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Article

Stimulating Effect of High Concentration of Calcium Ion on the Polymerization of the Tubulin-Colchicine Complex. −Relationship between Magnesium and Calcium−

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The polymerization reaction was reversible by the addition of calcium and/or EGTA and cooling. The extent of inhibition was affected by the pH and magnesium ion concentration. Maximum inhibition of polymerization was observed at a concentration of around 100μM calcium ion. The presence of calcium in a polymerization buffer caused the critical concentration to increase. At above 250μM calcium ion, no inhibition was observed. The inhibitory effect is due to the high-affinity sites of calcium to the tubulin-colchicine complex and the stimulating effect is due to low-affinity sites.

Abbreviations: EGTA, Ethlene-bis (oxyehylenenitrilo) tetraacetic acid; GTP, guanosine 5'-triphosphate.

Key words: tubulin; colchicine; calcium; binding; GTP

Microtubules, which are fundamentally composed of a protein called tubulin[1)], play many roles in cellular processes. The microtubule protein binds to metals[2-7)] as well as drugs[8-12)]. The roles of magnesium and calcium among cations are very important in biological systems. For microtubule assembly, magnesium ion is required, but some amount of calcium ion inhibits the microtubule assembly[13)]. These

facts indicate that divalent cations play different roles in microtubule assembly.

It is considered that the properties of the tubulin-colchicine complex are similar to those of tubulin. For example, both proteins polymerize in the presence of magnesium and GTP[14, 15]; in addition, they have the same sedimentation coefficient[16,17], very similar thermodynamic properties[14,15,16] and a GTPase activity[18, 19].

Calcium has important roles on tubulin. Currently some papers have been published on the interaction between tubulin and calcium[20,21], indicating that so many researchers are interesting in the interaction. We examined the polymerization of the tubulin-colchicine complex and calcium binding to the complex in the state of GTP in a BES buffer[22, 23].

Also we have reported the interactions between the complex in the states of GTP and GDP and calcium in an imidazole buffer[24]. The ultracentrifugal behavior of the complex in a BES buffer was different from that in an imidazole buffer[25].

In the case of tubulin, we should take care of the measurement condition due to the instability of tubulin as well as solvent conditions. Tubulin is a very unstable protein, and the stability is improved by forming a complex with colchicine[26].

In this paper, the effects of calcium ion on the polymerization of the tubulin-colchicine complex are examined in an imidazole buffer in the presence of magnesium ion.

MATERIALS AND METHODS

Chemicals

Colchicine was obtained from Aldrich (Milwaukee, WI, USA). Calcium carbonate, calcium chloride and EGTA were from the Fisher Scientific Co. (Hampton, NH, USA). GTP was from Sigma (St. Louis, MO, USA). All other reagents used were special or reagent grade. Distilled water was further purified through the system of charcoal and ion exchange resin columns, and another distillation was carried out using a glass distilling apparatus. Twice-distilled water was used in every experiment, including the purification of tubulin from calf brain.

Preparation of the Tubulin-Colchicine Complex

The preparation of calf brain tubulin was performed according to the established method described earlier [8, 27, 28].

The tubulin-colchicine complex was prepared by the method of Andreu and Timasheff[26]. The complex formation was confirmed spectroscopically from absorption at 353 nm. The determination of the bound nucleotide was carried out according to the procedure of Seckler and Timasheff using the Beckman

HPLC system. The result indicated that one molecule of the tubulin-colchicine complex contained 1.87 molecules of GTP and a 0.09 molecule of GDP. This means that the tubulin-colchicine complex used in this experiment was in the GTP state.

Polymerization and Microtubule Assembly

A polymerization reaction of the tubulin-colchicine complex was performed in a 10 mM imidazole-0.1 mM GTP buffer, pH 7.0 or 6.5, in the presence of magnesium ion without glycerol. The measurement of polymerization was carried out turbidimetrically, as described by Andreu *et al*[14].

RESULTS

Polymerization of the Tubulin-Colchicine Complex

This section describes the effects of magnesium ion concentration. Fig. 1 shows typical polymerization profiles of the tubulin-colchicine complex in a 10mM imidazole, 0.1 mM GTP buffer, pH 7.0, at different concentrations of magnesium in the absence and presence of calcium ion. The tubulin-colchicine complex was able to polymerize in an imidazole buffer. Furthermore, an increase in turbidity was reversible by cooling. The polymerization was inhibited by small amounts of calcium.

