Molecules in mammalian brain that interact with the colchicine site on tubulin

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ABSTRACT  Colchicine, a plant alkaloid, is a potent inhibitor of mitosis and other physiological processes that involve microtubules. These effects are mediated by the specific binding of colchicine to a high-affinity receptor site on tubulin, the major protein of microtubules. It seemed possible that the colchicine site on tubulin might also be the receptor for endogenous cellular molecules. We now report that mammalian brain, in fact, contain a class of molecules that interact with the colchicine site on tubulin. Tubulin-agarose affinity chromatography has been used to isolate factors from soluble extracts of bovine brain that interact with tubulin. Certain of these factors inhibit the binding of \(^{3}H\)colchicine to tubulin. Using the inhibition of colchicine binding as an assay, I have partially resolved at least two species. One is a protein and another may be a heat-stable peptide. In parallel to the action of colchicine, this smaller species inhibits the assembly of isolated microtubules. This class of molecules may include the endogenous ligands for which the colchicine site on tubulin is the receptor. They may have a significant role in the regulation of cellular microtubule function and assembly.

Tubulin, the major protein subunit of microtubules, has specific, high-affinity binding sites for a number of drugs, notably colchicine and the Vinca alkaloids, vinblastine and vincristine (1, 2). Such drugs block mitosis (3) and depolymerize cytoplasmic microtubules with consequent dramatic effects on cell shape and development, axoplasmic transport, and secretion (2, 4, 5). They are also potent inhibitors of microtubule assembly in vitro (6). Several of these "spindle poisons" are chemotherapeutic for leukemia and other cancers.

Colchicine, an alkaloid derived from the plant _Colchicum autumnale_, has been known for centuries as a treatment for gout. There is an extensive early literature concerning the effects of colchicine on cell growth, mitosis, and the induction of polyplody (7). The introduction of radioactive colchicine (8) led to the isolation of "colchicine-binding protein," the soluble cellular receptor for the drug (9). This protein was subsequently identified as tubulin (10, 11). The binding of colchicine to tubulin is slow, but highly specific and essentially irreversible (12). One mole of colchicine is bound per 120,000-dalton tubulin dimer (1). The native configuration of tubulin is required for the integrity of the colchicine-binding site since activity is lost upon treatment with denaturing agents (13). Although the colchicine site is accessible on the tubulin dimer, it is concealed in the intact microtubule. Hence, the site may lie in the region of tubulin–tubulin interactions (11).

Although the interaction of tubulin with colchicine has been studied for many years, the physiological significance of the colchicine site has hitherto been considered. It seemed unlikely that a protein found in mammalian cells should have evolved specific sites to interact with drugs from plants. Rather, we speculated that animal cells might contain endogenous compounds for which the colchicine site is the physiological receptor. We were encouraged in this idea by the recent discovery of endorphins, the endogenous ligands of the morphine receptors in brain (14, 15).

I now report the identification in mammalian brain of a class of cellular molecules that interact with the colchicine site on tubulin and suggest that these species are the physiological ligands of the colchicine site on tubulin. They may be of importance in the regulation of cellular microtubule assembly and function.

MATERIALS AND METHODS

Microtubules, Tubulin, and Tubulin-agarose. Microtubules were isolated from calf brains by cycles of polymerization as described (16). About 85% of the protein is \(\alpha\) and \(\beta\) tubulin, with 15% as various microtubule-associated proteins. Tubulin was isolated from twice-polymerized microtubules by phosphocellulose chromatography (17). Microtubules were depolymerized for 20 min at 4°C in cold buffer A [50 mM 4-morpholinoethanesulfonic acid (Mes), pH 6.8/0.5 mM MgCl\(_2\)/1 mM dithiothreitol]. The sample was centrifuged at 14,000 rpm for 20 min (Sorvall SS-34 rotor) and slowly applied to a phosphocellulose column (Whatman P11) previously equilibrated with buffer A. Protein was applied at 3 mg/ml of bed volume. Homogeneous tubulin was eluted with buffer A at a flow rate of 3 column volumes per hr. The eluate was brought to 1 mM GTP, and 0.5 mM MgCl\(_2\) was added to restore magnesium removed by the phosphocellulose. Tubulin was quick-frozen in liquid nitrogen at 7–15 mg/ml and stored in aliquots at −70°C.

Tubulin (with GTP omitted) was coupled covalently to agarose with Bio-Rad Affigel 10, an N-hydroxysuccinimide ester of succinylated aminoalgalgy agarose. Freshly prepared tubulin (150 mg in 5 ml of buffer A) was gently agitated for 2 hr at 4°C with 5 ml of Affigel in the same buffer. In some cases, 20% glycerol (vol/vol) was added as a stabilant. The slurry was poured into a column and washed with 0.1 M Tris-HCl, pH 7.0/0.5 mM MgCl\(_2\)/1 mM dithiothreitol, then with buffer A containing 20 mM KCl until the A\(_{260}\) returned to baseline. Usually 7–9 mg of tubulin was coupled per ml of gel.

