

# Short Window of Opportunity for Calpain Induced Growth Cone Formation after Axotomy of *Aplysia* Neurons

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Received 8 January 2002; accepted 6 May 2002

**ABSTRACT:** Our laboratory has established that local activation of calpain by a transient elevation of the free intracellular calcium concentration is crucial for the induction of growth cone (GC) formation in cultured *Aplysia* neurons. The mechanisms and stages in which calpain is involved in the formation of a GC are not known. We began to study these questions by determining the nature of calpain's action and the stages in which calpain activity affects the cascade of events that leads to the formation of the GC and its extension. We report that the calpain-dependent transformation of an axonal segment into a GC occurs within a narrow window of opportunity that lasts approximately 5 min. If calpain is inhibited during this window of opportunity, GC formation does not occur. Inhibition of calpain after the window of opportu-

nity slows down the rate of lamellipodial extension but doesn't arrest it. The proteolysis of spectrin, a calpain substrate and a major component of the membrane skeleton, occurs within this window of opportunity, in agreement with the hypothesis that spectrin proteolysis is an early step in the formation of the GC. If the onset of proteolysis is deferred, spectrin remains unchanged and GC formation is compromised. We suggest that calpain participates in two different processes: it is critical for the triggering of GC formation and plays a modulatory role during the extension of the GC's lamellipodia. © 2002

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**Keywords:** calpain; spectrin; growth cone formation; nerve regeneration; cytoskeleton; membrane skeleton; *Aplysia*

## INTRODUCTION

A crucial step in the regeneration of injured nerves is the formation of growth cones (GCs) at the tip of transected axons. GCs possess autonomic functions

that are essential for regeneration: the GC translates local signals provided by the microenvironment into growth patterns (O'Leary and Wilkinson, 1999; Song and Poo, 1999). Elements of the cytoskeleton are assembled in the GC (Suter and Forscher, 2000), and intracellular membrane is inserted into the GC's plasmalemma (Craig et al., 1995). Upon identifying a target, the GC or its trailing neurite undergoes structural and functional changes resulting in the formation of presynaptic terminals (Haydon and Drapeau, 1995; Jontes et al., 2000; Kalil et al., 2000). Although much is known about the behavior of GCs and their structure, little is known about the mechanisms that initiate their formation after injury, during the normal course of development, or during remodeling associated with learning and memory processes (Bailey and Kandel, 1993; Glanzman et al., 1990).

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Contract grant sponsor: German-Israeli Foundation for Scientific Research and Development; contract grant number: I-0598-162.01/98.

Contract grant sponsor: USA-Israeli Binational Science Research Foundation; contract grant number: 97-297.

Contract grant sponsor: Israeli National Science Foundation; contract grant number: 620/98.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/neu.10084

Our laboratory has shown that axotomy, or a localized and transient elevation of the free intracellular calcium concentration ( $[Ca^{2+}]_i$ ) in intact cultured *Aplysia* neurons, induces GC formation and neuritogenesis (Spira et al., 1999; Ziv and Spira, 1997; and see Ziv and Spira, 1998; Ashery et al., 1996). During axotomy, the plasma membrane is breached, allowing the formation of a steep  $[Ca^{2+}]_i$  gradient along the axon, reaching the millimolar level at the tip. The plasma membrane reseals and the  $[Ca^{2+}]_i$  returns to control levels within approximately a minute. Typically, 10–20 min following axotomy, a GC in the form of a flat lamellipodium emerges 50–150  $\mu$ m proximally to the transection point, at the site where the  $[Ca^{2+}]_i$  reached maximal values of 300–500  $\mu$ M. The GC's lamellipodium rapidly expands in area for approximately an hour and subsequently splits to form numerous neurites, each possessing a smaller GC at its tip. The neurites extend and continue splitting, forming a dense neuritic tree at the site of injury.

By using concurrent imaging of  $[Ca^{2+}]_i$  and of intra-axonal proteolytic activity, we found that axotomy or localized elevation of the  $[Ca^{2+}]_i$  activates proteolytic processes in the region in which a GC is formed (Gitler and Spira, 1998; Spira et al., 2000). Because the proteolytic activity is induced by calcium and is blocked by the calpain inhibitor calpeptin we attributed this 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, illustrating that calpain activity is essential for the formation of GCs. Finally, we identified the proteolysis of the submembrane spectrin network as a possible link between calpain activation and GC formation (Gitler and Spira, 1998).

