

Calmodulin Stabilization of Kinetochore Microtubule Structure to the Effect of Nocodazole

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Abstract. To investigate the function of calmodulin (CaM) in the mitotic apparatus, the effect of microinjected CaM and chemically modified CaMs on nocodazole-induced depolymerization of spindle microtubules was examined. When metaphase PtK₁ cells were microinjected with CaM or a CaM-TRITC conjugate, kinetochore microtubules (kMTs) were protected from the effect of nocodazole. The ability of microinjected CaM to subsequently protect kMTs from the depolymerizing effect of nocodazole was dose dependent, and was effective for ~45 min, with protection decreasing if nocodazole treatment was delayed for more than 60 min after injection of CaM.

The CaM-TRITC conjugate, similar to native CaM, displayed the ability to activate bovine brain CaM-dependent adenylate cyclase in a Ca⁺⁺-dependent manner and showed a Ca⁺⁺-dependent mobility shift when subjected to PAGE. A heat-altered CaM-TRITC con-

jugate also protected kMTs from the effect of nocodazole. However, this modified CaM was not able to activate adenylate cyclase nor did it display a Ca⁺⁺-dependent mobility shift when electrophoresed. In a permeabilized cell model system, both CaM analogs were observed to bind to the spindle in a Ca⁺⁺-independent manner.

In contrast, a performic acid-oxidized CaM did not have a protective effect on spindle structure when microinjected into metaphase cells before nocodazole treatment. The oxidized CaM did not activate adenylate cyclase and did not exhibit Ca⁺⁺-dependent mobility on polyacrylamide gels.

These results are interpreted as supporting the hypothesis that CaM binds to the mitotic spindle in a Ca⁺⁺-independent manner and that CaM may serve in the spindle, at least in part, to stabilize kMTs.

SINCE the discovery of calmodulin (CaM)¹ in the mitotic spindle (Welsh et al., 1978), many investigators have speculated about its role there. Several groups have shown that CaM can potentiate the depolymerizing activity of Ca⁺⁺ on microtubules (MTs) in vitro (Marcum et al., 1978; Job et al., 1981). Because of these observations, the most prominent hypothesis concerning CaM's role is that it acts in the spindle to regulate MT disassembly (for review see Welsh and Sweet, 1988).

This hypothesis is supported by studies which demonstrate that raising intracellular Ca⁺⁺, by direct microinjection or treatment of cells with caffeine, will cause the depolymerization of cellular MTs (Kiehart, 1981; Salmon and Segall, 1980). Microinjection of Ca⁺⁺-bound CaM has been reported to depolymerize cytoplasmic MTs in interphase cells (Keith et al., 1983) and a recent paper suggests that Ca⁺⁺/CaM injection can prolong metaphase in mitotic cells, ostensibly by causing a transient depolymerization of spindle MTs. (Keith, 1987).

1. *Abbreviations used in this paper:* BSA-TRITC, TRITC-conjugated bovine serum albumin; CaM, calmodulin; CaM-TRITC, TRITC-conjugated calmodulin; HA-CaM-TRITC, heat-altered TRITC-conjugated calmodulin; kMT, kinetochore microtubule; MA, mitotic apparatus; MT, microtubule; nkMT, nonkinetochore microtubule.

Conversely, there are many reports of CaM association with MT structures that are not exhibiting net disassembly. The distribution of CaM in the mitotic apparatus (MA) is identical to that of the kinetochore MTs (kMTs), which are preferentially stable to the depolymerizing conditions of cold (Welsh et al., 1979). CaM has been found in association with the cold-stable subset of the cytoplasmic MT complex of interphase cells and with interphase MTs that are repolymerizing after release from cold treatment (Deery et al., 1984). Furthermore, CaM is present on taxol-stabilized MT structures (DeBrabander et al., 1986).

In this report we describe experiments which suggest that CaM may have Ca⁺⁺-independent stabilizing effects on the MA. When CaM was injected into living metaphase PtK₁ cells, the kMTs were resistant to the effect of the MT inhibitor, nocodazole. Because similar results were obtained with an apparently Ca⁺⁺-insensitive analog of CaM, we also examined the Ca⁺⁺ dependence of the incorporation of CaM into the MA. In a permeabilized cell system, CaM assumed its usual distribution in the mitotic spindle in the absence of Ca⁺⁺. These data support the hypothesis that the interaction of CaM with mitotic MTs is not dependent on the presence of Ca⁺⁺, and that CaM has a Ca⁺⁺-independent stabilizing effect on kMT structure.

Materials and Methods

Cell Culture

PtK₁ cells were a gift from Dr. J. Richard McIntosh, University of Colorado, Boulder, CO. They are grown in Ham's F12 medium supplemented with 10% characterized or defined FBS (HyClone Laboratories, Logan, UT) in a 37°C humidified incubator with a 5% CO₂, 95% air atmosphere. Culture medium is changed daily.

Protein Preparation

CaM was purified from bovine testis by Ca⁺⁺-dependent phenyl-Sepharose-affinity chromatography (Gopalakrishna and Anderson, 1982). TRITC (Research Organics, Inc., Cleveland, OH) was coupled to CaM (CaM-TRITC) as described by Welsh (1983). TRITC was conjugated to BSA (BSA-TRITC) in a similar fashion.

Heat-altered CaM-TRITC (HA-CaM-TRITC) was prepared by boiling a 2 mg/ml solution of CaM-TRITC in PBS, pH 12, for 2 h. The resulting solution was dialyzed against PBS, 1 mM Ca⁺⁺, pH 7.0, and mixed with 1 ml of phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) which had been equilibrated against the PBS/Ca⁺⁺ buffer. The mixture was gently centrifuged and the supernatant was collected and designated heat-altered (i.e., it failed to bind to phenyl-Sepharose in a Ca⁺⁺-dependent manner).

Performic acid-oxidized CaM was made by mixing 1.0 ml formic acid (Aldrich Chemical Co., Milwaukee, WI) with 0.1 ml 30% H₂O₂ (Mallinkrodt, Paris, KY) and incubating at room temperature for 15 min. 440 µl of this solution was added to 2 mg of lyophilized CaM and incubated at room temperature for 15 min. The reaction was stopped by addition of 2 ml H₂O, then frozen and lyophilized.

Before microinjection, all solutions were dialyzed against a buffer consisting of 40 mM KOH (Mallinkrodt), 100 mM monopotassium glutamate (Sigma Chemical Co., St. Louis, MO), 100 mM NaOH (Mallinkrodt), 1 mM EGTA (Sigma Chemical Co.), 1 mM MgSO₄ (MCB, Cincinnati, OH), adjusted to pH 7.0 with citric acid (Sigma Chemical Co.).

