Calcium and Gadolinium Ions Stimulate the GTPase Activity of Purified Chicken Brain Tubulin through a Conformational Change†

Claudio Soto, Patricio H. Rodríguez, and Octavio Monasterio*

Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

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ABSTRACT: Ca^{2+} and Gd^{3+} stimulated the GTPase activity of chicken brain tubulin 13- and 26-fold, respectively. Mg^{2+}, Tb^{3+}, and Na^{+} had no effect. This GTPase activity showed a saturation behavior with Ca^{2+} and Gd^{3+} with a maximal activity of 0.26 ± 0.026 and 1.15 ± 0.78 nmol min^{-1} per mg of tubulin and semisaturation constants, expressed as the concentration of the cation needed for 50% of saturation, of 0.32 ± 0.18 and 0.011 ± 0.007 mM, respectively. In the presence of Ca^{2+}, the GTPase activity was proportional to tubulin concentration in the range 0.9−31.8 μM. The semisaturation constants for the inhibition of tubulin polymerization and for the depolymerization of microtubules by Ca^{2+} were 0.71 ± 0.1 and 0.049 ± 0.043 mM, respectively. The similarity of the Ca^{2+} semisaturation constants for inhibition of tubulin assembly and stimulation of the GTPase activity suggests that these processes are correlated. These results support the hypothesis that the GTPase activity is related to but not directly involved in the mechanism of inhibition of Ca^{2+}-dependent tubulin assembly. This inhibition could be better explained by the formation of a nonfunctional conformational state of tubulin induced by Ca^{2+} that is responsible for the GTPase activity. Quenching of the intrinsic fluorescence of tryptophan induced by Ca^{2+} showed an apparent dissociation constant of 0.14 ± 0.005 mM, in the range of values determined through tubulin polymerization inhibition or through the induction of GTPase activity by Ca^{2+}. Acrylamide-induced quenching of the intrinsic fluorescence showed values of the Stern−Volmer constants of 5.4 ± 0.12 and 5.0 ± 0.15 M^{-1} in the absence and presence of Ca^{2+}, respectively. These results support the hypothesis that the inhibition of tubulin polymerization and the induction of the GTPase activity by Ca^{2+} is mediated by a conformational change. Ca^{2+} failed to induce depolymerization of GDP−AlF_4-microtubules; this could be explained by a model in which Ca−tubulin is unable to assemble into microtubules and the rate of dissociation of GDP−P_i−tubulin from the microtubule ends is extremely slow compared with the rate of GDP−subunit dissociation, supporting the concept that the GTP− and GDP−P_i−tubulin cap at the ends of microtubules regulates their dynamic instability.

Tubulin is an α−β heterodimer that self-assembles *in vitro* into microtubules in the presence of GTP,[1] while in the presence of GDP, the polymerization is inhibited [Weisenberg & Dery, 1976; for review, see Monasterio et al. (1995)]. Tubulin in microtubules will not exchange the nucleotide except at the microtubule plus end (Mitchison, 1993) or until disassembly occurs (Erickson & O'Brien, 1992). Tubulin has two guanosine nucleotide binding sites. One, the nonexchangeable site (N-site), contains Mg^{2+} and GTP that exchanges very slowly (Jacobs et al., 1974; Correia et al., 1988) and is not hydrolyzed during the polymerization process (Weisenberg & Deery, 1976); the other site, named the exchangeable site (E-site), contains GTP or GDP bound as a Mg^{2+}−nucleotide complex (Monasterio, 1987). GTP at the E-site is hydrolyzed with the liberation of P_i during the polymerization reaction (Jacobs et al., 1974; Weisenberg & Deery, 1976). The exchangeable nucleotide binding site of tubulin was localized by affinity labeling at β-tubulin (Geahlen & Haley, 1977). Thus, the GTP bound to the α subunit is nonexchangeable, while one exchangeable GTP molecule bound to the β subunit E-site is hydrolyzed to GDP and P_i following the polar assembly of the tubulin dimer into microtubules.

Tubulin in the presence of nonhydrolyzable GTP analogs retains its capacity to self-assemble into microtubules (Arai & Kaziro, 1976; Weisenberg & Deery, 1976), indicating that GTP hydrolysis is not a requirement for tubulin polymerization. This finding was studied further by following the time course of spontaneous tubulin polymerization and the accompanying hydrolysis of GTP. The lack of a tight temporal correlation between these two reactions indicates that they are not mechanistically coupled and that GTP is hydrolyzed in microtubules following polymerization (Carlier & Pantaloni, 1981). It has been suggested that the resulting microtubule, after tubulin polymerization, consists of a core of GDP subunits and stretches of GTP and GDP−P_i subunits at the ends of the growing microtubules (Kirschner & Mitchison, 1986; Melki et al., 1990). While GTP−tubulin is the active form of the protein, GDP−tubulin will not normally assemble into microtubules, although in the pres-

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* To whom correspondence should be addressed.

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[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; arsenazo III, 2,2′-[1,8-dihydroxy-3,6-disulfo-2,7-naphthylenebis(azo)]dibenzenearsonic acid; GTP, guanosine 5′-triphosphate; GMPCPP, 5′-(α,β-methylene)triphosphate; GDP, guanosine 5′-diphosphate; GTP(γS), guanosine 5′-(γ-thiotriphosphate).

