Role of Intracellular Calcium in NI-35–Evoked Collapse of Neuronal Growth Cones

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A myelin-associated protein from the central nervous system, the neurite growth inhibitor NI-35, inhibits regeneration of lesioned neuronal fiber tracts in vivo and growth of neurites in vitro. Growth cones of cultured rat dorsal root ganglion neurons arrested their growth and collapsed when exposed to liposomes containing NI-35. Before morphological changes, the concentration of free intracellular calcium ($[Ca^{2+}]_i$) showed a rapid and large increase in growth cones exposed to liposomes containing NI-35. Neither an increase in $[Ca^{2+}]_i$ nor collapse of growth cones was detected in the presence of antibodies to NI-35. Dantrolene, an inhibitor of calcium release from caffeine-sensitive intracellular calcium stores, protected growth cones from collapse evoked by NI-35. Depletion of these caffeine-sensitive intracellular calcium stores prevented the increase in $[Ca^{2+}]_i$ evoked by NI-35. The NI-35–evoked cascade of intracellular messengers that mediates collapse of growth cones includes the crucial step of calcium release from intracellular stores.

During development, neuronal growth cones interact with physical and chemical cues in their environment. These interactions guide the growth cones along specific pathways to their appropriate targets. Many factors such as soluble or substrate-bound growth factors and components of the extracellular matrix provide favorable environments that allow or promote motility of growth cones and neurite elongation (*1*). Repulsive and inhibitory factors also exist and may participate in the guidance of growth cones (*2–4*). We have isolated membrane proteins from myelin of the central nervous system (CNS) that inhibit growing neurons (*5*). These neurite growth inhibitors, NI-35 and NI-250, are exclusively expressed on the surface of differentiated oligodendrocytes, cells that myelinate the CNS (*5*). Binding of the specific monoclonal antibody IN-1 to NI-35 and NI-250 allows neurites to grow over myelin in vitro (*6*) and results in long-distance regeneration of lesioned CNS fiber tracts when applied in vivo (*7*). The presence of these inhibitory components on the surface of cultured oligodendrocytes is responsible for the arrest and collapse of growth cones in direct encounters of dorsal root ganglion (DRG) neurons with differentiated oligodendrocytes (*8*). Growth arrest is long-lasting and is not observed in the presence of the specific antibody IN-1.

Changes in the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) in growth cones have been related to growth cone motility and behavior (*9*). Exposure of growth cones to specific neurotransmitters can arrest neurite outgrowth (*10*). These effects are mediated in various neuronal cell types by increases in $[Ca^{2+}]_i$ (*11*). Binding of other surface molecules may cause changes in $[Ca^{2+}]_i$ (*12*). Therefore, we investigated the possible role of Ca^{2+} as a second messenger in the effects of NI-35.

To determine the effect of NI-35 on neuronal growth cones, liposomes (20 to 40 μl) containing the NI-35 protein (*13*) were applied with a fine pipette to cultures of rat DRG neurons growing on a substrate of poly-L-lysine and laminin in the presence of neuron growth factor (*14*). Growth cones responded within a few seconds with a rapid but transient twitch-like retraction of the lamellipodia (Fig. 1). This response was also obtained after the addition of protein-free liposomes or liposomes that contained NI-35 incubated with IN-1 antibodies (*15*) and is nonspecific. Growth cones were often smaller within 60 s after liposome contact, but their appearance was normal and their motility remained undisturbed. Between 2 and 4 min after addition of NI-35 liposomes, the growth cones entered a second phase of altered morphology that led to their collapse. Lamellipodia and filopodia retracted, surface area was severely reduced, and growth cones started to become rounded. Final collapse occurred within 10 to 15 min depending on the original size of the growth cone and was characterized by the absence of filopodia, by greatly reduced lamellipodia, and often by a club-shaped structure (Fig. 1). Motility of growth cones (forward displacement) was completely arrested and the actual tip of the extended neurites often retracted by several micrometers (Fig. 1). This collapse and arrest lasted more than 20 min, and only a few growth cones recovered motility within the following 20 to 30 min. Of 37 growth cones observed continuously under the microscope (*16*), 33 displayed collapse and retraction in response to addition of NI-35 liposomes. These responses are independent of any influence by the cell body because growth cones completely isolated from their cell body by transection of the neurites (*17*) collapsed in the presence of NI-35 liposomes in a manner indistinguishable from that of growth cones of intact neurons.

