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Effects of Tubulin-myosin Interaction on Guanosine-5′-Triphosphate Hydrolysis by Myosin

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Tubulin from porcine brain inhibited not only the Mg-ATPase activity but also the Mg-GTPase activity of actomyosin from rabbit skeletal muscle. The inhibitory effect of tubulin on GTPase, however, was weak and a large amount of tubulin was necessary for half-maximum inhibition as compared with ATPase inhibition by tubulin. Although tubulin inhibited GTP hydrolysis by actomyosin at higher concentrations of GTP, it was found that tubulin conversely accelerated the hydrolysis of GTP at low concentrations of substrate (<0.3 mM). The Km value for GTP fell from 2.5×10^{-3} to 1.1×10^{-3} M upon adding tubulin. Since tubulin slightly activated myosin Mg-GTPase activity, it might block the site of actin activation. The inhibition did not seem to be competitive, because excess actin did not completely eliminate the GTPase inhibition by tubulin. The addition of KCl (at 50—100 mM) activated the GTPase activity of actomyosin, but this activation was not observed when tubulin was present. Further addition of KCl (at 200—400 mM) decreased the GTPase activity both in the presence and absence of tubulin, although the GTPase activity in the presence of tubulin was higher than that in the absence of tubulin. The extent of inhibition of GTPase by tubulin was not significantly affected by the presence of colchicine or vinblastine.

Keywords——tubulin; actomyosin; myosin; interaction; GTPase; ATPase

Microtubules and actomyosin, which are representative contractile proteins, participate in several similar functions.[1] Accordingly, microtubules and actomyosin have been considered to interact with each other. Morphological techniques revealed that actin or myosin filaments were often in close apposition with microtubules.[1a,2] Since brain actomyosin-like proteins are similar to skeletal muscle proteins in several biochemical characteristics,[3] skeletal muscle actomyosin and brain microtubule proteins can be used in model experiments to examine the interaction of the proteins *in vitro*. Microtubule-associated proteins prepared from calf brain microtubules bound to actin from rabbit skeletal muscle as judged by viscometry and electron microscopy, and the interaction was inhibited by several nucleotides, including ATP and GTP.[4] Rat brain tubulin interacted with skeletal muscle myosin to inhibit actin-activated myosin Mg-ATPase activity and the corresponding turbidity change.[5] The interaction between myosin and tubulin was observed by electron microscopy to result in the formation of side-by-side aggregates of myosin filaments which were surrounded by tubulin particles, and it was also found that the amount of tubulin bound to myosin was decreased when tubulin was added after preincubation of myosin with ATP or GTP.[6] Subfragment-1 modified by treatment with *p*-chloromercuribenzoate inhibited the polymerization and depolymerization of microtubules, but heavy meromyosin modified by treatment with N-ethylmaleimide did not interfere with the polymerization of microtubules.[7]

We also considered that tubulin was a useful tool to examine some characteristics of the nucleotide triphosphatase activity of actomyosin. In this work, we investigated the effects of tubulin and GTP on the characteristics of the Mg–ATPase and Mg–GTPase activities of myosin and actomyosin.

Experimental

Preparation of Proteins——Microtubules were prepared from porcine brain homogenate by three cycles

of polymerization and depolymerization according to the procedure described by Shelanski *et al.*[8] with some modification.[9] From a microtubule preparation, tubulin was purified by phosphocellulose (Whatman P II) column chromatography as described previously.[5] Tubulin was dialyzed against 100 mM 2-(N-morpholino)ethanesulfonic acid (MES)-KOH (pH 6.5), 1 mM $Mg(CH_3COO)_2$, and 25% glycerol and stored at −80 °C until use. Myosin was prepared from rabbit skeletal muscle by Perry's method[10] and stored in 0.5 M KCl and 50% glycerol at −20°C. Actin was isolated from rabbit skeletal muscle by the method of Spudich and Watt.[11] Myosin and actin were dialyzed against 20 mM MES-KOH (pH 6.5) containing 0.5 M KCl and 100 mM MES-KOH (pH 6.5), respectively, and used within 10 days. The molecular weights of myosin, actin, and tubulin were taken to be 470000, 45000, and 110000, respectively.[3a,12] Myosin, actin, and tubulin used in this experiment were extremely pure, as determined by 7% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.[5]

