Tension and Compression in the Cytoskeleton of PC 12 Neurites

HARISH C. JOSHI,* DAN CHU,* ROBERT E. BUXBAUM,‡ and STEVEN R. HEIDEMANN*

*Departments of *Physiology and ‡Chemical Engineering, Michigan State University, East Lansing, Michigan 48823*

ABSTRACT We report in this article that the retraction of PC 12 neurites, unlike that of other cultured neurons, is due to tension within the neurite. Retraction is rapid and independent of metabolic energy. Transection of one arm of a branched neurite immediately causes the remaining arm to take up a new equilibrium position between attachment points. Similarly, detachment of one growth cone of a cell causes the cell body to move to a new equilibrium position between the remaining neurites. These observations provide direct evidence for the suspension of the cell soma among a network of tensioned neurites. We used retraction as an assay for neurite tension to examine the role of actin filaments and microtubules in neurite support and elongation. Our data suggest that microtubules (MTs) within PC 12 neurites are under compression, supporting tension within the actin network. Treatment of cells with drugs that disrupt actin networks, cytochalasin D or erythro-9-[3-(2-hydroxynonyl)]adenosine eliminates retraction regardless of the absence of MTs, lack of adhesion to the substratum, or integrity of the neurite. Conversely, stimulation of actin polymerization by injection of phalloidin causes retraction of neurites. Treatments that depolymerize MTs, nocodazole or cold, cause retraction of neurites, which suggests that microtubules support this tension, i.e., are under compression. Stabilization of MTs with taxol stabilizes neurites to retraction and under appropriate circumstances can drive neurite extension. Taxol-stimulated neurite extension is augmented by combined treatment with anti-actin drugs. This is consistent with the actin network's normally exerting a force opposite that of MT assembly. Cytochalasin and erythro-9-[3-(2-hydroxynonyl)] adenosine were found to increase slightly the dose of nocodazole required for MT depolymerization. This is consistent with the postulated balance of forces and also suggests that alteration of the compression borne by the microtubules could serve as a local regulator for MT polymerization during neurite outgrowth.

There is general agreement that axonal growth is intimately dependent upon the assembly and spatial organization of the neuronal cytoskeleton (3, 16). Despite this agreement, the role of the cytoskeletal elements in axonal growth is poorly understood. The mechanism for integrating the polymerization of actin and microtubules (MTs)[1] is completely unknown. However, several different lines of evidence point to a local, i.e. controlled within the axon, mechanism for the integration of cytoskeletal assembly with axonal growth: axons and neurites severed from the cell body can regrow (18, 24, 28); new growth cones appear on the normally quiescent sides of neurites in response to MT depolymerization (4, 28); neurite outgrowth can be guided by local changes in substratum adhesiveness (17); and isolated neurites or growth cones can respond to a gradient of nerve growth factor (NGF) (6, 23).

We recently reported that rate and extent of neurite outgrowth of PC 12 rat pheochromocytoma cells were positively correlated with the stability of the neurite MTs to depolymerization. These observations suggested to us that external

[1] *Abbreviations used in this paper:* EHNA, erythro-9-3-(2 hydroxynonyl) adenosine; MT, microtubule; N culture, cells grown in the presence of NGF only; NBD, *p*-nitrobenzoxadiazole; NC culture, cells grown in the presence of NGF and elevated cAMP levels; NGF, nerve growth factor.

force acting on MTs may control and integrate their assembly and disassembly (13). This speculation has also been put forward by Bray (2), who showed by vectorial analysis that the angles of neurites growing from cultured cells were consistent with neurites' being under tension (1). However, retraction of severed neurites of neurons in primary culture (4, 10, 24, 28) or of colchicine-treated neuroblastoma (25) did not suggest that tension was responsible. The retractions were slow and energy dependent, and the rate and extent of retraction depended upon the sharpness of the glass knife (28) or the pattern of laser irradiation (10). Unlike experiments with retraction in other neural cells, the experiments reported here indicate that retraction of PC 12 neurites is a direct result of tension on the neurite. We have used this manifestation of neurite tension to examine the role of actin filaments and microtubules in neurite support and elongation. Our data