Protein concentrations in injection solutions were for native CaM, 2.0 mg/ml; CaM-TRITC, 2.0 mg/ml; HA-CaM-TRITC, 1.5 mg/ml; Performic acid-oxidized CaM, 2 mg/ml; BSA-TRITC, 1 mg/ml.

Antibodies and Immunofluorescence

Antibodies to tubulin (α , β dimer) were raised in sheep and affinity purified using an Affi-gel-15 (Bio-Rad Laboratories, Richmond, CA) tubulin-affinity column. Indirect immunofluorescence was performed essentially as described by Welsh (1983), using fluorescein-labeled rabbit anti-sheep IgG (Cappel Laboratories, Cochranville, PA) as a secondary antibody.

Microinjection

For microinjection, cells were grown on 25-mm-diam #1 thickness glass coverslips (VWR Scientific, San Francisco, CA) affixed with a silicone adhesive to the underside of a 35-mm-diam plastic tissue culture dish (Falcon Labware, Oxnard, CA) in which a 20-mm-diam hole had been bored. With this construction, we were able to perform microinjection, imaging, and immunofluorescence processing without remounting the coverslip.

Micropipettes were pulled from self-filling capillary tubes (model IB100F; World Precision Instruments, Inc., New Haven, CT) using a Sutter Instruments Sachs-Flaming PC-84 micropipette puller. Micropipettes were positioned with a micromanipulator (E. Leitz, Inc., Rockleigh, NJ). Injection was accomplished by applying pressure to a 50-ml syringe connected to the micropipette with Silastic™ tubing (model 602-175; Dow Corning Corp., Midland, MI).

Permeabilization

Cells were lysed by a method modified from Cande et al. (1981) in order to allow interaction of CaM-TRITC with the MA under conditions in which the Ca⁺⁺ concentration could be controlled. Cells were grown in the microinjection dishes described above. Before permeabilization, the culture was rinsed twice with 2 ml of a solution containing 85 mM Pipes (Research Organics, Inc.), pH 6.94 (buffer A), and either 10 µM Ca⁺⁺ (A/C) or 10 mM EGTA (A/E). The cells were then lysed for 90 s with 2 ml of A/C or A/E containing 0.08% Brij 58 (ICI Americas, Wilmington, DE). The lysis solution was removed and replaced with 20 µl of CaM-TRITC which had

been dialyzed against the lysis solution. The solution was spread over the dish by placing an 18-mm-diam coverslip over the drop. After 90 s, this solution was rinsed off with 2 ml of A/C or A/E and the cells were either observed immediately or fixed with 3% formaldehyde in A/C or A/E.

Imaging

Cells were viewed through a Leitz 63× 1.4NA phaco 4 plan apo fluorescence objective mounted on a Leitz Diavert microscope equipped for epifluorescence illumination. The microscope is mounted on a micro-G vibration isolation table to minimize movement during microinjection.

Images of cells were obtained with a DAGE/MTI, Inc. ISIT video camera (Michigan City, IN) coupled to the microscope through a Leitz vario-orthomat zoom lens adapter. Video output from the camera was digitized by an image processor (model 75; International Imaging Systems, Milpitas, CA), interfaced to a Masscomp DP500 computer. All images consisted of a sliding average over 16 video frames, with an out-of-focus image interactively subtracted to enhance contrast and reduce background. Processed images were photographed either directly from a 13" monitor (Mitsubishi Int'l Corp., New York, NY) or with a Matrix Instruments recorder equipped with a 35-mm film back.

Drug Treatments and Experimental Protocol

For nocodazole treatment, nocodazole (Sigma Chemical Co.) was dissolved in DMSO (Sigma Chemical Co.) at 100 µg/ml. Aliquots of this stock solution were added directly to the culture medium to achieve the desired concentration.

For each experiment, cells were removed from the incubator and placed on the microscope stage at room temperature (23 ± 2°C). The dish was then searched, in a predefined pattern, for cells which appeared to be in metaphase; these cells were injected with CaM or various analogs. After a 30-min microinjection period, the medium was replaced with medium containing 1 µM nocodazole, and the dish was left at room temperature for 15 min. Finally, cells in the dish were fixed at room temperature for 30 min with a solution of 3% formaldehyde (Fisher Scientific Co., Pittsburgh, PA) in phosphate buffer (Welsh, 1983) and processed for indirect tubulin immunofluorescence.

Calculation of Spindle Index Distributions

In a typical experiment 50–100 cells per dish were injected. Following tubulin immunofluorescence processing, the microinjection region was relocated, and injected cells in this region were scored with a "spindle index" value ranging from 0 to 3, based on the tubulin immunofluorescence appearance of their MA (see Results, Fig. 4). The fraction of injected cells in each spindle index category was calculated and these values were denoted the "spindle index distribution."

Injected cells were relocated in one of two ways. In the case of TRITC-labeled analogs, microinjected cells retained sufficient TRITC-tagged protein after immunofluorescence processing to allow their identification. In other cases (e.g., free TRITC or unlabeled protein) we were not able to unequivocally identify injected cells. For these treatments, all metaphase cells within a scribed area were microinjected and counted. After immunofluorescence processing, any mitotic cells found within this area that had not entered anaphase were scored. In these cases, the number of cells scored usually exceeded the number of cells initially injected. Because in previous experiments with labeled protein, we had determined that 85 ± 5% of injected cells were relocatable, we estimated the overcount in cases where we could not identify injected cells by subtracting 85% of the number of cells injected from the total number of cells counted. The overcount represents the number of uninjected cells in our raw count. Because we had previously determined the spindle index category distribution of uninjected cells, we could correct the count for each category by subtracting the fraction of the total overcount calculated to be in that category (based on the distribution for uninjected cells) from the raw count. The corrected counts were used to calculate the final distribution.

Correlative Electron Microscopy

Treated cells were located for EM in the following manner: an injected cell was located after fixation by the presence of fluorescent analog. A square was scribed around this cell with a micropipette and fluorescence and phase-contrast video images were taken. Before embedding for EM, the cell location was recorded using the cell list function of an ACAS 470 (Meridian

Instruments, Okemos, MI). After embedding, cells were relocated on the ACAS 470. A thin film of ink was placed on the bottom surface of the plastic and the laser of the ACAS 470 was used at sufficient power output to ablate a region of the ink circumscribing the regions in the plastic containing each cell of interest. These regions could be visually located without magnification and were then cut out and remounted for EM sectioning.

