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Rapid disassembly of cold-stable microtubules by calmodulin

(tubulin/polymerization/mitosis/calcium)

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ABSTRACT **Purified cold-stable microtubules from the rat brain are insensitive to podophyllotoxin and to millimolar concentrations of free calcium. However, in the presence of calmodulin at concentrations substoichiometric to that of tubulin, calcium causes rapid microtubule disassembly. The half-maximal effective calcium concentration in the presence of calmodulin is 100 μM. With 800 μM free calcium, the half-maximal effective concentration of calmodulin is 1.0 μM (or one-tenth the tubulin concentration). Calmodulin is without effect in the absence of calcium. Troponin C is approximately one-fifth as effective as calmodulin, and parvalbumin is totally ineffective. Troponin I partially inhibits the calcium/calmodulin-induced disassembly of microtubules in the crude extract and blocks the calcium/calmodulin effect on purified cold-stable microtubules. A 5-fold excess of trifluoperazine does not inhibit the calcium/calmodulin-induced disassembly.**

Recycled cold-labile microtubules from mammalian brain tissue readily depolymerize in the presence of low concentrations of calcium (1–4). Microtubules in brain crude extract and within the cell are also calcium sensitive. Calmodulin, a M_r 17,000, thermostable, calcium-binding protein (for review, see ref. 5), has been reported to enhance the calcium sensitivity of purified brain microtubules (6). The effect, however, is weak and requires high calmodulin concentrations (6–8).

Microtubules that assemble in crude brain extracts contain a substantial subpopulation that is stable in the cold (0–4°C) (9, 10). This subpopulation is also stable (10) to drugs such as podophyllotoxin that potently inhibit microtubule assembly both *in vivo* and *in vitro* and cause the often-rapid disassembly of cold-labile microtubules. *In vitro* analysis of cold-stable microtubules in crude extract has shown, however, that they are rapidly disassembled by millimolar concentrations of calcium (9, 10).

Both cold-stable and cold-labile microtubules are present side by side in the cell, often with strikingly different distributions. Kinetochore-to-pole microtubules in the mammalian mitotic apparatus, for instance, are solely of the cold-stable type (11).

Calmodulin has been localized by immunofluorescence in the small pericentriolar region of the mitotic apparatus (12–14). This bound calmodulin is absent in spindles containing no microtubules (drug disassembled) and, further, seems specifically associated with the cold-stable subpopulation (14).

As the presence of calmodulin at the spindle poles coincides with the location of the [net disassembly end of] the spindle microtubules (15, 16), it is attractive to speculate that calmodulin might play a role in specifically regulating spindle assembly (microtubule assembly initiates at that site) (ref. 17; for review, see ref. 18) and disassembly. The controlled disassembly of kinetochore-to-pole microtubules is believed to be necessary to anaphase chromosome movement (19, 20), which is often characterized by a decrease in the kinetochore-to-pole distance (18). Furthermore, one model of mitosis predicts that controlled disassembly of microtubules at the poles should be a constant feature of all stages of mitosis (21).

We report here that the cold-stable subpopulation of brain microtubules assembled *in vitro* is stable to calcium at <5 mM and to high concentrations of calmodulin but rapidly disassembles when calmodulin and calcium are both present. Unlike what has been observed on purified cold-labile microtubules, the effect of calmodulin on the purified cold-stable subpopulation is strong and occurs at substoichiometric concentrations.

MATERIALS AND METHODS

Materials. Buffer materials and other reagents were obtained from Sigma, unless stated otherwise, and were of the purest grades available.

Calmodulin was purified from beef and pork brain by modifications of published methods (22). Troponin C was prepared according to Greaser and Gergely (23) or Ebashi *et al.* (24) with slight modifications. It was purified on a DE-52 column equilibrated with 50 mM Tris·HCl, pH 8.0, and eluted with 0–0.5 M NaCl in the same buffer (25). Troponin I (24) was lyophilized before use and, because of its insolubility, resuspended directly in the microtubule-containing preparation at the time of assay.