Analytical Procedures——ATPase and GTPase activities were determined by measuring the Pi liberation. The standard assays were carried out by incubating 2 mM ATP or GTP in a final volume of 0.4 ml containing 50 mM MES-KOH (pH 6.5), 2 mM $Mg(CH_3COO)_2$, and 2.5% glycerol. The concentrations of myosin, actin, and tubulin were 0.1, 0.05, and 0.2 mg/ml, respectively. Preincubations of protein mixtures were conducted for 3 min on ice and then the reaction was started by addition of the substrate. The reaction was stopped after incubation for 3 to 15 min at 37°C by adding 0.4 ml of 20% trichloroacetic acid, and inorganic phosphate was measured by the method of Martin and Doty.[13]

Protein was determined by the method of Lowry *et al.*[14] with bovine serum albumin as a standard.

Results

The Inhibition of Actomyosin ATPase and GTPase by Tubulin

Since the order of addition of proteins and ATP changed the extent of actomyosin ATPase inhibition by tubulin,[5] the present experiment was performed under the conditions which gave the most effective inhibition, *i.e.*, the reaction was started by adding the substrate after incubation of actin with myosin-tubulin mixture as mentioned in "Experimental," unless otherwise stated.

Porcine brain tubulin as well as rat brain tubulin[5] inhibited rabbit skeletal muscle actomyosin Mg–ATPase activity, as shown by curve A in Fig. 1a. Tubulin also inhibited the Mg–GTPase activity (curve B). The inhibitory effect of tubulin on both ATPase and GTPase increased with increasing concentration of tubulin, and the maximum inhibition of ATPase reached 80%, whereas that of GTPase was 40% as calculated from the double-reciprocal plot (Fig. 1b). The concentrations of tubulin which showed the half-maximum inhibitions of ATPase and GTPase were 0.05 and 0.19 mg/ml, respectively.

Since tubulin is known to have two binding sites for guanine nucleotides per mol of protein, and nucleotide on one of them is exchangeable with nucleotide in the medium, it was considered that tubulin-GTP complex generated in the assay of GTPase activity might exhibit a different mode of inhibition from tubulin in the assay of ATPase activity. Thus, the effect of the complex, [formed by mixing tubulin with a low concentration of GTP for 10 min at 4°C as described by Arai *et al.*[15]] on the ATPase activity was examined. However, the complex had almost the same effect on the ATPase activity as tubulin itself (Table I). Therefore, the reason for the lesser effect of tubulin on GTPase than on ATPase activity is unknown at present.

Tubulin also inhibited the turbidity chagne of myosin-actin-Mg–ATP mixture. The effect of tubulin on the turbidity change caused by GTP was not clear, because the turbidity increase of actomyosin was only slight when GTP was used instead of ATP.

The extent of the inhibition of actomyosin Mg–ATPase activity by tubulin was smaller at low ATP concentrations than at high ATP concentrations (Fujii *et al.*, unpublished observation). Thus, actomyosin Mg–GTPase activity was measured for 3 min at 37°C in the presence or absence of tubulin at various GTP concentrations from 0.1 to 2 mM. As shown in Fig. 2, it was observed that GTPase activity was inhibited by the addition of tubulin at high GTP concentrations, whereas below 0.3 mM the activity was stimulated by adding tubulin. The *K*m value for GTP fell from 0.25 to 0.11 mM in the presence of tubulin. The enhancement of GTPase activity by the addition of tubulin was marked at lower GTP concentrations, and

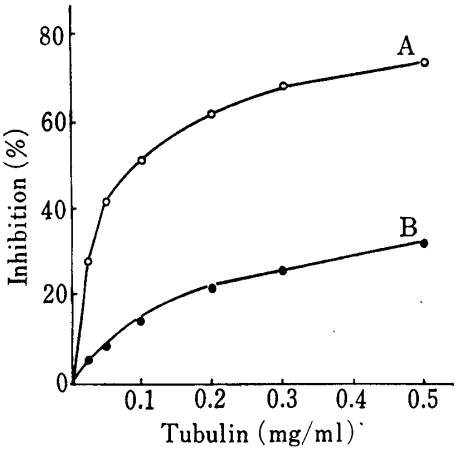

Fig. 1a. Dependence on Tubulin Concentration of Actomyosin Mg-ATPase and Mg-GTPase Activities

