Microinjection of Ca\textsuperscript{++}-Calmodulin Causes a Localized Depolymerization of Microtubules

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ABSTRACT The microinjection of calcium-saturated calmodulin into living fibroblasts causes the rapid disruption of microtubules and stress fibers in a sharply delimited region concentric with the injection site. This effect is specific to the calcium-bearing form of calmodulin; neither calcium-free calmodulin nor calcium ion at similar levels affects the cytoskeleton. If cells have previously been microinjected with calcium-free calmodulin, elevation of their intracellular calcium levels to 25 mM potentiate the disruption of microtubules throughout the cytoplasm. Approximately 400 mM free calcium is required to cause an equivalent disruption in uninjected cells. The level of calmodulin necessary to disrupt the full complement of cellular microtubules is found to be approximately in 2:1 molar ratio to tubulin dimer. These results indicate that calmodulin can be localized within the cytoplasm in a calcium-dependent manner and that it can act to regulate the calcium lability of microtubules at molar ratios that could be achieved locally within the cell. Our results are consistent with the hypothesis that calmodulin may be controlling microtubule polymerization equilibria in areas of high local concentration such as the mitotic spindle.

A large variety of cellular events including mitosis, cell elongation, and neurite outgrowth are believed to be related to the polymerization and depolymerization of microtubules in vivo (1). However, the mechanisms by which these events might be regulated are only beginning to be understood, with the bulk of the evidence drawn from in vitro biochemical studies.

A number of investigators (2) have found that microtubules in crude extracts are depolymerized by concentrations of calcium much lower than those required to depolymerize tubules made from purified microtubule proteins. This disparity suggested that some factor in crude extracts “sensitized” the tubules to calcium; calmodulin was an obvious candidate for this function. When calmodulin was added to purified microtubule proteins, Marcum et al. (3) found that the calcium concentration required for microtubule depolymerization was decreased by two orders of magnitude, to approximately the level observed in crude extracts. In addition it was found that the inhibition of microtubule polymerization was proportional both to calmodulin and to calcium concentrations (3). While this interaction occurs at calmodulin:tubulin in ratios much higher than the ratios of calmodulin:enzyme necessary to activate cellular enzymes, at least two factors argue in favor of its physiological relevance. First, the immunocytochemical localization of calmodulin between the chromosomes and spindle poles of mitotic cells (4, 5) and at the postsynaptic termini of basal ganglion neurons (6) correlates with the locations in the cell where microtubule depolymerization might be expected to occur. Second, it has been shown recently that increased calmodulin:tubulin ratios in transformed cells correlate well with the decreased levels of polymerized microtubules found in these same cells (7). It was therefore hypothesized that the in vitro system represented an incomplete system for the regulation of microtubule polymerization, lacking some factor present in the cell, and accordingly required these anomalously high ratios of calmodulin to tubulin (8). In support of this hypothesis, Schliwa et al. (9) found that microtubules in detergent-permeabilized cells show Ca\textsuperscript{++}-sensitivity comparable to that shown by microtubules in crude extract.

Here we present direct evidence pertaining to the interaction of calmodulin and microtubules in the living cell. By using pressure microinjection, we have introduced calmodulin into living fibroblastic cells at levels roughly stoichiometric with cellular tubulin. Calcium-calmodulin complexes, but not calcium-free calmodulin, potentiated depolymerization of microtubules. This disruption was strictly localized around the site of microinjection, indicating that the microinjected cal-
Calmodulin is tightly bound to some relatively immobile element of the cytoplasm. Immunocytochemical localization of calmodulin in injected cells revealed that the calcium-saturated form is localized in a pattern similar to the pattern of microtubule disruption. Stress fibers were also disrupted or disturbed by the injection of Ca"++-calmodulin, but intermediate filaments were unaffected. Experiments in which the calcium levels of the injection fluid and of the cell as a whole were manipulated indicate that the localization of the microinjected calmodulin is controlled by calcium concentrations.