We analyzed the specificity of the induction of growth cone collapse by NI-35 liposomes 20 min after liposome addition by counting the percent of collapsed growth cones in fixed cultures (*18*). Addition of NI-35 liposomes caused 86.5 ±

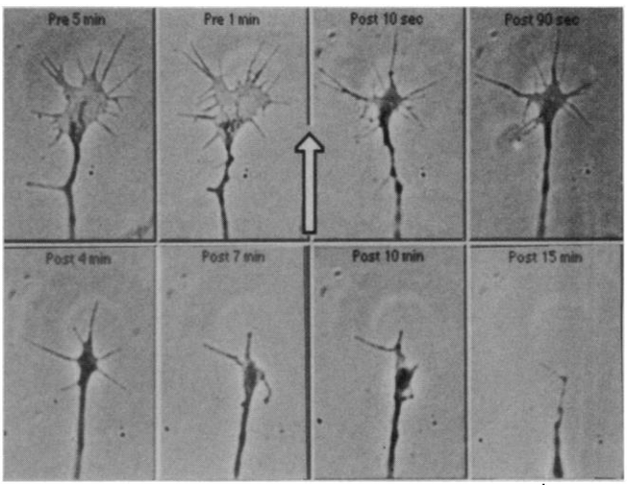

Fig. 1. Phase-contrast photomicrographs of response of a DRG growth cone to the application of liposomes containing NI-35. The first two frames indicate the degree of spontaneous change normally occurring over a 4-min interval before the addition of liposomes (Pre). Ten seconds after (Post) application of liposomes (open arrow), the twitch-like response was evident as a decrease in lamellipodial surface area. However, subsequent reexpansion of the lamellipodium was observed within 90 s. By 4 min, unmistakable signs of collapse were seen. Collapse was complete within 10 to 15 min and withdrawal of the neurite also was observed. Scale bar, 5 μm.

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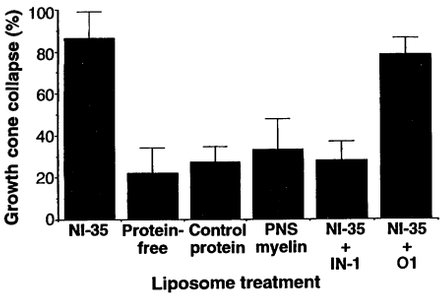

Fig. 2. Quantitative analysis of growth cone collapse in response to various treatments. Values represent the percent of collapsed versus uncollapsed growth cones 20 min after liposome addition. Data are shown as the mean ± SEM.

12.5% of DRG growth cones (n = 93) to collapse within 20 min (Fig. 2). This number might be a slight underestimate because a few collapsed growth cones may have recovered by this time. After the addition of protein-free liposomes, only 22 ± 12% (n = 87) of the growth cones collapsed (Fig. 2). Liposomes that contained a noninhibitory CNS myelin protein of 90 kD (*13*) caused 27 ± 7% (n = 69) of the growth cones to collapse (Fig. 2). To exclude a possible nonspecific effect on growth cones by interactions with proteins that bind to neurons, we also made liposomes containing myelin from the peripheral nervous system (PNS), which, like the CNS, contains relatively large amounts of myelin-associated glycoprotein (*19*). This final control gave no collapse response comparable to that of NI-35 (34 ± 13% collapsed; n = 78). We analyzed further the specificity of the collapse induced by NI-35 by exposing growth cones to NI-35 liposomes that had been incubated with the neutralizing monoclonal antibody IN-1. Under these conditions, only 28 ± 9% (n = 73) of the growth cones collapsed (Fig. 2). Liposomes with the NI-35 protein that were incubated with the control antibody O1 (*20*) evoked collapse of 78 ± 8% (n = 69) of the growth cones (Fig. 2). In some experiments, the response of growth cones was monitored continuously. During the first 10 s after addition of liposomes that lacked NI-35 or contained NI-35 blocked by IN-1 antibodies, the previously described rapid and transient contraction was observed. This response was never followed by an arrest of growth cone motility or complete collapse.

