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Interaction of Protein-Bound Polysaccharide (PS-K) with Microtubule Proteins. V.[1] Influence on Tubulin-Dependent Adenosine Triphosphatase (ATPase) Activity

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In the presence of 5 mM Ca^{2+} or 2 mM Mg^{2+}, a protein-bound polysaccharide (PS-K) isolated from Basidiomycetes inhibited the tubulin-dependent adenosine triphosphatase (ATPase) activity of microtubule-associated proteins (MAPs) in a concentration-dependent manner, though it had little effect on the activity in the absence of tubulin. The extent of inhibition decreased at lower concentrations of Ca^{2+} or Mg^{2+} and an excess amount of tubulin largely restored the inhibitory effect. When Mn^{2+} was added to the reaction mixture instead of these divalent cations, the presence of PS-K conversely stimulated the tubulin-dependent ATPase activity of MAPs at lower Mn^{2+} concentrations (< 1—2 mM) under the conditions tested, though PS-K inhibited the ATPase activity at high Mn^{2+} concentrations. Maximal stimulation and inhibition were about 160% and 70% of the original level (*i.e.*, in the absence of PS-K), respectively. PS-K could also enhance MAPs ATPase activity under conditions where it enhanced the activity in the presence of tubulin, indicating that PS-K itself interacts with MAPs ATPase as tubulin does. The addition of a large amount of ATP under experimental conditions where PS-K inhibited tubulin-dependent Mn^{2+}-ATPase activity of MAPs, on the other hand, led to stimulation by PS-K. Thus, the influence of PS-K on the ATPase activity was dependent on the molar ratio of metal ions to adenosine triphosphate (ATP).

Keywords——brain microtubule protein; PS-K; microtubule-associated protein; tubulin; ATPase; metal ion

PS-K, a protein-bound polysaccharide, has been shown to possess antimitotic activity against a variety of tumor cells.[2] Although it is assumed to restore the depressed functions of lymphocytes or macrophages in tumor-bearing hosts, the precise mechanism at the molecular level is not yet known. A number of antimitotic reagents including colchicine, Vinca alkaloids, maytansinoids, griseofulvin, and podophyllotoxin are known to mediate their antitumor effects through interaction with microtubules, which are fibrous organella found in almost all eukaryotic cells.[3] These drugs also inhibit the polymerization and induce the depolymerization of the reconstituted microtubules. Recently, we found that PS-K interacts with microtubule proteins and suppresses the polymerization in a dose-dependent manner.[1,4,5] Many functions of microtubules such as 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.[6] In addition, such functions would presumably require a special system to initiate the generation of force by transforming chemical energy (adenosine triphosphate (ATP)) into mechanochemical work. Recent reports have indicated that an ATPase is specifically associated with mammalian brain microtubule proteins.[7] When microtubule proteins were separated into the microtubule-associated proteins (MAPs) and tubulin by gel filtration, ammonium sulfate precipitation, and phosphocellulose column chromatography, adenosine triphosphatase (ATPase) activity was recovered in the MAPs fraction.[7a,b,8] Moreover, the ATPase activity was remarkably

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stimulated by adding purified tubulin.[9]

Earlier studies from this laboratory have indicated that PS-K inhibits the ATPase activity contained in porcine brain microtubule proteins.[10] In this report, we describe in detail the effect of PS-K on ATPase activity using MAPs and tubulin separated from microtubule proteins.

Materials and Methods

Materials——PS-K was purchased from Kureha Chemical Industry Co., guanosine triphosphate (GTP) and 2-(*N*-morpholino)ethanesulfonic acid (MES) were from Boehringer, pepstatin was from the Protein Research Foundation, phosphocellulose was from Whatman, and Aquacide II was from Calbiochem. Other chemicals used were of reagent grade.

