Inhibition by p-bromophenacyl bromide of microtubule assembly in vitro

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Introduction

Microtubules (MTs) play a central role in a variety of cellular phenomena including cell division, cell morphology and intracellular transport [1]. Pharmacological studies of MT function and assembly involve the use of a range of MT poisons. Such agents can disrupt the control of MT dynamics either by stabilizing, e.g. taxol [2, 3], or destabilizing e.g. MTs [4–7], usually through direct interactions with either tubulin [3–6] or microtubule-associated proteins (MAPs) [7].

Colchicine and some of its derivatives, podophyllotoxin and the benzimidazole compounds prevent MT assembly by binding directly to tubulin [1, 4–7]. Colchicine is the most studied MT poison and was instrumental in the early identification of tubulin as the major protein subunit of MT [8]. It is a tropolene derivative with a single binding site on the β-tubulin subunit. Podophyllotoxin interacts with the same binding site [9], unlike the Vinca alkaloids, which bind to another region of tubulin [10]. Concentrations of colchicine lower than 10⁻⁵M can cause mitotic arrest by blocking spindle formation. The disassembly by colchicine of interphase MT in cultured cells results in the collapse of vimentin filaments to form perinuclear aggregates [11].

Although colchicine arrests the division of cancer cells very effectively, its applications in cancer therapy are extremely limited due to its high level of toxicity in man [12, 13]; instead, podophyllotoxin and the Vinca alkaloids are the preferred anti-cancer agents [14]. However, colchicine has been used successfully in the treatment of inflammatory reactions associated with gout and rheumatism [15–17] suggesting that MTs represent a potential target for the development of new anti-inflammatory agents.

This study involves the characterization of the molecular events that underlie the anti-inflammatory action of the phospholipase A₂ inhibitor p-bromophenacyl bromide (pBPAB). Although previous work suggested that the pharmacological action of this drug was primarily due to its ability to inhibit phospholipase A₂ [18–20], recent studies have shown that it also has diverse effects at the cellular level [21–23] and may inhibit phospholipase C [24]. Furthermore, the fact that stimulation of Fcγ 2b receptor-mediated phagocytosis by pBPAB involved changes in cytoskeletal organization [23] raised the possibility that this agent might interact directly with a cytoskeletal protein(s). The aim of the present work was to determine whether this effect could reflect a direct interaction with the MT system.

Materials and methods

Preparation of MT protein

Porcine brain MTs were purified through two cycles of temperature-dependent disassembly–reassembly by the method of Shelanski et al. [24], except that the reassembly buffer (pH 6.9) consisted of 100 mM-Pipes, 4M-glycerol, 1 mM-MgSO₄, 1 mM-EGTA and 0.5 mM-phenylmethanesulphonyl fluoride (PMSF). Centrifuged two-cycle-purified MT pellets were stored at −70°C until required. Before turbidity experiments (see below), or further purification steps, they were resuspended by glass-Teflon homogenization in Mes buffer [10] which consisted of 100 mM-Pipes, 4 mM-glycerol, 1 mM-MgSO₄, 1 mM-EGTA and 0.5 mM-phenylmethanesulphonyl fluoride (PMSF). Centrifuged two-cycle-purified MT pellets were stored at −70°C until required. Before turbidity experiments (see below), or further purification steps, they were resuspended by glass–Teflon homogenization in Mes buffer [100 mM-2-(N-morpholino)ethanesulphonic acid, 1 mM-MgSO₄, 1 mM-EGTA, pH 6.8] and incubated for 30 min on ice. Cold-stable aggregates were then removed by centrifugation at 100000 gₛ for 20 min at 4°C, leaving the depolymerized MT proteins in the supernatant.

When necessary, tubulin free of MAPs was obtained by phosphocellulose chromatography [25]. Briefly, depolymerized two-cycle-purified MT proteins (see above) were applied to a phosphocellulose column (Whatman P11) pre-equilibrated with Mes buffer. Pure tubulin was eluted in the void volume whereas MAPs bound to the resin. The purity of MAP-free tubulin was demonstrated by SDS/PAGE [26].

