Biophysical Properties of Stable Microtubules in Neurites Revealed by Optical Techniques

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ABSTRACT. We tested the stability of microtubules in the neurites of cultured dorsal root ganglion cells by dissolving the cytoplasmic membrane with detergent and exposing them to defined extracellular medium under the microscope. Smooth cytoplasmic filaments visualized after membrane removal were suggested to be microtubules by the preservation of all of the filaments in the presence but not in the absence of taxol. They were further confirmed to be microtubules by immunostaining with anti-tubulin antibody. Significant number of microtubules in the established neurites remained longer than 1 hour after membrane removal. To investigate their stabilization mechanism, we transected the exposed microtubules by laser microbeam irradiation and observed their length changes with video-enhanced microscopy. Microtubule fragments started to shorten on both sides of the transection site, more rapidly from the newly generated plus ends than from the minus ends. The maximal rate as well as the pattern of shortening correlated with the time of transection; microtubules transected later than 30 min after membrane removal shortened at rates less than 20 µm/min and typically with intermittent pauses, while the more labile microtubules included in the earlier transections shortened continuously at higher rates. Microtubules in neurites were thus stabilized by (1) stopping disassembly at local sites including the plus ends, and (2) slowing disassembly along the length. Observations of the course of disassembly also suggested the presence of specialized points along microtubules which is involved in anchoring microtubules to the substratum or transiently stopping disassembly.

Key words: microtubule/microtubule stabilization/neurite/cultured neuron/laser transection/video-enhanced microscopy

Introduction

Microtubules (MTs) are major elements of the axonal cytoskeleton which provide structural support and serve as tracks for axonal transport. Compared with cytoplasmic MTs in dividing cells, MTs in the axon are shown morphologically to be highly stabilized. During outgrowth and elongation of neurites, MTs not only increase in quantity but also become progressively more stabilized, as demonstrated by their resistance against destabilizing agents (2, 3), slower turnover rates (8, 11), or higher levels of posttranslational modifications specific to tubulin in polymer (1, 3, 5). Biochemical analyses also revealed that a large proportion of axonal tubulin was insoluble under various MT-depolymerizing conditions such as low temperature and presence of Ca²⁺ (16, 24). In dorsal root ganglion (DRG) neurons in culture, the absolute amount of tubulin per cell increases during neurite outgrowth and elongation. At the same time, a dramatic increase in the proportion of insoluble tubulin is also observed, indicating that MT assembly and stabilization take place during this process (19).

To further investigate the nature of such insoluble tubulin and the mechanism of MT stabilization in the neurite, we developed a new method of directly observing and testing the stability of cellular MTs by the use of video-enhanced differential interference contrast (DIC) microscopy combined with a flow cell technique (25). When DRG neurons set in the flow cell were perfused with a detergent-containing buffer, cytoplasmic membrane was removed and long, smooth filaments were exposed which were identified as MTs. Many of the exposed filaments disappeared within a few minutes, but some persisted for more than 1 hour. Those MTs were

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further transected by laser microbeam irradiation at various time points after membrane removal to explore the mechanism of stabilization (10). Stable MTs were induced to disassemble from the site of transection at rates which correlated with their persistence time after membrane removal. Presence of specialized points along MTs was suggested where MTs were anchored to the substratum or transiently protected from disassembly.

Materials and Methods

Cell culture

DRG neurons prepared from 2-week-old Wister rats were plated onto coverslips coated with poly-lysine and cultured in serum-free medium for 6–7 days (19, 25).

Observation of neuritic MTs with video-enhanced DIC microscopy

DRG neurons were mounted into a flow cell (chamber volume of approximately 150 μl) (25), and were observed with a DIC microscope, equipped with a Plan Apochromat 63 × 1.4 NA oil-immersion objective lens and 4 × TV relay lens, and a 100-W halogen lamp. Images were detected with a Newvicon camera, enhanced with an image processor and recorded with a S-VHS video cassette recorder.

Under video-enhanced DIC observation, cell membrane was removed by perfusing buffer A kept at 37°C (60 mM Pipes, 25 mM Hepes, pH 6.9, 2 mM MgCl₂, 5 mM EGTA, 0.2% Triton X-100, 10% glycerol, 0.2 M NaCl, 0.25 mM PMSF and 200 μg/mL leupeptin). In some instances, taxol was included in buffer A at a final concentration of 10 μM.

