MAGNESIUM STIMULATION OF CALCIUM BINDING TO TUBULIN AND CALCIUM INDUCED DEPOLYMERIZATION OF MICROTUBULES

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1. Introduction

Microtubule assembly in vitro is inhibited by calcium, and it has been suggested that Ca is an intracellular regulator of microtubule assembly [1]. This suggestion has been supported by studies on human granulocyte chemotaxis [2]. However, questions have been raised about the ability of Ca to inhibit microtubule assembly at physiological concentrations and therefore about its ability to function as a regulator of assembly [3]. We now report that the inhibition of polymerization by Ca is a function of the concentrations of Mg ions present. At approx. physiological Mg concentrations (2 to 5 mM), significant inhibition of polymerization occurs at micromolar concentrations of Ca. Data has also been obtained indicating that Ca binds directly to tubulin, and that binding is enhanced by Mg.

2. Materials and methods

Tubulin was isolated from bovine brain by the method of Shelanski et al. [4], and was stored in 25% glycerol below 0°C. Prior to use, the protein was passed through G-25 Sephadex (medium) to remove most of the glycerol [5]. The buffer in all experiments was 0.1 M MES (2-*N*-morpholinoethane sulfonic acid) at pH 6.6 adjusted with sodium hydroxide. All chemicals were reagent grade.

Polymerization was determined by the increase in turbidity at 340 nm, 15 min. after the temperature was raised to 35°C [6] in a water jacketed cuvette. Protein concentration was determined by the method of Hartree [7], using bovine serum albumin as a standard. Free Ca concentrations were calculated from the association constant of EGTA with Ca [8]. For simplicity, the concentrations have not been corrected for competitive binding by Mg, or for binding to other components of the system. All major components, tubulin, EGTA, GTP, and MES buffer bind Ca and Mg to some extent. The major effect of this will be to lower the actual free Mg and Ca concentrations. Competitive binding of Mg to EGTA has been ignored because of the much greater affinity of EGTA for Ca over Mg [8]. Bound Mg will compete with the binding of Ca to GTP. However, under the conditions of most of these experiments (0.5 mM GTP and 1 mM EGTA) the amount of Ca bound to GTP will be only about 0.2% of that bound to EGTA based upon the difference in their affinity constants for Ca [8,9]. Displacement of the Ca bound to GTP by Mg would result in the release of no more than 0.004 mM Ca. Competitive binding to the buffer has been ignored since the buffer is in large excess, and only about 2% of the buffer will have bound ligand at the highest Mg and Ca concentration. The affinity constants of MES buffer for Mg and Ca are not known with precision, but the approximate binding constants of Good et al. [10] indicate that about half of the total Mg may be bound at 5 mM Mg.

For the calcium binding studies the protein was allowed to incubate at 4°C for one hour with ^{45}Ca (New England Nuclear Co.) in an Amicon Centroflo Membrane Cone type CF50A. GTP was present in all samples at a concentration of either 0.1 or 0.5 mM. After aliquots were removed in duplicate or triplicate for counting, the unit was spun for a total of 30 sec

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in a Sorval GLC-1 at 2000 rev/min at 4°C. Aliquots were again removed in duplicate or triplicate from both the filtrate and the supernatant. Counting was done in 10 ml. Brays solution, or in 10 ml of ACS (Amersham Searl Co.) with the addition of 1 ml of 10 mM $CaCl_2$.

3. Results

Polymerization in the presence of 1 mM EGTA and varying Mg concentrations alone indicated optimal conditions at about 0.5 mM Mg (fig.1). Mg at concentrations greater than about 2 mM Mg significantly inhibits polymerization. Polymerization in 0.5 mM Mg with no added Ca was therefore selected as 100% polymerization.