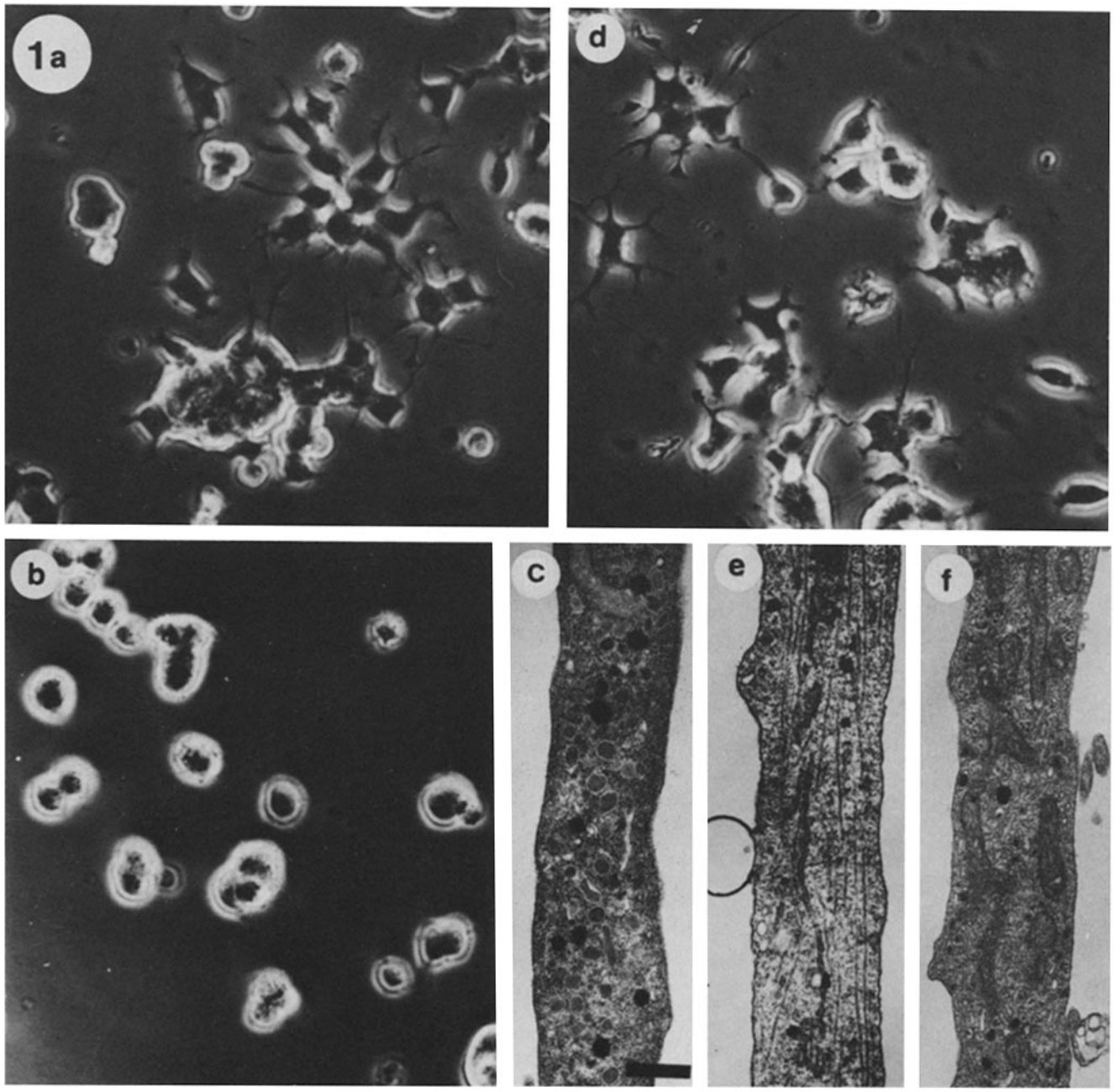

FIGURE 1 Retraction of PC 12 neurites in response to microtubule depolymerization. (*a*) Phase micrograph of N culture cells immediately before treatment. (*b*) Cells from same circled area as in *a* after 15 min in the presence of 0.1 μg/ml nocodazole. (*c*) Electron micrograph of a rare "persistent" neurite of an N culture fixed after 18 min in the presence of 0.1 μg/ml nocodazole. No microtubules are found in such neurites although the cell bodies contain a few MTs in the perinuclear region. (*d*) N culture cells after 16 min in 0.5 μg/ml cytochalasin D, 15 min in 1.0 μg/ml nocodazole. (*e*) Electron micrograph of an N culture neurite fixed after 16 min in 2.0 μg/ml cytochalasin D and 15 min 0.1 μg/ml nocodazole. 16/17 neurites examined from both N and NC conditions in the presence of cytochalasin D and threshold doses of nocodazole contained an apparently normal array of MTs. (*f*) Neurite from an NC culture cell fixed after 16 min in 0.5 μg/ml cytochalasin D and 15 min in 1.0 μg/ml nocodazole. At this dose of nocodazole MTs were absent in all 10 neurites examined from both N and NC cultures. All light micrographs, × 750. bar, 20 μm. All electron micrographs, × 21,500. Bar, 0.5 μm.

suggest that MTs within PC 12 neurites are under compression, supporting tension within the actin network. Pharmacological disruption of the actin network affects MT disassembly in vivo in a manner consistent with the complementary force interaction's playing a role in controlling MT assembly in neurites. These data support the hypothesis on the role of external force in cytoskeletal assembly and the more general hypothesis of Ingber and Jamieson that forces play an informational role in cell growth and differentiation (15).

MATERIALS AND METHODS

NGF was the kind gift of Dr. Mark Bothwell (Princeton University). Nocodazole was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Cytochalasin D, phalloidin, actinomycin, cyclohexamide, sodium azide, dinitrophenol, and sodium arsenate were obtained from Sigma Chemical Co. (St. Louis, MO). Taxol was a gift from the National Cancer Institute. Erythro-9-[3-(2 hydroxynonyl)] adenosine (EHNA) was obtained from Burroughs Wellcome & Co. (London). *p*-nitrobenzoxadiazole (NBD)-phallicidin was obtained from Molecular Probes Inc. (Junction City, OR).

Cells were grown in RPMI 1640 medium containing 10% horse serum and 5% fetal calf serum. 50 ng/ml NGF was added 7 d before the experiment. After 6 d of NGF "priming" (5) cells were triturated from the surface and replated (3×10^4 cells/plate) onto 35-mm Primaria tissue culture dishes (Falcon Plastics, Oxnard, CA) that had previously been treated with 1 mg/ml polylysine for 30 minutes then rinsed twice with water. Cells were allowed to regenerate neurites for 24 h under two conditions; with 50 ng/ml NGF (N cultures) or with 50 ng/ml NGF and elevated cAMP levels (either 1 mM dibutyryl cAMP or 0.1 μM 2-choloroadenosine), called NC cultures. Cells with regenerated rather than newly initiated neurites were employed because this decreased the number of experimental variables between N and NC cells (13). For the quantitative assay of neurite retraction, two 1.5-mm-diam circles were marked on the bottom of the culture dish immediately before treatments. Four photographs that completely surveyed each circled region were taken before and after treatment. Counts were made of neurites from all photographs for a given experimental treatment.

Phalloidin was injected into cells according to the method of Wehland et al. (27). 0.2 mM phalloidin in 0.14 M KCl containing 0.8% dimethylsulfoxide was injected into NC cells as above using needles pulled on micropipette puller (model m1, Industrial Science Associates, Flushing, NY). Control cells were injected with 0.14 m KCl containing 0.8% dimethylsulfoxide.

Neurites were severed with a laser beam moving at 50 μm/s by the ACAS 470 Workstation (Meridian Instruments Inc., Okemos, MI). This instrument integrates a 2-W argon ion laser focused through the objective of an inverted microscope with a computer controlled X–Y stage and a photodetector. NC cells were cultured as previously described except that the growing surface of the Primaria dish was blackened with ink extracted from a "Sharpie" (Sanford Corp., Bell Wood, IL), marker (one new pen in 100 ml 70% ethanol) before polylysine treatment. Only cells under NC conditions extended neurites to an appreciable degree on the blackened growth surface necessary for laser transection; i.e., N culture neurites were not used. Because laser experiments were not performed in a 10% CO_2 atmosphere, the culture medium was exchanged ~1 h before the experiment for medium buffered with 5 mM HEPES pH 7.4. During experimentation the cultures were maintained at 37°C with an air curtain incubator (Sage Instruments Div., Orion Research Inc., Cambridge, MA).

Fluorescent staining of PC 12 actin with NBD-phallicidin followed the procedure provided by Molecular Probes Inc.

RESULTS

Response of PC 12 Neurites to Anti-Cytoskeletal Drugs

We extended our previous results on the retraction of PC 12 neurites in response to microtubule depolymerization in N and NC culture conditions (13). The data are summarized in Table I. More than 90% of N culture neurites retract within 15 min after treatment with 0.1 μg/ml of the anti-MT drug Nocodazole (18) (Fig. 1, *a* and *b*). Approximately 70% retract after 2 h at 10°C. NC culture neurites are more stable, do not retract upon cold treatment (13), and require a higher concentration of nocodozole. However, 90% of NC culture neurites retract after 15 min at 0.2 μg/ml nocodazole. Time-lapse videotape of N cultures poisoned with nocodazole showed two different kinetics of retraction. Approximately 80% of the cells retracted gradually over a period of 30–40 s after an initial lag period of 3 to 7 min. The other cells remained fully extended while its sisters were retracting then suddenly re-

TABLE I. *Retraction of PC 12 Neurites*

Treatment	N cultures	NC cultures
0.1 μg/ml nocodazole, 15 min	67/1,123 (4)	1,126/1,179 (4)
0.2 μg/ml nocodazole, 15 min	—	102/1,091 (3)
10°C, 2 h	67/310 (1)	—
0.1 mM DNP, 10 mM azide, 0.1 mM arsenate 40 min, nocodazole* 15 min	94/1,324 (2)	155/1,278 (2)
2 μg/ml cytochalasin D only, 20 min	515/579 (2)	234/220 (1)
2 μg/ml cytochalasin D, 16 min; nocodazole* 15 min	414/452 (2)	218/207 (1)
0.5 μg/ml cytochalasin D, 16 min; nocodazole* 15 min	589/726 (3)	825/931 (2)
0.5 μg/ml cytochalasin D, 16 min; 1 μg/ml nocodazole 15 min	1,066/1,200 (2)	1,949/2,253 (2)
2 μg/ml cytochalasin D, 2.1 h; 10°C, 2 h	441/447 (2)	
0.2 mM EHNA 30 min, nocodazole* 15 min	1,009/1,074 (2)	996/1,250 (2)

The numerator is the total number of neurites in surveyed regions after treatment. The denominator is the total number of neurites in the same regions before treatment. The number of experimental repetitions is given in parentheses.

