Ca^{2+}- AND CALMODULIN-DEPENDENT FLIP–FLOP MECHANISM IN MICROTUBULE ASSEMBLY–DISASSEMBLY

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1. Introduction

In [1], tau (τ) factor, one species of the non-tubulin accessory proteins, was identified as a calmodulin-binding protein. The binding between two protein species occurred in a Ca^{2+}-dependent fashion. No other proteins from the microtubules were capable of binding calmodulin. Tau factor was discovered [2] as an essential factor for the assembly of the tubulin dimer into the microtubules. Microtubules, purified by assembly–disassembly cycles in vitro, are composed of tubulin and several microtubule-associated proteins. When the tubulin is freed of the microtubule-associated proteins by phosphocellulose chromatography, it can no longer assemble into the microtubules under standard polymerization conditions. Addition of the tau proteins back to the PC-tubulin fully restored its polymerizability [2–6]. In [3] it was concluded that tau is both necessary and sufficient for nucleation and elongation of microtubules from PC-tubulin.

These facts, in combination with our finding that calmodulin associates with tau factor in the presence of Ca^{2+} [1] lead to an attractive hypothesis that calmodulin regulates the microtubule assembly in such a way that, in the presence of Ca^{2+}, it inhibits the assembly or promotes the disassembly by forming a calmodulin · tau complex, thus depriving the tubulin of the factor (tau) which confers upon the tubulin the ability to polymerize. In the absence of Ca^{2+}, tau becomes available for the assembly of the tubulin as it is released from calmodulin. These results provide evidence that validates this hypothesis.

Abbreviations: PC-tubulin, phosphocellulose-purified tubulin; MES, 2-(*N*-morpholino)ethane–sulfonic acid; EGTA, ethyleneglycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid

2. Methods

PC-tubulin and tau factor were prepared from microtubules purified from bovine brain by 3 assembly–disassembly cycles. The detailed procedure for the preparations is described in [1]. Calmodulin was purified from bovine brain as in [7]. The concentrations of calmodulin and protein were determined as in [8]. Polymerization of PC-tubulin into the microtubules was determined at 37°C by the continuous measurement of the turbidity at 350 nm as detailed in [9]. A Shimazu model UV-200A spectrophotometer equipped with a chart recorder and a water-jacketed cuvette chamber was used for this measurement.

3. Results

In a preliminary experiment it was confirmed that the PC-tubulin we prepared was completely unable to assemble, and that addition of tau factor fully restored the capacity of the PC-tubulin to assemble at 37°C as measured by turbidity. The amount of tau factor used was ~30% (w/w) of PC-tubulin, which was adopted from [3]. This assembly-formation of PC-tubulin in the presence of tau factor was inhibited by the addition of calmodulin in a dose-dependent fashion as depicted in fig.1. Each curve reached a plateau in 20–40 min at a different A_{350}-value depending upon the concentration of calmodulin. The inhibitory effect of calmodulin was reversed by the addition of EGTA (curve (e)), indicating that the process is reversible depending upon the concentration of Ca^{2+}. The results also indicate that the interaction between proteins is stoichiometric and not catalytic.

Fig.2 shows the sensitivity of the calmodulin-

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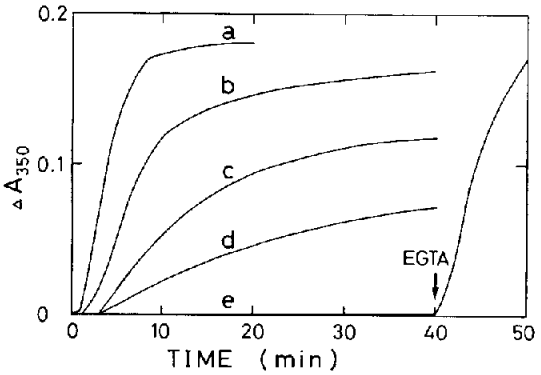

Fig.1. Inhibition of microtubule assembly by the addition of calmodulin. Incubation mixture contained, in a final volume of 1.0 ml, 20 mM MES buffer (pH 6.8), 80 mM KCl, 1 mM $MgCl_2$, 1 mM GTP, 1 mM 2-mercaptoethanol, 1×10^{-5} M free Ca^{2+} (a mixture of EGTA and $CaCl_2$), 1.06 mg PC-tubulin/ml, 308 μg tau factor/ml and various concentrations of calmodulin (see below). The incubation was carried out at 37°C and the tubidity measured at 350 nm was continuously recorded. Calmodulin concentration in μg/ml: (a) 0; (b) 63; (c) 188; (d) 375; (e) 750. At an arrow indicated on curve (e), a calculated amount of EGTA was added to decrease free Ca^{2+} to $\sim 1 \times 10^{-7}$ M. Ca^{2+} concentration of the mixture of EGTA and $CaCl_2$ was calculated according to [10].