Podophyllotoxin was obtained from Aldrich. Stelazine (trifluoperazine) was a generous gift from Smith, Kline & French. The buffer (designated MEM) was composed of 100 mM 2-(*N*-morpholino)ethanesulphonic acid/1.0 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid/1.0 mM $MgCl_2$, pH 6.75.

Assembly–Dissassembly Assays by Turbidimetric Measurements. These were carried out in 0.8-ml cuvettes. Changes in optical density of microtubule solutions were followed at 350 nm and 30°C, using a Varian Cary 219 recording spectrophotometer equipped with a constant-temperature chamber and a "cell programmer" for rapid successive assays of five different samples. At the end of each experiment, the baseline (control in the absence of microtubules) was determined for each sample by adding 20 mM $MgCl_2$ and 1.0 mM ATP, which rapidly causes the disassembly of all remaining microtubules, as confirmed by electron microscopy.

Purification of Cold-Stable Microtubules. Adult rats (inbred strain W/FU) were anesthetized with ethyl ether and decapitated. Brains were quickly removed and homogenized in MEM buffer (1.5 ml per brain) at 0°C using a motor-driven Potter–Elvehjem glass homogenizer fitted with a Teflon pestle (2400 rpm, three strokes, loose fitting pestle). Time and extent of homogenization are critical: too-extensive homogenization leads to preparations that assemble totally within 10 min and

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are unsuitable; best results are obtained with preparations that display a lag phase of ≈10 min before the start of assembly.

The homogenate was centrifuged for 30 min at 4°C (Beckman L5-50B centrifuge and 70.1 ti rotor; 200,000 × *g*). Microtubules in the supernatant were then allowed to assemble at 30°C. The turbidity of the rat brain crude-extract supernatant accurately measures the extent of microtubule assembly in the preparation; maximum assembly is observed in the absence of added nucleotide. Further, the microtubules that assemble become progressively more cold stable to a maximal extent (≈40%) of cold stability at 50 min (unpublished results). After 60 min of incubation, the crude extract was cooled to 0°C for 10 min and 25 μM podophyllotoxin was added. On rewarming, the extract, containing only cold-stable microtubules, was layered onto a 50% sucrose cushion (in MEM buffer) and the assembled material was collected by centrifugation at 200,000 × *g* for 2 hr at 25°C (26). The pellets were carefully washed as described (26) and resuspended at 30°C in MEM buffer to one half the initial volume. After a final centrifugation (2500 × *g*, 5 min, 0°C), the supernatant was adjusted to 1.3 mg/ml for use in subsequent assays. Polyacrylamide gel scans indicate that ≈70% of the protein present is tubulin. Typically, cold-stable microtubules in such preparations account for 80% of the total solution turbidity.

Free Calcium. Free calcium concentrations were calculated from the association constants for calcium–EGTA complexes (7). From the published association constants (27–29), other binding contributions (Mg^{2+} and H^+ to EGTA) were negligible (<1% of the total ligand bound to EGTA at the lowest Ca^{2+} concentration used). Free Ca^{2+} concentrations were independently checked by measurement with a calcium electrode. For the crude extract these measurements were important, as free calcium was already present.

Electron Microscopy. Electron microscopy was performed as described (26).

Gel Electrophoresis. Polyacrylamide slab gel electrophoresis was performed using 8% acrylamide/0.1% sodium lauryl sulfate according to Schier-Neiss *et al.* (30). Gels were stained with Coomassie blue before scanning.

RESULTS

Centrifugation of recycled microtubules through a 50% sucrose cushion frees the microtubules of unbound nucleotides, drugs, and contaminating protein (including tubulin subunit) (26, 31). Recently, this approach has been shown to be effective in purifying cold-stable microtubules assembled in brain crude extract (10) and applicable to the rat brain system used here, as confirmed by electron microscopy.