* In experiments employing nocodazole, 0.1 μg/ml was used for N cultures, 0.2 μg/ml for NC cultures unless otherwise noted.

TABLE II. *Occurrence of Microtubules in Neurites of Cells Treated with Anti-Cytoskeletal Drugs*

Treatment	Neurites containing MTs
N cells	
0.1 μg/ml nocodazole	0/3 (2)*
0.1 μg/ml nocodazole + 2.0 μg/ml cytochalasin D	9/9 (4)
0.1 μg/ml nocodazole + 0.2 mM EHNA	5/6 (2)
2 h at 10°C + 0.5 μg/ml cytochalasin D	4/4 (2)
1.0 μg/ml nocodazole + 2.0 μg/ml cytochalasin D	0/6 (4)
0.2 μg/ml nocodazole + 0.5 μg/ml cytochalasin D	0/2 (2)
0.2 μg/ml nocodazole + 2.0 μg/ml cytochalasin D	0/2 (2)
NC cells	
0.2 μg/ml nocodazole	0/2 (1)*
0.2 μg/ml nocodazole + 2.0 μg/ml cytochalasin D	7/8 (4)
0.2 μg/ml nocodazole + 0.2 mM EHNA	6/7 (2)
1.0 μg/ml nocodazole + 2.0 μg/ml cytochalasin D	0/4 (2)
0.3 μg/ml nocodazole + 0.5 μg/ml cytochalasin D	1/6 (4)

The numerator is the number of cells that have neurites containing MTs. The denominator is the number of cells observed. The numbers in parentheses are the numbers of experimental repetitions.

* These observations were made on those rare neurites that remained extended under these conditions (see Table I).

tracted; i.e., the neurite snapped back into the soma. Retractions induced by nocodazole were not affected by combined inhibitors of electron transport, dinitrophenol and Na azide; and of glycolysis, Na arsenate. Ultrastructural observations confirmed that MT depolymerization did accompany retraction. Microtubules were not observed in the rare persistent neurites (Fig. 1 *c*). We found very few MTs in the rounded, "retracted" cells, inconsistent with simple retraction of MTs into the cell body. We confirmed the observation of Solomon and Magendantz (25) that cytochalasin D rescues neurites from MT depolymerization-induced retraction (Fig. 1 *d*). Only 13% of neurites from N and NC cultures retract upon the addition of cytochalasin D at either 0.5 or 2.0 μg/ml 1 min before addition of the low dose (see Table I) of nocodazole previously 90% effective. Use of a considerably higher dose of nocodazole (1 μg/ml) did not alter the rescue by cytochalasin. Cytochalasin also rescued essentially all N cell neurites from cold-induced retraction. EHNA has recently been reported to disrupt actin networks within various cultured cells (21). The addition of 0.2 mM EHNA also rescued neurites from nocodazole induced retraction (Table I). For N cells 94% of the neurites remained extended after 15 min of 0.1 μg/ml nocodazole when EHNA was added 15 min before nocodazole. For NC cells, 80% of the neurites withstood 0.2 μg/ml nocodazole when pretreated with EHNA.

We wished to determine the effect of anti-actin drugs on MT depolymerization within the neurite. N and NC cells from the experiments above were examined ultrastructurally and scored for the presence or absence of MTs within neurites. The data are summarized in Table II. Ultrastructural obser-