Microtubule-Depleted Brain Extracts. Fresh calf brain was homogenized and subjected to high-speed centrifugation at 4°C followed by a single cycle of polymerization and centrifugation at 30°C. The supernatant was diluted 1:1 with buffer A containing 40 mM KCl. Approximately 500 mg of protein was applied to the tubulin–agarose column. The column was washed with 6 vol of 20 mM KCl in buffer A, then eluted successively with 0.15 M KCl and 0.5 M KCl in buffer A. Elution was

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Abbreviations: Mes, 4-morpholinoethanesulfonic acid; buffer A, 50 mM Mes, pH 6.8/0.5 mM MgCl\(_2\)/1 mM dithiothreitol.

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monitored by absorbance at 250 nm, and protein was determined by the Lowry method.

Colchicine-Binding Assay. The binding of \(^{3}H\)colchicine to tubulin was determined by the adsorption of the tubulin-colchicine complex to DEAE-impregnated filters (18). Reaction mixtures contained 0.1 M Mes (pH 6.8), 1.0 mM MgCl₂, 1 mM GTP, 0.12 mg of tubulin per ml, 0.2 \(\mu\)M \(^{3}H\)colchicine (New England Nuclear, 2 Ci/mmol; 1 Ci = 3.70 \(\times\) \(10^{10}\) Bq), and other additions as indicated. In some cases, the specificity of binding was confirmed through isolation of the \(^{3}H\)colchicine–tubulin complex by gel filtration on Bio-Gel P-6 (20). After incubation for 30 min at 30°C, the reaction mixture was chilled to 4°C and the tubulin–\(^{3}H\)colchicine complex was separated from free colchicine on a 1 \(\times\) 20 cm column of P-6 equilibrated with the reaction buffer.

RESULTS

Isolation of Tubulin-Binding Factors by Affinity Chromatography. The colchicine site is exposed on the tubulin subunit but hidden in the microtubule (11). Hence, with the exception of tubulin itself, proteins or other molecules that bind to this site might well be excluded from assembled microtubules. Consequently, I removed microtubules from a soluble brain extract by a cycle of assembly and centrifugation. The microtubule-depleted supernatant was adsorbed to a tubulin-agarose affinity column in order to identify tubulin-binding factors (Fig. 1). Tubulin covalently bound to agarose retains many properties of native tubulin. These include colchicine and GTP binding and self-association with free tubulin (19). The column also binds microtubule-associated proteins such as tubulin assembly protein (16). A class of tubulin-binding factors that was retained on the column could be eluted with a KCl step gradient (Fig. 1). Polyacrylamide gel electrophoresis under denaturing conditions indicated that some 15–20 proteins were present in the 0.5 M KCl eluate. These proteins represent a class of factors that bind to tubulin, but most likely are not present in the intact microtubule.

Identification of Endogenous Colchicine Competitors in Brain. Among the tubulin-binding factors isolated by tubulin-agarose chromatography are species that inhibit the binding of \(^{3}H\)colchicine to tubulin. To demonstrate this, aliquots of the tubulin-binding factors eluted with 0.5 M KCl were preincubated with tubulin for 15 min at 30°C and the incubation was continued for 1 hr at 37°C in the presence of \(^{3}H\)colchicine. Material from the affinity column strongly inhibited subsequent colchicine binding (Fig. 2). At sufficiently high

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Isolation of tubulin-binding factors by affinity chromatography on tubulin-agarose.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Tubulin-binding factors inhibit the interaction of \(^{3}H\) colchicine with tubulin. The ability of material absorbed to tubulin-agarose to prevent the binding of \(^{3}H\)colchicine to pure tubulin is shown as a function of factor concentration. One hundred percent binding is 6.3 nmol for 1.8 mg of tubulin. ○, Tubulin assembly protein (50 \(\mu\)g/ml) was added as indicated. ●, Tubulin-binding factors.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Effect of tubulin-binding factors on formation of a tubulin-colchicine complex. Reaction mixtures contained tubulin and colchicine except as indicated. (A) ○, No addition; ●, 30 \(\mu\)l of tubulin-binding factors; ×, 100 \(\mu\)l of tubulin-binding factors, tubulin omitted. (B) ○, Preincubation for 30 min at 30°C prior to addition of 30 \(\mu\)l of tubulin binding factors; ●, 30 \(\mu\)l of tubulin-binding factors, KCl (0.5 M) was added at the onset of reaction.
chicine by chromatography on Bio-Gel P-6 (Fig. 3A). Preincubation with tubulin-binding factors eluted from the affinity column strongly inhibited (>70%) the subsequent formation of a tubulin-[3H]colchicine complex (Fig. 3A). The tubulin-binding factors did not bind colchicine themselves (Fig. 3A). Also, if tubulin was preincubated with colchicine, subsequent addition of the factor preparation did not reduce tubulin-colchicine complex formation (Fig. 3B). This is reasonable in view of the irreversible nature of colchicine binding. In contrast to the colchicine-binding reaction itself, which is relatively independent of ionic strength, inhibition of binding by the colchicine competitors was abolished by 0.5 M KCl (Fig. 3B).

