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Calcium, Protease Activation, and Cytoskeleton Remodeling Underlie Growth Cone Formation and Neuronal Regeneration

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SUMMARY

The cytoarchitecture, synaptic connectivity, and physiological properties of neurons are determined during their development by the interactions between the intrinsic properties of the neurons and signals provided by the microenvironment through which they grow. Many of these interactions are mediated and translated to specific growth patterns and connectivity by specialized compartments at the tips of the extending neurites: the growth cones (GCs). The mechanisms underlying GC formation at a specific time and location during development, regeneration, and some forms of learning processes, are therefore the subject of intense investigation. Using cultured *Aplysia* neurons we studied the cellular mechanisms that lead to the transformation of a differentiated axonal segment into a motile GC. We found that localized and transient elevation of the free intracellular calcium concentration ($[Ca^{2+}]_i$) to 200–300 μM induces GC formation in the form of a large lamellipodium that branches up into growing neurites. By using simultaneous on-line imaging of $[Ca^{2+}]_i$ and of intraaxonal proteolytic activity, we found that the elevated $[Ca^{2+}]_i$ activate proteases in the region in which a GC is formed. Inhibition of the calcium-activated proteases prior to the local elevation of the $[Ca^{2+}]_i$ blocks the formation of GCs. Using retrospective immunofluorescent methods we imaged the proteolysis of the submembrane spectrin network, and the restructuring of the cytoskeleton at the site of GC formation. The restructuring of the actin and microtubule network leads to local accumulation of transported vesicles, which then fuse with the plasma membrane in support of the GC expansion.

KEY WORDS: *Aplysia*; axotomy; growth cone; calcium; calpain; spectrin; cytoskeleton; regeneration.

INTRODUCTION

The cytoarchitecture, synaptic connectivity, and physiological properties of neurons are determined during their development by the interactions between the intrinsic properties of the neurons and signals provided by the microenvironment through

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which they grow. The interactions are mediated and translated to specific growth patterns by specialized compartments at the tips of the extending neurites: the growth cones (GCs). This cellular compartment translates external signals into specific "maneuvers" such as turning, retraction, or the facilitation of the extension rate. The GC hosts the cellular machinery to identify the target neurons, the mechanisms to stop the growth, and finally the mechanisms to differentiate into a stationary presynaptic terminal. Thus, GCs play important roles during the normal course of neuronal network formation, as well as during the regeneration of neurons after injury (for review see Gordon-Weeks, 2000). Morphological restructuring of adult neurons and their regrowth in relation to some forms of learning and memory may also depend on the formation of new GCs in response to specific stimuli (Bailey and Kandel, 1993; Glanzman *et al.*, 1990).

The signaling pathways that lead to structural and functional remodeling of GCs are partially understood. Less is known about the cellular and molecular mechanisms that trigger GC formation at a specific site and at a given time by neuroblasts, by adult neurons after injury, or by neurons that undergo morphological changes in relation to learning processes.

Our laboratory began to explore the cellular mechanisms that underlie GC formation by examining a specific case in which GC formation is induced by axotomy. The induction of GC formation by axonal transection enables a clear definition of the points in the time and space in which the dramatic restructuring of differentiated axons into a motile GCs are triggered, thus allowing the analysis of spatiotemporal and causal relations between cellular events associated with the local remodeling process. We assume that the cascade of cellular events involved in the initiation of GC formation at a specific location after axotomy represent common cellular mechanisms that take part in the induction of GC formation during development and during activity-dependent neuroplasticity.

The results in this review are based on experiments performed in neurons isolated from *Aplysia* and maintained in culture, a preparation that has been used extensively to study different forms of neuronal plasticity (for a review see Kandel *et al.*, 1991). We wish to emphasize that the objective of this review is to describe the mechanisms that trigger, and take part in, GC formation at a specific time and site and not to examine the mechanisms that set the neuronal genome into growth mode. Cultured neurons in general, and *Aplysia* neurons in particular, appear to be primed for growth by the trauma of the culturing procedures. Thus, the molecular and cellular background of the neurons is preset for the formation of GC by localized signals (Ambron *et al.*, 1996; Dash *et al.*, 1998; Lankford *et al.*, 1998; Walters *et al.*, 1991; Walters and Ambron, 1995).

Using calcium-ratio–imaging methods we first examined the relations between free intracellular calcium concentration $[Ca^{2+}]_i$ gradients and GC formation. We found that localized and transient elevation of the $[Ca^{2+}]_i$ induced by axotomy or by local application of calcium ionophore to intact cultured neurons induces GC formation and neuritogenesis (Spira *et al.*, 1996, 2000; Ziv and Spira, 1997). By using concomitant imaging of $[Ca^{2+}]_i$ and of intraaxonal proteolytic activity, we found that such treatments activate proteases in the region in which a GC is formed (Gitler and Spira, 1998; Spira *et al.*, 2000). Since the proteases are activated by calcium and at

least one of them is blocked by the calpain inhibitor calpeptin, we attributed this proteolytic activity to calpain. Inhibition of calpain prior to axotomy or to the local elevation of the $[Ca^{2+}]_i$ in intact axons blocks the formation of GCs. Using retrospective immunofluorescent methods we identified the proteolysis of the submembrane spectrin network as a possible link between calpain activation and GC formation (Gitler and Spira, 1998).

