On-Line Confocal Imaging of the Events Leading to Structural Dedifferentiation of an Axonal Segment into a Growth Cone after Axotomy

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ABSTRACT
The transformation of a transected axonal tip into a growth cone (GC) after axotomy is a critical step in the cascade of events leading to regeneration. However, the mechanisms underlying it are largely unknown. In earlier studies we reported that axotomy of cultured Aplysia neurons leads to a transient and local increase in the free intracellular Ca\(^{2+}\) concentration, calpain activation, and localized proteolysis of the submembranal spectrin. In a recent ultrastructural study, we reported that calpain activation is critical for the restructuring of the microtubules and neurofilaments at the cut axonal end to form a compartment in which vesicles accumulate. By using on-line confocal imaging of microtubules (MTs), actin, and vesicles in cultured Aplysia neurons, we studied the kinetics of the transformation and examined some of the mechanisms that orchestrate it. We report that perturbation of the MTs’ polymerization by nocodazole inhibits the formation of an MT-based compartment in which the vesicles accumulate, yet actin repolymerization proceeds normally to form a nascent GC’s lamellipodium. Nevertheless, under these conditions, the lamellipodium fails to expand and form neurites. When actin filament polymerization is inhibited by cytochalasin D or jasplakinolide, the MT-based compartment is formed and vesicles accumulate at the cut axonal end. However, a GC’s lamellipodium is not formed, and the cut axonal end fails to regenerate. A growth-competent GC is formed only when MT restructuring, the accumulation of vesicles, and actin polymerization properly converge in time and space. J. Comp. Neurol. 494:705–720, 2006. © 2005 Wiley-Liss, Inc.

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Early studies revealed that axotomy leads to extensive ultrastructural damage along the cut axonal tip. The severity of the ultrastructural damage diminishes as the distance from the lesion site increases (Ballinger and Bittner, 1980; Gross and Higgins, 1987) and appeared to reflect the pathological processes activated by calcium and calcium-activated proteases (Schlaepfer and Bunge, 1973; Schlaepfer and Hasler, 1979; Zimmerman and Schlaepfer, 1984a,b; Schlaepfer, 1987; Hall and Lee, 1995). Subsequent studies revealed that the influx of calcium ions and the activation of calpain are necessary to initiate the formation of a membrane seal at the cut axonal end (for review see Fishman and Bittner, 2003; for specific information on cultured Aplysia neurons see Ziv and Spira, 1997; Gitler and Spira, 1998, 2002; Spira et al., 2003). Furthermore, the increases in the free intracellular calcium concentration ([Ca\(^{2+}\)]\(i\)) and calpain activation were found to play a critical role in the structural transformation of an axon into a GC after axotomy. In fact, a recent electron microscopic study in our laboratory revealed that, in cultured Aplysia neurons, axotomy leads to a highly orchestrated and characteristic restructuring cascade that transforms within tens of minutes the cut axonal end into a GC (Spira et al., 2003). This cascade involves the division of the microtubule (MT) skeleton at the cut axonal end into three zones: the proximal zone (PZ), in which MTs and neurofilaments are oriented in various directions; and a distal zone (DZ; 50 –150 µm long), in which cytoskeleton maintains its normal structure; a narrow (10-µm-long) transition zone (TZ), in which MTs and neurofilaments are oriented in various directions; and a distal zone (DZ; 50–150 µm long), in which MTs and neurofilaments form electron-dense aggregates. Vesicles accumulate within the TZ, which becomes the GC’s center, around which actin filaments assemble to form the leading edges of an expanding lamellipodium.

The mechanisms that coordinate in time and space the restructuring of an axon into a GC have not yet been elucidated. Here, we began to study the transformation by examining two principal mechanisms. The first assumes that the coordinated structural remodeling is achieved by continuous feedback among the participating cytoskeletal components (MTs and actin). In such a case, perturbation of the assembly kinetics of either one of the components would disrupt the assembly of the others. The second possibility assumes that axotomy triggers, in parallel, two independent restructuring cascades, one of MTs and the other of actin. In this case, the perturbation of one element would not affect the others. Through the use of an on-line confocal system, which allows for almost simultaneous imaging of three different structural components, along with pharmacological tools to perturb MTs or actin polymerization dynamics, we found that the axotomy triggers, in parallel, the independent restructuring of MTs and actin. Perturbation of the actin restructuring dynamics does not affect the initial and critical processes of MT restructuring and accumulation of vesicles. Perturbation of the MTs' restructuring does not interfere with the initial restructuring of the actin network. Nevertheless, a competent growth cone-organizing center (GCOC), which is essential for the regeneration of the cut end, is formed only when MT restructuring, vesicle accumulation, and actin polymerization properly converge in time and space. We propose, based on the present study and earlier reports, that injury-induced local calpain activation triggers and thus synchronizes in time and space the parallel subcellular cascades, which leads to the transformation of a cut axonal segment into a growth-competent GC.