**MATERIALS AND METHODS**

Calmodulin was the gift of Dr. C. B. Klee (National Institutes of Health). Antivimentin antiserum and monoclonal anti-a-tubulin were the gifts of Drs. E. Wang (The Rockefeller University) and S.Blobe (Cold Spring Harbor Laboratories), respectively. Rabbit antitubulin serum was raised in rabbits against phosphocellulose-purified tubulin (10) which was further purified by preparative PAGE. Nitrobenzoxxazoliodial(1NBD)-phallacidin was obtained from Molecular Probes (Plano, TX). Sheep anticalmodulin antibody was purchased from CABAEC (Inc. (Houston, TX). Fluorescently labeled secondary antibodies were obtained from Antibodies, Inc. (Davis, CA) or from Cappel Laboratories (WestChester, PA). Ovalbumin and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO), tetramethylrhodamine or fluorescein isothiocyanate (TRITC and FITC) from Research Organics and the collagen ionophore A23187 from Calbiochem-Behring Corp. (SanDiego, CA) (as the Ca'-Mg.2 (WestChester, PA). Ovalbumin and BSA were obtained from Antibodies, Inc. (Davis, CA) or from Cappel Laboratories (WestChester, PA). Ovalbumin and BSA were mixed with the injection solution as a tracer for injected cells. For double immunofluorescence staining was intended, FITC- or TRITC- coverslips (No. 1, 25-mm diameter) and were used 1-2 d after plating.

**Cell Culture and Fluorescent Staining:**

Microinjection and Fluorescent Staining: Micropipette-mediated microinjection was performed as described by Graneman and Graneman (11), using micropipettes with a tip diameter of ~1 μm. In all cases, except where double immunofluorescence staining was intended, FITC- or TRITC-BSA was mixed with the injection solution as a tracer for injected cells. For double-label studies, all cells in a marked region were injected. After injection, cells to be stained for indirect antitubulin immunofluorescence were fixed for 15 min in 3% formaldehyde solution in Dulbecco's phosphate-buffered saline (PBS). Coolant for indirect antitubulin immunofluorescence staining were unfixed. In both cases, cells were permeabilized in methanol at 20°C and rehydrated in PBS. Staining with antitubulin or antivimentin first antibodies was performed at antibody concentrations of ~50 μg/ml (serum antibodies) or 20 μg/ml (monoclonal antibody) for 30 min. After washing in PBS, fluorescent second antibodies were added at 100 μg/ml, and incubated for 30 min. The patterns of tubulin staining obtained with serum antitubulin and monoclonal anti-a-tubulin were identical, except that serum antitubulin stained the cell nucleus. (This staining of the cell nucleus by serum antitubulins has been described by other workers and can be adsorbed out independent of the antibody's activity against tubulin.) After final washing, the coverslips were mounted in a solution of 16% (wt/vol) polyvinyl alcohol (Sigma type II; Sigma Chemical Co., St. Louis, MO) in PBS containing 33% glycerol. Actin staining was performed after actin permeabilization by rehydration in NBD-phallacidin (0.03 μM in PBS) as described by Bank et al. (12).

To fix and stain microinjected calmodulin, we found it necessary to use glutaraldehyde as our primary fixative; injected cells fixed in formaldehyde gave no staining beyond that of uninjected ones. The procedure employed was modified from that described by Osborn and Weber (13) and is as follows: cells were fixed in 1% glutaraldehyde in PBS for 15 min at room temperature, and were then permeabilized in ~20°C methanol. Next, they were treated with four changes of fresh 0.5 mg/ml NaBH4 in PBS. Finally, they were washed extensively in PBS and treated with anticalmodulin antibody (100 μg/ml), followed by fluorescein-labeled second antibody (100 μg/ml), and were mounted as described above.

Fluorescence photomicrography on Kodak Tri-X film was performed with a Leitz Ortholux microscope, equipped with an epifluorescence illuminator and an Orthomat automatic camera.

**Quantitation of Injection Volumes:** In many experiments, the amount of calmodulin microinjected into each cell was determined on the basis of the fluorescence emission intensity of a tracer co-injected along with the calmodulin. This procedure is a modification of the method of Seeley et al. (14).

