Evidence That Calcium May Control Neurite Outgrowth by Regulating the Stability of Actin Filaments

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Abstract. We investigated the effects of calcium removal and calcium ionophores on the behavior and ultrastructure of cultured chick dorsal root ganglia (DRG) neurons to identify possible mechanisms by which calcium might regulate neurite outgrowth. Both calcium removal and the addition of calcium ionophores A23187 or ionomycin blocked outgrowth in previously elongating neurites, although in the case of calcium ionophores, changes in growth cone shape and retraction of neurites were also observed. Treatment with calcium ionophores significantly increased growth cone calcium. The ability of the microtubule stabilizing agent taxol to block A23187-induced neurite retraction and the ability of the actin stabilizing agent phalloidin to reverse both A23187-induced growth cone collapse and neurite retraction suggested that calcium acted on the cytoskeleton. Whole mount electron micrographs revealed an apparent disruption of actin filaments in the periphery (but not filopodia) of growth cones that were exposed to calcium ionophores in medium with normal calcium concentrations. This effect was not seen in cells treated with calcium ionophores in calcium-free medium or cells treated with the monovalent cation ionophore monensin, indicating that these effects were calcium specific. Ultrastructure of Triton X-100 extracted whole mounts further indicated that both microtubules and microfilaments may be more stable or extraction resistant after treatments which lower intracellular calcium. Taken together, the data suggest that calcium may control neurite elongation at least in part by regulating actin filament stability, and support a model for neurite outgrowth involving a balance between assembly and disassembly of the cytoskeleton.

THE neuronal growth cone is a structurally and functionally unique part of the developing neuron that plays a key role in the morphological differentiation of the neuron. The growth cone is the site of new membrane addition (6, 7, 46, 47) and recycling (11) and the major, if not exclusive, site of addition of new microtubule subunits to the elongating neurite (4). The growth cone is also the site of action of all known neurite outgrowth promoting or inhibiting influences, where the site of action has been established (for review of recent work in this field, see reference 25). This highly motile structure is also unique in that its behavior appears to be largely autonomous from direct control by the cell body (55) and, as such, can serve as a useful model system for understanding control of cell shape and motility.

Many studies have implicated calcium ions in the regulation of neurite outgrowth in both vertebrate (2, 13, 35, 36, 60) and invertebrate (17, 40, 41) neurons, as well as in cell division (23, 48), spreading (29, 45), shape changes (20, 22), and migration (14, 38, 42, 43) of other cell types (for reviews of factors affecting cell shape and motility, see references 21 and 56, and for reviews of regulatory functions of calcium, see 10 and 51). Recently, an inward directed calcium current has been identified in growth cones of actively extending neurites, but not spontaneously inactive growth cones (2) and calcium channel blockers or removal of extracellular calcium have been reported to inhibit neurite extension in a number of different cell types (2, 17, 60). Measurements of free calcium concentrations within growth cones have further shown that calcium levels in growth cones of elongating neurites are consistently higher than concentrations in spontaneously stopped growth cones (13, 26, 41), and that conversely, large rapid increases in growth cone calcium are associated with at least two classes of stimuli that inhibit neurite outgrowth (40). Taken together, the data indicate that both high and low growth cone calcium concentrations may be incompatible with neurite extension, but the mechanism of action of calcium in regulating outgrowth remains unclear.

Calcium ions are involved in a number of processes necessary for neurite extension. Calcium ions mediate cadherin based cell–cell and cell–substratum attachments (18), and lowered extracellular calcium concentrations could inhibit neurite outgrowth by reducing substrate attachment. Calcium ions are also involved in kinesin-based rapid transport of organelles (65) and in vesicle fusion to the plasma membrane (3, 61), and alterations in intracellular calcium concentration could therefore potentially disrupt transport or polarized insertion of new membrane. The actin–myosin force generation system, thought to be an important force in pull-

ing growth cones forward (8, 33, 58), is also influenced by calcium ions, and this system could become paralyzed or uncoordinated in the presence of greatly lowered or elevated intracellular calcium. In addition, calcium ions influence the modification (9) polymerization/depolymerization (20, 28, 54) and enzymatic degradation (5, 15, 24, 44) of many different cytoskeletal components (for reviews of actin and microtubule associated proteins and the effects of calcium on their functions, see references 16, 49, and 59), as well as altering the interactions between the cytoskeleton and plasma membrane (66). Alternatively, changes in intracellular or extracellular calcium concentrations could indirectly inhibit many developmental processes by placing increased demands on ion pumps and depleting ATP pools.

Although there is evidence that calcium can influence many cell functions, there has been little direct evidence to indicate which of these mechanisms might actually operate in living cells. In particular, the possibility that calcium might inhibit outgrowth by altering cytoskeletal organization is complicated by the fact that there are many different cytoskeletal components that are sensitive in different ways to changes in calcium concentration. For example, low calcium concentrations enhance filament nucleation from purified g-actin, but calcium also inactivates the actin bundling protein alpha actinin and activates the depolymerizing factor gelsolin (49). Without knowing the precise concentrations of actin and the various actin associated proteins, one could not predict what, if any, effect a given change in calcium concentration would have on actin networks. In this study, we have attempted to directly investigate the effects of conditions that alter intracellular calcium on the ultrastructural organization of growth cones.

Materials and Methods

Cell Culture and Culture Media

Freshly dissected dorsal root ganglia (DRG)[1] from 9-d-old white leghorn chick embryos were dissociated with trypsin and plated on polyornithine-coated glass coverslips attached to sterile petri dishes for light microscope observations (19) or polyornithine-coated, formvar-covered gold grids (62), for EM experiments. Culture medium consisted of bicarbonate buffered F14 medium (Gibco Laboratories, Grand Island, NY) supplemented with 50% heart conditioned medium, L-glutamate (2 mM), sodium selenite (5 ng/ml), sodium pyruvate (0.4 mg/ml), phosphocreatine (5 mM), and nerve growth factor (15 ng/ml) (19). Antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO) was also added at 1×. After 1 d in culture, neurons were exposed for 5–30 min to one or more of the following manipulations designed to alter intracellular calcium concentrations: calcium-free medium with EGTA (0.3 mM), calcium ionophores A23187 (2.5 μM, Sigma Chemical Co.), or ionomycin (100–200 nM; Behring Diagnostics, San Diego, CA) in calcium-containing medium (0.3 mM CaCl2), or calcium-free medium with EGTA. For some experiments, taxol (National Cancer Institute (Rockville, MD) (2 μM) or phalloidin (Sigma Chemical Co.) (6 or 13 μM) were also added alone or in combination with A23187.

Calcium-free, Hepes-buffered medium was made as for F12 with the exceptions that hypxanthine, linoleic acid, lipoic acid, phenol red, putrecine, and thymadine were omitted and soy bean lipids (80 μl/ml Boehringer Mannheim) were added. Individual inorganic salts were obtained from Sigma Chemical Co., and MEM essential amino acids, MEM nonessential amino acids, basal medium-Earl's vitamins, and sodium pyruvate concentrated solutions were obtained from Gibco Laboratories. The pH of the medium was adjusted to 7.4 with NaOH and HCl and osmolarity adjusted to 320 m OsM with additional Hepes. Antibiotic-antimycotic solution (1×, Sigma Chemical Co.) and L-glutamate (2 mM) were added and the medium was sterile filtered and stored at 4°C. Immediately before use 0.3 mM CaCl2 or 0.3 mM EGTA were added to the medium.

1. *Abbreviation used in this paper*: DRG, dorsal root ganglia.

A23187, ionomycin, monensin, and taxol were dissolved in absolute ethanol. Phalloidin was dissolved in methanol. Control media for experiments involving any of these drugs contained equal volumes of the ethanol and/or methanol vehicles. To assess the ion specificity of the effects some cells were also exposed to the monovalent cation ionophore monensin (25 μM). (Drug concentrations were selected based on reports of their ability to inhibit motile behavior, and/or elevate intracellular calcium in other cell types [30, 68]).

Behavioral Analysis

For observation of behavioral responses, culture medium was replaced with Hepes-buffered F12 medium (Gibco Laboratories) with 10% added calf serum (Gibco Laboratories) or the calcium-free, Hepes-buffered medium described above. In initial experiments, sodium selenate, sodium pyruvate, phosphocreatin, and nerve growth factor were also added to this medium, but these supplements did not appear to be necessary for short term neuronal health or process extension. Broken coverslip spacers were then placed on top of the cell bearing coverslip and a second coverslip placed on top of that. The resulting chamber was then sealed with stopcock grease except for small openings on two ends for exchanging medium. The chamber was placed on an inverted microscope (IM 35; Carl Zeiss, Inc., Thornwood, NY) and maintained at a constant 37°C with an air curtain incubator (ASI 400; Carl Zeiss Inc.). Medium was exchanged by adding it to one free end and drawing the medium through with capillary action using tissues (Kimwipe). One milliliter of medium was exchanged in this way through a chamber with a volume of ~0.2 ml. Neuronal behavior was monitored with phase-contrast optics or VEC-DIC microscopy using a video camera (MTI 65), a monitor (Trinitron; Sony Corp. of America, New York, NY), and an image processing system (QX9200; Quantex). Growth cone activity before and after each treatment was recorded on a time lapse video recorder (VTR NV-8300; Panasonic Company, Secaucus, NJ) at a speed of 18 h. Neurites were then photographed or traced from the screen at the beginning of each treatment condition and at 5- or 10-min intervals after each treatment. Growth cones were excluded from study if they showed significant fluctuations in motile activity or in rate of neurite outgrowth, or frequent changes in direction during a 10-min preexperiment observation period in control medium.