Measurement of MT assembly

MT polymerization was monitored by the change in absorbance at 350 nm [27] after the addition of 1...
mm-GTP (Sigma, type IIS). In a typical experiment, MTs were polymerized for 30 min at 37°C in the presence and absence of various amounts of pBPAB or its structural analogues p-chlorophenacyl chloride and acetophenone. The calcium sensitivity of the polymerized MT protein was then assessed by incubation for 5 min in the presence of 10 mM-CaCl₂. Since polymerized MTs are normally disassembled under these conditions [28, 29] any drug-induced change in the response to Ca²⁺ would be indicative of an altered structure of the polymerized MTs.

Further confirmation of the extent of microtubule assembly was obtained by SDS/PAGE analysis of polymerized material pelleted by centrifugation at 100000×g, for 20 min at 35°C in a Beckman TLA 100 bench-top ultracentrifuge. The structural integrity of MTs polymerized in the presence or absence of drug was assessed by electron microscopy after direct negative staining with 1% (w/v) aqueous uranyl acetate. Protein content of samples was estimated by the assay of Lowry et al. [30] using RSA as a standard.

Results
As shown in the turbidimetric study in Fig. 1, pBPAB inhibited MT assembly in a concentration-dependent manner. Polymerization was reduced to less than 50% of control values in the presence of 10 μg of drug/ml and the effect reached a maximum of 90% inhibition at approximately 100 μg/ml.

To assess the biochemical integrity of MTs polymerized in the presence of pBPAB their change in absorbance was recorded after 5 min incubation with 10 mM-Ca²⁺. In agreement with other studies [28, 31] there was a sharp decrease in the turbidity of control MTs (Fig. 2). However, MT proteins incubated under polymerizing conditions with pBPAB (≥ 5 μg/ml) exhibited a significant increase in absorbance in the presence of Ca²⁺ (Fig. 2). Neither the decrease in MT polymerization nor the altered Ca²⁺ sensitivity was observed in the presence of the two structural analogues of pBPAB.

To determine whether pBPAB interacts directly with tubulin, turbidity experiments were performed with phosphocellulose-purified tubulin. Table 1 shows that in the absence of Ca²⁺ there was no significant polymerization in either control or drug-treated samples, reflecting the absence of MAPs which are required as co-factors for MT formation [25, 29, 32]. However, while the addition of Ca²⁺ after 30 min incubation in polymerization buffer induced only a slight rise in absorbance of control tubulin, it caused a 10-fold increase in the turbidity of tubulin incubated with drug. Such an increase in turbidity could be caused by a number of factors including the formation of open MT sheets such as those induced by Zn²⁺ [33]. This possibility was investigated by performing a parallel experiment substituting Zn²⁺ for Ca²⁺. In this case there was no significant difference in the high turbidity values obtained in the presence or absence of drug.

![Fig. 1](image_url)

Effects of pBPAB on MT assembly

Polymerization of MT proteins (3–5 mg/ml) in the presence or absence of various amounts of pBPAB, was measured as the change in absorbance at 350 nm after 30 min incubation at 37°C with 1 mM-GTP. Results are expressed as a percentage of control values (no added drug) for each experiment (n = 4 to 7). All treated groups were significantly different from control values by Student’s 't' test for paired data (P < 0.05).

![Absorbance ratio (Ca²⁺/no Ca²⁺)](image_url)

Absorbance ratio (Ca²⁺/no Ca²⁺) in absorbance was recorded after 5 min incubation with 10 mM-Ca²⁺. In agreement with other studies [28, 31] there was a sharp decrease in the turbidity of control MTs (Fig. 2). However, MT proteins incubated under polymerizing conditions with pBPAB (≥ 5 μg/ml) exhibited a significant increase in absorbance in the presence of Ca²⁺ (Fig. 2). Neither the decrease in MT polymerization nor the altered Ca²⁺ sensitivity was observed in the presence of the two structural analogues of pBPAB.

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![Fig. 2](image_url)

Effects of pBPAB on turbidity of pre-formed MT treated with Ca²⁺

MTs were polymerized in the presence or absence of various amounts of pBPAB. Pre-formed MTs were then incubated for 5 min at 37°C in the presence of 10 mM-CaCl₂. The effects of calcium are expressed as the ratio of the change in absorbance obtained after incubation with cation to the change in absorbance without cation (n = 4). There was no significant difference between the Ca²⁺ effects at 1 μg of pBPAB/ml and control. However, all other values in the presence of drug were significantly different from controls (P < 0.02) by the Mann-Whitney 'U' test.
The effects of pBPAB and divalent cations on phosphocellulose purified tubulin