Possible nonspecific effects on colchicine binding—especially proteolysis—were excluded by forming a tubulin–colchicine complex and then incubating it for various times with the tubulin-binding factors. The rate of release of bound colchicine was not enhanced by this treatment (not shown). In separate experiments, HeLa cell tubulin, metabolically labeled with [35S]methionine and isolated by the method of Speigelman et al. (21), was incubated with the colchicine competitors and analyzed by fluorography after sodium dodecyl sulfate/polyacrylamide gel electrophoresis. No obvious proteolytic fragments were detected (not shown).

**Table 1. Properties of colchicine competitors**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peptide binding, relative %</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>RNase A</td>
<td>105</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>DNase I</td>
<td>102</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>94</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>65</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

One hundred percent binding is 6.8 nmol. Peaks 1 and 2 refer to the Bio-Gel P10 column (Fig. 4). Treatments were as follows: RNase A, 10 μg/ml; DNase I, 15 μg/ml; phospholipase C, 10 μg/ml; trypsin, 8 μg/ml. All treatments were for 10 min at 37°C. Trypsin was inactivated by addition of an 8-fold excess of soybean trypsin inhibitor. Treated or untreated factors (40 μl) were assayed in the colchicine-binding reaction by the DEAE-filter method.

**Table 2. Inhibition of microtubule assembly by a colchicine antagonist from brain**

<table>
<thead>
<tr>
<th>Colchicine antagonist</th>
<th>Microtubule assembly, % inhibition of assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(peak 1), μl 20 min at 37°C</td>
</tr>
<tr>
<td>No addition</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.24</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
</tr>
<tr>
<td>10</td>
<td>0.08</td>
</tr>
<tr>
<td>15</td>
<td>0.06</td>
</tr>
<tr>
<td>20</td>
<td>0.04</td>
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The effect of peak 1 from the P10 gel filtration column on microtubule assembly was assayed by light scattering. Reaction mixtures (0.4 ml) contained buffer A and 0.4 mg of phosphocellulose-purified tubulin. Concentrated fractions from the P10 column were added as indicated. After preincubation for 5 min at 37°C, polymerization was initiated by addition of 2 mM GTP and 50 μl of phosphocellulose-purified accessory proteins (17). Microtubule assembly was monitored turbidometrically by recording the increase in light scattering at 350 nm in a Guilford recording spectrophotometer equipped with a thermostated cuvette chamber.

**Partial Purification of Endogenous Colchicine Competitors.** I have used the inhibition of [3H]colchicine binding to tubulin as an assay to monitor the further purification of the endogenous colchicine analogs from the class of tubulin-binding factors.

Gel filtration on Bio-Gel P10 resolved two major peaks of activity (Fig. 4). One activity eluted with a molecular weight in excess of 15,000 (peak 2). The other activity was partially included (peak 1). Its elution position is consistent with a molecular weight of less than 5000. Both species were insensitive to ribonuclease, deoxyribonuclease, and phospholipase C (Table 1). The trypsin sensitivity of the larger species indicates that it is a protein or proteins. The smaller species proved to be heat stable (90°C, 5 min), but was only partially trypsin sensitive. It may be a peptide or peptides.

**Inhibition of Microtubule Assembly by Endogenous Colchicine Competitors.** The most striking effect of colchicine binding to tubulin is the inhibition of microtubule assembly. Therefore, I asked whether endogenous tubulin-binding factors...
that interact with the colchicine site might also block assembly. The results indicate that the smaller molecular weight factor is, indeed, an inhibitor of assembly (Table 2). In the presence of sufficient amounts of the factor, added before polymerization began, there was over 80% inhibition of assembly, as determined turbidometrically. Hence, this molecule may be a naturally occurring negative modulator of microtubule assembly which acts at the colchicine site on tubulin.

**DISCUSSION**

Colchicine, a plant alkaloid, has been recognized since the 1930s as a potent spindle poison and mitotic inhibitor (7). The drug also affects a wide range of other cellular processes, including motility, morphogenic movements, axonal transport, and certain secretory events (2). These physiological effects are a consequence of the inhibition by colchicine of cytoplasmic microtubule assembly. Such inhibition results from the specific interaction of colchicine with tubulin, the main structural protein of microtubules. This is the only known site of colchicine action in the cell (1). The binding occurs at a single high-affinity site ($k = 2-2.5 \times 10^7$ mol/liter) on each 65 tubulin subunit (22).

