Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 1037–1041, February 1981
Cell Biology

Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins

(detergent extraction/calmodulin/calmodulin inhibitors/immunofluorescence microscopy)

MANFRED SCHLIWA*, URSULA EUTENEUER*, JEANNETTE CHLOE BULINSKI†‡, AND JONATHAN G. IZANT*

*Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309; and †Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706

Communicated by Keith R. Porter, October 31, 1980

ABSTRACT **Detergent-extracted BSC-1 monkey cells have been used as a model system to study the Ca^{2+} sensitivity of *in vivo* polymerized microtubules under *in vitro* conditions. The effects of various experimental treatments were observed by immunofluorescence microscopy. Whereas microtubules are completely stable at Ca^{2+} concentrations below 1 μM, Ca^{2+} at greater than 1–4 μM induces microtubule disassembly that begins in the cell periphery and proceeds towards the cell center. At concentrations of up to 500 μM, both the pattern and time course of disassembly are not markedly altered, suggesting that, within this concentration range, Ca^{2+} effects are catalytic rather than stoichiometric. Higher (millimolar) Ca^{2+} concentration results in rapid destruction of microtubules. Of other divalent cations, only Sr^{2+} has a slight depolymerizing effect, whereas millimolar Ba^{2+}, Mg^{2+}, or Mn^{2+} is ineffective. Disassembly induced by micromolar Ca^{2+} is inhibited by pharmacological agents known to bind to calmodulin and inhibit its function, suggesting that calmodulin mediates Ca^{2+} effects. Both the addition of exogenous brain microtubule-associated proteins (MAPs) after lysis and the retention of endogenous cellular MAPs normally extracted during the lysis step stabilize microtubules against the depolymerizing effect of micromolar Ca^{2+}. The results indicate that, in this model system, microtubules are sensitive to physiological Ca^{2+} concentrations and that this sensitivity may be conferred by calmodulin associated with the microtubules. MAPs appear to have a modulating effect on microtubular Ca^{2+} sensitivity and thus may function as a discriminating factor in cellular functions performed by calmodulin. It is hypothesized that Ca^{2+}-stimulated microtubule disassembly depends on the relative amount of MAPs.**

Several important cellular functions depend on an ordered assembly and disassembly of microtubules. The factors controlling these processes are, however, not fully understood. Since the discovery of conditions for the reversible polymerization of microtubules *in vitro* (1) it has been suggested that the concentration of Ca^{2+} might be a regulator of the assembly/disassembly process. For the most part the evidence supporting this hypothesis is circumstantial. Kiehart and Inoue (2) microinjected Ca^{2+} into dividing sea urchin eggs and observed a local and transient reduction of spindle fiber birefringence. Likewise, ionophore-mediated Ca^{2+} entry has been shown to reversibly affect the cytoplasmic microtubule complex of cultured cells (3) and microtubule-containing heliozoan axopodia (4). The concentration of Ca^{2+} required to inhibit microtubule assembly *in vitro* apparently depends on the purification protocol used. Assembly in crude brain extracts is inhibited by near micromolar Ca^{2+} (1, 5, 6), whereas the assembly of purified protein is sensitive to Ca^{2+} concentrations in the millimolar range only (7). Interestingly, the sensitivity to micromolar Ca^{2+} of purified microtubule protein can be restored by a factor present in crude brain extracts (8).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

The discovery that calmodulin, a ubiquitous Ca^{2+}-binding protein that regulates a number of Ca^{2+}-dependent functions (for reviews, see refs. 9 and 10), influences microtubule assembly *in vitro* in a Ca^{2+}-dependent manner (11) has opened the possibility that Ca^{2+} effects on microtubules are mediated by this protein. Immunofluorescence microscopy with antibodies against calmodulin further showed an association of this protein with certain components of the mitotic spindle (12, 13), suggesting an important role in Ca^{2+}-dependent functions during mitosis.