Preparation of Proteins——Microtubule proteins were prepared from porcine brain by three cycles of temperature-dependent polymerization and depolymerization in buffer A [100 mM MES–KOH (pH 6.5), 0.5 mM $Mg(CH_3COO)_2$, 1 mM ethyleneglycol-bis (2-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), and 2 μg/ml pepstatin].[9,11]

MAPs and tubulin from microtubule proteins were prepared as follows. A microtubule preparation (400 mg) in buffer A and 25% glycerol was applied to a phosphocellulose column (2.6 × 20 cm) which had been equilibrated with the same solution. Tubulin was recovered as the first protein fraction at the exclusion limit. After the column had been washed with three column volumes of buffer A and 10% glycerol, the MAPs fraction was eluted with the above buffer in the presence of 0.6 M KCl. The MAPs fraction was concentrated by the use of Aquacide II and dialyzed against buffer A and 25% glycerol. Proteins were stored at −80 °C until use.

The purified tubulin showed no detectable band other than tubulin on 8% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate.[12] On the other hand, the MAPs fraction was heterogeneous, consisting of high-molecular-weight components and minor peptides.

Assay for ATPase——ATPase activity was measured by determining the amount of inorganic phosphate released by means of a colorimetric procedure.[13] The standard reaction mixture contained 60 mM MES–KOH (pH 6.5), 5 mM 2-mercaptoethanol, 12.5% glycerol, 2 mM ATP, 100 μg of MAPs, and 260 μg of tubulin in a final volume of 0.4 ml. The reaction was initiated by the addition of substrate and terminated by adding trichloroacetic acid at a final concentration of 10% (w/v) after incubation for 30 min at 37 °C. The rate of ATP hydrolysis was linear over this period.

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

Results

The effect of PS-K on ATP hydrolysis by MAPs in the presence or absence of tubulin is shown in Fig. 1. As previously reported,[9] purified tubulin was able to stimulate ATPase activity recovered in the MAPs fraction when microtubule proteins were separated into MAPs and tubulin by column chromatography on phosphocellulose. As shown in Fig. 1, PS-K

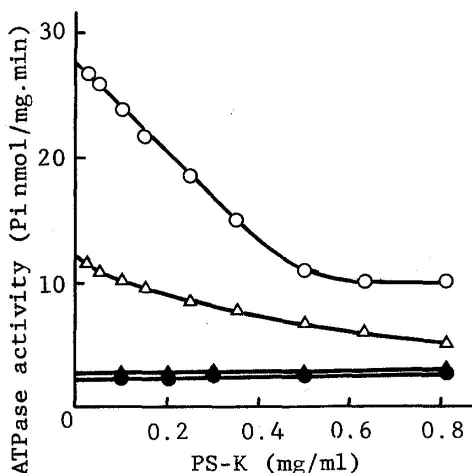

Fig. 1. Effect of PS-K on ATPase Activity in MAPs and MAPs-Tubulin Mixture

ATPase activity was measured as described in Materials and Methods, in media containing 60 mM MES–KOH (pH 6.5), 5 mM 2-mercaptoethanol, 12.5% glycerol, and 2 mM ATP, made up to contain the indicated concentration of PS-K. The concentrations of MAPs and tubulin were 0.25 and 0.65 mg/ml, respectively. ○, 5 mM $CaCl_2$ + MAPs + tubulin; ●, 5 mM $CaCl_2$ + MAPs; △, 2 mM $Mg(CH_3COO)_2$ + MAPs + tubulin; ▲, 2 mM $Mg(CH_3COO)_2$ + MAPs.

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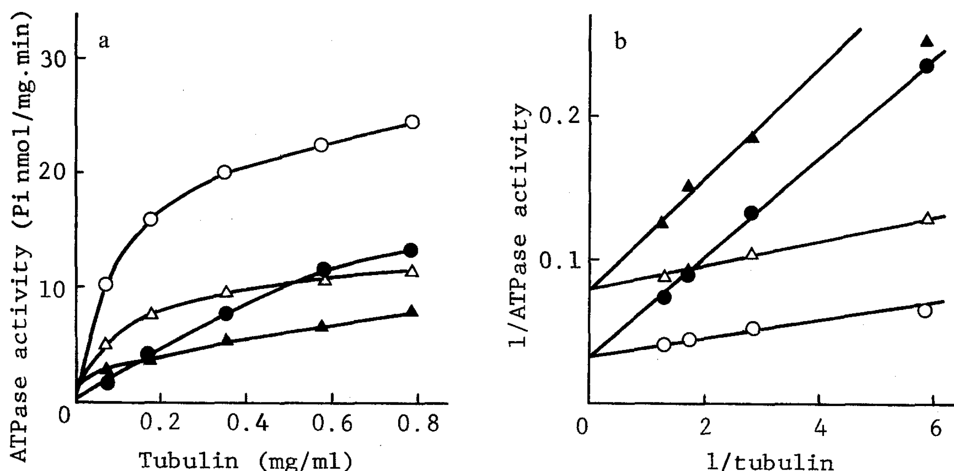