**MATERIALS AND METHODS**

**Solutions**

**L-15 supplemented for marine species.** Leibovitz’s L-15 medium (Gibco-BRL, Paisley, Scotland) was supplemented for marine species according to Schacher and Proshansky (1983) by the addition of 12.5 g/liter NaCl, 6.24 g/liter D(+) dextrose, 3.15 g/liter anhydrous MgSO\(_4\), 344 mg/liter KCl, 192 mg/liter NaHCO\(_3\), 5.7 g/liter MgCl\(_2\)·6H\(_2\)O, and 1.49 g/liter CaCl\(_2\)·2H\(_2\)O. Penicillin, streptomycin, and amphoterocin B (Biological Industries, Kibbutz Beit Haemek, Israel) were added to make final concentrations of 100 U/ml, 0.1 mg/ml, and 0.25 µg/ml, respectively.

**Culture medium.** Culture medium consisted of 10% filtered hemolymph obtained from Aplysia fasciata (the specimens were collected along the Mediterranean coast) diluted in L-15 supplemented for marine species (ms L-15).

**Artificial sea water.** Artificial sea water (ASW) consisted of NaCl 460 mM, KCl 11 mM, CaCl\(_2\) 10 mM, MgCl\(_2\) 55 mM, HEPES 10 mM, adjusted to pH 7.6.

**Pharmacological reagents**

Calpeptin (Calbiochem, San Diego, CA) was diluted in ASW to 133 µM from a 50-mM stock solution in dimethylsulfoxide (DMSO). This solution was diluted to a final concentration of 100 µM upon addition to the experimental bath. A stock solution of 20 mM Nocodazole (Sigma, Rehovot, Israel) in DMSO was diluted in ASW to a concentration of 5 µM. This was added to the experimental chamber, to reach a final concentration of 2–5 µM. Five millimolar jasplakinolide (Bubb et al., 1994; Molecular Probes, Eugene, OR) or 10 mM cytochalasin D (Sigma) DMSO stock solutions were dissolved in ASW to concentrations of 3 µM and 12.5 µM, respectively. These solutions were further diluted in the experimental bathing solution to 2.5 µM jasplakinolide and 10 µM cytochalasin D. RH237 [N-(4-sulfutyl)-4-(6-(p-dibutylamynophenyl)hexatrenyl)] pyridinium, inner salt, a gift from Dr. R. Hildeshem, the Weizmann Institute of Science (Grinvald et al., 1982), was diluted in ethanol to a concentration of 10 mM and further diluted before use in ASW to a concentration of 10 µM. Five milligrams per milliliter tetramethylrhodamine tubulin, from bovine brain (Molecular Probes), was loaded into a glass micropipette and pressure-injected into the cytoplasm of the cell body.

**Cell culture**

Neurons B1 and B2 from buccal ganglia of A. californica were isolated and maintained in culture as previously described (Schacher and Proshansky, 1983; Spira et al., 1993, 1996). In the present study, we refer to these neurons collectively as B neurons. Briefly, juvenile A. californica (1–10 g) were anesthetized by injecting isotonic nematic Proshansky (1–10 g) were anesthetized by injecting isotonic ASW to 133 µM upon addition to the experimental bath. A stock solution of 20 mM Nocodazole (Sigma, Rehovot, Israel) in DMSO was diluted in ASW to a concentration of 5 µM. This was added to the experimental chamber, to reach a final concentration of 2–5 µM. Five millimolar jasplakinolide (Bubb et al., 1994; Molecular Probes, Eugene, OR) or 10 mM cytochalasin D (Sigma) DMSO stock solutions were dissolved in ASW to concentrations of 3 µM and 12.5 µM, respectively. These solutions were further diluted in the experimental bathing solution to 2.5 µM jasplakinolide and 10 µM cytochalasin D. RH237 [N-(4-sulfutyl)-4-(6-(p-dibutylamynophenyl)hexatrenyl)] pyridinium, inner salt, a gift from Dr. R. Hildeshem, the Weizmann Institute of Science (Grinvald et al., 1982), was diluted in ethanol to a concentration of 10 mM and further diluted before use in ASW to a concentration of 10 µM. Four milligrams per milliliter tetramethylrhodamine tubulin, from bovine brain (Molecular Probes), was loaded into a glass micropipette and pressure-injected into the cytoplasm of the cell body.

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FROM AN AXON INTO A GROWTH CONE AFTER AXOTOMY

2.5 hours. After the protease treatment, the ganglia were pinned and desheated. The neurons were manually pulled out along with their original axon with the aid of a sharp glass microelectrode. The neurons were immediately plated in glass-bottomed dishes coated with poly-L-lysine (Sigma) containing culture medium. All experiments were performed 24–48 hours after plating, at room temperature (21–25°C), after replacing the culture medium with ASW.

**Axotomy**

Axonal transection was performed by applying pressure on the axon with the thin shaft of a micropipette under visual control, as previously described (Ziv and Spira, 1993; Spira et al., 1993, 1996, 2003).