GC FORMATION AND NEURITOGENESIS AFTER AXOTOMY

Axotomy of cultured *Aplysia* neurons is followed by striking morphological changes in which within 10–30 min the cylindrical axonal structure is transformed into a flat, dynamic GC in the form of a lamellipodium (Fig. 2 (A)). Following axotomy the cut end retracts and narrows down. Around 30–100 μm away from the transected tip a short segment of the axon swells, while the more proximal section of the axon maintains normal morphology. The swollen segment becomes the GC's center and is referred to as the transition zone. Minutes after axotomy, a flat lamellipodium vigorously extends (laterally) from the swollen region (Ashery *et al.*, 1996; Ziv and Spira, 1997), forming a giant GC. Within a period of 60–120 min the perimeters of the lamellipodia begin to break up into discrete branches that continue to elongate.

The nature of the cellular processes that underlie the transformation of a stationary, differentiated axon into a motile GC is the subject of the present review. As a first step in the study we examined the hypothesis that an influx of calcium ions (Ca^{2+}) into the injured axons initiate the process that leads to the dedifferentiation of an axonal segment into a motile GC. To that end we first imaged the $[Ca^{2+}]_i$ gradients formed by axotomy and then tested the role of Ca^{2+} in the initiation of GC formation.

INTRACELLULAR $[Ca^{2+}]_i$ GRADIENT AFTER AXOTOMY

The rupturing of the plasma membrane caused by axotomy leads to membrane depolarization, associated with the generation of action potentials. These activate voltage-gated Ca^{2+} channels, and elevate the $[Ca^{2+}]_i$ to the micromolar range within the neuron (Ziv and Spira, 1993). Concomitantly, Ca^{2+} diffuses from the external medium into the axoplasm through the ruptured axonal membrane. The influx of Ca^{2+} forms a steep $[Ca^{2+}]_i$ gradient between the cut end of the axon and the remaining parts of the neuron (Fig. 1). The $[Ca^{2+}]_i$ exceeds 1 mM at the tips of the transected axon and declines to a level of a few hundreds of micromollars, 100–200 μm away from the transected tips (Ziv and Spira, 1995, 1997).

The diffusional spread of Ca^{2+} ions into the axon is controlled by intracellular Ca^{2+} buffers. Fixed endogenous buffers tend to retard Ca^{2+} diffusion, whereas mobile buffers accelerate it (Neher, 1995). In cultured *Aplysia* neurons, the majority of the endogenous buffers were found to be stationary (Gabso *et al.*, 1997). Accordingly, the effective Ca^{2+} diffusion coefficient in the axons is low (16 $\mu m^2/s$). This may account for the slow rate at which the calcium front spreads from the point of transection into the axon. It should be noted that the ratio between mobile and stationary

