INHIBITION OF TUBULIN-DEPENDENT ADENOSINE TRIPHOSPHATASE (ATPASE) ACTIVITY IN MICROTUBULE PROTEINS FROM PORCINE BRAIN BY COLCHICINE

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Tubulin-dependent Mg^{2+}-ATPase activity of microtubule-associated proteins (MAPs) was stimulated by adding high concentrations of tubulin and dilute GTP. Preincubation of colchicine with tubulin for 30 min at 37°C suppressed the stimulation to some extent. The enhancement by GTP and the inhibition by colchicine were not observed containing Ca^{2+} instead of Mg^{2+}. These experimental conditions to enhance tubulin-dependent Mg^{2+}-ATPase activity of MAPs were very similar to those to induce tubulin polymerization. Furthermore, the enhancement seemed not to be caused by Pi release from microtubule polymerization.

INTRODUCTION

It has been demonstrated that cytoplasmic microtubules contain a unique ATPase which is insensitive to several ATPase inhibitors.[1] The ATPase activity was recovered in the microtubule-associated proteins (MAPs) fraction after separation of MAPs and tubulin using several methods.[2] The activity in the MAPs fraction was remarkably stimulated by the addition of tubulin.[3] Tubulin-dependent ATPase activity of MAPs was more active with Ca^{2+} than with Mg^{2+}. The ATPase seemed not to be related to tubulin polymerization, since Ca^{2+} was one of the most potent inhibitors of tubulin polymerization[4] and the ATPase activity reached a plateau when a small amount of tubulin (0.11mg/ml) below critical concentration of its polymerization (0.15-0.2 mg/ml) was added to the reaction mixture containing MAPs.[3a,5]

Vinblastine, a potent inhibitor of tubulin polymerization, stimulated tubulin-dependent MAPs ATPase activity through its binding to tubulin dimers.[6,7] The stimulation is thought to depend on tubulin aggregation by vinblastine, because the drug has been known to form oligomeric struc-

tures through its interaction with microtubule proteins.[7,8] Recently, we found that taxol which promotes microtubule polymerization stimulated tubulin-dependent Mg^{2+}-ATPase activity of MAPs.[9] Various functions of microtubules including axonal transport, cell division, receptor activity, secretion, and maintenance of cell shape are thought to depend on the controlled assembly and disassembly of microtubules in the cytoplasm.[10] Most of these functions are also considered to contain an energy-dependent process. Thus, it is important to determine the relationship between the ATPase activity and structural change of microtubules. We now present evidence that tubulin-dependent Mg^{2+}-ATPase activity of MAPs is more active under conditions that induce microtubule formation *in vitro*.

MATERIALS AND METHODS

Materials—GTP and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Boehringer, colchicine and ethyleneglycol-bis (2-amino-ethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) from Nakarai Chemical, phosphoenolpyruvate and pyruvate kinase from Oriental Yeast, pepstatin from Protein Foundation, and phosphocellulose from Whatman. Other chemicals used were of the highest purity available.

Preparation of Proteins—Porcine brain microtubule proteins were purified by three cycles of temperature-dependent polymerization and depolymerization in buffer A [100 mM MES-KOH (pH 6.5), 0.5mM Mg $(CH_3COO)_2$, 1mM EGTA, and 2μg/ml pepstatin]. The second and third polymerization steps were carried out in the presence of buffer A containing 10% glycerol, 0.2mM GTP, 0.5mM phosphoenolpyruvate and 0.5μg/ml pyruvate kinase, and the solution was centrifuged at 70000×g for 45 min at 25°C after laying on a cushion containing buffer A and 25% glycerol. MAPs and tubulin were prepared by using ion exchange chromatography on a phosphocellulose and dialyzed against buffer A and 25% glycerol as described previously.[3a,6,9] Proteins were stored at −80°C until use.

Analytical Procedures—ATPase activity was measured at 37°C for 30 min in terms of the release of inorganic phosphate.[6,9] The standard reaction mixture contained 60mM MES-KOH (pH 6.5), 0.5mM EGTA, 5mM 2-mercaptoethanol, 12.5% glycerol, 2mM ATP, 0.2mM GTP, and either 2mM $Mg(CH_3COO)_2$ or 5mM $CaCl_2$. The concentrations of MAPs and tubulin were 0.24 and 0.64mg/ml, respectively.