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ence of taxol, assembly is induced (Díaz et al., 1993). Thus, on the basis of kinetic evidence, it has been postulated that depolymerization of the body of a microtubule, which normally consists of the intrinsically unstable GDP-ligated protein, is prevented by interaction with the active GTP−tubulin terminal cap (Carlier, 1991). The stochastic loss and recovery of the stabilizing cap thus generate the length fluctuations characteristic of microtubule dynamic instability (Erickson & O'Brien, 1992; Kirschner & Mitchison, 1986; Chen & Hill, 1985; Martin et al., 1993). Nonhydrolyzable GTP analogs with γ-phosphate groups capable of proper coordination with Mg^{2+} support microtubule assembly (Shearwin & Timasheff, 1992; Monasterio & Timasheff, 1987; Hyman et al., 1992); on the other hand, nucleotide hydrolysis is believed to be required to destabilize microtubules (Carlier et al., 1988). This is supported by the differences in stability of microtubules made of tubulin ligated to GMPCPP, a very slow hydrolyzable GTP analog (Hyman et al., 1992; Caplow et al., 1994). Microtubules with a γ-phosphate (Vale et al., 1994) or a structural analog of phosphate are stiffer than nucleoside diphosphate microtubules (Venier et al., 1994). Microtubule instability, also observed *in vivo* (Cassimeris et al., 1988), is currently thought to be employed by the cell to regulate in space and time the assembly of these structures (Kirschner & Mitchison, 1986; Verde et al., 1992). Clearly, the interactions of the exchangeable nucleotide γ-phosphate and a coordinated magnesium ion with the protein (Shearwin et al., 1994; Carlier, 1991; Correia et al., 1988) control the molecular switch of tubulin functionality and microtubule dynamic instability, in analogy to other nucleotide binding proteins.

Ca^{2+} prevents tubulin polymerization and induces rapid depolymerization of preformed microtubules (Kirschner et al., 1974; Olmsted & Borisy, 1975). Both processes can be reversed by Ca^{2+} chelation. Two modes of action have been proposed to explain the effect of Ca^{2+}, one mediated by calmodulin and microtubule-associated proteins (Berkowitz & Wolff, 1981; Job et al., 1981; Lee & Wolff, 1984) and the other by direct interaction of Ca^{2+} with tubulin, which would behave like a Ca^{2+} binding protein (Berkowitz & Wolff, 1981; Solomon, 1977; Lee & Timasheff, 1977). With respect to the direct effect of Ca^{2+} on tubulin, it has been demonstrated that Ca^{2+} inhibits tubulin polymerization by inducing endwise depolymerization of bovine brain microtubules (Karr et al., 1980). However, the precise mechanism by which Ca^{2+} inhibits tubulin polymerization and induces microtubule depolymerization is unknown.

In the present work, the effect of Ca^{2+} and Gd^{3+} on the inhibition of tubulin polymerization and microtubule depolymerization was characterized. Our results show that both cations are specific to stimulate the GTPase activity of tubulin and that there is a good correlation between the stimulation of the GTPase activity, the inhibition of tubulin polymerization, and a conformational change of the protein. Furthermore, the effect of Ca^{2+} on microtubule depolymerization was studied in the presence of aluminum fluoride, an analog of P_i. The results show that under these conditions the stability of microtubules is less affected by Ca^{2+}.

MATERIALS AND METHODS

Reagents. MES, GTP (type III), EGTA, glycerol, guanidine hydrochloride, GTP(γS), Gd_2O_3, Sephadex G-25, DEAE-Sephadex A50, and Sephacryl S-300 were purchased from Sigma Chemical Co. $TbCl_3$ was from Aldrich Chemical Co. $MgCl_2$, $CaCl_2$, EDTA titrisol, and arsenazo III were obtained from Merck AG Darmstadt. Other chemicals were analytical grade. Ultrapure acrylamide was purchased from Gibco BRL.

Chicken Brain Tubulin Purification. Chicken brains (donated by Pollos Ariztía), for tubulin preparation, were dissected from freshly slaughtered animals, kept on ice, and used within 2 h. Tubulin was purified by the method described by Weisenberg et al. (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970) as modified by Lee et al. (1973). The stock protein was stored in the presence of 1 M sucrose (Frigon & Lee, 1972), and the experimental samples were prepared by batch equilibration with 5−10 volumes of dry-packed Sephadex G-25 columns equilibrated with the experimental buffer. Tubulin concentrations were determined spectrophotometrically in 6 M guanidine hydrochloride by using an absortivity value of 1.03 L g^{-1} cm^{-1} at 275 nm (Na & Timasheff, 1981).

Assembly Assays. Tubulin (2−4 mg/mL) in polymerization buffer [0.1 M MES (pH 6.4), 15 mM $MgCl_2$, 0.1 mM GTP, and 3.4 M glycerol] was induced to polymerize at 37 °C in a water-jacketed cuvette which was thermostatically regulated by a Haake D8 or Heto water bath circulator. The self-assembly of tubulin into microtubules was monitored by turbidity measurements at 350 nm (Gaskin et al., 1974) using a Gilford model 2000 spectrophotometer.

Atomic Absorption Spectroscopy. Concentrations of Ca^{2+} and Mg^{2+} were determined in a Perkin-Elmer model 360 atomic absorption spectrometer using a specific lamp for each metal, an air-acetylene flame, and commercial standard solutions.

Gadolinium Stock Solution. Solutions (0.1 M) of $GdCl_3$ were prepared by dissolving Gd_2O_3 in 0.3 M hydrochloric acid (Reuben, 1971). The concentrations of these solutions were determined by titration with EDTA using arsenazo III as indicator (Fritz et al., 1958).