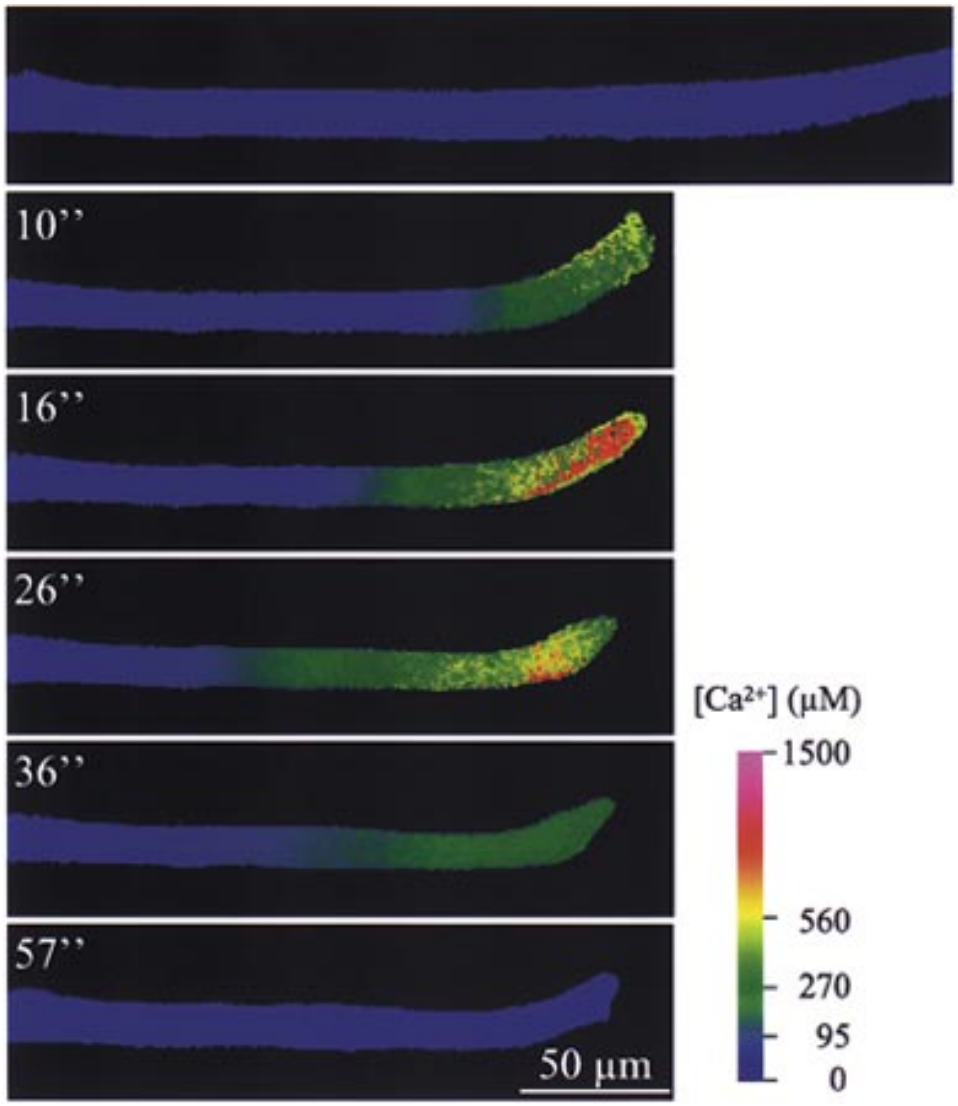


Fig. 1. Mag-fura-2 ratio images of the $[Ca^{2+}]_i$ after axotomy of a cultured *Aplysia* neuron. Ca^{2+} is observed to diffuse from the cut end toward the cell body (10, 16, 26 s). As a consequence a $[Ca^{2+}]_i$ gradient of more than 1000 μM is formed along an axonal segment of $\sim$250 μm. Following the resealing of the ruptured membrane, the $[Ca^{2+}]_i$ recovers to near control levels (36, 57 s). The kinetics of the $[Ca^{2+}]_i$ of this experiment is shown in Fig. 2(C). Time is given in seconds from axotomy.

calcium buffers and their relative concentrations differ in the various types of neurons (Kosaka *et al.*, 1993; Roberts, 1993). Thus, the parameteres described above cannot be applied to other neurons. This can be done only when the mobility, concentration, and affinity of the endogenous buffers to Ca^{2+} are known. In *Aplysia* neurons, a membrane seal is formed over the cut end 0.5–3 min after axotomy (Spira *et al.*,

1993, 1996). In other preparations that were tested under semi in vivo conditions, complete seal formation was estimated to occur over a longer time course (Borgens *et al.*, 1980; Eddleman *et al.*, 2000; Lichstein *et al.*, 2000; Strautman *et al.*, 1990; Yawo and Kuno 1983, 1985). In cultured *Aplysia* neurons, membrane seal formation leads to the recovery of the $[Ca^{2+}]_i$ within 1–5 min after axotomy (Fig. 1).

ECTOPIC GCs ARE FORMED BY TRANSIENT ELEVATION OF THE $[Ca^{2+}]_i$ IN INTACT NEURONS

Correlation between the site of GC formation after axotomy and the $[Ca^{2+}]_i$ gradients revealed that growth cones consistently extend from regions in which $[Ca^{2+}]_i$ was transiently elevated to 200–300 μM and that the extension of a GC's lamellipodium always occurred after the $[Ca^{2+}]_i$ recovered to the control level (Ziv and Spira, 1997; compare Figs. 1 and 2(A)).

To examine whether a transient elevation of the $[Ca^{2+}]_i$ provides a signal sufficient for inducing GC formation, we transiently elevated the axonal $[Ca^{2+}]_i$ to 300–500 μM by local microejection of calcium ionophore (ionomycin) onto the plasma membrane of intact neurons. We found that when the $[Ca^{2+}]_i$ was elevated to 300–500 μM, GC and neuritogenesis are induced. Greater Ca^{2+} concentrations were commonly associated with visible damage to the axon, manifested as beading and axonal degeneration. Conversely, lower Ca^{2+} concentrations did not noticeably affect the axonal morphology or ultrastructure (Ziv and Spira 1997; Spira *et al.*, 2000).

These findings strongly suggest that the transient elevation of $[Ca^{2+}]_i$ is a signal sufficient for inducing the dedifferentiation of an axonal segment into GCs. It is worth noting that the first signs of GC formation are only detectable after the $[Ca^{2+}]_i$ recovers to control levels. We interpret this observation to suggest that the transient elevation in $[Ca^{2+}]_i$ triggers the growth process but is not essential for its perpetuation.

$[Ca^{2+}]_i$ GRADIENT, ULTRASTRUCTURAL ALTERATIONS, AND GC FORMATION

To gain insight into the nature of the cellular processes that underlie the transformation of an axon into a GC, we next correlated the spatial distribution of $[Ca^{2+}]_i$ and the ultrastructural alterations induced by axotomy or ionophore application. To that end cultured neurons were loaded with mag-fura-2, their axons were transected, and the alterations in $[Ca^{2+}]_i$ caused by axotomy were recorded. After the recovery of $[Ca^{2+}]_i$ to near-control levels (1–5 min after axotomy), the neurons were fixed for EM examination by rapid superfusion with a gluteraldehyde fixation buffer (Benbassat and Spira, 1993; Ziv and Spira, 1997).

