soluble in water to the extent of 15 $\mu$M, whereas the true aqueous solubility of fura-2/AM is a mere 0.11 $\mu$M, nearly a 140-fold difference. Thus, whereas 1–10 $\mu$M BAPTA/AM gives an indication of 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 concentraion 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.

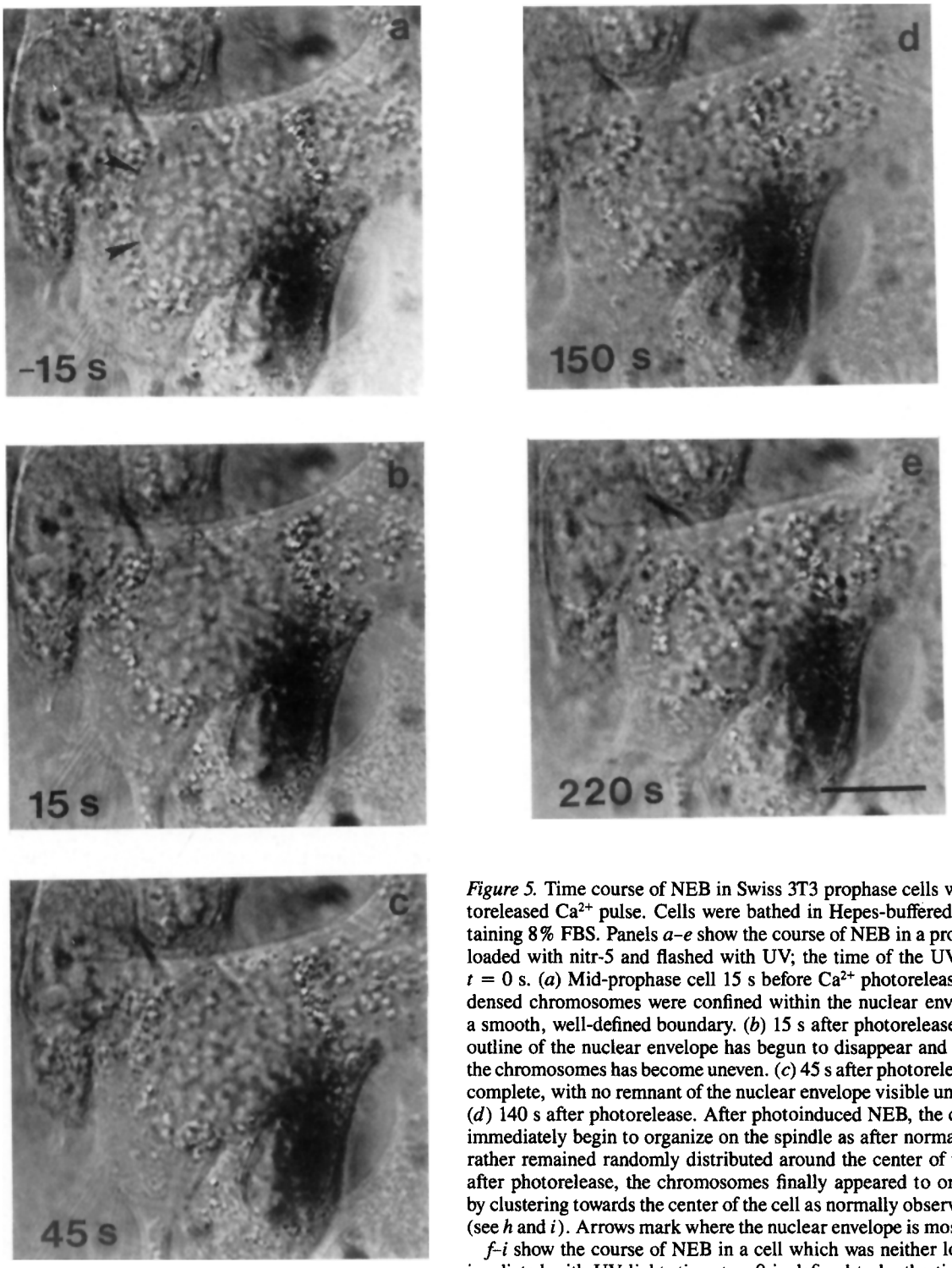



*Figure 5.* Time course of NEB in Swiss 3T3 prophase cells with and without photoreleased $Ca^{2+}$ pulse. Cells were bathed in Hepes-buffered DMA (pH 7.4) containing 8% FBS. Panels *a–e* show the course of NEB in a prophase Swiss 3T3 cell loaded with nitr-5 and flashed with UV; the time of the UV flash 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

