Two Opposing Effects of Calmodulin on Microtubule Assembly Depend on the Presence of Microtubule-associated Proteins*

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The effect of bovine brain calmodulin on the assembly of pure bovine brain tubulin has been examined in the presence and absence of microtubule-associated proteins (MAPs). In the absence of MAPs, calmodulin enhances the rate and extent of polymerization of pure tubulin, probably by sequestering Ca\(^{2+}\) from tubulin since the effect is mimicked by ethylene glycol bis(\(\beta\)aminoethyl ether)N,N,N',N'-tetraacetic acid and parvalbumin. From stoichiometric considerations, all 4 Ca\(^{2+}\) binding sites of calmodulin appear to participate in this effect. In the presence of MAPs, calmodulin confers increased Ca\(^{2+}\) sensitivity on the tubulin polymerization process, enhancing the inhibitory effect of Ca\(^{2+}\) on the rate and extent of assembly. The effect of calmodulin on the assembly of tubulin is dependent on the presence of Ca\(^{2+}\). The data suggest that calmodulin of both low (Ca\(^{2+}\);calmodulin) and high (Ca\(^{2+}\);calmodulin) Ca\(^{2+}\) occupancy may be involved in this enhancement of Ca\(^{2+}\)-induced inhibition of polymerization. Thus, calmodulin has dual and opposing actions on Ca\(^{2+}\) sensitivity of tubulin polymerization depending on the presence or absence of MAPs.

The discovery that calmodulin participates in a number of calcium-dependent regulatory processes led to the proposal that Ca\(^{2+}\)-mediated regulation of microtubule assembly and disassembly is also mediated by this protein (1-3). However, it was recently shown that the role of Ca\(^{2+}\) in tubulin polymerization is considerably more complex. Inhibition of polymerization exhibits two types of Ca\(^{2+}\) sensitivity (4): 1) a high intrinsic Ca\(^{2+}\) sensitivity, independent of calmodulin, that is characterized by a steep temperature dependence and a steep inverse tubulin concentration dependence operating primarily through the nucleation process; and 2) an enhancement of Ca\(^{2+}\) sensitivity in the presence of calmodulin that has also been observed by others; this enhancement, however, requires relatively high calmodulin/tubulin molar ratios. There has been some uncertainty regarding the locus of interaction between microtubules and calmodulin. Although binding of calmodulin to tubulin has been reported in several studies (2, 5), tubulin is not captured by calmodulin affinity columns (6-8), nor does it interfere with the activation of phosphodiesterase by calmodulin (2, 6). Since the enhancement of Ca\(^{2+}\) sensitivity was demonstrated in preparations that contained both tubulin and MAPs\(^1\) (1, 2, 3, 9), it was important to investigate the role of MAPs in the response to calmodulin. Such an interaction has been suggested (7, 8). In the present study, MAP-free 6 S tubulin is polymerized in the absence and presence of exogenous MAPs. This has led to the identification of two, disparate Ca\(^{2+}\)-related actions of calmodulin on microtubule assembly.

MATERIALS AND METHODS

Preparations of Microtubule Protein, Pure Tubulin, MAPs, and Calmodulin—Microtubule protein was isolated from calf brain by two cycles of temperature-dependent polymerization-depolymerization (10). The purified protein was stored at -80 °C in 20 mM Mers, pH 6.5, containing 70 mM NaCl, 0.5 mM MgCl\(_2\), and 2 mM glycerol. Pure tubulin was prepared by polymerizing the twice-cycled microtubule protein in 0.4 M Pipes, 10% Me2SO, 0.5 mM MgCl\(_2\), and 0.6 mM GTP, pH 6.9 (11). After centrifugation, the excess buffer was carefully removed by washing the pellet with water at room temperature. The pellet was resuspended in 20 mM Pipes, pH 6.9, and passed through a phosphocellulose column equilibrated with the same buffer. Pure tubulin from this column was concentrated to -10 mg/ml by centrifugation in Amicon ultrafiltration membrane cones. These preparations yielded a single band and showed no MAPs in overloaded sodium dodecyl sulfate polyacrylamide gels.