Following axotomy, microtubules and neurofilaments that are normally oriented in parallel to the longitudinal axis of the axon are no longer detected at the tip of the transected axon in regions that correspond to peak $[Ca^{2+}]_i$ elevations above

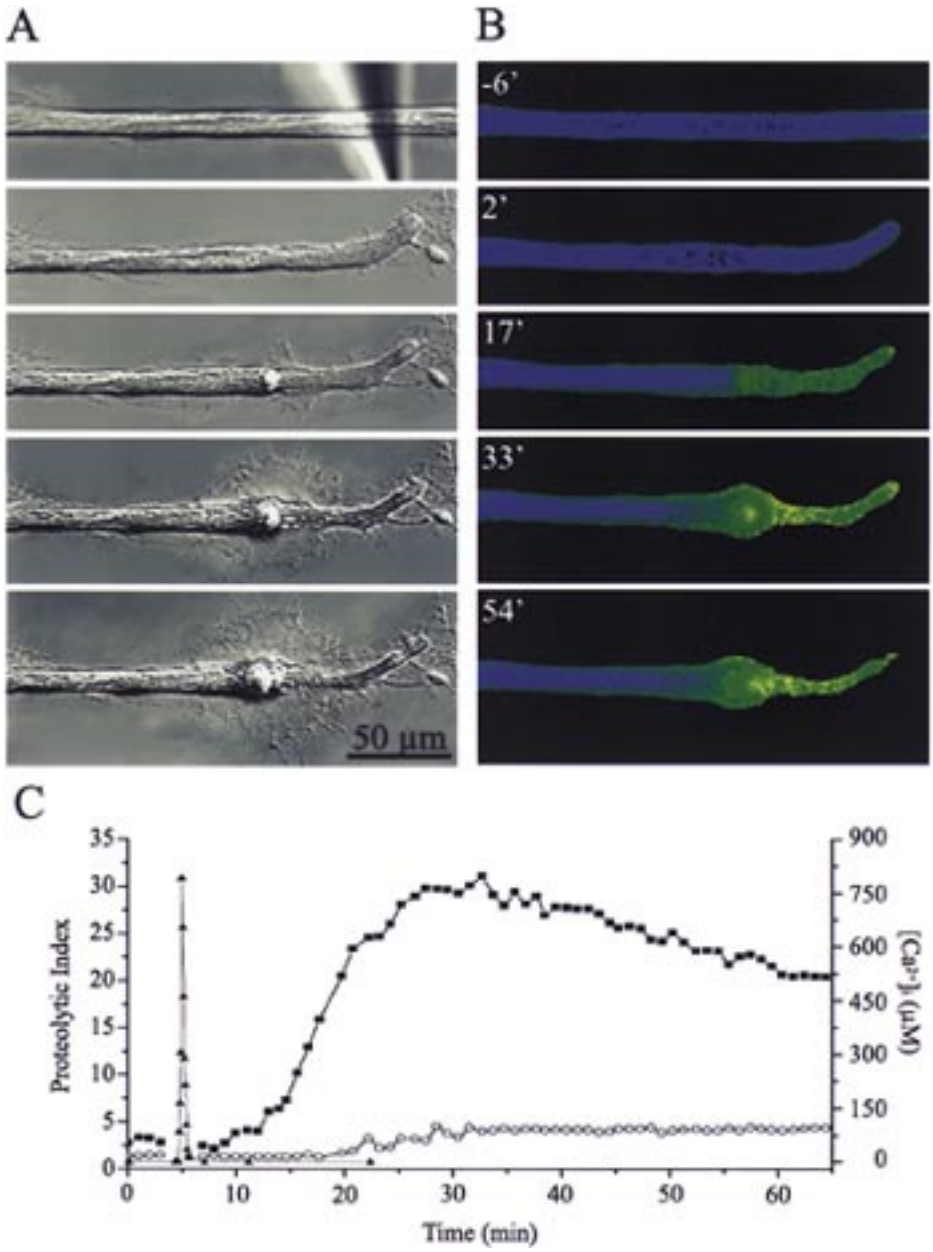


Fig. 2. Axotomy induces proteolytic activity, as evidenced by the cleavage of the exogenous proteolytic substrate (CBZ-Alnyl-Alnine)-R110. A neuron was transected in the presence of 10 μM (CBZ-alnyl-alnine)-R110. The morphology, $[Ca^{+2}]_i$ (shown in Fig. 1), and accumulation of the proteolytic product of (CBZ-Alnyl-Alnine)-R110 were imaged following axotomy. (A) Transmitted-light images of axonal transection and the extension of a growth cone in the form of a flat lamellipodium. Note the flattening of the axon's tip (2'), the formation of a transition zone (17') and the extension of a GC's lamellipodium (33' and 54'). The images shown in (A) correspond to those shown in (B). (B) Pseudocolor-ratio images of the accumulation of the proteolytic product R110, taken at different time points after axotomy. The R110 fluorescent signal increase within 10 min of axotomy, reaching a peak at ~30 min. (C) A graph describing the kinetics of the accumulation of R110 (squares measured at the transition zone and the circles at 200 μM proximal to it). The triangles depicts the transient increase in the $[Ca^{2+}]_i$ shown in Fig 1.