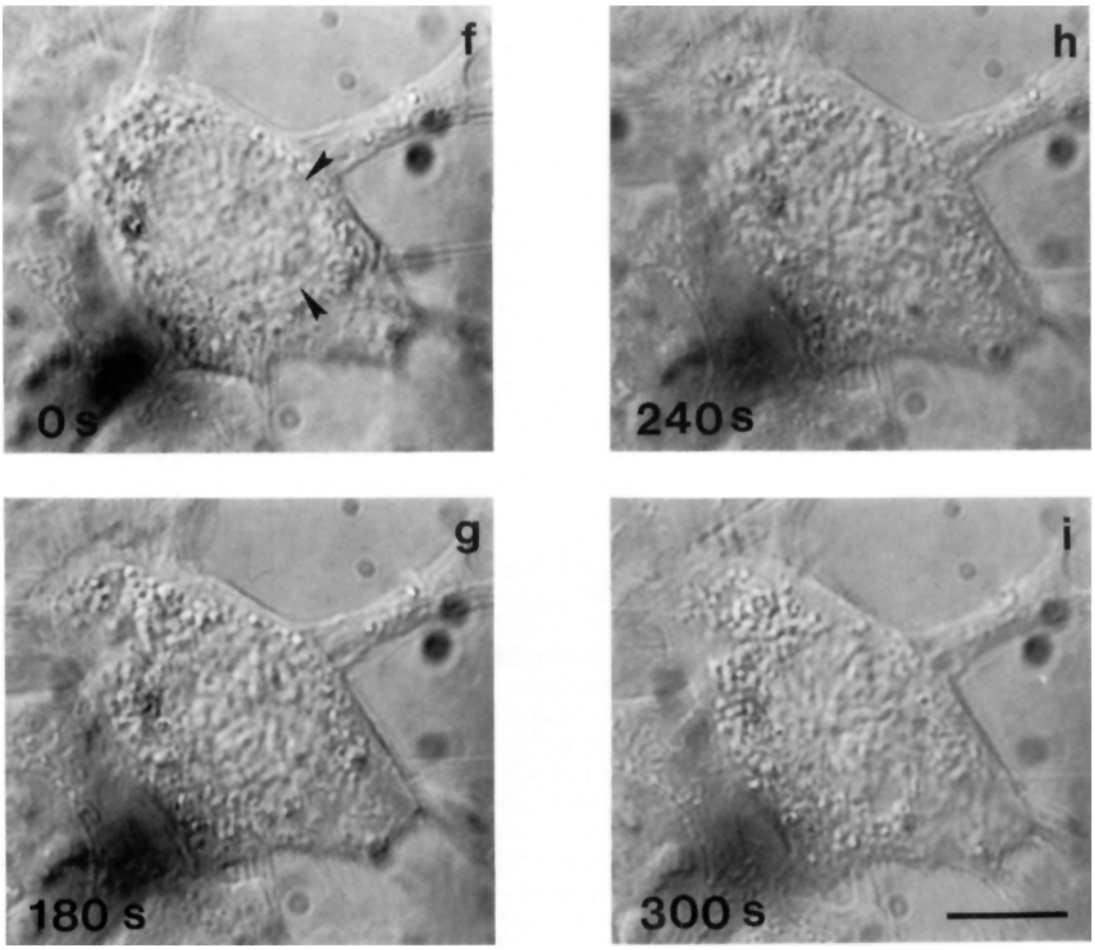



*Figure 5 (continued)*

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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ (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 $[Ca^{2+}]_i$ might be important for progression of mitosis. In view of this proposal, it is worth noting that in our cells the resting $[Ca^{2+}]_i$ 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 mitotis 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 | – |
| 0.5 ± 0.2 min | 5.3 ± 2.3 min |