Calcium Measurements

Calcium concentrations in growth cones of cultured cells were assessed using the calcium indicator dye Fura-2AM (Molecular Probes Inc., Junction City, OR). Briefly, cultures were incubated with Fura-2AM (6 μM in normal culture medium) for 1.5 h at 37°C, washed three times with culture medium and incubated for an additional 2–3 h before use to allow the dye to deesterfy. Fura-2-AM loaded cells were monitored with a microscope (IM 35; Carl Zeiss, Inc.) with quartz optics, a 1.3 NA fluorite objective (40×; Nikon Inc., Garden City, NY), and epifluorescence illumination from a 50-W Hg lamp. A video camera (MTI SIT 66) and computer (Apple II plus, New York, NY) equipped with a digitizing board (Digisector, Microsoft Works) were used to generate numerical values for fluorescence intensities at a single point in the middle of each growth cone. Fluorescence emission intensities at 340- and 380-nm stimulating wavelengths (filters ± 2 nm; Ditric Optics Inc., Hudson, MA) were used to estimate growth cone calcium concentrations using the formula:

$$[\mathrm{Ca}] = K_d \times \frac{R - R_{min}}{R_{max} - R} \times (Fo/Fs) \quad (13),$$

where R indicates the ratio of fluorescence at 340 nm:380 nm stimulating wavelengths. Reported values of 225 for K_d and 7.5 for Fo/Fs (13) were used in our calculations and R_{max} and R_{min} were obtained experimentally by permeabilizing cells with 300 nM ionomycin and measuring fluorescence intensities at 340- and 380-nm in 0.6 mM calcium-containing medium or calcium-free medium with 0.3 mM EGTA (68). Measurements to determine R_{max} and R_{min} were made at 2-min intervals after the addition of the ionophore and ratios were observed to plateau roughly 10 min after onset of treatment, with an apparent loss of dye in the cells and increase in fluorescence of the medium observed 20–30 minutes after treatment. In our studies, values of 8.2 and 0.13 were obtained for R_{max} and R_{min}, respectively. For each R value, background fluorescence from a cell free region of the same coverslip at 340- and 380-nm stimulating wavelengths were subtracted from the intensities measured inside the cell. To assess the effects of ionophores or other drugs on growth cone calcium concentrations, fluorescence intensities were measured immediately after an exchange of control medium, 10 min later, immediately after drug treatment, and 10 min

after drug treatment. For some experiments, measurements were also made at shorter intervals as well. For assessment of the effects of A23187 treatment on growth cone calcium, a nonfluorescent derivative of A23187, 4-bromo-A23187 (Molecular Probes Inc.) was used in place of A23187, because of the high fluorescence of the nonderivatized form of the ionophore.

Ultrastructural Analysis

For ultrastructural studies, grid bearing coverslips were transferred to dishes containing fresh culture medium or calcium-free Hepes-buffered medium (with 0.3 mM EGTA or 0.3 mM CaCl2) and appropriate ionophores or ethanol vehicle. After 5–30 min, cells were fixed with glutaraldehyde, stained with osmium and uranyl acetate, dehydrated in alcohol, critical point dried with carbon dioxide, and examined as whole mounts using a transmission electron microscope (model No. 100; JEOL USA; Cranford, NJ) operating at 100 KV (62–64). In initial experiments, the first 20–25 growth cones identified in each experimental condition were photographed and analyzed in greater detail. To assess the frequency of disruption of actin filaments in the growth cone periphery, ~100 neurons from each condition were scored for the presence or absence of at least one region of the growth cone periphery showing an apparent loss of actin filaments. Only growth cones with essentially intact cell membranes were scored, and areas with tears in membrane were not included in the analysis. Cells were only scored as positive for actin filament disruption if they showed the characteristic feature of loss of microfilaments in the growth cone periphery and intact microfilament networks in and leading into the filopodia. No attempt was made to quantify the extent of the actin filament disruption. To assess whether the apparent loss of actin filaments in the growth cone periphery of some cells was because of osmium destruction, in two experiments 0.2% tannic acid and 0.05% saponin were added to the fixative to better preserve actin filament (57), and in one experiment, cells were partially fixed with 4% paraformaldehyde and 0.04% glutaraldehyde and incubated with 3× 10-7 M phalloidin to stabilize actin filaments before conventional fixation and whole mount processing.

To test the hypothesis that cytoskeletal elements in growth cones with elevated calcium levels might be inherently less stable than growth cones in control medium, in two experiments, neurons exposed to 25 μM A23187 in calcium-containing medium were extracted with Triton X-100 for 2 min to remove the cell membrane and the less stable cytoskeletal elements before fixation (53). This procedure leaves the cell nucleus and a variable composition of cytoskeletal structures attached to the substrate. The cytoskeletons of the Triton X-100 extracted neurites were initially ranked into five categories ("typical," more complex, much more complex, less complex, or much less complex) according to the relative complexity of filament composition compared with a typical extracted control. Later the middle or typical category was divided in half and added to the pooled values for the two higher and lower complexity groups respectively to yield two categories; less than or equal to the complexity profiles typically observed in controls (≤ typical), and greater than or equal to the complexity profiles typically observed in controls (≥ typical).

Results

Growth Cone Behavior

In control medium, DRG growth cones typically exhibited continuous filopodial and lamellipodial activity and a relatively uniform rate of neurite extension. Simple exchanges of control medium produced brief (~1 min duration) increases in filopodial activity in roughly 1:5 of the neurites tested, but prolonged effects of exchanges were not noted. Both ethanol and methanol vehicles slightly reduced rates of neurite outgrowth when compared with controls without vehicle.

Removal of calcium resulted in a gradual and simultaneous reduction in both motile activity of filopodia and lamellipodia, and in neurite outgrowth. Neurite extension after 10 min in calcium-free medium was noticeably decreased compared to the preceding 10 min in control medium (Fig. 1), with outgrowth in calcium-free medium averaging only 34 ± 22% ($n = 9$) of that seen during the same period of time in control medium. Complete cessation of motile activity and outgrowth typically occurred within 10–15 min after changing to calcium-free medium with EGTA. No losses of filopodia or lamellipodia, or overall changes in growth cone shape were noted in calcium-free medium, and the overall impression was one of growth cone activity simply freezing. Similar to the reports of Goldberg (17) for Aplysia neurons, calcium removal did not cause losses of filopodia or obvious changes in the speed or amount of vesicle traffic within growth cones or neurites, and lamellipodia appeared to grow thicker after several minutes in calcium-free medium.

Exposure of cells to calcium-free medium containing 2.5 μM A23187 or 150 nM ionomycin produced responses that were more rapid, but essentially similar to calcium-free medium alone (not shown). Growth cone motility and neurite outgrowth typically stopped completely 2–5 min after changing to calcium-free medium with calcium ionophores, and no neurite retraction or net losses of filopodia or lamellipodia were noted.

After exposure to either 2.5 μM A23187 or 100 nM ionomycin in normal calcium-containing medium, neurites underwent a characteristic series of behavioral changes resulting in a significant reduction in neurite length (Fig. 2). Neurite outgrowth slowed detectably and the growth cone began to round up within 1–3 min after the addition of either ionophore. This was followed by a retraction of the neurite that became evident within 5–10 min after ionophore addition, and a gradual decrease in the number of filopodia and in filopodial movement. If continually exposed to calcium ionophores in calcium-containing medium, neurites typically retracted for 20–30 min before stopping. The net reductions in neurite length were typically equivalent to roughly twice the length of the original growth cone. Filopodial activity, however, often continued to be observed for an additional 20–30 min after neurite retraction was completed. (This activity was predominantly because of preexisting filopodia, as few new filopodia were extended after the addition of calcium ionophores.) As in the case of treatment with calcium-free medium, bidirectional movement of intracellular particles continued to be observed in calcium ionophore–treated cells at rates and levels similar to control medium. Growth cones remained attached to the substrate throughout all treatment conditions tested.

Behavioral responses to both calcium removal and calcium ionophore treatment were readily distinguishable from responses to control exchanges alone (compare Fig. 3 *A* with *B*, *C*, and *D*) and were highly consistent. Neurites continued to extend when exposed to repeated exchanges of control medium. By comparison, 18 out of 20 neurites tested ceased neurite extension or had their rates of extension greatly reduced within 10 min after calcium removal and 34 out of 43 neurites tested with 2.5 μM A23187 and 5 of 6 neurites tested with 100 nM ionomycin began retracting within 10 min after the addition of the drug.

