Induction of Growth Cone Formation by Transient and Localized Increases of Intracellular Proteolytic Activity

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Abstract. The formation of a growth cone at the tip of a transected axon is a crucial step in the subsequent regeneration of the amputated axon. During this process, the transected axon is transformed from a static segment into a motile growth cone. Despite the importance of this process for regeneration of the severed axon, little is known about the mechanisms underlying this transformation.

Recent studies have suggested that Ca^{2+}-activated proteinases underlay the morphological remodeling of neurons after injury. However, this hypothesis was never tested directly. Here we tested the ability of transient and localized increases in intracellular proteolytic activity to induce growth cone formation and neuritogenesis. Minute amounts of the proteinase trypsin were microinjected into intact axonal segments or somata of cultured *Aplysia* neurons, transiently elevating the intracellular protease concentration to 13–130 nM in the vicinity of the injection site. Such microinjections were followed by the formation of ectopic growth cones and irreversible neuritogenesis. Growth cones were not formed after external application of trypsin, microinjection of the carrier solution, or inactivated trypsin. Growth cone formation was not preceded by increases in free intracellular Ca^{2+} or changes in passive membrane properties, and was blocked by inhibitors of actin and tubulin polymerization. Trypsin-induced neuritogenesis was associated with ultrastructural alterations similar to those observed by us after axotomy.

We conclude that local and transient elevations of cytoplasmic proteolytic activity can induce growth cone formation and neuritogenesis, and suggest that localized proteolytic activity plays a role in growth cone formation after axotomy.

THE transection of an axon is often followed by the formation of a new growth cone near the tip of the amputated axon (Shaw and Bray, 1977; Bray et al., 1978; Wessells et al., 1978; Baas and Heidemann, 1986; Baas et al., 1987; Rehder et al., 1992; Ashery et al., 1996; Ziv and Spira, 1997). During this process, the differentiated, stable axonal segment is transformed into a motile, irregularly shaped growth cone. Although this structural and functional dedifferentiation is crucial for the successful regeneration of the amputated axon, little is known about the mechanisms that underlie this process.

In earlier studies we found that axotomy of identifiable cultured *Aplysia* neurons is followed by a transient influx of Ca^{2+} through the ruptured membrane (Ziv and Spira, 1995, 1997). The influx forms a steep Ca^{2+} concentration gradient along an axonal segment of ~150–200 μm, in which the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[1] can exceed 1 mM near the cut end. The influx is terminated within 1 to 2 min from transection when a membrane seal forms over the cut end. In the ensuing period (1–5 min), $[Ca^{2+}]_i$ gradually returns to control levels (Ziv and Spira, 1993, 1995; Spira et al., 1993, 1996).

The increases in $[Ca^{2+}]_i$ alter the axonal ultrastructure in a characteristic manner (Spira et al., 1993; Ziv and Spira, 1997) similar to that reported in other systems (Schlaepfer, 1974; Meiri et al., 1983; Roederer et al., 1983; Lucas et al., 1985; Emery et al., 1987; Gross and Higgens, 1987). The Ca^{2+}-induced ultrastructural alterations include dissociation of microtubules, detachment of the axolemma from the axoplasmic core, swelling of endoplasmic reticulum and mitochondria, and accumulation of vesicles at specific locations. Although these alterations are usually considered to be pathological in nature, we recently provided experimental evidence indicating that they may also play a role in the cascade of events that leads to the dedifferenti-

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1. *Abbreviations used in this paper*: ASW, artificial sea water; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; MAP, microtubule-associated protein.

ation of an axon into a motile growth cone (Ziv and Spira, 1997). In the aforementioned study, we showed that transient (1–3 min) and localized elevations of $[Ca^{2+}]_i$ to 300–500 μM in intact axons of cultured *Aplysia* neurons lead to the formation of ectopic growth cones and irreversible neuritogenesis. This process is associated with ultrastructural alterations similar to those observed in axotomized axons near the regions from which growth cones emerge.

Earlier studies have suggested that elevations in intracellular Ca^{2+} concentrations activate Ca^{2+}-dependent proteases known as calpains. The excess activation of calpains was proposed to induce pathological processes manifested as the formation of membrane blebs, varicosity formation, beading, degeneration, and neuronal death (Wang and Yuen, 1994). In contrast, several studies have suggested that calpains may play important roles in the recovery of neurons from injury (Gitler and Spira, 1996). For example, calpains seem to play a role in membrane resealing after injury (Xie and Barrett, 1991; Godell et al., 1997), and in the restoration of dendritic structure after excitotoxic injury (Faddis et al., 1997). Furthermore, the activation of calpains has been suggested to underlie certain forms of neuronal remodeling related to long-term memory (Lynch and Baudry, 1984; Lynch and Seubert, 1989). However, the ability of localized proteolytic activity to induce neuronal remodeling has not been tested in a direct manner.

In principle, the ability of calpains to induce neuronal remodeling could be directly tested by microinjecting calpain into intact axons and following its effects on neuronal morphology and ultrastructure. Unfortunately, these experiments are complicated by the fact that calpain activity is regulated by several intracellular factors that are difficult to control experimentally, such as the concentration of calpastatin (an endogenous calpain inhibitor; Goll et al., 1992), the phospholipid composition and, of course, the $[Ca^{2+}]_i$. The latter factor poses the greatest difficulty, as experimentally elevating $[Ca^{2+}]_i$ to levels required for calpain activation results in growth cone formation (Ziv and Spira, 1997), even without the introduction of exogenous calpain. As a result, the effects exerted by the microinjected calpains would be obscured.

Thus, we tested the hypothesis that a transient and localized increase in serine protease activity may be sufficient to transform a differentiated axonal segment into a growth cone by microinjecting trypsin into intact axonal segments of cultured *Aplysia* neurons. We report that this experimental procedure leads to the rapid extension of an ectopic growth cone from the injection site that is followed by the irreversible formation of new neurites.