* Controls included cells not loaded with nitr-5/AM and cells loaded with either nitr-9/AM or exhaustively prephotolyzed (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 which, when loaded into cells and illuminated with UV light, releases $Ca^{2+}$ (nitr-5 $K_d$ for $Ca^{2+}$: 0.145 $\mu$M prephotolysis, 6.3 $\mu$M postphotolysis; Adams et al., 1988). Because $Ca^{2+}$ release from photolyzed nitr-5 is very fast ($t_{1/2}$ = 218 $\mu$s; Adams et al., 1988), very rapid elevations in $[Ca^{2+}]_i$ can be effected by UV flash photolysis. Such UV flash-induced $Ca^{2+}$ rises can be visualized using a fluorescent $Ca^{2+}$ indicator that is excitable by visible light, fluo-3 (Minta et al., 1989; Kao et al., 1989), whose fluorescence quantum yield is 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/AM. The trace clearly demonstrates that UV flash photolysis of nitr-5 can very rapidly raise $[Ca^{2+}]_i$. Whereas the magnitude of the increase in $[Ca^{2+}]_i$ was a function of the extent of loading as well as the duration of UV photolysis, separate calibrations showed that in our experiments, after flash photolysis of nitr-5, $[Ca^{2+}]_i$ 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 compartments (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 observed in fact took place in the cytosol, rather than in subcellular compartments. Fig. 4 *b* shows the fluorescence intensity vs time trace for a cell loaded with fluo-3 and nitr-5. The cell was first permeabilized with 20 $\mu$M digitonin, which has been shown to be effective in releasing soluble cytosolic contents without significantly disrupting subcellular organelles (Kao et al., 1989; Kawanishi et al., 1989). As the cell was permeabilized, the initial influx of $Ca^{2+}$ gave rise to a large, fast increase in fluo-3 intensity, followed by a rapid decrease in fluorescence as intracellular fluo-3 escaped through the permeabilized plasma membrane. A 1-s flash of UV light delivered to the cell after permeabilization did not produce a response akin to that shown in Fig. 4 *a*. These results demonstrate that in cells loaded with fluo-3 and nitr-5, the increase in fluo-3 fluorescence after UV photolysis reflected an increase in cytosolic $[Ca^{2+}]$ resulting from photorelease of $Ca^{2+}$ from cytosolic nitr-5 and was not the result of photoreactions involving compartmentalized nitr-5 and fluo-3. Similar results were obtained when the cells were permeabilized in DME containing 3.5 mM EGTA (pH 7.4, free $[Ca^{2+}]$ <100 nM). In addition to the permeabilization controls, experiments wherein photoreleased Ins(1,4,5)$P_3$, a second messenger, caused transient increases in the $Ca^{2+}$-dependent fluorescence of intracellularly trapped fluo-3 also provide strong evidence that the fluo-3 indicator resides primarily in the cytosol (see below and Fig. 6 *c*).

***$Ca^{2+}$ Photoreleased from Intracellularly Trapped nitr-5 Causes Precocious NEB.*** We have examined the effect of these rapid $Ca^{2+}$ concentration jumps on the progression of NEB in Swiss 3T3 cells. Before NEB, the nuclear envelope appeared in Nomarski optics as a relatively smooth, closed boundary enclosing the chromosomes. After NEB has started to occur, the smooth boundary began to dissolve and the chromosomes began to drift and protrude out of their previously well-defined region of confinement by the nuclear envelope. We have taken the dissolution of the nuclear boundary and chromosome spill-out as the morphological markers for NEB (see Fig. 5, *c* and *h*). When middle to late prophase cells pre-loaded with nitr-5 via the AM ester and bathed in DME containing 8% FBS were given short UV pulses (50–500 ms duration), NEB occurred within 29 s (± 14 s, Table III). The occurrence of NEB in response to a photochemical $Ca^{2+}$ concentration jump was not dependent on the presence of FBS in the bathing medium, as essentially indentical results were obtained when the same experiments were performed with prophase cells loaded with nitr-5 but bathed in 0% FBS: NEB occurred within 41 ± 22 s ($n$ = 7) of the UV flash. Precocious NEB precipitated by photoreleased $Ca^{2+}$ is illustrated in Figs. 5, *a–e*. In control cells, the elapsed time between middle/late phophase and NEB is 5.3 min (± 2.3 min, Table III). For comparison with Fig. 5, *a–e*, we present in Fig. 5, *f–i* photomicrographs which illustrate the time course of NEB in a cell that has not experienced photochemical manipulation.