MAPs were prepared by boiling microtubule protein according to the method described by Fellous et al. (12) with the following modifications. Solid NaCl and dithiothreitol were added to the twice-cycled microtubule protein preparations to final concentrations of 0.75 M and 2 mM, respectively. The solution was dialyzed against 20 mM Pipes buffer containing 0.75 M NaCl and 2 mM dithiothreitol and then immersed in a boiling water bath for 5 min. The boiled solution was centrifuged at 40,000 X g for 30 min and the supernatant was dialyzed against either 20 mM Pipes, pH 6.9, or 0.1 M Mers, pH 6.6, containing 2 mM mercaptoethanol and concentrated by ultrafiltration.

Calmodulin was prepared from calf brain by a modification (4) of the method of Klee (13). Frog parvalbumin was generously supplied by Drs. J. Haiech and C. Klee (National Institutes of Health).

Assembly of Pure Tubulin—Tubulin was polymerized in assembly buffer containing 0.1 M Pipes (pH 6.9), 1 mM MgCl\(_2\), and 1 mM EGTA at 37 ± 0.2 °C measured by a thermistor in a cuvette in the cell changer, unless otherwise stated. Polymerization was initiated by the addition of GTP to 1 mM concentration and measured by following the increase in turbidity at 350 nm using a Cary 219 spectrophotometer equipped with a thermostated multiple cell changer. High protein concentrations were required under these conditions but were kept constant when possible to eliminate correcting for the concentration effect on Ca\(^{2+}\) sensitivity (4). The rate was determined from the steepest portion of the turbidity scans (A\(_{350}\) versus time).

Polymerized samples were spot-checked by electron microscopy (Phillips 300) by direct application to grids and negative staining with 2% uranyl acetate. We thank Dr. Howard A. Bladen (National Institutes of Health) for the electron microscopic observations.

Determination of Protein Concentration—The concentration of pure tubulin and calmodulin was determined using the extinction

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The abbreviations used are: MAP, microtubule-associated protein; Mes, 2-(N-Morpholino)ethanesulfonic acid; Pipes, 1,4-piperazineethanesulfonic acid; Me2SO, dimethyl sulfoxide; EGTA, ethylene glycol bis(\(\beta\)aminoethyl ether)N,N,N',N'-tetraacetic acid; CaM, calmodulin; Ca\(_{EGTA}\), free Ca\(^{2+}\) concentration in equilibrium with EGTA; Ca\(_{2+}\), free Ca\(^{2+}\) concentration in equilibrium with both EGTA and calmodulin.

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coefficients of 1.20 liters g⁻¹ cm⁻¹ at 280 μm (4) and 3300 m⁻¹ cm⁻¹ at 277 nm (13), respectively. The concentration of MAPs was determined by the method of Bradford (14) using crystalline bovine serum albumin as standard.

Calculation of Free Ca²⁺ Concentrations—In all experiments, the concentration of EGTA added was 1 mM. Two types of Ca²⁺ concentrations are depicted in the figures. In some, the free Ca²⁺ in equilibrium with EGTA is listed and termed Ca²⁺EGTA. In this case, the free calcium concentration was calculated by using the apparent association constant of EGTA with Ca²⁺ of 4.7 × 10⁹ M⁻¹ (15). However, because of the high molar ratios of calmodulin/tubulin required for the effects, free Ca²⁺ was also corrected for both EGTA and calmodulin equilibrium (termed Ca²⁺EGTA) using binding constants taken under conditions of 0.1 M KCl and pH 7.55 (16). Values were computed by using the MLAB system and we thank Dr. Byungkook Lee (National Institutes of Health) for these calculations. The absence of a need to correct for Ca²⁺ binding to GTP or GDP at these pH conditions has been detailed previously (4).

