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Axon Growth: Roles of Microfilaments and Microtubules*

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Abstract. The motile tips of elongating axons consist of growth cones from which microspikes protrude. Cytochalasin B causes retraction of microspikes, rounding-up of growth cones, and cessation of axon elongation. Drug withdrawal is followed by resumption of growth cone and microspike activity and of axon elongation. In contrast, colchicine causes shortening and retraction of axons, but it does not initially affect the tips. Growth cones and microspikes of elongating axons contain a network of 50 Å microfilaments, the pattern of which is altered by cytochalasin treatment. These experiments indicate that both structural integrity of the axon and continuing function of its motile tip are essential elements in axonal elongation.

The tips of elongating axons consist of expanded regions called "growth cones" from which project long slender microspikes (filopodia). These microspikes continually wave about, extend, and retract as the growth cone moves over a substratum.[1] Although it is assumed that growth cones and microspikes play a significant role in axon extension, it has not been previously possible to analyze their functions.

We have investigated the effects of cytochalasin B on axon elongation since this drug halts cell movement,[2] a process similar in many respects to growth cone movement. Cytochalasin B is also known to inhibit cytokinesis[3] and morphogenetic movements,[4] apparently by disrupting contractile 50 Å microfilaments. The drug's effects upon nerve cells are compared with those of colchicine and colcemid, which disrupt microtubules[5] and cause retraction of elongating axons.[6]

Methods. Nerve ganglion culture: Lumbosacral dorsal root ganglia from 8-day-old white leghorn chick embryos were cultured in Grobstein tissue culture dishes[7] (Microchemical Specialties). The semisolid culture medium consisted of 0.05 ml of 1% agar in Hanks' balanced salt solution plus 0.1 ml of medium 199 containing 10% embryo extract,[8] 20% fetal calf serum, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B). Nerve growth factor was present as a 1:300,000 homogenate of submandibular glands from highly inbred BALB/c adult male mice;[9] this concentration of homogenate was found to be optimal for producing rapid outgrowth of axons.

Cell cultures: Dorsal root ganglia and the ventral halves of spinal cords were dissociated by a modification of Scott's procedure.[10] Lumbosacral dorsal root ganglia (80–120) from 8-day-old embryos were incubated at 37°C in 0.25% trypsin solution (Grand Island Biological). After 1 hr, the trypsin was aspirated, culture medium containing serum was added, and the ganglia were repeatedly flushed through a narrow bore pipet. The resulting cell suspension was pelleted at 500 *g* for 2 min and resuspended. After two such washes, the final dilution was made to approximately 10^5 cells/ml in nerve

growth factor-containing culture medium (modified F12 with 10% fetal calf serum).[11] Cell suspension (4 ml) was placed in 30-ml Falcon plastic tissue culture flasks, which were gassed with 5% CO_2 and incubated in a 37°C room. Or, 2–3 ml portions of suspension were placed in 35-mm diameter Falcon Petri-style culture dishes, which were incubated at 37°C in a humidified 5% CO_2 incubator. The ventral halves of 6-day-old chick embryo spinal cords were dissociated similarly and the cells were plated in the same nutrient medium, but without nerve growth factor.

After incubation for 20–25 hr (dorsal root ganglion cells) or 2–7 days (ventral spinal cord cells), cultures were examined in a 37°C room with an inverted microscope using phase contrast optics. The cells were sketched and were periodically measured with a filar micrometer before and after drug application.

Drugs: Stock solutions contained 1 mg of cytochalasin B/ml of dimethylsulfoxide or 10 or 100 μg of either colchicine (Calbiochem) or colcemid (CIBA Pharmaceutical Products) per ml Hanks' solution. In order to add one of these drugs to a ganglionic culture, an aliquot was mixed with medium and 0.05 ml of the mixture was pipetted onto the culture. For cell culture, 2–3 ml of medium was removed and mixed with an aliquot of the drug stock solution; the mixture was then gently pipetted back into the culture vessel. Control cultures were either left untouched or received an equivalent amount of Hanks' solution or dimethylsulfoxide. To obtain "recovery" from cytochalasin treatment, ganglia were excised from the semisolid medium, washed three times in cytochalasin-free culture medium, and reincubated in fresh semisolid medium containing nerve growth factor. Cell cultures were washed 4–5 times and reincubated in cytochalasin-free medium with nerve growth factor.