Materials and Methods

Cell Culture

Buccal neurons B1 and B2 were isolated from juvenile specimens of *Aplysia californica*, or adult *Aplysia oculifera* collected from the northern section of the Gulf of Eilat, Israel, and maintained in culture conditions as previously described (Schacher and Proshansky, 1983; Benbassat and Spira, 1993; Spira et al., 1996). Briefly, buccal ganglia were isolated, incubated for 1.5–2.5 h in 1% protease (type IX; Sigma Chemical Co., St. Louis, MO) at 35°C. The ganglia were desheathed, and then the cell body of the buccal neurons B1 and B2, with their long axons, were pulled out with sharp micropipettes and placed on poly-L-lysine–coated (Sigma Chemical Co.) glass bottom culture dishes. The culture medium consisted of equal parts of filtered hemolymph from *Aplysia faciata* collected along the Mediterranean coast and L-15 supplemented for marine species. All experiments were done 8–48 h from plating, after replacing the culture medium with artificial sea water (ASW): 460 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$, 10 mM Hepes adjusted to pH 7.6.

Electrophysiology

Conventional intracellular recording and stimulation with single glass microelectrodes were done using an amplifier in bridge mode (Model Axoclamp-2A; Axon Instruments, Inc., Foster City, CA). The microelectrodes were pulled from 1.5/1.02-mm borosilicate glass tubes with filaments and filled with 2 M KCl. The transmembrane potential was recorded from single 5–12 MΩ microelectrodes inserted into the cell body and the input resistance was monitored by applying rectangular (0.1–0.3 nA, 300–500 ms) hyperpolarizing current pulses once every 2–3 s. The electrophysiological data was recorded to 1/2 inch video cassettes with a Neuro-Corder (Neuro Data Instruments Corp.).

Trypsin Microinjection

0.5–5 mg/ml trypsin (TPCK treated, type XIII; Sigma Chemical Co.) was dissolved in a carrier solution consisting of KCl 0.5 M, and Hepes 10 mM, adjusted to pH 7.4. The trypsin solution was prepared fresh before every experiment and stored on ice until used. Micropipettes (5–8 MΩ resistance) were back filled with the trypsin solution, and mounted in a microelectrode holder connected to the headstage of an intracellular recording amplifier (see above) and a picoinjector (Medical Systems). The micropipette was immersed in the bathing solution and 5–10 brief pressure pulses were applied to expel any saline that may have entered the micropipette. To confine the spread of trypsin along the axon, we found it necessary to microinject extremely small volumes of solution. To achieve this, we did not pressure-inject the solution into the axon. Rather, the micropipette was brought into contact with the neuronal membrane, and inserted by momentarily increasing the capacity compensation for the micropipette, causing high frequency current oscillations ("buzzing"). This procedure, which is routinely used by electrophysiologists to insert microelectrodes into cells, is associated with the ejection of a small amount of solution into the cytoplasm. The micropipette was then gently pulled back and removed.

Estimates based on video recordings of the injection procedure suggested that ~10 femtoliters of solution were injected in each experiment. Assuming that the trypsin spread along an axonal segment of ~50–100 μm, the average trypsin concentration in the vicinity of the injection site was in the range of 0.3–3 μg/ml (13–130 nM).

Control injections of heat-inactivated trypsin or trypsin-free carrier solution did not have any effects on axonal morphology. Pressure ejection of trypsin onto the external axonal surface did not affect axonal morphology either.

Cytochalasin B and Nocodazole Application

Cytochalasin B (Sigma Chemical Co.) was added from 10 mM stocks in DMSO to ASW to a final concentration of 10 μM ([DMSO] <0.1%). The preparation was superfused with 10 ml of the cytochalasin B solution. The neurons were incubated in the solution 20–30 min before the injection procedure.

Nocodazole (methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-YL) carbamate; Sigma Chemical Co.) was added from a 20 mM stock in DMSO to ASW, to a final concentration of 2 μM ([DMSO] <0.01%). The preparation was superfused with 10 ml of the nocodazole-containing solution. The neurons were incubated in the solution 2–3 h before the injection procedure.

Video Microscopy

The video microscope system used for video enhanced contrast differential interference contrast imaging consisted of a microscope (model Axiovert; Carl Zeiss, Inc., Oberkochen, Germany) set with differential interference contrast optics. A 100W halogen lamp (for low magnifications) or a 100W mercury arc lamp were used as light sources. The condenser, set for Koehler illumination, was equipped with a long-working distance 0.63 NA front lens. The specimens were illuminated for minimal periods to minimize photo dynamic damage. The objectives used were either a 20× 0.50 or 40× 0.75 NA Plan-Neofluar objectives, or an oil immersion 63×

1.4 NA Plan-Apochromat (all from Carl Zeiss, Inc.). In some instances, a 4× teleconverter was placed between the microscope projection lens and the video camera's face plate. The images were collected with a video camera (Vidicon; Hamamatsu Phototonics, Hamamatsu City, Japan) and stored to a 3/4 inch video cassette recorder (Sony Corp., Sony Corp., Tokyo, Japan). Images were then digitized using a PC-hosted frame grabber (Imaging Technology Inc., Bedford, MA) by averaging 16–64 video frames. An out of focus image of an empty area of the culture dish was then subtracted from the original images. In some cases, a local contrast enhancing algorithm developed by Narenda and Finch (described in "Digital Image Processing" by Gonzalez and Wintz, Sec. Ed., page 159) was used to enhance objects with little inherent contrast. Final images were prepared with commercially available software (Adobe Photoshop and Macromedia Freehand). Fura-2 Ca^{2+} loading, imaging, and calibrating were done as previously described (Ziv and Spira 1993, 1995).

Electron Microscopy

The neurons were fixed by perfusing the culture dish with 10 ml of fixative solution containing 3% glutaraldehyde in ASW at pH 6.9 (Forscher et al. 1987). After the initial fixation, the culture dishes were removed from the microscope, the fixative solution was substituted several times, and the fixed neurons were incubated in the fixative for an additional 30 min. The cells were then washed in ASW and cacodilate buffer, pH 7.4, postfixed by 0.5% osmium tetraoxide and 0.8% $K_3Fe(CN)_6$ and stained en block with aqueous 3% uranyl acetate solution for 30 min. Dehydration was carried out through a series of ethanol solutions, and finally the neurons were embedded in Agar 100. The blocks were sectioned by a microtome (LKB Instruments, Inc., Bromma, Sweden), and thin sections of ~70 nm were stained by lead citrate, tannic acid, and uranyl acetate.