[γ-^{32}P]GTP Synthesis. The radioactive nucleotide was kindly synthesized by M. Antonelli using the procedure described by Walseth and Johnson (1979).

Tubulin GTPase Activity Assays. Hydrolysis of the γ-phosphate of GTP was assayed essentially as described by Monasterio and Timasheff (1987). After separation of tubulin from aggregates by chromatography on a Sephacryl S-300 column equilibrated with 0.1 M MES (pH 6.4), 0.5 mM $MgCl_2$, and 0.1 mM GTP, the GTPase activity was determined by adding [γ-^{32}P]GTP and placing the tubes at 37 °C. The reaction was stopped by adding 1 volume of active charcoal suspension. P_i was determined counting the ^{32}P after nucleotide absorption to active charcoal (100 mg/mL) in 0.2 M KCl solution adjusted to pH 1.8 as described by Nieto et al. (1975).

Fluorescence Spectroscopy. The storage buffer of tubulin was exchanged by rapid gel filtration on Sephadex G-25 followed by chromatography in Sephacryl S-300 (25 × 1 cm) equilibrated with 50 mM PIPES (pH 6.0). Intrinsic steady-state fluorescence measurements were carried out on a Perkin-Elmer LS-50 spectrofluorometer equipped with a personal computer and software suitable for analysis of spectra. The emission spectra obtained in the calcium and acrylamide quenching experiments were produced with an excitation wavelength of 295 nm and a band width of 5 nm.

Table 1: Stimulation of Tubulin GTPase Activity by Ca^{2+} and Gd^{3+} [a]

addition	GTP hydrolyzed (nmol/min)	stimulation (-fold)
none	0.02	–
2 mM $CaCl_2$	0.26	13
8 μM $GdCl_3$	0.52	26

[a] Tubulin free of aggregates (1.7 and 2.2 mg/mL for Ca^{2+} and Gd^{3+}, respectively) was incubated at 37 °C in a reaction mixture containing 0.1 M MES (pH 6.4), 0.5 mM $MgCl_2$, and 0.1 mM [γ-^{32}P]GTP (specific radioactivity of 15 570 cpm/nmol), in a final volume of 0.1 mL. The GTPase activity was determined as described in Materials and Methods and expressed per milligram of tubulin.

The spectra were corrected for solvent and dilution effects. Titrations were made by adding small aliquots of calcium stock solution (or acrylamide) to 3 mL of tubulin solution (1 μM tubulin subunit), assuring that no more than a 3% dilution was made by the end of each experiment. All measurements were carried out at 20 °C, regulating the cuvette holder thermostatically with a Hakee G water bath circulator.

Data Analysis. Thermodynamic and kinetic parameters were determined by fitting the experimental data to the Hill equation, $y = YCa^h/(K_{0.5}{}^h + Ca^h)$, where Y is the maximal value of the variable y, h is the Hill coefficient, $K_{0.5}$ is the semisaturation constant, and Ca is the total concentration of Ca^{2+}. The values of Y, h, and $K_{0.5}$ were obtained using a nonlinear regression method available in the commercial computer programs ENZFITTER and Sigma Plot 5.0. Fluorescence data were fitted with the same computer programs to eq 1 (shown in Results).

RESULTS

Effect of Ca^{2+} on the Tubulin GTPase Activity. When the GTPase activity of chicken brain tubulin prepared by the method of Weisenberg and Timasheff (1970) was measured, higher values than those usually reported for tubulin from other sources were found. To determine if tubulin aggregates were responsible for this effect, the Sephadex G-25 filtration step for the equilibration with the experimental buffer (see Materials and Methods) was replaced by filtration on Sephacryl S-300, as previously described by Andreu and Timasheff (1981). Two peaks of proteins were obtained, one in the void volume which had a high GTPase activity that was not stimulated by Ca^{2+} and the other, corresponding to the tubulin dimer, with a lower GTPase activity that was stimulated by Ca^{2+}. The elution of the Ca^{2+}-dependent GTPase activity followed a profile identical to that of tubulin (not shown), indicating that no other GTPase activities with molecular weights different from that of tubulin were present. The protein peak ratio between tubulin and aggregates was similar to that reported for calf brain tubulin by Andreu and Timasheff (1981).

The effect of Ca^{2+} on the initial velocity of [γ-^{32}P]GTP hydrolysis was studied. As shown in Table 1, tubulin GTPase activity was stimulated by Ca^{2+}. The GTP hydrolysis rates per milligram of tubulin were determined under initial velocity conditions, in the presence and in the absence of 2 mM $CaCl_2$, and were 0.26 and 0.02 nmol min^{-1}, respectively. Ca^{2+} induced a 13-fold stimulation of the GTP hydrolysis rate.

In order to determine the specificity of Ca^{2+} to stimulate the GTPase activity of tubulin, the influence of different Ca^{2+} and Mg^{2+} concentrations on the hydrolysis of GTP was measured (Figure 1). The stimulation of the GTP hydrolysis by Ca^{2+} showed a saturation behavior between 0.1 and 2 mM. On the other hand, Mg^{2+} in the concentration range from 0.1 to 4.0 mM did not stimulate significantly this activity. Na^+ (1 mM NaCl) had no effect (not shown). These results indicate that the stimulation of the GTPase activity by Ca^{2+} was specific and saturable.