Calcium Sensitivities of Total Crude Extract and Purified Cold-Stable Microtubules. Cold-stable microtubules in the supernatant of rat brain crude extract are very sensitive to submillimolar levels of free calcium, undergoing rapid and almost complete disassembly at 400 μM free calcium (Fig. 1). Similar calcium sensitivities have been reported previously (1, 2, 9). The half-maximal concentration of free calcium causing disassembly is 100 μM.

By contrast, purified cold-stable microtubules are quite insensitive to free calcium; concentrations in the millimolar range are required. The slight response to 0.8 mM free calcium (see Fig. 3, curve b) is as large a response as we have ever obtained at any calcium concentration <5 mM.

Effect of Calcium and Calmodulin on Cold-Stable Microtubules. Purified cold-stable microtubules do not respond to calmodulin in the absence of calcium, even at high calmodulin concentrations (10-fold in excess of tubulin). We assayed for the effect of calmodulin on purified cold-stable microtubules at various calcium concentrations. Calmodulin alone at 4.0 μM causes

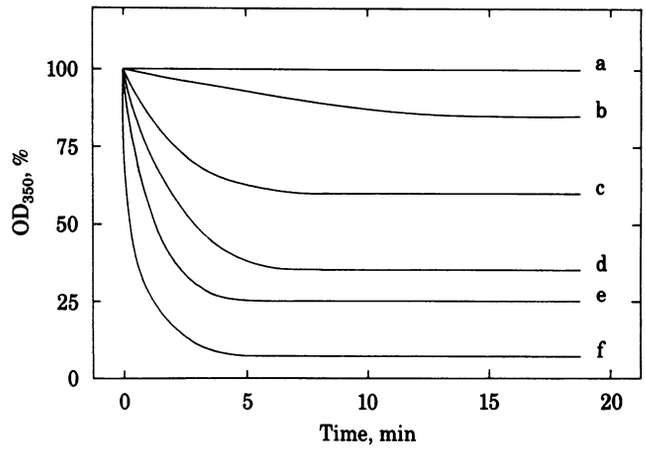

FIG. 1. Effect of $CaCl_2$ on crude-extract cold-stable microtubules. Microtubules were assembled from brain crude-extract supernatants and, after 60 min at 30°C, chilled to 0°C for 10 min, rewarmed in the presence of 25 μM podophyllotoxin, and then exposed to various free Ca^{2+} concentrations. Results are presented as percent of turbidity before Ca^{2+} addition remaining after addition. Curves: a, no Ca^{2+} (control); b–f, Ca^{2+} at 10, 200, 400, 600, and 800 μM, respectively.

no disassembly of microtubules but, in the presence of low concentrations of free calcium, it produces rapid disassembly (Fig. 2). Under these conditions, the half-maximal effect is obtained at a free calcium concentration of 100 μM. Microtubules totally disassemble in calmodulin at 800 μM free calcium.

Similarly, a concentration of calcium (800 μM) that causes rapid disassembly of all species of microtubules present in the crude extract does not destabilize purified cold-stable microtubules (Fig. 3). Addition of calmodulin alone is also without effect; it is evident that microtubules are only sensitive to the presence of both calmodulin and calcium. At this calcium concentration (800 μM) half-maximal disassembly of microtubules occurs in the presence of 1.0 μM calmodulin when the total tubulin concentration is approximately a 10-fold excess.

