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ATP-dependent regulation of cytoplasmic microtubule disassembly

(inhibitors of energy metabolism/colcemid/vinblastine/tubulin treadmilling/protein kinase)

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ABSTRACT **Indirect immunofluorescent staining with an antitubulin antibody was used for studying the role of ATP in the regulation of cytoplasmic microtubule disassembly. Depletion of the cellular ATP pool in cultured mouse fibroblasts with various inhibitors of energy metabolism leads to inhibition of the microtubule disassembly induced by colcemid or vinblastine. Glucose added to the inhibitor-containing incubation medium partially restores the cellular ATP content and abolishes the inhibition of microtubule disassembly. The metabolic inhibitors did not change [^{3}H]colcemid uptake by the cells; therefore, their action on the microtubule disassembly was not caused by the reduction in intracellular colcemid. Addition of ATP to the cytoskeleton preparations obtained by Triton X-100 treatment of the cells markedly stimulates microtubule depolymerization. This effect was specific for ATP; it was not observed in the presence of GTP, UTP, CTP, ADP, AMP, adenosine 5′-(β,γ-methylene)triphosphate (a nonhydrolyzable analogue of ATP), or inorganic pyrophosphate or tripolyphosphate. Therefore, depletion of the cellular ATP pool reduces the rate of microtubule disassembly whereas addition of ATP increases it. These results suggest that a certain ATP-dependent reaction [most probably, phosphorylation of some of the microtubule protein(s)] controls microtubule disassembly in the cells.**

Division, locomotion, and changes of the eukaryotic cell shape are accompanied and, probably, determined by the processes of assembly and disassembly of microtubules and other cytoskeletal structures. For example, in the prophase of mitosis, cytoplasmic microtubules are depolymerized and their subunits are used to form a mitotic spindle (1). In the late telophase, a reverse process takes place: i.e., spindle microtubules undergo depolymerization, and cytoplasmic microtubules form again. Therefore, assembly and disassembly of cytoplasmic microtubules are subjected to cellular control. *In vitro* studies have shown that the assembly of microtubules depends on the presence of GTP (2, 3) and is strongly inhibited by Ca^{2+} (2). Nevertheless, it is not clear yet how microtubule assembly and disassembly are regulated in the living cell.

The use of mitostatic alkaloids (e.g., colchicine and colcemid) makes it possible to study the kinetics of microtubule disassembly in cells. These drugs bind to the depolymerized tubulin molecules, and the resultant tubulin–drug complex blocks further assembly of microtubules (4). Therefore, the time of microtubule disassembly in the presence of colcemid or colchicine depends on the rate of dissociation of subunits from microtubules.

A different approach to the study of microtubule depolymerization is provided by the use of cells treated with Triton X-100 for solubilization of the plasma membrane (5–8). Microtubule assembly in the Triton-extracted cells is inhibited because depolymerized tubulin readily leaves the cells and is diluted by the incubation buffer to a concentration less than the critical one. Thus, in Triton-extracted cells, only the depolymerization of microtubules takes place (8). This allows us to study microtubule disassembly without using assembly inhibitors.

This paper describes experiments showing that microtubule disassembly in cultured mouse fibroblasts is an ATP-dependent process. It was shown that (*i*) the rate of microtubule destruction in colcemid- or vinblastine-treated cells sharply decreases in the presence of energy-metabolism inhibitors that reduce the cellular ATP pool and (*ii*) addition of ATP to cells demembranated with Triton X-100 caused a marked acceleration of microtubule destruction. We interpret the ATP requirement for microtubule disassembly as an indication of a possible role of microtubule protein phosphorylation in the regulation of this process.

MATERIALS AND METHODS

Cells. Secondary cultures of mouse embryo fibroblasts were used. The cells were plated at a density of 2×10^4 cells per cm^2 onto Petri dishes with slides and used 24 hr later. The cultures were grown in L-15 medium supplemented with 10% fetal calf serum. Incubation with inhibitors of energy metabolism, colcemid and vinblastine, was performed in Dulbecco's phosphate-buffered saline.

Immunofluorescent Staining. Microtubules in the cells were visualized by indirect immunofluorescent staining with a monospecific antibody against bovine brain tubulin. Before fixation, the cultures were extracted with 1% Triton X-100 in buffer M [50 mM imidazole, pH 6.8/50 mM KCl/0.5 mM $MgCl_2$/1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid/0.1 mM EDTA/1 mM 2-mercaptoethanol] at 20°C for 3 min. To preserve the microtubules during extraction, buffer M was supplemented with 4% polyethylene glycol 40,000. The procedures of fixation and antibody staining, as well as control of the specificity of staining, have been described (8, 9).