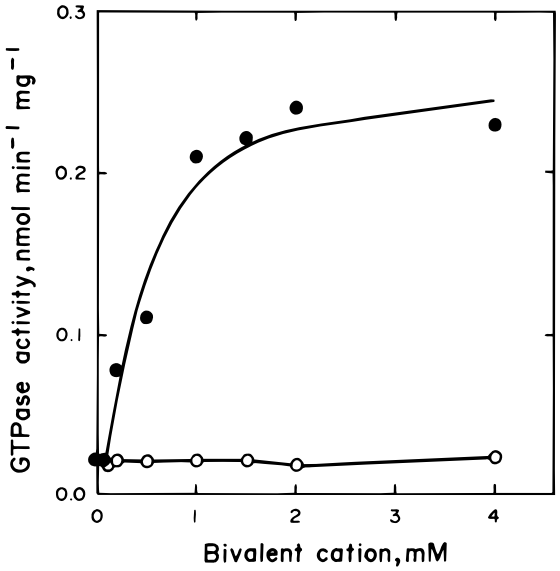

FIGURE 1: Effect of Ca^{2+} and Mg^{2+} concentrations on the tubulin GTPase activity. Tubulin (2.4 mg/mL) in the presence of 0.1 mM [γ-^{32}P]GTP (specific activity of 10 253 cpm/nmol) was incubated at 37 °C for 10 min with Ca^{2+} (filled circles) or Mg^{2+} (open circles) in the same buffer described in the footnote of Table 1, in a final volume of 0.1 mL. The GTPase activity was determined as described in Materials and Methods.

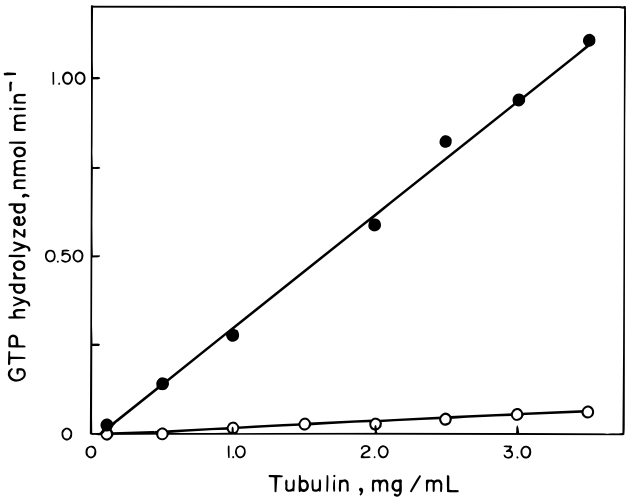

FIGURE 2: Influence of tubulin concentration on GTP hydrolysis. Mixtures of different tubulin concentrations, in a final volume of 0.08 mL, were incubated at 37 °C for 15 min under the same conditions described in the footnote of Table 1, with (filled circles) or without (open circles) 2 mM $CaCl_2$. The GTPase activity was determined as described in Materials and Methods.

The influence of tubulin concentration on the GTP hydrolysis rate in the presence or in the absence of 2 mM Ca^{2+} was assayed (Figure 2). The GTP hydrolysis stimulated by Ca^{2+} was proportional to tubulin concentration at values between 0.1 and 3.5 mg/mL, indicating that the stimulation of the GTPase activity was not mediated by tubulin polymerization. This conclusion is further supported by the fact

that, when tubulin (0.2 and 2 mg/mL) was incubated under the experimental conditions described in Figure 1, the same amount of aggregates was found in the absence of divalent cations and in the presence of 2 mM calcium or magnesium. The amount of aggregates was proportional to the initial concentration of tubulin. These results indicate that the aggregates were not responsible for the GTPase activity induced by calcium, because in the absence of bivalent metals and in the presence of 2 mM magnesium this activity was not induced (Figure 1), although the aggregates were present. This strongly suggests that tubulin dimer was responsible for the GTPase activity of tubulin.

Dependence of GTPase Activity, Tubulin Polymerization Inhibition, and Microtubule Depolymerization on Ca^{2+} Concentration. To establish if the GTPase activity stimulated by Ca^{2+} was associated with both inhibition of tubulin polymerization and microtubule depolymerization, the Ca^{2+} dependence of these three processes was studied. In all cases, a saturation behavior by Ca^{2+} was observed (Figure 3). The Ca^{2+} concentrations needed to reach half of the maximum of the GTPase activity and to inhibit polymerization by 50% were similar (Figure 3A,B), and it was lower for microtubule depolymerization (Figure 3C).

Table 2 shows the semisaturation constants for these three processes. These constants were defined as the Ca^{2+} molar concentration needed to reach 50% saturation. The results indicate similar values of the semisaturation constants for polymerization inhibition and Ca^{2+}-stimulated GTPase activity, within experimental error; they were at least 1 order of magnitude higher than that for microtubule depolymerization. The similarity in the values obtained for the semisaturation constants of the inhibition of tubulin polymerization and the stimulation of the GTPase activity suggests that these processes are coupled.

Effect of Gadolinium Ions on the GTPase Activity of Tubulin. The lanthanide ions, Gd^{3+} and Nd^{3+}, behave as Ca^{2+} analogs because they inhibit tubulin polymerization and induce microtubule depolymerization (Soto, 1993). On the other hand, Tb^{3+} behaves as a Mg^{2+} analog (Monasterio et al., 1993).

