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THE EFFECT OF POLYAMINES ON TUBULIN ASSEMBLY

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The assembly of cold solubilized microtubules prepared from calf brain and the polymerization of tubulin purified from this material are facilitated by polyamines at physiological concentrations. The number of free amino groups in the polyamine determines the ability of the polyamines to promote microtubule formation. Spermine with four amino groups was the most effective polyamine tested. Spermidine and N'-acetylspermine with 3 amino groups were less effective than spermine but more effective than N^8-acetylspermidine and putrescine which contain two free amino groups. Microtubule formation may therefore be controlled by alterations in the nature and amounts of polyamines present.

Polyamines are polycationic compounds that are an abundant constituent of dividing cells, although their mechanism of action remains unknown (1). Microtubules are ubiquitous constituents of eukaryotic cells that are involved, as a component of the cytoskeleton, in properties of cells involving motility including cell division (2). There are conflicting reports in the literature concerning the possible interaction of polyamines and microtubules. It has been reported that polyamines induce the disassembly of reconstituted microtubules *in vitro* (3). However, a large number of studies have indicated that polycations, including some naturally occuring polyamines, promote the assembly of purified tubulin (4). We have therefore examined the effects of a variety of polyamines found in mammalian cells on the assembly of microtubules purified from calf brain.

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Materials and methods

Microtubules were prepared from calf brain through two cycles of polymerization and microtubule associated proteins (MAPs) and tubulin were purified from this material by phosphocellulose chromatography (5). Polyamines, 2-(N-Morpholino) ethane sulphonic acid (MES), ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), and guanosine-5'-triphosphate (GTP) were obtained from Sigma Chemical Co.

Microtubule assembly was followed by monitoring turbidity at 350 nm (6) in a Perkin Elmer 124 Spectrophotometer maintained at 37° using a water jacketed cell holder. All assembly experiments were carried out in 0.1M MES buffer, pH 6.4, 1 mM EGTA, 0.5 mM $MgCl_2$ and 30% v/v glycerol with other additions as indicated in individual experiments. Purified proteins were stored at -20° in 0.1M MES, pH 6.4, 30% v/v glycerol. After centrifugation at 100,000 x g for 1 hr at 0°, microtubule and tubulin preparations were diluted to the appropriate concentration and EGTA to 1 mM and $MgCl_2$ to 0.5 mM were added and the preparations were incubated at 37° from 5 minutes before the addition of other substances from concentrated stock solutions. Protein was determined by dye binding or by using a modified Lowry procedure with bovine serum albumin as standard (6). Electron microscopy was carried out on samples fixed with glutaraldehyde and negatively stained with uranyl acetate (7).

Results

Fig. 1 shows the effects of the presence of polyamines on the polymerization of cold solubilized microtubules as determined by measuring the increase in turbidity at 350 nm. All polyamines tested increased both the rate and extent of polymerization of cold solubilized microtubules. The effectiveness of the polyamines in promoting polymerization was directly related to the number of free amino groups in the polyamines. Spermine with four positively charged amino groups at pH 6.4 was the most effective and the effect of other polyamines decreased with decreasing positive charge. The critical concentration for tubulin polymerization in the presence of polyamines was examined by varying the concentration of solubilized microtubules present at a fixed polyamine concentration and measuring the maximum absorbance at 350 nm obtained. At a concentration of 1 mM, spermidine and N'-acetyl spermine reduced the critical concentration for polymerization from 0.6 mg/ml to 0.4 mg/ml. At this concentration N^8-acetylspermidine and putrescine reduced the critical concentration for polymerization to 0.5 mg/ml. Spermine at a concentration of

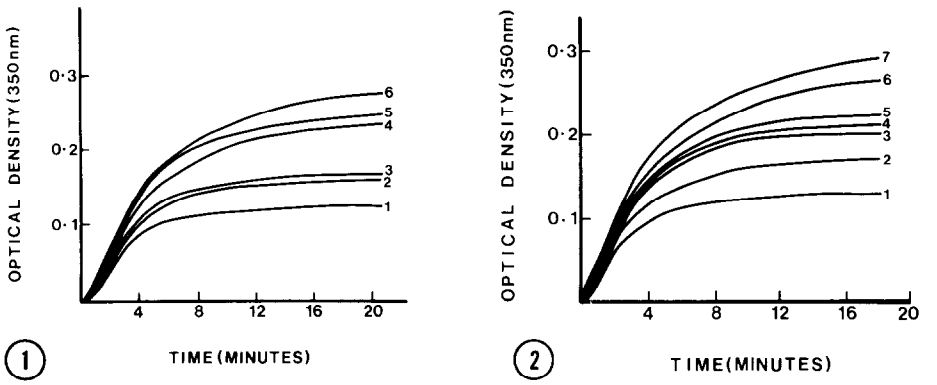

Figure 1. Cold solubilized microtubules (1.6 mg/ml) were preincubated at 37° in assembly buffer for 5 minutes. Polymerization was initiated by the addition of GTP to 1 mM final concentration and polyamines to final concentrations as indicated: (1) no polyamine (2) 1 mM putrescine (3) 1 mM N-acetylspermidine (4) 1 mM N-acetylspermine (5) 1 mM spermidine (6) 0.25 mM spermine.