The polymerization of tubulin was determined at 37°C by the continuous measurement of the turbidity at 350 nm. The reaction mixture was

same as in the standard condition of ATPase activity containing 2mM $Mg(CH_3COO)_2$. The assay was initiated by the addition of ATP-GTP mixture and incubation was extend for 30 min.

Protein was determined by the method of Lowry *et al.*[11] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Tubulin-dependent MAPs ATPase activity in the presence of Mg^{2+} was lower than the activity in the presence of Ca^{2+} at low concentrations of tubulin. However, Mg^{2+}-ATPase activity was appreciably close to Ca^{2+}-ATPase activity at high concentrations of tubulin (Table 1). The addition of 0.2mM GTP to the reaction mixture containing Mg^{2+}, which tends to induce tubulin polymerization, resulted in stimulation of hydrolytic activity of MAPs in the presence of tubulin alone, while the stimulation by GTP in the presence of Ca^{2+} was not observed. Although colchicine, a plant alkaloid which inhibits tubulin polymerization,[7] was not inhibitory to Ca^{2+}-ATPase of MAPs in the presence or absence of tubulin, it showed an inhibitory effect on tubulin-dependent Mg^{2+}-ATPase of MAPs. The degree of inhibition by colchicine was remarkable at high concentrations of tubulin under conditions where tubulin can polymerize into microtubules, compared with that at a low concentration of tubulin. The same phenomena were observed when podophyllotoxin was used instead of colchicine. When GTP

Table 1. Stimulation by Tubulin of Mg^{2+}-and Ca^{2+}-ATPase of MAPs in the Presence and Absence of Dilute GTP and Colchicine

Divalent cation	Tubulin conc. (mg/ml)	ATP (2mM)	ATP+GTP (2mM+0.2mM)	ATP+GTP+colchicine (2mM+0.2mM+0.01mM)
		Pi released (nmole/30min)		
Mg^{2+}	0	15.2	17.3	17.8
	0.11	29.1	33.7	28.0
	0.56	50.9	59.9	41.1
Ca^{2+}	0	3.9	4.0	4.3
	0.11	40.8	39.4	40.4
	0.56	59.0	59.0	57.8

Values are the amount of Pi released (nmole) during incubation for 30 min at 37°C. Enzyme activity was measured in the standard condition modified to contain the concentrations of tubulin indicated. The concentrations of GTP and colchicine were 0.2 and 0.01mM, respectively. Colchicine was preincubated with tubulin for 30 min at 37°C to form tubulin-colchicine complex.

(1mM) was used as a substrate, tubulin stimulated the hydrolytic activity of MAPs in the presence of Ca^{2+} or Mg^{+2}, but neither colchicine nor podophyllotoxin influenced detectably the activity (data not shown).

The release of Pi from ATP-GTP mixture may be due to tubulin polymerization because the polymerization is linked to GTP hydrolysis.[12] High molecular weight components and tau proteins, which were contained in the MAPs fraction and induced tubulin polymerization, have been shown to retain their activities after heat treatment of microtubule proteins for 4 min at 95°C in the purification procedure.[13] The inducing activity on tubulin polymerization by MAPs was still retained when MAPs were incubated for 3 min at various temperatures from 37 to 85°C, and furthermore the enhancement of the activity was observed above 65°C as shown in Fig. 1. The increased turbidity caused by polymerization was reversed by cooling. On the other hand, tubulin-dependent MAPs ATPase activity in the presence of Ca^{2+} or Mg^{2+} was completely inactivated by heating above 65°C.

A small amount of Pi release was observed at a constant rate for 30 min

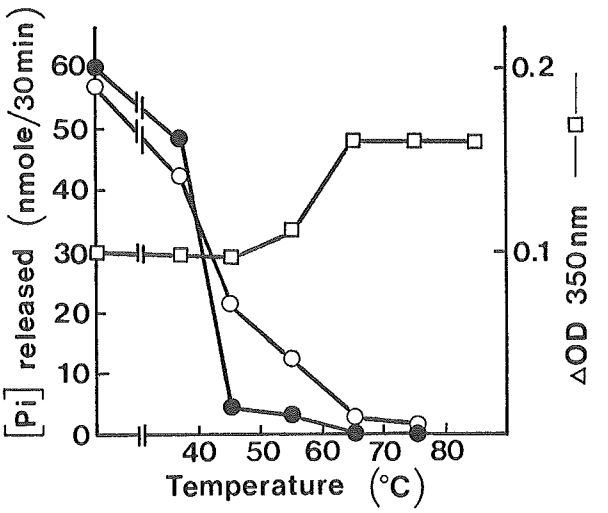

Fig. 1. Effect of Heat Treatment of MAPs on ATPase Activity and Tubulin Polymerization

