Growth of Neurites without Filopodial or Lamellipodial Activity in the Presence of Cytochalasin B

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ABSTRACT   To examine the role in neurite growth of actin-mediated tensions within growth cones, we cultured chick embryo dorsal root ganglion cells on various substrata in the presence of cytochalasin B. Time-lapse video recording was used to monitor behaviors of living cells, and cytoskeletal arrangements in neurites were assessed via immunofluorescence and electron microscopic observations of thin sections and whole, detergent-extracted cells decorated with the S1 fragment of myosin. On highly adhesive substrata, nerve cells were observed to extend numerous (though peculiarly oriented) neurites in the presence of cytochalasin, despite their lack of both filopodia and lamellipodia or the orderly actin networks characteristic of typical growth cones. We concluded that growth cone activity is not necessary for neurite elongation, although actin arrays seem important in mediating characteristics of substratum selectivity and neurite shape.

Elaborate networks and bundles of actin filaments are prevalent throughout the growth cone regions, and particularly characterize the filopodial extensions, of neurite tips in culture. These filamentous arrays are readily demonstrated in whole-mount and detergent-extracted cytoskeletal preparations in which actin filaments are only occasionally identified more proximally along neurites (15–17, 26). Thus it seems that there is a state of neuronal actin that is rather unique to growth cones and that presumably is reflected in the dynamic motile activities of these structures.

The growth cones of living nerve cells appear to actively explore their environments, cyclically protruding and retracting (1) to make successive tentative contacts with their substrata and with other cells (14). They may be observed, upon establishing cellular contacts, to exert forces capable of displacing those processes to which they adhere (14), and they may be observed to make "choices" among variably adhesive substrata, such that their preferred sites of attachment select the paths to be followed by growing fibers (13–15).

It is thus intuitively appealing to suppose that filopodial/lamellipodial activity is essential for neurite initiation and extension, and it is proposed that actin-mediated tensions within adherant growth cones may be mechanically translated into more proximal cytoskeletal rearrangements, thereby initiating appropriate cell shape changes and/or directing transport processes necessary for growth (3, 4, 13–17, 26). Indeed a cessation of neurite growth has been reported to accompany the disorganization of actin filaments and retraction of filopodia induced by cytochalasin B (CB)[1] (26). However, the growth of neurons on sufficiently adhesive substrata is here shown to be not inhibited by CB, and indeed, neurons initially plated in the presence of CB are shown to extend profuse neuritic arborizations, unaided by morphologically identifiable filopodia or lamellipodia, or by any actin organization resembling their characteristic filament bundles. Pulling actions of growth cones are thus apparently not requisite for nerve fiber elongation per se. Morphological peculiarities and stringent substratum requirements of neurons cultured with CB, however, suggest more subtle ways in which actin filaments might participate in neurite extension.

MATERIALS AND METHODS

Cell Cultures: Dorsal root ganglia from 9–11-d-old chick embryos were trypsin-dissociated and cultured as previously described (13) in an equal mixture of Ham's F12 and heart conditioned medium (9), supplemented with 10% calf serum (Sterile Systems, Inc., Logan, UT) and 10 ng/ml nerve growth factor (a gift of Dr. Eric Shooter, Stanford University). For fluorescent staining and detergent extraction, cells were plated in Petri dishes on, respectively, glass coverslips or gold electron microscope grids overlayed with Formvar films, which had been carbon-coated and treated with polyornithine (13, 15). Other cells were cultured directly on either polyornithine-treated or untreated Petri and tissue culture plastic.

A stock solution of CB (Sigma Chemical Co., St. Louis, MO) was prepared

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[1] *Abbreviations used in this paper:*   CB, cytochalasin B.

in DMSO at 1 mg/ml; further dilutions were made with F12 culture medium. 0.01–10.00 µg/ml (final concentration) of CB was added to experimental cultures, either initially or after 1 d in culture, with control cultures receiving equivalent doses of DMSO. Cells were cultured for 1–4 d in 5% CO_2 at 37°C.

Observation of Living Cells: For observation of living cells, glass coverslips were glued over 22-mm holes drilled in Falcon 1006 50-mm Petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA), and cultures were prepared as above. Immediately before viewing, culture medium was replaced with a small amount of warm HEPES-buffered F12 (similarly supplemented with serum and nerve growth factor), and a second coverslip was mounted over chips of glass to create a small chamber, which was then sealed with silicone grease. The microscope stage was warmed to 37°C by an air-stream incubator (Nicholson Precision Instruments, Inc., Gaithersburg, MD), and either neurites were photographed directly on Tech-Pan film, or neurite behavior was recorded with a model 65 SIT camera (Dage-MTI, Inc., Michigan City, IN) and a Panasonic NV-8030 time-lapse video recorder. Individual frames from videotapes were photographed from the video monitor on Pan-X film with a 35-mm SLR camera.