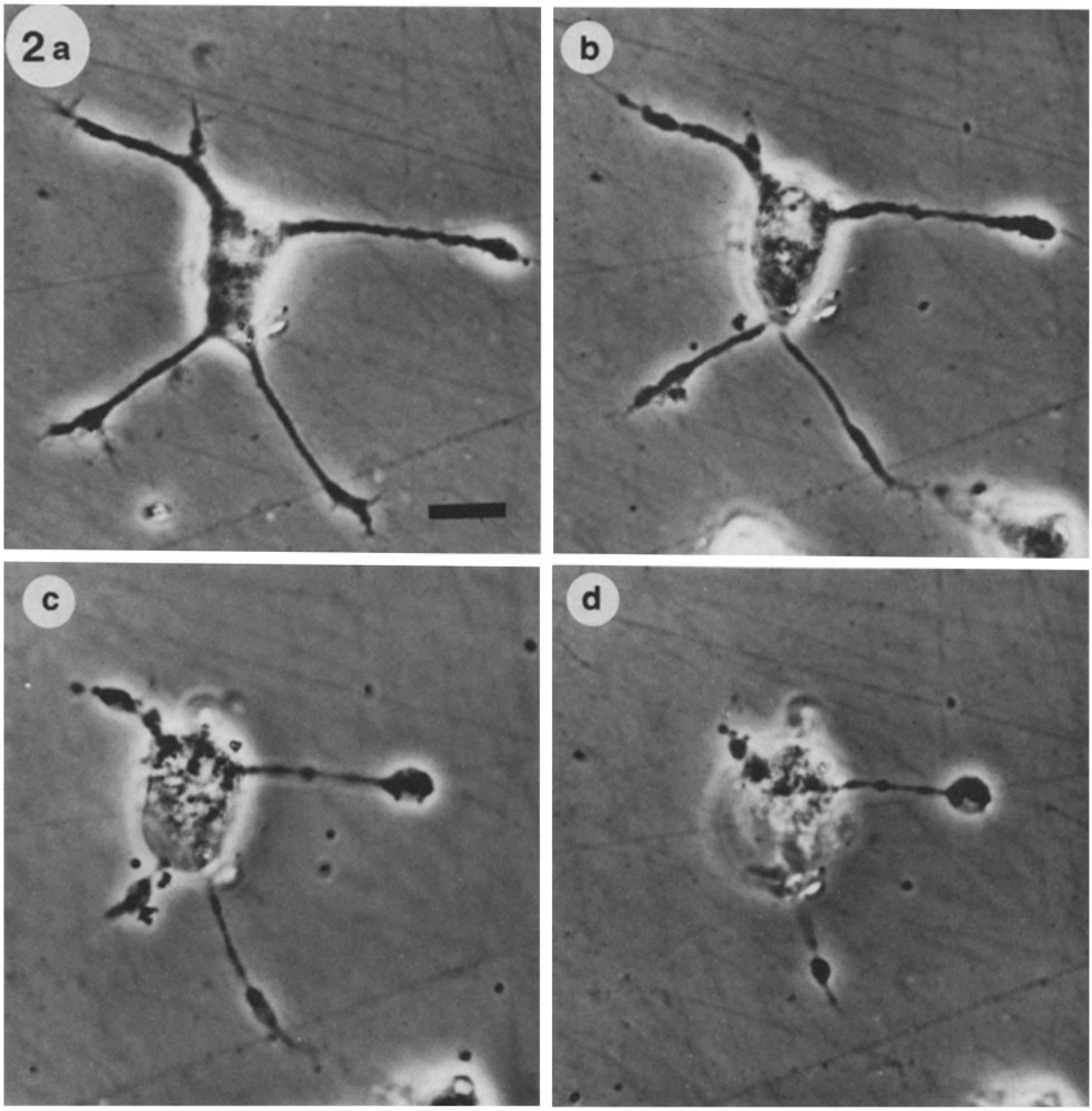

FIGURE 2 Retraction of PC 12 neurites in response to injection of phalloidin. Phase micrograph of an NC cell injected with 0.2 mM phalloidin in 0.14 M KCl containing 0.8% dimethylsulfoxide. (*a*) Immediately after injection. (*b–d*) 9, 28 min, and 32 min after injection, respectively. The two retractions seen here, retraction of neurite into cell body and retraction of axoplasm into neurite beads, were typical of cells injected with phalloidin. Bar, 10 μm. × 1,200.

vation of N cells and NC cells treated with either cytochalasin or EHNA and a low dose of nocodazole that was effective at MT depolymerization without anti-actin drugs (0.1 and 0.2 μg/ml, respectively) showed that these cells now contained an apparently normal array of microtubules (Fig. 1*e*). N cells treated with 0.5 μg/ml cytochalasin D and subjected to 10°C for 2 h also contained MTs. However, doses of nocodazole ≥0.2 μg/ml for N cells or ≥0.3 μg/ml for NC cells completely eliminated MTs in 19/20 cells treated with either 0.5 or 2.0 μg/ml cytochalasin (Fig. 1*f*). Apparently, treatment of cells with cytochalasin or EHNA slightly raised the threshold for induced depolymerization. These results also indicated that MTs were not required for neurite maintenance in the presence of cytocholasin. As seen in Fig. 1*c* and *f*, axoplasm within neurites without MTs was similar in appearance to that typically found within cell soma. This alteration was true for all examined neurites without MTs and apparently occurred rather rapidly. The cell in Fig. 1*f* was fixed 16 min after the first addition of any drug. The only observed difference at the light microscopic level was that the sides of the neurites became less smooth during this period.

Phalloidin has been shown to promote actin polymerization in vivo (27) and in vitro (29). All neurites of cells injected with 0.2 mM phalloidin retracted in one of two ways. The cell in Fig. 2 shows both. Most neurites retracted into the soma over a 30-min period. However, in some neurites the axoplasm appeared to break within the axonal membrane envelope and the axoplasm retracted into a bead at the growth cone or along the neurite. These retractions were noticeably slower than others we had observed. This may be a result of the time course of phalloidin action in vivo; Wehland et al. (27) waited three hours before assessing the effect of phalloidin on cultured cells. Cells injected with the same solution without phalloidin showed no reaction whatever to the injection during the one hour they were observed.

Laser Transection and Dislodgement of PC 12 Neurites

Neurites of PC 12 cells grown in NC conditions on surfaces blackened with ink were severed by the moving beam of an argon ion laser. In five separate experiments, 24/27 unbranched neurites transected anywhere between the cell body and terminal growth cone showed complete retraction of both proximal and distal segments within 2 min (Fig. 3, *a* and *b*). In five of the cells, both proximal and distal portions retracted immediately, i.e., snapped back. In the remaining 19 cells, the proximal portion snapped back into the soma and the distal portion retracted gradually over 30–40 s, occasionally appearing beaded during the retraction. Retraction after transection was not affected by combined inhibitors of electron transport and glycolysis; after 10–30 min of combined treatment with 10 mM Na azide, 0.1 mM dinitrophenol, and 0.1 mM Na arsenate 12/14 neurites showed complete retraction 2 min after transection. Branched neurites showed a similar response to transection except that proximal and distal retraction stopped at the nearest branch point. Those neurite portions that remained took up new positions consistent with neurites' being taut fibers suspended between attachment points (Fig. 3, *c* and *d*). Less easily documented observations, the pattern of movement of neurites disturbed by agitating the medium or by gentle micromanipulation, also suggested that PC 12 neurites were taut fibers attached to the substratum only at growth cones and the cell body. We confirmed the