The activities were measured under the standard conditions with 0.1 mg/ml of myosin and 0.05 mg/ml of actin. The incubation times were 5 min for ATPase and 10 min for GTPase assay. Specific activites of actomyosin ATPase and GTPase activities in the absence of tubulin were 450—530 nmol Pi/min/mg and 150—180 nmol Pi/min/mg of myosin, respectively. ○, ATPase activity; ●, GTPase activity.

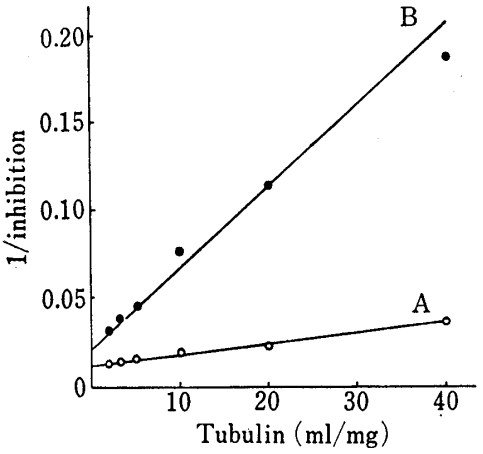

Fig. 1b. Double Reciprocal Plot of the Data in Fig. 1a

TABLE I. Effects of Dilute GTP on Actomyosin Mg-ATPase Inhibition by Tubulin

Concentration of GTP (M)	ATPase activity (%)
None	100
2×10^{-5}	105
2×10^{-4}	106

Preincubation was performed for 10 min at 4°C. The activity was measured as described in Fig. 1.

the activity reached 150% of the control level when the concentration of GTP used was 0.01 mM. The effect of tubulin concentration on actomyosin Mg-GTPase activity was also examined under conditions giving activation. When the reaction was performed by adding 0.2 mM GTP for 5 min at 37°C, the activation of actomyosin Mg–GTPase activity increased linearly until the molar ratio of tubulin to myosin in the assay mixture reached to 4 as shown in Fig. 3. At the molar ratio of 2, tubulin might remain in an unpolymerized state, because myosin and actomyosin did not induce the polymerization of tubulin, in contrast to the effect with microtubule-associated proteins, and the concentration of tubulin (0.05 mg/ml) was far below the critical concentration for polymerization (1.3—1.5 mg/ml).[16] Therefore, the activation cannot be ascribed to the polymerization of tubulin.

Although tubulin had no effect on Mg- or Ca–ATPase activity of myosin, it slightly activated myosin Mg–GTPase activity (Fig. 4), as it did actomyosin Mg–GTPase activity at low GTP concentrations. The activation by tubulin occurred at concentrations of GTP between 0.01 to 2 mM and the addition of tubulin had no effect on the *K*m value for GTP, in contrast to the case of actomyosin Mg–GTPase activity (data not shown). The activation was not diminished even when heat-treated tubulin was used instead of native tubulin. Accordingly, tubulin was considered to inhibit actin activation of myosin Mg–GTPase activity at high GTP concentrations.

Fig. 5 shows the effect of actin concentration on tubulin-inhibited actomyosin Mg–GTPase activity at 2 mM GTP concentration. The maximum activation of myosin Mg–GTPase activity by actin was about 2.5-fold, although that of Mg–ATPase activity by actin was 12- to 15-fold under the same conditions. The addition of excess actin, even when the molar ratio of myosin/tubulin/actin was 1/4/20, did not eliminate the inhibitory effect of tubulin. Tubulin barely changed the apparent affinity of myosin for actin in the presence of GTP.