**mRNA preparation and injection**

mRNAs were in vitro transcribed by using the recombinant transcription system, as described from our laboratory (Sahly et al., 2003). Briefly, 10 μg of enhanced green fluorescent protein (EGFP)-Aplysia actin (provided by Dr. DesGrosiler, Montreal University) or EGFP-human α-tubulin (Clontech, Palo Alto, CA) pCS2 constructs were linearized with NotI and purified using the DNA clean-up system (Promega, Madison, WI). One to three micrograms of linearized DNA were transcribed by using a RiboMax-sp6 kit (Promega P1280). A typical reaction contains 8 μl transcription buffer; 8 μl rNTPs mix containing 25 mM CTP, ATP, and UTP and 12 mM GTP; 4 μl of 15 mM Cap analog (Roche 85846029); 1 μl rRnasin (Promega N251A); and 4 μl enzyme mix. A final volume of 40 μl was incubated for 2–4 hours at 37°C. RNA was purified by using a RNeasy mini kit (Qiagen 74104), and the clean RNA was eluted to a final volume of 25–40 μl and kept at −80°C until use.

The transcribed mRNAs were pressure injected into the cytoplasm of the cultured neurons 4–24 hours after plating. In preparing the injections, 0.5–5 μg/μl mRNA was added to 80 mM KCl. We estimated the injected volume to be approximately 10% of the cell’s body volume. Throughout the injection, the input resistance and transmembrane potential of the neuron were recorded by the injection micropipette. At the end of the injection, the micropipette was removed from the cell.

**Microscopic imaging**

The system used for confocal imaging consisted of an Olympus microscope IX70 and a Bio-Rad Radiance 2000/AGR-3 confocal imaging system. The objective used was an Olympus planApo ×60 1.4 NA oil objective. Images of EGFP-labeled proteins were acquired by excitation at 488 nm (argon laser), and the emitted fluorescence was collected at 500–560 nm. For sequential imaging, RH237 and EGFP fusion proteins, RH237 was excited by 488 nm, and the emitted light was collected at 660 nm. When EGFP, RH237, and tetramethyl-rhodamine tubulin were simultaneously imaged, the EGFP fluorescence was collected at 500–530 nm. The argon laser excitation intensity was usually lowered to 5–10%. The pinhole was set to 1.6–2.5 mm. Images were collected and processed using LaserSharp and LaserPix Bio-Rad software, respectively. The figures were prepared in LaserVox and Adobe Photoshop software. Supplemental movies (S) can be found at Wiley InterScience (http://www.interscience.wiley.com/jpages/0021-9967/suppmat). Calcium imaging was carried out by Mag-fura-2 (Molecular Probes), as previously described from our laboratory (Ziv and Spira, 1995, 1997). Ratio imaging of proteolytic activity was done by continuous incubation of the neurons in ASW containing 10 μM bis (CBZ-alanyl-alanine amine) rhodamine 110 (Molecular Probes), as we described previously (Gitler and Spira, 1998).

**Electron microscopy**

Electron microscopy was performed as previously described (Spira et al., 1993, 2003; Ashery et al., 1996; Ziv and Spira, 1997). The neurons were fixed by 3% glutaraldehyde (EMS, Fort Washington, PA) in ASW at pH 6.9. The cells were then washed in ASW and cacodylate buffer, pH 7.4 (EMS), and were postfixed in 0.5% osmium tetroxide (Next Chimica, Centurion, South Africa) and 0.8% K4Fe(CN)6. Dehydration was carried out through a series of ethanol solutions, and finally the neurons were embedded in Agar 100 (Agar Scientific, Stansted, England). Next, the blocks were sectioned with a microtome (LKB, Stockholm, Sweden). Thin sections of about 70 nm were stained by lead citrate, tannic acid, and uranyl acetate and then examined with a Jeol 100CX electron microscope.

**RESULTS**

**Morphological transformation of an axon into a GC after axotomy**

The transformation of a stable axon into a motile GC after axotomy of cultured Aplysia neurons can be subdivided by morphological criteria into five phases. 1) Within 30–60 seconds of axotomy, the plasma membrane seals the cut end (Spira et al., 1993; Ziv and Spira, 1995, 1997, Ashery et al., 1996; Fig. 1B). 2) In the course of 5–30 minutes following axotomy, a small axonal segment located 50–150 μm from the cut end swells (Fig. 1C). The segment located proximal to the swelling retains its cylindrical shape, while distal to the swelling the axon retracts and then gradually assumes a flat form (Spira et al., 2003). 3) Ten to thirty minutes after axotomy, a flat lamellipodium extends laterally from the swollen compartment described above (Fig. 1D). Retrospective examination reveals that the swollen segment (Fig. 1C, TZ) forms the center of the GC. We refer to it as the GCOC. 4) For 20–40 minutes after axotomy, the area of the lamellipodium continues to increase (Fig. 1D,E). 5) Thereafter, the GC’s lamellipodium breaks up into branches, each led by a motile GC (see Fig. 6). Whereas the GCs are motile structures that translocate along the substratum, the GCOC’s lamellipodium expands laterally over the culture dish but does not lead to a net movement of the main axon’s tip. The present study focuses on analyzing the critical steps that underlie the transformation of a transected axonal segment into a GCOC.
Axotomy induces rapid restructuring of the MTs and subdivides the cut end into three distinct zones