RESULTS

6 S Tubulin—The effect of calmodulin on the assembly of pure tubulin dimer is shown in Fig. 1. Relatively high concentrations of Ca²⁺ were required because the polymerization of pure tubulin requires high concentrations of this protein, thus lowering the Ca²⁺ sensitivity (4). When using ~27 μM tubulin, 45 μM Ca²⁺EGTA caused extensive inhibition of microtubule assembly. The rate of assembly was more severely affected than the extent in those experiments in which plateau values were attained during the experimental period. To our surprise, addition of 18 μM calmodulin partially reversed the Ca²⁺ induced inhibition of polymerization (Fig. 1). The extent of reversal varied as a function of calmodulin concentration when tubulin and Ca²⁺ concentrations were held constant. In four experiments, the extent of reversal ranged from 10 to 50% at a Ca²⁺EGTA concentration of 65 μM and a calmodulin/tubulin ratio of 0.8. Higher molar ratios (to 3:1) did not overcome the residual inhibition of polymerization (data not shown). This effect of calmodulin was exerted through the Ca²⁺ present in the system. In the absence of Ca²⁺, calmodulin had no effect on the progress curve for polymerization (compare curves 1 and 2 of Fig. 1) even with calmodulin/tubulin ratios of 2 (data not shown). Microtubules formed in the presence or absence of calmodulin were indistinguishable by electron microscopic examination and disappeared in the cold.

The stimulatory effect of calmodulin on the polymerization of pure 6 S tubulin in the presence of Ca²⁺ was observed over a wide range of calcium concentrations (Fig. 2). When the free Ca²⁺ concentration was calculated merely on the basis of the equilibrium with EGTA (termed Ca²⁺EGTA) as shown in Fig. 2A, there was a shift to the right along the abscissa of the rate versus concentration curve when 24 μM calmodulin was added. When the free Ca²⁺ concentration was corrected for binding to calmodulin according to binding constants published by Haiech et al. (16), the points without and with calmodulin fell or the same line (Fig. 2B). This strongly suggests that the effect of calmodulin in these experiments was as a sequestering agent, merely lowering the free Ca²⁺ concentration. To test this hypothesis, we compared the effect of calmodulin with two other Ca²⁺ chelating agents, EGTA and parvalbumin. As shown in Fig. 3, both EGTA and parvalbumin reversed the Ca²⁺ induced inhibition of the polymerization of pure 6 S tubulin in a manner analogous to that observed with calmodulin. At high enough Ca²⁺ concentrations, in experiments not shown here, this sequestering action amounted to about 4 times the calmodulin concentration, as would be expected from the four Ca²⁺ binding sites on that protein (16-19).

6 S Tubulin with MAPs—In contrast to the results obtained with pure tubulin dimer, the inhibitory effect of calcium is known to be enhanced by calmodulin when microtubule proto-
FIG. 3. The effect of calcium binding agents on polymerization of pure tubulin. Polymerization of 33 μM (3.6 mg/ml) pure tubulin was performed in the presence or absence of calcium and calcium binding agents as indicated. The concentrations of CaCl₂, EGTA, calmodulin, and parvalbumin (PA) were, respectively, 45, 80, 15, and 10 μM.

FIG. 4. The effect of Ca²⁺-calmodulin on the assembly of tubulin in the presence of MAPs. In the presence of 80 μg/ml of MAPs, pure tubulin (2.4 mg/ml, 22 μM) was polymerized at 31 °C in 0.1 M Pipes buffer, pH 6.9, containing 1 mM concentration each of MgCl₂, GTP, and EGTA, and various concentrations of calcium with or without 117 μM MAPs, pure tubulin (2.4 mg/ml, 22 μM) and various concentrations of calcium with or without calmodulin. Values reported are expressed as percentage of the control value carried out in the absence of calcium.

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<th>CaCl₂ (μM)</th>
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<tr>
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FIG. 5. Calmodulin sensitivity of tubulin assembly in the presence of MAPs. A, pure tubulin (12 μM) was polymerized as in Fig. 4 at 37 °C in the presence of 0.24 mg/ml of MAPs, 100 μM CaCl₂, and increasing concentrations of calmodulin. The control rate of polymerization (ΔA₅₀₀/100 s) in the absence of calmodulin was 0.042. At 15 μM calmodulin, the concentration of CaCl₂, was calculated to be 68 μM. B, pure tubulin (12.5 μM) was polymerized at 37 °C in the presence of 10 μM CaCl₂ with 0.08 (●), 0.12 (●), 0.28 (●) mg/ml of MAPs with increasing concentrations of calmodulin. The control rates of polymerization (ΔA₅₀₀/100 s) in the absence of calmodulin were 0.0096, 0.049, and 0.108 in the presence of 0.08, 0.12, and 0.28 mg/ml of MAPs, respectively. At 12 μM calmodulin, the concentration of CaCl₂, was calculated to be 8.4 μM.