We stress that the precocious NEB occurred in response to photoreleased $Ca^{2+}$ and was not the result of UV exposure per se, as no precocious NEB was observed when cells loaded with inactivated nitr-5 or no nitr-5 at all were subjected to UV flash photolysis. Furthermore, we know that precocious NEB was not an artifact resulting from the action on cellular structures by reactive photochemical intermediates or products generated in the photolysis of nitr-5, because UV flashes delivered to prophase cells loaded with the control compound nitr-9 elicited no similar response. The results of experiments on $Ca^{2+}$ photorelease and NEB are summarized in Table III.

Note that although the disassembly of the nuclear envelope could be drastically accelerated by the pulse of photoreleased $Ca^{2+}$, 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 cytoplasm, 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 photorelease 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 crisscross 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 experimenter. To address these concerns, we have performed double-blind experiments wherein metaphase Swiss 3T3 cells were loaded with mixtures (prepared and number-

*Table IV. Effect of Photoreleased $Ca^{2+}$ on the Timing of the Metaphase–Anaphase Transition**

| | Middle/mid-late metaphase‡ | | | Late metaphase§ | | |
|---|---|---|---|---|---|---|
| | From flash to appearance change | From appearance change to poleward movement | Total time of transit | From flash to appearance change | From appearance change to poleward movement | Total time of transit |
| Cells loaded with nitr-5 | 12.0 ± 7.0 s<br>0.20 ± 0.12 min | 4.9 ± 2.0 min | 5.1 ± 2.1 min | 10.8 ± 5.1 s<br>0.18 ± 0.09 min | 3.1 ± 2.0 min | 3.3 ± 2.1 min |
| Control cells | – | – | 6.7 ± 3.0 min | – | – | 3.9 ± 2.0 min |

* For middle/midlate metaphase cells: nitr-5/AM-loaded, $n = 24$; control, $n = 24$.
For late metaphase cells: nitr-5/AM-loaded, $n = 18$; control, $n = 28$.
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.
‡ Middle metaphase is defined as that stage at which ≥50% of the chromosomes are aligned at the equatorial plate; mid-late metaphase is defined as that stage at which ≥75% of the chromosomes are aligned.
§ Late metaphase is defined to be the stage at which ≥90% of the chromosomes are aligned.

coded by one experimenter) containing nitr-5/AM, nitr-9/AM, exhaustively photolyzed (inactivated) nitr-5/AM, or no nitr compound at all. The second experimenter who activated UV flash photolysis and made observations at the microscope had no prior knowledge of the composition of the various loading mixtures, but faithfully recorded the experimental observations. At the end of each day of experimentation, the results of microscope observation were matched to the coding sheet that specified loading sample number and composition. In 13 out of the 15 double-blind experiments performed, there was complete agreement between the coding sheet and the results of UV photolysis and microscopic observation: only cells loaded with active nitr-5/AM exhibited changes in chromosome appearance in response to UV irradiation; cells loaded with nitr-9/AM, preinactivated nitr-5/AM or no nitr compound at all showed no comparable effect. In 2 of the 15 experiments, the opacity of the 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 ± 7.0 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 was 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 $[Ca^{2+}]_i$ 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 $Ca^{2+}$ jumps, poleward migration of the chromatids did not start until 5.1 ± 2.1 min after photorelease of $Ca^{2+}$. In the control cells, where the UV flash produced no $Ca^{2+}$ 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 $Ca^{2+}$ 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 late metaphase cells, and 3.9 ± 1.8 min in the controls; see Table IV).