The mechanisms by which calpain is involved in the formation of a GC after axotomy or following a local elevation of the  $[Ca^{2+}]_i$  in intact neurons are not known. Here we began to study this question by examining at what stage of GC formation calpain activity is essential. Two models could explain the dependence of GC formation on calpain. Either calpain acts as a trigger, swiftly altering the axon's structure and allowing subsequent remodeling processes, culminating in the emergence of a GC, or it could participate continuously in the assembly of the GC, facilitating the extension of the lamellipodium. A prediction of the first model is that inhibition of calpain should block the formation of the GC only during a short period following the influx of  $Ca^{2+}$ . Thereafter, inhibition of calpain should not interfere with the extension of the GC. According to the second model, calpain activity is necessary for the duration of the extension of the GC.

In the present study, we define the period during which calpain activity is critical for the formation of a new GC by examining the effect of varying the timing of calpain inhibition on GC formation. We found that inhibition of calpain blocks the formation of a GC only if it is in effect within a narrow window of time that lasts up to 5 min, starting with the transient elevation of the  $[Ca^{2+}]_i$ . We also observed that the submembrane spectrin network is locally proteolyzed only during this short period. After this critical window of time, inhibition of calpain does not block GC formation. However, it does slow down the extension of the GC's lamellipodium. If the onset of proteolysis is deferred, spectrin remains unchanged and GC formation is compromised.

## MATERIALS AND METHODS

### Solutions

*L-15 Supplemented for Marine Species (msL-15)*. 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/L NaCl, 6.86 g/L D(+) glucose  $\cdot$  H<sub>2</sub>O, 3.15 g/L anhydrous MgSO<sub>4</sub>, 344 mg/L KCl, 192 mg/L NaHCO<sub>3</sub>, 5.7 g/L MgCl<sub>2</sub>  $\cdot$  6H<sub>2</sub>O, and 1.49 g/L CaCl<sub>2</sub>  $\cdot$  2H<sub>2</sub>O. Penicillin, streptomycin, and amphotericin B (Biological Industries, Kibbutz Beit Haemek, Israel) were added up to final concentrations of 100 units/mL, 0.1 mg/mL and 0.25  $\mu$ g/mL, respectively.

*Culture Medium*. Culture medium consisted of 5–20% filtered hemolymph obtained from *Aplysia faciata* (specimens were collected along the Mediterranean coast) diluted in ms-L15.

*Artificial Sea Water (ASW)*. ASW consisted of NaCl 460 mM, KCl 10 mM, CaCl<sub>2</sub> 10 mM, MgCl<sub>2</sub> 55 mM, HEPES 10 mM, adjusted to pH 7.6.

*Calpeptin-Containing ASW*. Calpeptin (Calbiochem, San Diego, CA) was diluted in ASW to 133  $\mu$ M from a 50 mM stock solution in dimethyl sulfoxide. This solution was diluted to a final concentration of 100  $\mu$ M upon addition to the experimental bath.

### Cell Cultures

Neurons B1 and B2 from buccal ganglia of *Aplysia californica* were isolated and maintained in culture as previously described (Schacher and Proshansky, 1983; Spira et al., 1996, 1999). Briefly, juvenile *A. californica* (1–10 g) were anesthetized by injection of isotonic MgCl<sub>2</sub> solution (380 mM) into the animal's body cavity. Buccal ganglia were dissected and incubated in ms-L15 containing 1% protease

(type IX; Sigma, Rehovot, Israel) at 34°C for 1.5–2.5 h. Following the protease treatment the ganglia were washed with ms-L15, pinned, and desheathed. The identified 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-bottom dishes coated with poly-L-lysine (Sigma) containing culture medium. All experiments were performed 8–36 h from plating, at room temperature (21–25°C) after replacing the culture medium (msL-15) with ASW.

## Video Microscopy

The system used for video enhanced contrast-differential interference contrast (VEC-DIC) microscopy consisted of a Zeiss Axiovert microscope equipped with DIC optics, a long working distance condenser set for Köhler illumination, and a 100 W halogen light source. A Zeiss 40X 0.75 NA Plan-Neofluar objective was used. Images were collected by grabbing and averaging 32 video-frames produced by a Vidicon video camera (Hamamatsu, Japan). Grabbing was done on-line (Imaging Technologies).