Results

Trypsin Microinjections Result in the Rapid Formation of Ectopic Growth Cones

The formation of a new growth cone induced by an intraaxonal microinjection of trypsin is illustrated in Fig. 1. A micropipette filled with a solution containing 0.5 M KCl, 10 mM Hepes, and 0.5–5 mg/ml trypsin was brought into contact with the axonal membrane, and a small volume (~10 femtoliters) of this solution was microinjected into the axon. The micropipette was then gently pulled away (Fig. 1 *A*). We estimated that this procedure resulted in a transient intraaxonal trypsin concentration of 13–130 nM (refer to Materials and Methods).

Trypsin microinjection was followed by the lateral extension of filopodia and lamellipodia from the injection site, usually within 5 to 15 min from the microinjection procedure. In the experiment of Fig. 1, the initial extension of an ectopic growth cone was detected within 8 min from the trypsin injection. This growth cone as well as others induced in this manner closely resemble growth cones formed after axotomy and in response to transient elevations of $[Ca^{2+}]_i$ in intact axonal segments of cultured *Aplysia* neurons (Ashery et al., 1996; Ziv and Spira, 1997).

The ectopic growth cones subsequently gave rise to numerous neurites that extended from the injection site, attaining lengths of hundreds of micrometers within 6 to 12 h (Fig. 2). Trypsin microinjection into axonal segments located between the soma and the distal tip of the axon did not prevent the extension of neurites from the distal tip of the neuron (Fig. 2). This observation suggests that trypsin microinjections did not block the transport of substances required for neuronal growth from the soma to distal regions of the axon.

The appearance of neurites formed after trypsin microinjections was somewhat different from that of neurites formed at the distal tip of the axon: trypsin-induced neurites were usually straighter and thicker than the neurites at the distal tip, and they seemed to have a reduced tendency to branch.

We observed some variability in the axonal response to trypsin microinjections (n = 35). Whereas 63% of trypsin microinjections resulted in irreversible neurite outgrowth, in 11% of the experiments trypsin microinjection caused little or no obvious alterations to axonal morphology. In the remaining 26% of the experiments, trypsin microinjection led to pathological morphological alterations, such as a reduction in axonal diameter, blebbing, or complete degeneration. Because these pathological responses could be reliably induced by intentionally microinjecting excess

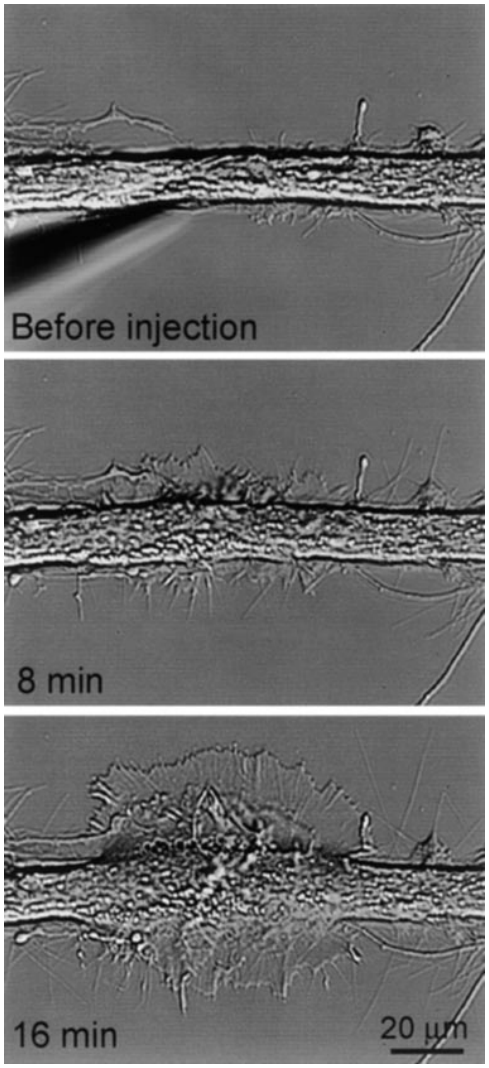

Figure 1. Microinjections of trypsin induce the formation of ectopic growth cones. The formation of an ectopic neuronal growth cone was induced by microinjecting a minute amount of trypsin into the intact axon. The micropipette used for injecting the proteinase is seen pointing at the injection site (*top*). Note the rapid formation of a growth cone in the form of an extending lamellipodium on both sides of the axon (8 and 16 min after trypsin injection, *middle* and *bottom*, respectively).

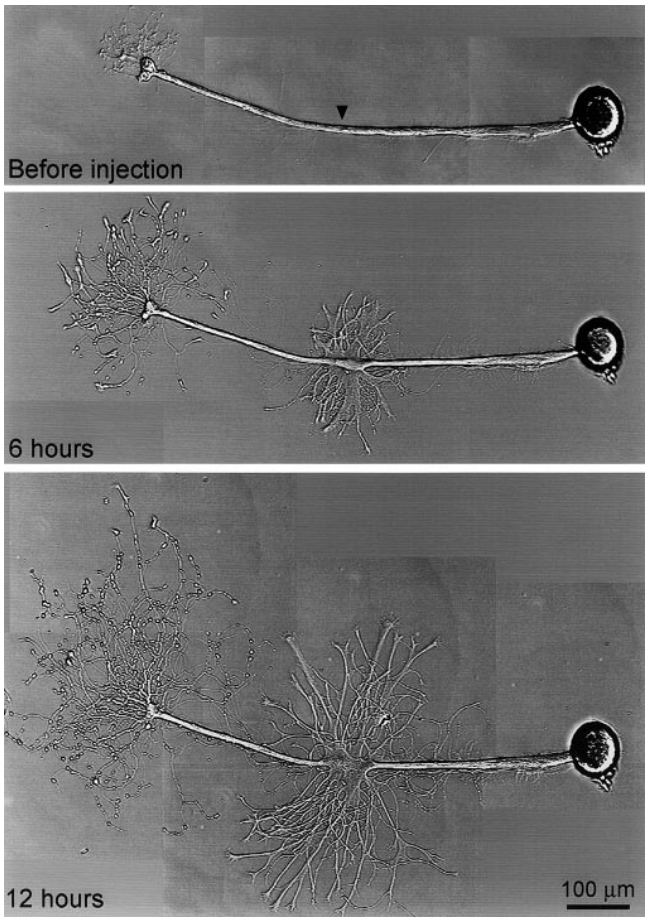

Figure 2. Neuritogenesis induced by microinjection of trypsin. The formation of ectopic neuronal outgrowth was induced by microinjecting a minute amount of trypsin into the axon of an intact neuron. The injection site is marked by an *arrowhead* (*top*). Note that although new neurites extended from the injection site, the neurites at the distal tip of the axon continued to elongate (6 and 12 h after trypsin injection, also see Fig. 3 *A*).

quantities of trypsin, we attribute the pathological responses observed in some experiments to excessive intraaxonal concentrations of trypsin. The procedure we used for trypsin microinjection (refer to Materials and Methods) did not allow precise control of the microinjected volume, and consequently, the final intraaxonal trypsin concentration varied from one experiment to another. However, as morphological alterations were never induced by microinjections of heat-inactivated trypsin or trypsin-free carrier solution ($n > 10$), the changes in axonal morphology were clearly induced by the proteinase and not by the microinjection procedure itself.

