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Intracellular free calcium levels are reduced in mitotic Pt K2 epithelial cells

(quin-2/microtubule/microscope spectrofluorometry)

CHARLES H. KEITH*, FREDERICK R. MAXFIELD, AND MICHAEL L. SHELANSKI†

Department of Pharmacology, New York University School of Medicine, 550 First Avenue, New York, NY 10016

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ABSTRACT **Using a fluorescence ratio method, we have studied the intracellular free calcium levels in individual quin-2-loaded mitotic cells under the microscope. We have found that intracellular free calcium concentrations in Pt K2 epithelial cells drop by ≈50% as they pass through mitosis. Calcium levels in interphase cells were 53 ± 7 nM. During prophase, free cytoplasmic calcium begins to decrease, reaching 28 ± 3 nM in prometaphase. Calcium levels remain low until the nuclear envelope is re-formed in late telophase, when they increase again to interphase levels. This decrease in overall free calcium in mitosis suggests that the mitotic cell has mechanisms for the general sequestration, and perhaps local release, of calcium ions.**

A variety of studies have implicated calcium as a signal in the modulation of a multiplicity of cellular functions, as outlined in recent reviews (1–3). Numerous observations suggest that microtubular equilibria, particularly during mitosis and meiosis, are among the events controlled by free calcium concentrations. Microtubule depolymerization *in vitro* is effected by calcium ions, and the levels of calcium required to achieve this depolymerization are lowered from millimolar to micromolar levels in the presence of calmodulin (4–7). In addition, mitotic spindles are sensitive to free calcium levels in the micromolar range both in living cells (8, 9) and *in vitro* (10, 11). Calmodulin is localized in the mitotic spindle of dividing cells (12, 13), and calcium-saturated calmodulin potentiates the localized depolymerization of microtubules when microinjected into living cells (14). Fluorescence observations of chlortetracycline-loaded cells have suggested that spindles contain significant amounts of membrane-adjacent free calcium (15, 16), and a variety of spindles contain an elaborate network of membrane-bound tubular (17) or vesicular (16, 18) elements. In sea urchin eggs, these vesicles have been shown to accumulate calcium (19), suggesting that a mechanism exists within the dividing cell for the sequestration of calcium (8, 20). Finally, studies have indicated that lowered extracellular calcium levels may accelerate mitosis (21) and that increased calcium levels may delay the onset of metaphase in activated sea urchin eggs (22).

In this paper, we report the use of the fluorescent calcium indicator methoxyquinolinebis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (quin-2) (23, 24) to measure the free calcium levels of individual cells on the microscope stage. We find that mammalian (Pt K2) cells show a 50% decrease in intracellular free calcium levels as they enter mitosis and an increase in free calcium to interphase levels as they finish mitosis.

MATERIALS AND METHODS

Cells and Reagents. Pt K2 (rat kangaroo kidney epithelial) cells were plated at 50%–90% confluence in coverslip-bottomed tissue culture dishes. Cells were maintained in Dulbecco's modified Eagle's medium (DME medium; GIBCO), supplemented with 10% fetal calf serum, in a humidified incubator with a 9% CO_2 atmosphere. One hour before the start of an experiment, the medium was changed to minimum essential medium (ME medium) without phenol red and sodium bicarbonate (GIBCO), supplemented with 10% fetal calf serum (KC Biological, Lexena, KS) and 25 mM Hepes (Sigma). A coverslip was sealed over the top of the dish with silicone grease to prevent evaporation.

Quin-2 and quin-2 A/M (the acetoxymethyl ester of quin-2) were obtained from Lancaster Synthesis (Eastgate, England). 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was purchased from Molecular Probes (Junction City, OR), EGTA was from Sigma, and calcium chloride and EDTA were from Fisher.