Immunofluorescent staining

The structures remaining 30 min after membrane removal was fixed by perfusing the flow cell with 1% glutaraldehyde, and then processed with anti-β-tubulin mAb (DM1B) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig in the same flow cell for fluorescent microscopy.

Laser transection of neuritic MTs

Laser transection of neuritic MTs was performed using the second harmonic 532-nm picosecond pulses from a mode-locked Nd:YAG laser. The laser beam was introduced through the epifluorescence port, and focused on the sample plane through an objective. The spot of irradiation was fixed at the center of the field of view. The effective spot size of the focused laser beam was less than 1 μm in diameter. The minimum laser power needed to transect MTs within a second was determined to be about 10 mW. With MTs exposed from the neurites, transection was complete by ~1/8 s of laser irradiation. Similar irradiation focused on buffer near MT (~2 μm away) had no effect.

At various time intervals after membrane removal, a single MT to be transected was selected and its polarity and distance from the cell body were checked. The behavior of MTs after transection was followed under DIC microscopy. Usually, only one of the two newly generated MT ends was followed since they were no longer kept in a single focal plane and difficult to observe simultaneously. Routinely, transected MTs were followed either for the shortening distance of at least 5 μm or the duration of at least 30 s after transection.

Dilution experiment of MTs reassembled in vitro from purified tubulin and tau

The purified tubulin and tau were prepared from bovine brain as previously described (12, 13, 20, 22). Flagellar axoneme fragments were prepared from sea urchin sperm (4).

For experiments on in vitro reassembled MTs, a smaller volume flow cell (chamber volume of approximately 25 μl) was constructed. The axoneme suspension, diluted with BRBB80 buffer (80 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, pH 6.9), was introduced into a flow cell and incubated to secure attachment to the coverslip surface. After washing with BRBB80 buffer, the mixture of purified tubulin (10 μM) and tau (3 μM) in BRBB80 containing 1 mM GTP was introduced into the flow cell and incubated at 37°C until the MTs grew to more than 100 μm in length (~1 h). The MTs were then diluted by perfusing buffer A and the disassembly was followed with video-enhanced DIC microscopy.

Determination of MT-shortening rates

In transection and dilution experiments, MT length was measured at intervals of 1–5 s using a fixed reference point in the observation field and plotted as a function of time. The accuracy of the length measurement was estimated to be <1 μm. The rate of shortening was calculated from the maximum slope on the plot determined by the least squares regression analysis. In transection experiments, shortening behavior within the first 2 μm from the laser irradiation point was excluded from analysis because of possible direct damage caused by irradiation. Thus, shortening rates were estimated to be zero when changes in MT length during observation was less than 2 μm.

Results and Discussion

Real-time observation of established neurites by video-enhanced DIC microscopy during and after membrane removal

In Figure 1A, the most proximal region of a major thick neurite at 6 days after plating is shown intact. By
Fig. 1. Membrane removal and exposure of cytoplasmic filaments from the neurites of 6-day-old cultures by perfusion with detergent-containing buffer. The flow cell chamber containing DRG neurons were perfused with buffer A, with (D–F) or without (A–C) 10 μM taxol. (A); the most proximal region of an intact neurite adjacent to the cell body. (B–C); same region of the neurite at the end of buffer flow (B; 2 min after the start of perfusion at 21:52:00), and after 32 min (C) at 37°C in buffer without taxol. Another neurite is shown intact (D), immediately after membrane removal in the presence of taxol (E; 2 min after the start of perfusion at 15:49:30), and after 30 min (F) at 37°C in the same buffer containing taxol. These exposed cytoplasmic filaments were confirmed to be MTs by specific immunostaining with anti-tubulin antibody (G; fluorescence image, H; DIC image). Bar, 5 μm.
perfusing the flow cell chamber with buffer A kept at 37°C, cytoplasmic membrane was dissolved and many smooth filaments became clearly visible as shown in Figure 1B immediately after the end of the flow (total duration of flow was 2 min). The bundled filaments splayed out with time, and gradually disappeared up to 30 min. A significant proportion of filaments, however, remained even after 30 min of incubation in the extracellular medium (Fig. 1C).

When similar observation was made with taxol (10 μM) present in the perfusion buffer (Fig. 1D–F), there was no apparent loss of filaments during incubation for 30 min (Fig. 1F) and longer (up to 2 h; data not shown). This suggested that most of these filaments were MTs and that the loss of filaments in the absence of taxol was due to MT-disassembly.