FIG. 6. The effect of calmodulin on the sensitivity of the rate of tubulin assembly to increasing concentrations of CaCl₂ in the presence of MAPs. Pure tubulin was polymerized at 37 °C in the presence (●, ▲) and absence (○, △) of calmodulin (24 μM) with increasing concentrations of CaCl₂. The figure was obtained from two separate experiments. The protein concentrations of tubulin and MAPs were, respectively, 11 μM (1.2 mg/ml) and 0.48 mg/ml for experiment 1 (△, ▲) and 12.5 μM (1.4 mg/ml) and 0.36 mg/ml for experiment 2 (○, ●). The control rates of polymerization (ΔA₅₀₀/100 s) in the absence of added CaCl₂ were 0.106 and 0.032 for experiment 1 (△, ▲) and experiment 2 (○, ●), respectively.
extent of polymerization (Table I). The effect on the rate is consistently greater than that on the extent.

That the calmodulin-mediated enhancement of Ca"+ inhibition required the presence of MAPs is shown in Fig. 5B where increasing concentrations of MAP proteins were added to 6 S tubulin. At 10 μM Ca++/calmodulin, the fractional inhibition of polymerization was increased by increasing the concentration of MAPs from 0.08 to 0.12 mg/ml even though the total level of polymerization had increased. When still higher concentrations of MAPs were used, there was no additional change in the per cent of inhibition of polymerization produced by calmodulin. It is of interest that increasing the calmodulin above 7.5 μM (calmodulin/tubulin ratio of 0.62 and a Ca"+ sites/Ca"+ ratio of 3) did not increase the inhibition of polymerization measured under these conditions. By contrast, when excess Ca"+ was present (Fig. 5A), inhibition of polymerization was a linear function of the calmodulin concentration over the range tested.

The requirement for Ca++-calmodulin as inhibitor of tubulin polymerization in the presence of MAPs can also be shown by the converse experiment wherein the Ca++/calmodulin is varied at fixed calmodulin levels (Fig. 6). In the absence of calmodulin, inhibition of polymerization, commenced at 6-10 μM Ca++/calmodulin. By contrast, the presence of calmodulin at these Ca++ concentrations caused substantial inhibition of polymerization. Thus, 24 μM calmodulin enhances Ca++-induced inhibition starting in the physiologic (low micromolar) range of Ca++ concentrations and extends well beyond that, as shown in Fig. 6 where the values are normalized to the rate in the absence of Ca"+. Even at low molar ratios of Ca++ to calmodulin, there is relatively high enhancement of the inhibition.

**DISCUSSION**

Two potentially antagonistic actions of calmodulin on tubulin polymerization are demonstrated in the present study. When no MAPs are present, calcium, which has an *intrinsic* inhibitory effect on tubulin polymerization (4) (presumably through a single, high affinity Ca++ binding site (20)), is sequestered from the system by the addition of calmodulin. Even though the binding constants and salt sensitivities differ for the Ca++ sites on calmodulin (16-19), this sequestering occurs with a stoichiometry approaching 4 Ca++/calmodulin. Thus, calmodulin acts competitively with tubulin for the binding of Ca++ and promotes polymerization. The reactions may be depicted as follows:

\[ \text{Ca}^{++} + (6 \, \text{S}) \text{tubulin} = \text{Ca}^{++}\text{-tubulin} \quad (1) \]

\[ \text{Ca}^{++} + \text{calmodulin} = \text{Ca}^{++}_{\text{calmodulin}} \quad (n = 4) \quad (2) \]

Such an effect would be relatively independent of the value of n in reaction 2 and depends primarily on the affinity constants of the four Ca++ binding sites of calmodulin under our incubation conditions. It should be pointed out that electron microscopic pictures of microtubules that are formed in the presence of calmodulin were indistinguishable from those formed without calmodulin and both disappeared in the cold. Therefore, it is unlikely that the stimulatory effect seen by turbidity is due to anomalous structures. MAPs are thus not required to elicit this polymerization-promoting effect of calmodulin. To what extent calmodulin may provide a Ca++-sequestering function in *in vitro* microtubule assembly remains to be assessed.