Microscopy. All measurements were performed on a Diavert microscope (Leitz) equipped with an MPV photometer and a Dage–MTI (Michigan City, IN) SIT camera or a Zeiss–Venus TV3 intensified vidicon (Carl Zeiss, Thornwood, NY). The fluorescence illuminator was a 100 W mercury arc lamp. All lenses, except for the objective (see below), in the excitation path of this microscope were made of quartz so the 340-nm exciting radiation would not be absorbed. For measurements of quin-2 fluorescence, 340- or 360-nm narrow band-pass UV filters (Oriel, Stamford, CT) were inserted in the excitation path before the 400-nm dichroic prism, and a 500-nm broad band-pass (70-nm full width at half maximum) filter (Oriel) was inserted on the emission side. For time-course measurements of individual cells, an additional 0.5 OD quartz neutral density filter (Corion, Holliston, MA) was inserted in the exciting beam to reduce photobleaching. In some experiments, a glass 25% transmission neutral density filter (Leitz) was used along with the 360-nm filter to equalize the intensities of the 340- and 360-nm illumination. Measurements of HPTS fluorescence were performed with two filter systems: (*i*) the Leitz A filter block (340- to 380-nm excitation filter, 400-nm dichroic prism, and 430-nm long-pass barrier filter) and (*ii*) the Leitz I2 block, with the excitation filter replaced by a 450-nm narrow band-pass filter (giving a 450-nm excitation filter, a 510-nm dichroic prism, and a 515-nm long-pass barrier filter). (The I2 filter block is not ideal for measurements of the fluorescence emission of HPTS, which has an emission maximum at 510 nm, but the long-wavelength shoulder of the emission peak provides sufficient intensity for accurate measurement.) The calibration curves were taken on the microscope stage, using exactly the same filter sets, so the ratio of the fluorescence intensities observed with these two filter sets could be converted accurately to pH. Objectives used

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Abbreviations: HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid; quin-2, methoxyquinolinebis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; quin-2 A/M, acetoxymethyl ester of quin-2.

*Present address: Department of Zoology, University of Georgia, Athens, GA 30602.
†To whom reprint requests should be addressed.

were a Zeiss 25× Neofluar, for location and identification of mitotic cells under phase contrast, and a 100× Nikon UV-fluor for fluorescence measurements. (The Nikon objective is a fluorite rather than quartz objective and passes 65% of illumination at 330 nm.) For all measurements, 30-μm-diameter measuring and illumination apertures, centered on the nucleus or chromosomes, were used.

Background Measurements and Quin-2 Loading. Before cells were loaded with quin-2 or injected with HPTS, measurements were made of background fluorescence on each dish. Five to seven cells, in all stages of the cell cycle, were chosen at random and measurements of their fluorescence at 340 and 360 nm, or at 380 and 450 nm in the case of HPTS, were made on the microscope stage. Background fluorescence was fairly uniform between cells, showing a standard deviation of 10%–15% of the average intensity. Thereafter, for calcium measurements, the cells were incubated for 30 min with a 30 μM solution of quin-2 A/M in the same medium at 37°C. After loading with quin-2, the cells were washed and allowed to equilibrate with fresh phenol red-free medium for 15 min before measurements were begun. For pH measurements, selected early prophase cells in phenol red-free medium were injected with HPTS (10 μM in 137 mM KCl) using an air pressure-driven glass microelectrode (25). Measurements were begun 10 min later, a time when both pH and pCa^{2+} of injected interphase cells had equilibrated to levels that were unchanged 1 hr later. For all experiments, cells were maintained at 37°C on the microscope stage by using an air stream incubator (Arenberg Sage, Boston).

Calibration of Quin-2 Fluorescence. Quin-2 fluorescence is sensitive to $[Ca^{2+}]$ when excited at 340 nm but is independent of it when excited at 360 nm (26). To determine free calcium levels in quin-2-loaded cells, the cells' 340/360 (excitation) fluorescence ratio values were determined. By reference to a calibration curve relating quin-2 340/360 ratios to $[Ca^{2+}]$, these ratios were converted to free calcium levels. The calibration curve was made by taking 340/360 fluorescence ratios of a series of quin-2-containing calcium buffers (27) on the microscope stage (see figure 2 of ref. 26). Calibration measurements were made weekly, so that any changes in lamp output or other system properties (e.g., filter solarization) could be detected.