In contrast to neuritic filaments, similar filaments from Schwann cell processes completely disappeared within 1 min of membrane removal (data not shown).

To confirm the identity of the stable filaments with MTs, filaments remaining 30 min after membrane removal were fixed and carried through immunostaining procedures with anti-β-tubulin antibody in the same flow cell. Direct comparisons between fluorescent and DIC images of the immunostained material clearly indicate that the filaments remaining 30 min after membrane removal were immunostained (Fig. 1G, H). The immuno-positive filaments were smooth and straight in the DIC image, and could be distinguished from other types of filaments such as thick filaments coated with granules or wavy filaments which were immuno-negative. We thus concluded that smooth, straight filaments observed by DIC microscopy after membrane removal were MTs.

**Laser transection of cytoplasmic MTs exposed from the neurites**

We transected the exposed cytoplasmic MTs by laser microbeam irradiation to see whether they were stabilized mainly by securing their ends or by protection along their lengths. A transection site was chosen on a single MT, and its orientation and distance from the cell body were recorded. In the case shown in Figure 2, transection site (+ mark in Fig. 2A) was 43 μm from the cell body which was located at the lower left corner of the observation field. In most cases, transected MT fragments sprang straight and started to shorten on both sides of the transection site. Since MTs in these

![Fig. 2. Laser transection of cytoplasmic MTs exposed from the neurites of DRG neurons. (A): At 30 min after membrane removal, one of the exposed MTs was transected by laser irradiation at the site marked with (+) which was located 43 μm from the cell body. (B–F): The newly generated end of the proximal MT fragment (plus end; arrowhead) was followed and shown at (B) 1 sec, (C) 9 sec, (D) 20 sec, (E) 35 sec, and (F) 40 sec after laser transection. Bar, 5 μm.](image-url)
neurites are uniformly oriented with their plus ends distal to the cell body, the new free end of the proximal MT fragment generated by transection is the plus end, while that of the distal MT fragment is the minus end. In the case shown in Figure 2, the new plus end (arrow head) was observed to shorten at a rate of 11 μm/min.

By following and comparing the shortening of MTs after transection, three different patterns were noted as shown in Figure 3; (1) continuous shortening at a constant rate, (2) stepwise shortening with intermittent pauses, and (3) no shortening including less than 2 μm of shortening during observation. To evaluate the rates of MT shortening independent of the shortening pattern, we determined the shortening rate from the maximal slope on the plot of MT length vs. the time after transection as shown in Figure 3.

The plus-end shortening rates (n=75) showed a clear correlation with the transection time after membrane removal (Fig. 4). Among earlier transections, the rates were distributed over a wide range. With time after membrane removal, MTs exhibiting shortening rates over 20 μm/min were progressively decreased and were no longer observed later than 30 min. Thus MTs persisting longer in the extracellular medium consisted only of those exhibiting slower shortening rates after transection, indicating that they were quantitatively more stabilized. In addition to such stable MTs, MTs which would spontaneously depolymerize within 30 min were also included among earlier transections and were responsible for the occurrence of high shortening rates. Compared with the plus ends, minus-end shortening rates (n=29) were distributed over the narrower range. They also showed a tendency to decline with time after membrane removal.

We compared the shortening patterns between the two groups of transections as suggested from the above results; transections performed within 30 min after membrane removal (<30 min) or more than 30 min after membrane removal (>30 min). Relative proportions of the three shortening patterns are shown for both groups in Figure 5. For plus ends, proportions of pattern 1 and pattern (2+3) were significantly different between the two groups (χ² test for independence: P<0.02). Similar tendency was observed with minus ends. Thus, highly-stabilized MTs persisting more than 30 min were characterized by the low shortening rate and the occurrence of pauses during shortening (pattern (2+3)), while the labile MTs which were included only in the earlier transections exhibited continuous shortening at high rates (>20 μm/min).