Although levels of partial disassembly are reached in these titration experiments, plateaus do not seem to represent simple equilibria between subunits and assembled microtubules; podophyllotoxin, an assembly inhibiting drug, does not influence the data. In addition, there should be no equilibrium because these experiments were conducted in the absence of guanine

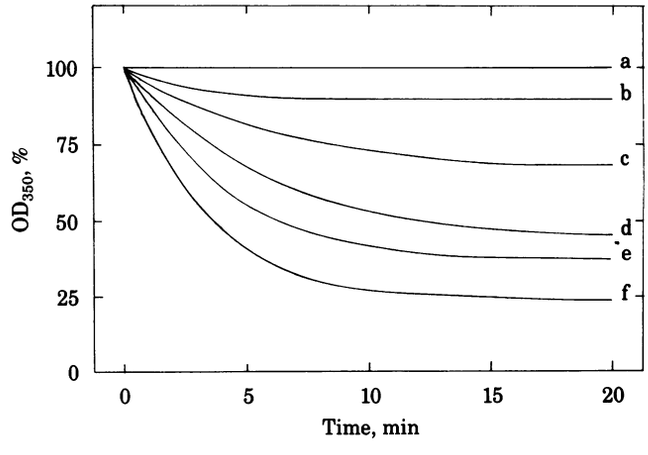

FIG. 2. Effect of $CaCl_2$ on purified cold-stable microtubules in the presence of calmodulin. Purified cold-stable microtubules were assayed for stability to various concentrations of free Ca^{2+} by measurement of their percent loss of turbidity at 30°C. All samples contained 5 μM calmodulin. Curves: a, no Ca^{2+}; b–f, Ca^{2+} at 25, 100, 200, 400, and 800 μM, respectively.

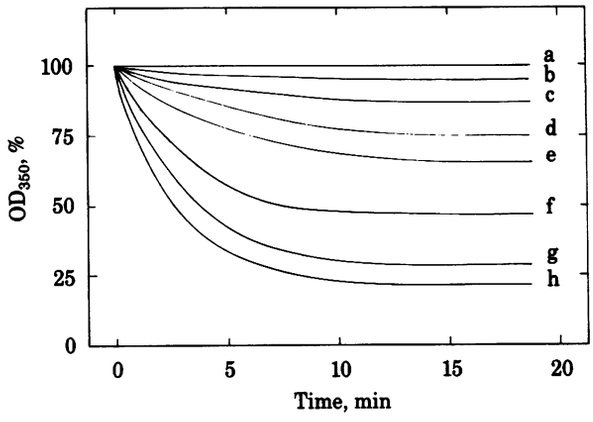

FIG. 3. Effect of calmodulin on purified cold-stable microtubules. Purified cold-stable microtubules were assayed for stability to various concentrations of calmodulin by measurement of their percent loss of turbidity at 30°C. All samples contained 10 μM tubulin. Curves: a, no Ca^{2+} or calmodulin (control); b, 800 μM free Ca^{2+} and no calmodulin; c–h, 800 μM Ca^{2+} and calmodulin at 0.2, 0.5, 1.0, 2.0, 3.0, and 6.0 μM, respectively.

nucleotide, which is required for assembly. Perhaps calmodulin is being sequestered by unpolymerized microtubule protein as the reaction proceeds. It is also possible that a microtubule-associated protein is reassociating with the remaining microtubules more and more densely as the reaction proceeds.

Effects of Troponin C and Parvalbumin on Cold-Stable Microtubules. Skeletal muscle troponin C, a calcium-binding protein similar in sequence to calmodulin (22, 32–34), can substitute for calmodulin in some calmodulin-requiring mechanisms, although its potency is 0.1–1% that of calmodulin itself (5, 35). The results of an experiment in which the effects of troponin C and calmodulin on purified cold-stable microtubule disassembly were compared are shown in Fig. 4. The half-maximum efficiency of troponin C is 5 μM (at 20 μM free Ca^{2+})—i.e., an efficiency approximately one-fifth that of calmodulin.

In a similar experiment, parvalbumin (another homologous Ca^{2+}-binding protein) was without effect on cold-stable microtubules, up to 20 μM.