Addition of CB to Previously Established Cultures: We identified cells growing directly on tissue culture and petri dishes by etching the bottoms of the dishes with a diamond objective and then photographed them before addition of 10 µg/ml of CB to the culture medium. The same cells were photographed again 1 d later.

Actin Immunofluorescent Staining: Cells on coverslips were fixed at 37°C for 30 min with 1% glutaraldehyde in PBS, rinsed, and quenched with 0.5 mg/ml NaBH₄ in Ca⁺⁺/Mg⁺⁺-free PBS for 15 min. A solution of 0.4% Triton X-100 in PBS with 10 mg/ml BSA was used to permeabilize the fixed cells for 10 min before a 30-min incubation with rabbit antiactin antibodies (a gift of Dr. Judith Schollmeyer, Roman Hruska Meat Research Center) diluted 1:200 with the same solution (16, 17). Cells were then rinsed thoroughly and incubated another 30 min with fluorescent goat anti-rabbit antisera (Cappel Laboratories, Inc., Cochranville, PA), similarly diluted.

E/C8 Immunofluorescent Staining: Cultures were stained with a reportedly neuron-specific monoclonal antibody, E/C8 (provided by Drs. Gary Ciment and James Weston, University of Oregon) (8), to verify their neuronal character. After a 30-min fixation at 37°C with 4% paraformaldehyde in Ca⁺⁺/Mg⁺⁺-free PBS, they were incubated for 60 min with 0.2% Triton X-100 in Ca⁺⁺/Mg⁺⁺-free PBS that contained 5 mg/ml BSA, then for 45 min with E/C8-conditioned medium diluted 1:50 with the permeabilizing solution, rinsed, incubated 15 min more with the same solution, and finally incubated for 30 min with a 1:80 dilution of fluorescent rabbit anti-mouse antisera (Cappel Laboratories, Inc.).

Phase-contrast and Fluorescent Microscopy: Following fixation and staining, coverslips were rinsed several times and mounted in Elvanol for examination with a Zeiss IM microscope or GFL microscope equipped for fluorescence observations with epi-illumination. Photographs were made on 35-mm Tri-X film, using a 63× planapochromat objective.

Cytoskeleton Preparation and S1 Decoration: Extraction and fixation for electron microscopy were carried out in a cytoskeleton-stabilizing "PHEM" buffer (23) consisting of 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl₂. Cells on electron microscope grids were rinsed with the PHEM buffer and extracted for 4 min with 0.2% Triton X-100 in PHEM containing 20% glycerol and 1 mg/ml of the S1 fragment of heavy meromyosin (a gift from Dr. David Thomas, University of Minnesota). They were then rinsed twice more and fixed for 30 min with 0.2% glutaraldehyde in PHEM. Fixed cytoskeletons were stained with 0.4% tannic acid in water for 30 min and with 2% aqueous uranyl acetate for 30 min, and then rinsed, dehydrated through ethanol, critical-point dried, and coated with carbon. Grids were examined at 80 kV with a JEOL 100 CX electron microscope.

Thin-section Electron Microscopy: For transmission electron microscopy, cells in polyornithine-treated culture dishes were fixed for 30 min with 1% glutaraldehyde and for 1 h with 1% OsO₄, then dehydrated through ethanol and embedded in resin (Polybed, Polysciences, Inc., Warrington, PA). Blocks were then removed from the dishes, and sections that were cut parallel to the substratum were stained with 2% aqueous uranyl acetate and lead citrate. Observation was made at 60 kV.