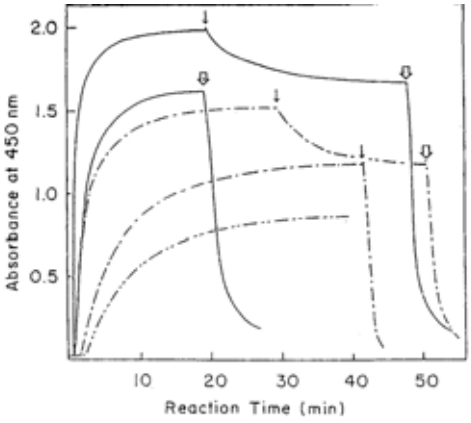

Fig.1. Polymerization profiles of the tubulin-colchicine complex in 10mM imidazole-0.1mM GTP buffer, pH7.0, containing 6, 8, or 12 mM $MgCl_2$. 100 μM calcium ion was added after reaching plateau of polymerization or before heating a reaction mixture. Protein concentration was 1.50 mg/ml. Arrow ↓ indicates addition of 100 μM calcium ion and arrow⇒ does cooling of 10℃.

-·-·- 6mM $MgCl_2$: — · — 8mM $MgCl_2$: ——12mM $MgCl_2$

Turbidity obtained in the presence of 100μM calcium was the same as that of the plateau turbidity after addition of 100μM calcium to maximum polymerization.

Effect of the Magnesium Ion Concentration on the Polymerization

The effect of the magnesium ion concentration on the polymerization was examined at the protein concentrations of 1.50 and 1.20 mg/ml at pH 7.0 and 6.5, respectively, in the absence and presence of 100μM calcium ion. The results are shown in Fig. 2. The plateau turbidity of polymerization is dependent on the magnesium ion

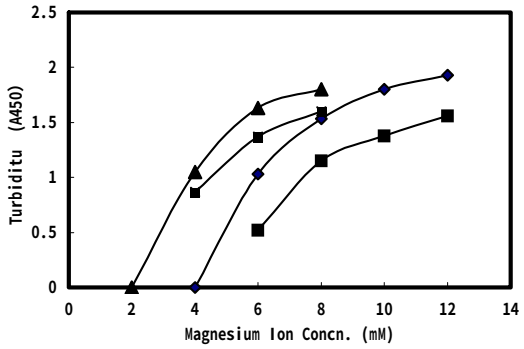

Fig.2. Effect of Magenesium Ion Concentration on the Polymerization of the Tubulin-Colchicine Complex in 10 mM Imidazole-0.1 mM GTP Buffer, in the Absence and Presence of 100 μM Calcium Ion at the Protein Concentration of 1.50 mg/ml (pH7.0) or 1.20 mg/ml (pH6.5).

▲ no calcium at pH7.0; ■ presence of 100 μM calcium ion at pH7.0; ◆ no calcium at pH6.5; ■ presence of 100 μM calcium ion at pH6.5.

concentration. The polymerization of the tubulin-colchicine complex was not observed in a 10mM imidazole, 0.1 mM GTP buffer, pH 7.0, containing 4 mM $MgCl_2$. As the magnesium ion concentration became higher, more polymerization was observed. Although polymerization did not occur at 4 mM $MgCl_2$ at pH 7.0, a reduction of pH from 7.0 to 6.5 introduced polymerization at 4 mM $MgCl_2$. Gaskin *et al.*[29] observed, using porcine tubulin, that a greater extent of microtubule assembly occurred at pH 6.5 than at 7.0.

The presence of 100μM calcium ion decreased the turbidity of polymerization from 1.551 to 1.164 at a protein concentration of 1.50 mg/ml at pH 7.0 in the presence of 8 mM $MgCl_2$ (Fig. 2). In addition, the inhibition by calcium ion was observed at pH 6.5. At a low concentration of magnesium, calcium ion inhibited polymerization much more than at a high concentration. The extent of inhibition was affected by the pH and magnesium ion concentration. For example, the turbidity of polymerization at 6 mM $MgCl_2$ was reduced from 1.617 to 1.426 by 100 μM Ca^{2+} at pH 6.5 (11.8% of inhibition), while it was reduced from 0.855 to 0.515 at pH 7.0 (39.8% of inhibition).

Table I. Critical Concentrations for the Tubulin-Colchicine Complex in 10 mM Imidazole Buffer Containing 0.1 mM GTP in the Absence and Presence of 100μM Calcium Ion.

pH	$MgCl_2$ Concn. (mM)	Ca^{2+} Concn. (μ M)	Critical Concn. (mg/Mℓ)
7	8	0	0.608
		100	0.792
6.5	4	0	0.432
		100	0.577

A critical concentration of the tubulin-colchicine complex in a 10mM imidazole, 0.1mM GTP, 8 mM $MgCl_2$ buffer, pH 7.0, was determined in the absence and presence of 100μM calcium ion. Table Ⅰ indicates the critical concentration for the polymerization of the tubulin-colchicine complex at 8 and 4 mM $MgCl_2$ at pH 7.0 and 6.5, respectively. The substitution of imidazole for

phosphate resulted in a lower critical concentration in microtubule assembly.[30] The addition of 100μM calcium ion led to a higher critical concentration from 0.608 to 0.792 mg/ml. A trial measurement of the critical concentration was performed in the absence and presence of calcium ion. The existence of a critical concentration for the tubulin-colchicine complex polymer was confirmed.