To study the dynamics of MT remodeling after axotomy, we labeled the MTs either by intracellular microinjection of tetramethyl-rhodamine-labeled tubulin monomers (Keating et al., 1997) or by intracellular microinjection of EGFP-α-tubulin mRNA. The injection of the probe did not alter the behavior of the neurons or their response to axotomy in any noticeable way.

Axotomy in ASW (control conditions) induces a retrograde wave of MT disassembly as revealed by the dissipation of the linearly oriented structures (Fig. 2A; see also S1). This process starts at the point of axonal transaction and propagates retrogradely at a rate of 7–16 µm/second (n = 10), along an axonal segment of 50–150 µm (Fig. 2A, axotomy, 6–24 seconds; see also S1). The retrograde wave of MT disassembly propagates at a rate similar to that of the calcium concentration front that originates at the ruptured end of the axon (Ziv and Spira, 1995, 1997).

The termination of the retrograde MT disassembly wave corresponds in time to the formation of a membrane seal over the cut end and the recovery of the free intraxonial calcium ion level (Spira et al., 1993; Ziv and Spira, 1995, 1997).

The termination of the MTs’ disassembly is followed, within minutes of axotomy, by a repolymerization phase. The repolymerization wave progresses proximodistally starting at the point at which the MTs maintain their integrity (Fig. 2A, 24 seconds; see also S1). This is followed by MTs’ repolymerization along the cortical region of the DZ (Fig. 2A, 6–15 minutes; see also S1). With time, a narrow TZ is formed between the proximal and the DZs. The TZ is confined by the repolymerized MTs of the PZ on one side, and initially by low-density MTs of the DZ on the other side (Fig. 2A, 10 and 15 minutes). With time, the density of the MTs at the DZ increases and the subdivision of the cut end into PZ, TZ, and DZ become clearer (Fig. 3A). Retrospective examination of the results reveals that the TZ is the site at which the vesicles accumulate (described below and see Fig. 3AII) and from which the lamellipodium extends (Figs. 2, 3, 5). The TZ, as revealed by confocal imaging of EGFP-tubulin, corresponds to the TZ described by us in an earlier ultrastructural study (Spira et al., 2003). These electron micrographs revealed that, whereas the PZ is composed mainly of parallel longitudinally oriented MTs, the DZ contains MT bundles composed of tightly packed MTs, and the TZ contains fewer MT fragments that orient in various directions.

**Actin restructuring after axotomy**

The actin skeleton was labeled by intracellular microinjection of EGFP-actin mRNA. In the intact axon, the EGFP-labeled actin network appeared to be distributed within the bulk of the axoplasm (Figs. 2B, 3AIII, and 7AIII). EGFP-actin hot spots are seen along the plasma membrane of the main axon facing the glass substrate (Figs. 2B, control, 5I; see also S2). These actin puncta most likely represent adhesion complexes (Hynes and Lander, 1992; Schoenwaelder and Burridge, 1999).

The actin puncta dissipate, after axonal transection, in parallel to the retrograde depolymerization wave of the MT (Fig. 2B, control, 30 seconds, 1.5 and 3 minutes; see also S2). Thereafter, when the calcium concentration is down-regulated (not shown), large EGFP-actin puncta assemble in a proximodistal direction along the plasma membrane facing the substratum (Fig. 2B, 3–15 minutes; see also S2). These actin puncta probably reestablish the plasma membrane adhesion to the culture substrate. In parallel to and within minutes of axotomy, intense actin fluorescence is detected along the axoplasmic cortex of the transition and DZs (Figs. 2B, 9.5 minutes, 3AIII, 5II; see also S3). Close to the surface of the glass substrate, the actin assemblies into radially oriented bundles at the leading edge of the GCOC’s lamellipodium (Figs. 2, 15 minutes, 5; see also S3, S5).

**Accumulation of vesicles within the TZ**

To examine the kinetics of the accumulation of the vesicles and its relation to the restructuring of the cytoskeleton, we first labeled the cytoskeleton by microinjection of fluorescent probes, as described above, and then stained the intracellular vesicles by bathing the neurons for 30 minutes in the styryl dye RH237. Initially, RH237 partitions into the plasma membrane (not shown); thereafter,
it is internalized and stains the endocytotic vesicles (see below). Twelve to twenty-four hours after staining, the RH237 fluorescence is distributed in the lipid membranes of subcellular organelles.