## Mag-Fura-2 Ca<sup>2+</sup> Imaging

Mag-fura-2 (Molecular Probes, Eugene, OR) loading, imaging, and calibration were done as previously described (Ziv and Spira, 1995, 1997). The fluorescence microscopy system consisted of a Zeiss Axiovert microscope equipped with a 75 W Xenon arc lamp, a Zeiss 40X 0.75 NA Plan-Neofluar objective, 340 ± 5 nm and 380 ± 5 nm bandpass excitation filters set in a computer-controlled, Lambda10 position filter changer (Sutter, Novato, CA), a dichroic mirror with a cut-off threshold of 505 nm and a 545 ± 25 nm band pass emission filter. The images were collected with an intensified CCD video camera (Hamamatsu, Japan), stored as computer files, and processed using a software package written in our laboratory.

## Proteolytic Activity Imaging

Ratio imaging of proteolytic activity was performed as described previously (Gitler and Spira, 1998). Neurons that were previously loaded with mag-fura-2 were continuously incubated in ASW containing 10 μM bis(CBZ-alanyl-alanine amine) rhodamine 110 (bCAA-R110; Molecular Probes) and were imaged for the production of fluorescent rhodamine 110 (R110). Ratio imaging was used to correct for volumetric changes and was performed as described for mag-fura-2 except that the excitation wavelengths used were 490 ± 6 nm, which excites R110, and 350 ± 5 nm, which is the isosbestic point of mag-fura-2.

## Axotomy

Axonal transection was performed by applying pressure on the axon with the thin shaft of a micropipette under visual

control, as has been previously described (Spira et al., 1993, 1996; Ziv and Spira, 1993).

## Elevation of the Free Intracellular Ca<sup>2+</sup> Concentration by the Application of Ionomycin

A local and transient elevation of the [Ca<sup>2+</sup>]<sub>i</sub> was achieved by locally applying ionomycin from a micropipette onto the axonal membrane as was previously described (Ziv and Spira, 1997). Briefly, ionomycin (calcium salt; Sigma) from a stock solution of 10 mM in dimethyl sulfoxide was diluted with ASW to a final concentration of 0.5–1 mM and focally applied by pressure ejecting the solution onto the axonal membrane with a micropipette. The alterations in the [Ca<sup>2+</sup>]<sub>i</sub> were monitored in real time by mag-fura-2 imaging.

## Measurement of the Extension Rate of the GC and Definition of Arrest of GC Formation

Because the vast majority of axotomized B1/2 neurons produce GCs in the form of a flat lamellipodium within 10 to 30 min of axotomy, success of GC formation was defined as the extension of either lamellipodia or of thicker stable protrusions during the first hour after axotomy. Arrest of GC formation includes instances in which either no change in morphology occurred except for those caused directly by the axotomy procedure, or the axon thickened without subsequently forming a lamellipodium, or only thin, short filopodia were formed. In some experiments, we tested the effect of calpeptin in situations in which the initial phase of GC formation might already have occurred. In this case, GC emergence was considered successful if the lamellipodium continued extending and did not stall or collapse.

The rate of extension of the GC's lamellipodium was defined as the increase of the apparent surface area of the lamellipodium in square micrometers per minute. This parameter is only an approximation because it doesn't take into account the GC's three-dimensional structure. However, it has been shown in previous studies that this approximation is adequate (Ashery et al., 1996). Tracing was carried out manually on enhanced images using Adobe Photoshop.

## Immunocytochemistry

Cultured neurons were fixed by incubating them in ASW containing 4% paraformaldehyde and 400 mM sucrose for 15 min (Forscher and Smith, 1988), washed thoroughly with phosphate buffered saline (PBS: NaCl 8 g/L, Na<sub>2</sub>HPO<sub>4</sub> 1.44 g/L, KCl 0.2 g/L, KH<sub>2</sub>PO<sub>4</sub> 0.24 g/L, at pH 7.4), and permeabilized by incubating the neurons for 5 min in PBSTr1% (1% Triton X-100 in PBS), 15 min in PBSTr0.2%, or 1 h in PBSTr0.1%. No differences were observed between these different preparations. The neurons were then washed with PBSTw (0.2% Tween-20 in PBS) and then