Fig. 2a. Dependency on Tubulin Concentration of MAPs ATPase Activity in the Presence and Absence of PS-K

Tubulin-dependent ATPase activity of MAPs was measured under the standard conditions modified to contain the indicated concentration of tubulin. ○, 5 mM $CaCl_2$; ●, 5 mM $CaCl_2$ + 0.6 mg/ml PS-K; △, 2 mM $Mg(CH_3COO)_2$; ▲, 2 mM $Mg(CH_3COO)_2$ + 0.6 mg/ml PS-K.

Fig. 2b. Double-Reciprocal Plots of the Data in Fig. 2a

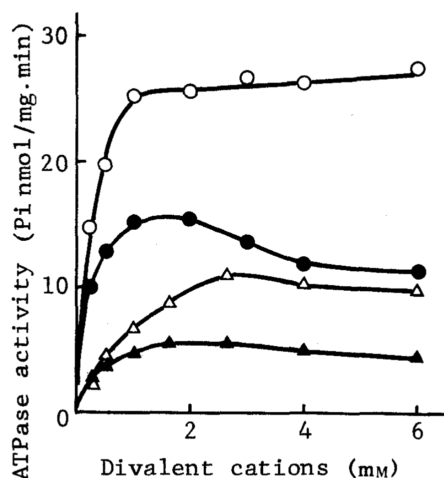

Fig. 3. Effect of Ca^{2+} and Mg^{2+} Concentrations on Tubulin-Dependent ATPase Activity of MAPs in the Presence and Absence of PS-K

Enzyme activity was measured under the standard conditions modified to contain the indicated concentrations of Ca^{2+} and Mg^{2+}. ○, $CaCl_2$; ●, $CaCl_2$ + 0.6 mg/ml PS-K; △, $Mg(CH_3COO)_2$; ▲, $Mg(CH_3COO)_2$ + 0.6 mg/ml PS-K.

inhibited the tubulin-dependent Ca^{2+}- and Mg^{2+}-ATPase activity of MAPs, but had no effect in the absence of tubulin. The inhibitory effect was found to be dose-dependent, and the concentration of PS-K required for half-maximal inhibition was about 0.23 mg/ml for both tubulin-dependent Ca^{2+}- and Mg^{2+}-ATPase.

MAPs ATPase activity in the presence of Ca^{2+} or Mg^{2+} is illustrated as a function of tubulin concentration in Fig. 2a. The inhibitory effect of PS-K on both activities was observed whenever tubulin was present in the reaction mixture. However, double-reciprocal plots show that a large amount of tubulin was able to overcome the inhibition (Fig. 2b). With 0.6 mg/ml PS-K, the K_m values for tubulin were approximately 5 times higher than the values without it.

When the concentrations of MAPs and tubulin were held constant and those of Ca^{2+} and Mg^{2+} were increased, PS-K inhibited the tubulin-dependent ATPase activity of MAPs at over 0.5 mM metal ions (Fig. 3). The Ca^{2+}-ATPase activity showed a biphasic character in the presence of PS-K, whereas the activity in its absence did not exhibit this characteristic. PS-K did not effectively inhibit the ATPase activity at low Mg^{2+} concentrations, but rather stimulated the activity at 0.25 mM Mg^{2+}.