Trypsin Microinjection Does Not Damage the Axonal Membrane

We have previously shown that localized and transient elevations in $[Ca^{2+}]_i$ to 300–500 μM induce ectopic growth cone formation along intact axonal segments of cultured *Aplysia* neurons (Ziv and Spira, 1997). To exclude the possibility that the formation of growth cones after trypsin microinjections resulted from Ca^{2+} influx through damaged axolemma, we monitored the $[Ca^{2+}]_i$, the transmembrane potential, and the input resistance before, during, and after the microinjection procedure.

The experiment of Fig. 3 (*A*) shows that trypsin injection did not alter the transmembrane potential or input resistance of the neuron ($n > 15$). While trypsin microinjection was followed by growth cone formation from the injected site (Fig. 3, *B* and *D*) the outgrowth was not preceded by a large elevation in $[Ca^{2+}]_i$ (Fig. 3 *C*). In all experiments in which $[Ca^{2+}]_i$ was monitored ($n = 5$), the microinjection procedure was followed by a small (50–100 nM) and transient increase in $[Ca^{2+}]_i$, most likely caused by the microinjection procedure and the subsequent removal of the microinjection pipette. However, as we have previously shown, even elevations of $[Ca^{2+}]_i$ to several micromolars (an order of magnitude greater than those observed after trypsin injections) do not induce the formation of growth cones (Ziv and Spira, 1997).

These findings suggest that the permeability of the axonal membrane is not compromised by trypsin microinjections, and exclude the possibility that the induction of growth cone formation by trypsin microinjections is mediated by transient elevations of $[Ca^{2+}]_i$.

Growth Cone Induction by Trypsin Injections Requires Actin and Tubulin Polymerization

To investigate the roles of actin filaments and microtubules in the process of trypsin-induced growth cone formation, we injected trypsin into the axons of neurons that were preincubated in the actin polymerization inhibitor cytochalasin B, or in nocodazole, a microtubule polymerization inhibitor. In the presence of 10 μM cytochalasin B, trypsin injections did not induce the extension of any filopodia or lamellipodia ($n = 3$; data not shown). Trypsin microinjections in the presence of 2 μM nocodazole ($n = 3$) were followed by the lateral extension of many filopodia (Fig. 4). However, this response was followed neither by extension of a new growth cone nor the formation of new neurites. These results are consistent with previous studies that suggest that actin polymerization is required for the extension of the growth cone's leading edge, and that microtubules play important roles in the stabilization of the newly formed structures and in their subsequent differentiation into cylindrical neurites (Goldberg and Burmeister, 1989; Bray, 1992; Lin and Forscher, 1993; Bentley and O'Connor, 1994; Lin et al., 1994; Tanaka and Sabry, 1995).

Trypsin Injections into the Somata Induce the Formation of Growth Cones and Neuritogenesis

The cytoskeletal filaments composing the axoskeleton of cultured *Aplysia* neurons are oriented in parallel to the longitudinal axis of the axon (Spira et al., 1993; Ziv and Spira, 1997). It is conceivable that trypsin microinjections induce ectopic growth cone formation by locally disrupting the continuity of microtubule arrays, thereby creating an accumulation site for vesicles transported in ortho- and retrograde directions along these microtubules. Increased vesicle density at a given site may accelerate vesicle fusion with the plasma membrane, and, as a consequence, cause a localized extension of ectopic growth cones.