RESULTS

Morphology and Behavior

Cells were consistently observed to extend long, neurite-like processes on polyornithine-treated substrata in the presence of CB in concentrations of up to 10 µg/ml, for periods of up to 4 d in culture. (No attempt was made to sustain
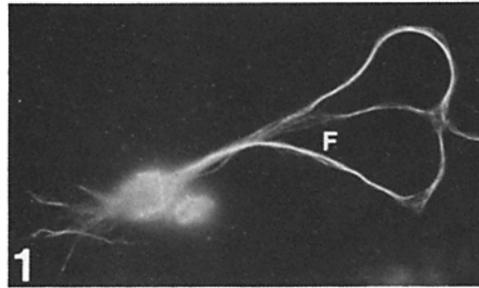

FIGURE 1 Fibers (F) initiated by nerve cells in the presence of CB (5 µg/ml) stained intensely with the neuron-specific antibody, E/C8. Other cells, lacking such processes, were unlabeled. Note (see also Figs. 2–5) the large number of fibers fraying from the cell body and the looping neurites. Filopodia and lamellipodia, or growth cone-like structures, are not evident. × 870.

cultures for longer periods.) Their distinct expression (Fig. 1) of the neuronal antigen, E/C8 (8), was taken as evidence that such cells were indeed neurons. (Work of Ciment, G., A. H. Ressler, P. C. Letourneau, and J. A. Weston, in preparation, indicates that the antigen is a neurofilament-associated protein.) Neurites achieved lengths of several hundred micrometers (Fig. 2), displaying elaborate loopy contortions and an excessive degree of branching, but no filopodia or lamellipodia.

The previous demonstration (26), on untreated plastic, of CB-induced paralysis of growing nerve fibers was verified here. That is, further growth was not apparent following the addition of CB to cultures already established on unmodified tissue culture or Petri dishes. Indeed, as noted in the earlier study, the majority of such neurites actually retracted. Likewise, neurites were rarely produced by cells initially plated in CB-containing medium on untreated surfaces. However polyornithine-treated surfaces undoubtedly did support continued neurite elongation after addition of CB (Fig. 3). Neurites thus subjected to cytochalasin following their initial growth in its absence tended to acquire rounded tips and looping shapes like those continuously exposed to the drug.

Time-lapse films of living neurons in the presence of CB clearly demonstrated the elongation of these blunt-ended processes (Fig. 4), without any apparent tensile activity suggestive of growth cones. Thus, in contrast to the flailing about and substratum "sampling" which filopodia and lamellipodia typically do (14), and the neurite stretching that commonly appears subsequent to the establishment of filopodial adhesions, the tips of neurites in CB gave the impression of simply extruding material. This sometimes seemed to occur in a pulsatile fashion, but with no suggestion of the cyclic pattern of protrusion and retraction typical of filopodia- and lamellipodia-bearing growth cones (1).

Certain of the peculiar looping processes typical of these cells appeared to be thus generated by the simple deposition of material along circular paths. But curiously, others evolved not from "ends" at all, but from flattened out areas at more proximal positions along neurites. Fig. 5 illustrates a video sequence in which one such loop was generated by the apparent tearing of a hole in a sheet of membrane.

Saltatory, bidirectional, intracellular particle movements were commonly evident all along neurites, around circular processes, and across flattened extensions.

Clearly these sorts of morphological and behavioral effects of CB do not readily lend themselves to precise characteriza-
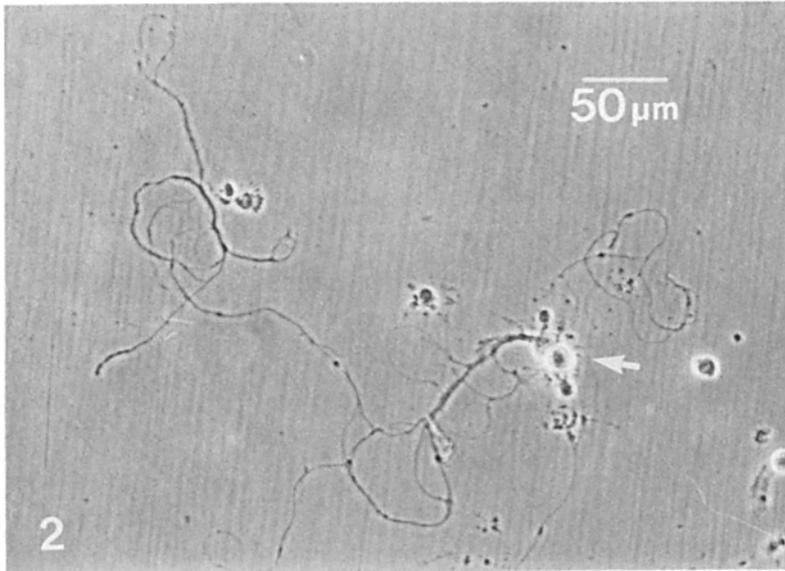
FIGURE 2 Neurons cultured for 4 d in the continuous presence of 10 μg/ml CB generated elaborate neuritic formations several hundred micrometers long. Illustrated is the arborization of a single nerve cell; the arrow indicates the cell body. × 230.
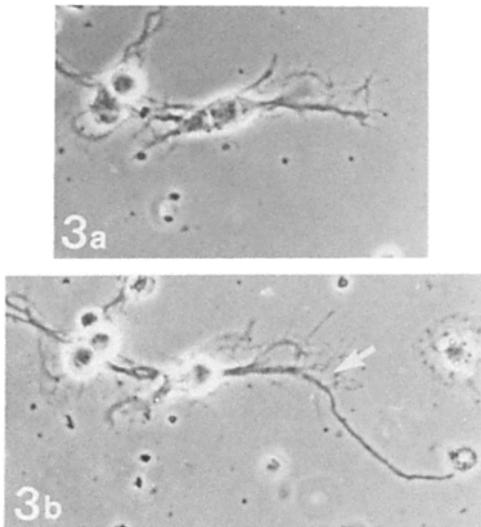