300 μM (Fig. 3(A)–(C)). At the very tip of the axon, in regions where the $[Ca^{2+}]_i$ exceeds 1500 μM, dissociated microtubules and neurofilaments form large, amorphous electron-dense aggregates (Fig. 3(B)). Further away from the cut end, at an axonal segment that corresponds to $[Ca^{2+}]_i$ levels of 300–1500 μM, the dissociated cytoskeletal elements form elongated electron-dense aggregates (Fig. 3(A), (C), and upper part of (D)). No intact neurofilaments are observed along this segment.

At larger distances from the transected tip, at regions that correspond to $[Ca^{2+}]_i$ of $\sim$300 μM (Fig. 3(A) and lower part of (D)), clusters of relatively short fragments of microtubules can be seen. These fragments are no longer oriented exclusively in parallel to the longitudinal axis of the axon.

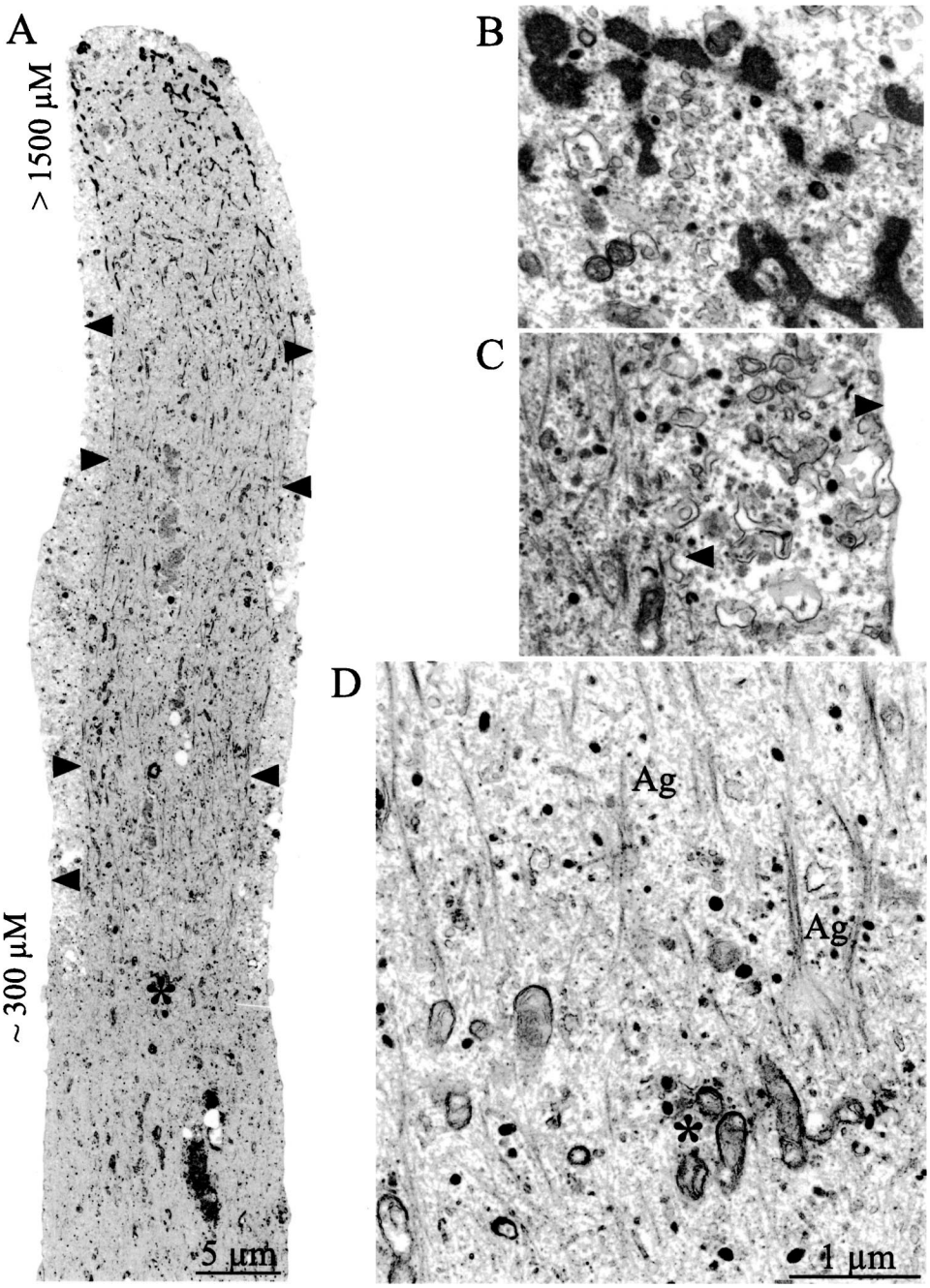
Another feature characteristic to transected axons is that along segments of $\sim$100 μm in which the Ca^{2+} concentrations transiently exceed 300 μM, the plasma membrane detaches from the axoplasmic core (Fig. 3(A) and (C), arrowheads). The space between the core of the axoplasm and the axolema is filled with amorphous material, vesicles, and swollen subsurface cisterns. The detachment of the plasma membrane is most likely the outcome of the calcium-induced proteolysis of spectrin, a filamentous key component of the membrane skeleton (see below).