The effects of both calcium removal and calcium ionophores could also be readily reversed by returning to control medium 10 min after the initial treatment (Fig. 3, *B*, *C*, and *D*). Recovery from calcium removal was more rapid and reinitiation of neurite outgrowth was observed within 2–5 min after return to control medium in 9 out of 11 neurites tested. Ten out of fifteen neurites that retracted after exposure to A23187 and 5 out of 5 neurites that retracted in response to ionomycin reextended within 20 min after return

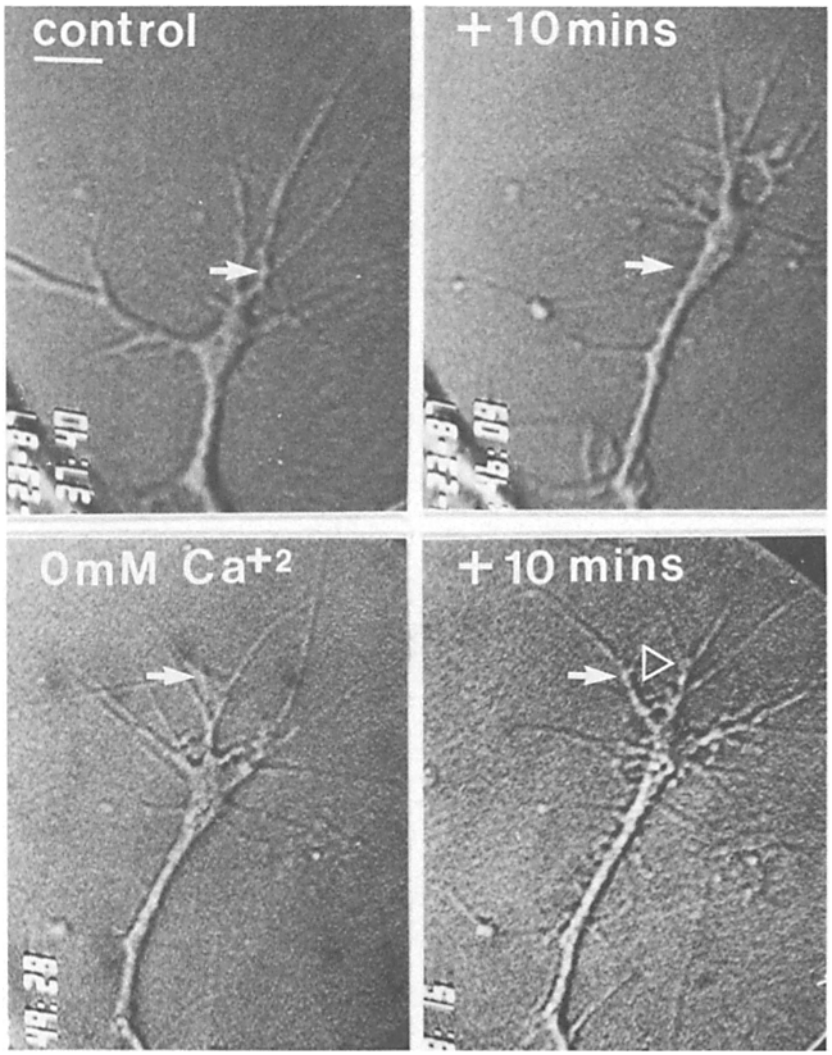


Figure 1. Removal of extracellular calcium inhibits neurite outgrowth. VEC-DIC micrograph of a typical cultured DRG neurite immediately after an exchange of control medium (*top left*), after 10 min in control medium (*top right*), immediately after changing to calcium-free medium with 3 mM EGTA (*bottom left*), and 10 min after changing to calcium-free medium (*bottom right*). Note that the neurite extends significantly during the 10-min period in control medium (*top*), but extends very little during a 10-min period in calcium-free medium (*bottom*). Arrows indicate the position of the leading lamellipodial edge immediately after changing medium. Open triangles indicate the leading edge 10 min after the exchange. Bar, 10 μm.

to control medium, and all neurites that were inhibited from growing by calcium removal reinitiated outgrowth within 30 min after return to control medium. In five separate cases, neurites were observed to retract and then reextend after two successive calcium ionophore treatments, and in four cases tested, outgrowth was reinitiated after two successive treatments with calcium-free medium. Neurite outgrowth could be reinitiated after 30 min in calcium-free medium but could only rarely be recovered after 30 min calcium ionophore exposures. Recovery from calcium ionophore treatment in calcium-free medium was erratic and inconsistent. Neurites of cells returned to control medium after exposure to calcium ionophores in calcium-free medium often produced rapid bursts of motile activity and outgrowth alternating with periods of inactivity or retraction, and only 4 out of 10 neurites tested recovered significant growth following this treatment.

Gross features of growth cone responses to 2.5 μM of the monovalent cation ionophore monensin were similar to the responses to the two divalent cation ionophores (compare Fig. 3 *E* with *C* and *D*); neurite outgrowth abruptly stopped and neurites began to retract 5–10 min after drug addition; filopodial motility persisted during neurite retraction. 13 out of 14 neurites tested responded in this manner, and 7 out of 10 recovered neurite outgrowth within 30 min after returning to control medium. On a subtler level, it was noted that the overall shape of the retracting growth cone was flatter in monensin treated cells, and tongues of lamellipodia often persisted during monension-induced retraction.

To assess whether either depolymerization of microtubules or actin filaments or both played a significant role in calcium ionophore-induced neurite retraction, we exposed neurites to the microtubule stabilizing agent taxol (33) or the actin stabilizing agent phalloidin (67) in either control medium or calcium ionophore containing medium. Both cytoskeletal stabilizing agents were observed to inhibit neurite outgrowth and partially antagonize the effects of calcium ionophore treatment in calcium-containing medium, but the effects of the two agents differed in many respects.

Taxol alone caused a widening of neurites and a slight decrease in rate of neurite extension with no noticeable effect on filopodial activity. The addition of 2 μM taxol before and during exposure to A23187 prevented subsequent neurite retraction in each of three cells tested that had previously been observed to retract in response to A23187, although the characteristic rounding of the growth cone and loss of lamellipodia was still noted. Addition of taxol after A23187-