Establishment of Mitotic Stages. The stage of mitosis in Pt K2 cells was assigned on the basis of observations of the cells under phase contrast optics at ×250. The criteria for assignment of cells to the various stages of mitosis are as follows: (*i*) Prophase: Condensation of chromosomes is clearly visible, and nucleoli have disappeared. The nuclear envelope is still intact. (*ii*) Prometaphase: Nuclear envelope has broken down, but chromosomes have not yet fully progressed to the metaphase plate. (*iii*) Metaphase: Chromosomes are aligned on the metaphase plate (see Fig. 1*A*). (*iv*) Anaphase: Separation of sister chromatids is in progress, but there is no sign of constriction at the middle of the cell. (*v*) Telophase: Cleavage furrow between the daughter cells is apparent, but they have not yet flattened out onto the substrate. (*vi*) Late telophase: Daughter cells have flattened, and the midbody between them is maximally constricted, but the nuclear envelopes have not yet re-formed. (*vii*) Interphase: Nuclear envelope is intact, and nucleolus (or nucleoli) is formed.

Multicell Measurements. After determining background, cells were loaded with quin-2, and the culture was scanned under phase contrast optics for mitotic cells. After noting the stage of mitosis, the microscope objective was changed to the UV-fluor objective, and fluorescence measurements were taken under 340- and 360-nm excitation. After subtraction of average background fluorescence values, a 340/360 ratio was calculated. This ratio was converted to free calcium levels as described above. Measured calcium levels were averaged for all cells of a given mitotic stage (10–30 cells per stage of mitosis, and 17 interphase cells), and standard errors of the mean calcium level were calculated.

Single-Cell Measurements. After background measurements and quin-2 loading, cells in prophase were located. Measurements of 340/360 ratios were then made on the same cell through the various stages of mitosis. The exciting beam was controlled by a programmable shutter and attenutated by a 0.5 OD quartz neutral density filter to reduce bleaching to <2% per measurement. After telophase measurements were made, cells were reloaded with quin-2, and interphase fluorescence ratios were recorded. Reloading was necessary because the intracellular quin-2 concentration appears to drop slowly in these cells and because the original mitotic cell had doubled, halving the quin-2 content of each daughter cell. The individual 340/360 ratios were then converted to free calcium levels.

Measurements of Changes in Cytoplasmic pH. To determine whether mitotic cells show changes in intracellular pH sufficient to affect the quin-2 fluorescence ratios, the fluorescence of microinjected HPTS at 380 and 450 nm was measured at intervals throughout the course of mitosis (28). After subtraction of the corresponding average background fluorescence values, the ratio of fluorescence emission on excitation at these two wavelengths was calculated. By reference to a calibration curve of 380/450 fluorescence ratios of HPTS solutions at various pH values, taken on the microscope stage, these quantities could be converted to intracellular pH values.

RESULTS

Effect of Quin-2 on Mitosis. Since quin-2 is a calcium chelator, the introduction of high intracellular quin-2 concentrations might significantly increase intracellular calcium buffering capacity and perturb mitosis. Indeed, when quin-2 A/M, at final concentrations between 1 and 30 μM, was added to Pt K2 cells in Hanks' buffered salt solutions containing lowered amounts of $CaCl_2$ (<0.2 mM), or for extended periods in calcium-containing medium, cells appeared to be retarded in their transit through mitosis. However, if loading is restricted to <1 hr in calcium-containing medium (ME medium supplemented with 10% fetal calf serum) and cells are given 15 min in the same medium without quin-2 to reequilibrate, mitosis appears to proceed normally. Under these conditions, metaphase transit time (the time from the breakdown of the nuclear envelope to the beginning of anaphase), anaphase duration (time from sister chromatid separation until the beginning of cytokinesis), and mitosis transit time [time from the breakdown to the reestablishment of nuclear envelope(s)] are comparable to those of untreated cells in the same medium and at the same temperature (Table 1).