Tubulin (2–3 mg/ml), purified by phosphocellulose chromatography, was incubated with 1 mM-GTP for 30 min at 37°C in the presence or absence of 36 μM-pBPAB. The change in absorbance was then recorded after a further 5 min incubation with 10 mM-CaCl₂ or 10 mM-ZnCl₂. Data (mean ± s.d., n = 7) are presented as the change in absorbance from the value at time zero. There was a significant increase in turbidity between samples incubated with both Ca²⁺ and pBPAB compared with controls (Ca²⁺). Whereas Zn²⁺ induced the same response as pBPAB, the most abundant structures being tentatively identified as aggregates of tubulin oligomers (results not shown).

<table>
<thead>
<tr>
<th>Added cation</th>
<th>Control</th>
<th>pBPAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.026 ± 0.007</td>
<td>0.026 ± 0.004</td>
</tr>
<tr>
<td>10 mM-Ca²⁺</td>
<td>0.074 ± 0.012</td>
<td>0.250 ± 0.040</td>
</tr>
<tr>
<td>10 mM-Zn²⁺</td>
<td>0.865 ± 0.032</td>
<td>0.732 ± 0.060</td>
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We conclude that pBPAB is not only an inhibitor of phospholipase activity but also an effective MT poison. It may be that MT disruption plays an important role in the anti-inflammatory action of this agent. Work is underway to examine these possibilities in cultured cells.

We are grateful to F. H. Mullins for providing some of the MT proteins used in this study, and to Ms S. Oliphant for typing the manuscript. This work was supported by the Wellcome Trust (U.K.).

Introduction

The marked changes seen in the organization of the neuronal cytoskeleton are characteristic of mammalian nerve regeneration [1]. The molecular basis of these events is poorly understood but is known to correlate with alterations in the expression, synthesis and axonal transport of tubulin and other cytoskeletal proteins [2-10]. Tubulin, the structural sub-unit of microtubules, is of particular interest in studies of nerve regeneration, since microtubules are important in the maintenance of neuronal integrity, through their involvement in axonal transport, growth-cone advance and polarity [11, 12].

Tubulin has been shown to be axonally transported in two distinct groups of slowly transported proteins, slow component 'a' and slow component 'b' (SCa and SCb) [13, 14]; SCb is enriched in cold-labile tubulin, while SCa contains essentially stable forms [15]. Following axonal injury there is an increase in the proportion of tubulin carried in SCb [2, 6], indicating a shift in tubulin stability. While this may be partly attributable to the increases in expression and synthesis of specific isoforms of tubulin [e.g. α1 and β (class II)] that follow axonal injury [9, 10], these phenomena may not be the only factors contributing to the shift in stability of the pre-existing neuronal microtubule network.

Abbreviations used: SCa, slow component a; SCb, slow component b; PMSF, phenylmethanesulphonyl fluoride; TCA, trichloroacetic acid; TBS, Tris-buffered saline.

Tubulin: tyrosine ligase activity in regenerating peripheral nerve
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Our recent work has concentrated on the role of post-translational modifications of tubulin, which provide mechanisms for dynamically altering the biochemical properties of tubulin and microtubules in cytoplasmic areas remote from the cell body. There are three main post-translational modifications of tubulin: phosphorylation of β-tubulin by casein kinase II [16-18], tyrosination/detyrosination of α-tubulin by tubulin-tyrosine ligase and tubulin-tyrosine carboxypeptidase [19-22] and acetylation of the ε-amino group of the lysine-40 residue of α-tubulin by tubulin acetylase [23]. There is evidence that post-translational processing of tubulin is associated with the level of microtubule stability. For example, detyrosinated and acetylated tubulins have been found to be co-localized with sub-populations of stable microtubules [24], whereas tyrosinated tubulin is associated with more dynamic populations [25].

Post-translational modification of tubulin is developmentally regulated and altered during neurite extension in cultured cells. Phosphorylation of β-tubulin occurs during neurite extension in cultured neuronal cells, possibly promoting microtubule assembly and stabilization [16, 18, 26, 27]; a comparable phosphorylation pattern has been found during development [28]. Acetylation of α-tubulin increases during axonal maturation [30, 31] corresponding to the formation of stable microtubule populations. The tyrosination of α-tubulin is found to increase at the onset of the formation of...