Despite the extensive literature on colchicine and its effects on microtubules, the possible physiological significance of the colchicine-binding site on tubulin has not been considered.

In this report, I demonstrate that mammalian brain tissue contains endogenous molecules that interact with the colchicine site on tubulin. Our approach was to isolate a class of tubulin-binding factors from soluble, microtubule-depleted extracts of bovine brain by affinity chromatography on columns of tubulin covalently linked to agarose. Tubulin-agarose affinity chromatography is widely applicable for the identification and isolation of proteins or other cellular molecules that interact with tubulin. For example, we have used affinity chromatography for the isolation of tubulin assembly proteins ("tau" factors) (16) and for purification of the high molecular weight microtubule-associated proteins (unpublished data). Among the species retained by tubulin-agarose, I detected an activity that prevents the binding of $[^3$H]colchicine to tubulin. Using the inhibition of colchicine binding as an assay, I have resolved two major colchicine competitors by gel filtration. One, with a molecular weight in excess of 15,000, is a protein or proteins, as shown by trypsin sensitivity; the other has a molecular weight less than 5000, is heat stable, and may be a peptide. Our preliminary studies suggest that the higher molecular weight fraction contains at least two proteins.

Colchicine binds to tubulin in a reaction that is slow, temperature-dependent, and essentially irreversible (1). Thermodynamic parameters and the lack of an ionic strength effect suggest that colchicine binds in a hydrophobic pocket on tubulin (1, 22). In contrast, the endogenous colchicine competitors are eluted from tubulin-sepharose by increasing the ionic strength, and they do not inhibit colchicine binding in the presence of 0.5 M KCl. These observations suggest that their binding to tubulin is reversible and, most likely, ionic. Reversible interaction with the colchicine site is not surprising since at least two other drugs that bind at this site, Colcemid and podophyllotoxin, do so reversibly. More unusual is that the binding of the colchicine competitors to tubulin is not hydrophobic. The most straightforward explanation is that these molecules, which are considerably larger than colchicine, do have a hydrophobic region with a stereochemistry analogous to colchicine, but that the major binding forces occur with adjacent regions of tubulin and are hydrophilic in nature. Other explanations are that the colchicine site is concealed but not occupied by these factors or that the factors bind tubulin at a remote site and inhibit colchicine binding allosterically. Although direct occupation of the colchicine site is most appealing, we cannot at present exclude other mechanisms.

The most dramatic consequence of the interaction of colchicine with tubulin is the inhibition of microtubule assembly (6). Inhibition occurs stoichiometrically. Complete blockade of polymerization takes place when as few as 1 in 250 tubulin subunits have bound colchicine (6, 20). In the cell, dissolution of the mitotic spindle occurs when only 3-5% of the tubulin is complexed with colchicine (8). We have found that at least one of the endogenous colchicine competitors in brain, the small putative peptide, is an inhibitor of microtubule assembly in vitro. Thus, the colchicine site may be a locus for physiological inhibition of microtubule polymerization in the cell itself.

The discovery in animal cells of molecules that interact with the colchicine site on tubulin is reminiscent of the identification of peptides and proteins in mammalian brain that seem to be the physiologically important ligands of the morphine receptor (14, 15). By analogy with "endorphins," we postulate a physiological role for the colchicine site on tubulin and for the cellular molecules with an affinity for that site.

What is the biological function of endogenous colchicine competitors? At least one of them may act like colchicine. That is, it may bind to tubulin subunits to form a complex that subsequently caps the growing end of a microtubule to prevent further assembly (20). With such a mode of action, very low intracellular concentrations would be effective in disrupting a microtubule network. Conversely, it could be the removal of a colchicine competitor from a soluble pool of inactive tubulin that initiates polymerization. More speculatively, a number of long-standing complexities about the cytoskeleton might be explained by the presence of colchicine analogs in cells. These include the rapid disassembly of cytoplasmic microtubules just prior to mitosis and the rapid disassembly of the spindle just afterwards, the presence in cells of different stability classes of microtubules (23) together with substantial amounts of unpolymerized tubulin above the critical concentration, and the unusual observation that colchicine rapidly depolymerizes cytoplasmic microtubules in vitro even though it does not depolymerize preformed microtubules in vitro. Based on the observation that colchicine treatment of cells induces formation of cilia and basal bodies, we might even postulate a role for the endogenous colchicine analogs in shunting pools of tubulin between different supramolecular structures (24).

The identification of endogenous molecules in mammalian brain that interact with the colchicine site on tubulin suggests a new cellular pathway for the control of microtubule assembly and raises the possibility that other microtubule-active drugs, such as the anti-lymphocytic Vinca alkaloids, may also have cellular analogs.

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