Until it becomes possible experimentally to manipulate Ca^{2+} concentrations inside a living cell, the hypothesis that Ca^{2+} and calmodulin regulate microtubule assembly can be tested only *in vitro*. Here we describe the use of an *in vitro* system that resembles the *in vivo* situation. We report on the Ca^{2+} lability of *in vivo* polymerized microtubules in detergent-extracted cells and present evidence for the involvement of calmodulin mediating the Ca^{2+} effect. In addition, we show that the Ca^{2+} lability of these microtubules is modulated by microtubule-associated proteins (MAPs). Part of the data have been presented at the Second International Symposium on Microtubules and Microtubule Inhibitors, Beerse, Belgium, 1980 (14).

MATERIALS AND METHODS

Cell Culture. African green monkey kidney cells (BSC-1) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were seeded into 35-mm plastic Petri dishes that contained four round glass coverslips. After 24–48 hr, coverslips were removed from the Petri dishes and used for experiments.

Detergent Extraction. Cells were washed twice for 5 sec each at room temperature with a buffer containing 60 mM 1,4-piperazinediethanesulfonic acid (Pipes), 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 1 mM $MgCl_2$ (extraction buffer; pH 6.9). Cells were lysed in extraction buffer supplemented with 0.15% Triton X-100 or Brij 58 for 30–120 sec. The supernatant was removed and the extracted cells on the coverslip were washed with extraction buffer before application of the Ca/EGTA buffer.

Preparation of Ca/EGTA Buffers. Ca/EGTA buffers with free Ca^{2+} in the range of 0.1–100 μM were made by using a computer program prepared by John Gilkey that took into account the pH of the final solution, ionic strength, and Mg^{2+} concentration. The buffers were made up in 60 mM pipes/25 mM Hepes/1 mM $MgCl_2$, pH 6.9.

Abbreviations: MAP, microtubule-associated protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

‡ Present address: Dept. of Biochemistry, University of Washington, Seattle, WA 98195.