Adenylate Cyclase Assay

CaM and CaM analogs were assayed for the ability to stimulate partially purified CaM-sensitive adenylate cyclase from bovine cortex. 5 μ g of CaM or CaM analog and varying amounts of Ca^{++} were added to a reaction mixture containing 20 mM HEPES, pH 7.5; 3 mM MgCl_2 ; 0.5 mM EGTA; 2 mM cAMP; 4 mM phosphoenolpyruvate; 0.12 mM isobutyl methyl xanthine; 0.5 mM α - ^{32}P ATP (1 μCi /assay tube); 10 μ g pyruvate kinase and 3.5 μ g cyclase. After incubation at 30°C for 15 min, ^{32}P cAMP was isolated by Dowex-50 anion exchange chromatography followed by $\text{Zn}^{++}/\text{Ba}^{++}$ precipitations (Krishna et al., 1968).

Results

kMT Stabilization

When metaphase cells were treated for a short period of time with a moderate dose of nocodazole (1 μM for 15 min at $23 \pm 2^\circ\text{C}$), and then processed for tubulin immunofluorescence, the normal spindle structure was significantly altered (Fig. 1, compare *a* and *b*). The nonkinetochore MTs (nkMTs) were no longer apparent. A few MTs remained which were attached at one end to a kinetochore and apparently free at the other. They are presumed from their appearance to be remnants of kMTs. In general, no orientation of MTs toward recognizable spindle poles was observed. In serial EM sections, the only MTs observed were a few fragments located near or attached to the kinetochores (not shown). Fragments of MTs attached only at the pole end were not observed. When anaphase cells were treated under similar conditions, recognizable spindle structure was usually retained, and although it was difficult to determine whether the few MTs remaining in the pole to chromosome region were kMTs or nkMTs, at least some interzonal MTs, which are presumably precursors of the midbody, were retained (Fig. 1, compare *e* and *f*).

In contrast, metaphase cells which were injected with CaM-TRITC before nocodazole treatment and immunofluorescence processing show a different morphology. The distribution of MTs in these cells was not completely normal, but resembled cold-treated cells: nkMTs were not apparent, but the kMT arrays usually appeared normally oriented toward clearly defined pole regions (Fig. 1 *c*). Spindles were often, but not always, shorter than normal. When injected cells were examined by serial sectioning and EM, the same results were found: in nocodazole-treated cells that had been injected with CaM, nkMTs were not apparent but kMTs were retained; the kMTs appeared normal, and converged toward a pole region (Fig. 2). Mitotic spindles in cells which had been injected with CaM-TRITC but not treated with nocodazole appeared essentially normal (Fig. 3).

The appearances of the cells in Fig. 1, *a* and *c*, are the most common for the respective treatments. However, each population of cells contained cells whose kMTs ranged in appearance from completely disorganized to relatively normal. For this reason, we chose to analyze our experiments and controls by assigning each microinjected cell a value based on the tubulin immunofluorescence appearance of its kMTs

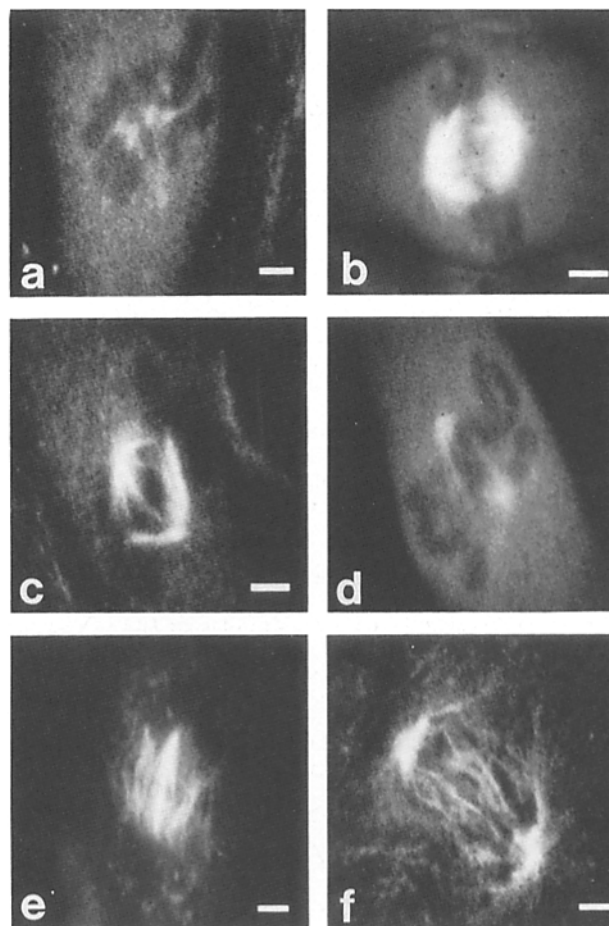


Figure 1. (a) Metaphase cell treated with nocodazole (1 μM , 15 min, $23 \pm 2^\circ\text{C}$). Tubulin immunofluorescence. (b) Metaphase control cell. Tubulin immunofluorescence. (c and d) Metaphase cell injected with CaM-TRITC before treatment with nocodazole. (c) Tubulin immunofluorescence. (d) CaM-TRITC fluorescence. (e) Anaphase cell treated with nocodazole. Tubulin immunofluorescence. (f) Anaphase control cell. Tubulin immunofluorescence. Bars, 5 μm .

(Fig. 4). We then prepared histograms of these values for spindles found in each treatment group in order to assess the effects of the treatments on the population as a whole.

Fig. 5 illustrates the results of an analysis of several cell populations. When cells were not injected, or were injected with free TRITC, BSA-TRITC, or with performic acid-oxidized CaM, and then treated with nocodazole, the most common morphology observed was a completely disorganized spindle (Fig. 5 *a*). On the other hand, when cells were injected with native CaM, CaM-TRITC, or HA-CaM-TRITC, the most common configuration had kMT arrays that appeared essentially normal (Fig. 5 *b*). When these distributions were analyzed with a chi-squared analysis, it was found that cultures microinjected with native CaM, CaM-TRITC, and HA-CaM-TRITC differed significantly from microinjected controls (free TRITC, BSA-TRITC, or oxidized CaM) at the 99% confidence level. The chi-squared statistics from comparisons between the distributions observed for native CaM, CaM-TRITC, and HA-CaM-TRITC did not indicate significant differences at the 90% confidence level.