Loading of Quin-2. That Pt K2 cells actually become loaded with quin-2 when exposed to the ester is indicated by the fact that their fluorescence, when excited at either 340 or 360 nm, increases substantially upon exposure to quin-2. In a

Table 1. Timing of mitosis in PtK2 cells

	Quin-2-loaded cells	Control cells
Metaphase transit time, min	29 ± 6	26 ± 2
Anaphase duration, min	7 ± 1	9 ± 2
Mitosis transit time, min	61 ± 3	65 ± 10

Cells were loaded for 30 min with 30 μM quin-2 A/M in ME medium, washed for 15 min in Hepes-buffered ME medium before the start of an experiment. Mitoses were followed on the stage of an inverted microscope, and the stage temperature was maintained at 37 ± 1°C, using an air curtain incubator. Timings represent mean of six cells loaded separately with quin-2 on different days.

typical experiment, the average fluorescence of interphase cells increased by a factor of 3 at 340 nm and by a factor of 2 at 360 nm when they were loaded with quin-2. Since the standard deviation of the background fluorescence is only 5% of the fluorescence intensities of loaded cells, the variation in background fluorescence will not adversely affect measurements of quin-2 fluorescence. Fig. 1 shows phase contrast and 360-nm fluorescence micrographs of a quin-2-loaded cell at metaphase. Intracellular [Ca^{2+}] was then determined by referring the 340/360 fluorescence ratio to a calibration curve, as described in *Materials and Methods*.

Calcium Levels During Mitosis. When average calcium levels of quin-2-loaded interphase cells are measured as described above, they are found to be 53 nM, with a standard error of 7 nM. In prophase, free calcium concentration drops to 38 ± 2 nM, and in prometaphase to 28 ± 3 nM. Calcium levels remain fairly constant at 25–28 nM in telophase. Thereafter, they increase again to the interphase level (Fig. 2, solid line).

When single cells are followed through the course of mitosis, their pattern of calcium variation follows that found in multicell observations. The experimental scatter of single-cell measurements is relatively high compared to multicell measurements, because the illumination levels were reduced to avoid photobleaching and cell damage during repeated exposure. In a typical experiment (solid squares), the calcium level in prophase was found to be 84 nM. During prometaphase, the free calcium dropped to 24 nM, where it remained until the late stages of telophase, when it increased to 40 nM.

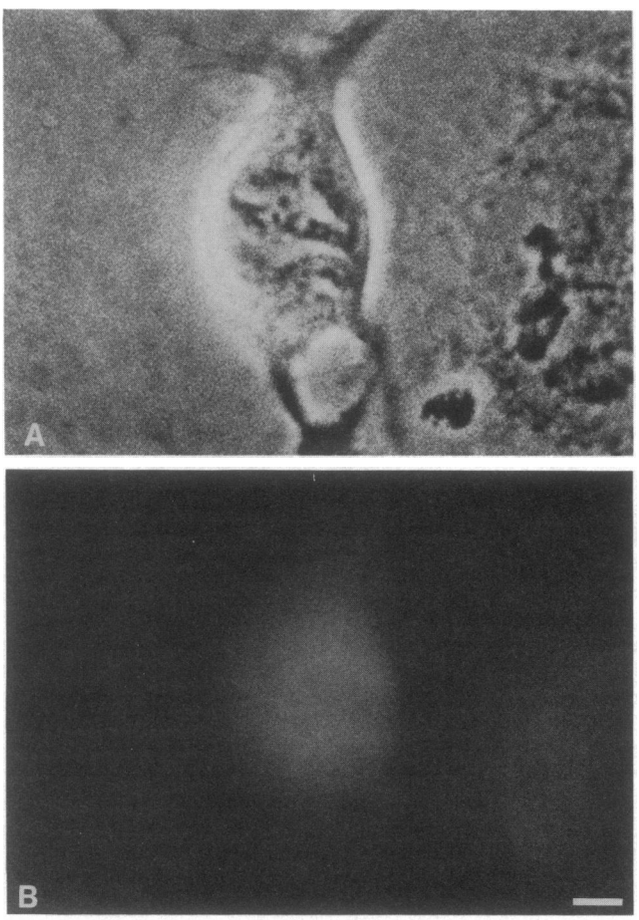

FIG. 1. (*A*) Phase and (*B*) 360-nm fluorescence photomicrographs of a Pt K2 cell at the metaphase/anaphase transition. These photomicrographs were taken at an exposure index of 3200. The fact that adequate fluorescence is present to make the fluorescence photomicrograph illustrates that 360-nm fluorescence is sufficiently intense to be measured accurately. (Bar = 10 μm.) (×640.)