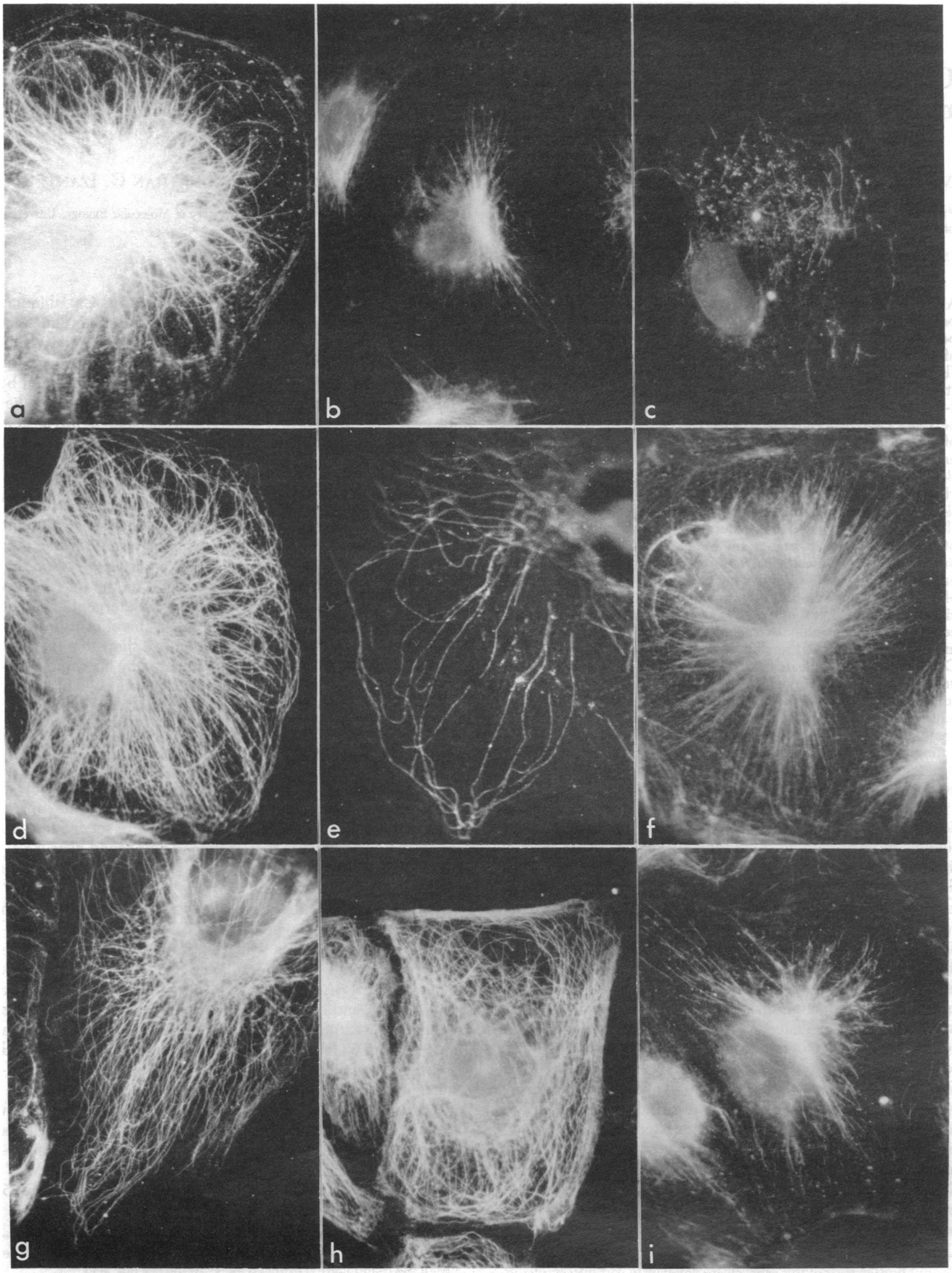

FIG. 1. *(Legend appears at the bottom of the next page.)*

Antisera. The tubulin antiserum was a generous gift of Keigi Fujiwara. It was prepared according to procedures described elsewhere (15). The antiserum against a M_r 210,000 assembly MAP isolated from HeLa cells was prepared as described (16). Procedures for the isolation of a monoclonal antibody against MAP_2 have been outlined (17).

Preparation of MAPs. MAPs were separated from three-times-cycled beef brain microtubules in a DEAE-Sepharose column according to Vallee and Borisy (18). As judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, the MAP fraction contained the high molecular weight MAPs and a number of low molecular weight proteins but very little tubulin (<10%).

Immunofluorescence Microscopy. Cells were fixed for immunofluorescence microscopy with 0.3% glutaraldehyde for 10 min in the buffer used in the step immediately preceding fixation. Coverslips were washed with phosphate-buffered saline and treated with $NaBH_4$ (1 mg/ml) for 6–8 min (19). After two additional washes with phosphate-buffered saline, cells were incubated at 37°C for 45 min with the respective antisera, washed thoroughly, and allowed to react for 30–45 min with fluorescein-conjugated goat immunoglobulins specific for rabbit immunoglobulin. Coverslips were mounted on slides and viewed in a Leitz Orthoplan light microscope equipped with epifluorescence illumination.

Reagents. Trifluoperazine was obtained from Smith, Kline & French. Pimozide was a gift of McNeil Laboratories, Fort Washington, PA. The synthetic drug R24571 was a generous gift of Marc de Brabander, Janssens Pharmaceutica. All other reagents were obtained from Sigma.

RESULTS

Extraction of BSC-1 cells with Triton leaves a residue consisting mainly of actin filaments, intermediate filaments, and microtubules in a three-dimensional arrangement very similar to that of intact cells. Extracted cells can be kept in extraction buffer for at least 45 min without major effects on the integrity and stability of microtubules, as judged by immunofluorescence microscopy. Only during the lysis step are high (10 mM) concentrations of EGTA necessary; once extraction is completed, cells can be transferred to a low-EGTA medium without affecting microtubule stability. Stabilizing agents such as polyethylene glycol or Me_2SO_4, proteolysis inhibitors, or nucleoside triphosphates (ATP, GTP) are not essential.