FIGURE 3 (a) A nerve cell cultured for 1 d on polyornithine-treated plastic was photographed before the addition of 10 μg/ml CB. (b) The same cell, photographed 15 h later, has grown ~90 μm (from arrow) in the presence of the CB. × 480.

FIGURE 4 Two frames from a video sequence illustrate the growth of a blunt-ended nerve fiber (arrows) in the presence of 5 μg/ml CB. Time is displayed at the upper left. The process extended 15 μm over a period of 41 min, and at no time did it protrude any semblance of lamellipodia or filopodia. × 640.

tion of their dose dependency. However it was qualitatively noted that lower concentrations of CB permitted closer approximations to typical growth cone structures and activities (Fig. 6). Thus, with increasing doses of CB, neurite tips tended to appear more round and smooth, and the dramatic ruffling and probing activities of filopodial and lamellipodial protrusions tended to give way to localized twitching and quivering along neurites and at their tips.

Immunofluorescent Localization of Actin

Whereas in control cells actin fluorescence was weak and diffuse along neurites and concentrated only at growth cones (Fig. 7, inset, and references 16 and 17), neurons cultured with CB were peppered with discrete concentrations of actin along their processes (Fig. 7). This atypical actin distribution in neurons was correlated with a lack of typical stress fiber staining in fibroblast-like cells, which displayed similar blotches of actin fluorescence (Fig. 7). Actin concentrations
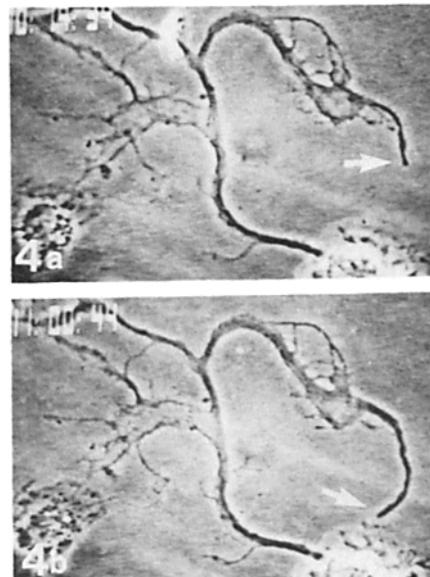
were sometimes, but not consistently, observed at the tips of CB-cultured neurons, despite the absence there of filopodia. Again, the CB concentration dependence of actin distribution was subtle and continuous; a critical concentration for anomalous staining was not detectable.