Increasing Ca concentrations in the presence of 5.0 mM Mg and 1 mM EGTA results in increasing inhibition of polymerization (fig.2). At a concentration of about 0.035 mM Ca, polymerization is approximately 50% of that seen without any added Ca. A 10–20% inhibition is seen to occur in the presence of concentrations as low as 0.003 mM Ca. If the same experiment is repeated, but at a ten-fold lower Mg concentration (fig.2), no significant inhibition is

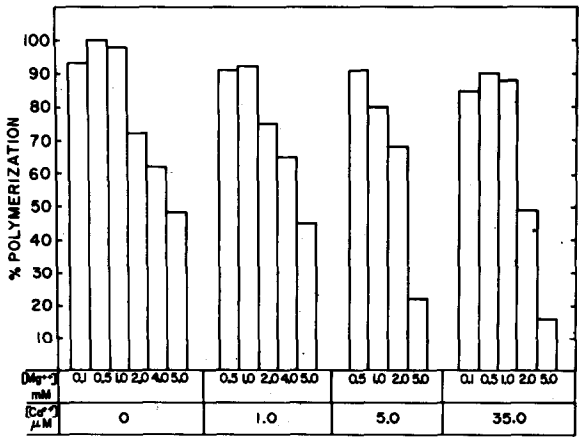

Fig.1. Microtubule assembly at varying Mg concentrations in the absence and presence of Ca. Assembly in 0.5 mM Mg in the absence of any added Ca was taken as 100% polymerization. The concentrations of Ca were determined as indicated in Materials and Methods. The protein was polymerized in 0.1 M MES buffer, pH 6.6 in the presence of 5×10^{-4} M GTP and 1 mM EGTA.

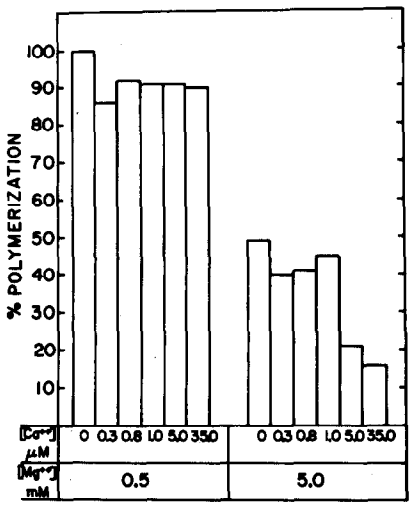

Fig.2. Microtubule assembly at varying Ca concentrations in the presence of either 5.0 mM Mg or 0.5 mM Mg. Assembly in 0.5 mM Mg in the absence of any added Ca was taken as 100% polymerization. The concentration of Ca was determined as indicated in Materials and methods. The protein was polymerized in 0.1 M MES, buffer, pH 6.6 in the presence of 5×10^{-4} M GTP and 1 mM EGTA.

observed for the corresponding values of Ca. At millimolar concentrations of Ca, however, inhibition of polymerization can be observed at this Mg concentration. At these concentrations ring-shaped aggregates of tubulin [11] are observed to be the prevalent structure, more rings being observed at higher Ca concentrations. Millimolar concentrations of Ca can also induce the depolymerization of preexisting microtubules.

In the presence of 0 to 0.035 mM Ca less polymerization is observed at higher Mg concentrations and at 5.0 mM Mg polymerization is about 10% of that seen with 0.5 mM Mg (fig.1). At the higher concentrations of Mg (greater than 2 mM) rings are again the prevalent structure observed.

With no added Mg, and the addition of 1 mM EDTA and 1 mM EGTA to the assembly medium, verly little polymerization occurs. If, however, Mg is added after incubation has begun, polymerization will proceed almost immediately. The addition of Ca alone to achieve a free Ca ion concentration of about 0.001 mM in the presence of both 1 mM EDTA and 1 mM EGTA also promotes polymerization. In the absence of added ions, near maximal polymerization

occurs in the presence of EGTA alone, but little or no polymerization occurs if EDTA alone is used, indicating that our protein preparations contain sufficient levels of Mg to stimulate polymerization.

The inhibitory activity of Ca suggests a direct interaction of Ca with tubulin and we have therefore determined the extent of binding using ultrafiltration to separate bound from free Ca. The extent of binding was calculated from the difference in ^{45}Ca activity above and below the filter. Only a fraction of the initial volume is spun through the filters so that little concentration of the protein occurs. Our results clearly indicate binding of Ca to tubulin, but more significantly they also demonstrate an effect of Mg on binding which is consistent with its effect on the sensitivity of polymerization to Ca (fig.3). The highest level of Ca binding occurs when the Mg concentration is between 5 and 7 mM. Ca binding is decreased at very low (0 to 0.5 mM) and very high (10 mM) Mg concentrations. In four experiments the minimum binding observed (at 0 or 0.5 mM) averaged 0.37 ± 0.20 moles per mole of tubulin and the maximum binding (at 5 or 7 mM) averaged 0.72 ± 0.15. All of these experiments were performed at 0.01 mM Ca. Variation of the Ca concentration gave complicated results which indicate cooperative binding at low Ca and binding to a large number of low affinity sites at high Ca. Binding to the low affinity sites is decreased by addition of Mg.