Microtubules of Triton-extracted BSC-1 cells are Ca^{2+} labile; upon the addition of Ca/EGTA buffers with free Ca^{2+} at greater than 1–4 μM, microtubules depolymerize. This process is time dependent and vectorial—i.e., it starts in the cell periphery and proceeds towards the cell center (Fig. 1 *a* and *b*). It appears as if the microtubule complex gradually "melts" towards the cell center. By 5 min, only short microtubule segments are found near the nucleus, often associated in a radial fashion with a brightly fluorescent dot, probably the centrosome (Fig. 1*b*). However, microtubules do not depolymerize strictly from their distal ends, as is indicated by the presence of frequent breaks and microtubule fragments in the cell periphery, although fragments do disappear first before more central portions of a microtubule are affected.

Table 1. Concentrations of calmodulin inhibitors required to attain virtually complete inhibition of microtubule depolymerization induced by 100 μM Ca^{2+} in lysed tissue culture cells

Inhibitor	Conc., μM
R24571	5
Pimozide	20
Trifluoperazine	50
Chlorpromazine	200

Within the range of 5–500 μM Ca^{2+}, the pattern and time course of microtubule disassembly appears to be independent of the Ca^{2+} concentration, suggesting that within this concentration range Ca^{2+} effects are catalytic rather than stoichiometric. Only at 1 mM Ca^{2+} or above is microtubule breakdown accelerated and more irregular; after 1 min, only a few short fragments without any apparent connection to the centrosome are present (Fig. 1*c*). Microtubules are essentially stable at Ca^{2+} concentrations below 1 μM (Fig. 1*d*).

The depolymerizing effect is highly specific for Ca^{2+}. Of other divalent cations tested, only Sr^{2+} at 0.5 mM induces microtubule disassembly; Mg^{2+} at 10 mM or Ba^{2+} and Mn^{2+} at 1 mM are ineffective. The inclusion of a near-physiological concentration of KCl (100 mM) in the lysis medium and the Ca/EGTA buffer does not alter microtubule stability or Ca^{2+} sensitivity.

The pattern of microtubule breakdown induced by other agents such as cold or Colcemid treatment (10 μM) differs markedly from that induced by Ca^{2+}. As already noted by others (20), microtubule depolymerization does not occur with the degree of synchrony observed with the use of Ca^{2+}. Rather, relatively long microtubules are observed throughout the cytoplasm, some of which may still reach the cell periphery (Fig. 1*e*).

Calmodulin Inhibitors. To test whether a calmodulin-like molecule confers Ca^{2+} sensitivity to microtubules in lysed cells, four different pharmacological agents known to interfere with calmodulin-mediated processes were tested for their effect on Ca^{2+}-induced disassembly. All agents used inhibited disassembly, although the concentration required to attain virtually complete inhibition varied (Table 1). The order of potency of the drugs in inhibiting microtubule depolymerization conforms with their relative effectiveness in other assay systems (21). All agents block microtubule disassembly induced by Ca^{2+} concentrations between 5 and 100 μM but show little or no effect at Ca^{2+} concentrations equal to or greater than 1 mM.

Effect of MAPs. As a possible factor involved in the control of Ca^{2+} sensitivity of microtubules, the role of MAPs has been investigated. By using an antibody directed against a M_r 210,000 assembly MAP isolated from HeLa cells that is specific for primate cells (22), it has been shown that cell lysis induced by Triton largely removes this high molecular weight MAP from microtubules (see also ref. 23). It is possible, therefore, that microtubules in lysed BSC-1 cells have little high molecular weight MAPs associated with them. MAP influence on Ca^{2+}