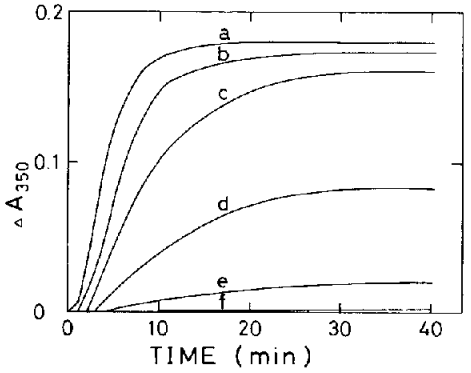

Fig.2. Dependence of the inhibition of the microtubule assembly upon the Ca^{2+} concentration. The assembly was determined as in fig.1, except that a fixed concentration (750 μg/ml) of calmodulin and various concentrations of Ca^{2+} (see below) were added. To each assay tube, 0.1 ml each of mixtures of 10 mM EGTA and appropriate concentrations of $CaCl_2$ were added to produce the desired concentration of free Ca^{2+}: the concentration of free Ca^{2+} was calculated according to [10]. Ca^{2+} concentration: (a) EGTA control (1 mM EGTA); (b) 3×10^{-7} M; (c) 1×10^{-6} M; (d) 3×10^{-6} M; (e) 6×10^{-6} M; (f) 1×10^{-5} M.

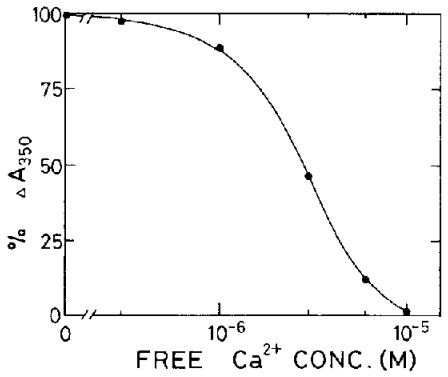

Fig.3. Degree of the assembly of tubulin as a function of Ca^{2+} concentration: 40 min values in fig.2, converted to percentage values taking the EGTA control (curve (a)) as 100%, were replotted against the Ca^{2+} concentration.

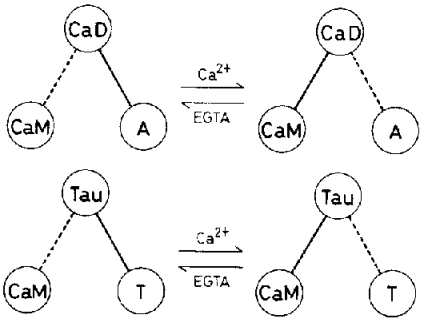

Fig.4. Diagrammatic presentation of the flip–flop mechanism working in the calmodulin–caldesmon–actin system and the calmodulin–tau–tubulin system: CaM, calmodulin; CaD, caldesmon; A, actin; T, tubulin.

dependent inhibition of the assembly to the Ca^{2+} concentration. In this experiment a fixed amount of excess calmodulin, corresponding to curve (e) in fig.1, was used. In fig.3, 40 min values of fig.2 were replotted as a function of the Ca^{2+} concentration: the value for EGTA alone (curve (a) of fig.2) served as 100% value. The concentration range of Ca^{2+} that exerts regulatory effect on the assembly was 10^{-7}–10^{-5} M. A half-maximum inhibition (or activation) was attained at $\sim 3 \times 10^{-6}$ M.

4. Discussion

Since the first successful microtubule assembly in vitro, by chelating Ca^{2+} using EGTA [11], Ca^{2+} has been thought to be a physiological regulator govern-

ing microtubule assembly–disassembly [12,13]. Consequently, a possibility arose that this effect of Ca^{2+} may be mediated by calmodulin, a ubiquitous Ca^{2+}-dependent regulator in both the animal and plant kingdoms. Thus, in [14] a characteristic localization of calmodulin was observed in the chromosome-to-pole region of the mitotic apparatus visualised by immunofluorescence. Calmodulin was subsequently found to both inhibit and reverse microtuble assembly in vitro in the presence of 10^{-6}–10^{-5} M Ca^{2+} [15,16]. Although these results indicate the interaction of calmodulin with the microtubules, the mechanism of the molecular events leading to the 'Ca^{2+}- and calmodulin-dependent' microtubule disassembly has not been elucidated yet.

Based on these results and those in [1], we now present a novel mechanism for the regulation of the microtubule assembly–disassembly. While tau factor is essential for the tubulin to assemble [2–6], Ca^{2+}-dependent association of calmodulin with tau factor prevents the tau from interacting with tubulin thus inhibiting or reversing tubulin assembly. At decreased Ca^{2+} levels, calmodulin–tau interaction ceases, leading to the interaction of tau–tubulin, with the consequent assemby-formation. In this respect, Ca^{2+} acts as a flip–flop switch toward assembly and disassembly depending upon its concentration. Ca^{2+} at 3×10^{-6} M was found for the half-maximum inhibition (or activation) of the tubulin assembly.

We have found a similar flip–flop mechanism for the interaction of calmodulin with another cytoskeletal protein, actin fiber, with a 150 000-M_r protein called caldesmon as a key protein [17,18]. In this case, caldesmon interacts with calmodulin and actin in the presence and absence, respectively, of Ca^{2+}. A comparison of both systems, i.e., calmodulin–caldesmon–actin and calmodulin–tau–tubulin, is summarized in fig.4. It is attractive to hypothesize that such flip–flop mechanism as shown in fig.4 may be extended to a general principle working in the system where calmodulin is implicated in the regulation of the cytoskeleton and contractile systems.

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