Ultrastructure

As in the neurites of untreated cells and those exposed to CB subsequent to neurite extension (26), microtubules and neurofilaments were numerous throughout the lengths of neurites generated in the presence of CB. These were evident in electron micrographs of both thin sections (Figs. 8 and 9) and cytoskeletons (Fig. 10c), consistent with intense immunofluorescent demonstrations of the purported neurofilament-associated E/C8 protein (Fig. 1) and of tubulin (not
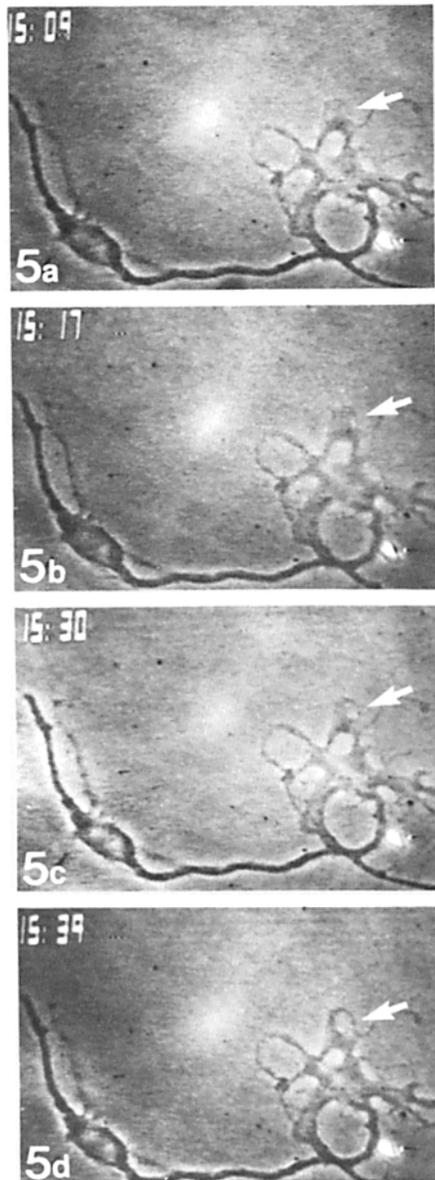
FIGURE 5   A sequence spanning 30 s illustrates the generation of a loop via the tearing of a flattened region (arrows) along a neurite growing in the presence of CB (2 μg/ml). Time appears at the upper left. (a) Material has accumulated in a broad lamellar extension, in which (b) a tiny hole appears and (c) rapidly widens, until (d) only the boundary remains. × 640.

shown). Also, as in typical growth cones and in those blunted by CB-induced filopodia retraction (26), bundled microtubules and neurofilaments terminated somewhat proximally to the neurite tip (Fig. 9). However, the microfilament lattice, which in untreated neurons dominates the growth cone margin and its protrusions (16, 17), was absent in cells grown with CB, and in its place an indistinct granular matrix enclosed a heterogeneous collection of vesicles. This organization, too, was reminiscent of that described following treatment of previously extended growth cones with CB (26).

In extracted cytoskeletons, the S1 myosin fragment failed to identify orderly networks of actin in either neurons or fibroblast-like cells cultured with high doses (5 μg/ml) of CB, although short decorated filaments were occasionally observed in both. In such cases, the filaments tended to be

associated with aggregates of extraction-resistant material (Fig. 10, a and b) whose distribution resembled that of the above-noted concentrations of actin fluorescence (Figs. 7 and 10c). Similar aggregates were never noted in control cells.

DISCUSSION

It seems clear that under certain conditions, neurons in culture are quite capable of elaborating neuritic processes without the participation of filopodia or lamellipodia. Furthermore, the exhibition of particulate motion and the undisputable deposition of material at their distal ends, by neurons devoid of their usual arrangements of filamentous actin, indicate that
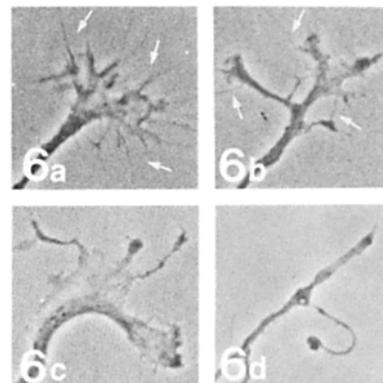

FIGURE 6   Neurite tips displayed increasingly unusual morphologies in the presence of increasing doses of CB. (a) Cells in 0.01 μg/ml CB had quite typical growth cones, bearing lamellipodia and filopodia (arrows) which demonstrated vigorous ruffling and protrusion/retraction activities in video recordings. (b) Cells cultured with 0.10 μg/ml CB retained filopodia-like structures (arrows), but appeared "shriveled" and lacking in veil-like lamellipodia. Ruffling activity was less dramatic in time-lapse recordings. (c) Cells cultured with 0.30 μg/ml CB showed little semblance of filopodia, and only flacid flattened areas spreading out along neurites and at termini. Activity was evident only at the ends of processes, and resembled pulsatile "twitching." (d) At CB concentrations of up to 10 μg/ml, neurons generated long, blunt-ended fibers, with quivering at neurite tips. × 870.
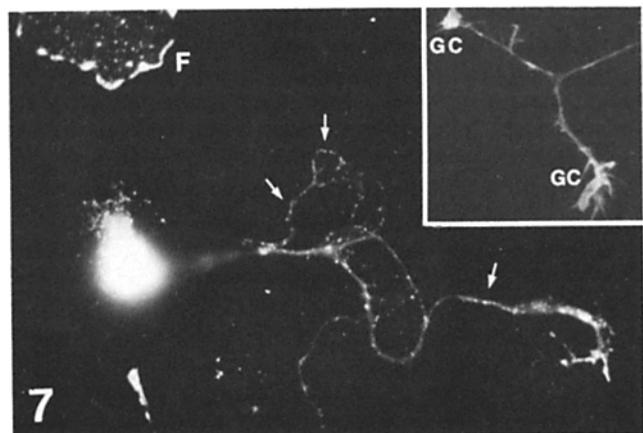