FIG. 1 (*on preceding page*). Monolayer cultures of BSC-1 cells stained for immunofluorescence microscopy with antibodies against tubulin (*a–e*, *g–i*) or MAP_2 (*f*). (×530.) Cells in *a–g* were extracted with Triton X-100. (*a*) Treatment with 10 μM Ca^{2+} for 1 min. Microtubule depolymerization starts in the cell periphery. (*b*) Treatment with 10 μM Ca^{2+} for 5 min. (*c*) Cell treated with 5 mM Ca^{2+} for 5 min. (*d*) Microtubules are unaffected by treatment with 0.5 μM Ca^{2+} for 10 min. (*e*) Cell kept at 0°C for 30 min. (*f*) Cell treated with a MAP fraction isolated from beef brain microtubules by DEAE-Sepharose chromatography, final concentration 0.2 mg/ml. Immunofluorescence microscopy with a monoclonal antibody against MAP_2 demonstrates association of this MAP with cellular microtubules. (*g*) Cell treated with MAPs at 0.2 mg/ml and then exposed to 100 μM Ca^{2+} for 5 min. Microtubules are unaffected. (*h*) Cell extracted with 0.15% Brij 58 for 2 min and then treated with 100 μM Ca^{2+} for 5 min. Microtubules are unaffected. (*i*) Cell extracted with 0.15% Brij 58 for 2 min, followed by extraction with 0.15% Triton X-100 for 1 min and treatment with 100 μM Ca^{2+} for 5 min. Microtubules depolymerize.

sensitivity of microtubules has been tested in two different ways: (*i*) readdition of exogenous MAPs to microtubules of lysed cells, and (*ii*) retention of preexisting endogenous MAPs during the lysis process.

In the first approach, MAPs isolated from brain microtubules were added to the cytoskeletons at final concentrations of 0.1–0.2 mg/ml after Triton extraction. By using a monoclonal antibody directed against MAP_2, it was shown that at least MAP_2 did associate with BSC-1 cell microtubules (Fig. 1*f*). Free Ca^{2+} at a concentration of up to 500 μM had little or no effect on the integrity of these microtubules (Fig. 1*g*). Only Ca^{2+} at greater than 1 mM was capable of inducing depolymerization of MAP-decorated microtubules.

In the second approach, two conditions were discovered that help to retain preexisting cellular MAPs on microtubules of lysed cells. One is provided by the use of 4% (wt/vol) polyethylene glycol (M_r 20,000) in the lysis medium and the Ca/EGTA buffer. Microtubules of cells treated in this way will stain with antibodies against the M_r 210,000 HeLa MAP, and depolymerization induced by 10 or 100 μM Ca^{2+} is substantially slowed down or completely prevented. That in fact the retention of MAPs and not an unspecific protein stabilizing effect is the mechanism of polyethylene glycol action is suggested by experiments in which polyethylene glycol has been added to cytoskeletons after lysis and, hence, after removal of preexisting cellular MAPs. In that case, μM Ca^{2+} will induce microtubule disassembly. The second condition involves the use of Brij 58 instead of Triton X-100 as detergent without any change in the composition of the lysis buffer or the extraction protocol. Like Triton, this detergent removes the plasmalemma, but the organization of the cytoplasm remains largely unchanged when compared to whole intact cells (24). Also under these conditions the M_r 210,000 MAP is retained, and microtubules are stable in the presence of up to 100 μM Ca^{2+} (Fig. 1*h*). In cells first extracted with Brij 58 and subsequently treated with Triton X-100, micromolar Ca^{2+} will induce microtubule depolymerization (Fig. 1*i*), indicating that the treatment with Brij 58 did not remove or inactivate the Ca^{2+}-sensitizing factor.