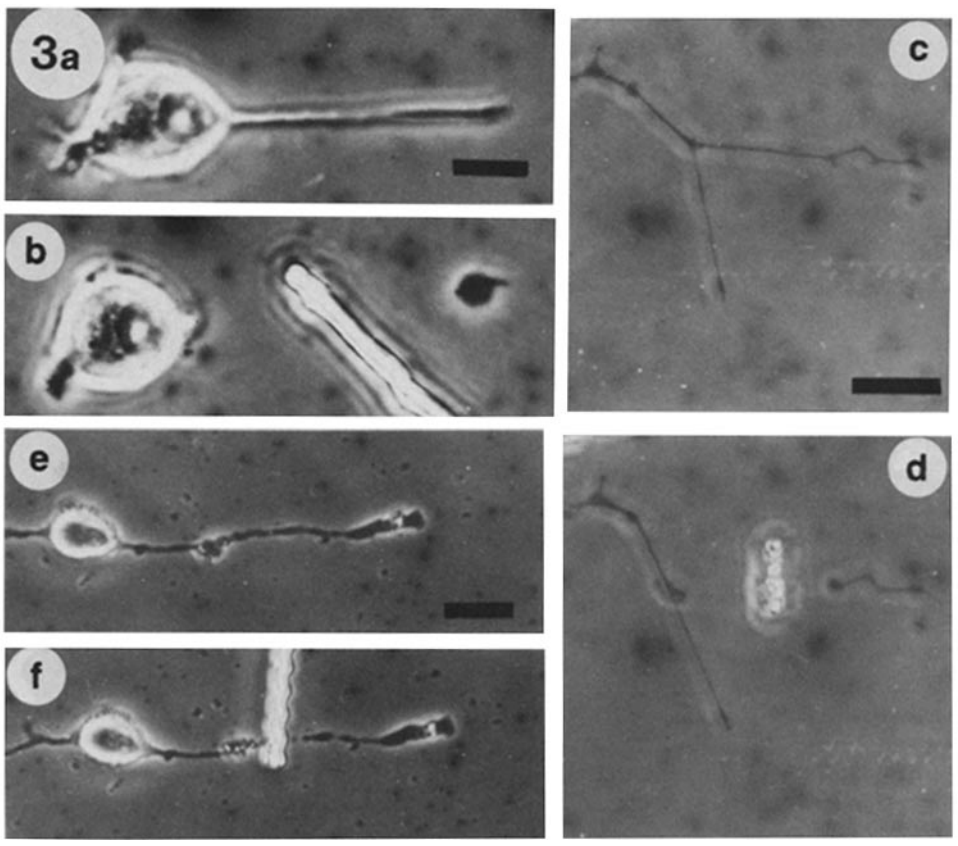

FIGURE 3 Laser transection of PC 12 neurites. (*a*) NC culture cell before transection. (*b*) The same cell as in Fig. 2*a* 2 min after laser transection. The laser scar is clearly visible on the dish and indicates that the neurite was severed midway between growth cone and soma. Note the complete retraction of both proximal and distal portions. (*a* and *b*) Bar, 10 μm. × 1,000. (*c*) NC cells with branched neurites before transection. (*d*) The same cell as in Fig. 2*c* ~4 s after transection of one branch of neurite. This micrograph shows that branched neurites move upon transection as if they were taut fibers suspended between growth cone and soma. This micrograph also illustrates the immediate retraction of the proximal portion of transected neurites, the distal portion is still in the process of retracting. (*c* and *d*) Bar, 40 μm. × 300. (*e*) NC cell in 0.5 μg/ml cytochalasin D immediately before transection. (*f*) Same cell as in *e*, 2 min after laser transection. As described in the text, such neurites remained extended for as long as observed. Bar, 20 μm. × 600.

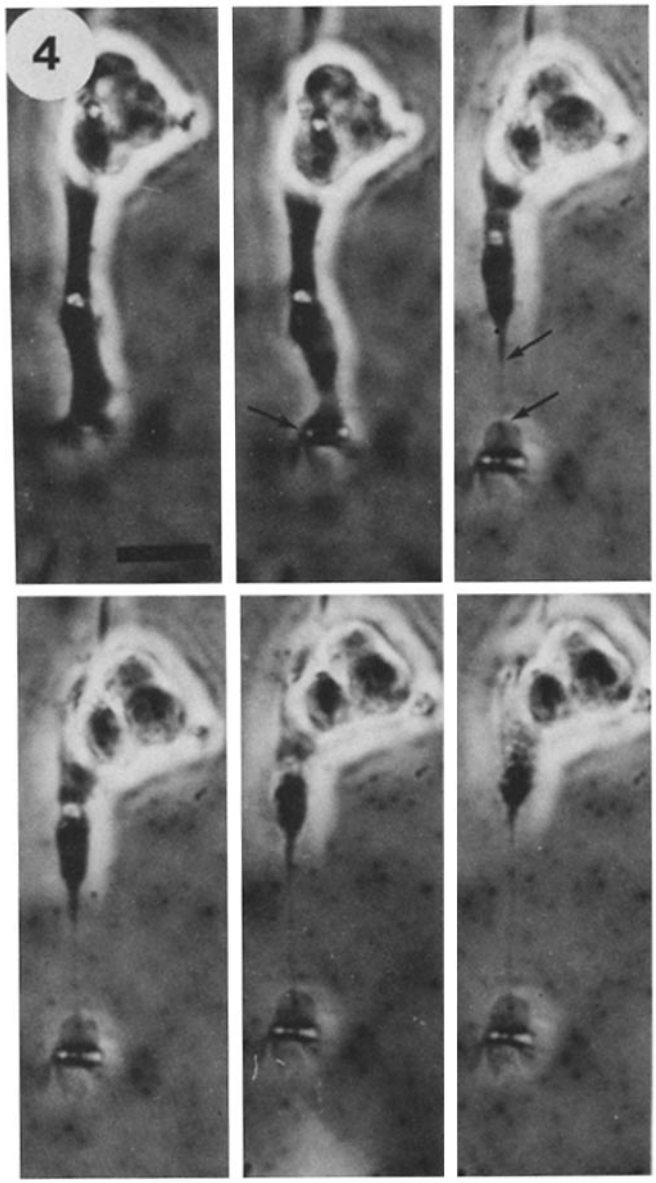

FIGURE 4 Sequence of photographs of a cell that had received an attenuated laser "cut" across its growth cone (arrow). The first panel shows the cell before treatment, the second panel immediately after lasing. The cytoplast retracts from a point behind the growth cone leaving a cell remnant, presumably the plasma membrane (double arrows). The entire sequence here occurred over ~40 s. Bar, 10 μm. × 1,200.

observation of Shaw and Bray (24) that transected neurites are rescued from retraction by treatment with cytochalasin; 37/40 no longer showed retraction of either the proximal or distal segments after 5–20 min in the presence of 0.4 μg/ml cytochalasin D (Fig. 3, *e* and *f*). Treatment of cells with 1 μM taxol for 5 to 20 min also prevented retraction; none of 13 showed any retraction. Neurites treated with cytochalasin or taxol were stable after transection for as long as they were observed, up to 40 min, if the culture dish was not jarred or removed from the stage of the microscope.

We could observe two other reproducible retractions using the laser without severing the neurite. Application of attenuated laser beam (50% of the energy used above) across the growth cone of seven cells caused the retraction of axoplasm within a cell remnant as shown in Fig. 4. In all cases the retraction began proximal to the point of laser treatment, where the growth cone first broadens from the neurite. Retraction required 30–40 s, as for several other retractions. The cell remnant was exceedingly fragile; in most cases the recoil of the camera shutter caused it to part during attempts to photograph it. All attempts to add fixitive also caused it to part. As above, combined inhibitors of energy metabolism had no effect on this retraction. Retraction was also observed in those cells that had their growth cones dislodged by a shock wave produced by laser irradiation of the plate near the growth cone. The cell itself was not irradiated. All 13 cells treated in this way retracted unbranched neurites proximally into the soma within 2 min. Again, retraction was gradual over the course of 30–40 s. Translation of the cell body was usually observed when one of the neurites was dislodged. Fig. 5 shows a tripolar cells before and after dislodgement of one neurite. As shown in the micrographs, the movement of the cell body was always such as to suggest that the cell body occupied an equilibrium position in a network of tensioned neurites.