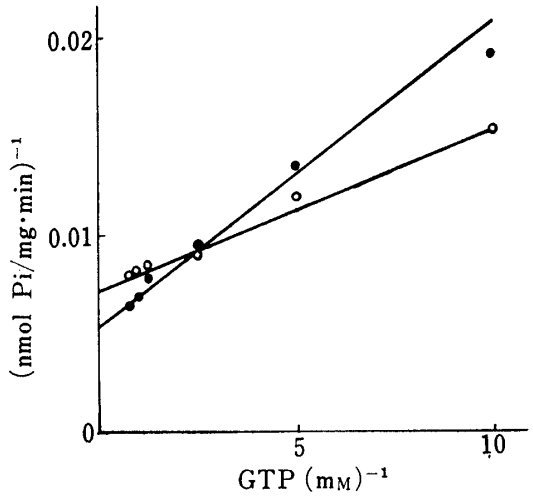

Fig. 2. Dependence on GTP Concentration of Actomyosin Mg-GTPase Activity and its Modification by Tubulin

The incubation was performed for 3 min at 37°C and the reaction mixture was the same as described in "Experimental" except that the concentration of tubulin was 0.3 mg/ml. ●, (−) tubulin; ○, (+) tubulin.

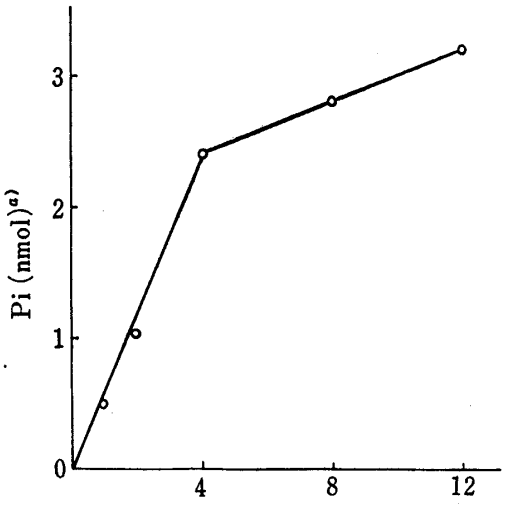

Fig. 3. Dependence on Tubulin Concentration of Actomyosin Mg-GTPase Activity

The reaction mixture was same as for Fig. 1 except that the GTP concentration was 0.2 mM. *a*) (activity with tubulin)-(activity without tubulin).

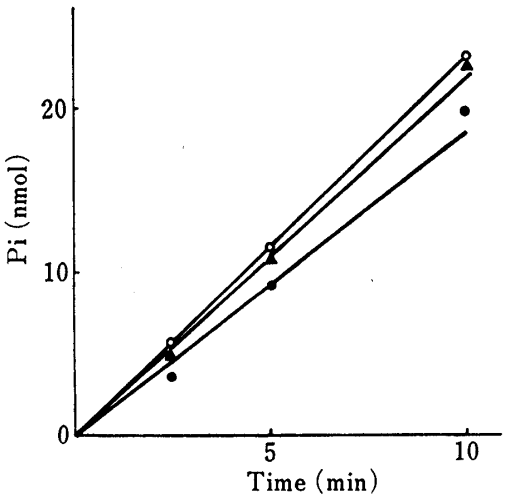

Fig. 4. Time Courses of Pi Liberation after Addition of 0.2 mM GTP to Myosin and Myosin-tubulin Mixture

The concentrations of myosin and tubulin were 0.1 mg/ml and 0.2 mg/ml, respectivily. Denaturation was performed by incubation of tubulin for 5 min at 70°C. ●, (−) tubulin; ○, (+) tubulin; ▲, (+) denatured tubulin.

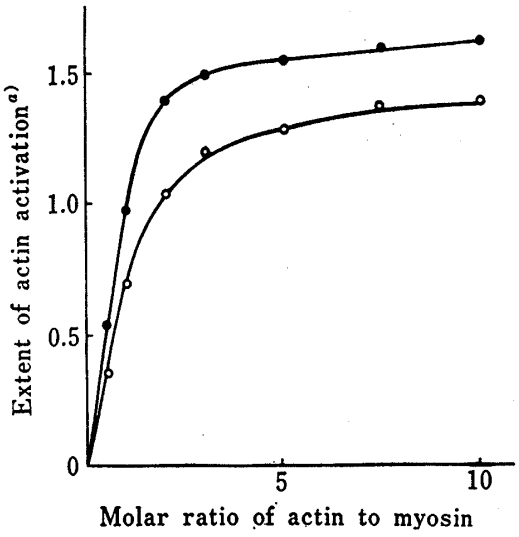

Fig. 5. Dependence of Actin Concentration of Actomyosin Mg-GTPase Inhibition by Tubulin