FIGURE 7   Control neurons (inset) exhibited weak and diffuse actin staining along fibers, with more intense labeling at their growth cones (GC). Neurons cultured with CB (5 μg/ml) displayed flecks of actin fluorescence (arrows) along their processes and throughout flattened regions. Actin concentrations were sometimes, but not always, apparent at the ends of neurites. Fibroblast-like cells (F) in the CB-containing cultures also displayed blotchy concentrations of actin, as opposed to typical stress fiber formations. × 870.
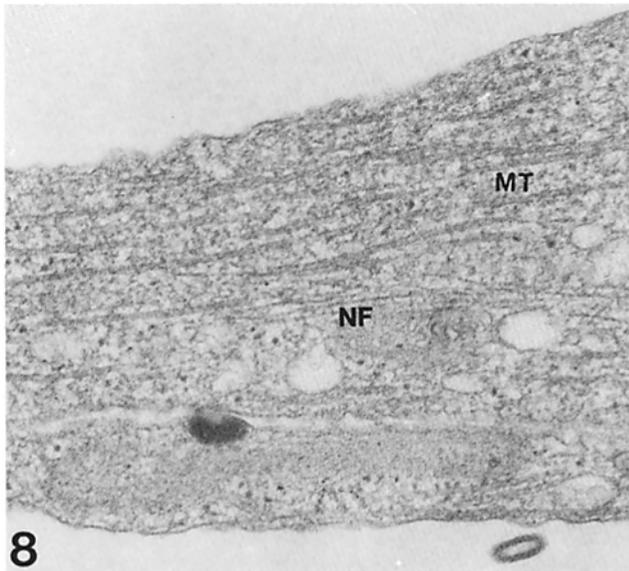
FIGURE 8 Neuronal processes generated in the presence of CB were ultrastructurally similar to typical neurites. Neurofilaments (NF) and microtubules (MT) were evident throughout their lengths. × 55,500.

at least certain components of the neuronal transport mechanism are not dependent upon a CB-sensitive actin assembly. Nonetheless, the grossly atypical configurations assumed by these filament-deficient neurites do suggest some manner of participation by actin filaments in the regulation of cell shape, and their requirement for a highly adhesive substratum would seem to confirm that cell surface and cytoskeletal organizations are intimately interrelated.

Certain non-neuronal cells cultured with CB assume arbored, neuron-like morphologies in a manner said to depend upon the integrity of microtubules (2, 24) and possibly intermediate filaments (21). The capacity for extending cylindrical processes, then, is clearly not uniquely intrinsic to neurons nor does it require growth cone-like specializations. Rather, it may simply reflect cytoskeletal activities of growing, adherant cells which are subject to moderation by actin-mediated tendencies for spreading and flattening.

However arborization in the presence of CB is not the rule among non-neuronal cells; it was rarely exhibited by the non-neuronal cells in these primary cultures. Where reported in fibroblasts (21), CB-initiated processes are distinct from neurites in their dense and orderly arrays of intermediate filaments and their sparcity of microtubules. Other reports (2, 24), involving cell lines, do not offer ultrastructural data, but suggest, on pharmacological grounds, that process extension requires microtubules. It is thus unnecessary to presume that a common mechanism of process extension exists among non-neurons or that fibroblastic arbors share any features with neurites other than their cylindrical shapes and their lack of actin networks. Certainly neurites, with or without growth cones, are at least set apart from the CB-induced processes of other cells by the overwhelmingly greater lengths they attain.

The unusually profuse elaboration of neuritic fibers by neurons cultured with CB would perhaps be consistent with the notion that actin-mediated tensions play some restrictive role in neurite formation. One could imagine, for example, that actin arrays might regulate cytoplasmic turgor or membrane integrity in ways that can modulate the transport and

insertion of nascent membrane or protrusion of cytoskeletal branches. For example, subjective impressions prompted by behaviors such as those illustrated in Fig. 5 are that the membranes of neurons cultured with CB may be unusually fluid and fragile, and that their excessive branching may reflect a lack of intramembrane tensions, as opposed to transmembrane pulling forces.