The GTP hydrolysis rate per milligram of tubulin in the presence of 8 μM Gd^{3+} was 0.52 nmol of GTP hydrolyzed per minute (Table 1). This value, when compared to the control without Gd^{3+}, showed 27-fold stimulation, which was twice the stimulation produced by 2 mM Ca^{2+}.

Figure 4 shows the influence of Gd^{3+} and Tb^{3+} concentrations on the tubulin GTPase activity. Gd^{3+}, but not Tb^{3+}, stimulated the GTPase activity between 1 and 10 μM. Concentrations over 10 μM Gd^{3+} produced a decrease in the GTPase activity (not shown), probably due to the formation of aggregates which do not have GTPase activity. These aggregates induced turbidity in the reaction mixture, and were not produced by tubulin denaturation, because they were dissolved with EGTA and tubulin was polymerized again in the presence of Mg^{2+}. Gd^{3+} has a higher affinity for tubulin than Ca^{2+} (Soto, 1993), and this is supported by the observation that tubulin stimulation of GTP hydrolysis occurred at lower Gd^{3+} concentrations. The value of the Gd^{3+} semisaturation constant for the stimulation of the GTPase activity of tubulin, obtained from Figure 4, is 0.011 $\pm$ 0.07 mM.

Quenching of the Intrinsic Fluorescence Induced by Ca^{2+}. Figure 5 shows the Ca^{2+} titration curve plotted according to

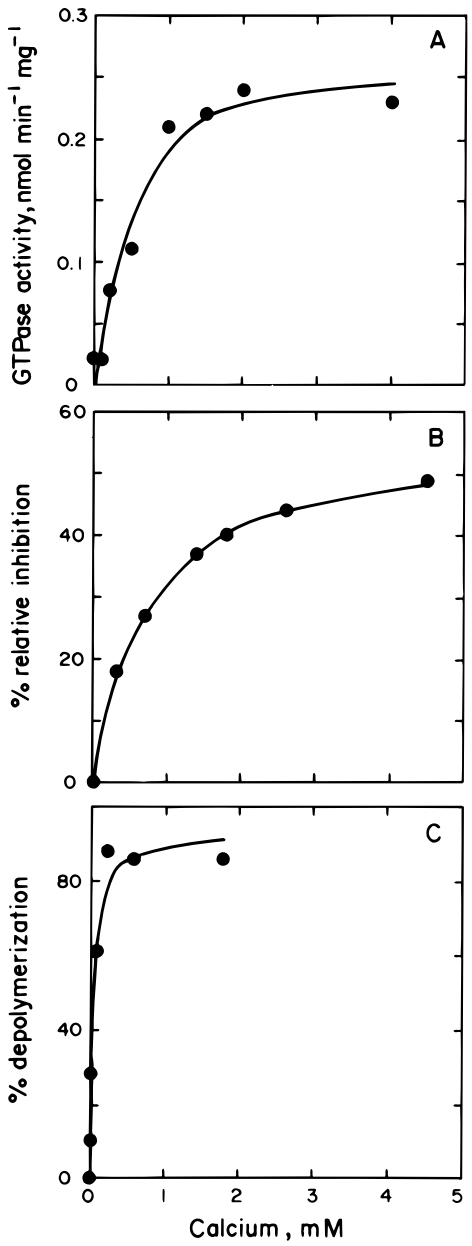

FIGURE 3: Effect of Ca^{2+} on the GTPase activity of tubulin (A), tubulin polymerization (B), and microtubule depolymerization (C). The results showed in A were obtained under the experimental conditions described in Figure 1. In B, tubulin (2.8 mg/mL) was incubated at 0 °C for 15 min in polymerization buffer, with the $CaCl_2$ concentrations indicated in the Figure. The polymerization reaction was induced by jumping the temperature to 37 °C and recording the turbidity at 350 nm. The inhibition of tubulin polymerization, expressed as a percentage, corresponds to one minus the turbidity plateau value (expressed as the fraction of the value in the absence of Ca^{2+}) for each Ca^{2+} concentration multiplied by 100. In C, microtubules were polymerized by incubating tubulin (2.3 mg/mL) at 37 °C for 20 min in the polymerization buffer (see Materials and Methods). Ca^{2+}, at the concentrations indicated in the Figure, was added to each sample, and the change of turbidity at 350 nm was recorded. The percentage of depolymerization corresponds to the decrease in the turbidity measured 5 min after the addition of Ca^{2+}, divided by the turbidity without Ca^{2+}, and multiplied by 100.

the fluorescence equivalent of the Scatchard (1949) binding equation

$$(-\Delta F/F_o)/\mathrm{Ca} = KF_{\mathrm{CaQ}} - K(-\Delta F/F_o) \qquad (1)$$

where ΔF is the decrease in the relative fluorescence intensity

Table 2: Semisaturation Constants for Ca^{2+} Effect on Different Tubulin Functions[a]

function	$K_{0.5}$[b]
polymerization inhibition	0.71 ± 0.1
microtubule depolymerization	0.049 ± 0.043
stimulation of GTP hydrolysis	0.32 ± 0.18

[a] The values for the semisaturation constants for tubulin polymerization inhibition, microtubule depolymerization, and stimulation of GTP hydrolysis were obtained from the results shown in Figure 3 using the commercial computer programs ENZFITTER and Sigma Plot 5.0 to fit the curves to the experimental data. [b] $K_{0.5}$ is the millimolar concentration of total Ca^{2+} necessary to reach 50% of saturation.