As soon as the microtubular pattern subdividing the axoplasm into the three zones is formed after axotomy, RH237 fluorescence begins to accumulate within the TZ (Figs. 3AIL, 3B, 4A; see also S4). To establish that the accumulated RH237 signal represents the accumulation of vesicles, we compared the distribution of RH237 fluorescence with the ultrastructural composition of the axon at various time points after axotomy (n > 10). As shown in Figure 4B–D, the RH237 fluorescence signal localizes at regions in which vesicles and tubular structures concentrate. Relatively small RH237 fluorescence pockets are also detected within the DZ (Fig. 4B–D).
Since the translocation of individual RH237-labeled vesicles cannot be visualized, the source of RH237-labeled vesicles could not be determined. The vesicles that accumulate within the TZ (Fig. 3) could arrive by anterograde transport from the cell body or be formed by a surge of membrane retrieval induced by axotomy at the cut axonal end (Fishman and Bittner, 2003). The use of styryl dyes does not allow us to differentiate between these potential membrane resources. With time after axotomy, the RH237 fluorescence gradually declines from the GCOC concomitantly with the extension of the GC’s lamellipodium (Figs. 3AII, 6).

**Extension of a GC’s lamellipodium**

The leading edge of the extending GCOC’s lamellipodia is enriched with actin bundles (Fig. 5; see also S3, S5). As time progresses and the GC’s lamellipodium extends far-

Fig. 3. Spatiotemporal distribution of microtubules, actin, and vesicles during the transformation of an axon into a growth cone after axotomy. A B neuron was cultured for 4 hours, then microinjected with tetramethyl-rhodamine tubulin and EGFP-actin mRNA. Thereafter, the neuron was bathed for 30 minutes in the styryl dye RH237, and the excess dye was thoroughly washed away. Twelve hours later, the neuron was transected, and the distributions of microtubules (AI), RH237-labeled vesicles (AII), and actin network (AIII) were imaged 3 μm above the substrate level. Prior to axotomy, the microtubules are oriented in parallel to the longitudinal axis of the axon (AI, control). The vesicles (AII) and actin (AIII) are evenly distributed within the axoplasm. Axotomy leads within minutes to subdivision of the cut end into proximal (PZ), transition (TZ), and distal (DZ) zones (AI, second image from the top). RH237-labeled vesicles concentrate within the transition zone (AII). EGFP actin assembles within minutes of axotomy at the perimeters of the transition and the distal zones (AIII). The time after axotomy is given in minutes on the left side of AI. B: A merged pseudocolor image of microtubules (red), actin (green), and RH237-labeled vesicles (blue) of the neuron shown in A taken 5 minutes after axotomy. Scale bars = 20 μm in A; 10 μm in B.

Fig. 4. Accumulation of vesicles at the transition zone. A neuron cultured for 3 hours was bathed for 30 minutes in RH237. The excess dye was thoroughly washed away from the bathing solution. Twelve hours later, the neuron was transected and the distribution of RH237 fluorescence was imaged 3 μm above the substrate level. A: Consecutive confocal images of RH237 distribution before axotomy (control) and 24 seconds and 3, 4, 6, and 9 minutes after axonal transection (see also S4). B–D: The RH237 fluorescent signal is colocalized with vesicles concentration at the transition zone. Twenty-four minutes after axonal transection (B), the RH237 fluorescent signal concentrated at the transition zone (arrow) and to a lesser extent at the distal zone and at the tip of the axon (arrowhead). The neuron was then fixed and prepared for electron microscopic observation (C,D). The low-magnification micrograph shown in C was enlarged (D), revealing that RH237 fluorescence corresponds to areas in which vesicles accumulate (D1 and D2, also marked in C). The area where no fluorescence was detected is devoid of vesicles (D3). Scale bars = 5 μm in A–C, 0.5 μm in D.
ther, a delicate actin meshwork forms between the GC’s center and the actin bundles at its leading edge (Fig. 5; see also S3, S5). This actin meshwork is invaded by radially oriented MTs (Figs. 6A,D; see also S5–S7). Throughout the process of lamellipodium expansion, actin-rich puncta are formed along the lamellipodial plasma membrane facing the substratum (Figs. 5, 6C; see also S3, S5). When the radius of the lamellipodium reaches 30–60 μm, the lamellipodium breaks up into branches, each led by a motile GC (Fig. 6). This is associated with assembly of actin-rich leading edges (Fig. 6C,D) and the translocation of vesicles from the GCOC into the tips of the motile GCs (Fig. 6B,D).

Independent parallel polymerizations underlie the restructuring of the MT and actin network

The observations reported above demonstrate that, within minutes of transection, the cut axon undergoes robust structural remodeling to form a GCOC. Two principal mechanisms could account for this highly orchestrated transformation: continuous feedback among the main participating elements is required to ensure coordination of the transformation in time and space. Alternatively, axotomy triggers independent polymerization cascades of MT and actin. These parallel events converge in time and space to allow regrowth. These hypotheses are examined below.