The reaction mixture was the same as for Fig. 1 except that the concentration of tubulin was 0.1 mg/ml and that of actin was varied as shown. ●, (−) tubulin; ○, (+) tubulin. *a*) Extent of actin activation

$$= \frac{\text{(activity with actin)} - \text{(activity without actin)}}{\text{(activity without actin)}}$$

Table II. Effects of Colchicine and Vinblastine on Actomyosin Mg-GTPase Activity in the Presence and Absence of Tubulin

Concentrations of drugs (M)		Mg-GTPase activity (%)	
		(−)Tubulin	(+)Tubulin
None		100	100
Colchicine	2×10^{-5}	96	101
	2×10^{-4}	97	104
Vinblastine	2×10^{-5}	98	98
	2×10^{-4}	97	96

Colchicine was preincubated with tubulin for 30 min at 37°C to form colchicine-tubulin complex.
The activity was measured as described in Fig. 1.

Effects of Various Compounds on Mg-GTPase Inhibition by Tubulin

As shown in Fig. 6, the addition of 0.05 M KCl activated actomyosin Mg–GTPase activity to the extent of 130% but did not affect the GTPase activity in the presence of tubulin. Both in the presence and absence of tubulin, the addition of 0.2—0.4 M KCl reduced the GTPase activity. Above 0.2 M KCl, however, the rate of GTP hydrolysis was higher in the presence of tubulin than in its absence. Colchicine and vinblastine at 2×10^{-5} and 2×10^{-4} M, which are suitable concentrations for interaction with tubulin and inhibition of the polymerization of tubulin, had almost no effect on actomyosin Mg–GTPase activity in the presence and absence of tubulin under conditions where tubulin inhibited the GTPase activity (Table II).

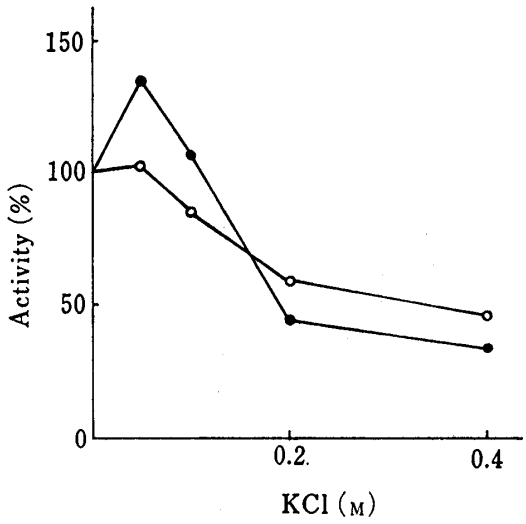

Fig. 6. Effects of KCl on Actomyosin Mg-GTPase Inhibition by Tubulin

The reaction conditions were the same as for Fig. 1. The concentration of KCl was varied as shown. ●, (−) tubulin; ○, (+) tubulin.

Discussion

The present study demonstrated that the interaction between myosin and tubulin affects the hydrolysis of GTP, which is the second most abundant free nucleotide (after ATP) in the brain[17] and which is involved in the polymerization and depolymerization of microtubules.[15,18] It was found that there were two differences in the inhibitory effects of tubulin on Mg–ATPase and Mg–GTPase activities of actomyosin (Fig. 1). 1) The GTPase activity of actomyosin was less readily inhibited than the ATPase activity by tubulin. 2) The amount of tubulin which was required for half-maximum inhibition of actomyosin GTPase activity was about 4 times that in the case of actomyosin ATPase activity. The possibility that tubulin-GTP complex produced in the assay mixture of GTPase caused the decrease in the inhibitory effect of tubulin can be ruled out (Table I). Tubulin caused slight activation of the Mg–GTPase activity of myosin (Fig. 4). Therefore, the difference of inhibition may due to the extent of actin activation. The extent of actin activation of myosin GTPase activity was 1/5—1/4 of that of ATPase activity, as mentioned in "Results."