incubated in blocking solution (PBSTw + 1% bovine serum albumin + 10% powdered skimmed milk) for 1 h at room temperature, followed by incubation in the primary antibody solution [polyclonal rabbit antihuman spectrin (Sigma); 1:300 in blocking solution] for 12 h at 4°C. The neurons were washed thoroughly with PBSTw and were then incubated in the staining solution for 1 h at room temperature in the dark. The staining solution contained the secondary antibody [sheep antirabbit IgG, Cy3 conjugated (Sigma); 1:500] diluted in blocking solution. In several experiments 220 nM Alexa 488-conjugated Phalloidin (Molecular Probes) was added to the staining solution. The dishes were washed thoroughly with PBSTw followed by washing with PBS, and finally filled with a 2% *n*-propyl-galate solution in 1:1 glycerol:DDW. Controls for unspecific binding of the secondary antibody, performed by omitting the primary antibody, as well as various batches of rabbit preimmune serum, showed insignificant fluorescence.

## Confocal Microscopy

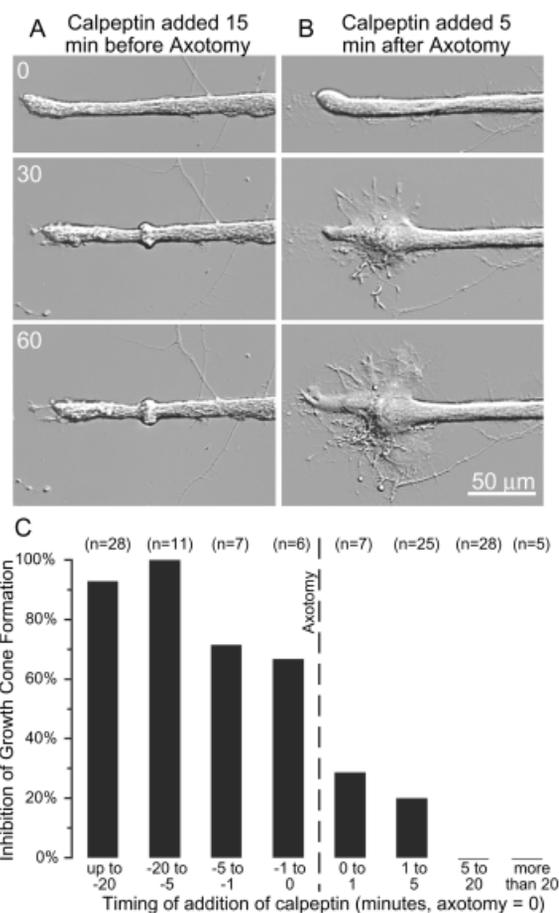
The confocal microscopy system consisted of a Bio-Rad MRC-1024 confocal head coupled to a Zeiss Axiovert 135M inverted microscope equipped with a Zeiss 40X 1.3 NA Plan-Neofluar oil immersion objective. The preparations were excited with the 488 nm band of a 100 mw air cooled argon ion laser (Ion Laser Technologies, Salt Lake City, UT). One percent or 3% of the laser power was used. Two channels of emission were utilized: Cy3 fluorescence was observed with a  $570 \pm 15$  nm bandpass filter, while Alexa 488 fluorescence was observed with a  $525 \pm 20$  nm bandpass filter. The resulting images were corrected for bleed through from the green to the orange channel, when necessary. The final images were prepared using Adobe Photoshop.

## RESULTS

### Calpeptin Inhibits the Formation of a GC Only if Present during Axotomy

To resolve the relationship between calpain activity and the processes that trigger GC formation we inhibited calpain activity at different stages of GC formation (Fig. 1). This was done by applying the cell permeable calpain inhibitor calpeptin (100  $\mu$ M; Tsujinaka et al., 1988) to the experimental chamber at various times in relation to axotomy [Fig. 1(A,B)]. Arrest of GC formation was defined either as a failure of the transected axon to extend a lamellipodium or as a complete stop of the further expansion of any structures existing at the time of the addition of calpeptin (see Materials and Methods section).