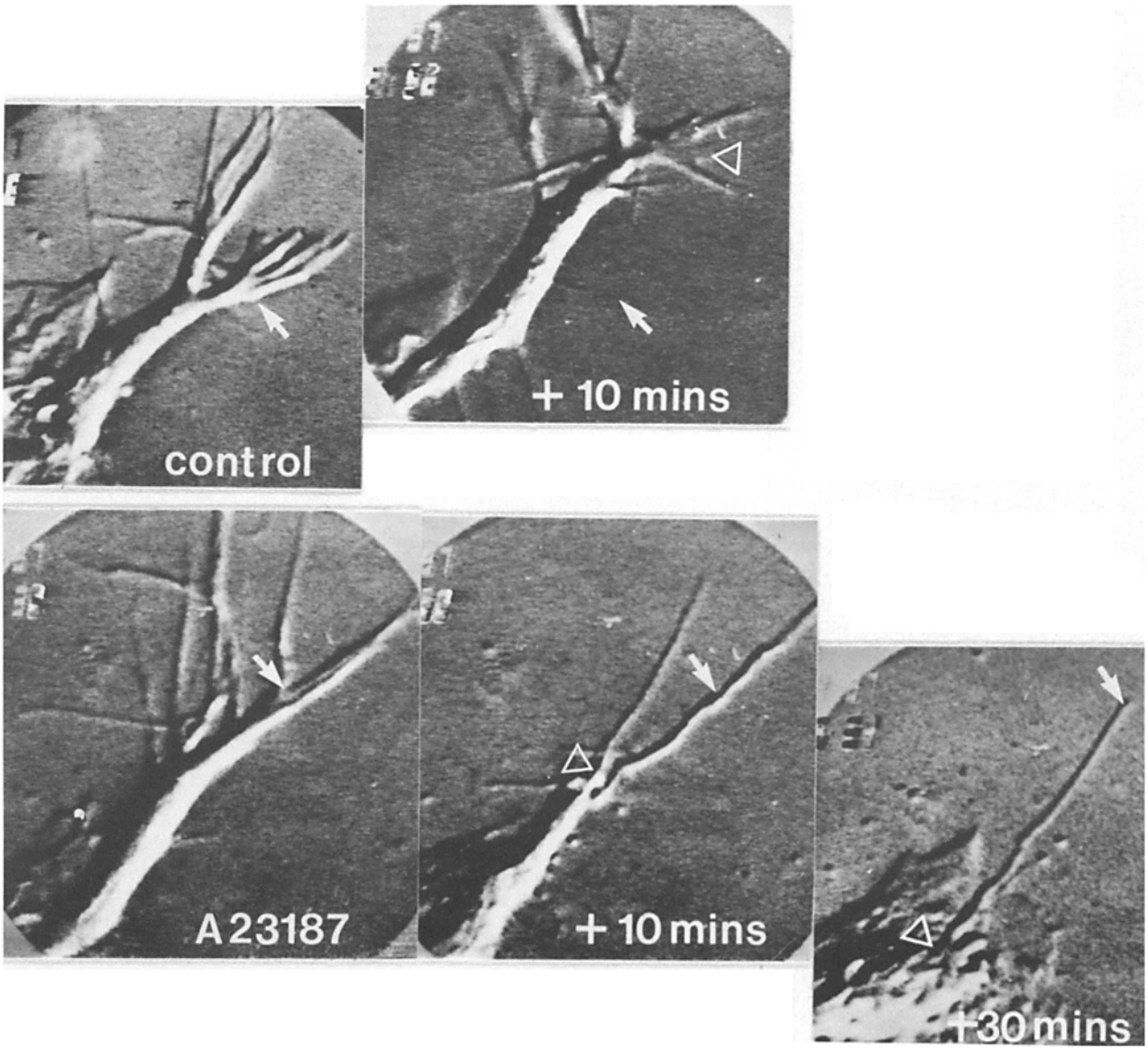


Figure 2. A23187 inhibits outgrowth and induces neurite retraction. VEC-DIC micrograph of a typical neurite immediately after an exchange of control medium (*top left*), 10 min after the control exchange, immediately after the addition of 2.5 μM A23187 (*bottom left*), 10 min after the addition of A23187 (*bottom middle*), and 30 min after the addition of A23187 (*bottom right*). Note that the neurite extends significantly during a 10-min period in control medium, but the growth cone collapses and the neurite has begun to retract at 10 min after the addition of A23187. Filopodial movement is indicated by the blurring of some filopodia in this still frame. 30 min after the addition of A23187, the neurite has retracted significantly, but one filopodium still remains. Arrows indicate the position of the leading lamellipodial edge immediately after changing medium. Open triangles indicate the leading edge 10 or 30 min after the exchange as indicated. Bar, 10 μm.

induced retraction did not cause reinitiation of neurite outgrowth in any of five neurites tested. (Taxol data not shown.)

In contrast, phalloidin alone inhibited both neurite outgrowth and filopodial activity, producing responses that were similar to the responses observed after removal of extracellular calcium. Interestingly, phalloidin was also observed to reverse A23187-induced neurite retraction in the continuous presence of the ionophore in 3 out of 5 neurites exposed to phalloidin 5–10 min after A23187 treatment. The time course and general characteristics of the recovery of neurite outgrowth after the addition of phalloidin were similar to the recovery observed after return to control medium (Fig. 4 *A*). After the addition of 13 μM phalloidin, growth cones resumed the flattened appearance typical of growth cones in control medium, showed extensive filopodial and lamellipodial activity, and began elongating neurites within 10–15 min (Fig. 4 *A*). (Recovery of neurite outgrowth was not observed in neurites exposed to calcium ionophores for 30 min before phalloidin addition and spontaneous recovery was never observed with continuous A23187 exposure without phalloidin.) The reciprocal experiment showed that A23187 was also capable of reversing phalloidin-induced inhibition of neurite outgrowth (Fig. 4 *B*). In 3 out of 5 cells tested, neurites that had ceased extending in the presence of 13 μM phalloidin, reinitiated outgrowth after the addition of 2.5 μM A23187, although reextension was sometimes preceded by a brief retraction phase.

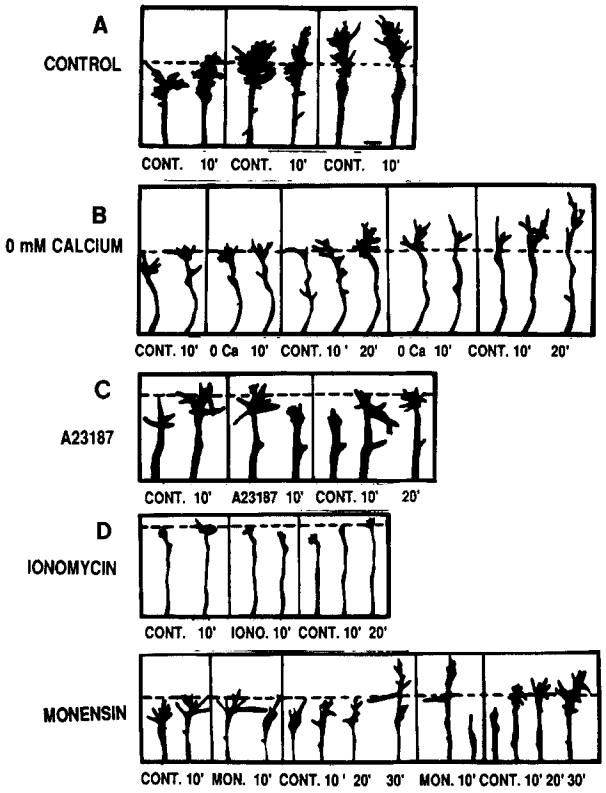


Figure 3. Reversible inhibition of neurite outgrowth in response to calcium removal, 25 μM A23187, 100 nM ionomycin, and 25 μM monensin. Neurites were traced from video micrographs at successive time points after control exchanges or ionophore treatments. Vertical lines separating groups of tracings within a box indicate a change of condition (either a control exchange or experimental manipulation) and within each group each successive tracing from left to right shows the neurite 10 min after the preceding time point. Dashed lines indicate the farthest point of neurite extension at the start of each experiment and serve as references for assessing changes in neurite length. (Reference position markers, such as dead cells and scratches in the substrate that were used to align tracing are not shown.) *A*, Neurite continues to extend after three successive exchanges of control medium. *B*, Neurite extends in control medium, slows or stops extension in calcium-free medium (with EGTA), extends once more in control medium, halts again in calcium-free medium, and recovers a second time. *C* and *D*, Neurites extend in control medium, retract after the addition of calcium ionophores, and reextend upon returning to control medium. In *E*, neurite is shown extending in control medium, retracting in response to monensin, recovering in control medium, retracting in response to a second monensin treatment, and recovering a second time. Bar, (*A*) 10 μm.

Effects of Treatments on Growth Cone Calcium Levels

To estimate the magnitudes of changes in growth cone calcium induced by calcium removal or calcium ionophore treatments, and to assess whether the behavioral effects of monensin or phalloidin might be because of trivial actions on cell calcium, growth cone calcium concentrations were measured by the Fura-2AM method before and after experimental manipulation. Calcium measurements were made in the centers of growth cones: immediately after a change of control medium, 10 min after the control exchange, and 10 min after exposure to ionophores and/or phalloidin.

Growth cones of chick dorsal root ganglion neurons showed a wide range of calcium concentrations in control medium, similar to the results reported by Connor (13) for chick central nervous system neurons. For 120 growth cones examined in a preliminary screening, fluorescence ratios at 340:380 nM stimulating wavelengths in Fura-2AM loaded cell ranged from 0.32 to 3.70, translating to estimated calcium concentrations of 40 nM to 1340 nM, with a mean value of 500 ± 300 nM. The majority of growth cones examined (>60%) had apparent calcium concentrations of 200–800 nM. Growth cones with calcium levels in this range and showing relatively stabile morphologies were selected for further experiments. (No attempt was made to correlate growth state with calcium concentration.) It should be stressed that calcium concentrations obtained in this manner are only estimates of intracellular calcium levels, and although the relative relationships between values are highly reliable, any errors in the determination of R_{max} and R_{min} would result in systematic errors in estimates of the actual intracellular calcium concentrations.

Repeated fluorescence measurements in control medium indicated that calcium concentrations at a given point in the growth cone could fluctuate significantly over periods of several minutes. These fluctuations were observed in controls with no vehicle added, as well as in the presence of ethanol or methanol vehicles. During a 10-min period in control medium, the average absolute change in apparent calcium concentrations was 80 ± 60 nM (n = 46). Both increases

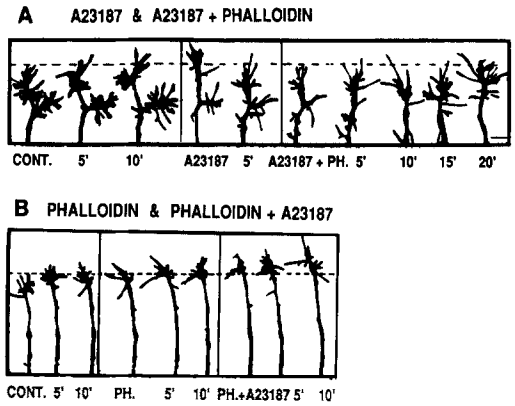


Figure 4. Phalloidin inhibits neurite outgrowth and reverses A23187-induced neurite retraction. Tracings of two neurons are shown at successive time intervals after an exchange of control medium or the addition of an experimental agent. In *A*, the neurite is shown immediately after a control exchange, 5 and 10 min later, immediately after the addition of 2.5 μM A23187, 5 min after the addition of A23187, immediately after the addition of 13 μM phalloidin in the continuous presence of A23187, 5, 10, 15, and 20 min later. The neurite retracts in response to A23187 and reextends after the addition of phalloidin in the continued presence of A23187. In *B*, a neurite is shown in control medium, 5 min later, 10 min later, immediately after the addition of 13 μM phalloidin, 5 min after phalloidin addition, 10 min after phalloidin addition, immediately after the addition of 2.5 μM A23187 in the continued presence of phalloidin, 5 and 10 min after the addition of A23187 in the continued presence of phalloidin. Neurite outgrowth is inhibited after the addition of phalloidin, but rapid neurite extension is reinitiated after the addition of A23187 in the continued presence of phalloidin. Bar, (*A*), 10 μm.

and decreases in calcium levels were roughly equally common, and the average net change during a 10-min period in control medium was 0 nM ± 100 nM (n = 46).