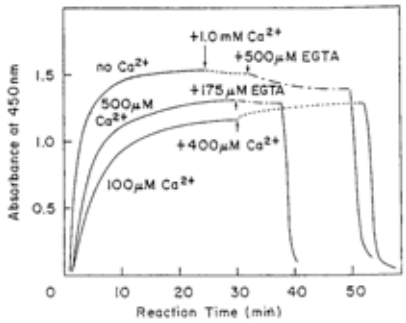

Fig.4. Time Course of Polymerization of the Tubulin- Colchicine Complex in 10 mM Imidazole-0.1 mM GTP Buffer, pH 7.0, Containing 8 mM $MgCl_2$ and Efects of the Addition of EGTA and Calcium Ion during Polymerization.

Effect of Calcium Ion Concentration on the Polymerization

Many reports show that many divalent cations induce microtubule assembly.[3, 5-7] Both magnesium and calcium are divalent cations. It is very interesting to clarify the differences between magnesium and calcium from the cell biological point of view.

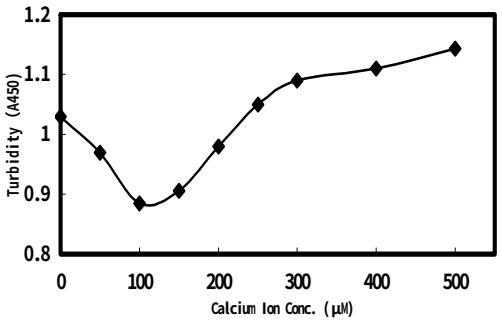

Fig. 3. Effect of Calcium Ion Concentration on the Polymerization of the Tubulin-Colchicine Complex in 10 mM Imidazole-0.1mM GTP Buffer, pH 6.5, Containing 4 mM $MgCl_2$.

We tried to examine the effects of low and high concentrations of calcium on polymerization because there have been different reports on the calcium levels required for inhibition.

The effects of calcium and magnesium on polymerization are very complicated, and the inhibition of polymerization by calcium is limited at a low concentration in the case of pH 7.0[24].

Furthermore, a similar effect was confirmed at pH 6.5 (Fig. 3). The experiment was carried out at pH 6.5 at a protein concentration of 1.20 mg/ml in the presence of 4 mM $MgCl_2$. When a small amount of calcium was used, the inhibition effect on polymerization could be observed; however, at above 250 μM Ca^{2+}, no inhibition was observed. Although 100μM calcium inhibited polymerization by 13.7%, turbidity with 500 μM calcium increased

12.0% compared with that without calcium ion. It is very interesting that a small amount of calcium inhibits polymerization and that a large amount of calcium stimulates the reaction.

In order to confirm these findings, experiments using EGTA were carried out in 10mM imidazole, 0.1mM GTP buffer, pH 7.0, containing 8 mM $MgCl_2$ (Fig. 4). As shown in Fig. 4, further addition of 400μM calcium to the reaction system containing 100μM calcium introduced an increase in turbidity. The addition of enough EGTA caused the polymerization to recover to the extent of that without calcium, while a small amount of EGTA did not remove the effect of calcium. Though the addition of 1 mM calcium ion did not affect turbidity, a further addition of 500 μM EGTA led to a decrease in turbidity. These results indicate that the effect of calcium on the polymerization reaction is reversible.

DISCUSSION

This paper discusses the stimulating effect of calcium ion on the polymerization of the tubulin-colchicine complex in the presence of magnesium ion. Tubulin exists in the form of microtubules, which is formed by the polymerization. It is well-known that many metal ions affect microtubule assembly. There are many metal ions in a living cell. For example, magnesium ion exists around 0.5 mM and calcium ion does at the range between 10^{-7} and 10^{-5} M. In this paper, the concentration of each metal ion was high compared with that in a living cell. These concentrations used in this paper are the same as those used in the *in vitro* experiments by many others.

There are many papers on the interaction between tubulin and magnesium.[31-34] On the other hand, the effect of calcium has recently reported as described above.[20, 21] Also we reported the calcium binding.[22-24] so, we are interesting in the relationship between magnesium and calcium on the polymerization of tubulin.