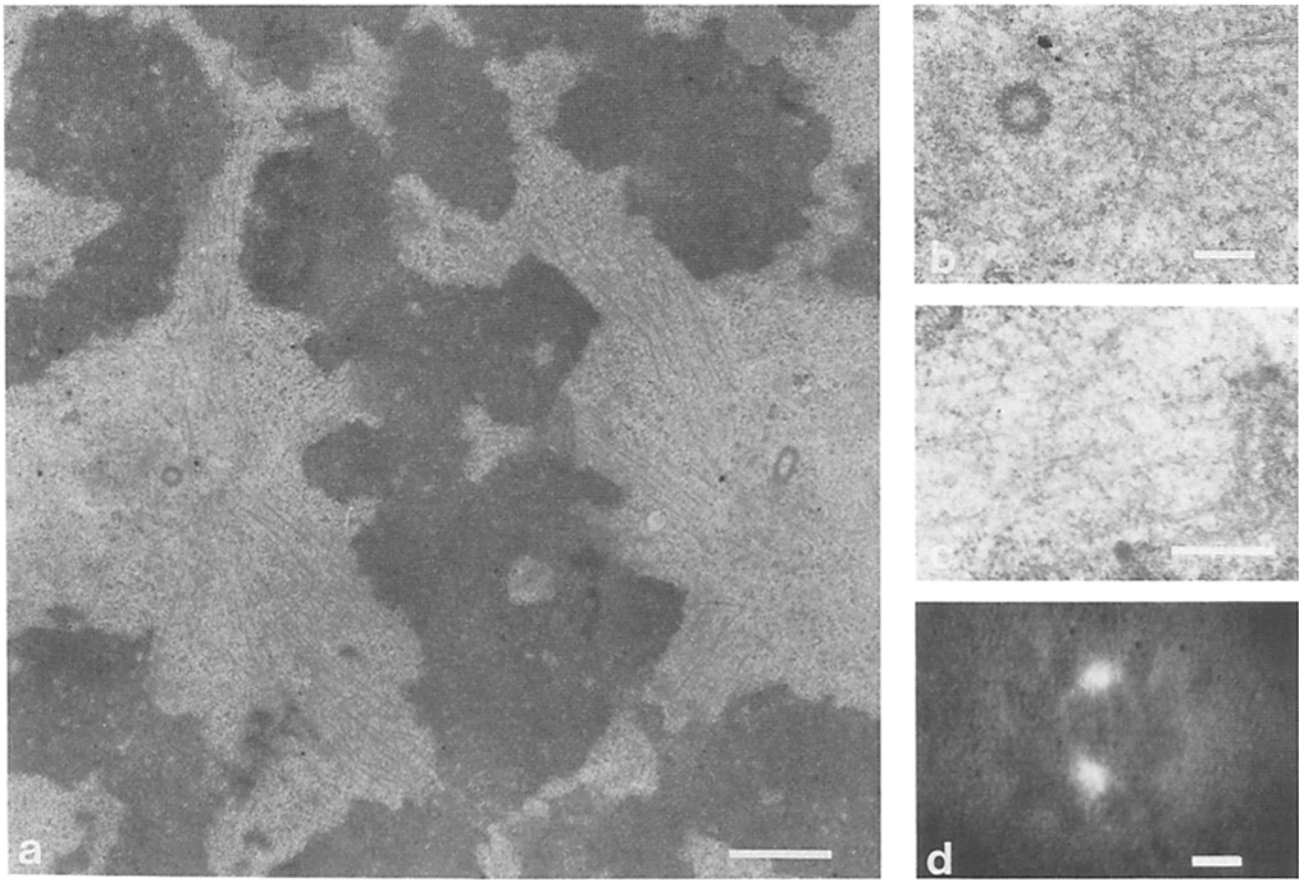


Figure 2. (a) Transmission electron micrograph from serial sections of a metaphase cell injected with CaM-TRITC, then treated with nocodazole (1 μM , 15 min, $23 \pm 2^\circ\text{C}$). (b) Spindle pole region. (c) Kinetochore and proximal portion of a kMT bundle. (d) CaM-TRITC fluorescence image of this cell. Bars: (a and c) 0.5 μm ; (b) 0.25 μm ; (d) 5 μm .

The above results suggested that the addition of exogenous CaM to a mitotic cell increases the stability of the kMTs. This effect is dose dependent (Fig. 6). Although precise doses are difficult to calculate because we cannot control the exact amount injected, we estimate that the half-maximum effective concentration in the microinjection solution is ~ 0.5 mg/ml. Because microinjection is in effect a perturbation, we expected that the stabilization effect would be transient; as the cell presumably acts to return CaM levels to their preinjection state, or as a new functional equilibrium is attained, the stabilization effect might be expected to disappear. To test this hypothesis, cultures were injected as above and then held at 25°C for varying times before adding nocodazole. Fig. 7 shows the results of this experiment. The stabilizing effect of microinjected CaM disappeared after roughly 60 min.

Ca⁺⁺-Independent Interaction of CaM with the Spindle

HA-CaM-TRITC, which was purified from CaM-TRITC based on its inability to bind to phenyl-Sepharose in the presence of Ca^{++} , would also stabilize kMTs. This observation suggested that the interaction of CaM with the MA might not be dependent on the presence of Ca^{++} .

The CaM-TRITC used in this study has been shown to be active in standard biological assays for CaM (Zavortink et

al., 1983). The HA-CaM-TRITC analog was assayed for its Ca^{++} -dependent mobility shift on SDS-PAGE in the presence and absence of Ca^{++} (Fig. 8). In contrast to native CaM or CaM-TRITC, HA-CaM-TRITC did not show any component with an increased mobility in the presence of Ca^{++} . This suggests that compared to CaM-TRITC, HA-CaM-TRITC has a much less significant conformational change in the presence of Ca^{++} .

The CaM analogs were also assayed for their ability to stimulate partially purified CaM-dependent adenylate cyclase from bovine cortex in a Ca^{++} -dependent manner (Fig. 9). Native CaM and CaM-TRITC showed Ca^{++} -dependent stimulation of cyclase while HA-CaM-TRITC and performic acid-oxidized CaM showed no significant stimulation.

To address the hypothesis that CaM might interact with spindle MTs in a Ca^{++} -independent fashion, we examined the ability of CaM-TRITC to concentrate in the spindles of permeabilized cells. PtK₁ cells were lysed in buffers containing 10 μM Ca^{++} or 10 mM EGTA. At 10 μM Ca^{++} , we would expect to find significant Ca^{++} binding to CaM (Crouch and Klee, 1980) but little or no Ca^{++} /CaM-dependent MT depolymerization (Job et al., 1981). With 10 mM EGTA present, we would expect that there would be virtually no free Ca^{++} . In both cases, CaM-TRITC entered permeabilized cells, concentrated in the spindle, and was retained