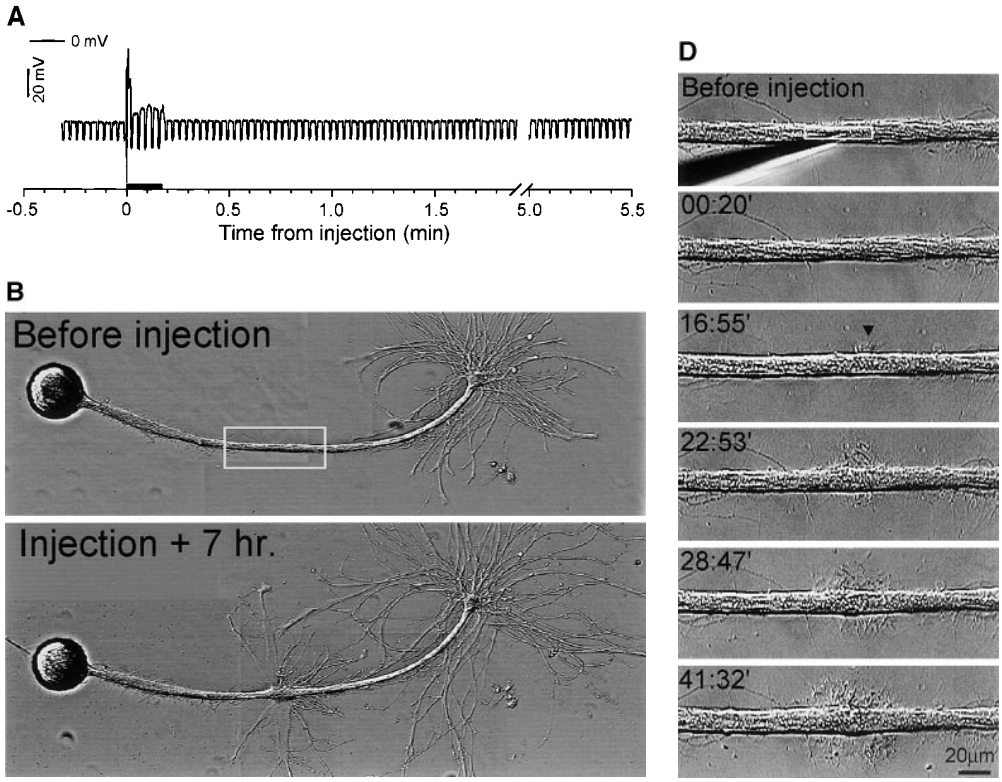

Figure 3. Trypsin injection does not significantly alter membrane permeability. Electrophysiological recordings of passive membrane properties and fura-2 ratio imaging were used to determine if trypsin-induced growth cone extension is preceded by changes in membrane permeability or large elevations of $[Ca^{2+}]_i$. (*A*) The transmembrane potential of a microinjected neuron was recorded by a microelectrode inserted into the cell body and then the input resistance was monitored by applying rectangular hyperpolarizing current pulses once every 2 s. Except for the transient depolarization caused during the injection procedure (*horizontal bar*), no significant changes in the neuron's passive membrane properties were recorded. (*B*) Low magnification images of a trypsin-microinjected neuron before (*top*) and 7 h after (*bottom*) trypsin microinjection. Note the numerous neurites that extended from the injected site (the center of the rectangle in the *top panel*). (*C*) Alterations in $[Ca^{2+}]_i$ caused by the microinjection of trypsin into the axon of the neuron shown in *B*. The $[Ca^{2+}]_i$ was determined for the region enclosed in the rectangle shown in *D* (*top*). Apart from a small (50–100 nM) and transient increase in $[Ca^{2+}]_i$ that followed the microinjection procedure, no significant alterations in $[Ca^{2+}]_i$ were recorded before or during the extension of the growth cone. (*D*) The growth cone induced by the microinjection of trypsin into the axon of the neuron shown in *B*. The first signs of growth cone formation were detected ~17 min after the injection procedure (*arrowhead*, *third panel from top*). Time is given in minutes from the microinjection of trypsin.

To examine whether trypsin-induced growth is dependent on the presence of linear arrays of microtubules, we tested whether microinjected trypsin induces growth cone formation along the cell body membrane, where microtubule orientation is much less uniform. To that end, we microinjected trypsin into the soma cytoplasm of cultured neurons at a plane just above that of the culture dish. Intrasomal trypsin injections induced the extension of a large lamellipodium along the perimeter of the soma, such as that shown in Fig. 5. The lamellipodia continued to extend and eventually branched into discrete, elongated neurites (Fig. 6). These experiments (n = 4) suggest that trypsin microinjections can also induce neuronal outgrowth from compartments that do not contain uniformly oriented microtubules.

Ultrastructural Alterations Caused by Trypsin Microinjections

To determine the changes in axonal cytoarchitecture induced by microinjections of trypsin, neurons were fixed at various times after intraaxonal trypsin microinjections (n >20), and then thin sections of their axons were examined by electron microscopy.

The most significant alterations were observed in parallel to the initial extension of the growth cone. These characteristic alterations in axonal ultrastructure are illustrated in the experiments of Figs. 7–9. In these experiments the neuron was fixed and processed for electron microscopy at an early stage of lamellipodium extension (Fig. 7, ~18 min after injection). The ultrastructure of this axon is shown in Fig. 8. A low magnification longitudinal section through the trypsin-injected axon reveals that a large number of dense vesicles had accumulated along a segment of ~35 µm (*arrowheads*), centered around the injection site. Closer examination revealed that the linear arrangement of the axoplasmic cytoskeleton in this region was disrupted and that microtubules were fragmented into small, randomly oriented fragments (Fig. 8 *C*). Although neurofilaments had also lost their uniform orientation, they appeared to be intact, and so did the endoplasmic reticulum and mitochondria in this region. In segments proximal and distal to the injection site, where no vesicle accumulation was observed, the axoskeleton retained its linear organization (Fig. 8, *B* and *D*).

In some experiments, cross-sections and longitudinal sections of trypsin-injected axons revealed that densely packed microtubule arrays had formed along the perime-

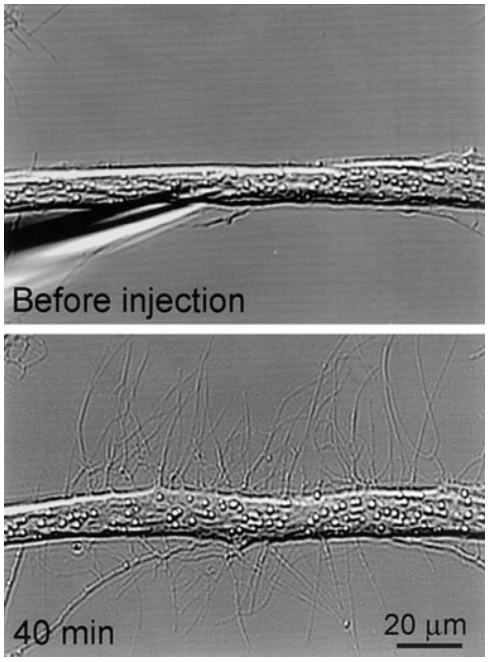

Figure 4. Trypsin-induced growth cone formation is blocked by an inhibitor of microtubule polymerization. Trypsin injections in the presence of 2 μM nocodazole led to the extension of many fine filopodia on both sides of the injection site. These filopodia, however, did not develop into a growth cone structure.

ter of the axoplasmic core (Fig. 9). The distance between adjacent microtubules in these arrays was much smaller than that observed in control axons (compare Fig. 9, *A* and *C*). As the minimal distance between microtubules is determined in part by microtubule-associated proteins (MAPs; Brown and Berlin, 1988; Hirokawa, 1994), it is possible that the close packing of these microtubules is related to the degradation of MAPs by trypsin. It is interesting to note that the appearance of these microtubule arrays is very similar to that of microtubule arrays formed in in vitro microtubule preparations after digestion with trypsin (Vallee and Borisy, 1977).