Fluorescence measurements before and after calcium manipulations indicated that calcium removal resulted in a slight decrease, and calcium ionophore addition resulted in a larger increase in growth cone calcium. Each of 6 growth cones exposed to calcium-free medium with EGTA for 10 min showed a decrease in growth cone calcium compared with control values. The magnitude of the decrease was highly variable however with the average net response being −190 nM ± 120 nM (n = 6). Both the calcium ionophores 4-bromo-A23187 (the nonfluorescent derivative of A23187) and ionomycin produced large and consistent increases in growth cone calcium concentrations. 10 min after calcium ionophore addition, calcium levels in growth cones treated with 2.5 μM 4-bromo-A23187 increased an average of 340 ± 140 nM (n = 7) and levels in 100 nM ionomycin treated growth cones increased an average of 330 ± 80 nM (n = 6). (Responses to calcium-free medium and calcium ionophores differed from controls at the 0.001 level of significance using the unpaired t test.)

By comparison, neither monensin nor phalloidin produced statistically significant changes in growth cone calcium. 10 min after treatments with 2.5 μM monensin or 13 μM phalloidin, the average net changes in growth cone calcium levels were found to be 0 ± 50 nM (n + 6) and +10 ± 120 nM (n = 12), respectively. Similarly, the addition of 13 μM phalloidin in combination with calcium ionophores resulted in changes in growth cone calcium that were not different from the effects of the calcium ionophore alone. 10 min after the addition of phalloidin + A23187 or phalloidin + ionomycin, growth cone calcium levels increased an average of 270 ± 80 nM (n = 7) and 320 ± 110 nM (n = 6), respectively, compared with values of 340 ± 140 nM and 330 ± 60 nM, respectively, for the ionophores alone. (Values for ionophore treatment in the presence of phalloidin differed from controls at the 0.001 level of significance using the unpaired t test, but did not differ significantly from ionophore treatment alone.) These data argue against the possibility that the behavioral effects of monensin and phalloidin were because of previously unidentified effects of these drugs on calcium metabolism.

Growth Cone Ultrastructure in Unextracted Whole Mounts

Ultrastructural features of unextracted whole mounts of DRG growth cones exposed only to changes of control medium were similar to those described previously for chick retina neuronal growth cones (62–64) (Fig. 5, *A* and *F*). Individual cytoskeletal elements typically appeared as discrete nontapering filaments interconnected to one another and to the plasma membrane and membranous organelles, and forming an uninterrupted network throughout the growth cone. Microtubules and large membranous organelles were confined primarily to the central region of the growth cone, while the periphery and filopodia were filled with a network of microfilaments, small vesicles, and electron dense particles. Previous studies have established that cytoskeletal elements in the growth cone periphery are almost exclusively actin filaments (31, 32, 34, 69).

Ultrastructural features of growth cones exposed to calcium-free medium (Fig. 5 *B*) or calcium-free medium with calcium ionophores (not shown) were, in general, similar to controls. Cytoskeletal networks tended to be more densely packed and large membranous organelles were more often observed in lamellipodial regions than was the case in typical controls, but these trends were noticeable only after screening large numbers of growth cones.

Consistent with light microscope observations of growth cone behavior, growth cones exposed to A23187 or ionomycin in calcium-containing medium before fixation typically had a more rounded or collapsed appearance than growth cones in control medium. This collapsed shape was more pronounced in growth cones exposed to ionophores for longer periods of time (30 min as opposed to 5 or 10 min). Growth cones exposed to ionophores for 30 min, also had a denser appearance and seemed to contain higher concentrations of small membranous vesicles than controls. As ultrastructural features were more readily visualized in cells exposed to the ionophore for shorter periods of time, the majority of cells were examined 10 min after exposure to ionophores.

The most noticeable ultrastructural change in cells treated with calcium ionophores in calcium-containing medium was an apparent loss of actin filaments in the growth cone periphery of a majority of treated cells (Fig. 5, *C*, *D*, *G*, and *H*). Apparent losses of actin filaments in portions of the growth cone periphery were also observed in some control cells, but the percentage of growth cones affected was smaller, and although no attempt was made to measure each area, the size of the regions showing losses of actin filaments also tended to be smaller in the control condition. The appearances of growth cones treated with A23187 (Fig. 5, *C* and *G*) and ionomycin (Fig. 5, *D* and *H*) were very similar, and the apparent disruption of peripheral microfilament organization could be readily distinguished from common fixation artifacts by the fact that actin filament bundles within, and leading into, filopodia remained intact while intervening regions were devoid of filaments.

Consistent with reports that the monovalent cation ionophore monensin inhibits membrane insertion and recycling (30), growth cones exposed to 25 μM monensin tended to have increased densities of small (40–100-nm-diam) vesicles (Fig. 5 *E*). As was observed at the light microscope level, growth cones of monensin treated cells had few filopodia, but broader, lamellipodia-like protrusions were fairly common. Unlike growth cones treated with calcium ionophores, no changes in actin networks were noted after monensin treatment.

Percentage of Growth Cones Showing Apparent Peripheral Microfilament Disruption

Although most noticeable in cells exposed to calcium ionophores in calcium-containing-medium, areas of apparent peripheral actin filament disruption were also observed less frequently in other treatment conditions. To quantify this observation, ~100 neurites from each treatment group were scored for the presence or absence of this feature in a series of separate experiments. The percentages of growth cones showing peripheral filament disruption were highly consistent for each treatment condition across many experiments,