Effects of nocodazole on MT restructuring and the formation of a GCOC. For the experiments, neurons were incubated for 15–40 minutes in 2–5 μM of the MT-depolymerizing reagent nocodazole and then transected in its presence. Electron microscopic observations revealed that incubation of neurons in 10 μM nocodazole for 30–40 minutes does not affect the structural integrity of the MTs (not shown). We found that, in the presence of nocodazole, the cut axonal end reseals, and the increase in [Ca^{2+}]_{i} is down-regulated at rates similar to those observed in con-
Fig. 6. Microtubules, the actin network, and the redistribution of vesicles after branching of the growth cone lamellipodium. A neuron was cultured for 4 hours and labeled as described for Figure 3. The axon was transected 14 hours later and imaged at the substrate level. The images were taken 3.5 hours after axotomy (from the same experiment shown in Fig. 3). A: Microtubules. B: RH237-labeled vesicles. C: Actin. D: Merged image of microtubules (red), vesicles (blue), and actin (green). Note that, at this stage of growth, the growth cone’s lamellipodium branched to form several motile GCs. The microtubules extended from the GCOC into the perimeter. RH237-labeled vesicles translocated from the GCOC into the newly formed motile GCs, which are enriched at the leading edges by actin. Scale bar = 30 μm in D (applies to A–D).

trol experiments. MTs depolymerize, and calpain is activated, as revealed by imaging of tetramethyl-rhodamine tubulin and bis(CBZ-alanyl-alanine) rhodamine 110, respectively. However, the characteristic restructuring of the cut axonal end is inhibited (Fig. 7). Thus, in nocodazole, the MTs do not subdivide the cut axonal end into a
Fig. 7. Nocodazole prevents microtubule polymerization and the accumulation of vesicles but does not interfere with the initial processes of actin restructuring. A neuron was cultured and prepared for confocal imaging as described for Figure 3. Prior to axotomy, the neuron was incubated for 30 minutes in ASW containing 2 μM nocodazole. The axon was then transected in the presence of nocodazole. The images shown in A were taken 3 μm above the substrate. Columns I, imaging of tetramethyl-rhodamine tubulin; II, imaging of RH237; III, imaging of EGFP-actin. The images were collected before nocodazole application (control), after 30 minutes in nocodazole (nocodazole), and at different points in time after axotomy (indicated in minutes postaxotomy). Note that, in nocodazole, the MTs fail to subdivide the cut axonal end into PZ, TZ, and DZ (I) and that RH237-labeled vesicles do not accumulate (II). Nevertheless, axotomy is followed by accumulation of actin at the perimeters of the cut end (column III). B: Merged images of tubulin (red), RH237 (blue), and actin (green) of an axon transected 46 minutes earlier were taken at the substrate level (I) and 3 μm above it (III). II: Computer reconstructions of virtual cross-sections made at the levels indicated by the yellow lines (1–7). The collection of the confocal images at steps 0.3 μm apart to produce the virtual cross-sections lasted for 15 minutes. Scale bars – 20 μm in A, 15 μm in BII (applies to BI,BIII); 10 μm in BII.
Fig. 8. Effect of cytochalasin D on the restructuring of the cut axonal end. A neuron was prepared for confocal imaging as described for Figure 3. Prior to axotomy, the neuron was incubated for 30 minutes in ASW containing 10 μM cytochalasin D. The axon was transected and imaged in the presence of the drug. The images shown in A were taken 3 μm above the substrate. Columns I, imaging of tetramethyl-rhodamine-labeled tubulin; II, imaging of RH237; III, imaging of EGFP-actin. The images were collected before cytochalasin D application (control), after 30 minutes in cytochalasin D, and at different times after axotomy (indicated in minutes postaxotomy). Note that, in cytochalasin D, the MTs subdivide the cut axonal end into PZ, TZ, and DZ (I) and that RH237-labeled vesicles accumulate at the TZ (II). However, axotomy is not followed by the polymerization of actin at the TZ and DZ perimeters (III). The merged images (tubulin, red; RH237, blue; and actin, green) shown in B were taken at the substrate level (I) and 3 μm above it (III). II: Computer reconstructions of virtual cross-sections made at the levels indicated by the yellow lines (1–8). The collection of the confocal images at steps 0.3 μm apart to produce the virtual cross-sections lasted for 15 minutes. Scale bars = 20 μm in A; 5 μm in B.
crease in the concentration of the [Ca2+]i is down-regulated at rates similar to those of the control experiments (not shown). In the presence of cytochalasin D or jasplakinolide, axotomy leads to the restructuring of the MTs into the three axonal zones (Fig. 8AII), and vesicles labeled with RH237 accumulate in the TZ (Fig. 8AII). However, the characteristic assembly of actin filaments in the perimeter of the TZ and DZ does not take place (Fig. 8AIII,B). Moreover, under these conditions, a lamellipodium is not formed, and the MTs do not polymerize laterally for over 24 hours.