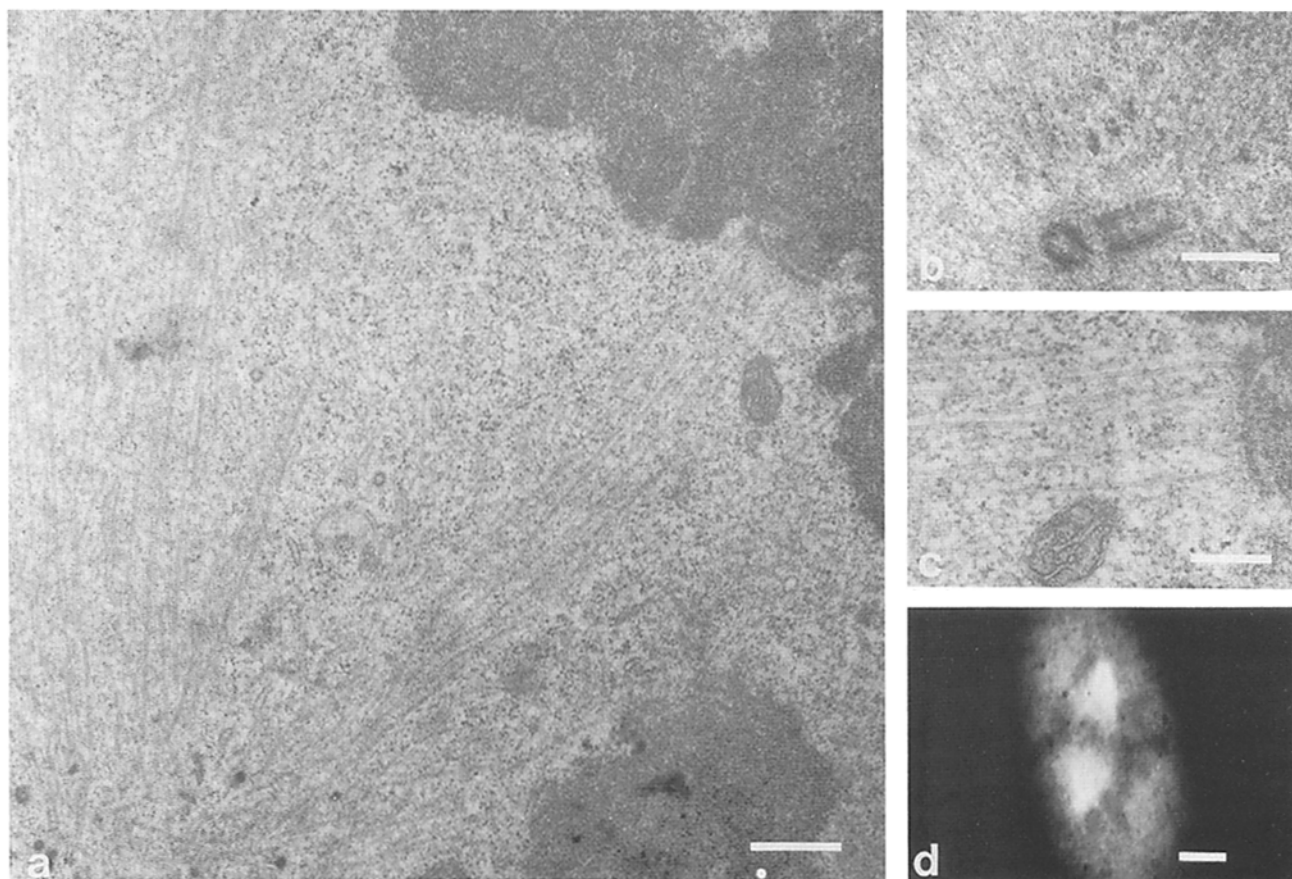


Figure 3. (a) Transmission electron micrograph from serial sections of a metaphase cell injected with CaM-TRITC. (b) Spindle pole region. (c) Kinetochore and proximal portion of a kMT bundle. (d) CaM-TRITC fluorescence image of this cell. Bars: (a and c) 0.5 μm ; (b) 0.25 μm ; (d) 5 μm .

after the fluorescent protein solution was washed away. The CaM-TRITC distribution in the MA was indistinguishable from that observed by direct microinjection of the CaM-TRITC analog or immunofluorescence (Fig. 10). However, CaM-TRITC fluorescence in interphase cells appeared to be somewhat higher when cells were permeabilized in the presence of 10 μM Ca^{++} (Fig. 10 e, regions bordering mitotic cell). We also observed that HA-CaM-TRITC would concentrate in the spindle in this system in a pattern indistinguishable from that of CaM-TRITC (Fig. 11), while BSA-TRITC and free TRITC failed to concentrate in the MA (not shown).

Discussion

We have shown that microinjection of native CaM, CaM-TRITC, or HA-CaM-TRITC can transiently make the kMTs resistant to the effect of nocodazole, and that CaM-TRITC can concentrate in the spindle in an apparently Ca^{++} -independent manner.

There are several assumptions inherent in the conclusion that CaM can stabilize MTs. First, we assume that nocodazole and CaM-TRITC each act independently in the MA. Nocodazole is presumed to work by binding to free tubulin dimers, thus lowering the free tubulin concentration and inhibiting first nucleation and then, at higher concentrations,

polymerization (Hoebeke et al., 1976). For CaM to directly inhibit nocodazole, it would have to bind to the drug molecule and prevent its binding to free tubulin dimers. CaM-TRITC was microinjected into selected cells while nocodazole was present in the culture medium and able to cross cell membranes. It is unlikely that the limited amount of CaM-TRITC present in ~ 50 – 75 injected cells could bind to a significant fraction of the nocodazole molecules present in the culture dish. Furthermore, the stabilization effect was limited to the injected cells. If CaM were interacting directly with nocodazole, acting to lower its effective concentration, one would have expected to see stabilization of MTs in both injected and noninjected cells in the culture. This was not observed. The conclusion that the stabilization effect is not a result of direct interaction with nocodazole is supported by the observation that similar results were obtained when colcemid was used as the MT inhibitor and the drug treatment was carried out at 37°C (data not shown).

We also assume that our method of quantitating the stabilization effect is reasonably accurate. During a typical experiment, all of the metaphase cells within a marked region would be microinjected. A significant number of prophase and prometaphase cells would also be found in this region but not injected because their spindles were not sufficiently organized; in such cells the poles are not distinctly separate

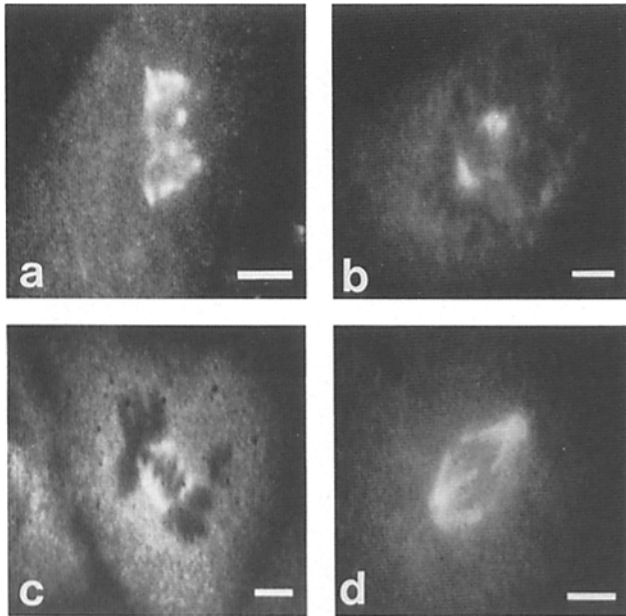


Figure 4. Examples of metaphase spindles fitting each index category. (a) Spindle index 0. Disorganized remnants of kMTs. (b) Spindle index 1. Remnants of kMTs. Possibly some organization into parallel arrays. (c) Spindle index 2. Well-defined parallel arrays of kMTs with clearly defined poles remaining within or bordering the chromosome mass. (d) Spindle index 3. Well-defined parallel kMT arrays oriented towards discrete poles distinctly separate from the chromosome mass. Bars, 5 μ m.