The axotomy-induced alterations to the axon's cytoarchitecture end abruptly 50–150 μm from the cut end, resulting in the formation of a sharp transition zone between the axonal segment in which the cytoarchitecture is altered and the rest of the axon in which the cytoarchitecture appears normal (Fig. 3(A) and (D)). Examination of the spatiotemporal $[Ca^{2+}]_i$ distribution does not reveal any sharp drop in the $[Ca^{2+}]_i$ gradient that parallels the sharp transition in the axonal cytoarchitecture. In fact, the $[Ca^{2+}]_i$ gradually decreases through the transition region from $\sim$300 μM to the micromolar range. The transition zone is characterized by the disappearance of the microtubular aggregates and the reappearance of intact neurofilaments and microtubules. It is of particular interest to note that at this transition zone the detached axolema consistently reattaches to the axoplasmic core (Fig. 3(A)) and that several minutes after axotomy large numbers of vesicles accumulate at the transition zone. This region corresponds to the site from which a GC lamellipodium extends after axotomy.

To examine whether the elevation in $[Ca^{2+}]_i$ underlies the ultrastructural alterations described above, or whether other injury-related events are involved, we focally applied ionomycin to axonal segments of intact *Aplysia* neurons and examined its effects on the axon's cytoarchitecture (Ziv and Spira, 1997). Ionomycin applications that elevated the $[Ca^{2+}]_i$ to 300–500 μM at the application point, and to decreasing values at increasing distances from the site of application, induced ultrastructural alterations identical to those documented in transected axons.

The observations described above demonstrate two major points.

a. The ultrastructural alterations induced by axotomy or by focal applications of calcium ionophores are identical. Thus, the transient elevation in the $[Ca^{2+}]_i$, rather than the mechanical injury, triggers the formation of a GC.
b. The ultrastructural alterations that are characterized by the formation of the transition zone do not follow the $[Ca^{2+}]_i$ gradient, suggesting that other factors may be involved in the process that lead to the ultrastructural modifications.


A
> 1500 µM
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B
C
D
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A TRANSIENT ELEVATION OF $[Ca^{2+}]_i$ INDUCES LOCALIZED ELEVATION OF PROTEOLYTIC ACTIVITY

The findings presented above raise questions as to the nature of the molecular processes that link the elevated Ca^{2+} levels and the massive restructuring that underlies the transformation of a differentiated axon into a growth cone. Our studies suggest that Ca^{2+}-dependent proteases provides the link between $[Ca^{2+}]_i$ and GC formation (Gitler and Spira, 1998).

The spatiotemporal relationship between axotomy, $[Ca^{2+}]_i$, and the activation of a protease are illustrated in Fig. 2(B) and (C) (the same experiment as that shown in Fig. 1). To examine these relations we simultaneously imaged the $[Ca^{2+}]_i$, using mag-fura-2, and proteolytic activity, using the fluorogenic membrane permeable proteolysis indicator bis(CBZ-L-Alanyl-L-Alanine amine)-Rhodamine 110 (bCAA-R110). bCAA-R110 is practically nonfluorescent, while the products of the cleavage of its amide bonds, rhodamine 110 (R110) and its monoamides, are highly fluorescent (Leytus *et al.*, 1983a,b). In these experiments, the basal proteolytic activity in neurons was imaged for approximately 20 min and then their axons were transected. As a result, the $[Ca^{2+}]_i$ levels transiently increased. The $[Ca^{2+}]_i$ gradient declined to control values within a few minutes. Axotomy was followed by a significant increase in the level of the proteolytic activity, as evidenced by the increase in the fluorescent signal of R110 (Fig. 2(B)). Such increase was detected approximately 2 min after axotomy (not shown). Initially, the fluorescent signal increased uniformly within the axoplasm of the transected axonal tips. Thereafter, discrete peaks of R110 fluorescent signals are detected. After reaching peak levels, the fluorescent signal gradually decreased (Fig. 2(C)). In the present review we will relate to the significance of the early proteolytic processes and not discuss the nature of the R110 fluorescent hot spots or their dynamics. This aspect will be discussed in a separate paper.

To determine if this proteolytic activity was induced by the transient $[Ca^{2+}]_i$ elevation, rather than by other injury-related processes, $[Ca^{2+}]_i$ was focally elevated by ionomycin application to an axon of an intact neuron. We found that proteolytic activity was induced along restricted axonal segments in which the $[Ca^{2+}]_i$ had been elevated to 300–400 μM. The time course of the $[Ca^{2+}]_i$ transient and that of the