Fig. 4. Correlation between the maximal shortening rate and the time of MT transection after membrane removal. Maximal shortening rates determined as shown in Figure 3 for plus ends (filled circles; n=75) and minus ends (open circles; n=29) are plotted against the time of transection.
For the spontaneous shortening phase of plus ends exhibiting dynamic instability intracellularly (6, 17, 18, 21, 23), shortening rates in the range of 5–33 μm/min have been obtained. The mean plus-end shortening rates of transected neuritic MTs determined in the present study for patterns 1 and 2 in both early (≤30 min) and late (>30 min after membrane removal) transections are all within this range of values. Since the plus-end shortening rate of even the most stable neuritic MTs (>30 min group with pattern 2 behavior; 9.6 μm/min) was comparable to that of the relatively stable MTs in other cells, it is difficult to account for their unusual stability in the extracellular medium by the shortening behavior alone. Protection at the ends of MTs may contribute significantly to the formation of highly-stabilized MTs in neurites. Shortening rate of

the labile neuritic MTs (≤30 min group with pattern 1 behavior; 25.6 μm/min), on the other hand, was comparable to the rate of the most dynamic intracellular MTs and the in vitro shortening rates of reassembled MTs (26, 27), indicating that they were not significantly stabilized along their length.

To further investigate the characteristics of the highly-stabilized MTs, longer-term observations were made on the plus ends of MTs transected later than 30 min after membrane removal. Seven examples of the life history of individual proximal MT fragments after transection are shown in Figure 6. The results clearly show that none of the MTs were absolutely stable. All of them shortened after transection albeit with longer or shorter intermittent pauses. Pattern 3 defined previously is actually a variation of pattern 2 with a longer pause. Though most of the pauses observed in the 7 examples were less than 1 min in duration, three incidences of pauses longer than 1 min were noted (*) mark in Fig. 6) which would have been classified into pattern 3 under short-term observation.

Since the most stable MTs started to disassemble from the transection site, they were not fully stabilized along the length and were originally protected from disassembly at their ends.

**Dilution-induced disassembly of in vitro reconstituted MTs**

To find out the role of MT-associated proteins (MAPs) in producing the stable MTs found in neurites, we tested the stability of in vitro reconstituted MTs under similar experimental conditions. When MTs were reassembled onto sperm axonemes using purified tubulin (10 μM) and tau (3.0 μM), they were very straight and often bundled. When these MTs were per-
fused with buffer A as in the case of DRG neurons, all of them started to shorten spontaneously and completely disappeared within 30 min (data not shown). This result indicates that they did not reach the level of stabilization found in neuritic MTs. Their mean shortening rate was 10 μm/min. Pauses in the shortening course were also observed in some cases, but their duration was much shorter.

In vitro, brain MAPs suppress the dynamic instability of MTs by reducing the rate and frequency of rapid shortening as well as increasing the frequency of rescue (7, 14, 15). Both the slow shortening rate and the occurrence of pauses can therefore be accounted for by the binding of MAPs. However, this is only applicable to the steady-state conditions where tubulin and MAPs in the MT polymer are at equilibrium with those in the soluble compartment. Under the present experimental conditions where soluble proteins are diluted out by perfusion, MAPs probably do not remain associated with MTs. Since perfusion resulted in the spontaneous disassembly of MTs polymerized from purified tubulin and a high concentration of tau, either the mode of binding of MAPs to the stable cellular MTs is different from what observed with in vitro reassembled MTs or that additional mechanisms are involved.

Existence of MT anchoring points suggested by transection

From their fluctuations in the extracellular medium, MTs seemed to be anchored only at specific points to the substratum (data not shown). The presence of such anchoring points became particularly evident when curved MTs were transected. These MTs sprang straight after transection with the newly generated ends free in solution, and the original anchoring points could be located from the orientation of the remaining fragments.

Since even the most stable MTs started to disassemble after transection and their disassembly rates were not slow enough to account for their persistence time after membrane removal, protection at the original MT ends and at specific sites along MTs must be considered. Though the original MT ends were difficult to find by the present method, local points were observed at which disassembly was transiently halted. Some of local points which anchor MTs to the substratum served as pausing points, but in most cases, disassembly continued past these points without retardation. Exposed stable MTs were curved and oriented in the original form of neurites through attachments to these points, suggesting their relation to the cell adhesion sites originally present in the intact neurites. MTs seemed to be curved under compressive force applied at these firm attachment sites since they sprang straight when transected between the anchoring points or freed from the anchoring point during shortening. The straightness of the MTs also indicate that they were rigid similar to the in vitro reassembled MTs (9). Identification of the molecules involved in the formation of these specific sites on MTs should provide further insights into the mechanism of MT stabilization in neurites.

The cell-free preparation of stable MTs described in the present study should serve as a unique system for further exploring the origin of the specific sites along MTs and testing the possible mechanisms of MT stabilization.

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