Upon re-formation of the nuclear envelope and significant flattening of the daughter cells, the free calcium level was found to be 78 nM. When the results of seven single-cell time courses were averaged, they agreed very well with the multicell measurements (dashed line).

To determine whether the observed changes in 340- and 360-nm fluorescence could be accounted for by variations in cellular autofluorescence through mitosis, we took 340- and 360-nm fluorescence measurements on cells not exposed to quin-2, following both the multicell and single-cell protocols. In neither case was any significant change in 340/360 ratio found in the various stages of mitosis. Thus, in multicell measurements, no change in either average 340/360 ratios or the 340-nm fluorescence alone exceeded the standard error of the measurement. The variations that were found did not follow any consistent pattern.

To determine whether changes in the quin-2 fluorescence of mitotic cells could be related to changes in intracellular pH, we measured the variation in the 380/450 fluorescence ratios of mitotic cells injected with HPTS. We found no variation of intracellular pH by >0.15 pH units through mitosis. Since pH changes of up to 0.2 pH units would not produce a significant change in the 340/360 ratio (26), changes in the quin-2 340/360 ratios during cell division must not be due to changes in cytoplasmic pH.

DISCUSSION

It has been suggested that calcium plays a significant controlling role in mitosis. The evidence in support of this hypothesis is largely indirect, such as the sensitivity of the mitotic spindle to micromolar calcium and the observation of calmodulin localization at the spindle poles. We have developed methods, using the calcium indicator quin-2, that allow us to measure free calcium levels in the mitotic cell. By measuring the ratio of quin-2 fluorescence at a wavelength where it is calcium dependent to that at a wavelength where it is calcium independent, we obtain a value that is independent of quin-2 concentration but is dependent on calcium concentration. Because we have removed the quin-2 concentration dependence, this method is more suitable for use on the microscope stage than are methods that measure an absolute fluorescence at 340 nm (24, 27, 29, 30). For processes such as mitosis, where cells change in size and shape, the ratio method offers the best available method for quantifying free calcium by using quin-2 fluorescence microscopy.

Using the quin-2 fluorescence ratio method, we have detected a 50% decrease in free intracellular calcium levels of Pt K2 cells as they pass through mitosis. Intracellular calcium concentrations of ≈53 nM drop during prophase and prometaphase to ≈27 nM. Calcium concentrations remain at this low level until the late stages of telophase, when they increase back to interphase values. The decrease in calcium levels is highly reproducible and is detected both in randomly selected cells at the various stages of mitosis and in time-course observations of individual quin-2-loaded cells passing through mitosis.

A number of potential artifacts must be considered in this or any other study of free calcium concentrations in quin-2-loaded cells. In the first place, quin-2 is a calcium chelator, with a dissociation constant for calcium of ≈115 nM (27) in buffers designed to simulate the intracellular environment. Since cells will take up levels of quin-2 of up to millimolar (27, 29, 31), they may significantly increase their intracellular calcium buffering capacity. One must determine whether such buffering, which will certainly affect calcium transients within the cell, will affect the process under consideration. By the criteria of appearance (at the light microscopic level) and timing of events, quin-2 loading does not appear to influence the course of mitosis in Pt K2 cells.