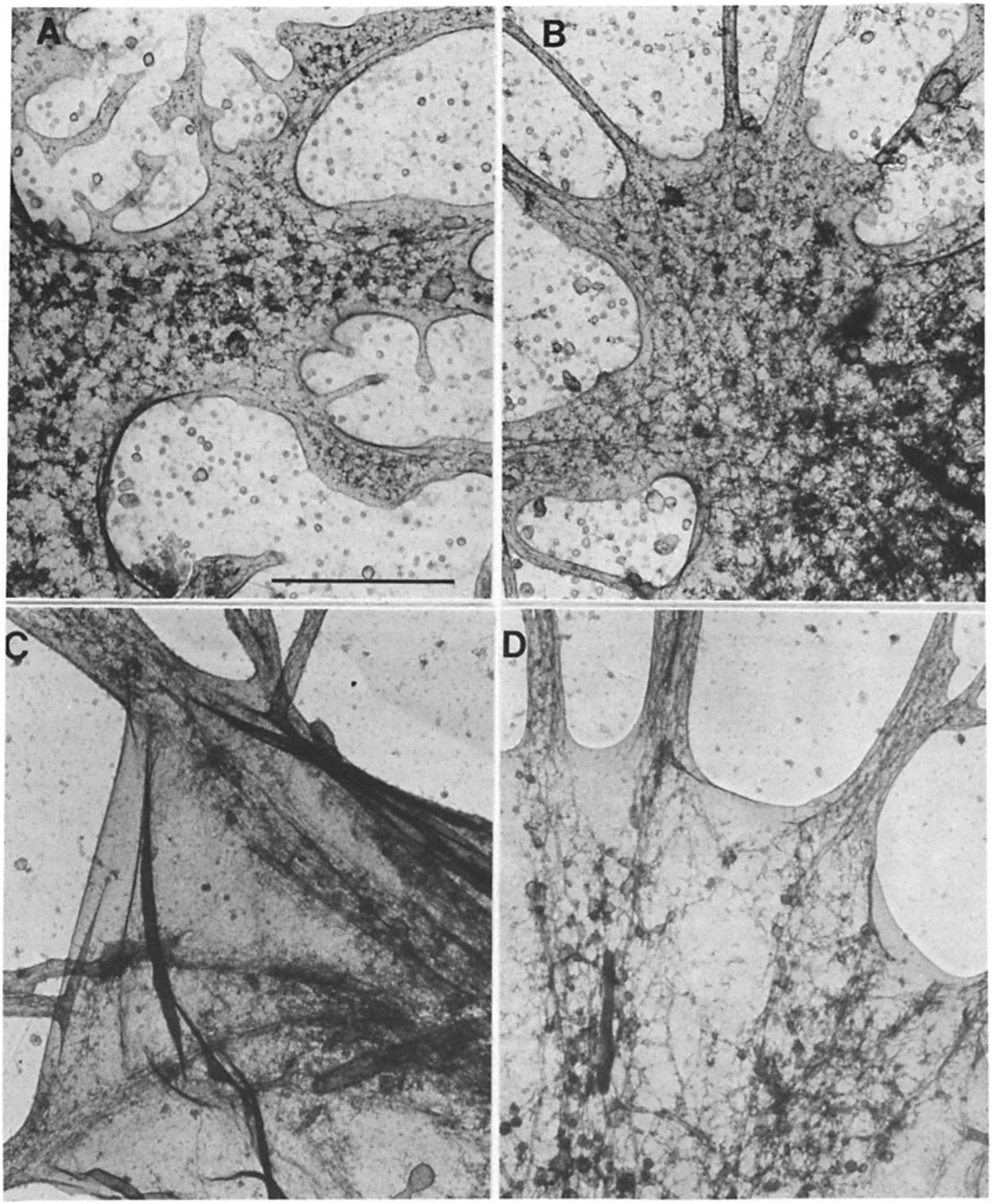
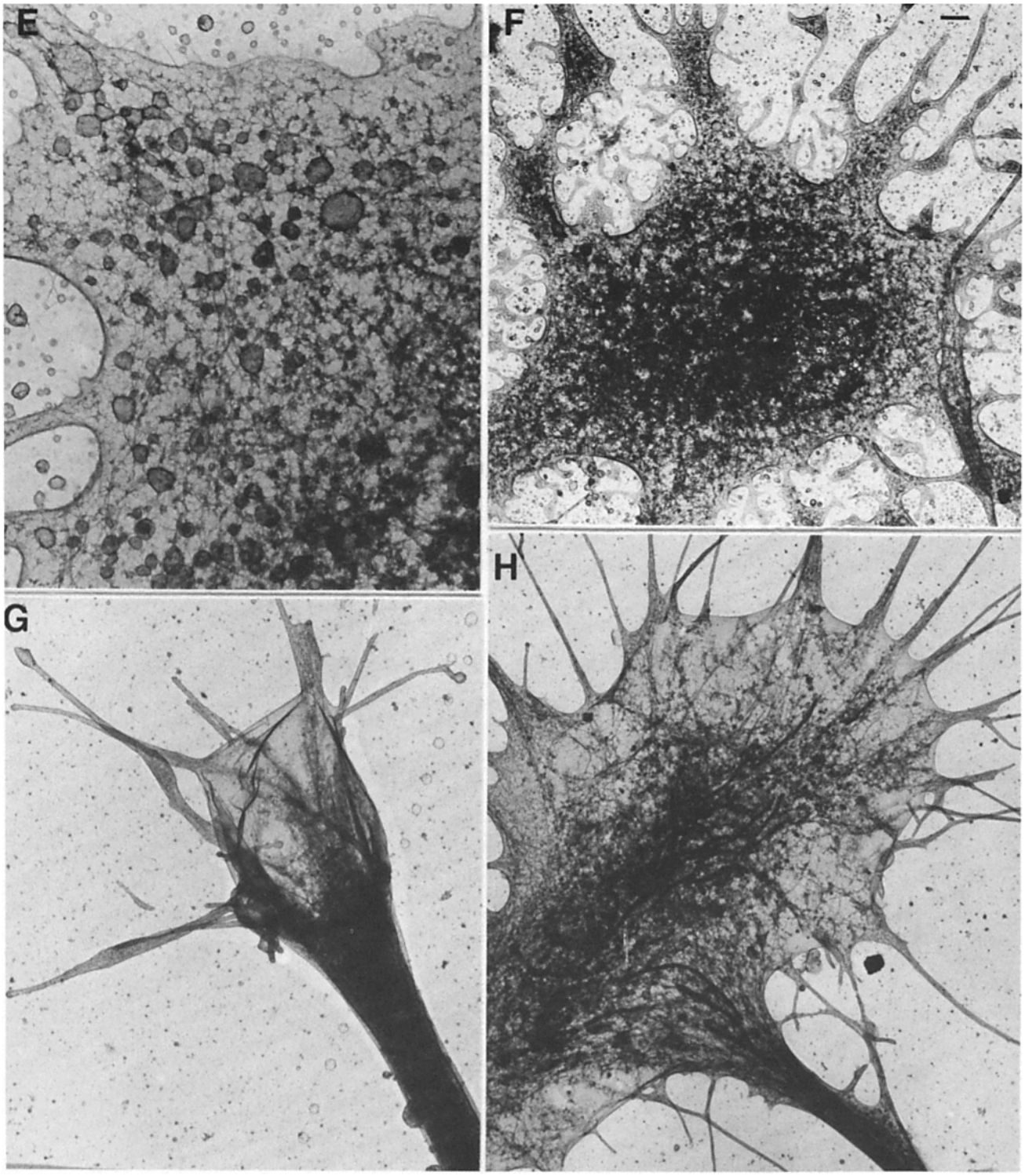


Figure 5. Whole mount electron micrographs show an apparent loss of actin filaments in the periphery of growth cones exposed to calcium ionophores for 10 min. *A–E*, Ultrastructural details of growth cones exposed to different treatment conditions for 10 min before fixation. *A*, Control medium. *B*, Calcium-free medium with EGTA. *C*, A23187. *D*, Ionomycin. *E*, Monensin. *F*, *G*, and *H* are low magnification micrographs of growth cones shown in *A*, *C*, and *D*, respectively. *A–E* and *F–H* are shown at the same magnifications. Bars, (*A* and *F*) 1 μm.

and no statistically significant difference was noted between the percentages of affected growth cones at 5- 10-, and 30-min exposures. All data for each drug treatment were therefore pooled and averaged (Table I).

In untreated controls, areas of apparent actin filament disruption were observed in at least part of the growth cone periphery of 32 ± 5% of the growth cones examined, and in calcium-free medium, the percentage of growth cones showing this feature were only very slightly lower (29 ± 6% or 1–3% lower for each of the three experiments). By compari-



son, an average of 60 ± 5% of growth cones exposed to A23187 for 5–30 min and 59 ± 6% of growth cones treated with ionomycin in calcium-containing medium showed an absence of microfilaments in at least part of the growth cone periphery. As noted earlier, the sizes of the regions showing apparent losses of actin filaments also tended to be larger in growth cones treated with calcium ionophores in calcium-containing medium. When cells were treated with A23187 or ionomycin in calcium-free medium, the percentage of growth cones with areas of disrupted filament networks was even lower than controls (21 ± 7% and 22 ± 5%, respectively), indicating that the increased numbers of growth cones showing this feature in ionophore treated cultures was because of enhanced calcium influx rather than the ionophores themselves. The incidences of growth cones with apparently disrupted peripheral actin filament networks were

Table I. Abundance of Growth Cones with Disrupted Peripheral Actin Filament Networks after Different Treatment Conditions

	No drug		Treatment condition (A23187)		Ionomycin		Monensin	
Experiment	W Ca^{++}	O Ca^{++}	W Ca^{++}	O Ca^{++}	W Ca^{++}	O Ca^{++}	W Ca^{++}	O Ca^{++}
1 (5 min)	30% (108)		55% (110)					
2 (10 min)	28% (125)		67% (108)					
3 (30 min)	30% (126)		63% (103)					
4 (10 min)	27% (100)						26% (102)	
5 (10 min)			58% (96)				26% (102)	
6 (10 min)	37% (146)		55% (103)		51% (217)		35% (150)	
7 (10 min)	39% (119)				61% (101)			
8 (10 min)	38% (126)	36% (114)					31% (110)	
9 (10 min)	35% (135)						34% (103)	
10 (30 min)	30% (119)		57% (51)	16% (103)				
11 (30 min)	30% (100)				55% (20)	24% (100)		26% (98)
12 (10 min)	24% (112)	21% (103)	56% (104)	26% (102)				
13 (10 min)			54% (67)	19% (101)	56% (102)	20% (46)		
14 (10 min)			63% (91)	33% (129)				
15 (10 min)					67% (77)	27% (147)		
16 (10 min)	29% (113)		68% (123)	13% (61)	64% (72)	15% (104)		
(Averages,	32% ± 5%	29% ± 6%	*60% ± 5%	21% ± 10%	*59% ± 6%	22% ± 5%	31% ± 3%	26%)

Neurons were cultured for 1 d and exposed for 5–30 min to ethanol vehicle, A23187 (2.5 μM), ionomycin (150 nM), or monensin (2.5 μM) in either calcium-containing medium or calcium-free medium with EGTA before fixation and processed as whole mounts for EM. For each experiment, ~100 neurite tips were examined for each condition and scored as either positive or negative for the presence of areas of apparent actin filament disruption in the growth cone periphery. Percentages of neurites with areas of apparent peripheral actin filament loss are shown for each experiment with the numbers of neurites examined in parentheses. Data from all experiments were pooled to yield average percentages of growth cones showing peripheral actin filament disruption for each experimental condition.
* Differs from control at the 0.001 level of significance using the paired *t* test.

not increased in monensin-treated cultures relative to controls (31 ± 3% compared with 32 ± 5% in controls), further arguing for a calcium specificity of the effect.

Growth cones treated with calcium ionophores in calcium-containing medium and fixed in the presence of tannic acid or with the addition of phalloidin to better preserve actin filaments showed frequencies of actin filament disruption intermediate between controls and ionophore-treated cells processed with the standard protocol (Table II). Cultures exposed to A23187 or ionomycin and fixed in the presence of actin stabilizing drugs showed increases in the abundance of growth cones with apparently disrupted peripheral actin filament networks of 11 ± 1% and 13 ± 2%, respectively, over controls, compared with increases of 31 ± 6% and 24 ± 8% for cells treated with the calcium ionophores processed in the conventional manner.