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TABLE I. Effect of Divalent Cations on Tubulin-Dependent ATPase Activity in the Presence and Absence of PS-K

Metal ions	ATPase activity (Pi nmol/mg·min) 0.5 mM		5 mM	
	(−) PS-K	(+) PS-K	(−) PS-K	(+) PS-K
Ba^{2+}	2.24	3.12	3.23	2.56
Co^{2+}	3.97	4.89	2.01	2.31
Mn^{2+}	9.86	12.75	11.59	3.12
Sr^{2+}	3.04	3.74	5.28	3.39
Zn^{2+}	3.93	4.39	0.97	1.31

Enzyme activity was measured under the standard conditions modified to contain the indicated concentrations of divalent cations. The concentration of PS-K was 0.6 mg/ml.

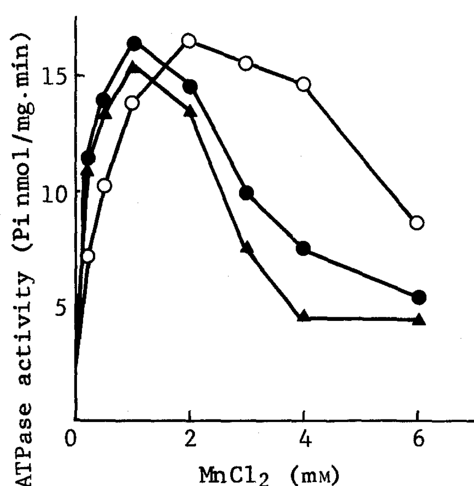

Fig. 4. Effect of Mn^{2+} Concentrations on Tubulin-Dependent ATPase Activity of MAPs in the Presence and Absence of PS-K

Enzyme activity was measured under the standard conditions modified to contain the indicated concentrations of Mn^{2+}. ○, (−) PS-K; ●, 0.3 mg/ml PS-K; ▲, 0.6 mg/ml PS-K.

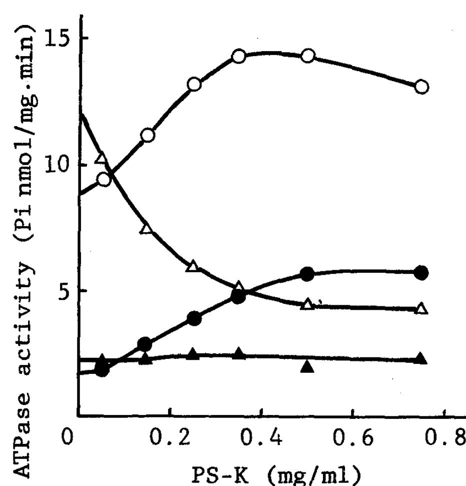

Fig. 5. Effect of PS-K on Mn^{2+}-ATPase Activity in MAPs and MAPs-Tubulin Mixture

Enzyme activity was measured under the standard conditions modified to contain the indicated concentrations of PS-K. ○, 0.5 mM $MnCl_2$ + MAPs + tubulin; ●, 0.5 mM $MnCl_2$ + MAPs; △, 5 mM $MnCl_2$ + MAPs + tubulin; ▲, 5 mM $MnCl_2$ + MAPs.

As described previously,[15] tubulin-dependent MAPs ATPase requires metal ions for the activity, because addition of ethylenediamine tetraacetic acid (EDTA)-EGTA mixture to the reaction solution markedly lowers the activity (2—4 nmol/mg·min). Five divalent cations, Ba^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+}, and Zn^{2+} were tested for their effects on the ATPase activity in the presence and absence of PS-K at two metal concentrations (Table I). Activity in the presence of Mn^{2+} was comparable to that in the presence of Mg^{2+}, whereas the other divalent cations did not significantly enhance the activity.

Figure 4 shows the effect of PS-K on the tubulin-dependent ATPase activity of MAPs at various concentrations of Mn^{2+}. In the absence of PS-K, near-maximal ATPase activity was obtained with Mn^{2+} concentrations in the range of 1.5 to 2.5 mM, but a gradual reduction occurred at concentrations over this range. Since addition of PS-K reduced the Mn^{2+} concentration required for maximal activity to about 1 mM and the activity declined steeply at still higher concentrations, a remarkable inhibition was observed at high Mn^{2+} concentrations. In contrast, at low Mn^{2+} concentrations (less than 0.5—1 mM), PS-K enhanced