### *The Effect of $Ca^{2+}$ Deprivation and of Discharging Intracellular $Ca^{2+}$ Stores on Mitosis in Swiss 3T3 Cells*

***$Ca^{2+}$ Deprivation by Treatment with Ionomycin in EGTA-containing Medium Halts Mitosis in Swiss 3T3.*** We have performed experiments wherein mitotic Swiss 3T3 cells were placed in DME containing sufficient EGTA to keep the extracellular $[Ca^{2+}]_i$ 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 $Ca^{2+}$ stores of cells bathed in EGTA-containing medium were then discharged by treatment with one or two 0.5–10 μM doses of the $Ca^{2+}$ ionophore ionomycin. Ionomycin was very effective at depleting the cells of internal $Ca^{2+}$ 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 $[Ca^{2+}]_i$, due to discharge of intracellular stores, before all the $Ca^{2+}$ 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 $[Ca^{2+}]_i$, suggesting that intracellular $Ca^{2+}$ stores have been depleted.

The $Ca^{2+}$ 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

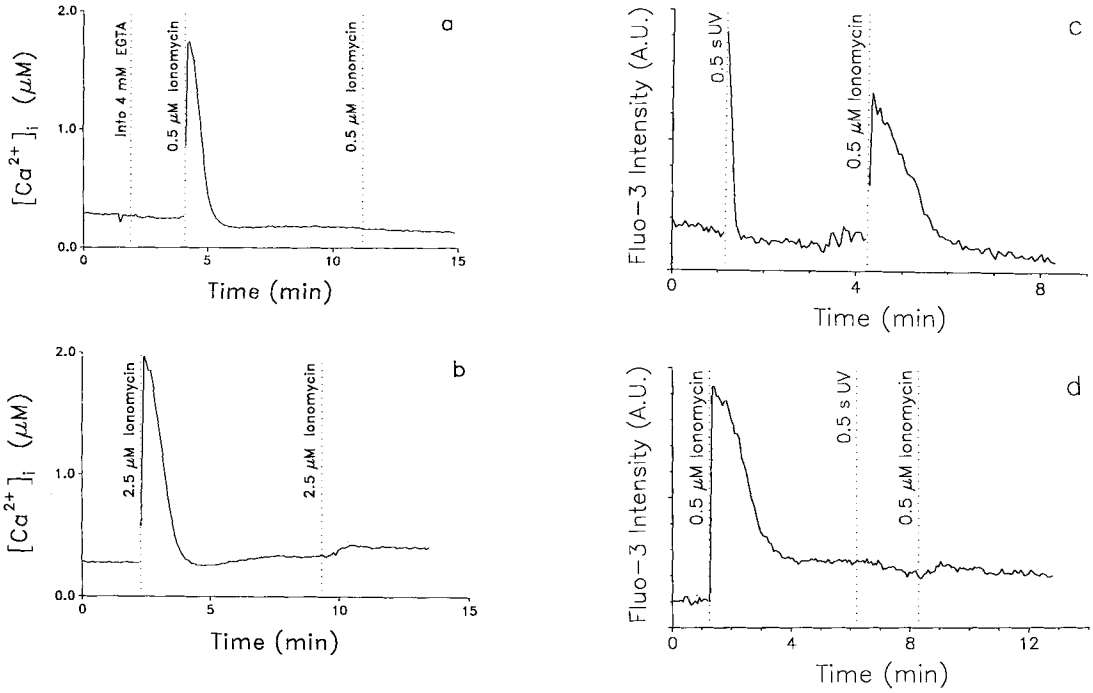



*Figure 6.* Ionomycin is effective in dissipating intracellular $Ca^{2+}$ stores. (*a*) A metaphase Swiss 3T3 cell loaded with fura-2 was placed into DME containing 4 mM EGTA and treated with 0.5 μM ionomycin two times, at ~7 min apart. (*b*) A metaphase Swiss 3T3 cell loaded with fura-2 and bathed in regular DME was given two treatments with 2.5 μM ionomycin at ~7 min apart. In both cases, the first ionomycin treatment discharged intracellular stores, as evidenced by the sharp but transient rises in $[Ca^{2+}]_i$. The stores apparently remained discharged in the continued presence of ionomycin, as a second treatment with ionomycin did not produce corresponding sharp rises in $[Ca^{2+}]_i$. (*c*) A Swiss 3T3 cell injected at room temperature in Hepes-buffered DME with a solution containing 2.55 mM 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 μM ionomycin produced a large, more protracted rise in $[Ca^{2+}]_i$, 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 μM ionomycin, which produced a large rise in $[Ca^{2+}]_i$. When $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$, but rather a gradual approach to a slightly higher $[Ca^{2+}]_i$, as was seen in *b*.

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.

***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 $[Ca^{2+}]_i$ 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 μM ionomycin. The first dose of ionomycin produced a large rise in $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ to a plateau value some-

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

| | Ionomycin/ EGTA* | Ionomycin/ Ca‡ | Control |
|---|---|---|---|
| Number of cells undergone metaphase-anaphase transition | 1/17 | 13/13 | 14/14 |
| Time between metaphase and start of poleward movement (*min*) | 13 | 7.8 ± 3.8 | 3.7 ± 3.0 |

* 10 cells in DME/0% FBS/4mM EGTA treated with 0.5 μM ionomycin, and 7 cells in DME/8% FBS/5mM EGTA treated with 10 μM ionomycin.
‡ Cells in DME/0% FBS treated with 2.5 μM ionomycin.