Ultrastructure of Extracted Neurite Tips

Cytoskeletons of extracted neurite tips exposed to control medium typically consisted of 5–15 microtubules interconnected with many finer filaments, but with few finer filaments extending beyond the microtubules (Fig. 6 *B*). Individual growth cones were scored as typical, more complex, much more complex, less complex, or much less complex than this standard, based on the numbers of microtubules and

Table II. Percentage of Growth Cones Fixed with Actin Stabilizing Protocols Showing Apparent Disruption of Peripheral Actin Filaments

	No drug		Treatment condition (A23187)		Ionomycin	
Experiment	W Ca^{++}	O Ca^{++}	W Ca^{++}	O Ca^{++}	W Ca^{++}	O Ca^{++}
17 (10 min) (Tannic acid)	34% (110)		46% (132)			
18 (30 min) (Tannic acid)	21% (109)		32% (118)			
19 (10 min) (Phalloidin)	18% (114)		30% (61)		30% (128)	
20 (10 min) (Fixed as 19)	31% (137)				46% (85)	
(Averages,	24% ± 9%		*36% ± 9%		38% ± 11%	

Cells were exposed to calcium ionophores or calcium-free medium and then fixed with one of three different fixation protocols. To enhance preservation of actin filaments, tannic acid was included in the fixation protocols of experiments *17* and *18*, and phalloidin was included in experiment *19* (see Materials and Methods). Cell in experiment *20* was cultured at the same time and treated identically to cells in experiment *19* except that phalloidin was omitted from the fixation protocol. Percentages of ionophore-treated growth cones showing peripheral actin filament disruption are intermediate between the values for control cells, and those for ionophore-treated cells were processed conventionally.
* Differs from control at the 0.001 level of significance using the paired *t* test.

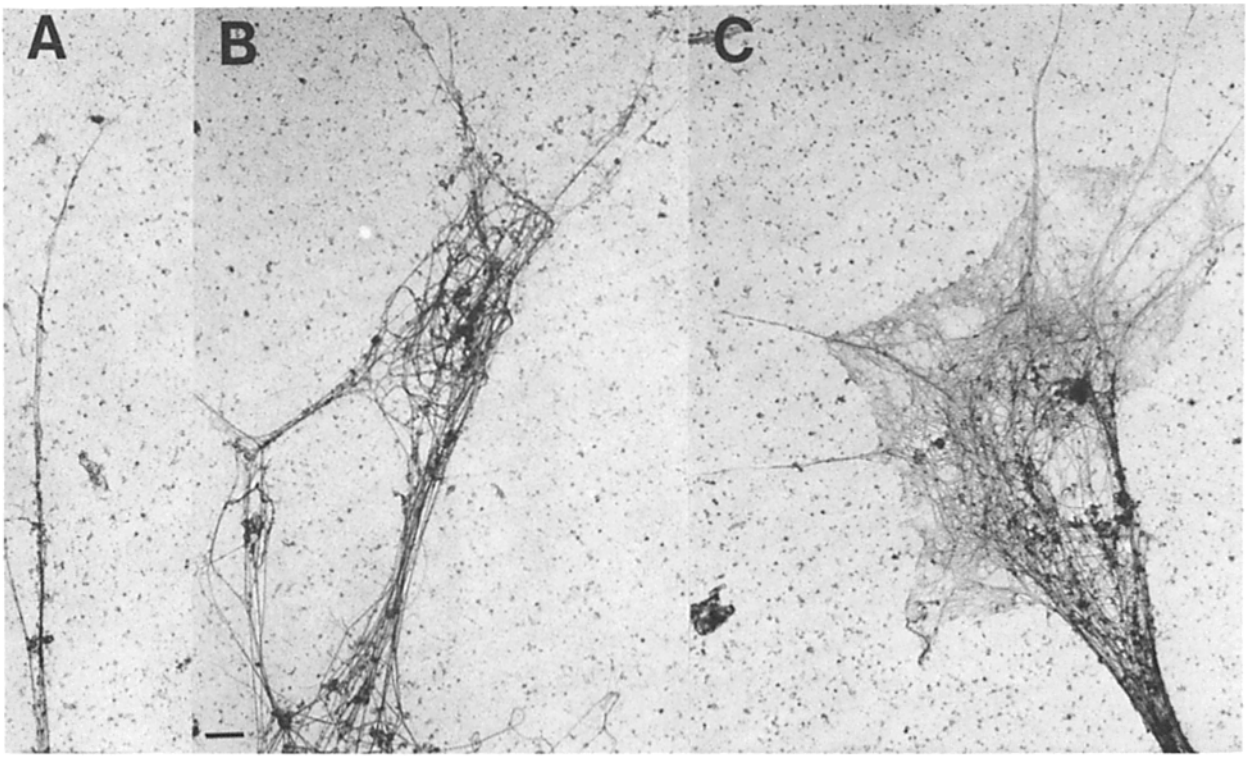


Figure 6. Complexities of growth cone cytoskeletons exposed to different treatment conditions and extracted with Triton-X 100. *A*, Growth cone exposed to A23187 in calcium-containing medium representing the lowest complexity group with only a few microtubules remaining. *B*, Growth cone treated with control vehicle only representing the typical condition with many microtubules that interconnected by finer filaments and a few microfilaments extending beyond the microtubules. *C*, Growth cone treated with A23187 in calcium-free medium and representing the highest complexity group. Note the weblike filamentous structures extending between microfilament bundles and beyond the microtubules. Bar, 1 μm.

the quantity of other cytoskeletal elements. In the highest category of complexity, bundles of actin filaments marked probable sites of filopodia in living growth cones and webs of actin filaments, often including some membrane fragments, extended between microfilament bundles and beyond the microtubules (Fig. 6 *C*). In the lowest category of complexity, only one or two microtubules and few, or no, other filaments remained to mark the position of the neurite tip (Fig. 6 *A*).

Extracted neurite tips exposed to A23187 in calcium-free or calcium-containing medium showed increases and decreases respectively in complexity of cytoskeletal composition (Table III). Eighteen percent of neurites exposed to A23187 in calcium-free medium for 30 min scored in the highest category for complexity of the remaining cytoskeleton, compared to 13% of control neurites and only 2% of neurites exposed to A23187 in calcium-containing medium. Similar data was obtained for neurites exposed to experimental conditions for only 10 min.

Pooling data into two groups, ⩽ typical and ⩾ typical (as described in Materials and Methods), showed that the criterion used did, in fact, rather closely define a mean point in controls, with 48% and 49% of the growth cones in two experiments falling into the ⩾ typical category (Table III). By comparison, higher percentages of growth cones exposed to calcium ionophores in calcium-free medium (57%), and a lower percentage of growth cones exposed to either of the two ionophores in calcium-containing medium (30% and 29%) fell into this ⩾ typical group. Thus, both the integrity of the peripheral actin filament network and the stability of the growth cone cytoskeleton as a whole were found to be decreased under conditions that enhanced calcium influx and induced neurite retraction, and increased under conditions

Table III. Complexity of Growth Cone Cytoskeleton Remaining after Standard Triton X-100 Extraction after Exposure to Control Medium or Calcium Elevating or Depleting Conditions

Exposure time	Conditions	Typical	Highest complexity
10 min	0 mM Ca w/A23187	57%	23% (111)
	Control	49%	21% (136)
	0.3 mM Ca w/A23187	29%	8% (115)
20 min	0 mM Ca w/A23187	57%	19% (103)
	Control	48%	13% (93)
	0.3 mM Ca w/A23187	30%	2% (105)

Growth cones exposed to different calcium conditions for 10 or 20 min and extracted with Triton X-100 were ranked into categories based on the relative complexity of the remaining cytoskeleton compared with the typical configuration seen in controls (see Fig. 5, *middle*). Growth cones scored as more complex than the typical condition, and one half of the typical group were grouped together as ⩾ *typical*. The percentage of growth cones representing the ⩾ *typical* category and the percentage showing the highest level of complexity (see Fig. 5, *right*) are shown for each condition for both experiments. Note that the percentages of neurites in both the ⩾ *typical* and the *Highest complexity* group increase in the calcium depleting conditions and decrease in the calcium elevating conditions relative to controls. (Numbers of growth cones scored are indicated in parentheses.)

that reduced calcium influx and inhibited neurite outgrowth without causing neurite retraction.

Discussion

Taken as a whole, the data presented here indicate that the stability of growth cone cytoskeletal elements, and particularly of the actin microfilament network in the growth cone periphery is profoundly altered under conditions that alter calcium influx and inhibit neurite outgrowth. Under conditions that increase calcium influx, actin filaments in the periphery appear to be disrupted or destabilized compared to control conditions or conditions that lower intracellular calcium. Similarly, increased calcium influx correlated with a decrease in the quantity and complexity of composition of cytoskeletal elements remaining after standardized extraction. The abilities of the cytoskeletal stabilizing drugs taxol and phalloidin to partially block or reverse the effects of elevated intracellular calcium levels further argue that the behavioral effects of calcium are mediated by changes in the cytoskeleton. The changes in cytoskeletal organization observed after manipulation of calcium influx and the antagonistic effects of cytoskeletal stabilizing drugs on the behavioral effects of calcium manipulations suggest a model in which calcium ions control neurite outgrowth primarily through regulation of actin filament assembly–disassembly or degradation, and secondarily through regulation of microtubules.