To use this technique to accurately quantify the amount of calmodulin injected into a cell that had been fixed and processed for immunofluorescence, we found it necessary to determine the amount of tracer fluorescence that was retained during processing. Accordingly, cells were grown on grided coverslips, and one cell per grid square was injected with a mixture of FITC-BSA and TRITC-ovalbumin (each 1 mg/ml). After 20 min, the microinjected living cells were located in each grid square by fluorescence, and their net rhodamine and fluorescein fluorescence emission intensities were measured using a Leitz MPV microscope fluorometer. The cells were then fixed and sham processed as if for indirect antitubulin immunofluorescence, using unlabeled second antibody, and they were remeasured in the same fashion as before. In this manner, it was found that 48 ± 5% of FITC-BSA fluorescence is retained during processing, whereas only a small fraction (~10%) of TRITC-ovalbumin fluorescence is retained. Accordingly, in subsequent experiments when quantification of injection volume was intended, FITC-BSA was used as tracer, and its measured fluorescence was multiplied by a factor of 2.05, to give an estimate of the amount of FITC-BSA actually injected into the cell.

In experiments where the effect of a known amount of microinjected calmodulin was to be determined, the solution to be injected was mixed with a known amount of FITC-BSA. After microinjection and processing of the cells for immunofluorescence (using rhodamine-labeled second antibody), the tracer fluorescence from each injected cell was measured, and the condition of the cytoskeleton was noted; the entire sequence was also videotaped to give a permanent record. After scanning all the microinjected cells, using the same apertures, the fluorescence of a series of FITC-BSA dilutions in PBS (pH 7.4) was measured in a hemocytometer, and the diameters of the (matched) illuminating and measuring apertures were then measured by comparison to a stage micrometer.

To establish that, under the convergent illumination geometry of the epifluorescence microscope, measurements taken of a solution in a 100-μm hemacytometer could be compared to measurements on a cell, the same set of microinjected cells were scanned with a 10X, 0.25 NA objective and a 63X, 1.4 NA objective. Using the same objectives and aperture settings, the fluorescence intensity from out FITC-BSA standards was measured, and the amount injected into the cell was calculated on the basis of measurements taken with each objective. The correlation between the calculated injection amounts as determined with the two objectives was excellent. Since the illumination path from a 1.4 NA objective is very highly convergent, and that from a 0.25 NA objective is not, the correlation of these sets of results indicates that illumination geometry is not an important factor when measuring the emission of a uniform solution in a hemocytometer.

In a typical experiment, the average volume injected per cell was 2.76 ± 0.9 pl. On the basis of the measurement the diameter of detached cells we can calculate a cell volume of ~20 pl. Thus the injection volume is approximately 14% of the cell's volume.

**Determination of Intracellular Calmodulin:**

Determination of the intracellular calmodulin in unperturbed gerbil fibroblasts was performed by radioimmunounassay (RIA), using a calmodulin radioimmunounassay kit purchased from New England Nuclear Laboratories. Cells were homogenized in an assay buffer identical to the one provided, except that it contained 1 mM phenylmethylsulfonyl fluoride and 1 mM aprotinin. The accuracy of the determination was checked both internally, by adding known amounts of calmodulin to aliquots of the cell lysate, and externally, by performing parallel determinations on a cell type (Swiss mouse 3T3) whose calmodulin levels have been determined by other workers. It was found that determinations performed using the calmodulin standards provided with the kit consistently gave calmodulin levels that were forty times lower than those reported in the literature. After discussions with the supplier it was reported (New England Nuclear technical bulletin number 2.5M1285-2102) that the antibody supplied with the RIA kit is standardized against calmodulin that has been heat denatured. The presence of EGF in the medium was measured using native calmodulin standards gave calmodulin levels of 100 fg calmodulin per Swas 3T3 and 340 fg calmodulin per gerbil fibroblast.
Tubulin dimer levels in gerbil fibroma cells were determined by the [\textsuperscript{3}H] colchicine rapid filter binding method for tubulin (15), corrected for the time delay of colchicine binding activity as described by Bamberg et al. (16).