from the chromosomes, so any stabilization of MTs which might occur could not be easily evaluated within our scheme. The presence of uninjected cells in the microinjection region does not present a problem when injected cells can be identified by the presence of fluorescently labeled protein in the cell. However, in cases where sufficient labeled protein was not retained after immunofluorescence processing, or when the injection solution did not contain a fluorescent marker, unequivocal identification of injected cells was not possible. We chose to address this problem by scoring every mitotic cell in the injection region which might have been injected, and then adjusting the data as described in Materials and Methods to correct for the presence of uninjected cells. Without adjustment, the data would be falsely biased toward the uninjected distribution; thus a correction is mandatory in order to validate the controls.

Based on this analysis, we can conclude that the stabilizing effect was due to the injected CaM (or analogs), and not an artifact of the protocol. We did not observe stabilization when free TRITC, BSA-TRITC, or oxidized CaM were injected. Hence, the effect was not due to the injection process, TRITC alone, or a nonspecific protein effect. In contrast, the stabilizing effect was observed when native CaM, CaM-TRITC, and HA-CaM-TRITC were injected. Therefore CaM was required for stabilization. CaM-TRITC shows slightly more effective stabilization than native CaM. It is possible that the presence of TRITC in this analog might contribute to the stabilization effect, but TRITC cannot be responsible for the entire effect. The fact that the effect is dependent on

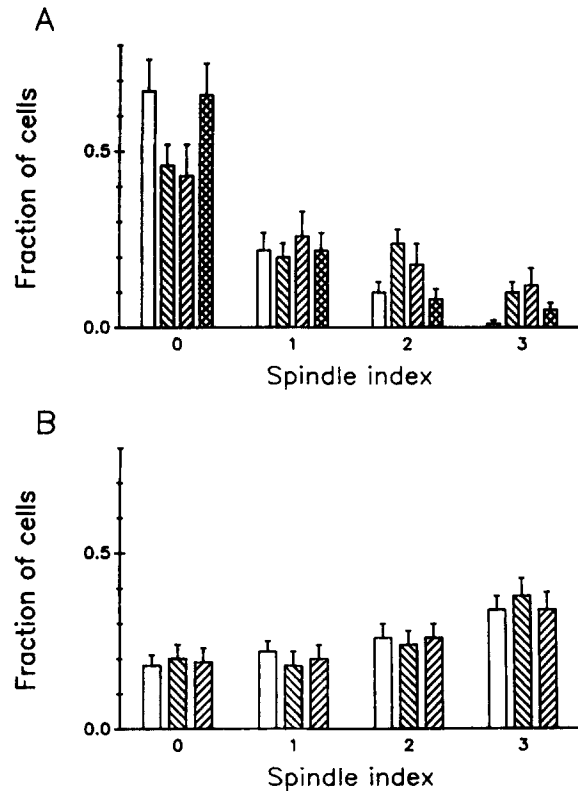


Figure 5. Spindle index distribution in populations of cells after various injections and nocodazole treatment. (A) Treatments without stabilizing effect. (□) No injections ($n = 89$). (▨) Cells injected with free TRITC ($n = 176$). (▧) Cells injected with BSA-TRITC ($n = 66$). (▩) Cells injected with a performic acid-oxidized analog of CaM ($n = 97$). (B) Treatments with stabilizing effect. (□) Cells injected with native CaM ($n = 239$). (▨) Cells injected with CaM-TRITC ($n = 142$). (▧) Cells injected with HA-CaM-TRITC ($n = 137$). Results are presented as fraction of cells \pm SD.

CaM concentration further supports the assumption that the observed stabilization is not an artifact.

A final assumption is that the effect observed for CaM is due to an interaction with the kMTs in the MA. The appearance of MTs in CaM-TRITC-injected, nocodazole-treated

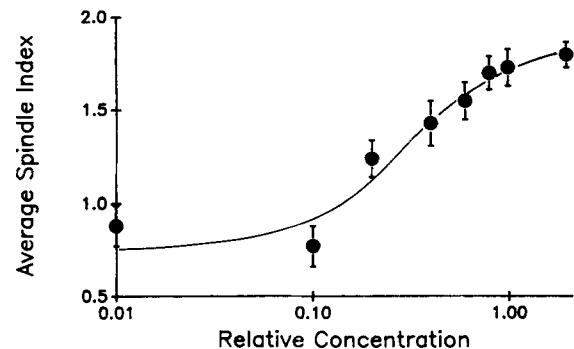


Figure 6. Dependence of spindle stabilization effect on concentration of CaM-TRITC in the injected solution. Cells were injected with various concentrations of CaM-TRITC followed by nocodazole treatment. The average spindle index value \pm SEM ($n = 100$) at each concentration is plotted vs. relative CaM-TRITC concentration (concentrations relative to 1.5 mg/ml).

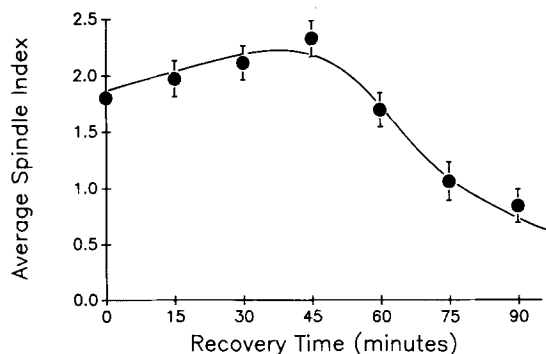


Figure 7. Duration of spindle stabilization effect. Cells were injected with CaM-TRITC, then left at 25°C for various times before treatment with nocodazole. The average spindle index value \pm SEM ($n = 40$) at each time point is plotted vs. length of time delay.

cells is very similar to the MT distribution in cold-treated cells, which contain only kMTs (Brinkley and Cartwright, 1975). CaM appears to be primarily associated with kMTs during mitosis (Welsh et al., 1979) and based on our serial sectioning EM observations, nkMTs were not apparent in our cells after CaM-TRITC injection and nocodazole treatment (e.g., Fig. 2 a). Nonetheless, it is impossible to rule out a mechanism which involves actions of CaM on other cellular components. If this were the case, however, one might expect that the stabilizing effect on spindle MTs would not be restricted to kMTs.