Although we have not rigorously investigated whether calcium manipulations affect such parameters as axonal transport, membrane insertion, or substrate attachment, the absence of any obvious reduction in vesicle transport or detachment of growth cones from the substrate indicates that any changes in these parameters are probably not great enough to account for the complete cessation of neurite outgrowth. Some, or all, of the aforementioned mechanisms may well contribute to the observed behavioral phenomenon, but no large and obvious changes were noted, compared to the rather large and obvious effects on neurite extension. The persistence of filopodial motility and bidirectional vesicle transport further argues that ATP levels are not critically depleted, and the reversibility of the responses indicates that the levels of the drugs used are not toxic to these cells, at least for short exposures. Likewise, the abilities of taxol and phalloidin to promote outgrowth in the presence of calcium ionophores in calcium-containing medium, indicated that vesicle transport and insertion of new membrane were sufficiently functional under these conditions to permit neurite extension. An apparent increase in vesicle density was noted in growth cones exposed to calcium ionophores for 30 min, implying a possible partial inhibition of membrane insertion or retrograde transport; however, these effects were not observed at earlier time points when retraction was apparent at the light microscope level, and this observation could also be explained by the reduction in growth cone volume. In any case, the effect did not appear to be calcium specific as it was observed more dramatically in monensin-treated growth cones.

The only significant changes in growth cones or neurites observed in this study that occurred within the same time frame as the behavioral responses and that could account for the main features of the responses, were changes in the cytoskeleton. In particular, the apparent disruption of peripheral actin filaments was observed at least as early as 5 min after the addition of calcium ionophores; a time when significant behavioral effects of the treatment could be noticed. The apparent lack of effect of calcium manipulations on microfilaments in filopodia was also consistent with the observed lack of effect of calcium ionophores on filopodia at early time points. The ability of the actin-stabilizing agent phalloidin to reverse calcium ionophore induced growth cone collapse and neurite retraction and the apparent reciprocal antagonism of A23187 and phalloidin provide further strong support for the idea that calcium ions inhibit neurite outgrowth by destabilizing actin filaments. The observation that phalloidin did not lower growth cone calcium concentrations or inhibit calcium ionophore-induced elevation of calcium, argues against trivial mechanisms of phalloidin action such as blocking calcium channels. It should be noted however that we have not yet directly established whether phalloidin does enter the cell under our experimental conditions and it is still possible that phalloidin could act through some as yet unidentified mechanism.

A model for calcium control of growth cone activity and neurite outgrowth by acting primarily through alterations in peripheral actin stability would also be consistent with the high buffering capacity of many growth cones for calcium (1), but strong behavioral sensitivity to conditions that alter calcium influx (2, 13, 17, 35, 36, 40, 41). In the model implied by this study, the effects of calcium ionophores or calcium removal would be primarily through local changes in calcium concentration at the growth cone periphery. Large changes in calcium concentration within the growth cone as a whole would not be necessary to produce changes in motility. The possibility that the apparent loss of peripheral actin filaments in growth cones exposed to calcium elevating conditions could be because of rapid expansion of the plasma membrane by calcium-mediated fusion of vesicles to the plasma membrane seems unlikely. Unlike other systems where plasma membrane expansion was observed after enhanced calcium influx (29, 45), chick DRG growth cones in this study collapsed and became narrower and more rounded under calcium elevating conditions.

Since neurite outgrowth is inhibited under conditions of altered calcium influx that result in either an increase or a decrease in actin filament disruption and cytoskeletal stability, the results suggest that both assembly of new cytoskeletal structures and disassembly of existing structures may be important for neurite extension. It should be remembered that the drug phalloidin, which stabilizes actin filaments, inhibited growth cone motility and neurite outgrowth in a manner similar to the effects of calcium removal. The detection of regions of apparent actin disruption in roughly 30% of growth cones in control medium is also consistent with the idea that disassembly of actin filaments in portions of the growth cone periphery may be part of the normal mechanism for controlling the extent and or direction of neurite outgrowth. Although sufficient to explain the responses observed, it has not yet been established whether changes in actin filament structures are necessary for these responses.

Although not as clear cut as the changes in microfilament networks, changes in microtubule polymerization and/or stability also appears to play a role in the calcium-mediated behavioral response. Extracted neurites exposed to calcium-elevating conditions showed reductions in numbers of micro-

tubules as well as in microfilament complements. Furthermore, the microtubule-stabilizing drug taxol was able to block the neurite retraction normally seen at later stages of calcium ionophore exposure, implying that the retraction component of the calcium response probably involved depolymerization of microtubules. Studies examining the combined effects of taxol and cytochalasins on neurite outgrowth, however (33, 39), indicate that enhancing microtubule stability can compensate in an indirect manner for outgrowth inhibiting effects of actin filament disruption, and the relative roles of changes in actin versus microtubules in the observed behavioral responses are therefore difficult to establish. More extensive tests of the combined effects of microtubule stabilizing or disrupting drugs and microfilament stabilizing and disrupting drugs under conditions of elevated or lowered calcium will be necessary to further clarify the relative roles of these two cytoskeletal elements in calcium-mediated behavioral responses.

The ultrastructural effects of elevated intracellular calcium appear to be ion specific. Although the behavioral responses to the monovalent cation ionophore monensin were grossly similar to those to the two calcium ionophores, disruption of peripheral actin filaments was not observed in cells treated with monensin. Fura-2 measurements of growth cone calcium concentrations confirmed that ionomycin and the nonfluorescent derivative of A23187, 4-bromo-A23187, were both capable of inducing large increases in growth cone calcium concentrations on the order of >300 nM, while monensin, which did not disrupt actin filaments, also did not alter growth cone calcium levels. Similarly, both calcium ionophores showed qualitatively similar effects on the cytoskeleton, and apparent losses of peripheral actin filaments were not noted in cells treated with calcium ionophores in calcium-free medium, indicating that the effects were not because of the properties of the ionophores themselves or to nonspecific changes in cation concentrations. Taken together, these results strongly support a link between elevated growth cone calcium and a disruption of peripheral actin networks.

It is not known at present whether elevated intracellular calcium completely degrades actin filaments in living cells or simply destabilizes them. Since osmium fixation used in the preparation of these samples is known to contribute to the destruction of actin filaments (57), it is possible that the apparent loss of filaments actually represents a destabilization or enhanced sensitivity to osmium destruction. The intermediate levels of actin filament disruption in calcium ionophore treated growth cones fixed in the presence of tannic acid or phalloidin, compared with controls and standard fixation, suggest that both microfilament degradation and destabilization may contribute to the ultrastructural appearance of the growth cone periphery.

It is also unclear whether the observed loss of actin filaments, and implied shortening of microtubules are caused by depolymerization or enzymatic degradation of these cytoskeletal elements. Several calcium-activated proteases are known to attack actin, actin-associated proteins, or microtubule-associated protein (5, 15, 24, 44). Currently, the rapid recovery of growth cones from elevated calcium conditions upon return to control medium or after the addition of phalloidin implies that large pools of cytoskeletal subunits are available for assembly and favors the depolymerization theory. The use of specific protease inhibitors would be necessary to resolve this issue however.

It is not certain why roughly 40% of growth cones treated with calcium ionophores in the presence of calcium did not show the characteristic disruption of peripheral actin filaments by EM, when $<20\%$ of the neurites monitored at the light microscope level failed to retract in response to ionophore treatment. One possibility is that the number of growth cones with disrupted actin filament networks may be underestimated because of a high percentage of affected growth cones being damaged in processing, and in fact more growth cones in the drug treatment conditions could not be scored because of damaged plasma membranes. Alternatively, the extent of disruption of actin filaments necessary to produce a detectable behavioral response may be relatively small and not require the complete loss of filaments in any one region.

An intriguing observation that may shed some light on the biochemical basis for the action of calcium was the apparent lack of effect of calcium manipulations on filopodial actin filaments. This observation suggests that differences in composition between actin filaments in the filopodia and growth cone proper might be responsible for the stability of filopodial microfilaments to elevated calcium. Recent fluorescence antibody labeling of DRG growth cones have indicated that filopodia are enriched in alpha actinin, filamin, myosin, and tropomyosin but depleted in spectrin and MAP2 compared to the growth cone and the neurite (32).

The data presented here suggest a model for calcium regulation of neurite outgrowth in which actin, and perhaps other cytoskeletal elements, are in a state of dynamic instability. The ultrastructural data indicate that changes in microfilament stability do occur within growth cones as a result of manipulating calcium influx, and that these changes are sufficient to account for the key characteristics of the response, including the time course. The ability of phalloidin to reverse A23187-induced neurite retraction and to promote a return to normal growth cone morphology and neurite outgrowth further imply that disruption of actin filament networks may be necessary for the behavioral effects of calcium. These data provide the first evidence that we know of that calcium can act as a regulator of actin filaments in a living cell and suggest one model for explaining the effects of calcium fluxes on cell shape and motility. The differential effects of elevated calcium influx on actin networks in the growth cone periphery versus the filopodia, which have different complements of actin-associated proteins, also suggests a possible criterion for speculating whether and how a given actin structure may be altered by changes in intracellular calcium.

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