RESULTS

To investigate the intracellular effects of calmodulin on the cytoskeleton, we microinjected calmodulin into gerbil fibroma cells. After various periods of incubation, we assayed the effects of this treatment on cytoskeletal elements by indirect immunofluorescence staining techniques.

The injection of calmodulin (0.10–0.24 mM, giving 0.3–0.6 fmol of added calmodulin per cell) and calcium chloride (0.5–1 mM, yielding ~4 Ca\textsuperscript{2+}/calmodulin) caused the rapid (within 5 min) loss of microtubules in a limited area around the injection site. The radius of the area of microtubule depolymerization was sharply defined, with the free ends of the fibrils readily apparent at the margins (Figs. 1 and 3b). The area of loss had a diameter of 100 \textmu m in a cell 200 \textmu m across (Fig. 1). Microtubules outside the affected area were normal in appearance. In a typical experiment, of 40 cells injected with 0.1 mM calcium-saturated calmodulin plus tracer FITC-BSA, 22 were later identified by their tracer fluorescence. Of these cells, two showed no disruption of their microtubules, 18 showed local disruption, and two showed disruption globally. This disruption of microtubules persisted for at least 75 min after injection (Fig. 2), demonstrating that the effect was not a transient artifact of the process of microinjection. At 20 min postinjection, the stress fibers were also disrupted around the site of injection, albeit in a much smaller radius than the microtubules (Fig. 3a). The vimentin filaments remained polymerized, although their arrangement may have been disturbed (Fig. 4).

If the perturbation of the microtubular cytoskeleton is Ca\textsuperscript{2+}-calmodulin dependent, the injection of calcium-free calmodulin should have no effect on the microtubules. To test this hypothesis, we used calmodulin which had been pre-equilibrated with EGTA (1.0 mM). Observation of cells injected with this material demonstrated that calcium-free calmodulin has no effect on the microtubules (Fig. 5). Similarly, injection of CaCl\textsubscript{2} (1 mM) caused no disruption of the microtubules or

![Figure 1](image1.jpg)

**Figure 1.** Antitubulin immunofluorescent staining of a gerbil fibroma cell, 20 min after injection of 200 \textmu M calmodulin + 1 mM CaCl\textsubscript{2}. Microtubules are disrupted in a sharply delimited area surrounding the injection site. Bar, 20 \textmu m. \times 440.

![Figure 2](image2.jpg)

**Figure 2.** Antitubulin immunofluorescence staining of a gerbil fibroma cell 75 min after its injection with 200 \textmu M calmodulin + 1 mM CaCl\textsubscript{2}. A total of 6 \times 10\textsuperscript{-12} gm (0.35 femtomole) of calmodulin was injected into this cell. Note that both the disruption of the microtubules and the localization of this effect around the injection site persist even with 75 min incubation. Bar, 20 \textmu m. \times 500.
stress fibers, indicating that cytoskeletal breakdown was due neither to a direct effect of calcium nor to an artifact of microinjection (Fig. 6). In addition, the injection of balanced Ca**-EGTA solutions, containing 10 mM each of CaCl2 and Na2EGTA, or of a similar solution containing 25 μM excess CaCl2, had no effect on the microtubular cytoskeleton of these cells. To determine whether the absence of change on the injection of Ca**-free calmodulin was due solely to the lack of calcium ions, we exposed cells which had been previously injected with EGTA-calmodulin to calcium ionophore A23187 (5 μM) for 5 min in the presence of 25 μM free calcium. This treatment caused microtubule disruption throughout the entire cell (Fig. 7a), rather than locally around the injection site. Stress fibers were also lost throughout the cell with only occasional, wispy elements seen (data not shown). Uninjected cells in the same dish showed normal microtubular cytoskeletons (Fig. 7b). Uninjected cells required 400 μM Ca** for microtubule depolymerization, but tubules in injected cells were depolymerized by 10–25 μM Ca**. When cells that had been injected with Ca**-calmodulin were exposed to ionophore and calcium in the same manner as those injected with EGTA-calmodulin, microtubule depolymerization was still limited to an area around the injection site (Fig. 7c).