Electron microscopy: Control and cytochalasin-treated ganglia and ganglionic cell cultures were fixed in glutaraldehyde-paraformaldehyde and postfixed in osmium using previous methods.[12] Individual fixed ganglia and their surrounding areas of axon outgrowth were cut out of the semisolid agar matrix of ganglionic cultures, and the resulting blocks were embedded in epon for sectioning. Cell cultures were embedded in epon directly in the culture dishes. After polymerization, cells to be examined were marked using the inverted phase microscope. Epon blocks were cut, mounted, and sectioned parallel to the original floor of the Petri plate.[11]

Amino acid incorporation: Ganglionic cultures were incubated in 0.2 μCi/ml [^{14}C] amino acid mixture (New England Nuclear). For long-term labeling, the [^{14}C] amino acids were added to the medium at the time of explanation. For 2-hr pulses, the label was added to cultures in 0.05 ml of medium. After incubation, the cultures were chilled to 4°C on ice, sonicated, precipitated with cold 5% trichloroacetic acid, heated 30 min at 90°C, cooled and filtered through glass fiber filters (Whatman GF/C), and washed seven times with cold 5% trichloroacetic acid. The sonication, trichloroacetic acid precipitation, and first two washes contained unlabeled amino acids at 1000 times the concentration of each [^{14}C] amino acid. The precipitates were solubilized in 0.7 ml of NCS (Nuclear Chicago) and counted; disintegrations per minute were calculated.[13]

Results and Discussion. In ganglionic cultures, a dense halo of nerve axons grew from the dorsal root ganglia within 18 hr, as previously described by Levi-Montalcini.[14] All axon elongation was halted by both cytochalasin B (7 μg/ml medium; Fig. 1) and by colchicine (0.1 μg/ml) when these drugs were added to cultures either at the time of explantation or after outgrowth had begun.

Although such ganglion cultures demonstrated clearly that both drugs inhibit axon initiation and elongation, the agar matrix did not permit the colchicine-induced axon retraction seen in cell culture.[6] Furthermore, numerous axon-Schwann cell interactions in the cultures could have been the primary site of drug action, causing the inhibition of axon elongation secondarily. Therefore an alternate technique was adopted, involving cell culture in fluid medium; this

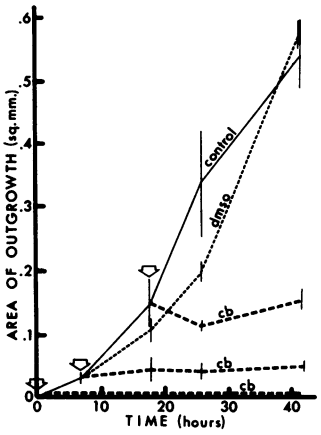

FIG. 1.—Effects of cytochalasin B and dimethylsulfoxide addition on axon outgrowth. The area of outgrowth was determined from camera lucida tracings, obtained with a dissecting microscope, of the halo of nerve axons growing out from cultured dorsal root ganglia. Arrows indicate time of these additions. Bars indicate ±1 SD.

provides a simplified system in which growing axons are free from all other cell and matrix contacts (except those with the plastic substratum).

The following results are a summary of sketches and periodic measurements of over 200 axons in ganglion and in ventral spinal cord cell cultures. Axons of ganglion cells grown in control medium elongate at 20–40 μm/hr; at the tips of all such axons are growth cones from which continually active microspikes protrude (Fig. 2*a*).

Addition of 7 or 10 μg/ml of cytochalasin B to cultures affects the tips of the axons first: 30 sec–3 min after addition, virtually all the microspikes "wilt" and begin to retract, leaving nearly bare growth cones (Fig. 2*b* and *c*); axon growth seases immediately. After 5–10 min, a peristaltic wave often passes proximally along the axon, leaving the distal portion of the axon thinner. In many cells, additional waves pass down the axon, and with each such event, the axon appears thinner and thinner, until it disappears. Of greater interest are the 10–20% of axons in any cytochalasin-treated culture which survive the early peristaltic events; of the axons remaining at 6 hr, 90% are still present and of exactly the same lengths after 12 more hr. Such axons demonstrate that cytochalasin B can halt axon elongation for 18 hr without causing axon retraction.