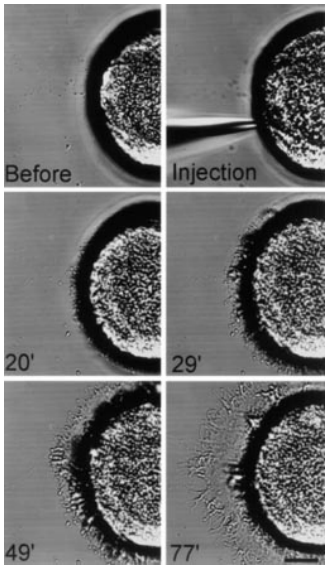

Figure 5. Trypsin microinjections into the somata of cultured *Aplysia* neurons induce rapid lamellipodia extension. The microinjection of trypsin into the soma of a cultured *Aplysia* neuron resulted in the extension of a lamellipodium from the entire perimeter of the cell body. Time is given in minutes from trypsin injection. Same experiment as that of Fig. 6. Bar 20 μm.

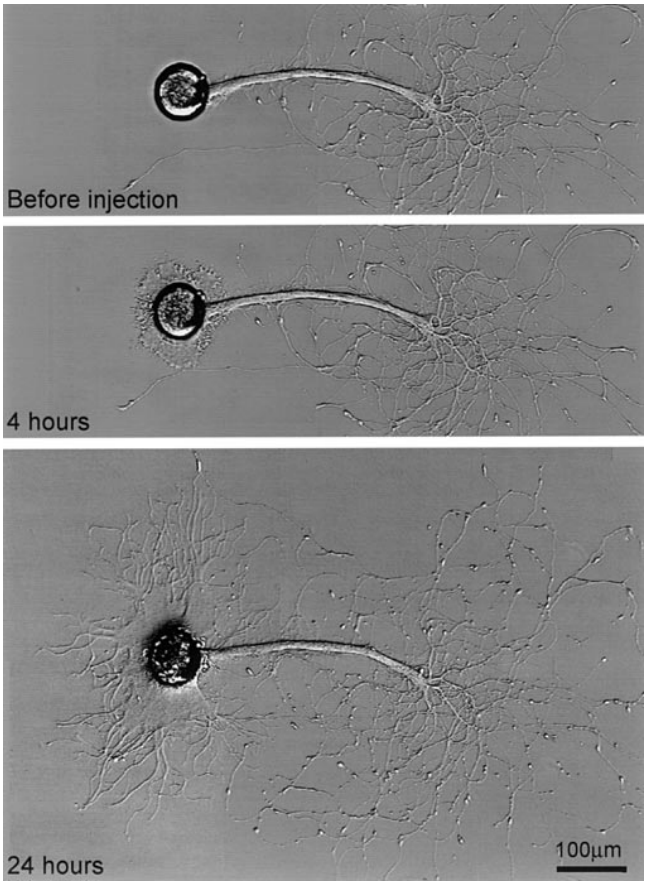

Figure 6. Trypsin-induced neurite outgrowth from the cell body. Trypsin-induced outgrowth from the somata of cultured *Aplysia* neurons developed into an elaborate array of elongating neurites. Same experiment as that of Fig. 5.

Discussion

The experiments described here were designed to test an hypothesis suggesting that a transient and localized increase in cytoplasmic proteolytic activity is sufficient to induce growth cone formation and neuritogenesis along otherwise intact axons. We found that brief microinjections of trypsin into intact axons or somata of cultured *Aplysia* neurons, resulting in transient trypsin concentrations of 13–130 nM, induce the formation of ectopic growth cones that subsequently give rise to new neurites. This outgrowth is not triggered by an increase in $[Ca^{2+}]_i$ or alterations in the passive membrane properties of the microinjected neurons. Ultrastructural examination revealed that trypsin microinjection is followed by alterations in axonal ultrastructure, which include localized microtubule fragmentation and the accumulation of vesicles. The ability of microinjected trypsin to induce the dedifferentiation of differentiated neuronal compartments into growth cones supports the hypothesis that transient increases in cytoplasmatic proteolytic activity are sufficient to induce the transformation of amputated axonal segments into growth cones.

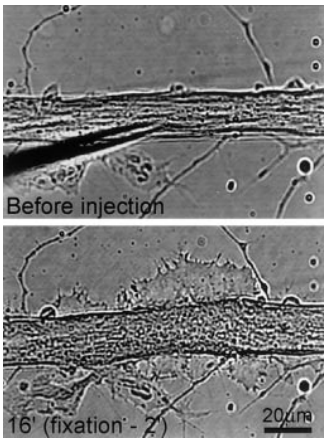

Figure 7. Electron microscopy analysis of trypsin-injected axons. The formation of a growth cone was induced by injecting trypsin into the axon, and the neuron was subsequently fixed and processed for electron microscopy. *Top*: the axon before the trypsin injection. The micropipette used for the trypsin injection is seen on the left-hand side. *Bottom*: The same axon 16 min after the microinjection procedure and ~2 min before the neuron was fixed. Note the lateral extension of a flat lamellipodium from the injection site. The electron microscope data for this experiment is shown in Fig. 8.