Extension of Cellular Processes in the Presence of Taxol and Cytochalasin

Corvaja et al. (7) found that PC 12 cells treated with NGF and taxol grew out bulbous, abnormal neurites within ~7 d. However, we found that PC 12 cells treated with taxol or taxol and cytochalasin could extend such neurites within a matter of hours under some circumstances. NC, but not N, cells treated with 1 μM taxol 1 h after replating for neurite regeneration extended significant cellular processes within 1 h. Such cellular processes continued to initiate and elongate for at least 2 h more. As Corvaja et al. (7) described, these processes differed in appearance from those initiated or regenerated in the presence of NGF or cAMP, most obviously by the large, spherical "bleb" at the terminus of the neurite. The addition of 2 μg/ml cytochalasin D in addition to taxol augmented abnormal neurite growth, N cells also extended similar "neurites" with the same rapidity. N or NC cells treated with 2 μg/ml cytochalasin D alone did not extend such neurites. Corvaja et al. reported that stimulation of PC 12 cells with NGF was required for taxol-induced neurite extension. We were surprised to find that PC 12 cells that had never been exposed to any growth factors other than those contained in RPMI 1640 and normal sera responded to the combined presence of taxol and cytochalasin by rapidly extending these abnormal cellular processes (Fig. 6). The addition of 0.5 μM actinomycin D and 10 μg/ml cycloheximide, shown by Gunning et al. (11) to inhibit >80% of RNA synthesis and >97% of protein synthesis in the same PC 12 cell line used here, did not alter the extension of the cellular processes. The addition of either taxol or cytochalasin alone to "unstimulated" PC 12 cells did not produce similar neurite outgrowth. When micromanipulated, all taxol "neurites" easily detached from the substratum, remained extended, and moved like a stiff fiber with a joint at the junction of the process and cell body. Ultrastructural observation of such processes showed a neurite with large numbers of MTs, as reported earlier (7). Flourescent staining of actin by NBD phallicidin showed actin located only in the enlarged, distal tip of the neurite and in "blebs" located along the neurite (Fig. 7*a*). Similar staining of typical N or NC neurites showed actin located throughout the neurite in a submembranous array (Fig. 7*b*) as expected from previous reports (22, 30).

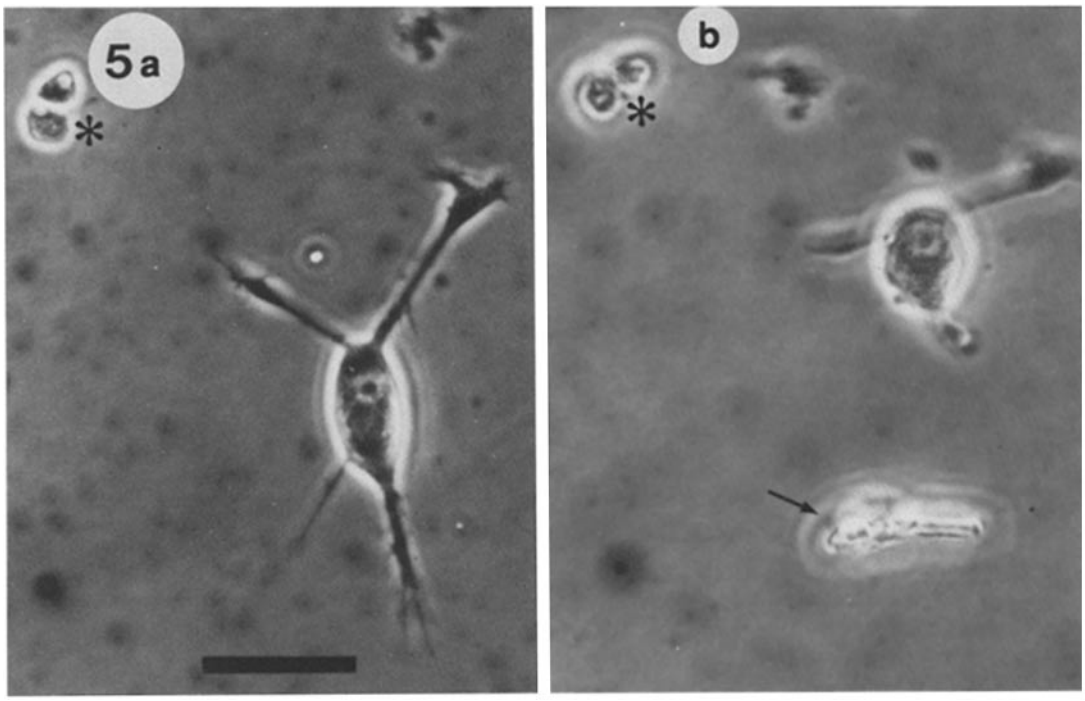

FIGURE 5 Detachment of growth cone from substratum causes rapid neurite retraction and translation of cells body. (*a*) Before laser irradiation. (*b*) 2 min after laser irradiation of region of the dish near the growth cone caused the growth cone to detach from the substratum. Note that the cell body took up a new equilibrium position between the remaining neurites. The asterisk marks a stationary reference point. Bar, 20 μm. × 800.

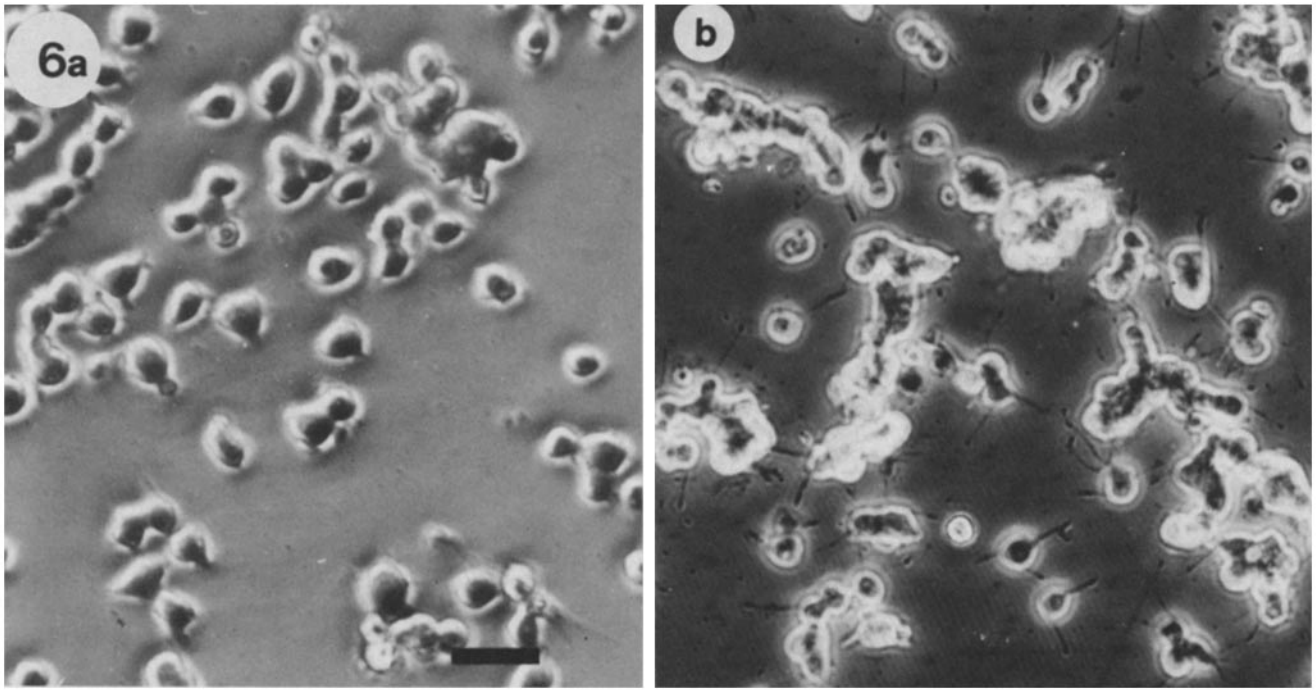

FIGURE 6 PC 12 cells that had not been exposed to NGF or elevated cAMP (*a*) before and (*b*) 3 h after treatment with 1 μM taxol and 2 μg/ml cytochalasin for 3 h. Abnormal, bulbous neurites are rapidly extended in such conditions. In this particular figure the cells had been treated with 0.5 μM actinomycin D and 10 μg/ml cycloheximide for 1 h before the addition of taxol and cytochalasin. The extension of neurites was unaffected by the RNA and protein synthesis inhibitors. Bar, 40 μm. × 300.