Replacement of the cytochalasin-containing medium with fresh, cytochalasin-free medium allows new growth cones and microspikes to form (Fig. 2*d*), and axon elongation starts anew within 4 hr. Furthermore, cells that had completely retracted their axons during this treatment initiate new axons tipped with growth cones and microspikes. Recovery occurs even in the presence of levels of cycloheximide (20 μg/ml) that decrease protein synthesis by 95%, as determined by autoradiographic analysis (grain counts over individual nerve cells) of [^{3}H]-leucine incorporation. Thus, both reinitiation of growth cone and microspike activity and resumption of axon elongation can occur in the absence of virtually all new protein synthesis.

Similar results are obtained with ventral spinal cord cell cultures, i.e., the earliest effects of cytochalasin B addition are on the microspikes and growth cones, axon growth ceases, and the axons remaining after 6 hr remain fixed in length for over 12 more hr.

Cytochalasin B (and its dimethylsulfoxide solubilizing agent) does not appear to be cytotoxic at the concentrations used: Cell bodies and axons of treated cells show no degenerative changes detectable with light or electron microscopy (Figs. 5 and 6; also see below); long-term and pulse [^{14}C] amino acid incorporation into hot trichloroacetic acid-insoluble material continues in ganglion cultures treated with cytochalasin B for 24 hr (Table 1; the explanation for the reduction from control values is currently under investigation)‡; and, finally, neurons in both ganglionic and cell cultures treated with cytochalasin B for 24 hr resume normal growth after removal of the drug and reincubation in normal medium.

The results of the cytochalasin experiments, in combination with the known effects of the drug on other cell types,[3,4] suggested to us that axon elongation might depend on 50 Å diameter contractile microfilament systems in the growth cone and microspikes.

The growth cones, microspikes, and peripheral cytoplasm of untreated axons are found to contain a network of fine filaments (Figs. 3 and 4). This network is the only organelle found in the microspikes and outer layer of the growth cone, except for occasional membrane-bounded vesicles, which may originate from the smooth endoplasmic reticulum of the growth cone. The network is composed of 40–60 Å filaments arranged in a polygonal (frequently hexagonal) pattern. It inserts upon the plasma membrane at its periphery, and upon the walls of neurotubules (microtubules), and perhaps upon 100 Å neurofilaments, internally; thus the filamentous network can be thought of as a framework linking the main longitudinal skeletal elements of the axon (neurotubules and neurofilaments) with the cell surface. In microspikes, the symmetric polygonal pattern of filaments is usually distorted to a linearly oriented pattern in which the polygons are highly elongated, with their long axes parallel to the microspike axis (Fig. 4). In scattered places near the bases of microspikes, "dense" regions occur in the network; in such regions the diameter of the polygons is reduced and minute circular structures are often seen.

Nerve cells fixed after 8–10 min in cytochalasin B possess growth cones which are rounded and have few microspikes (Fig. 5). In contrast to the straight microspikes of control cells, the few remaining on cytochalasin-treated cells are invariably curved, shortened, and broad in appearance. Coincident with these gross alterations is the virtual disappearance of the elongated polygonal filament pattern seen in control microspikes. Instead, the microspikes contain either a symmetrical pattern of polygons or extensive dense regions of minute polygons. In these cells, or in cells fixed after treatment for 8 or 16 hr in cytochalasin B, such dense regions also appear to be more frequent in the outer layer of the growth cone. These dense regions in growth cones and microspikes resemble the masses of granular and fibrillar material seen in cytochalasin-treated epithelia of mouse salivary gland and of chick oviduct.[4] Apparently, cytochalasin treatment of nerve cells does not completely disrupt the polygonal pattern of filaments, but does alter the shape of portions of the network in both microspikes and growth cones. Since this microfilament network is the only visible organelle—in addition to plasma membrane—of the peripheral growth cone and microspikes, it can be presumed that the observed alterations are linked in some