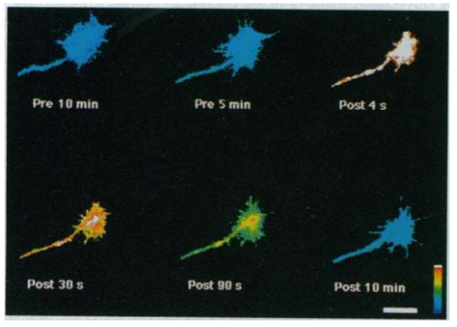

Fig. 3. Concentration of free intracellular $[Ca^{2+}]_i$ in a growth cone loaded with Fura 2 before (Pre) and after (Post) application of liposomes containing NI-35. The first two frames show the characteristically stable calcium base line concentrations in the absence of external stimuli over a 5-min period before application of liposomes. Within 4 s after application of NI-35 liposomes, $[Ca^{2+}]_i$ increased by over 20-fold. By 10 min, the growth cone was restored to nearly resting $[Ca^{2+}]_i$ values. Color scale indicates pseudocolor equivalents for $[Ca^{2+}]_i$ ranging from 50 nM (dark blue) to >3000 nM (white). Scale bar, 5 μm.

To assess whether growth cone collapse induced by NI-35 is correlated with changes in $[Ca^{2+}]_i$, we directly measured $[Ca^{2+}]_i$ in growth cones containing the calcium-sensitive fluorescent dye Fura 2-AM (*21*) before, during, and after application of NI-35 liposomes. These liposomes evoked a large and rapid rise in $[Ca^{2+}]_i$ (Fig. 3), with maximal concentrations observed within 20 s. In 17 growth cones, $[Ca^{2+}]_i$ increased from 157 ± 12 nM to peak values of 2132 ± 259 nM within 8 s (Fig. 4). The $[Ca^{2+}]_i$ subsequently returned (*22*) to a concentration similar to that in resting cells. In cells loaded with Fura 2, the changes evoked by NI-35 in growth cone morphology were different from those observed in cells without Fura 2: Collapse was not complete. This may have occurred because Fura-2 can act as a buffer of $[Ca^{2+}]_i$ or because it affects the specific site of Ca^{2+} action. A response to NI-35 could also be elicited in neuronal somata. Application of NI-35 to cell bodies resulted in an increase in $[Ca^{2+}]_i$ from 138 ± 2.4 nM to 880 ± 76 nM (n = 36) with a time course similar to that of growth cones. When a higher dose of a solution containing NI-35 liposomes was added (40 to 50 μl/ml) (*13*), a rapid and large increase in $[Ca^{2+}]_i$ occurred that saturated the Fura-2 signal and resulted in the loss of neuronal integrity. This disruption of neuronal processes resembled the effects observed after application of high concentrations of the calcium ionophore A23187.

Like the morphological changes induced by NI-35, the increase evoked by NI-35 in $[Ca^{2+}]_i$ was specific to NI-35 and not seen in growth cones treated with protein-free liposomes (176 ± 12 nM to 206 ± 14 nM; n = 12) or in liposomes incubated with the IN-1 antibody (174 ± 12 nM to 210 ± 26 nM; n = 26; P = 0.15). Abolition of the NI-35 effect by IN-1 was due to the specific inhibition of NI-35 because incubation of NI-35 liposomes with O1 antibodies caused increases in $[Ca^{2+}]_i$ similar to those observed with NI-35 liposomes not treated with antibodies. Treatment with IN-1 antibodies also blocked an increase evoked by NI-35 in $[Ca^{2+}]_i$ in neuronal somata. Thus, a close correlation exists between the long-lasting collapse of growth cones and the increase in $[Ca^{2+}]_i$ evoked by NI-35 liposomes.