While this work was in progress, Spero et al. (26) reported that cytochalasin and taxol induce abnormal neurite extension in neuroblastoma cells. However, in neuroblastoma these neurites formed sinuous turns, a response markedly different from that of taxol-induced neurites of PC 12.

DISCUSSION

It is clear that retraction of neurites requires that some force act on the neurite. Our observations indicate that the force of retraction in our experiments is provided by tension of PC 12

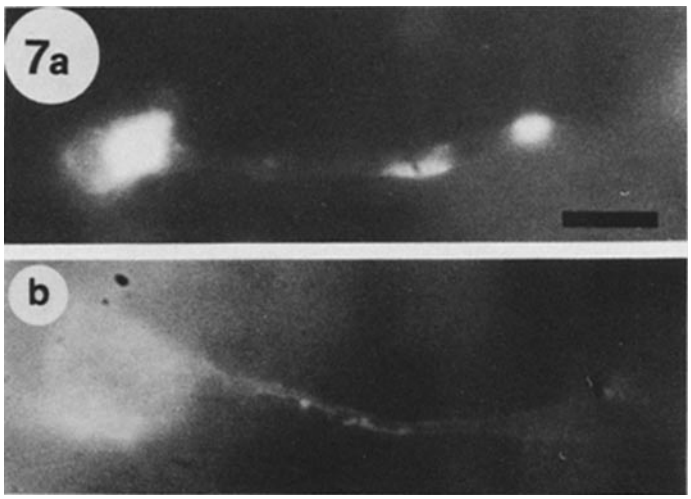

FIGURE 7 Comparison of actin network in neurites extended in the presence of taxol and cytochalasin and neurites extended by NGF stimulation. (*a*) Fluorescence micrograph of cell as in Fig. 6 stained with NSD-phallicidin. Actin in such neurites was concentrated in the cell body, in the bulbous terminal of neurite, and at bulges along the neurite. (*b*) N cell treated with NBD phallicidin shows the delicate staining along neurite. The concentration of fluorescence along the boundaries of such cells suggests that the actin network is closely associated with the axolemma. Bar, 10 μm. × 1200.

neurites. The rapidity of retraction both of lased neurites and of neurites subjected to MT depolymerization supports this claim. The proximal portion of lased neurites routinely underwent "instantaneous" retraction, similar to the rubber band released from tension. Time-lapse videotape of N culture cells treated with nocodazole also showed a number of cells that suddenly retracted completely. Those neurites that did not snap back nevertheless underwent rather rapid retraction over a period of 30–40 s after a lag of several minutes. Observation of this phenomena gave the impression that the retraction could occur only as fast as some support member was dissolving, presumably MTs. Combined inhibitors of glycolysis and electron transport had no effect on either laser- or MT depolymerization-induced retraction of Pc 12 neurites. We observed that neurites behaved like taut fibers tensioned between adhesion points. Indeed, we were able to obtain dynamic evidence for the conclusion of Bray (1) that the cell body occupies an equilibrium position in a network of tensioned neurites. Dislodgment of one neurite caused the cell body to rapidly take up a position of equilibrium with respect to the remaining neurites (Fig. 5). The rapid retraction of "axoplasm" without actual severing of the neurite (Fig. 4) indicates that retraction is not an artifact of neurite transection, nor does it require local dislodgement from the substratum since the growth cone remained extended. The location, appearance, and fragility of the cell remnant in this experiment suggests that it was the neurite plasma membrane. We postulate that lasing the growth cone severed the connection of the tensile element to the substratum, causing retraction of the cytoplasm within the still-extended membrane envelope. The growth cone region remains extended due to its attachment to the substratum at the filopodia.

We investigated the role of actin and microtubules in the support of neurites by using retraction as an assay for the tensile state of the neurite and assessing the effects of anticytoskeletal drugs on retraction. We attempted to control for drug side effects through the use of more than one treatment known to have the activity of interest, e.g. cold and nocodazole for MT depolymerization, cytochalasin and EHNA for actin network disruption. Our results suggest that MTs and actin are engaged in a complementary force interaction, a submembranous actin network as a tensile component within the neurite that is partially supported by compression of the MTs in the central region of the axoplasm. Cells treated with cytochalasin or EHNA no longer retracted their neurites regardless of the absence of MTs, lack of adherence to the substratum, or the integrity of the neurite. These results suggest that the force that previously moved the neurite is dissipated by treatment with these drugs. These poisons are known to disrupt actin networks (9, 20, 21), the form of actin found in neurites (22, 30). Conversely, we observed that the injection of phalloidin at a concentration previously reported not to cause retraction of other cell types (27) caused retraction of PC 12 neurites. We conclude that augmenting the density of the actin network by stimulating polymerization with phalloidin increased the tensile force within the neurite. The compressive state of neurite MTs is supported by several experiments. Treatments that depolymerize MTs, either nocodazole or cold, cause the retraction of neurites. Not all support is provided by internal MTs, however, We found that dislodgement of the growth cone also causes retraction, which suggested that some necessary support is also provided by the substratum. However, stabilizing MTs with taxol (12, 19) allowed the neurites to remain extended upon laser transection, indicating that they no longer required the support of the substratum. Apparently, stabilization of MTs allows them to support fully the tension on the neurite. Treatment of NC cells with taxol causes neurites to "grow" with astonishing rapidity. Presumably, MT polymerization pushes out the abnormal neurite. These results with taxol also suggest that MTs are compressive elements. Destabilization of the actin network by cytochalasin stimulated the extension of taxol-induced neurites. N cells grew neurites only when both drugs were added. PC 12 cells that had not been stimulated by NGF or cAMP also extended these neurites only in the presence of cytochalasin and taxsol, not with either substance alone. Our interpretation is that an intact actin network exerts a force opposite that of MT assembly, thereby inhibiting MT assembly–driven neurite extension.

Of particular interest, our evidence is consistent with the idea that the complementary force interaction between actin and MTs provides a local regulator for MT assembly. This proposed "tensegrity" mechanism (15) is an equilibrium process and would be a particularly economical way to integrate assembly of MTs with the actin-based motility of the growth cone. Compression of MTs should destabilize them, favor disassembly, and inhibit assembly, relative to no force (14). Release of compression, normally by the advance of the growth cone or here by disruption of the actin network, should stabilize MTs, i.e., decrease the critical concentration of tub-

ulin for assembly (14). If the compression of MTs is of an appropriate magnitude to affect MT assembly in vivo, then this decrease in the critical concentration should be observable as a stabilization of MTs to threshold depolymerization. Indeed, the great majority of examined N and NC neurites treated with a low dose of nocodazole that eliminated MTs in the absence of actin drugs now contained MTs in the presence of cytochalasin or EHNA. If the force is a sensitive regulator, then depolymerization stimuli slightly greater than threshold should cause depolymerization. Treatment of cells with slightly higher concentrations of nocodazole (0.2 μg/ml for N cells and 0.3 μg/ml for NC cells) in the presence of cytochalasin eliminated MTs. Although we cannot be certain that the increase in the threshold is due to drug-induced dissipation of tension in the actin network, our data do not support several other interpretations. Cytochalasin "rescued" MTs from both nocodazole and cold, so the rescue does not appear to depend on the method used for MT depolymerization. Furthermore, the rescue is obtained with cytochalasin or EHNA, so the effect is probably not due to some side effect of one anti-actin drug only. Nevertheless, the actin drugs we have employed may have important effects other than disruption of actin networks. We plan more direct experimentation to test our interpretation.