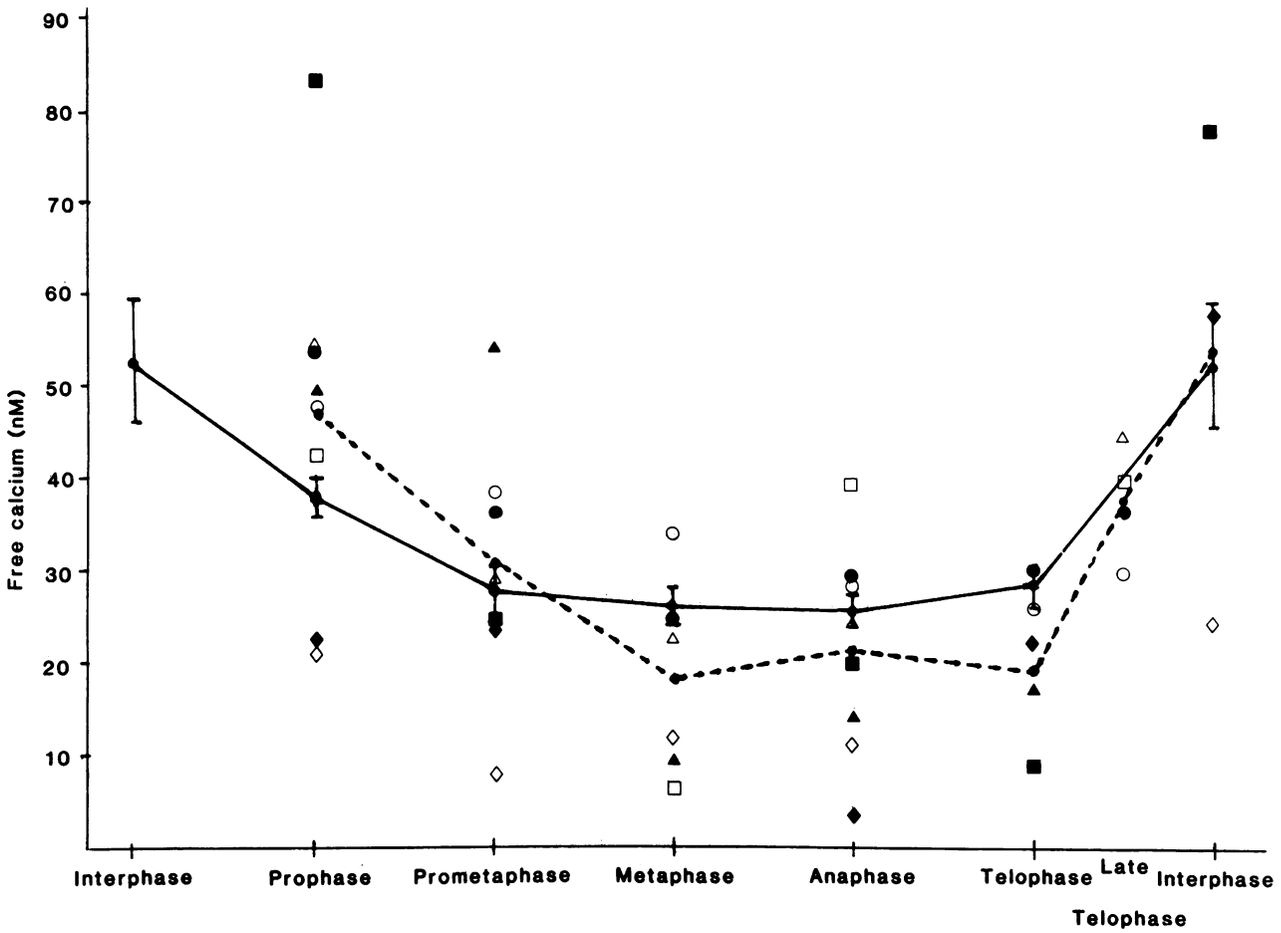

FIG. 2. Calcium levels, as determined from 340/360 ratios, of mitotic Pt K2 cells. Solid line represents the averaged calcium levels of a number of quin-2-loaded cells, selected as being in a given stage of mitosis by visual observation. Error bars represent SEM of the mean calcium value at that stage of mitosis. The individual graphics (circles, squares, triangles, and diamonds) represent calcium levels measured from individual cells as they pass through mitosis. Dashed line represents mean of seven individual time-course measurements.