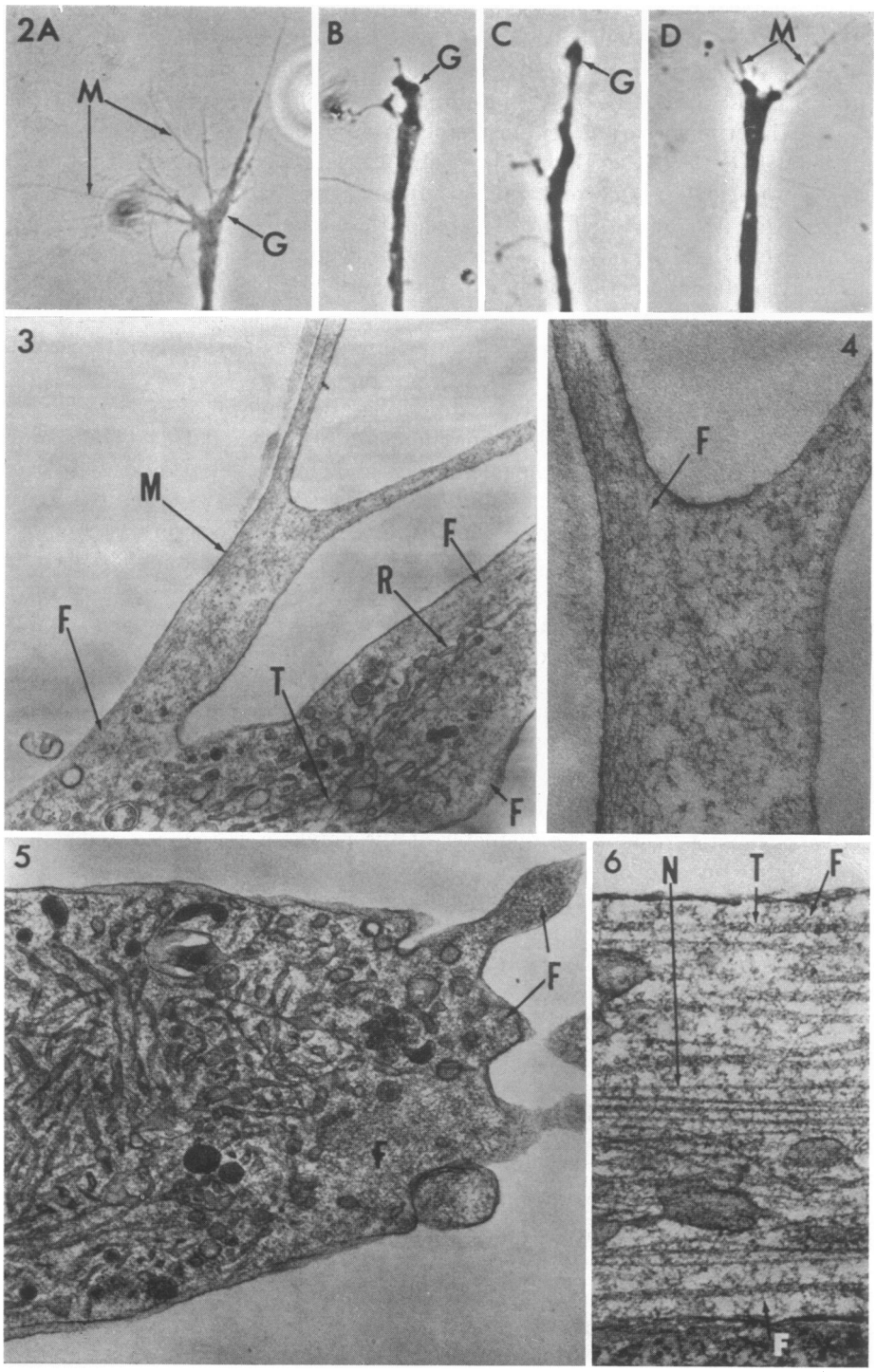
2A
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TABLE 1. [^{14}C] *amino acid incorporation in 24 hr ganglion cultures.*

Label	Control	Dimethylsulfoxide	Cytochalasin B
Pulse (22–24 hr)	251 ± 30 dpm*	250 ± 16 dpm	207 ± 34 dpm
Long term (0–24 hr)	1360 ± 210 dpm	1300 ± 200 dpm	842 ± 150 dpm

* Disintegrations/min per ganglion, mean ± 1 standard deviation.
Pulse labeling: 10 ganglia per culture; 300 ganglia total.
Long-term labeling: 5 ganglia per culture; 265 ganglia total.

way to the cytochalasin-induced retraction of microspikes, rounding of growth cones, and cessation of axonal elongation. All other cytoplasmic organelles, including neurotubules and neurofilaments, appear normal in structure and distribution in cell and ganglionic cultures fixed after cytochalasin treatment for as long as 18 hr (Fig. 6).