We conclude that we have observed the independent effects of CaM and nocodazole on the MA. In the case of nocodazole, we believe that the drug treatment provides a measure of the relative turnover rates of MTs in the MA. There is no evidence that nocodazole can act directly to depolymerize existing MTs. When MTs disappear from cells being treated with nocodazole (and for that matter other anti-MT drugs), one assumes that the MTs are being removed as part of the normal turnover of the cell's complement of MTs. They are not replaced because the action of the drug is to inhibit MT regrowth. The idea that the cell has an active role in removing MTs that disappear during drug treatment is supported by the observation that metabolic inhibitors block the action of anti-MT drugs in vivo (DeBrabander et al., 1981; Spurck et al., 1986a, b). Similarly, anti-MT drugs have little or no effect on MTs in permeabilized cell systems in the absence of ATP (Spurck et al., 1986a, b).

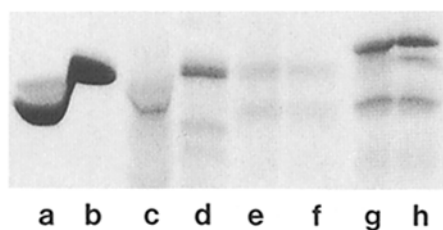


Figure 8. SDS-PAGE of CaM and CaM analogs in the presence and absence of Ca⁺⁺. (a and b) Native CaM; (c and d) CaM-TRITC; (e and f) HA-CaM-TRITC; (g and h) performic acid-oxidized CaM. (a, c, e, and g) 1 mM Ca⁺⁺ added. (b, d, f, and h) 1 mM EGTA added.

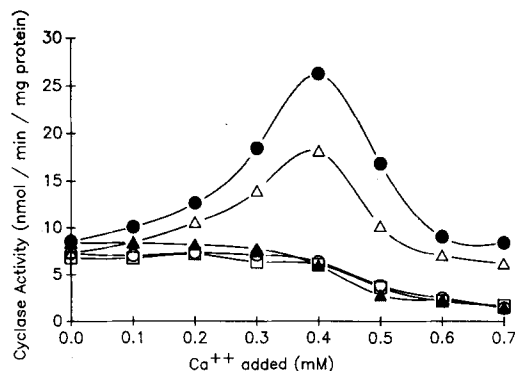


Figure 9. Ability of CaM and CaM analogs to stimulate partially purified CaM-dependent adenylate cyclase from bovine cortex in a Ca⁺⁺-dependent manner. (○) Control; (●) native CaM; (Δ) CaM-TRITC; (▲) HA-CaM-TRITC; (◻) performic acid-oxidized CaM. Data points represent the mean of duplicate samples.

Based on this argument, we conclude that differences in resistance to nocodazole observed between mitotic MT subclasses reflect different turnover rates of these MTs. We have observed that the relative resistance of spindle MT sub-

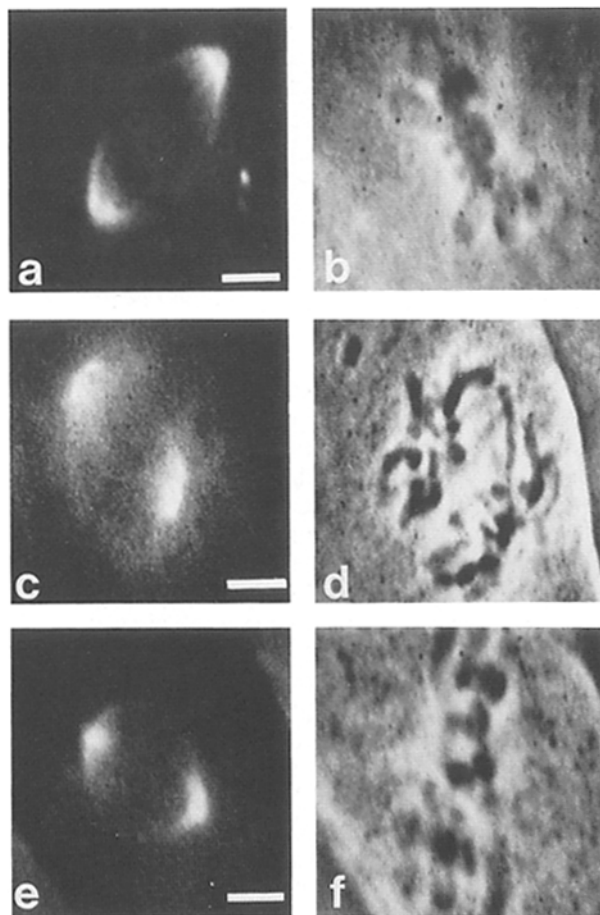


Figure 10. Incorporation of CaM-TRITC into permeabilized cells in the presence and absence of Ca⁺⁺. (a-d) Permeabilization in the presence of 10 mM EGTA. (a, c, and e) CaM-TRITC fluorescence. (b, d, and f) Phase-contrast images. (a and b) Metaphase cell. (c and d) Anaphase cell. (e and f) Permeabilization in the presence of 10 μM Ca⁺⁺. Bars, 5 μm.

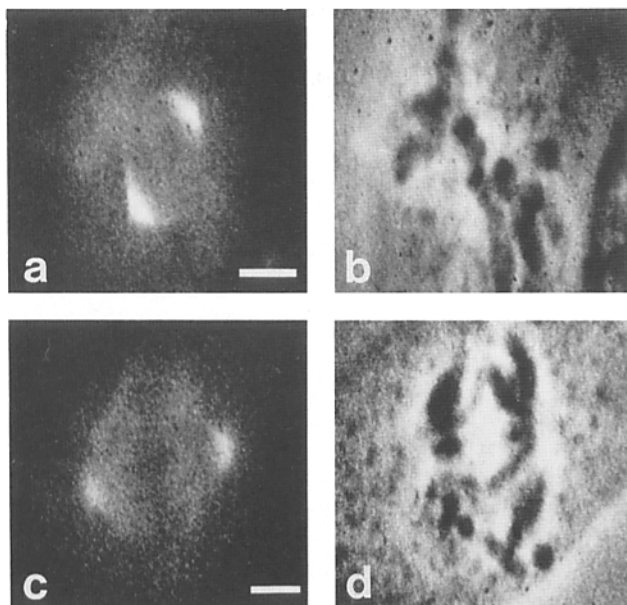


Figure 11. Incorporation of HA-CaM-TRITC into permeabilized cells in the presence of 10 mM EGTA. (a and b) Metaphase cell. (c, d) Anaphase cell. (a and c) HA-CaM-TRITC fluorescence. (b and d) Phase-contrast images. Bars, 5 μ m.

classes to nocodazole is as follows (from most to least stable): interzonal-anaphase, kinetochore-anaphase, kinetochore-metaphase, nonkinetochore-metaphase (data not shown). In the case of kMTs, under the treatment conditions used in this work, net disassembly appeared to occur at the spindle pole; MTs were frequently seen attached to kinetochores at one end but apparently not associated with a pole at the other. Conversely, spindle poles with MT fragments attached were not observed.