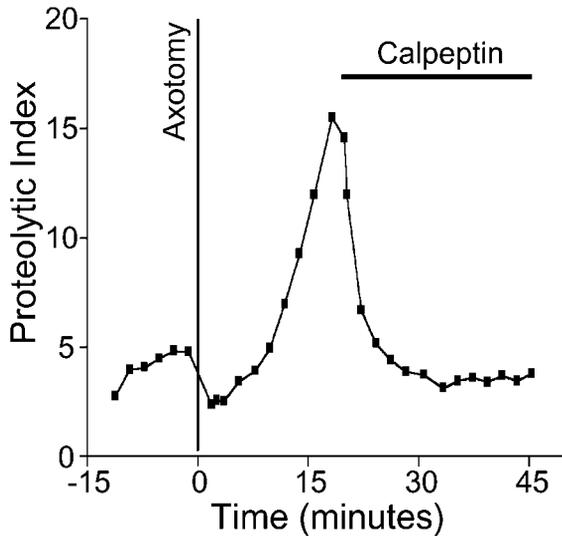
The histogram shown in Figure 1(C) illustrates that if calpeptin is added more than 5 min before axotomy, calpeptin completely inhibits GC formation. In con-



**Figure 1** Determination of the critical period for the initiation of GC formation by calpain activation. The critical window of time during which calpain activity induces GC formation was determined by adding 100  $\mu$ M calpeptin to the bath at various starting points. (A) DIC images illustrating the blockage of GC formation when a neuron is axotomized in the presence of calpeptin. (B) Example of the formation of a GC when calpeptin is applied after axotomy. Calpeptin was added to the medium 5 min after axotomy, concurrently with the initial appearance of the lamellipodia. The time elapsed from axotomy is indicated in minutes on the left of (A). (C) Histogram describing the percentage of axons in which GC formation was arrested, as a function of the timing of the addition of calpeptin in respect to axotomy. The number of experiments included in each column is indicated above the columns.

trast, if calpeptin is added 5 min after axotomy or later, it does not prevent the formation of the GC. The later calpeptin is added during the 10-min-spanning axotomy, the more of the transected neurons succeed in forming GCs.

To establish the temporal resolution of this experimental procedure, we examined the kinetics of the inhibition of ongoing calpain activity by bath application of 100  $\mu$ M calpeptin (Fig. 2). This was



**Figure 2** Determination of calpain inhibition dynamics by calpeptin. An axon was transected in the presence of bCAA-R110 ( $t = 0$ ). After the fluorescence signal started increasing, calpeptin was added to the bathing medium (bar). Calpeptin caused an immediate decay in the fluorescence intensity of R110, which could be fit by a single exponential.

achieved by real time imaging of calpain activity by the use of the fluorogenic indicator of proteolytic activity bCAA-R110 (see Gitler and Spira, 1998). In bCAA-R110, the fluorescence of R110 is quenched by two short identical peptides. Upon the cleavage of one or both amide bonds, fluorescence increases dramatically. Axotomy and GC formation invariably cause volume changes in the axon. To correct for the effect of volume changes on the fluorescence intensity measurements, the R110 fluorescence is ratioed against that of mag-fura2, which is microinjected into the neuron ahead of axotomy (for details see Gitler and Spira, 1998). We found that calpeptin readily inhibited calpain activity in axons that had been transected prior to its addition. Calpeptin caused the R110 fluorescent signal produced by proteolysis to decay immediately upon its application. The time course of the decay of the fluorescence signal encompasses the rate of influx of calpeptin, the inhibition of calpain by calpeptin, and the efflux and dispersal of the already-produced fluorescent proteolytic products. The decay of R110 fluorescence is fitted by a single exponent, characterized by a time constant of  $160 \pm 37$  s ( $n = 6 \pm$  S.D.; Fig. 2). Therefore, the temporal resolution of the experiments described above is at least in the range of 3–5 min (theoretically reflecting at a minimum 67–85% inhibition of calpain, respectively).