We are particularly indebted to Dr. Mark Bothwell for his more than generous gifts of nerve growth factor. We thank the Department of Microbiology at Michigan State for the use of their electron microscope facility, Stuart Pankratz for his invaluable assistance and John Duffendack of Meridian Instruments Inc. for the use of the ACA5470.

This work was supported by National Science Foundation grant BNS 84-01904. Dr. Heidemann is a recipient of a Research Career Development Award from the National Institutes of Child Health and Human Development.

Received for publication 22 January 1985, and in revised form 27 April 1985.

REFERENCES

1. Bray, D. 1979. Mechanical tension produced by nerve cells in tissue culture. *J. Cell Sci.* 37:391–410.
2. Bray, D. 1982. Filopodial contraction and growth cone guidance. *In* Cell Behavior. R. Bellair, A. Curtis, and G. Dunn, editors. Cambridge University Press, Cambridge. 299–317.
3. Bray, D., and D. Gilbert. 1981. Cytoskeletal elements in neurons. *Annu. Rev. Neurosci.* 4:505–523.
4. Bray, D., C. Thomas, and G. Shaw. 1978. Growth cone formation in cultures of sensory neurons. *Proc. Natl. Acad. Sci. USA.* 75:5226–5229.
5. Burstein, D. E., and L. A. Greene. 1978. Evidence for both RNA-synthesis dependent and independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 75:6059–6063.
6. Campenot, R. B. 1977. Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 74:4516–4519.
7. Corvaja, N., A. Di Luzio, S. Biocca, A. Cattaneo, and P. Calissano. 1982. Morphological and ultrastructural changes in PC 12 pheochromocytoma cells induced by combined treatment with NGF and taxol. *Exp. Cell Res.* 142:385–395.
8. DeBrabender, M., G. Guens, R. Van de Veire, F. Thone, F. Aerts, L. Desplanter, J. DeCree, and M. Borgers. 1977. The effects of R17934, a new antimicrotubular substance on the ultrastructure of neoplastic cell in vivo. *Eur. J. Cancer.* 13:511–528.
9. Godman, G. C., and A. F. Miranda. 1978. Cellular contractility and the visible effects of cytochalasin. *In* Cytochalasins: Biochemical and Cell Biological Aspects. S. W. Tanenbaum, editor. North Holland Publishing Co., Amsterdam. 277–429.
10. Gross, G. W., J. H. Lucas, and M. L. Higgins. 1983. Laser microbeam surgery: ultrastructural changes associated with neurite transection in culture. *J. Neurosci.* 3:1979–1993.
11. Gunning, P. W., G. E. Landreth, M. A. Bothwell, and E. M. Shooter. 1981. Differential and synergistic action of nerve growth factor and cylic AMP in PC 12 cells. *J. Cell Biol.* 89:240–245.
12. Heidemann, S. R., and P. T. Gallas. 1980. The effect of taxol on living eggs of *Xenopus laevis*. *Dev. Biol.* 80:489–494.
13. Heidemann, S. R., H. C. Joshi, A. Shechter, J. R. Fletcher, and M. Bothwell. 1985. Synergistic effects of cyclic AMP and nerve growth factor on neurite outgrowth and microtubule stability of PC 12 cells. *J. Cell Biol.* 100:916–927.
14. Hill, T. L., and M. W. Kirschner. 1982. Bioenergetic and kinetics of microtubule and actin filament assembly-disassembly. *Int. Rev. Cytol.* 78:1–125.
15. Ingber, D. E., and J. D. Jamieson. 1985. Cells as tensegrity structures: architectural regulation of histodifferentiation by physical forces transduced over basement membrane. *In* Gene Expression during Normal and Malignant Differentiation L. C. Andersson, C. G. Gahmberg, and P. Ekblom, editors. Academic Press Inc., NY. In press.
16. Landis, S. C. 1983. Neuronal growth cones. *Annu. Rev. Physiol.* 45:567–580.
17. Letourneau, P. C. 1975. Cell-to-substratum adhesion and guidance of axonal elongation. *Dev. Biol.* 44:92–101.
18. Mason, A., and K. J. Muller. 1982. Axon segments sprout at both ends: tracking growth with fluorescent D-peptides. *Nature (Lond.).* 296:655–657.
19. Schiff, P. B., J. Fant, and S. B. Horwitz. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature (Lond.).* 277:665–667.
20. Schliwa, M. 1982. Action of cytochalasin D on cytoskeletal networks. *J. Cell Biol.* 92:79–91.
21. Schliwa, M., R. M. Ezzell, and U. Euteneuer. 1984. Erythro-9-[3-(2 hydroxynonyl] adenine is an effective inhibitor of cell motility and actin assembly. *Proc. Natl. Acad. Sci. USA.* 81:6044–6048.
22. Schnapp, B. J., and T. S. Reese. 1982. Cytoplasmic structure in rapid frozen axons. *J. Cell Biol.* 94:667–679.
23. Seeley, P. J., and L. A. Greene. 1983. Short-latency local actions of nerve growth factor at the growth cone. *Proc. Natl. Acad. Sci. USA.* 80:2789–2795.
24. Shaw, G., and D. Bray. 1977. Movement and extension of isolated growth cones. *Exp. Cell Res.* 104:55–62.
25. Solomon, F., and M. Magendantz. 1981. Cytochalasin separates microtubule disassembly from loss of asymmetrical morphology. *J. Cell Biol.* 89:157–161.
26. Spero, D. A., E. T. Browning, and F. J. Roisen. 1985. Modification of microtubule-microfilament interactions during neurite outgrowth. *Ann. NY Acad. Sci.* In press.
27. Wehland, J., M. Osborn, and K. Weber. 1977. Phalloidin-induced actin polymerization in the cytoplasm of cultured cells interferes with cell locomotion and growth. *Proc. Natl. Acad. Sci. USA* 74:5613–5617.
28. Wessels, N. K., S. R. Johnson, and R. P. Nuttall. 1978. Axon initiation and growth cone regeneration in cultured motor neurons. *Exp. Cell Res.* 117:335–345.
29. Wieland, T. 1977. Modifications of actin by phallotoxins. *Naturwissenschaften.* 64:303–309.
30. Yamada, K., B. Spooner, and N. Wessels. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* 49:614–635.