To learn whether calcium-saturated calmodulin is localized in the same manner as the cytoskeletal disruption, cells were injected with calcium-saturated calmodulin (0.25 mM) in the presence of A23187 and external calcium (0.1 mM), incubated for 20 min, and processed to visualize calmodulin immunocytologically. The visualization of calmodulin localization required this maintenance of high intracellular calcium concentrations during incubation and fixation procedures using calcium ionophore and external calcium. Under these conditions, the distribution of calmodulin visualized was localized around the injection site in a pattern similar to the pattern of microtubule disruption observed in other experiments (Fig. 8).

To accurately correlate the extent of microtubule depolymerization induced with the amount of calmodulin injected, we injected cells first with calcium-free calmodulin, mixed with a known amount of tracer FITC-BSA. After 1–2-h incubation, the cells were exposed to calcium ionophore (A23187-5 μM) and external calcium (25 μM) for 5 min. After fixation and processing for antitubulin immunofluorescent staining of the same gerbil fibroma cell, 20 min after the injection of 240 μM calmodulin + 1.2 mM CaCl2. The disruption of stress fibers occurs in a smaller region than the loss of microtubules. Bars, 20 μm. x 360.

FIGURE 3 (a) NBD-phallacidin and (b) antitubulin immunofluorescent staining of the same gerbil fibroma cell, 20 min after the injection of 240 μM calmodulin + 1.2 mM CaCl2. The disruption of stress fibers occurs in a smaller region than the loss of microtubules. Bars, 20 μm. x 360.

FIGURE 4 Antivimentin immunofluorescent staining of a gerbil fibroma cell, 20 min after injection with 240 μM calmodulin + 1.2 mM CaCl2. No loss of intermediate filaments was observed following injection of calcium-saturated calmodulin. Bar, 20 μm. x 545.
cence, a second investigator scored the cells for microtubule disruption, and then measured the tracer fluorescence from each cell. By varying the volume and concentration of the material injected, a range of measured calmodulin levels from 1 to 15 pg/cell was assayed. When the extent of microtubule disruption was analyzed relative to the amount of calmodulin injected, it was found that above a threshold of ~7 pg (0.4 fmol) of injected calmodulin, most cellular microtubules were disrupted.

The colchicine binding assay for tubulin revealed that gerbil fibroma cells contain, on the average, 0.2 fmol of tubulin dimer per cell, which is approximately four times the amount found by radioimmunoassay in the considerably smaller Swiss mouse 3T3 fibroblasts (17).

**DISCUSSION**

Precise local control of microtubule disassembly is required by models of mitotic mechanisms (18) that invoke depolymerization of spindle fibers at the poles during mitosis. Similar controls might be required to mediate the breakdown of axonally transported microtubules at the nerve endings. The finding of Weisenberg (2) that calcium inhibited microtubule assembly in vitro suggested that the control mechanisms might be calcium-mediated. However, the concentrations of calcium required (1 mM) are far higher than the free calcium concentrations in cells, and the ion would be expected to diffuse rapidly through the cytoplasm requiring either that it be locally released at very high concentration or that it be restricted to the site of action. If the actions of calcium were to be mediated by a calcium-binding protein such as calmodulin, however, the concentrations of calcium needed would be expected to be lower by almost two orders of magnitude based on studies on microtubules in vitro (3).