**Effect of calpain inhibition on the transformation of an axon into a GC**

We confirmed here that, in the presence of calpeptin, axotomy does not lead to subdivision of the cut axonal end into PZ, DZ, and TZ; vesicles do not accumulate, and a GCOC's lamellipodium does not extend (Fig. 9). Nevertheless, actin hot spots reappear along the DZ facing the substratum. Consistently with the report of Gitler and Spira (2002), if calpeptin is applied 4–5 minutes after axotomy, the extension of a GC's lamellipodium proceeds, although at a slower rate (n = 10; not shown). This window of time can now, based on the present observations, be related to the critical formation of the TZ within minutes of axotomy. The above experiments thus confirm the conclusion that the activation of calpain after axotomy is necessary to allow proper structuring of the TZ as well as for the polymerization of actin filaments at the leading edge of the lamellipodium.
DISCUSSION

On-line confocal imaging and pharmacological perturbations, complemented by electron microscopic examinations, allowed us to extend the understanding of the mechanisms that underlie the transformation of a differentiated axon into a GC after axotomy. We discuss below, based on the present observations and studies published in the literature, the cascades of events that leads to the transformation and its orchestration. Figure 10 provides a flow diagram of the described events. The numbers in the diagram (1–14) correspond to the numbers in the text.

Transient increase in the free intracellular calcium concentration

Several published studies showed that axotomy (1) is followed by \( \text{Ca}^{2+} \) influx into the axoplasm through the cut axonal end (for review see Fishman and Bittner, 2003). In cultured Aplysia neurons, the steep \([\text{Ca}^{2+}]_i\) gradient formed along approximately 500 \( \mu \text{m} \) of the cut axonal end relaxes within minutes (Ziv and Spira, 1995, 1997; Gabso et al., 1997; Spira et al., 2003) as soon as a membrane seal is formed over the cut end (Spira et al., 1993; Ashery et al., 1996). In the present study, on-line confocal imaging revealed that the calcium wave induces, in parallel, the disassembly of the MTs and the actin puncta (3 and 4, respectively; see Fig. 2). In addition, it was shown that the elevated \([\text{Ca}^{2+}]_i\), activates calpains (5; Gitler and Spira, 1998, 2002). As soon as the \([\text{Ca}^{2+}]_i\), is down-regulated, the actin puncta recover (6; see Fig. 2B) independently of the calpain activation. The elevated \([\text{Ca}^{2+}]_i\), and activation of calpain play an important role in sealing off the cut end. This aspect was reviewed recently by Fishman and Bittner (2003) and thus is not discussed here.

Activation of calpain

Earlier studies revealed that, in cultured Aplysia neurons, the calpain inhibitor calpeptin does not block the formation of a membrane seal over the cut axonal end but inhibits the transformation of the cut axonal end into a GC (Gitler and Spira, 1998, 2002; Spira et al., 2003). Calpeptin was found to inhibit: 1) the subdivision of the cut end into PZ, TZ, and DZ (Spira et al., 2003; 8; see Fig. 9) and therefore inhibits the accumulation of vesicles at the GCOC (11, 12); 2) the assembly of actin fibers along the perimeters of the transition and DZs (9; see Fig. 9); and 3) the cleavage of the submembrane spectrin skeleton (Gitler and Spira, 1998, 2002). It thus appears that axotomy-induced local calpain activation initiates in parallel several processes that converge in time and space to form the GCOC (12).

Formation of a TZ by the repolymerization of MTs

Two distinct zones of MTs are formed by the calcium wave: the DZ, in which the MTs are initially depolymerized or fragmented, and a PZ, in which the MTs are not affected (Fig. 2A; see also S1). Within minutes after the recovery of the \([\text{Ca}^{2+}]_i\), the MTs of the PZ repolymerize in a proximodistal direction (Fig. 2A; see also S1). Concomitantly, a narrow, axoplasmic band is formed by repolymerization of MTs at the DZ (Figs. 2A, 3A; see also S1). The TZ is the site where the vesicles accumulate (Figs. 3–5; see also S4), and its formation is crucial for promoting the growth processes (see below). The mechanisms by which the vesicles accumulate at the TZ are still not understood. Tentatively, it was proposed that the MT bundles along the DZ do not provide the appropriate substrate for the transport of vesicles, and so the vesicles accumulate at this site (Spira et al., 2003).

Actin assembly

In the presence of calpeptin, actin filaments do not assemble to form radially oriented bundles at the perimeters of the distal and TZs (9; see Fig. 9). This could be due to the direct involvement of calpain in the restructuring of the actin network (Potter et al., 1998) or the indirect effects of calpain on other elements, such as spectrin (10), which in turn influence the behavior of actin. It was demonstrated, for example, that spectrin influences the molecular behavior of actin by facilitating cross-linking. Consequently, the absence of spectrin may allow actin to form longer filaments (Stromqvist, 1987).