There are several possible mechanisms which could account for differing turnover rates of mitotic MTs. First of all, differences in free tubulin concentrations at the kinetochore vs. the spindle equator and cell margin could alter MT polymerization kinetics. The physical structure of the MTs could differ between subclasses. It has been reported that addition of MT-associated proteins to MTs *in vitro* can increase their stability (Murphy and Borisy, 1975; Sloboda et al., 1976; Job et al., 1985). Studies using antibodies to posttranslationally altered tubulin subunits suggest the MTs containing acetylated α -tubulin may also be more stable (Piperno et al., 1987). Subclass-specific regulation of GTPase activity could create different levels of dynamic instability (Mitchison and Kirschner, 1984). Finally, associations between MTs and other spindle structures such as the kinetochore might create a physical cap of the MT which could inhibit depolymerization.

The injection of CaM into metaphase cells before nocodazole treatment apparently decreased the turnover rate of the kMTs such that they showed less net disassembly during the drug treatment period. The precise amount of CaM injected is difficult to estimate because of the variability in injection volume and cell size. Based on previous work using nearly identical equipment (Zavortink et al., 1983), we estimate that the injections increased the amount of CaM in the cell

by 60–100%. We conclude that the increased CaM concentration was responsible for the stabilization effect, and the time-dependent disappearance of the effect reflected the cell reaching a new functional equilibrium.

Our observation that CaM can stabilize kMTs is in agreement with previous evidence that CaM associates with stable MTs. CaM has been shown to be associated with cold- and drug-stable mitotic MTs (Welsh et al. 1979), cold-stable interphase MTs (Deery et al., 1984), taxol-stabilized MT arrays (DeBrabander et al., 1986), and MTs forming at the centrosomes and kinetochores in mitotic cells recovering from nocodazole treatment (Sweet, S. C., and M. J. Welsh, unpublished observations).

However, because CaM can mediate the depolymerizing effect of Ca^{++} *in vitro* (Marcum et al., 1978; Job et al., 1981), CaM is also thought to have the same action *in vivo*. There are two reports which support this hypothesis (Keith et al., 1983; Keith, 1987). These latter studies however, make the assumption that a Ca^{++} -saturated form of CaM retains its Ca^{++} -bound conformation for up to 15 min in the face of the cytoplasmic buffering of Ca^{++} . Nonetheless, it is clear that Ca^{++} can act *in vivo* to depolymerize MTs (Kiehart, 1981; Salmon and Segall, 1980), and therefore it is reasonable to consider that CaM might mediate a similar effect *in vivo*.

To account for these two apparently opposite effects of CaM in the MA, we considered the possibility that CaM would stabilize MTs in the absence of Ca^{++} and destabilize MTs in the presence of Ca^{++} . This hypothesis was suggested by the observation that HA-CaM-TRITC was able to protect kMTs from the effect of nocodazole.

HA-CaM-TRITC was isolated by its failure to bind to phenyl-Sepharose in the presence of Ca^{++} . CaM molecules which were able to expose a hydrophobic domain upon binding Ca^{++} would be removed from the solution during this step. HA-CaM-TRITC fails to activate CaM-dependent adenylate cyclase in the presence of Ca^{++} , even though the amount of protein used was such that native CaM would give more than 99% of maximum stimulation of the enzyme (Fig. 9). Furthermore, when examined by SDS-PAGE in the presence and absence of Ca^{++} (Fig. 8, lanes e and f), the protein shows no increase in mobility upon addition of Ca^{++} . Although a component with a mobility similar to Ca^{++} -bound CaM appears in the HA-CaM-TRITC lanes, because of the purification scheme and the inability of the HA-CaM-TRITC to stimulate adenylate cyclase, we conclude that this component does not demonstrate Ca^{++} -bound CaM activity. Thus, HA-CaM-TRITC was able to stabilize kMTs in spite of failing to demonstrate the Ca^{++} -dependent properties of CaM, suggesting that CaM can interact with the MA independent of the presence of Ca^{++} . Although the idea that CaM might bind to MTs in a Ca^{++} -independent manner has been mentioned before (Rebhun et al., 1980; Deery et al., 1984), it had never been explicitly tested.

We have tested this hypothesis, as described in the second portion of the Results section. By permeabilizing cells, we were able to control the Ca^{++} concentration in the spindle environment. In this system, under conditions (10 mM EGTA) where we would expect that there was virtually no free Ca^{++} , CaM incorporated in the MA in an apparently normal fashion, indicating that Ca^{++} is not required for the initial interaction of CaM with the spindle. In addition, the

heat-altered analog of CaM also concentrated normally in the MA. Thus neither Ca⁺⁺ nor Ca⁺⁺-bound CaM activity appear to be required for CaM to concentrate in the MA.

We believe that our data indicate that CaM initially associates with kMTs in the MA in the Ca⁺⁺-free conformation. This association appears to reduce the net kMT disassembly observed at the spindle pole during nocodazole treatment, either directly or possibly by stabilizing the kMT to spindle pole interaction. In normal cells, this effect could contribute to the ability of the kMT subclass to exhibit net assembly during prometaphase in the face of cytoplasmic conditions in which other MTs undergo net disassembly. Furthermore, bound to the MA independently of Ca⁺⁺, CaM would be in a position to mediate a Ca⁺⁺-dependent alteration in MT stability. We believe that the Ca⁺⁺ pulse observed at the onset of anaphase (Ratan et al., 1986) might result in a Ca⁺⁺/CaM-dependent destabilization of kMTs, which would allow net depolymerization of the kMTs as the chromosomes move poleward.

Evidence for such a system exists. The 145K STOP protein described by Margolis and co-workers confers cold stability to brain MTs (Margolis et al., 1986). MTs stabilized by the STOP protein depolymerize upon treatment with Ca⁺⁺ and CaM (Job et al., 1982). This line of evidence suggests that a CaM/STOP protein complex might be an integral part of the kMTs, contributing to the differential stability of this MT subclass during mitosis.

In summary, we believe that our observations support a model in which CaM acts to influence the stability of the kMTs. Before anaphase, the presence of Ca⁺⁺-free CaM apparently stabilizes kMTs. Upon entry into anaphase, CaM could mediate a Ca⁺⁺-dependent destabilization of these MTs, allowing chromosome movement toward the spindle pole to occur.

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