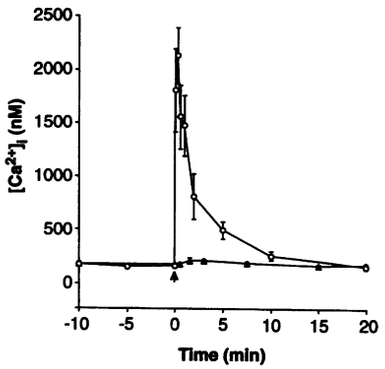

Fig. 4. Time course of changes in $[Ca^{2+}]_i$ evoked by liposomes containing NI-35 in the presence or absence of the monoclonal antibody IN-1. A large $[Ca^{2+}]$ increase is specifically evoked by NI-35 liposomes (open circles) and occurs in less than 8 s. NI-35 liposomes incubated with the neutralizing antibody IN-1 (closed triangles) produce no significant changes in $[Ca^{2+}]_i$. Arrow indicates time of liposome addition. Each value represents the mean ± SEM.

Large increases in $[Ca^{2+}]_i$ could result from an increased influx of Ca^{2+} from the extracellular environment (*23*), an increase in release of Ca^{2+} from internal stores (*24*), a decrease in Ca^{2+} clearance capacity (*22*), or any combination of these changes. Given the extremely rapid nature of the event induced by NI-35, a decreased clearance capacity does not seem likely. The reaction of DRG growth cones to elevated $[Ca^{2+}]_i$ was examined in two ways: by application of the calcium-specific ionophore A23187, which causes influx of Ca^{2+} from the medium (*25*), and by caffeine-induced release of Ca^{2+} from intracellular stores (*26*). When applied to DRG cultures, A23187 (10^{-6} M) caused 97% of the growth cones to collapse (n = 47). This response often occurred within 30 to 90 s and was characterized by blebbing and eventual deterioration of the neurites. This reaction of the neurites and growth cones resembles that seen when a high dose of solution containing NI-35 liposomes (80 to 100 μl/2 ml) was added but is unlike that seen with our usual experimental concentration of NI-35 (20 to 40 μl/2 ml). Application of caffeine (10 mM) to DRG cultures resulted in the

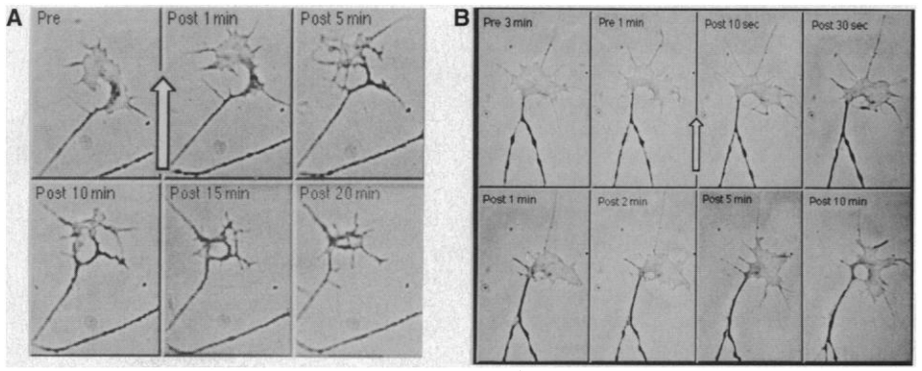

Fig. 5. Phase-contrast photomicrographs of DRG growth cones. (**A**) Addition of caffeine (40 mM) (open arrow) inhibits motility of growth cones and results in a decreased growth cone surface area. (**B**) Neurons were treated with dantrolene (40 μM) throughout the experiment starting 15 min before application of NI-35 liposomes. No collapse or arrest of motility occurred on application of NI-35 liposomes (open arrow), although slight retraction of filopodia was seen 1 min after application. Scale bar, 5 μm.