DISCUSSION

The results demonstrate that microtubules of extracted tissue culture cells are depolymerized by μM Ca^{2+}. The value of this model system resides in its close resemblance to the situation *in vivo* while allowing controlled experimentation *in vitro*. Lysis procedures have been chosen that allow stabilization of cytoplasmic microtubules during cell lysis and yet maintain Ca^{2+} lability. Ca/EGTA buffers were used to test for the Ca^{2+} lability of microtubules in this model sytem. Microtubules are completely stable at concentrations of free Ca^{2+} below 1 μM and are depolymerized at Ca^{2+} concentrations in the micromolar range. These values are in good agreement with a possible regulatory role of this ion in microtubule assembly. Cells normally maintain free Ca^{2+} in the cytoplasm at a level below 1 μM (25). The increase of Ca^{2+} to 1–10 μM by local and transient release from intracellular stores is believed to be the mechanism that activates many Ca^{2+}-dependent functions in a variety of cells (26, 27). With respect to microtubule regulation, fluctuations in cytoplasmic Ca^{2+} in the physiological range could either prevent or allow for microtubule assembly, thus regulating the state of the microtubule system in the cell.

Many Ca^{2+}-dependent processes require the presence of a Ca^{2+}-sensitizing factor such as calmodulin (9, 10). The involvement of calmodulin in conferring Ca^{2+} sensitivity in the μM range to microtubules in our model system is suggested by the action of pharmacological agents known to bind to calmodulin and inhibit its function. Possibly, calmodulin is associated with microtubules tightly enough to survive the lysis procedure used. Without this factor, millimolar Ca^{2+} is required to depolymerize microtubules. It is conceivable that the mechanism of depolymerization induced by millimolar Ca^{2+} is different from that mediated by calmodulin, because microtubules stabilized against micromolar Ca^{2+} by a calmodulin inhibitor are depolymerized by millimolar Ca^{2+}. In the latter case, Ca^{2+} may directly interact with the tubulin molecule at its Ca^{2+}-binding sites (28).

The mechanism of Ca^{2+}-induced calmodulin-mediated microtubule disassembly is not understood. *In vitro*, millimolar Ca^{2+} induces disassembly of microtubules from both ends (29). The more or less orderly centripetal disassembly of microtubules in extracted cells as described here is consistent with a process that occurs preferentially at the distal end. In the initial stages of Ca^{2+}-induced depolymerization, more proximal portions of the microtubules are apparently unavailable. There are major differences between the pattern and time course of microtubule depolymerization induced by Ca^{2+} or cold treatment, suggesting fundamental differences in the mechanisms involved.

The current data further provide evidence for a modulating effect of MAPs on the Ca^{2+} sensitivity of microtubules. This role can probably be ascribed to the high molecular weight MAPs and not the τ proteins, because microtubules of Triton-extracted cells are largely free of the former but may still contain the latter (23, 30). Both the addition of exogenous MAPs and the retention of endogenous MAPs render microtubules stable against the depolymerizing influence of up to 100 μM Ca^{2+}. This effect does not seem to involve unspecific stabilization of microtubules or removal of Ca^{2+}-sensitizing factors inadvertently caused by the procedures employed in these experiments.

Although Ca^{2+} sensitivity of microtubules in the micromolar range has been demonstrated, this sensitivity seems to be expressed only in the absence of specific MAPs; in the presence of a normal complement of endogenous cellular MAPs or exogenous brain MAPs, sensitivity to micromolar Ca^{2+} is decreased. If calmodulin confers Ca^{2+} sensitivity on microtubules, and the evidence presented here and in studies *in vitro* (11) supports this hypothesis, then the amount of MAPs associated with the microtubules may be a modulating factor in this regulatory scheme. Calmodulin is known to regulate a wide variety of Ca^{2+}-dependent processes, posing the question of how discrimination in space and time is achieved. With respect to the regulation of microtubule assembly, MAPs could be a "discriminating factor." In other words, whether a given state of calmodulin activation will result in microtubule disassembly is dependent on the presence or absence of MAPs. This concept is supported by the observation that Ca^{2+}-induced depolymerization of microtubules in heliozoan axopodia is preceded by the removal of intermicrotubule links, which probably are a special class of MAPs (31). However, this hypothesis will remain speculative until more is known about the regulation of MAP binding and function.