These results illustrate that calpeptin inhibits es-

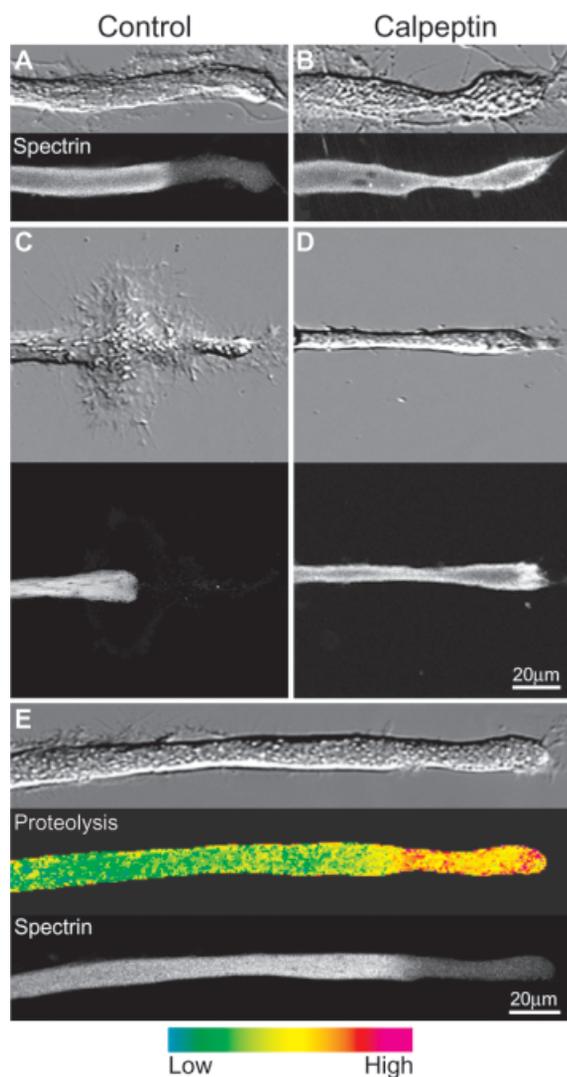
sential early steps in the cellular process that underlies the transformation of an axonal segment into a GC after axotomy. Once the initial and critical steps are complete, within a few minutes of axotomy, the inhibition of calpain cannot stop the formation of the GC. Therefore, calpain has a critical, short-lived role to play during the few minutes following axotomy.

### Spectrin Is Proteolyzed in the Transected Tip Immediately after Transection

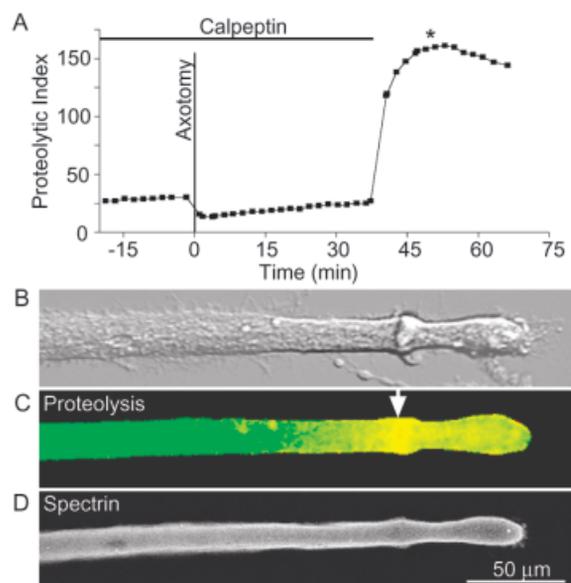
Spectrin is one of the main components of the membrane skeleton (Bennett, 1990). As such, it links the plasma membrane to the cytoskeleton, and confers structural integrity and elasticity to the plasma membrane. Spectrin, a well-known calpain substrate (Johnson et al., 1991), is proteolyzed in the transected end of *Aplysia* neurons after axotomy in a calpeptin-sensitive manner (Gitler and Spira, 1998).