Figure 2. Cold solubilized microtubules (1.6 mg/ml) were preincubated at 37° in assembly buffer for 5 minutes. Polymerization was initiated by the addition of GTP to a final concentration of 1 mM and polyamines to the concentrations indicated: (1) no polyamine (2) 1 mM putrescine (3) 2 mM putrescine (4) 4 mM putrescine (5) 0.5 mM spermidine (6) 1 mM spermidine (7) 2 mM spermidine.

0.25 mM reduced the critical concentration for polymerization to 0.3 mg/ml.

Figure 2 shows the effect on polymerization of increasing polyamine concentration for a fixed concentration of cold solubilized microtubules. The ability of a given polyamine to promote polymerization appears to be limited. Secondary effects were observed when spermidine or N'-acetyl spermine concentrations greater than 2 mM or spermine concentrations greater than 0.25 mM were used. Increased turbidity in the absence of GTP and the formation of a precipitate which settled out of solution over time were observed. At concentrations below these the polyamines had no effect on turbidity in the absence of GTP when mixtures were incubated at 37°.

Figure 3 shows the effects of polyamines on the polymerization of tubulin which had been freed of microtubule associated proteins (MAPs) by passage through phosphocellulose (PC tubulin). In the absence of

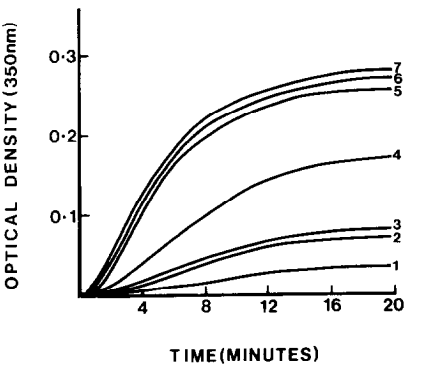

Figure 3. Tubulin (1.1 mg/ml) prepared by phosphocellulose chromatography was preincubated at 37° for 5 minutes. Polymerization was initiated by the addition of GTP to 1 mM and MAPs or polyamines as indicated: (1) GTP only (2) 1 mM putrescine (3) 1 mM N-acetylspermidine (4) MAPs (0.2 mg/ml) (5) 1 mM N-acetylspermine) (6) 1 mM spermidine (7) 0.25 mM spermine.

added MAPs, there was very little increase in turbidity of this material upon incubation at 37° after the addition of GTP. However, the addition of MAPs or polyamines to the GTP-containing mixtures caused an increase in the rate and extent of polymerization as measured by observing the increase of turbidity at 350 nm. As with the promotion of polymerization of cold solublized microtubules, the relative ability of polyamines to promote the polymerization of PC tubulin was directly related to the number of positively charged groups in the polyamine. Spermine was again the most effective.

In order to verify that the increases in turbidity observed were due to polymerization, mixtures were examined by electron microscopy. After negative staining, structures that were indistinguishable from microtubules formed from cold solublized microtubules or from PC tubulin to which MAPs had been added were obtained when incubations of mixtures containing GTP and polyamines were examined. Figure 4 shows a comparison of the structures obtained when PC tubulin was polymerized in the presence of spermidine or MAPs.

Further evidence that the presence of polyamines promoted the assembly of microtubules was obtained by carrying out incubations in