We are grateful to Dr. Keith R. Porter for generous support of this study and to Dr. Keigi Fujiwara for a gift of the tubulin antiserum. This work was supported by National Institutes of Health Grant GM 27324-01 to Keith R. Porter, American Cancer Society Grant CD-8E to J. R. McIntosh, and a Heisenberg Fellowship from the Deutsche Forschungsgemeinschaft to M.S.

1. Weisenberg, R. C. (1972) *Science* **177**, 1104–1105.
2. Kiehart, D. P. & Inoue, S. (1976) *J. Cell Biol.* **70**, 230a.
3. Fuller, G. N. & Brinkley, B. R. (1976) *J. Supramol. Struct.* **5**, 497–514.
4. Schliwa, M. (1976) *J. Cell. Biol.* **70**, 527–540.

5. Haga, T., Abe, T. & Kurokawa, M. (1974) *FEBS Lett.* **39**, 291–295.
6. Rosenfeld, A. C., Zackroff, R. V. & Weisenberg, R. C. (1976) *FEBS Lett.* **65**, 144–147.
7. Olmsted, J. B. & Borisy, G. G. (1974) *Biochemistry* **14**. 2996–3005.
8. Nishida, E. & Sakai, H. (1977) *J. Biochem.* **82**, 303–306.
9. Cheung, W. Y. (1980) *Science* **207**, 19–27.
10. Means, A. R. & Dedman, J. R. (1980) *Nature (London)* **285**, 73–77.
11. Marcum, J. M., Dedman, J. R., Brinkley, B. R. & Means, A. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3771–3775.
12. Andersen, B. A., Osborn, M. & Weber, K. (1978) *Cytobiologie* **17**, 354–364.
13. Welsh, M. J., Dedman, J. R., Brinkley, B. R. & Means, A. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1867–1871.
14. Schliwa, M. (1980) in *Microtubules and Microtubule Inhibitors*, eds. de Brabander, M. & de Mey, J. (Elsevier/North Holland, Amsterdam), pp. 57–70.
15. Fujiwara, K. & Pollard, T. D. (1978) *J. Cell Biol.* **77**, 182–195.
16. Bulinski, J. C. & Borisy, G. G. (1980) *J. Cell Biol.* **87**, 792–801.
17. Izant, J. G. & McIntosh, J. R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4741–4745.
18. Vallee, R. B. & Borisy, G. G. (1978) *J. Biol. Chem.* **253**, 2834–2845.
19. Weber, K., Rathke, P. & Osborn, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1820–1824.
20. Weber, K. & Osborn, M. (1979) in *Microtubules*, eds. Roberts, K. & Hymans, J. (Academic, New York), pp. 279–313.
21. Levin, R. M. & Weiss, B. (1976) *Mol. Pharmacol.* **12**, 581–589.
22. Bulinski, J. C. & Borisy, G. G. (1980) *J. Cell Biol.* **87**, 802–808.
23. Conolly, J. A. (1979) Dissertation (Univ. of Toronto, Toronto, Canada).
24. Schliwa, M. (1980) *Proceedings of the 38th Annual Meeting of the Electron Microscopy Society of America*, ed. Bailey, G. W. (Claitor's, Baton Rouge, LA), pp. 814–817.
25. Baker, P. F. (1976) *Symp. Soc. Exp. Biol.* **30**, 67–88.
26. Kinoshita, S. & Yazaki, I. (1967) *Exp. Cell Res.* **47**, 449–457.
27. Hepler, P. K. (1977) in *Mechanisms and Control of Cell Division*, eds. Rost, T. L. & Giffords, E. M. (Hutchison & Ross, Stroudsberg), pp. 212–232.
28. Solomon, F. (1977) *Biochemistry* **16**, 358–363.
29. Summers, K. & Kirschner, M. W. (1979) *J. Cell Biol.* **83**, 205–217.
30. Solomon, F., Magendantz, M. & Salzman, A. (1979) *Cell* **18**, 431–438.
31. Schliwa, M. (1977) *J. Submicrosc. Cytol.* **9**, 221–227.