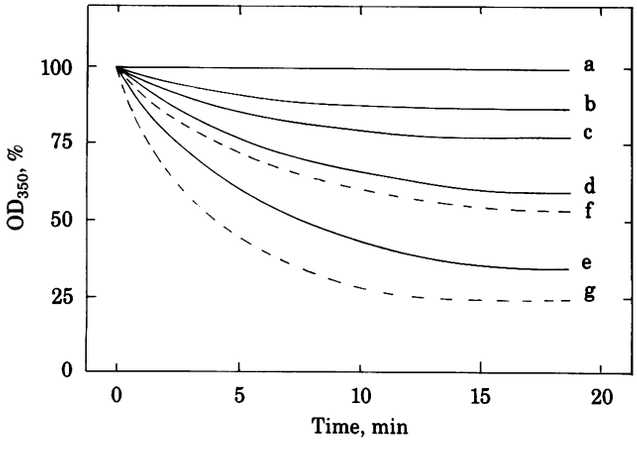

FIG. 4. Effect of troponin C (——) vs. effect of calmodulin (– – –) on purified cold-stable microtubules. Purified cold-stable microtubules were assayed for stability to various concentrations of troponin C by measurement of their percent loss of turbidity at 30°C. Curves: a, no troponin C and no Ca^{2+}; b–e, 800 μM free Ca^{2+} and troponin C at 1.3, 2.5, 5.0, and 10.0 μM, respectively; f and g, 800 μM Ca^{2+} and calmodulin at 1.5 and 5.0 μM, respectively.

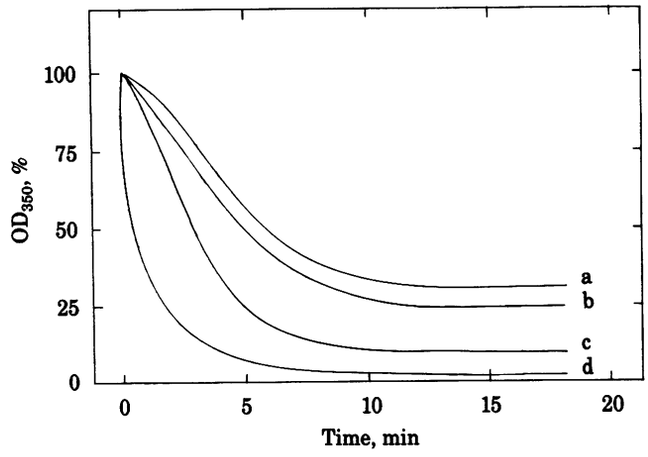

FIG. 5. Effect of troponin I on crude-extract cold-stable microtubules. Crude-extract cold-stable microtubules were prepared and assayed as described in the legend to Fig. 1. Troponin I was added to the samples before addition of $CaCl_2$. All samples contained 800 μM free Ca^{2+}. Curves: a–d, troponin I at 10.0, 5.0, 2.5, and 0 μM, respectively.

Effects of Troponin I and Trifluoperazine on Calmodulin-Induced Disassembly. Troponin I, a regulatory muscle protein that interacts strongly with troponin C, inhibits calmodulin-dependent mechanisms *in vitro* (32, 36). We find that troponin I at 3 μM (maximal) inhibits by 50% the Ca^{2+}-induced disassembly of microtubules in the crude extract (Fig. 5). As troponin I is highly insoluble, its final concentration in solution is uncertain, but must be only a fraction of the total added. Troponin I appears to have an especially marked effect on the initial rates of disassembly.

In the purified cold-stable microtubule system, troponin I blocks calmodulin-induced microtubule disassembly at concentrations stoichiometric to Ca^{2+}/calmodulin (data not shown).

Trifluoperazine, a drug reported to bind to Ca^{2+}/calmodulin and inhibit its activity, has no effect on calmodulin-induced microtubule disassembly at concentrations up to 10 μM. This result is similar to that reported for the purified cold-labile microtubule system (8).