The significance of the apparent actin aggregates in the CB-cultured cells is unclear. Similar aggregates appear after treatment of previously spread fibroblasts with cytochalasins (11, 22, 25), and CB induces a condensation of the actin lattice in previously extended neurites (26). In such cases, the actin accumulations are commonly supposed to represent the foci of a hypercontraction of formerly extended actin networks, which occurs within minutes of exposure to the drug. It is thus particularly curious that a similar distribution is established by cells grown in the continuous presence of CB.

The mechanisms of cytochalasin action and of actin assembly in cells are not well characterized, but there is considerable evidence that cytochalasin interacts directly with actin fila-
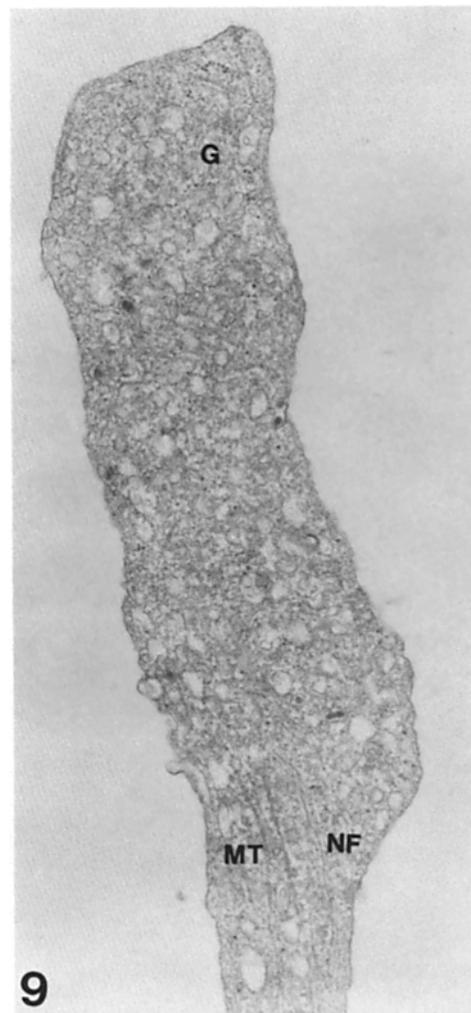