A second potential artifact in these studies would be systematic changes in cellular autofluorescence in dividing cells, due to the inclusion of various amounts of fluorescent substances in the measuring field. However, measurements of the fluorescence of mitotic Pt K2 cells that had not been loaded with quin-2 revealed that there is no statistically significant variation of autofluorescence, expressed either as a 340/360 ratio or as an absolute 340-nm fluorescence, during the course of mitosis.

A third source of potential error in these measurements arises because the calcium levels being detected are low relative to the binding constant of quin-2 for calcium (25%–50% of the dissociation constant), so the fluorescence intensities are fairly low, and random errors of measurement may be substantial. The error from this cause is probably part of the reason that the single-cell measurements show so much experimental scatter. However, when time-course measurements from a number of cells are averaged together, this type of randomly distributed error is averaged out, so that calcium levels are reliably indicated. In multicell measurements many measurements are taken at each stage of mitosis, so this source of potential error is overcome.

As with all studies using quin-2, artifacts might also arise if quin-2 fluorescence does not accurately reflect cytoplasmic calcium. This might occur either if quin-2 were differentially compartmentalized within the cell or if the intracellular ionic milieu differed significantly from that of the calibration buffer. The available published evidence indicates, however, that quin-2 is free in the cytoplasm of loaded cells and is not significantly taken up by organelles such as mitochondria, so that quin-2 fluorescence measures free cytoplasmic calcium (27, 29). In addition, analysis of our 360-nm images of interphase and mitotic cells (e.g., see Fig. 1) reveals a uniform distribution of quin-2 within the cytoplasm. Variations in the concentrations of intracellular Mg^{2+}, Na^+, or H^+ might also affect quin-2 fluorescence ratios. However, studies of quin-2 fluorescence ratios under a variety of such ionic conditions reveal only small changes (26), which would not alter the general form of these results.

The mechanisms responsible for chromosome translocation during mitosis have been studied for many years, and a number of models for mitotic mechanisms have been proposed. Although these models differ significantly in their proposal for the force-generating mechanism, or motor, behind chromosome movement, most of them are consistent with the hypothesis that the dynamic equilibrium of microtubules is linked to chromosome movement (32). Thus, most current mitotic models would stipulate that local depolymerization of microtubules would either drive or act as a governor for chromosome translocation (32). Because microtubules are known to be sensitive to micromolar calcium in crude extracts (4), and because calmodulin both is localized in the mitotic spindle (12, 13) and can restore micromolar calcium sensitivity to purified microtubules (5–7), calcium is an excellent candidate for a regulator of microtubule equilibria during mitosis.

In solution, calmodulin, like many intracellular calcium binding proteins, shows affinities for calcium that are 10^{-5} to 10^{-6} M (1, 2, 33). However, when bound to another protein, calmodulin's calcium affinity can be considerably higher

(34), so the intracellular changes in calcium in the range of pCa ≈7 might well control its activity as far as microtubules are concerned. Recent observations have shown that calcium concentrations in this range control the balance of beating between the two flagella of demembranated models of *Chlamydomonas* (35).

It is possible that the observed decrease in free intracellular calcium reflects the sequestration of free intracellular calcium into a form or compartment not sampled by indicator. Some of this sequestered calcium might then be released locally, to achieve spatially limited control of microtubular equilibria. Such a model would be quite consistent with observations that mitotic spindles contain numerous membrane-bound vesicles or tubular elements that contain and actively concentrate calcium (8, 19, 20). Under such a scheme, mitosis would be controlled by local release of calcium ion, generating local calcium concentrations up to a micromolar level, while overall calcium levels in the cell go down. These locally released calcium ions would transiently associate with effector proteins, such as calmodulin, which would act on the spindle to drive mitosis. At the same time, the low overall calcium concentration would tend to stabilize the rest of the spindle and would favor spindle formation in the early states of mitosis. The quin-2 ratio method, used on the microscope stage together with image processing techniques, gives us the ability to study local changes in free intracellular calcium. Future studies may use this method to determine whether localized calcium release occurs during the course of mitosis.

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