In contrast to cytochalasin B, colchicine and colcemid are thought to disrupt microtubules.[5] When added to cultures of dorsal root ganglion cells, these drugs have no early effects on either the shape or the activity of growth cones and microspikes; instead, these drugs appear to affect the axons first. Axon elongation appears normal for 20–30 min after addition of colchicine or colcemid (0.1 or 1 μg/ml medium); some axons have been measured to elongate up to 30 μm during this period of no apparent drug effect. After this lag, the axons begin to shorten. During the initial phases of this retraction, the growth cones and microspikes of shortening axons remain normal in appearance and activity. Later, as axon retraction continues, the tip regions become badly distorted, and the microspikes disappear. The majority of axons in a culture retract by 1 hr, over 95% retract by 6 hr, and virtually none can be found after 18 hr. Ventral spinal cord cell axons treated with colchicine behave similarly.

The electron micrographs of control and cytochalasin-treated cells demonstrate that the large numbers of neurotubules in the axons terminate within the growth cones and never extend into the 50 Å filament networks of microspikes and of the outer layers of growth cones. Our observations that colchicine and colcemid affect axons first and have no early effects on growth cones and micro-

FIG. 2.—(*A*) A growth cone (*G*) and microspikes (*M*) of an elongating dorsal root ganglion nerve cell. (*B*) A similar axonal ending, 6 min after addition of cytochalasin. Most microspikes have retracted. (*C*) A similar axonal ending, 5 min after cytochalasin application. (*D*) An example of recovery from cytochalasin treatment. This cell had been treated with 10 μg/ml cytochalasin B for 18 hr; at that time the ending appeared similar to that in (*C*). Cytochalasin B was removed, and the culture was reincubated for 17 hr. Microspikes have reappeared and axon elongation has started anew (×900).

FIG. 3.—A typical growth cone region showing the outer filamentous zone (*F*) and a bifurcating microspike (*M*) containing filaments. Smooth endoplasmic reticulum (*R*); microtubule (*T*) (×19,000).

FIG. 4.—The microspike of Figure 3, showing the filamentous network (*F*) and absence of microtubules and neurofilaments (see Fig. 6). The asymmetric polygonal pattern of the filaments is evident on the left side of this thin section (×63,000).

FIG. 5.—A typical "rounded" growth cone of a cytochalasin-treated cell. Microspikes are retracting, and the filamentous network (*F*) appears more dense than in untreated cells. Other growth cone organelles appear normal (×27,000).

FIG. 6.—A portion of an axon in the presence of cytochalasin B for 16 hr prior to fixation. Microtubules (*T*), neurofilaments (*N*), and the filamentous network (*F*) are intact. Note the numerous interconnections of the fine filaments with plasma membrane, microtubules, and neurofilaments (×38,000).

spikes provide additional evidence for the hypothesis that neurotubules provide support for the axon,[15] and that these drugs cause retraction of axons by disrupting the framework of neurotubules,[6] possibly by binding to the neurotubular protein subunits.[16] Although it is possible that such retraction is an indirect result of inhibition of axonal transport, a process dependent upon microtubules,[17] the speed of retraction is so great that it seems more reasonable to assign a structural role for the microtubules in the maintenance of axon shape.

These experiments suggest that at least two cytoplasmic elements are necessary for axon elongation. Networks of 50 Å microfilament are apparently required for structural integrity and function of the growth cone and its microspikes. Similar cytochalasin-sensitive microfilaments are involved in sea urchin cytokinesis,[3] mouse salivary gland morphogenesis, and chick oviduct gland formation,[4] all cases in which interference with the presumed contractile microfilament systems has drastic developmental effects.

The other cytoplasmic element required for axon elongation, the neurotubule (microtubule), appears to be an essential skeletal member of the cell, similar to the microtubular cytoskeletons of the long axopodial arms of the protozoan *Actinosphaerium*.[18] Neither the growth cone, as an active motile organelle, nor the axon, with its rigid neurotubular skeleton, can function alone to produce axon elongation; both must act if this important developmental process is to occur.

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‡ *Note added in proof:* Cytochalasin B has no effect upon the incorporation of [^{3}H] leucine by individual nerve cells. Thus, in autoradiograms of cell cultures, the number of grains over individual control nerve cells does not differ significantly from the number of grains over cytochalasin-treated ones.

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