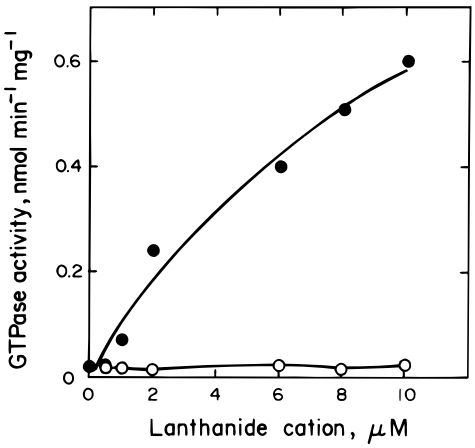

FIGURE 4: Effect of Gd^{3+} and Tb^{3+} on the GTPase activity of tubulin. Tubulin (1.1 mg/mL), in the same buffer described in Table 1, was incubated at 37 °C for 20 min with Gd^{3+} (filled circles) or Tb^{3+} (open circles). GTPase activity was determined as described in Materials and Methods.

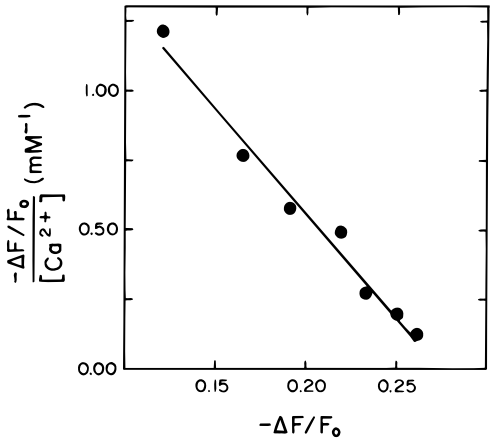

FIGURE 5: Quenching of the intrinsic fluorescence of tubulin induced by Ca^{2+}. Tubulin (1 μM) in a solution containing PIPES (50 mM, pH 6.0) was titrated with stock solutions of calcium chloride, and the corrected emission spectra were obtained. The emission intensities at 338 nm were measured using an excitation wavelength of 295 nm and a band width of 5 nm, at 20 °C.

F produced by the total Ca^{2+} concentration (Ca), F_o is F before the addition of Ca^{2+}, K is the Ca^{2+} binding constant, and F_{CaQ} is the percentage of F_o quenchable by a saturating concentration of Ca^{2+}. The results show that the binding of Ca^{2+} to tubulin is not cooperative and it has a dissociation constant of 0.14 ± 0.005 mM. Furthermore, binding of Ca^{2+} to tubulin induced a protein conformational change which is responsible for the quenching of approximately 25% of the tryptophan fluorescence. The conformational change induced by Ca^{2+} was confirmed by quenching of the intrinsic fluorescence with acrylamide. The direct Stern−Volmer plot showed monophasic curves between 0 and 200 mM acrylamide, with Stern−Volmer constants of 5.4 ± 0.12 and 5.0 ± 0.15 M^{-1} in the absence and in the presence of 2 mM Ca^{2+}, respectively (not shown). These values are in agreement with the values for the Stern−Volmer constants obtained by Steiner (1980) for the quenching of human platelet tubulin induced by acrylamide.

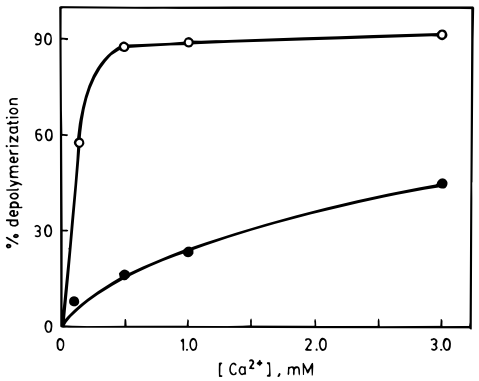

FIGURE 6: Ca^{2+} induces depolymerization of microtubules incubated with AlF_4^-, a structural analog of P_i. Two samples of tubulin (2.7 mg/mL) in polymerization buffer were induced to polymerize by jumping the temperature to 37 °C. After 20 min, AlF_4^- [2 mM NaF and 10 μM $Al(NO_3)_3$] was added to one sample (filled circles) and polymerization buffer to the control (open circles). Incubation continued for 20 min. Ca^{2+} was added to 0.7 mL aliquots of each sample, and after 5 min, the turbidity was recorded at 350 nm. The percentage of depolymerization was obtained as described in Figure 3C.

Depolymerization of Microtubules Induced by Ca^{2+} in the Presence of AlF_4^-. It has been demonstrated that AlF_4^- and BeF_4^- complexes, structural analogs of P_i, replace phosphate in the tubulin−GDP−P_i complex at the ends of microtubules (Carlier et al., 1988, 1989). In addition, these compounds bind 1000-fold more strongly than phosphate (Carlier et al., 1988). In order to study the effect of Ca^{2+} over the P_i liberation from the ends of microtubules as the result of their disassembly, depolymerization of preformed microtubules in the presence of AlF_4^- was measured. Figure 6 shows that microtubules incubated with AlF_4^- depolymerized at higher Ca^{2+} concentrations than microtubules incubated without it. This result indicates that microtubules that contain tubulin−GDP-AlF_4^- at their ends were less disassembled by Ca^{2+}. This is supported by the findings of Arai and Kaziro (1976) and Weisenberg and Deery (1976) that Ca^{2+} is unable to induce disassembly of microtubules when they have been assembled in the presence of nonhydrolyzable analogs of GTP.