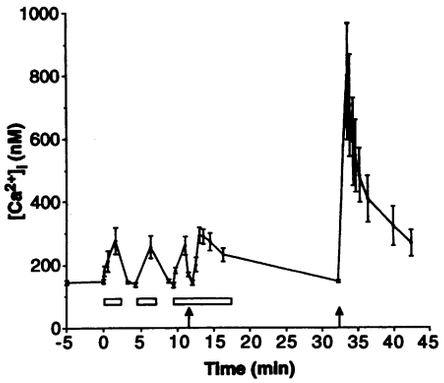

Fig. 6. Effect of sequential caffeine application on the increase in $[Ca^{2+}]_i$ evoked by NI-35. Three 10 mM caffeine pulses (open bars) each evoke a modest rise in intracellular Ca^{2+}. During the third pulse, application of NI-35 liposomes (first arrow) fails to yield the expected large increase in $[Ca^{2+}]_i$. Subsequently, caffeine is removed, and after an interval of 15 min (which presumably allows the replenishment of Ca^{2+} stores), the reapplication of NI-35 liposomes (second arrow) results in a large Ca^{2+} response. Data were obtained on DRG cell bodies loaded with Fura 2 and represent the mean ± SEM.

arrest of growth cone motility and pronounced morphological changes, such as filopodial retraction and a slow reduction in the surface area of growth cones. These changes resulted in thin, spiderweb-like growth cone structures (Fig. 5A). In 11 out of 12 growth cones, these changes occurred at times ranging from 5 to 20 min. Neurite retraction was not observed (Fig. 5A). These results show that induction of Ca^{2+} release from internal stores is sufficient to arrest motility of growth cones and cause partial growth cone collapse.

Because Ca^{2+} from both extracellular and intracellular sources can cause collapse of growth cones and arrest growth cone motility, we assessed whether blocking pathways associated with Ca^{2+} influx or release from intracellular Ca^{2+} stores could prevent collapse of growth cones evoked by NI-35. Dihydropyridine L-type calcium channel blockers (*27*) had no effect on the growth cone collapse induced by NI-35: 14 out of 17 collapsed. Similarly, addition of the N-type channel blocker ω-conotoxin (*28*) failed to prevent a reaction mediated by NI-35: 11 out of 14 collapsed. We do not rule out, however, some involvement of Ca^{2+} from extracellular sources through other transmembrane channels (*29*). Interesting results with cobalt, an agent that cannot be used with growth cones because of its deleterious effects, suggest a possible role of Ca^{2+} from extracellular sources. Large doses of NI-35 liposomes (80 to 100 μl/2 ml), which caused blebbing and disintegration of neurons, did not have such effects (three experiments; 32 cells monitored) when cobalt (4 mM), a general calcium channel blocker (*30*), was present. Washing out cobalt subsequently resulted in the usual striking effect of NI-35 addition.

If internal Ca^{2+} stores sensitive to caffeine are important to evoke collapse of growth cones by NI-35, it should be possible to prevent this collapse by blocking Ca^{2+} release from these sources by dantrolene (*31*). Indeed, preincubation of DRG cultures with dantrolene (40 μM) for 15 min prevented growth cone collapse evoked by NI-35 (Fig. 5B). Growth cones exposed to NI-35 in the presence of dantrolene responded within 1 to 2 min with a small shrinkage of the lamellipodia and retraction of some filopodia. Neither arrest of growth cone motility nor the characteristic complete collapse was observed in the presence of dantrolene in 12 out of 17 continuously monitored growth cones. To compare these effects directly with other experiments, we assayed the effect of dantrolene at 20 min on a larger number of growth cones (n = 63). Addition of liposomes containing NI-35 in the presence of dantrolene caused only 24 ± 11% of the growth cones to collapse, a response equivalent to that caused by addition of protein-free vesicles. This reaction means that dantrolene essentially negates the effect of NI-35 on growth cones.