It was Weisenberg who discovered the inhibition effect of calcium ion in an *in vitro* microtubule assembly at low concentration[13]. The inhibition effect of calcium on the microtubule formation from tubulin was observed at the range of micromolar order by Weisenberg[13]. In the case of the so-called cycle tubulin prepared by the polymerization and depolymerization method, the calcium sensitivity was lower than that of pure tubulin[35], and the microtubules formed by tubulin in the presence of 1 mM $CaCl_2$ had different properties from those formed in 1 mM EGTA[36]. This difference is assumed to come from the protein preparation. In other words, the effect of calcium on tubulin was weakened by the presence of microtubule-associated proteins.

The effects of magnesium and calcium on microtubule protein containing microtubule-associated proteins has been reported in some papers[31, 35, 37-39]. Though calcium ion is assumed to inhibit microtubule assembly of tubulin in two ways[35, 40], we investigated the intrinsic effect of calcium on the tubulin-colchicine complex rather than on tubulin. Microtubule assembly *in vivo* is assumed to be partially regulated by magnesium and calcium. Considering the concentration of metal in living cells, magnesium is the major divalent action. Since microtubule assembly is usually done in the presence of magnesium, the relationship between magnesium and calcium was investigated in the polymerization of the tubulin-colchicine complex.

The tubulin-colchicine complex can polymerize in the presence of magnesium at pH 7.0 (Fig. 1), and the polymerization was inhibited by 100μM calcium ion. The polymerization of the tubulin-colchicine complex has been reported independently by Saltarelli and Pantaloni[15] and Andreu *et al*[14]. Both reports indicate the *in vivo* polymerization under similar conditions of microtubule assembly from pure tubulin. Their buffers were MES (4-morpholineethane-sulfonate) and phosphate buffers. In addition, the requirement of magnesium and GTP was reported, and no glycerol was required.

The dependence of polymerization on magnesium ion was examined (Fig. 2). Polymerization measured by turbidimetry was observed at pH 7.0 at above 5 mM $MgCl_2$, and turbidity was in proportion to the concentration of magnesium, while only 4 mM $MgCl_2$ induced polymerization at pH 6.5. These facts indicate that the polymerization of the tubulin-colchicine complex is dependent on pH, as shown in the case of microtubule assembly from porcine tubulin by Gaskin *et al*[29]. The presence of 100μM calcium ion inhibited polymerization at both pH 7.0 and 6.5. The extent of inhibition by calcium at pH 7.0 was larger than that at pH 6.5 (Fig. 2).

In order to clarify this discrepancy, detailed experiments of the effect of calcium ion were carried out (Fig. 4). First, for polymerization, we used a 10mM imidazole buffer, pH 7.0, containing 0.1 mM GTP and 8 mM $MgCl_2$. The presence of 100μM calcium ion induced minimum polymerization under the condition used, and we could not observe perfect inhibition by calcium at any concentration. At more than 100μM calcium ion, the polymerization increased in proportion to the concentration of calcium, and no inhibition was then observed in the presence of 1 mM $CaCl_2$ turbidimetrically. However, the increase of magnesium concentration from 8 to 12 mM spread the concentration range of calcium,

which gave minimum turbidity[24] as reported previously. These results mean that the affinity of calcium is affected by the buffer conditions, including magnesium. Some researchers[36,41] reported that magnesium, as well as calcium, inhibited the microtubule assembly. The effect of calcium ion concentration on the polymerization was investigated under the condition of 4 mM $MgCl_2$ at pH 6.5 (Fig. 3). Higher turbidity was obtained at above 250μM calcium ion. As shown above, polymerization of the tubulin-colchicine complex was considerably affected by the magnesium ion concentration and pH in the absence of calcium, and the effect of magnesium on polymerization was affected by the pH value. Those results suggested that the binding affinity of magnesium to the tubulin-colchicine complex at pH 6.5 is greater than that at pH 7.0. The experimental results concerning the effect of calcium indicate that calcium ion inhibits and stimulates polymerization (Fig. 3). The result of Fig. 3 is confirmed by the result of Fig. 4. In our experiment, the presence of calcium in a polymerization buffer caused the critical concentration to increase, while Saltareli and Pantaloni[15] have reported that the critical concentration decreased in the presence of calcium ion and that calcium ion could replace magnesium ion. Andreu *et al* [14]. showed the partial inhibition of the polymerization by calcium ion at the concentration of 10^{-4} M, when the magnesium ion concentration was 1.6×10^{-2} M. The maximum inhibitory effect of calcium was always observed at a concentration of around100μM calcium ion, and the inducing effect was readily affected by the magnesium ion concentration and pH. This means that the inhibitory effect is due to the high-affinity sites of calcium to the tubulin-colchicine complex and the inducing effect is due to low-affinity sites.

The calcium binding to the tubulin-colchicine complex should be examined in an imidazole buffer containing different concentrations of magnesium ion. The results will be reported in a near future.

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