FIGURE 9 The tips of neurites produced in the continuous presence of CB resembled growth cones pictured by others (26) subsequent to CB-induced retraction of their filopodia. Microtubules (MT) and neurofilaments (NF) terminate in the proximal region, and numerous vesicles populate the terminal area, where an indistinct granular matrix (G) replaces the microfilament lattice typical of untreated growth cones. × 27,000.
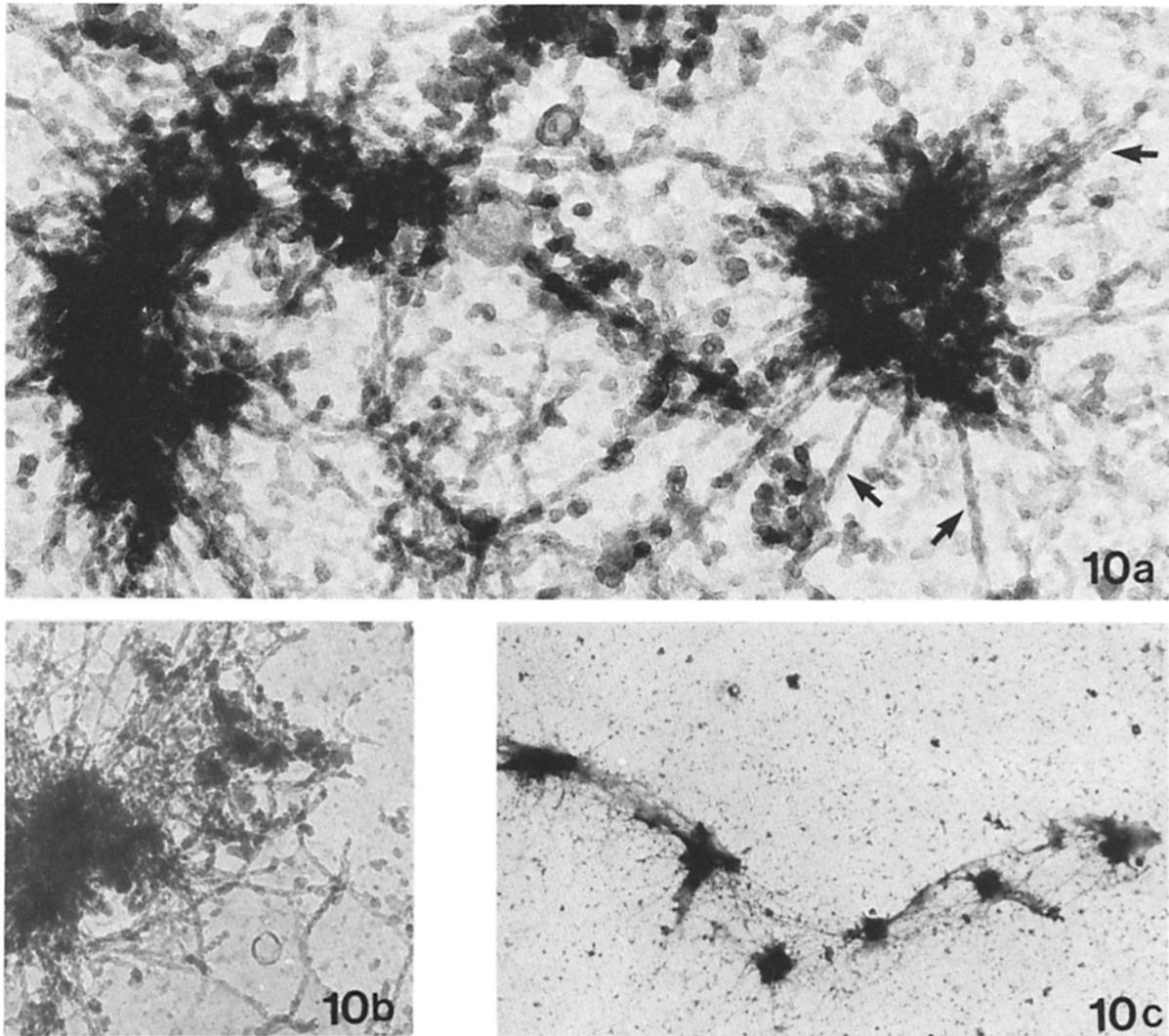
FIGURE 10 (a) The S1 decoration of a neurite generated in the presence of 5 μg/ml CB reveals sparse, short actin filaments (arrows) associated with clumps of detergent-resistant material along the extracted nerve fiber. (b) In the cytoskeleton of a fibroblast-like cell from a CB-containing culture, numerous short S1-labeled filaments radiate from a detergent-resistant aggregate. (c) The extracted terminal segment of a neuronal process generated in the presence of CB (5 μg/ml) displays a periodic distribution of extraction-resistant aggregates such as in a, which may correspond to the fluorescent actin peppering noted in Fig. 7. Microtubules and neurofilaments extend between the aggregates and frequently fray out along the sides of the neurite. (a) × 74,750; (b) × 41,500; (c) × 6,600.

ments (5–7, 10, 12, 18–20), and at least in vitro, inhibits further monomer addition at the "preferred" end for polymerization (5, 6, 10, 18, 19). Polymerization at the "less preferred" end, however, is thought not to be cytochalasin sensitive (5, 19). Perhaps, then, the actin aggregates along neurites that grow in the presence of CB represent nuclei for some aborted filament assembly process—whether they be newly established or the transported remnants of a formerly expanded network.

The question, of course, remains, for what are filopodia and lamellipodia used, if not for pulling out growing fibers? The failure of growth cone-deficient neurites to grow on less than optimally adhesive substrata certainly suggests some anchoring function for these structures. Additionally, their lack of substratum sampling and selecting behaviors, and their tortuous shapes, suggest that sensing and steering functions of filopodia and lamellipodia may be necessary for properly orienting neurites as they grow. Furthermore, the three-dimensional protrusion of filopodia and lamellipodia,

not restricted to the cell's substratum, may enable a growth cone to cross physical or chemical boundaries, and thus reach areas otherwise inaccessible to a cell limited to flowing along a continuously adhesive surface. Thus, while filopodial and lamellipodial activities may typically select adhesive contacts and determine paths along which nerve fibers are assembled, the undaunted growth of neurites without filopodia or lamellipodia clearly indicates that tensions generated by these projections are not necessary for eliciting neurite assembly.

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