Our observation that microinjected Ca**\(^{2+}\)**-calmodulin potentiates the depolymerization of microtubules in a sharply defined radius around the injection site implies both the limitation of calmodulin mobility and that the effect on microtubule polymerization has a sharp threshold. This is precisely the behavior that would be required of a local regulator of microtubule disassembly. The distribution of calcium-saturated calmodulin visualized by immunohistochemical techniques is also localized around the site of injection, although the boundaries of the area are not so sharp as the boundaries of the region of cytoskeletal disruption.
When Ca**-free calmodulin was injected into cells, followed by exposure to 25 μM Ca** and calcium ionophore A23187, we found that the microtubules were depolymerized throughout the cell. Cells injected with Ca**-calmodulin followed by exposure to A23187 and 25 μM Ca** still had depolymerization in a limited region. This implies that the Ca**-free calmodulin has greater mobility within the cell than the calcium-saturated form.

As shown in Fig. 3, Ca**-calmodulin also disrupts cellular stress fibers in a limited region around the site of injection. That the injection of free calcium alone shows no similar effect indicates that this effect is not due merely to mechanical stresses induced by microinjection, or due to a calcium-activated protease. It is not known whether the observed disruption represents the depolymerization or merely the spatial reorganization of the stress fibers. To our knowledge, no studies performed in vitro have shown an effect of calmodulin on the polymerization state of actin. However, recent studies have demonstrated that the actin-binding proteins fodrin, spectrin, and TW240 all share a conserved calmodulin-binding site (19); these studies, combined with our own observations, suggest that calmodulin might act to regulate stress fibers in intact systems.

Our experiments indicate that the injection of ~7 pg (0.4
fmol) of calcium-free calmodulin is sufficient to potentiate the disruption of the full complement of cellular microtubules when calcium is added later. Our measurements of tubulin dimer levels in gerbil fibroma cells, using the time-corrected colchicine binding assay, indicate that these cells contain ~0.2 fmol of tubulin dimer per cell. Therefore we can conclude that microinjected calmodulin potentiates the disruption of cellular microtubules in response to moderate levels of free calcium at a 2:1 molar ratio of calmodulin to tubulin dimer. Since in microinjection experiments only the largest cells are chosen, whereas the tubulin levels are a culture-wide average: the actual molar ratio in the cell is probably closer to 1:1. On the basis of in vitro studies, Marcum et al. (3) found that a calmodulin: tubulin ratio of 8:1 was necessary to drive microtubule depolymerization. The difference between the results in vitro and in the cell may reflect the fact that the microtubular cytoskeleton in the cell contains factors (perhaps those elements responsible for Ca**+-calmodulin immobilization), not present in purified tubulin preparations, which participate in the regulatory process.

The amount of calmodulin (7 pg) that we must microinject to fully disrupt the microtubular cytoskeleton is in considerable excess over the cell's native calmodulin (0.34 pg). These results suggest that when calmodulin is evenly distributed in the cytoplasm (as in interphase) it would have little effect on the extent of microtubule depolymerization. However, if high local concentrations of calmodulin can be achieved, with calmodulin: tubulin ratios of approximately 1:1, then calcium-calmodulin control of microtubule depolymerization can occur at physiological calcium levels. For example, during mitosis, when the calmodulin may be playing a role in potentiating microtubule depolymerization, it is condensed in the mitotic spindle (4, 20), which occupies a small portion of the cell's volume. The observation that Ca**+-calmodulin disrupts microtubules in a limited region indicates that there is a mechanism in cells to localize activated (Ca**+-saturated) calmodulin, as would be required to achieve high local concentrations.

In summary, we found that Ca**+-calmodulin produces a localized loss of microtubules at locally attainable molar ratios of calmodulin to tubulin, that the calcium form of calmodulin appears to be relatively immobilized within the cytoplasm, and that the calcium-free form is more mobile. Taken together with existing data on the effects of calmodulin on microtubules in vitro and with the reports of calmodulin localization at putative sites of microtubule disassembly in cells (4, 5), these findings argue strongly for the role of calmodulin in the regulation of microtubule-mediated functions in the cell.

This research was supported by grants from the American Cancer Society (CD-129) and the National Institutes of Health (NIH) (NS 15076) to M. L. Shelanski and from the NIH (AM 27083) and the Irma T. Hirschl Charitable Trust to F. R. Maxfield.

Received for publication 7 September 1982, and in revised form 11 August 1983.

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