Fig. 3. Ultrastructural alteration induced by calcium influx following axotomy. An axon was transected while imaging the free intracellular calcium concentration. The axon was fixed for electron microscopic observations three minutes after axotomy. (A) A longitudinal section, of the transected proximal tip of the axon. Note the disruption of the microtubules and neurofilaments and the precipitation of cytoskeleton aggregates from the cut end to, proximally, the point of transition (asterisk). Away from the cut end where the $[Ca^{2+}]_i$ was <1.5 but >0.3 mM, the cytoskeleton collapses to form longitudinal aggregates (Ag, enlarged in the upper section of D). The microtubules and neurofilaments regain normal orientation and structure in the transition zone (asterisk) where the $[Ca^{2+}]_i$ gradually declines to values below 0.3 mM. The plasma membrane detaches from the core of the axoplasm, starting at the very tip of the transected axon down to the area in which the transition zone is formed (arrowheads in A and C). The structure of the microtubules appears normal proximal to the transition zone (compare the upper and lower parts of D). (B) High magnification of the axoplasm close to the tip of the transected axon. Note the amorphous electron-dense aggregates of depolymerized cytoskeletal elements. (C) High magnification showing a region in which the plasma membrane detached the core of the axon. (D) High magnification of the transition zone where the cytoskeleton structure is reestablished.

proteolytic activity was similar to that observed following axotomy. Eventually, a GC emerged from the region where the $[Ca^{2+}]_i$ and the proteolytic activity were increased, within time periods similar to those measured following axotomy. These results illustrate that a transient increase in $[Ca^{2+}]_i$ is sufficient to induce both proteolytic activity and GC formation.

Preincubation of the neuron in 100 μM calpeptin completely inhibited both the proteolysis of the exogenous proteolytic substrate and GC formation induced by axotomy or by ionomycin applications. Calpeptin did not affect the recovery of $[Ca^{2+}]_i$ to control levels once the membrane resealed, suggesting that in cultured *Aplysia* neurons calpeptin does not alter neuronal Ca^{2+} handling characteristics.

The spatiotemporal relations described above and the inhibitory action of calpeptin on GC formation suggested causal relationships between the transient elevation of $[Ca^{2+}]_i$, proteolysis, and GC formation.

PROTEOLYSIS OF THE SUBMEMBRANE SPECTRIN CORRELATES WITH THE FORMATION OF THE GC

Aunis and Bader (1988) and Perrin *et al.* (1992) suggested that removal of spectrin makes the inner surface of secretory cells accessible for fusion with intracellular membranes. As spectrin is a substrate of calpain, and since the growth process requires the insertion of intracellular membranes into the neurolema, we studied the relations between Ca^{2+}-induced proteolysis, the distribution of axonal spectrin, and the formation of GC. For this purpose, we measured both the $[Ca^{2+}]_i$ transients and the ensuing proteolytic activity after axonal transection, and then immunolabeled the same neurons with antibodies against spectrin and *m*-calpain (Fig. 4(A) and (B)). Confocal microscope images of control neurons revealed that spectrin is abundant at the submembranal domain, and less so in the inner axonal space. We found that axotomy is followed by the proteolysis of the submembrane spectrin in regions where the $[Ca^{2+}]_i$ was elevated and in which proteolytic activity was induced (Fig. 4(A) and (B)). Calpeptin, which was found to inhibit Ca^{2+}-induced proteolytic activity and GC formation, also inhibited proteolysis of spectrin from beneath the plasma membrane (Gitler and Spira, 1998). Furthermore, consistent with the views that upon activation by Ca^{2+} calpains translocate from the cytosol to the plasmamembrane (Saido *et al.*, 1994), immunofluorescent labeling of *m*-calpain revealed preferential labeling of the submembrane region where $[Ca^{2+}]_i$ was elevated after axotomy (Fig. 4(C) and (D)).

INTRACELLULAR MICROINJECTION OF EXOGENOUS PROTEASES BYPASS THE Ca^{2+} SIGNAL AND INDUCES GC FORMATION

In principal, the role that calpain play in GC formation could be tested by direct microinjection of the protease into intact axons. Unfortunately, these experiments are complicated by the fact that calpain activity is regulated by several factors such