Because dantrolene is autofluorescent (*32*), it was not possible to demonstrate with Fura 2 that it blocked increases evoked by NI-35 in $[Ca^{2+}]_i$. We therefore monitored the effect of NI-35 on $[Ca^{2+}]_i$ after depletion of intracellular Ca^{2+} stores by pretreatment with caffeine (*33*). Quantitative Ca^{2+} measurements in growth cones were not possible because of morphological changes induced by caffeine and so were performed in neuronal cell bodies. After a series of three sequential caffeine pulses (2 min) to deplete Ca^{2+} stores, NI-35 liposomes were added in the presence of caffeine (three separate experiments; 17 cell bodies). Concentrations of Ca^{2+} induced by NI-35 did not increase above values normally detected in the presence of caffeine alone. Peaks were 335 ± 37 nM for caffeine, 322 ± 34 nM for the addition of NI-35 liposomes after caffeine treatment, and 880 ± 76 nM for NI-35 alone. In one representative experiment (Fig. 6), we followed the effect of both depleting and refilling internal Ca^{2+} stores on the increase in Ca^{2+} induced by NI-35. After three pulses of caffeine, the effect of NI-35 was minimal as compared to the effect induced by NI-35 on the same neurons after stores were allowed to replenish (Fig. 6). These results strongly suggest that NI-35 acts by releasing Ca^{2+} from caffeine-sensitive intracellular stores.

Changes in $[Ca^{2+}]_i$ in the growth cone have been thought to regulate motility and neurite elongation by their influence on assembly and stability of cytoskeletal elements (*9*, *11*). Here, a large increase in $[Ca^{2+}]_i$ preceded, by more than 1 min, the first signs of growth cone collapse after addition of NI-35 liposomes. Large increases in $[Ca^{2+}]_i$ like the one in response to NI-35, are already known to alter both actin (*34*) and tubulin (*35*) polymerization states, affect the activity of growth cone myosin (*36*) and calcium-dependent proteases such as calpains (*37*), or activate phosphokinases and phosphatases (*38*). Any of these effects could alter the cytoskeletal architecture of growth cones and lead to their arrest and collapse. The neurite growth inhibitor NI-35, a membrane protein specific to oligodendrocytes, may inhibit neurite growth through a sequence of events including interaction with a receptor and release of Ca^{2+} from

intracellular stores. Given the widespread occurrence of Ca^{2+}-induced Ca^{2+} release (*39*) and the alternate possibility of coupling of Ca^{2+} release and influx (*40*), an additional role for extracellular calcium cannot be ignored. The knowledge that large rises in intracellular calcium are necessary for growth cone collapse evoked by NI-35 may provide new means to alter the response of neurons to the inhibitory environment in the adult CNS and thus to increase their capacity for regeneration.