Effect of ATP on Detergent-Extracted Cells. The cells were extracted with Triton X-100 as described above and then incubated in buffer M with or without nucleotide at 37°C. After incubation, the cultures were fixed and stained with an antitubulin antibody.

Miscellaneous Biochemical Procedures. To study the effect of inhibitors on the colcemid uptake, 24-hr-old cultures of the same density as in the depolymerization experiments were incubated at 37°C with 10 μM [*ring C,methoxy*-^{3}H]Colcemid [New England Nuclear; specific activity 8.96 Ci/mmol (1 Ci = 3.7×10^{10} becquerels)] diluted to a specific activity of 0.10 Ci/mmol with unlabeled drug. Cell-bound radioactivity was extracted with 3% trichloroacetic acid and determined by liquid scintillation counting.

The cellular ATP content was determined by the luciferin-luciferase method, as modified (10). ATP was extracted from the cells by 3% trichloroacetic acid at 0°C, and the amount of ATP

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in the extract was measured on a Pico-ATP luminometer (Jobin Yvon) using LKB reagents.

RESULTS

Experiments with Living Cells. The immunofluorescent staining of the control cells showed numerous microtubules spreading from perinuclear regions of the cells (Fig. 1A) as described (1, 6, 11). Colcemid induced a rapid destruction of the microtubule network. After 30 min of incubation of the cells with 10 μM colcemid, only a limited number of microtubule fragments was seen in the cytoplasm. These fragments were as a rule connected with the cell center (Fig. 1B). We chose a 30-min incubation because it allows us to observe acceleration and deceleration of microtubule depolymerization.