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 $[Ca^{2+}]_i$, suggestive of the discharged state of the internal $Ca^{2+}$ stores. There was, however, a slow approach to a new, somewhat higher $[Ca^{2+}]_i$ 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 internal 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 $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ but rather only a slow approach to a slightly higher $[Ca^{2+}]_i$ 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 repletion 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 $[Ca^{2+}]_i$ 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 largely eschewed 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 $[Ca^{2+}]_i$ at the onset of anaphase (Table VI). In those cells where a $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $PtK_2$ 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 acquistion rates, we failed to observe a $Ca^{2+}$ transient in those cells undergoing the metaphase-anaphase transition. The second possible explanation is that the $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$, 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 μm²/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 ana-

*Table VI. Number of PtK$_1$ and REF52 Cells that Underwent Metaphase–Anaphase Transition with and without Global $Ca^{2+}$ Transients*

| | With $Ca^{2+}$ transient | Without $Ca^{2+}$ transient |
|---|---|---|
| $PtK_1$* | 3 | 18 |
| REF52‡ | 6 | 10 |

* In Ham's F-12 containing 10% (vol/vol) FBS.
‡ In DME containing 10% (vol/vol) FBS.

phase generally without an attendant global transient rise in $[Ca^{2+}]_i$. 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 (pancellular) $Ca^{2+}$ 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 did 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 Ins(1,4,5)$P_3$ induced premature chromatin condensation and NEB in early sea urchin embryos. The same workers also found that the Ins$P_3$ effect could be blocked by the intracellular presence of EGTA, suggesting that the principal effect of Ins$P_3$ was to generate a rise in $[Ca^{2+}]_i$, 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 $Ca^{2+}{}_i$ 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 artifically 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ appear not to be essential. The fact that global rises in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$, but as the released $Ca^{2+}$ is diffusionally diluted into the bulk cytoplasm, the global $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$, 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, $PtK_1$, showed that global $Ca^{2+}$ transients were not always observed during all mitotic stages in these cells. Our findings on $PtK_1$ cells are not inconsistent with results on $PtK_2$ cells reported by Ratan et al. (1988), who found $Ca^{2+}$ transients in a much larger percentage of the mitotic $PtK_2$ 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 nonuniversality 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.

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