DISCUSSION

The data presented in this work show that the binding of Ca^{2+} or Gd^{3+} to tubulin stimulates tubulin GTPase activity. Ca^{2+}-dependent GTPase activity was observed in the same concentration range where this cation inhibited tubulin assembly. Furthermore, a conformational change of the protein was induced in the same range of concentration of Ca^{2+}.

The role of GTPase activity in the mechanism of tubulin polymerization has been studied extensively since the discovery of tubulin assembly *in vitro* by Weisenberg (1972). It has been established that hydrolysis of the γ-phosphate

of GTP is not necessary for assembly of tubulin, and polymerization can take place in the presence of nonhydrolyzable GTP analogs (Weisenberg et al., 1976; Purich & MacNeal, 1978) but it cannot be initiated in the presence of GDP alone (Carlier & Pantaloni, 1978; Díaz & Andreu, 1993). Further investigation has been carried out on the hydrolysis of GTP associated with tubulin polymerization. The uncoupling between the GTP hydrolysis and microtubule assembly support the hypothesis of a dynamic GTP cap at the ends of microtubules (Carlier et al., 1987). Howard and Timasheff (1986) suggested that, in the cold disassembly of microtubules, rings formed of GDP subunits could be generated directly from protofilaments of GDP microtubules without prior dissociation into dimers. Kinetic data indicate the existence of an equilibrium between two conformations of tubulin, "straight" (microtubule-forming) and "curved" (ring-forming), under the allosteric control of bound nucleotide (Melki et al., 1989). The GTPase activity induced by Ca^{2+} would then be able to induce a curved conformational state of tubulin favoring ring formation. Our results show that no rings or other larger structures are formed in the presence of Ca^{2+}, suggesting that a conformational state different from that of tubulin−GDP is responsible for inducing GTPase activity, inhibition of tubulin assembly, and microtubule disassembly. A conformational change has also been found with colchicine (Andreu & Timasheff, 1982), another inhibitor of tubulin that induces GTPase activity (David-Pfeuty et al., 1979).

The presence of GDP produced by the Ca^{2+}-dependent GTPase reaction was not responsible for the Ca^{2+} inhibition of tubulin polymerization because the inhibition was abolished when EGTA was added to the polymerization reaction mixture 5 or 10 min after the beginning of incubation with Ca^{2+}. When EGTA was added after 20 min, the inhibition was irreversible, indicating that at this time the presence of GDP was responsible for the inhibition of tubulin assembly (C. Soto and O. Monasterio, unpublished results). Thus, the inhibition of tubulin polymerization is produced by a conformational change of the protein in the presence of Ca^{2+}, and this conformational state induces the GTPase activity. On the other hand, the fact that the semisaturation constant for microtubule depolymerization is lower than those for the other processes studied (Table 1) could be explained by the presence of GDP and tubulin−GDP as a product of microtubule assembly. One explanation for microtubule depolymerization by Ca^{2+}, based on the hypothesis of tubulin−GTP and tubulin−GDP−P_i caps (Melki et al., 1990), would be that this cation interacts with the subunits of tubulin located at the ends of the microtubules removing the cap, or the exchange of tubulin by calcium−tubulin at the end of the microtubule eliminates the cap. Another possible explanation for the effect of Ca^{2+} is that this cation could replace Mg^{2+} at the E-site in tubulin by exchange of the nucleotide−metal complex. To check this possibility, the equilibrium concentrations of the complexes and the free components of the experimental solutions were calculated using the affinity constants of the complexes and a computer program for multiple equilibrium (Storer & Cornish-Bowden, 1976). The concentrations of the calcium and magnesium complexes were determined at two concentrations of Mg^{2+}, 15 mM (used in tubulin polymerization and inhibition studies) and 0.5 mM (used in the GTPase activity studies), keeping the Ca^{2+} concentration constant at 2 mM. At high magnesium concentrations, the concentration of the tubulin−Mg^{2+}−nucleotide complex with respect to the complex with Ca^{2+} was 1 order of magnitude higher, and at 0.5 mM Mg^{2+}, the concentration of the Mg^{2+} complex was 4 times lower than that observed with Ca^{2+}. Under the assumption that the affinity constant of tubulin for Ca− and Mg−GTP complexes is similar, these results indicate that the concentration of the Ca^{2+}−nucleotide complex cannot be fitted to the saturation behavior shown in Figure 3. Therefore, the Ca^{2+}−nucleotide complex is not directly involved in the GTPase activity or polymerization inhibition because both processes were affected in a similar range of concentrations of Ca^{2+}. The results of Buttlaire et al. (1980) showing that Ca^{2+} binds to a site different from that for magnesium support our assumption.