A second, and opposite, effect of calmodulin on polymerization occurs in the presence of MAPs where Ca++-induced inhibition of polymerization is enhanced. Since calmodulin is ineffective in the absence of calcium, the reacting species is presumably Ca"+-calmodulin as in Equation 2. Although we do not know the precise numbers of Ca++ ions per calmodulin required for this effect, the data presented in Fig. 5B and 6, as well as those previously reported (Fig. 9 of Ref. 4), show substantial inhibition at Ca++/calmodulin molar ratios >10.

This suggests that, unlike previous findings with phosphodiesterase (18, 19), and myosin light chain kinase from skeletal muscle (21) Ca++-deficient calmodulin complexes, i.e. Ca++, calmodulin and Ca++-calmodulin, may be able to inhibit microtubule assembly in the presence of MAPs. That Ca++-calmodulin are the only active species, as they appear to be in other systems (18, 19, 21), seems unlikely since at low Ca++ to calmodulin ratios little of these species would be present (16-19). Moreover, if this were the case, there should be markedly greater inhibition when higher Ca++/calmodulin ratios prevail. This does not occur. On the other hand, Fig. 6A shows that at high Ca++/calmodulin ratios when the fraction of Ca++-calmodulin is low and that of Ca++-calmodulin is high, there is excellent inhibition, suggesting that the calcium-saturated calmodulin complexes are also inhibitory.

The present results suggest that one or more of the microtubule-associate proteins are required in the assembly system for Ca++/calmodulin to express its inhibitory effect on polymerization. They are consistent with the behavior of sea urchin microtubule protein which is devoid of MAPs after several cycles of assembly/disassembly (22). It has been shown that this tubulin preparation is insensitive to inhibition by brain or sea urchin calmodulin. Since completion of the present study, Sobue et al. (23) have presented evidence that τ factor is the calmodulin-binding MAPs.

We speculate that the basis for a MAP requirement may be a combination of calmodulin with MAPs, as in:

\[ \text{Ca}^{++}\text{-CaM} + \text{MAPs} = \text{Ca}^{++}\text{-calmodulin-MAPs} \quad (3) \]

thereby inactivating the protein for its role in polymerization and thus competing with tubulin for the available MAPs as in:

\[ \text{Tubulin} + \text{MAPs} = \text{tubulin-MAPs} \quad (4) \]

or by other pathways. One can envisage an interaction in which the product of reaction 4 combines with Ca++-calmodulin to form a ternary complex that is either unable to polymerize or one that decomposes to tubulin and Ca++-calmodulin-MAP or other forms. Finally, Ca++-calmodulin could bind to the completed microtubule in the presence of MAPs and promote depolymerization as has been shown in cold-stable microtubules (24). Several of these pathways are amenable to experimental analysis and are currently being studied.

Viewed from the perspective of MAPs, these proteins, or one of them, *can also* be considered as having two opposing actions on the Ca++ sensitivity of microtubule assembly. 1) Since MAPs promote nucleation and elongation (25, 26), they antagonize the *intrinsic* inhibitory effect of Ca++ (4). 2) On the other hand, they appear to be the locus for the *extrinsic* calcium/calmodulin interaction on polymerization (Fig. 5) and thus increase Ca++ sensitivity. In *vitro* microtubule assembly in the presence of limiting concentrations of Ca++ is thus a delicate balance of opposing effects of both calmodulin and of MAPs. To what extent these factors play a role in vivo is not clear at present. It should be pointed out, however, that the relatively low affinity of the microtubule system for Ca++/calmodulin might make it more subject to regulation by calmodulin than the high affinity enzymes such as cyclic nucleotide phosphodiesterase which might be expected to be nearly saturated under the concentrations prevalent in the brain and binding constants of the order of 107 M^-1^-.
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Two opposing effects of calmodulin on microtubule assembly depend on the presence of microtubule-associated proteins.

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