Above 0.35 mM GTP concentration (Fig. 2) and at low KCl concentrations (Fig. 6), tubulin inhibited actomyosin GTPase activity. On the other hand, tubulin stimulated actomyosin

GTPase activity at low GTP concentrations (Fig. 2) and high KCl concentrations (Fig. 6), and it also stimulated myosin GTPase activity (Fig. 4). Furthermore, the inhibition and activation depended on tubulin concentration (Figs. 1 and 3). GTP hydrolytic activity was low under conditions where tubulin stimulated the GTPase activity as compared with that under conditions where the inhibition was observed, and the stimulation was not found when ATP was used as a substrate. Therefore, it is possible that the binding of tubulin to myosin not only inhibited the actin-activated nucleotide triphosphatase activity of myosin but also induced a GTP hydrolytic activity of tubulin. One cycle of GTP hydrolysis occurs at the exchangeable site of tubulin when it polymerizes into microtubules.[18] However, tubulin could not polymerize, as mentioned in "Results," and actually it *per se* hardly showed GTPase activity under our experimental conditions. The binding of GTP to tubulin was reported to be diminished when it was incubated for 5 min at temperatures above 65°C.[15] Heat-treated tubulin which showed also the ability to bind to myosin retained the ability to activate myosin GTPase activity to the same extent as native tubulin (Fig. 4). In a preliminary study, N-ethylmaleimide-modified myosin (NEM-myosin) prepared by the method of Meeusen and Cande[7b] decreased the Mg–ATPase and Mg–GTPase activities of myosin to about 10% and still retained the ability to interact with tubulin. In contrast to the absence of tubulin, the extent of GTP hydrolysis by NEM-myosin and NEM-myosin-actin mixture in the presence of tubulin was not changed at 0.2 and 2 mM GTP. Therefore, the stimulation may be caused by myosin through which it interacts with tubulin.

Davit-Pfeuty *et al.*[19] reported that colchicine induced a GTPase activity of tubulin independently of polymerization, and that vinblastine inhibited the colchicine-stimulated GTPase activity. Colchicine and vinblastine had little effect on the extent of the inhibition here (Table II).

Relaxing proteins of skeletal muscle such as tropomyosin, troponin-I, and troponin-T were reported to change the actomyosin Mg–ATPase activity. Tropomyosin activated actomyosin Mg–ATPase activity at low ionic strength in the presence of low Mg–ATP concentrations and inhibited it in the presence of high Mg–ATP concentrations (0.1—1 mM); this is like the effect of tubulin on actomyosin Mg–GTPase activity.[20] On the other hand, troponin-I and troponin-T activated actomyosin Mg–ATPase activity at high Mg–ATP concentrations and inhibited it at low Mg–ATP concentrations (0.01—0.1 mM).[21] Microtubule-associated proteins prepared from rat microtubules by ion exchange chromatography on a phosphocellulose column showed an ATPase activity which was activated several-fold by addition of tubulin.[9] The tubulin-dependent activity was also found when GTP was used instead of ATP (Y. Ihara, personal communication). Hoshino reported that porcine brain tubulin remarkably stimulated the ATPase activity of 30 S dynein from *Tetrahymena* cilia and the stimulation occurred when GTP was used in place of ATP as a substrate.[22] However, the stimulative effects of tubulin occurred in the alkaline pH range and disappeared at temperatures above 25°C. Recently, Shimo-Oka *et al.*[23] reported that microtubule-associated proteins prepared from porcine brain activated the Mg–ATPase activity of actomyosin from rabbit skeletal muscle. Hayashi[24] indicated that myosin and heavy meromyosin, which also showed Mg–GTPase activities, brought about the partial depolymerization of microtubules by exhaustion of GTP in the solution and not by direct action of myosin or heavy meromyosin. Our results suggest that polymerization and depolymerization of microtubules may be affected in a complicated manner in the presence of actomyosin, because the extent of actomyosin Mg–GTPase activity in the presence of tubulin, a main component of microtubule proteins, was altered by GTP concentration.

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