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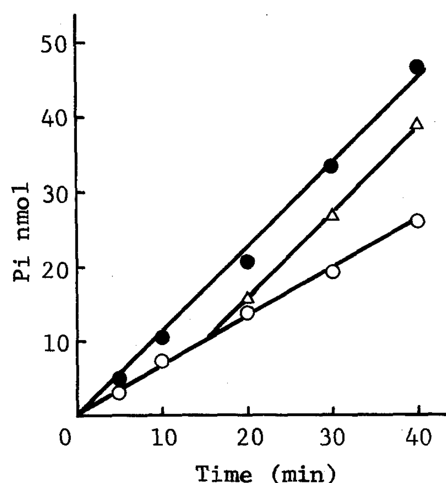

Fig. 6. Time Course of MAPs ATPase in the Presence and Absence of PS-K

The reaction mixture contained 0.5 mM $MnCl_2$ and 0.67 mg/ml MAPs. ○, (−) PS-K; ●, 0.6 mg/ml PS-K; △, PS-K (0.6 mg/ml) was added to the reaction mixture after 15 min.

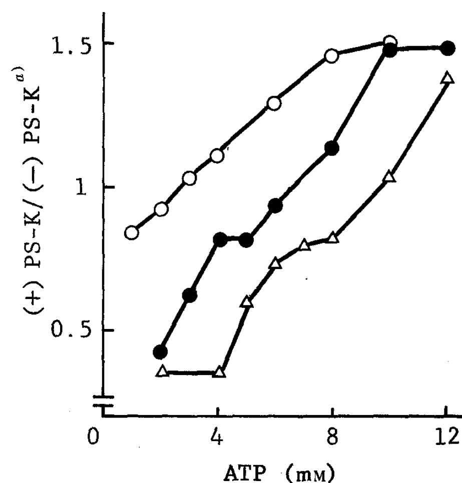

Fig. 7. Effect of ATP Concentration on Tubulin-Dependent ATPase Activity of MAPs

Enzyme activity was measured under the standard conditions modified to contain the indicated concentrations of ATP. The concentration of PS-K was 0.6 mg/ml. ○, 2 mM $MnCl_2$; ●, 4 mM $MnCl_2$; △, 6 mM $MnCl_2$. *a*) The rate of activity with PS-K/activity without PS-K.

the ATPase activity.

PS-K inhibited tubulin-dependent ATPase activity of MAPs in a reaction mixture containing 5 mM Mn^{2+}, but it had no effect in the absence of tubulin, as indicated in Fig. 5. Maximal inhibition was about 70% of the original level in the absence of PS-K. On the other hand, at 0.5 mM Mn^{2+} PS-K enhanced MAPs ATPase activity both in the absence and in the presence of tubulin, indicating that PS-K may be somewhat analogous to the tubulin molecule as regards MAPs ATPase activation. Maximal stimulation by PS-K reached about 340% and 160% in the absence and presence of tubulin, respectively.

The time course of ATP hydrolysis by MAPs at 0.5 mM Mn^{2+} is shown in Fig. 6. The ATPase activity proceeded at a constant rate for at least 40 min at 37 °C. When PS-K was added after 15 min incubation, the activity was stimulated immediately and at the same initial rate as when PS-K was added at the onset of the reaction.

The effect of PS-K on the tubulin-dependent ATPase activity of MAPs is illustrated as a function of substrate concentration in Fig. 7. At low ATP concentrations, the presence of PS-K inhibited the ATPase activity depending on the concentration of Mn^{2+}. Increasing the ATP concentration in the reaction mixture eliminated the inhibition and resulted in a stimulatory effect. Similar phenomena were observed when GTP was used as a substrate instead of ATP (data not shown). The transitional ATP concentration increased with increasing Mn^{2+} concentrations added and corresponded to a molar ratio of ATP to Mn^{2+} of about 1.5.