MAPs (1.3mg/ml) in buffer A containing 25% glycerol were heated for 3 min at the indicated temperature and rapidly cooled. The standard medium for ATPase activity was added to test tube containing heat-treated MAPs, and the activity was measured. The polymerization was expressed in the value of 350 nm for the initial 30 min. ○, 2mM Mg $(CH_3COO)_2$; ●, 5mM $CaCl_2$.

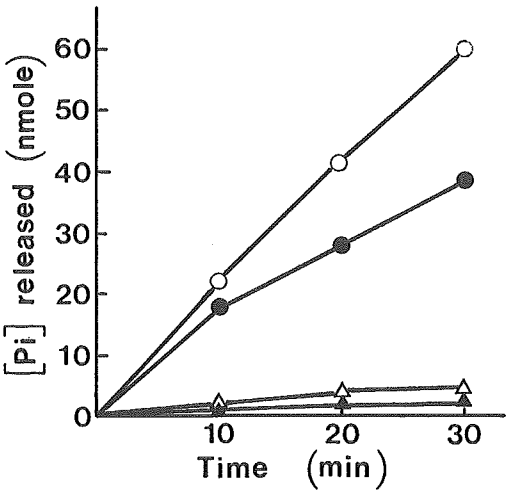

Fig. 2. Time Course of Pi Liberation by Tubulin-Dependent Mg^{2+}-ATPase of Native and Heat-Treated MAPs

Enzyme activity was measured as described in Fig. 1. Colchicine (0.01mM) was preincubated with tubulin (1.3mg/ml) in buffer A containing 25% glycerol for 30 min at 37°C. Heat treatment was performed by incubation of MAPs for 3 min at 75°C as described in Fig. 1. ○, Native MAPs ; ●, native MAPs+colchicine ; △, heat-treated MAPs ; ▲, heat-treated MAPs+colchicine.

by the addition of heat-treated MAPs to the reaction mixture of ATPase assay under conditions in which tubulin polymerization was induced (Fig. 2). This Pi liberation seems to be due to GTP hydrolysis during polymerization of tubulin. The addition of colchicine to the reaction mixture reduced Pi liberation from 4.7 to 2.6 nmole during a period of 30 min. In this experimental condition, microtubule polymerization was scarecely observed in the presence of colchicine (data not shown). On the contrary, a large amount of Pi was released in the reaction mixture containing native MAPs, which are also capable of forming microtubules, and 20.7 nmole Pi was reduced during the same period by adding colchicine. The decrease in Pi release in the latter case did not account for GTP hydrolysis by tubulin polymerization alone. Polymerized tubulin, therefore, is considered to be more effective than 6S tubulin in the stimulation of Mg^{2+}-ATPase of MAPs.

The motive force of cilia and flagella has been established to depend on the presence of dynein (ATPase) associated with outer doublet microtubules.[14] Dynein-like ATPase has been also identified in the cytoplasm of sea urchin eggs.[15] Hoshino[16] showed that porcine brain tubulin markedly stimulated the ATPase activity of 30S dynein from Tetrahymena cilia. It is

well known that F-actin is more active than G-actin in the stimulation of myosin ATPase activity. Contractile proteins are considered to contain a filamentous structure which should be closely associated with a force generating system transducing chemical energy (ATP) into mechanochemical work. Recent evidence suggests that membrane vesicles and granules in squid giant axon are moved along the microtubules by axonal flow and, further, a force generation protein is involved in the microtubule-based motility.[17] At present, however, we do not know the physicochemical features and precise function of tubulin-dependent ATPase. The surface of brain microtubules are covered by filamentous projections comprised of MAPs which tentatively perform a role analogous to that of dynein.[5a,18] The composition of MAPs is different in the regions of the nervous tissues.[19] Therefore, tubulin-dependent MAPs ATPase may play a significant role intracellular motion in a manner similar to actomyosin and dynein-tubulin systems.

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