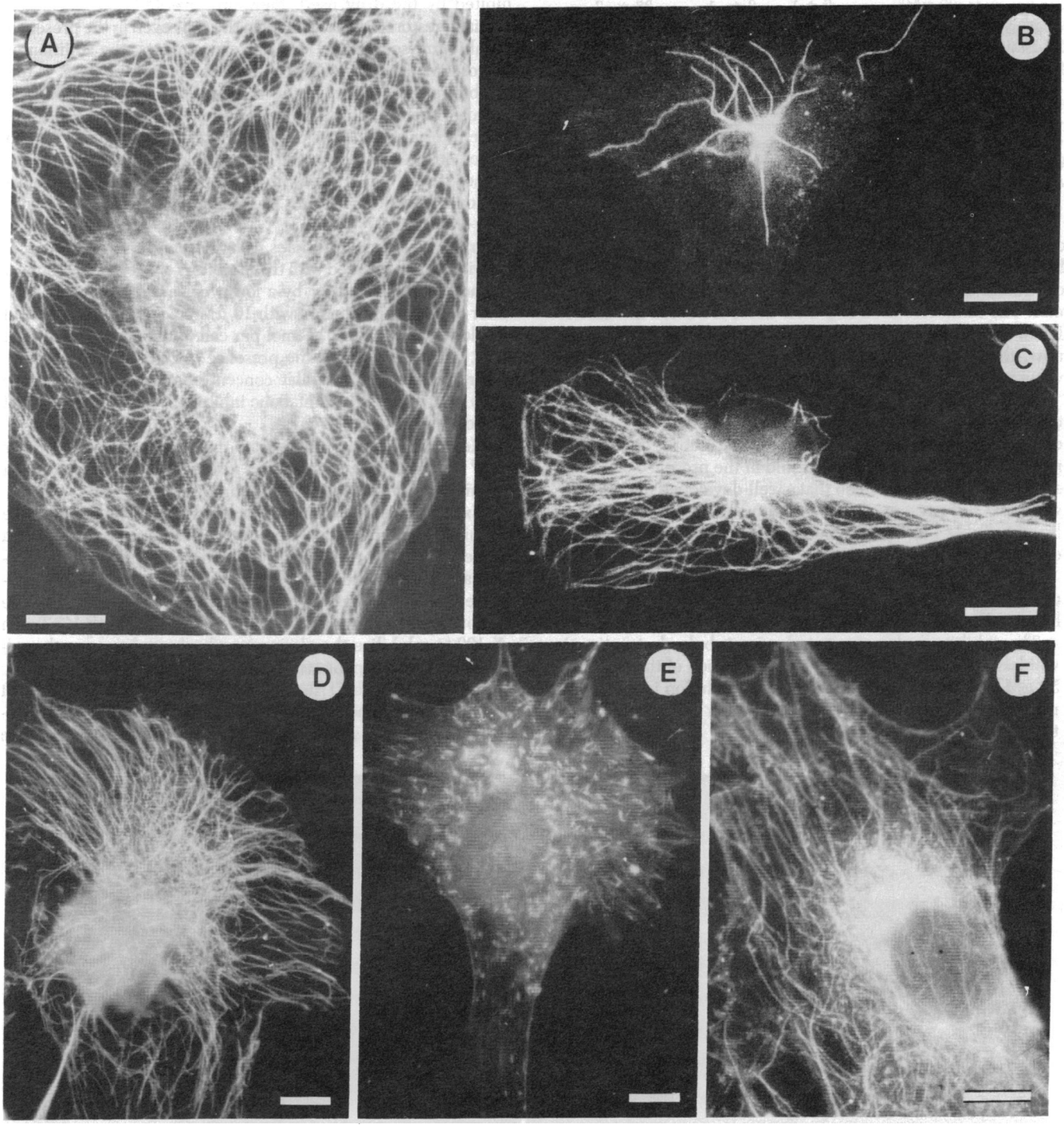

FIG. 1. Blocking of microtubule disassembly in mouse embryo fibroblasts treated with energy-metabolism inhibitors; indirect immunofluorescent staining with antibodies against tubulin. (*A*) Control: A well-developed microtubule network. (*B*) Cell exposed to 10 μM colcemid for 30 min. Note sharp reduction of both amount and length of microtubules. (*C*) Cell treated for 15 min with 20 mM sodium azide and then incubated for 30 min with 10 μM colcemid and 20 mM sodium azide. No destruction of microtubules is observed (cf. *B*). (*D*) Cell incubated with 20 mM sodium azide only for 45 min. (*E*) Cell exposed to 50 μM vinblastine sulfate for 30 min. Microtubules are completely destroyed. Numerous vinblastine-induced tubulin paracrystals can be seen. (*F*) Cell treated for 15 min with 20 mM sodium azide and then incubated for 30 min with 50 μM vinblastine sulfate and 20 mM sodium azide. Microtubules are partially preserved (cf. *E*). Scale bars, 10 μm.

Table 1. ATP content of mouse embryo fibroblasts treated with inhibitors of energy metabolism

Inhibitor	Incubation conditions: Dulbecco's saline 15 min	Dulbecco's saline 45 min	Dulbecco's saline + 30 mM glucose (45 min)
None	97 ± 6	110 ± 10	125 ± 13
Sodium azide (20 mM)	7 ± 1	2 ± 1	38 ± 7
Oligomycin (2.5 μM)	33 ± 1	8 ± 2	46 ± 2
2,4-Dinitrophenol (1 mM)	24 ± 3	3 ± 2	59 ± 4
Carbonyl cyanide *m*-chlorophenylhydrazone (10 μM)	12 ± 5	5 ± 2	33 ± 5

Values are expressed as percent of control value (mean ± SEM). Control cells (incubated in growth medium only) contained 6.6 fmol of ATP per cell.

To study the effect of energy metabolism inhibitors on microtubule destruction, the cells were pretreated with the inhibitors for 15 min, and then colcemid was added to a final concentration of 10 μM and the incubation was continued for 30 min. The inhibitors used and their doses are listed in Table 1. It can also be seen in Table 1 that in the presence of the inhibitors, the cellular ATP pool decreased to 7–33% of the control value after 15 min of incubation and to 2–8% after 45 min.

The inhibitors of energy metabolism dramatically reduced the rate of microtubule destruction in the colcemid-treated cells. Fig. 1*C* shows a typical pattern of microtubule distribution in cells treated with 10 μM colcemid in the presence of 20 mM sodium azide. It can be seen that a well-developed microtubule system is still present. Other inhibitors of energy metabolism listed in Table 1 also protected microtubules from colcemid-induced disassembly.

The inhibitors of energy metabolism made no significant changes in either the number or the distribution of microtubules in the cells (Fig. 1*D*).

The inhibitors also prevented microtubule destruction in vinblastine-treated cells. A 30-min incubation of the cells with 50 μM vinblastine sulfate in Dulbecco's solution induced degradation of the microtubules and formation of numerous small tubulin-containing paracrystals (Fig. 1*E*). If the cells were treated with vinblastine in the presence of 20 mM sodium azide, the microtubules were preserved (Fig. 1*F*). In some of the cells treated with vinblastine and sodium azide, a few tubulin paracrystals are seen in the cytoplasm together with the microtubules. These paracrystals probably resulted from precipitation of soluble tubulin in the cells.