Causal relationships between MT restructuring and actin network reorganization

To examine possible relationships between the assembly of the actin network and MTs after axotomy, we perturbed the polymerization kinetics of one element at a time and examined the effects on the other and on the formation of a GC (Rodriguez et al., 2003). In the presence of 2–5 \( \mu \text{M} \) nocodazole (13), axotomy induces the initial calcium-dependent MT depolymerization and activation of calpain. This is not followed, however, by repolymerization of the MTs and the formation of a TZ or by the accumulation of RH237-labeled vesicles (Fig. 7). In contrast, the actin skeleton initially undergoes restructuring similar to that observed in the control experiments. However, the GCOC’s lamellipodium fails to extend. These observations suggest that the initial actin restructuring along the perimeter of the DZ after axotomy is independent of MT polymerization. Its spatiotemporal coordination may be coordinated by the direct effects of calpain or, more likely, indirectly, by the cleavage of spectrin as described above (Fig. 10).

The observation that, in nocodazole, the nascent GCOC lamellipodium does not extend suggests that continuous polymerization of the actin network within the lamellipodium (Figs. 5, 6) depends on the interactions with MTs (14). This is consistent with many reports demonstrating that actin filaments and MTs influence each other’s polymerization and that these interactions are essential for GC motility and restructuring in response to external cues (Waterman et al., 1999; Gordon-Weeks, 2000, 2004). Perturbation of actin dynamics by cytochalasin D or jasplakinolide (15) prevents the formation of GCOC’s lamellipodia but does not inhibit the initial restructuring of the MTs or the accumulation of RH237-labeled vesicles in the TZ after axotomy.

Actin polymerizes at the perimeters of the TZ to support the extension of a GCOC lamellipodium immediately after axotomy. This is followed by the invasion of radially oriented MTs (Fig. 6). The invasion of the MTs to the lamellipodium may be related to the fact that only some forms of actin serve as MT polymerizing promoters, whereas others do not (Sabry et al., 1991; Tanaka and Kirshner, 1995; Schaefer et al., 2002). The newly polymerized MTs that extend into the lamellipodium then support the pro-
trusive activity of actin microfilaments at the perimeters and allow the translocation of vesicles from the GCOC to the motile GCs formed by the branching of the GCOC lamellipodium (Bentley and O'Connor, 1994; Lin et al., 1994; Letourneau, 1996).

**Accumulation of vesicles at the TZ**

The confocal imaging of RH237-labeled vesicles in our study revealed that initially vesicles accumulate at the TZ (11; see Figs. 3, 4; see also S4). The mechanisms that initially localize the majority of the vesicles to the TZ are not currently understood and are the subject of ongoing studies in our laboratory.

One of our earlier studies revealed that the submembrane spectrin is cleaved in the TZ and DZ (Gitler and Spira, 1998). We suggested that, when vesicle accumulation and spectrin cleavage converge in time and space, the vesicles can fuse with the plasma membrane, allowing the expansion of the GC lamellipodium. However, this proposal was not confirmed by any direct observations, and the mechanisms that regulate the fusion of vesicles with the plasma membrane of regenerating neurons are not known.

In summary, it appears that the orchestrated transformation of a differentiated axonal segment into a competent GCOC after axotomy unfolds in two main stages: 1) a calcium-dependent, synchronized disassembly of the MTs and an actin-based cytoskeleton and 2) activation of calpain. This is followed by a calpain-dependent repolymerization of the MTs and the actin network to form a GCOC. However, the restructuring of the MTs, which leads to the accumulation of vesicles, on the one hand, and the polymerization of the actin network, on the other, unfolds in parallel and independently of each other. A competent GCOC is formed only when MT restructurization, accumulation of vesicles, and actin polymerization converge in time and space.

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**LITERATURE CITED**


Spira ME, Oren R, Dormann A, Gitler D. 2003. Critical calpain-dependent...
ultrastructural alterations underlie the transformation of an axonal
segment into a growth cone after axotomy of cultured Aplysia neurons.
J Comp Neurol 457:293–312.
Stromqvist M. 1987. Brain spectrin fragments and crosslinks actin fila-
Tanaka E, Kirschner MW. 1995. The role of microtubules in growth cone
Udvadia AJ, Koster RW, Skene JH. 2001. GAP-43 promoter elements in
transgenic zebrafish reveal a difference in signals for axon growth during
Waterman Storer CM, Salmon E. 1999. Positive feedback interactions
between microtubule and actin dynamics during cell motility. Curr
Zimmerman UJ, Schlaepfer WW. 1984a. Calcium-activated neutral pro-
Zimmerman UP, Schlaepfer WW. 1984b. Multiple forms of Ca-activated
Ziv NE, Spira ME. 1993. Spatiotemporal distribution of Ca2+ following
axotomy and throughout the recovery process of cultured Aplysia neu-
Ziv NE, Spira ME. 1995. Axotomy induces a transient and localized eleva-
tion of the free intracellular calcium concentration to the millimolar
Ziv NE, Spira ME. 1997. Localized and transient elevations of intracellular
Ca2+ induce the dedifferentiation of axonal segments into growth