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13. Rat CNS and PNS myelin was prepared as described (*6*). Proteins from myelin of the CNS were fractionated on preparative polyacrylamide gels (11%) under reducing conditions. Gel regions containing NI-35 or control proteins migrating at approximately 90 kD were dissected, and the proteins were electroeluted in the presence of 0.1% SDS. To remove the SDS, we precipitated gel-extracted proteins overnight in a tenfold excess volume of acetone at −20°C. If small amounts of protein (<5 μg) were eluted from the gels, insulin (50 μg/ml; Sigma) was added as a carrier protein. Precipitated proteins were collected by ultracentrifugation at 40,000 rpm for 40 min at 4°C in a TLA 100.3 rotor (Beckman). Proteins were resuspended in 2.5% sodium cholate in Hank's balanced salt solution supplemented with 10 mM Hepes at a protein concentration of 50 μg/ml. Liposomes containing NI-35 or other proteins from CNS and PNS myelin were prepared by the cholate-column method [J. Brunner, H. Hauser, G. Semense, *J. Biol. Chem.* **235**, 7538 (1978)]. This procedure has been shown to result in single-layered–lipid protein vesicles of approximately 10 to 60 nm in diameter. The estimated concentration of incorporated protein is 25 μg/ml of liposome solution.
14. Dissociated DAG cells of newborn Lewis rats were processed according to the methods of M. E. Schwab and H. Thoenen [*J. Neurosci.* **5**, 2415 (1985)]. To reduce the amount of non-neuronal cells, we plated cells for 2 to 3 hours on plastic culture dishes. Neurons were grown for 10 to 24 hours on laminin-coated dishes (10 μg/ml; Collaborative Research, Bedford, MA) supplemented with fetal calf serum (10%) (Gibco) and neuron growth factor (20 ng/ml).
15. Liposomes containing protein were diluted (1:1, v:v) with hybridoma supernatant containing either IN-1 (*6*) or O1 (*20*).
16. To monitor precisely the morphological growth cone changes during application of liposomes, we washed DRG cultures twice in Dulbecco's modified Eagle's medium–Hepes (10 mM) and put them on the stage of an inverted Nikon Diaphot microscope. To provide a constant temperature of 37°C in the culture medium, we kept the microscope in a heated tent. The dish was equilibrated for at least 5 to 10 min before growth cones were selected for further observations. Growth cones were viewed continuously for 30 to 60 min with a ×100 Fluor/phase Nikon oil-immersion objective. Phase-contrast photomicrographs were acquired with a cooled charge-coupled device (CCD) camera (Photometrics) and processed with a Macintosh IIfx computer.
17. Growth cones were physically isolated by transection of the neurite with the tip of a glass micropipette attached to a micromanipulator in low (200 nM) concentrations of Ca^{2+}. After 10 to 15 min, medium containing normal concentrations of Ca^{2+} (1.4 mM) was added to the dish and growth cones were allowed to equilibrate for 5 to 10 min before addition of liposomes containing NI-35.
18. To evaluate larger numbers of growth cones, we slowly added 10 to 50 μl of NI-35 liposomes to a DRG culture dish. Only dishes where at least 50% of the fibers had growth cones were used. After a 20-min incubation at 37°C and in 10% CO_2, cultures were fixed by the slow addition of 2 ml of prewarmed 4% paraformaldehyde in 5% sucrose. After 2 to 4 hours, the fixed cultures were washed twice with phosphate-buffered saline (PBS) and mounted in glycerol-PBS. Collapsed and uncollapsed growth cones were counted under an inverted microscope.
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20. The monoclonal antibody O1 recognizes galactocerebroside (galC), a glycolipid specific for oligodendrocytes and myelin [I. Sommer and M. Schachner, *Dev. Biol.* **83**, 311 (1981)].
21. Neurons were loaded with 5 μM Fura 2-AM (Molecular Probes) for 40 min. Cells were then washed several times and incubated for 40 to 90 min before $[Ca^{2+}]_i$ was measured. All experiments were performed in Hepes (10 mM) (pH 7.4) in room air at 37°C. Images were viewed with a ×100 fluor/phase oil-immersion objective, which allowed us to focus on the growth cone in phase, thus minimizing the total amount of ultraviolet exposure. Images of phase as well as of wavelength of 350 nm and 380 nm were acquired with a cooled CCD camera (Photometrics). The ratio of the single wavelength fluorescence images was calculated on a MacIntosh IIfx computer, and quantitative values were obtained according to the calcium equation by G. Grynkiewicz *et al.* [*J. Biol. Chem.* **260**, 3440 (1985)]. Calibration parameters were derived from a least-squares fit to ratios measured for standard solutions of free Ca^{2+}.
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27. Solution of L-type calcium channel blockers contained Verapamil (10 μM), Nitrendipine (10 nM), Diltiazam (10 μM), and PN200-110 (100 nM) (Sandoz Pharmaceutical). All other drugs were purchased from Sigma. The continuous observation of growth cones here demonstrates that preliminary experiments where collapsed growth cones were counted after a fixed period (20 min) had elapsed after liposome addition [M. F. Schmidt, C. E. Bandtlow, T. D. Hassinger, M. E. Schwab, S. B. Kater, *Soc. Neurosci. Abstr.* **17**, 927 (1991)] offered an unacceptable degree of variability.
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41. We thank P. Atkinson for his skillful help in the cell culture work, J. Kapfhammer and M. Wright for critical comments on the manuscript, and V. Rehder for helpful discussions. We also thank L. Detweiler and S. Sternberg for providing help with the imaging technology, D. Giddings for help in preparing the figures, H. Thoenen for neuron growth factor, and C. E. Eden for a solution of L-type calcium channel blockers. Supported by the Swiss National Science Foundation (31-29981.90), the American Paralysis Association, Regeneron Pharmaceuticals, the Spinal Cord Research Foundation (SCRF840-01-02), and NIH grants NS28323 and NS24683.

11 May 1992; accepted 8 September 1992