To test the specificity of the inhibitors, we added them to the cells together with glucose. The glycolytic pathway was not inhibited by the drug used; therefore, the addition of glucose partially restored the ATP level in the cells even in the presence of inhibitor (see Table 1). In our experiments, the addition of glucose abolished the effect of the metabolic inhibitors. If the inhibitors were added to the cells in the presence of 30 mM glucose, the microtubules were destroyed by colcemid at the same rate as in the absence of inhibitors. Glucose itself had no effect on colcemid-induced microtubule depolymerization. Hence, the action of the inhibitors on microtubule depolymerization is specific and results from the reduction of the ATP content of the cells.

Protection of microtubules from colcemid-induced destruction in the cells treated with the inhibitors of energy metabolism cannot be accounted for by a lower colcemid uptake. In fact, after 30 min of incubation with 10 μM colcemid, the uptake of alkaloid was 1.26 ± 0.14 fmol per cell in the absence and 1.33 ± 0.14 fmol per cell in the presence of 20 mM sodium azide. Therefore, the intracellular concentration of colcemid in the cells treated with the metabolic inhibitors was identical to that in nontreated cells.

Hence, these data demonstrate that depletion of the cellular ATP pool by metabolic inhibitors results in a strong suppression of microtubule depolymerization in cells.

Experiments with Triton-Extracted Cells. The dependence of microtubule depolymerization on ATP may be a result of a direct interaction between nucleotide and microtubules. Alternatively, this effect may be mediated by a soluble cell component(s). To choose between these possibilities, we studied the action of ATP on microtubules in cells treated with 1% Triton X-100 to solubilize the plasma membrane and remove the soluble cellular components.

Incubation of the Triton-extracted cells in buffer M without ATP results in gradual depolymerization of cytoplasmic microtubules. Fig. 2*A* shows a cell extracted with Triton and then

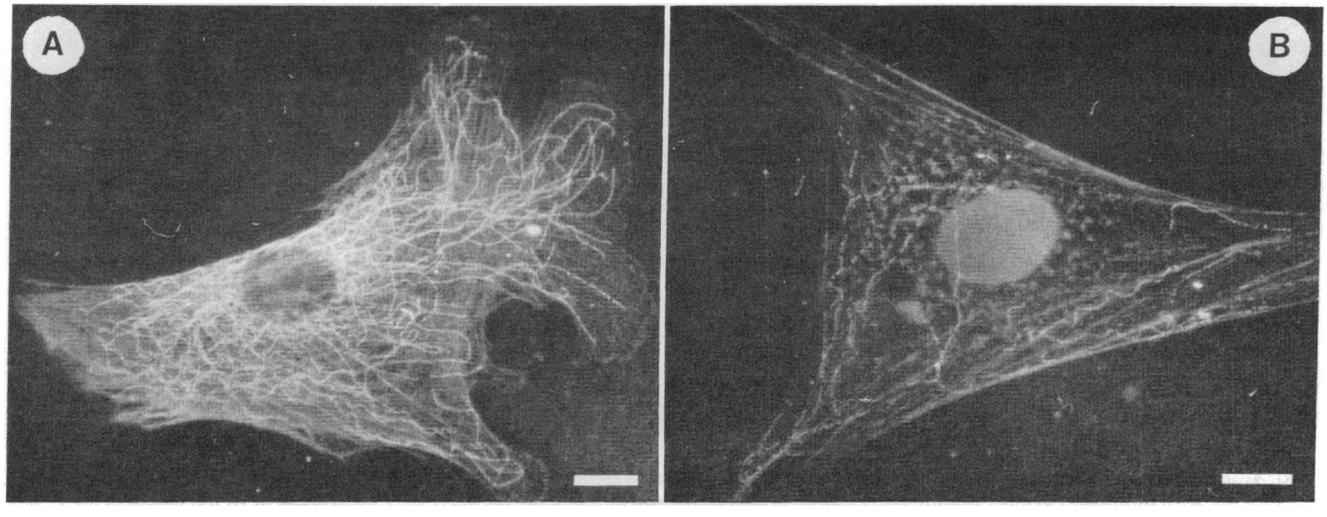

FIG. 2. ATP-induced acceleration of microtubule disassembly in Triton-extracted cells. Indirect immunofluorescent staining with antitubulin antibody. (*A*) Mouse embryo fibroblast extracted with 1% Triton X-100 and then incubated for 30 min at 37°C in ATP-free buffer M. Numerous microtubules are still present. (*B*) This cell was treated as in *A*, but incubation was performed in buffer M supplemented with 2 mM ATP. The microtubule network is completely destroyed. Some microtubule fragments are seen in the cytoplasm. Scale bars, 10 μm.

incubated for 30 min in buffer M without ATP. It can be seen that 30 min of incubation only slightly diminished the number and length of microtubules (cf. Fig. 1A). For complete depolymerization of the microtubule system under these conditions, the cells must be incubated for more than 3 hr (8). The addition of ATP to buffer M strongly stimulated the microtubule disassembly. Thus, 2 mM ATP induced almost complete depolymerization of microtubules after only 30 min of incubation (Fig. 2B).