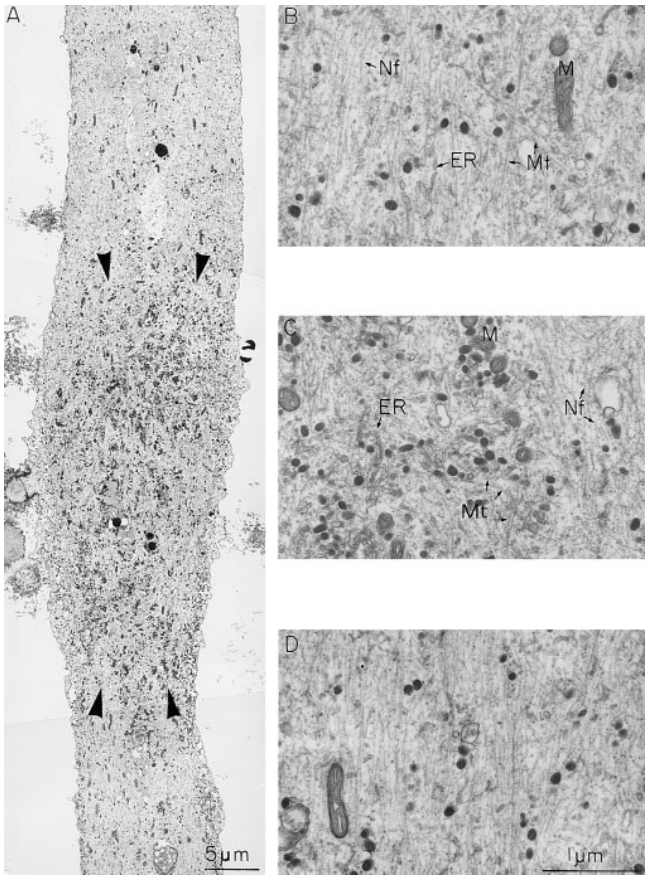

Figure 8. Trypsin induces alterations in the axonal cytoarchitecture. Electron microscope micrographs of the axon shown in Fig. 7. (*A*) A low magnification longitudinal section through the trypsin-injected axon showing the axonal ultrastructure along a segment of ~400 μm of the injected axon. The top (proximal) side of this micrograph corresponds to the right-hand side in Fig. 7. Note the accumulation of electron-dense vesicles and the extension of the lamellipodium membrane in the segment enclosed between the arrowheads. (*B*) A high magnification of axoplasm 30 μm proximal to the injection site reveals that the linear organization of the cytoskeleton in this region was maintained to a large degree. Many neurofilaments *Nf* and microtubules *Mt* are observed. (*C*) A high magnification of the axoplasm in the injection site showing the accumulation of electron dense vesicles and the presence of relatively short, randomly oriented microtubule fragments *Mt.* Endoplasmic reticulum profiles *ER* appear to be unaffected, and the appearance of mitochondria is normal. (*D*) A high magnification of axoplasm 30 μm distal to the injection site. The appearance of the axoplasm in this region is similar to that seen on the proximal side of the injected site.

The Identity of Proteins Degraded by Trypsin

At present, the identity of the proteins degraded by microinjected trypsin is unknown. Gilbert and Sloboda (1984) reported that treating vesicles isolated from extruded squid axoplasm with trypsin resulted in the loss of four major bands and six minor ones. One of the major bands comigrated with brain MAP2. The degradation of MAPs by trypsin is also supported by the study of Vallee and Borisy (1977), which showed that in vitro trypsin digestion of a microtubule preparation leads to the loss of high mol wt proteins associated with microtubules, resulting in a denser packing of the microtubules. The possible degradation of MAPs by the microinjection of trypsin is intriguing because of the role they play in stabilizing microtubules (reviewed in Hirokawa, 1994; Maccioni and Cambiazo, 1995). Interestingly, MAPs are extremely susceptible to degradation by Ca^{2+}-activated proteinases (Fischer et al., 1991; Friedrich and Aszodi, 1991; Johnson et al., 1991) as is fodrin (Johnson et al., 1991; Siman et al., 1984), a protein that serves to couple the plasma membrane to the cytoskeleton (Bennett and Gilligan, 1993).

Although the identity of proteins degraded by trypsin microinjection is unknown, indirect experiments suggest that key proteins involved in trypsin-induced neuronal outgrowth are relatively susceptible to proteolytic degradation, as similar results were obtained in experiments in which trypsin was substituted with protease type IX (data not shown). Further work is needed to identify the key proteins degraded by these exogenous proteinases.

The Mechanisms Underlying the Effects of Trypsin

The injection of exogenous proteinases into intact neurons has been previously used to destroy specific neurons under in vivo conditions (Parnas and Bowling, 1977; Bowling et al., 1978). In these studies, however, substantially greater quantities of proteolytic enzymes were microinjected (0.5% pronase; micropipette tip diameter, 1–2 μm; pressure injection duration, 1–5 s; pressure, 1–5 pounds per square inch) as compared to the minuscule amounts of trypsin microinjected in our experiments. We, too, have observed that the injection of excessive amounts of proteinase leads to visible damage. The dependence of the outcome on the quantity of microinjected trypsin suggests that trypsin-induced neuronal growth is the result of limited proteolysis, in the sense that only a subset of particularly labile proteins are degraded, and that the effects are confined both spatially and temporally. It is likely that the exogenous intracellular proteinase triggers cascades that result in neuronal outgrowth, and once these cascades are set in motion, the proteolytic activity is no longer required.

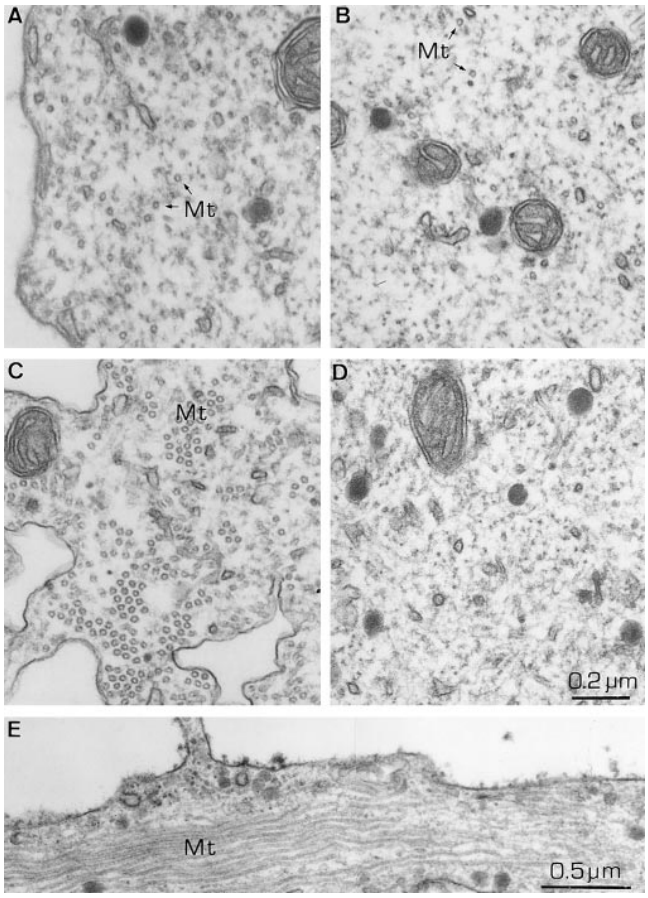

Figure 9. Trypsin alters the distribution of microtubules. A cross-section through a trypsin-injected axon reveals that trypsin leads to the disappearance of microtubules from the core of the axoplasm and to the appearance of packed arrays of microtubules along the perimeter of the axon. (*A* and *B*) A cross-section through the axon ~200 μm proximal to the injected site. Note the uniform distribution of microtubules Mt in peripheral regions of the axon (*A*) and near the center of the axon (*B*). (*C* and *D*) A cross-section through the axon at a point near the injection site. Note the packed arrays of microtubules formed along the periphery of the axon (*C*) and the decrease in microtubule density near the center of the axon (*D*). These packed arrays of microtubules are also apparent in longitudinal sections of trypsin-microinjected axons (*E*).