Discussion

Recently, we suggested that the tubulin-dependent ATPase activity of MAPs might be linked to polymerization and depolymerization of microtubules, since the activity is enhanced under conditions where microtubules are formed.[16] The addition of colchicine and podophyllotoxin, potent inhibitors of microtubule polymerization, inhibited the Mg^{2+}-ATPase activity under conditions suitable to induce tubulin polymerization. However, they did not have any significant effect on the Ca^{2+}-ATPase activity when microtubule polymerization was

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completely suppressed by the presence of Ca^{2+} (mM order) required for the ATPase activity (manuscript in preparation). The present results indicate that PS-K, an inhibitor of microtubule polymerization, inhibited the tubulin-dependent ATPase activity of MAPs, but did not inhibit ATPase activity without tubulin (Figs. 1 and 2). The inhibitory effect was also found over a wide range (0.25—6 mM) of Ca^{2+} concentrations (Fig. 3). Therefore, PS-K seemed to suppress the activation of MAPs ATPase activity by tubulin irrespective of microtubule formation. More recently, we reported that the drug showed a greater inhibitory effect on the Mg^{2+}-ATPase activity of brain microtubule proteins than on the Ca^{2+}-ATPase activity.[10] Opposite results were obtained in this experiment, *i.e.*, the extent of inhibition was greater with Ca^{2+} than with Mg^{2+} when tested with a mixture of MAPs and tubulin fractions. The reason for this discrepancy is unknown at present.

At higher Mn^{2+} concentrations, PS-K remarkably inhibited the tubulin-dependent ATPase activity of MAPs, but did not affect MAPs ATPase activity in the absence of tubulin, as in the cases with Ca^{2+} and Mg^{2+} (Figs. 1, 4, and 5). However, addition of an excess amount of ATP to the reaction mixture reduced the inhibitory action and, finally, resulted in stimulation by PS-K when the molar ratio of ATP to Mn^{2+} was over 1.5 (Fig. 7). Reduction of PS-K inhibition by increasing ATP concentration was also found when Ca^{2+} or Mg^{2+} was added to the assay mixture. However, the effect was not as prominent as in the case of Mn^{2+}. Various polysaccharides composed of glucose, mannose, xylose, *etc.* have been reported to form complexes with metal ions.[17] PS-K has been also reported to be a protein-bound polysaccharide mainly composed of glucose.[2a] Therefore, it can be presumed that PS-K shows affinity for metal ions, especially for Mn^{2+}, and the complex, if formed, might exhibit different characteristics in the interaction with microtubule proteins from those of PS-K alone. Since ATP is well known to complex metal ions and the affinity of metal ions for ATP appears to be higher than that for PS-K, a certain amount of free divalent cations not bound to ATP seems to be required for the appearance of ATPase inhibition by PS-K.

Previously, we reported that vinblastine, a reagent inhibitory to mitosis, stimulated the tubulin-dependent ATPase activity of MAPs and its mechanism was attributed to the capacity to induce tubulin to form oligomeric structures.[15] In addition, taxol, a new antineoplastic reagent isolated from *Taxus brevifolia*, induces microtubule assembly, and also stimulates the ATPase activity under the conditions required to form microtubules.[16,18] It is considered that these drugs act through their binding to the tubulin molecule and increase the extent of tubulin enhancement of MAPs ATPase activity. In contrast, the stimulation of PS-K is considered to be caused by its interaction with ATPase contained in the MAPs fraction, because PS-K *per se* stimulated MAPs ATPase activity as tubulin did (Fig. 5) and the stimulation was not due to its apparent stabilizing effect (Fig. 6). In fact, we found recently that the addition of exogenous MAPs or tubulin prevented the inhibitory effect of PS-K on microtubule polymerization to some extent and the extent of cancellation was greater by MAPs than by tubulin.[1] Furthermore, it has become apparent by using PS-K Sepharose 4B affinity column chromatography that PS-K interacts with not only tubulin but also several components of MAPs.[5]

At present, we do not know whether or not the ATPase is involved in microtubules other than brain microtubule proteins and plays any role in cellular functions. However, the copurification of ATPase with brain microtubules and the dependency of its activity on tubulin strongly suggests its structural and functional association with microtubules.[9,19] The enhancement of MAPs ATPase activity is considered to be specific to native tubulin, since bovine serum albumin, calmodulin, chicken gizzard F-actin, rabbit skeletal muscle F-actin, and denatured tubulin could not replace tubulin for activation.[15,19,20] As far as we know, PS-K is the first substance, other than tubulin, reported to enhance MAPs ATPase activity in itself in the presence of Mn^{2+}.