It should be mentioned here that in the cells with depolymerized microtubules, the antitubulin antibody often stains the nuclei, some particles in the perinuclear region, and, sometimes, stress fibers. This staining pattern has never been observed in cells with an intact microtubule system. None of these structures was stained with an antibody preincubated with an excess of soluble tubulin. Therefore, we think that the fluorescence of nuclei and other intracellular structures in cells with depolymerized microtubules is due to a nonspecific adsorption of tubulin on these structures.

It is not likely that the stimulation of microtubule depolymerization by ATP was caused by chelation of free Mg^{2+} from the solution because increasing the $MgCl_2$ concentration in buffer M from 0.5 to 5 mM did not protect the microtubules from depolymerization by 2 mM ATP. On the other hand, even 5 mM EDTA in buffer M is not sufficient to stimulate microtubule depolymerization.

Destruction of the microtubules occurs only in the presence of ATP; it does not take place in buffer M supplemented with 2 mM GTP, UTP, CTP, ADP, AMP, adenosine 5′-(β,γ-methylene)triphosphate (a nonhydrolyzable ATP analogue), or inorganic pyrophosphate or tripolyphosphate.

Hence, ATP induces microtubule depolymerization by direct interaction with a cytoskeletal protein.

DISCUSSION

The present communication has demonstrated that the processes of microtubule destruction in cultured fibroblasts are strongly inhibited if the cellular ATP pool is depleted by metabolic poisons.

It is well established that microtubule growth both *in vitro* (2, 3) and *in vivo* (12) is an energy-dependent process. On the other hand, we have shown (9), and confirmed here, that even after prolonged treatment of cells with inhibitors of energy metabolism, there were no significant changes in either the quantity or the average length of the microtubules. This result suggested that the reduction of intracellular ATP level inhibited not only the growth but also the depolymerization of microtubules. To check this possibility, we investigated the effects of various inhibitors of energy metabolism on the processes of microtubule depolymerization. It has been shown that microtubule depolymerization both in colcemid- and vinblastine-treated cells can be effectively prevented by these inhibitors. Similar results were obtained recently by Moskalewski *et al.* (13), who studied the effect of colchicine on rat chondrocytes.

Our results show that the effect of ATP on microtubules is not mediated by soluble proteins or small molecules of the cells; the rate of depolymerization of microtubules in the cells extracted with Triton X-100 is still dependent on ATP.

In addition, we showed that this effect is specific for ATP; microtubule disassembly was not accelerated by any other nucleoside triphosphate. These data are in good agreement with the results of Margolis and Wilson (14), who showed that ATP stimulates microtubule treadmilling *in vitro*. It is evident that both the *in vitro* effects of ATP described by Margolis and Wilson and the *in vivo* results reported here are due to the same mechanisms and that these mechanisms regulate microtubule disassembly in cells.

We do not believe that the state of microtubules in cells is directly controlled by ATP level. However, it is quite probable that phosphorylation of some of the microtubule protein by microtubule-associated protein kinase (15) is required for microtubule disassembly. If this is the case, then the depletion of ATP would result in the inhibition of phosphorylation and protect microtubules from depolymerization. These conclusions are supported by recent evidence (16) that phosphorylation of microtubule-associated proteins inhibits overall microtubule assembly *in vitro*.

We think that a similar mechanism of regulation operates in the control of the state of other cytoskeletal structures—e.g., actin microfilament bundles. This conclusion is based on our data showing that depletion of the ATP pool in cells protects the bundles from cytochalasin-induced destruction (9). It is known that cytochalasins B and D inhibit elongation of actin filaments (17–19). Therefore, the rate of destruction of microfilament bundles in the presence of cytochalasins probably reflects the rate of naturally occurring depolymerization of actin microfilaments. Thus, the suppression of the cytochalasin-induced destruction of actin microfilament bundles in the presence of energy-metabolism inhibitors is evidence that the depletion of ATP results in the inhibition of depolymerization of F actin *in vivo*.

We came to the conclusion that depolymerization of microtubules and, possibly, microfilaments in cells is an ATP-dependent process. Further studies are necessary to determine the molecular mechanisms of depolymerization control.

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