Previous studies suggested that adult neurons maintain the potential to form motile growth cones, and that this potential is normally suppressed by cytoskeletal integrity (Wessells et al., 1978) and extensive cross-linking of cytoskeletal elements (Joshi et al., 1986). It is possible that the temporally and spatially restricted proteolytic degradation of stabilizing and cross-linking proteins such as MAPs and fodrin, relaxes the structural constraints imposed on the cortical cytoskeleton, and that this may result in the expression of the inherent, but normally suppressed, potential for growth.

A proteolytic digestion of the cortical cytoskeleton could also expose the inner face of the plasma membrane, and this could increase its accessibility for fusion with intracellular vesicles (Burgoyne and Cheek, 1987; Perrin et al., 1987; Aunis and Bader, 1988). Such fusion may provide the intracellular membrane required for the rapidly extending growth cone (Cheng and Reese, 1987; Ashery et al., 1996). The fusion of vesicles with the plasma membrane could be further augmented by the accumulation of vesicles near the injection site. Interestingly, ectopic neuronal growth did not noticeably block neurite extension from the distal regions of the axon, arguing against a complete block of vesicle transport (Figs. 2 and 3 *B*). Furthermore, as trypsin microinjections into somata of *Aplysia* neurons (where microtubules are not uniformly oriented) also induced neuronal outgrowth (Figs. 5 and 6), the direct relationships between perturbations in vesicle transport and growth cone formation are not clear. On the other hand, microtubule disruption may be important for facilitating microtubule recruitment by the nascent growth cone, promoting its stabilization and subsequent transformation into an array of cylindrical neurites (Goldberg and Burmeister, 1989; Lin and Forscher, 1993; Bentley and O'Connor, 1994; Lin et al., 1994; Yu et al., 1994; Tanaka and Sabry, 1995).

Although trypsin injections were followed by microtubule fragmentation, electron micrographs revealed that trypsin microinjections could also induce the formation of densely packed microtubule arrays around the perimeter of the axoskeletal core. These apparent opposing effects of trypsin on microtubule organization may suggest that the effects of proteinases on cytoskeletal structure are rather complex. For example, the degradation of stabilizing proteins such as MAPs would be expected to destabilize microtubules (Hirokawa, 1994). On the other hand, the reduced cross-linking of cortical and axoplasmic cytoskeletal networks (in essence, a gel to sol transition) would reduce the compression forces acting on microtubules and as a result, promote microtubule polymerization (Heidemann and Buxbaum, 1994).

In the discussion above, it was implicitly assumed that structural proteins are the major substrates of trypsin microinjected into the neurons. It is almost certain, however, that other, nonstructural proteins are affected as well, and it is possible that their degradation leads to growth cone formation and ectopic neuritogenesis in ways not considered here. Further information as to the identity of proteins degraded by the microinjected proteinases may provide additional clues as to the mechanisms through which proteinase microinjections lead to growth cone formation.

Do Endogenous Intracellular Proteinases Play a Role in Neuronal Remodeling?

The formation of a growth cone after axotomy (or in response to focal applications of ionomycin) is preceded by a large and transient increase in $[Ca^{2+}]_i$ (Ziv and Spira, 1997). In a preliminary report it was shown that this $[Ca^{2+}]_i$ transient is followed by a transient increase in proteolytic activity that is spatially limited to regions where $[Ca^{2+}]_i$ was greatly elevated (Gitler and Spira, 1996). Furthermore, calpeptin, a specific inhibitor of calpain, abolished the formation of growth cones that usually follows such elevations in $[Ca^{2+}]_i$ (Gitler and Spira, 1996). The experiments described in the current study further support the hypothesis that Ca^{2+} activated proteinases play important roles in axotomy-induced growth cone formation as they directly

demonstrate that a spatially confined increase in cytoplasmic proteolytic activity can be sufficient to induce the transformation of a differentiated axonal segment into a growth cone.

This conclusion is also supported by the similarities between the morphological and ultrastructural alterations induced in intact axons by transient elevations of $[Ca^{2+}]_i$ to 300–500 μM (Ziv and Spira, 1997) and trypsin microinjections (this study): both experimental manipulations lead to the formation of ectopic growth cones that develop into elaborate neuritic trees; both treatments disrupt the linear organization of the cytoskeleton; and both can cause microtubule fragmentation. It is clear, though, that the ultrastructural alterations caused by such $[Ca^{2+}]_i$ elevations are more severe than those caused by trypsin microinjections, suggesting that trypsin injections do not fully mimic the affects of large $[Ca^{2+}]_i$ transients. Indeed, if the three manipulations known to induce growth cone formation (axotomy, $[Ca^{2+}]_i$ elevation, and proteinase injections) act through common pathways, it is conceivable that a minimal set of conditions must be met for new growth to occur, and that any manipulation capable of recreating these conditions may have the capacity to induce new neuronal outgrowth.

Our results are consistent with previous studies that proposed a role for endogenous proteinases in neuronal remodeling. For example, Lynch and his associates have suggested that calpain activation by Ca^{2+} influx through *N*-methyl-D-aspartate (NMDA)-type glutamate receptors may provide a mechanism through which patterned physiological activity could be translated into lasting modifications of synaptic structure (Lynch and Baudry, 1984; Lynch and Seubert, 1989). Other studies have proposed a role for calpains in neurite pruning (Song et al., 1994), in synapse elimination (Connold et al., 1986) and in synaptic plasticity (Hell et al., 1996). Furthermore, eukaryotic cells express additional cytosolic, nonlysosomal proteases whose activity is controlled by mechanisms other than Ca^{2+}. The profound alterations to neuronal morphology induced by trypsin microinjections suggest that the limited proteolysis of intracellular components can be a very potent mechanism for altering neuronal architecture. It is thus tempting to speculate that future studies may reveal new roles for endogenous proteinases in the determination of neuronal architecture during development or in remodeling processes that occur during adulthood.

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