DISCUSSION

Previous demonstrations of the effect of calmodulin on microtubule assembly have shown that it has a reproducible but weak enhancing effect on calcium inhibition of purified cold-labile microtubule assembly (6–8). Calmodulin concentrations between 2- and 8-fold in excess of that of tubulin were required for a substantial effect (6, 8). Calcium alone is capable of disassembling cold-labile microtubules (1–4). Here we present evidence that the cold-stable microtubule subpopulation behaves uniquely. It is almost totally resistant to either calcium (at millimolar concentrations) or calmodulin alone but disassembles rapidly in the presence of both at apparently physiological concentrations.

The intracellular concentration of calmodulin in the rat brain is ≈40 μM (37). The free calmodulin concentration in our crude extracts is 5 μM, close to the concentration yielding a maximal effect on purified cold-stable microtubules.

There are two important points to make with respect to this reaction. First, it is not stoichiometric, in that the microtubule response is half-maximal at a calmodulin concentration one-tenth the total tubulin concentration. Second, calmodulin causes the disassembly of microtubules that are not at equilibrium. It therefore appears not to function by interfering with a subunit-addition reaction.

The concentration range over which calcium changes from minimally to maximally effective in the presence of calmodulin is high (100–800 μM) relative to the amount required to saturate the four calcium binding sites on calmodulin (present at 4 μM). This result suggests that calcium has two distinct effects: first, it could activate calmodulin to destabilize microtubule end caps and, then, it could function independently of calmodulin at higher concentrations to directly promote dimer dissociation from calmodulin-destabilized polymers. The argument for a two-stage effect is buttressed by evidence that purified cold-stable microtubules are rendered cold labile in the presence of calmodulin at calcium concentrations just sufficient to saturate the calmodulin binding sites (unpublished observations).

The substoichiometric effect may be due to the formation of a tight complex of calmodulin with a microtubule-associated protein present at a concentration less than that of tubulin. This protein could presumably act as an end cap on the microtubule, preventing equilibrium loss from the cold-stable population. The fact that Ca^{2+} promotes a strictly end-wise depolymerization of microtubules (38) reinforces the hypothesis that calmodulin acts on a microtubule end cap. We have previously observed the presence of a substoichiometric 64,000-dalton "switch protein" on crude extract microtubules that appears to be responsible for forming a microtubule cap when dephosphorylated (39). Perhaps calmodulin directly influences the binding of this switch protein to the microtubule. It is possible that a search for a calmodulin-binding protein in cold-stable microtubule preparations might prove fruitful.

As noted by Marcum *et al.* (6), troponin C could replace calmodulin in the Ca^{2+}-dependent disassembly of microtubules. By using a different preparation of rat brain tubulin in a reconstituted *in vitro* system, these authors found troponin C to be even more effective than calmodulin whereas, under our experimental conditions, it was one-fifth as effective. In either case, these data indicate that the relative potency of troponin C in affecting microtubule assembly is far greater than observed with enzymes such as cyclic AMP phosphodiesterase or myosin light chain kinase, in which a 100- to 1000-fold excess of troponin C is required to elicit the same response.

The experiments reported here were conducted in the absence of any nucleoside triphosphate. Thus, it does not seem likely that calmodulin would exert its influence in microtubule stability indirectly—e.g., through enhancement of a calmodulin-dependent kinase activity. We have found (39) that a separate mechanism, possibly involving phosphorylation of the switch protein, independently destabilizes cold-stable microtubules.

It is apparent that there are different rates of disassembly of the crude and the purified cold-stable microtubules (cf. Fig. 1 and Fig. 2). It may be that the faster rate obtained in the crude extract results from the combined actions of the kinase and the Ca^{2+}–calmodulin complex.

The location of calmodulin at the mitotic poles (12–14), where all microtubule net disassembly ends are found (15, 16), suggests that it regulates the end-wise depolymerization of the spindle microtubules, especially those that are cold-stable (kinetochore-to-pole microtubules). Calmodulin-regulated microtubule disassembly could thus promote chromosome movement toward the poles in anaphase and possibly help to establish a constant poleward microtubule treadmilling reaction throughout mitosis (21).

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