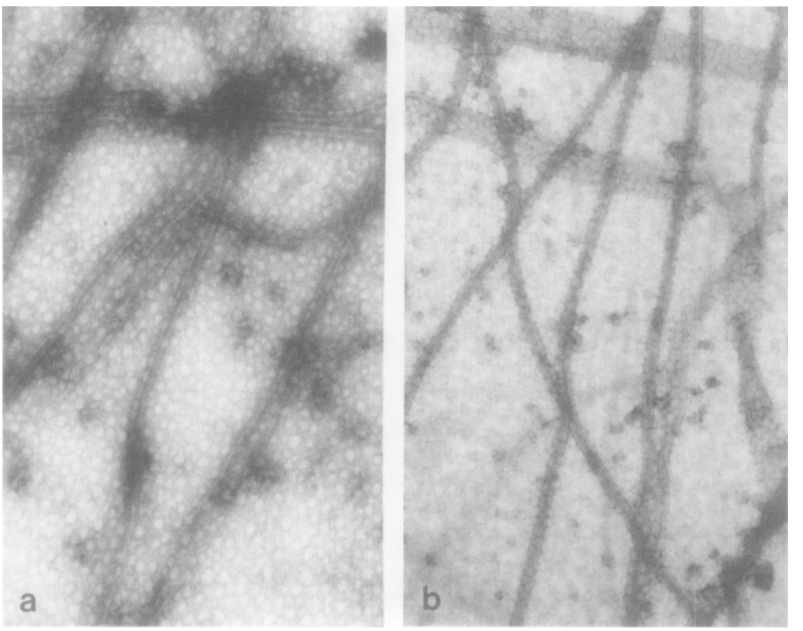

Figure 4. Electron micrographs (60,000 x) of the structures obtained when PC tubulin (2mg/ml) was polymerized in the presence of 0.4 mg/ml MAPs (4A) or 1mM spermidine (4B).

the presence of two inhibitors of microtubule formation. Figure 5 shows that the presence of Ca^{++} or of colchicine inhibits the increase in optical density observed upon the addition of spermidine to GTP-

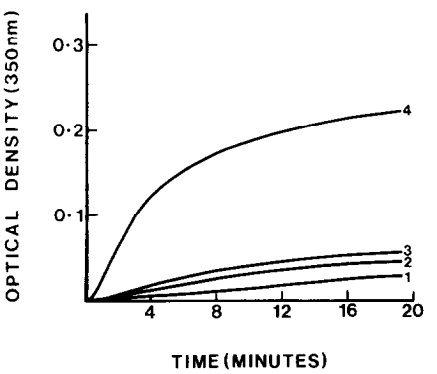

Figure 5. Tubulin (1.6 mg/ml) prepared by phosphocellulose chromatography was preincubated for 5 minutes. In sample (2) $CaCl_2$ was present at a concentration of 2 mM and in sample (3) colchicine was present at a concentration of 2 μg/ml. Polymerization was initiated by the addition of GTP to a final concentration of 1 mM and spermidine to 1 mM final concentration as indicated: (1) GTP only; (2), (3), (4) GTP and spermidine.

solutions of MAP free tubulin. Optical density increases in the presence of other polyamines were similarly inhibited.

To determine the extent of polymerization and the degree of reversibility, PC tubulin was polymerized in the presence of MAPs or 0.5 mM spermidine. The protein content was measured following 60 minutes incubation at 37° before and after pelleting by centrifugation at 100,000 x g for 1 hr at 37°. The pellets were dissolved by homogenization in cold 0.1M MES, pH 6.4 and the protein content of the supernatants after centrifugation at 100,000 x g for 1 hr at 0° was determined. From Table 1 it can be seen that under the conditions used a very similar extent of reversible polymerization was obtained in the presence of MAPs or spermidine.

Preliminary experiments with 1,4-^{14}C spermidine indicated that polyamine binding to tubulin was reversible. ^{14}C-spermidine pelleted with microtubules could be dialyzed away from tubulin after cold solubilization of the pellets.

Discussion

The polyamines are naturally occuring polycations that have been shown to increase in amount in proliferating cells (1). The controlled assembly and disassembly of tubulin into microtubules

Table 1

Sample	% of protein in 37° pellet	% of pellet cold soluble
PC tubulin	4	-
PC tubulin + spermidine	74	60
PC tubulin + MAPs	79	49

Reversible polymerization of tubulin. Phosphocellulose purified tubulin (2.4 mg/ml) was allowed to polymerize at 37° for 1 hr in the presence of 1 mM GTP. Spermidine (1 mM) or MAPs (0.4 mg/ml) were added at the start of incubations as indicated. Protein was determined before and after centrifugation at 100,000 x g for 1 hr at 37° and before and after centrifugation at 100,000 x g for 1 hr at 0° of the resulting pellet solubilized at 0° in 0.1M MES buffer, pH 6.4.

appears to be an essential aspect of cell division (2). In the present study it has been shown that at concentrations approximating levels found in mammalian liver and brain (9), the polyamines promote assembly of cold solublized microtubule preparations from calf brain and of tubulin prepared from this material free of assembly promoting proteins. This finding is in contrast to a report that putrescine, spermidine and spermine induce the *in vitro* disassembly of microtubules (3). However, it supports observations by many workers that polycations promote tubulin assembly (4). In the present study it has been found that the ability of polyamines to promote tubulin assembly is enhanced by the increasing numbers of amino groups in the polyamines. Thus the normal metabolic pathway of conversion of putrescine to spermidine and subsequently spermine (1) would be expected to promote microtubule assembly *in vivo*. Modification of polyamines by N-acetylation, a normal catabolic pathway for polyamines (10), would be expected to moderate these effects. This could account for *in vivo* observations that microtubule formation is dependent on polyamines in polyamine-auxotrophic CHO cells (11) and that both polyamines (12) and microtubules (13) increase in stimulated lymphocytes.

Acknowledgement

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