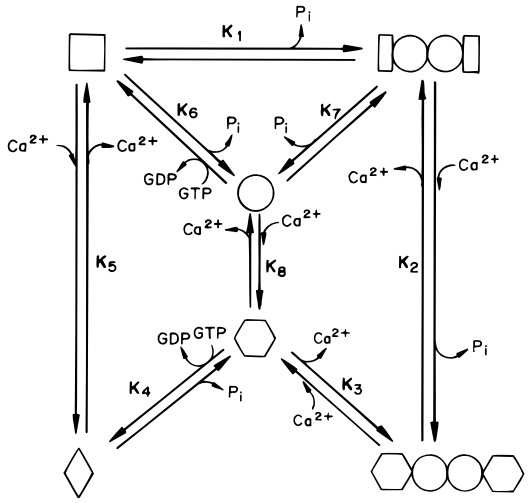

FIGURE 7: Model for the action of Ca^{2+} on tubulin and microtubules. Different conformational states of tubulin−nucleotide with and without Ca^{2+} are represented with the following symbols: square, tubulin−GTP; rhombus, Ca^{2+}−tubulin−GTP; hexagon, Ca^{2+}−tubulin−GDP; circle, tubulin−GDP; and rectangle, tubulin−GDP−P_i. The equilibria should involve more steps than those indicated in the scheme. For simplicity, one equilibrium constant for each step was assigned. Details are discussed in the text.

The effect of Ca^{2+} on tubulin and microtubules suggested by our data is summarized in the scheme presented in Figure 7. GTP−tubulin, represented by the square, is able to polymerize to a GTP microtubule form. In this form, GTP is then hydrolyzed to GDP, the process being accompanied by a conformational change in which the GDP at the E-site is not exchangeable, and at the ends of microtubules, a pair of caps with GTP or GDP−P_i stabilize microtubules. This state of microtubules is represented in the scheme by two circles between two rectangles, and the overall equilibrium constant is indicated by K_1. Upon depolymerization (K_7), tubulin returns to a conformation (circle) in which GDP can be replaced by GTP and tubulin can be recycled in assembly (K_6). In the presence of Ca^{2+}, tubulin−GTP suffers a conformational change (rhombus, K_5) that induces the GTPase activity, giving the GDP−tubulin−Ca^{2+} complex (hexagon, K_4). Upon depolymerization of microtubules by Ca^{2+} (K_2), the GTP or GDP−P_i tubulin caps change to a conformation that induces endwise depolymerization, producing the ternary complex GDP−tubulin−Ca^{2+} (hexagon, K_3). Ca^{2+} would induce a conformational change of the GDP−tubulin complex (circle to hexagon, K_8).

The model proposed in Figure 7 is supported by the following evidence: (1) Ca^{2+} and its analog Gd^{3+} induce a

tubulin GTPase activity mediated by a conformational change. It has been found that the binding of Ca^{2+} to Ca^{2+}-binding proteins produces a conformational change (Strynadka & James, 1989; Reid et al., 1981). (2) In the same range of concentrations, the binding of Ca^{2+} to tubulin is responsible for the stimulation of tubulin GTPase activity and the inhibition of tubulin polymerization, giving a Ca^{2+}−tubulin−GDP state. (3) GTPase activity, the inhibition of tubulin assembly, and microtubule disassembly is a consequence of the conformational change induced by Ca^{2+}. (4) Microtubules incubated with AlF_4^-, which binds 1000-fold more strongly than phosphate to the GTP site, were less disassembled by Ca^{2+}. This indicates that GTP hydrolysis and release of P_i are involved in the mechanism of action of Ca^{2+} and suggests that tubulin in the presence of nonhydrolyzable analogs of GTP may be resistant to the action of Ca^{2+}. This is supported by the following results: the inhibition of tubulin polymerization by Ca^{2+} is abolished in the presence of GTP(γS) (unpublished results), and microtubules assembled in the presence of nonhydrolyzable GTP analogs are not depolymerized by Ca^{2+} (Arai & Kaziro, 1976; Weisenberg & Deery, 1976). These results could be explained if Ca^{2+} does not bind to tubulin−GTP(γS) as suggested by the work of Arai and Kaziro (1976), where tubulin was polymerized in the presence of GMP−PNP, or by the existence of a conformational state different from that of tubulin−GTP in the presence of the nonhydrolyzable analogs. (5) The lower value of the semisaturation constant for microtubule depolymerization induced by Ca^{2+} with respect to that for the inhibition of tubulin polymerization (Table 1) indicates that binding of Ca^{2+} to the ends of microtubules induces endwise depolymerization. Another explanation would be that the endwise depolymerization would be induced by diminishing the concentration of tubulin capable of exchanging at the ends of the microtubule. However, this possibility is ruled out because the concentration of Ca^{2+} needed to depolymerize microtubules is lower than that for tubulin polymerization inhibition. Karr et al. (1980) have demonstrated that Ca^{2+} induces endwise depolymerization of bovine brain microtubules. These facts agree with the results of Carlier et al. (1988, 1989) and Melki et al. (1990), who demonstrated that only the ends of microtubules contain tubulin molecules with GDP−P_i in their E-site.

The proposed model, firmly based on experimental evidence, allows us to conclude that Ca^{2+} induces a conformational change of tubulin that is responsible for microtubule depolymerization, tubulin polymerization inhibition, and the induction of GTP hydrolysis. It allows us to conclude that at shorter times the effect of Ca^{2+} should be abolished by chelating agents, like EGTA, but at longer times the formation of microtubules should be inhibited by the presence of Ca^{2+} and GDP and, accordingly, addition of EGTA should have no effect. Therefore, in the presence of EGTA, only after the exchange of GDP by GTP should the assembly be restored. These conclusions were supported by the preliminary experimental data discussed above. It is possible to speculate that the conformational change responsible for the GTPase activity induced by Ca^{2+} allows a water molecule to form a pentavalent coordination complex at the exchangeable nucleotide binding site in a manner similar to that decribed for the conformational change induced on $G_{t\alpha}$ when a ligand interacts with the receptor (Sondek et al., 1994).

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