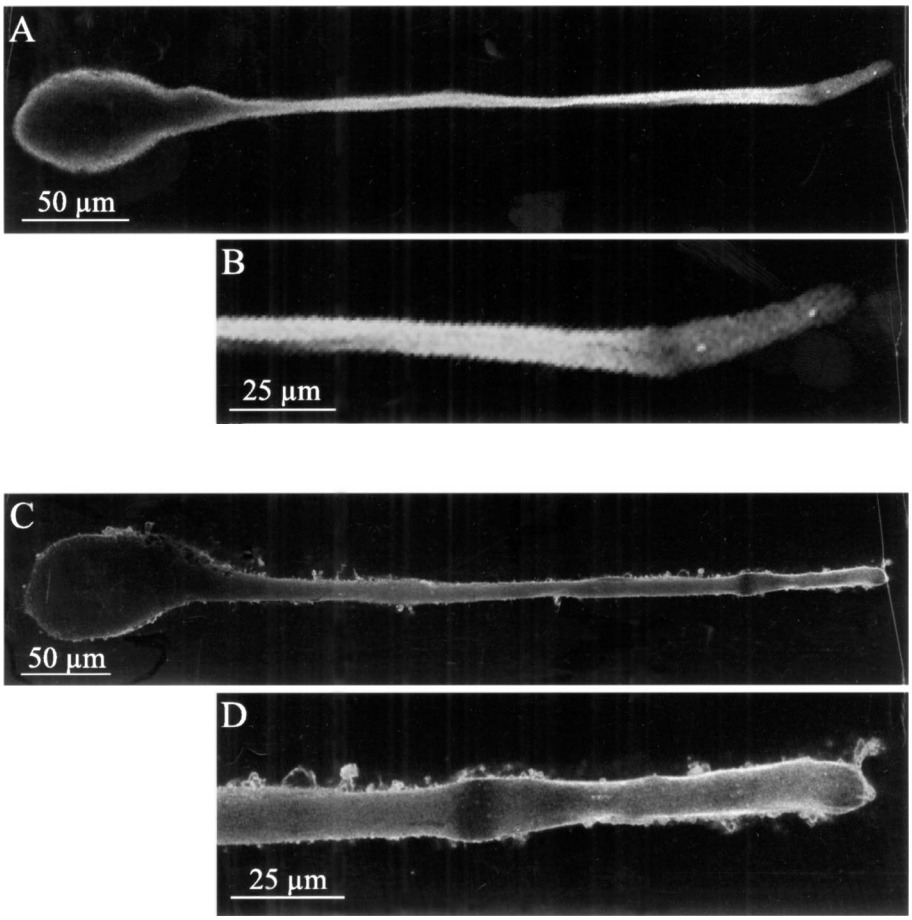

Fig. 4. The distribution of immunofluorescent-labeled spectrin and *m*-calpain in axotomyzed neurons. The neurons were axotomyzed and then fixed by 4% paraformaldehyde, approximately 10 min after axotomy. The neurons were permeabilized by 0.1% triton and 0.025% saponin respectively. The neuron was immunolabeled for spectrin with rabbit anti-human spectrin polyclonal antibodies (Sigma) and a secondary Cy3-conjugated sheep anti rabbit IgG ((A) and (B)). Confocal images of the spectrin immunofluorescent signal reveal that the spectrin fluorescent signal is reduced at the transected tip of the axon. The area in which the spectrin fluorescent signal is missing overlaps with the region in which the $[Ca^{2+}]_i$ activated calpain. A transected axon was immunolabeled for domain III/IV of *m*-calpain with mouse anti bovine *m*-calpain monoclonal antibodies (Calbiochem) and a secondary Cy2-conjugated goat anti mouse IgG (C) and (D). Confocal images of the calpain immunofluorescent signal reveals that calpain predominantly decorates the plasma membrane at the tip of the transected axon and is less pronounced along the plasma membrane of the rest of the axon. The area in which calpain is more pronounced overlaps with the region in which $[Ca^{2+}]_i$ activated calpain.

as the endogenous regulator calpastatin (Saido *et al.*, 1994) and by $[Ca^{2+}]_i$. The latter factor poses the greatest difficulty, as experimental elevation of the Ca^{2+} levels required to activate calpains is sufficient for inducing GC formation even without the introduction of exogenous calpain. To examine whether local and transient increase in intracellular protease activity may be sufficient to trigger GC formation, we

microinjected minute amounts of exogenous proteases, including trypsin, chemotrypsin, elastase, and papain, into intact axons (Leibovitch, 2001; Ziv and Spira, 1998). We found that this procedure leads to rapid formation of ectopic growth cone and irreversible neuritogenesis. Since in these experiments the $[Ca^{2+}]_i$ remained significantly below the levels needed to induce GC formation, we conclude that local and minute amounts of the exogenous proteases are sufficient to mimic the cascade of events triggerd by localized increase in the $[Ca^{2+}]_i$. These findings suggest that downstream activation of cytosolic proteases may be involved in the regulation of neuroplasticity.

CONCLUDING REMARKS

The studies described in this review establish sequence of cellular events that participate in the transformation of an axonal segment of cultured *Aplysia* neurons into a motile GC.

We identified a sequence of six steps that trigger GC formation. (a) The transient and localized increase in the $[Ca^{2+}]_i$ to 200–300 μM activate several proteases (b). These in turn (c) proteolyze key elements of the membrane skeleton (spectrin) and cytoskeleton (actin and microtubules). The limited and local proteolysis (d) restructures the cytoskeleton. (e) The structural modification of the cytoskeleton creates the conditions to locally trap anterogradly and retrogradly transported vesicles. (f) The trapped vesicles fuse with the exposed inner face of the plasma membrane, a process that is facilitated by the local proteolysis of spectrin. Our experiments demonstrate that the cascade of GC formation can be initiated by intracellular microinjections of minute amounts of exogenous proteases, suggesting that, in principle, GC formation may be triggered downstream to Ca^{2+} signals.

It is expected that additional enzymes compliment the components described above. For example, in addition to the Ca^{2+}-dependent proteases other Ca^{2+}-dependent enzymes are expected to participate. These include actin-severing enzymes (Welch *et al.*, 1997), phospholypases (Eberhard and Holz, 1988), kinases, and phosphatases. The activation of these enzymes is expected to take part in the massive cytoarchtectural restructuring that underlies GC formation by direct modulation of the stability of the cytoskeletal elements, or by fine-tuning the affinity of the involved biochemical processes. It is premature to generalize our findings to other neuronal types or to other forms of neuroplasticity. Nevertheless, the cascade of cellular events described above may represent common cellular mechanisms that participate in different forms of neuoplasticity and may be now tested in less-accessible neuronal systems.

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