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References

1) T. Fujii, Y. Akabane, S. Odori, M. Fujii, Y. Kondo, and K. Ohki, *Chem. Pharm. Bull.*, **32**, 4518 (1984).
2) *a*) S. Tsukagoshi and F. Ohashi, *Gann*, **65**, 557 (1974); *b*) K. Nomoto, C. Yoshikumi, K. Matunaga, T. Fujii, and K. Takeya, *ibid.*, **66**, 365 (1975); *c*) S. Abe, M. Yamazaki, and D. Mizuno, *ibid.*, **69**, 223 (1978).
3) L. Wilson, *Life Sci.*, **17**, 303 (1975); R. F. Ludueña, "Microtubules," ed. by K. Roberts and J. S. Hyams, Academic Press Inc., London, 1979, pp. 65—116; S. Ikeyama and M. Takeuchi, *Biochem. Pharmacol.*, **30**, 2431 (1981).
4) Y. Kondo, T. Fujii, T. Koyama, M. Fujii, and K. Ohki, *Chem. Pharm. Bull.*, **31**, 2920 (1983).
5) T. Fujii, Y. Akabane, S. Odori, M. Fujii, Y. Kondo, and K. Ohki, *Igaku No Ayumi*, **130**, 19 (1984).
6) J. B. Olmsted and G. G. Borisy, *Ann. Rev. Biochem.*, **42**, 507 (1973); P. Dustin, "Microtubules," Springer-Verlag, Berlin, Heidelberg, New York, 1978; S. N. Timasheff and L. M. Grisham, *Ann. Rev. Biochem.*, **49**, 565 (1980).
7) *a*) V. I. Gelfand, F. K. Gyoeva, V. A. Rosenblat, and N. A. Shanina, *FEBS Lett.*, **88**, 197 (1978); *b*) H. D. White, B. A. Coughlin, and D. L. Purich, *J. Biol. Chem.*, **255**, 486 (1980); *c*) G. A. Sharp, J. T. R. Fitzsimons, and G. A. Kerkut, *Comp. Biochem. Physiol.*, **66A**, 415 (1980).
8) H. Larsson, M. Wallin, and A. Edström, *J. Neurochem.*, **33**, 1249 (1979); B. C. Webb, *Arch. Biochem. Biophys.*, **198**, 296 (1979).
9) Y. Ihara, T. Fujii, T. Arai, R. Tanaka, and Y. Kaziro, *J. Biochem.* (Tokyo), **86**, 587 (1979).
10) T. Fujii, Y. Akabane, M. Fujii, Y. Kondo, and K. Ohki, *Chem. Pharm. Bull.*, **32**, 2460 (1984).
11) T. Fujii and R. Tanaka, *Life Sci.*, **24**, 1683 (1979).
12) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
13) J. B. Martin and D. M. Doty, *Anal. Chem.*, **21**, 965 (1949).
14) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15) T. Fujii, Y. Kondo, M. Kumasaka, and K. Ohki, *J. Neurochem.*, **39**, 1587 (1982).
16) T. Fujii, Y. Kondo, M. Kumasaka, T. Suzuki, and K. Ohki, *J. Neurochem.*, **41**, 716 (1983).
17) G. T. Grant, E. R. Morris, D. A. Rees, P. J. C. Smith, and D. Thom, *FEBS Lett.*, **32**, 195 (1973); J. A. Rendleman, *Food Chem.*, **3**, 47 (1978).
18) P. B. Schiff, J. Fant, and S. B. Horwitz, *Nature* (London), **277**, 665 (1979); N. Kumar, *J. Biol. Chem.*, **256**, 10435 (1981); E. Hamel, A. A. del Campo, M. C. Lowe, and C. M. Lin, *ibid.*, **256**, 11887 (1981).
19) S. Tominaga, K. Hirosawa, and Y. Kaziro, *FEBS Lett.*, **144**, 112 (1982).
20) S. Tominaga and Y. Kaziro, *J. Biochem.* (Tokyo), **93**, 1093 (1983).

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