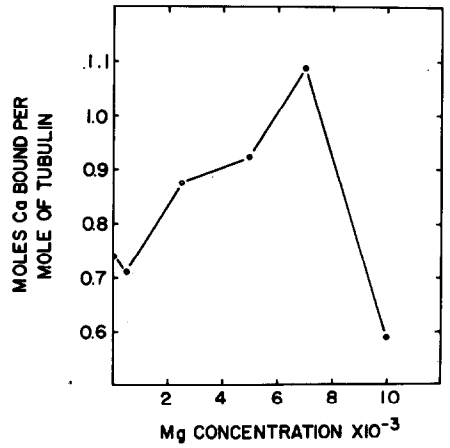

Fig.3. Effect of Mg on binding of Ca to tubulin. Binding was determined by ultrafiltration. The Ca concentration was 0.01 mM and the protein concentration was 0.2 mg/ml. The solution also contained 0.1 M MES buffer and 0.1 mM GTP.

4. Discussion

The data presented here demonstrates that both Ca sensitivity and Ca binding of tubulin is dependent upon the Mg concentration. At 5 mM Mg significant inhibition occurs at about 0.01 mM Ca, a value consistent with that observed by Haga et al. [12], although somewhat higher than that reported by Weisenberg [1]. The actual amount of free Mg is probably considerably lower than that added. At 5 mM Mg about 10% of the total Mg will be bound to GTP (at 0.5 mM) and the MES buffer may bind about 40% of the remaining Mg.

There is disagreement as to the actual concentration of free intracellular Mg and concentrations as low as 0.5 mM and as high as 4 mM Mg [13–17] have been reported. Thus at concentrations of Mg which are probably close to physiological, Ca can inhibit polymerization at concentrations consistent with its possible role in microtubule regulation.

The argument can be made that the inhibition observed by low concentrations of Ca occurs only at suboptimal conditions for polymerization (5 mM Mg concentration rather than 0.5 mM) and is therefore not significant. However, optimal conditions for in vitro polymerization used in many laboratories do not necessarily reflect what occurs in vivo. Only a fraction of the tubulin in the cell probably polymerizes at any given time [18]; therefore, the level of in vitro inhibition seen with low concentrations of Ca may be sufficient to account for in vivo inhibition.

Microtubules do not form in high Ca or Mg concentrations. Temperature shift experiments in the analytical ultracentrifuge reported by Weisenberg [11], indicate that the ring boundary breaks down to subunit or smaller aggregates concurrent with microtubule assembly. The addition of 2 mM Ca prevents the disappearance of the ring boundary. The increased stability of rings may explain the inhibition of microtubule assembly observed at high concentrations of Ca or Mg [19].

Results in our laboratory indicate stoichiometric binding of Ca to tubulin supporting the idea of specific ion-protein interaction. This is contrary to results reported by Staprans et al. [20]. Their assay depends on the differential competition for ^{45}Ca between Chelex-100 and tubulin. However, the stability constant for Ca binding to tubulin is only

about 10^5 which is at least two orders of magnitude less than that of Chelex-100. Tubulin may therefore not effectively compete for binding to the resin.

Olmsted and Borisy [21] have also demonstrated increased inhibition of polymerization by Ca at higher concentrations of Mg. However, Olmstead and Borisy required higher concentrations of Mg and Ca than we have used to obtain significant inhibition of polymerization. This is presumably a result of differences in experimental conditions. The ability of Ca to serve as an intracellular regulator of microtubule assembly appears to depend upon the activity of Mg ions in the cytoplasm and the extent to which other conditions in the cell are optimized for microtubule assembly.

While this work was in preparation a paper has appeared by Hayashi and Matsumura [22] which also reports binding of Ca to tubulin. Our results are consistent with theirs, at 0.5 mM Mg they observed 0.1 mol of bound Ca per mole of tubulin, as compared to our result of 0.37 mol.

Acknowledgements

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