If the proteolysis of spectrin is essential for the initiation of GC formation, it is reasonable to expect it to occur during the time when calpain inhibition can abolish GC formation. To examine this prediction, we studied the distribution of spectrin in axons fixed within a few seconds of axotomy. We found that spectrin immunolabeling is attenuated sharply in the transected tip during this period. In all neurons fixed less than a minute after transection [ $n > 10$ ; Fig. 3(A)], spectrin labeling was markedly reduced from the cut end up to the location where the GC is expected to emerge. Spectrin immunolabeling did not recover at the transected tip throughout the emergence of the new GCs lamellipodium [Fig. 3(C)]. In axons transected in the presence of calpeptin, this effect was not observed, so that at no time was the spectrin immunolabeling reduced, even at the transected tip itself [Fig. 3(B,D)]. If calpeptin was applied after transection, the spectrin labeling at the tip was reduced to the same extent as in axons not treated with calpeptin at all (not shown). The proteolytic activity as reported by bCAA-R110 becomes visible a few minutes after axotomy, when enough product is produced to be clearly observable by this technique (see Discussion). Comparison of the initial R110 signal to the spectrin distribution in the same neuron illustrates the spatial correlation of axotomy-induced proteolytic activity and the rapid proteolysis of spectrin [Fig. 3(E)].

These observations imply that the proteolysis of spectrin occurs within a minute of axotomy, in agreement with the hypothesis that processing of spectrin by calpain is one of the initial steps that enables the neuron to form a GC. Because calpain is known to



**Figure 3** Timing of the proteolysis of spectrin after axotomy. Neurons were axotomized and immunolabeled for spectrin. (A) A neuron was perfused with fixative  $\approx 10$  s after axotomy. The staining of spectrin at the tip is significantly lower than in the rest of the axon. (B) The axon was transected in the presence of  $100 \mu\text{M}$  calpeptin and was perfused with fixative 30 s after axotomy. Spectrin can be observed all along the axon. (C) The distribution of spectrin in a well-developed GC, fixed 43 min after axotomy was performed under control conditions. Spectrin is absent from the GC and the underlying axonal segment. (D) The axon was transected in the presence of calpeptin and fixed 40 min after axotomy. Spectrin can be clearly observed up to the axon's tip. Notice that the image is essentially equivalent to that shown in (B). (E) Proteolytic activity was monitored in a neuron by the use of bCAA-R110. The neuron was fixed as soon as proteolytic activity could be clearly discerned (10 min after transection in this instance) and processed for spectrin immunolabeling. Shown is the distribution of the fluorescence indicating proteolysis and the distribution of spectrin. A reduction in spectrin immunostaining correlates with the location where the protease indicator was observed.



**Figure 4** Spectrin is not proteolyzed in an axon transected in the presence of calpeptin, even after calpeptin is removed. A neuron was axotomized in the presence of calpeptin, while proteolytic activity was monitored with  $10 \mu\text{M}$  bCAA-R110. Calpeptin was washed off 38 min after axotomy. The neuron was fixed after an additional 36 min and was then immunolabeled for spectrin. (A) Time course of the changes in the ratio-corrected fluorescent intensity of the proteolytic product R110 (proteolysis index). The rates of increase of the proteolysis index prior to and after axotomy ( $t = 0$ ) are similar, demonstrating that calpeptin inhibited the proteolytic activity induced by axotomy. Upon the removal of calpeptin, an immediate increase in the proteolysis index was observed. Shown is the proteolysis index at the swelling in the axon marked in (C) by an arrow. (B) DIC image of the axon 74 min after transection. Notice that the axon did not form a GC. (C) Pseudocolor image of the proteolysis index 12.5 min after the removal of calpeptin [time marked by asterisk in (A)]. Notice that the proteolysis index increased throughout the axon and especially within the swelling in the axon (arrow). (D) The distribution of spectrin is not altered in spite of the increase in the proteolytic activity.

cleave spectrin in a limited fashion (Boivin et al., 1990), the decrease in spectrin immunolabeling at the transected tip could be due to the proteolysis of all spectrin comprising the membrane skeleton or due to the weakening of the skeleton by its partial cleavage and the subsequent redistribution of spectrin into the axoplasm.

A scale bar indicating the intensity of the proteolytic index is supplied below. The color scale was enhanced to facilitate the viewing of the faint proteolysis signal.

### Spectrin Is Not Proteolyzed When Calpain Activity Is Temporally Dissociated from Axotomy

We observed that when the proteolytic activity of calpain is delayed in respect to axotomy, full blown GCs are not formed. These experiments were performed by transecting the axons in the presence of calpeptin and then washing away the inhibitor from the dish after a delay (Fig. 4). In these experiments, GC formation either does not take place within an hour [Fig. 4(B)], a period within which GCs invariably emerge in control conditions ( $n = 7/14$ ), or only small and stunted GCs are formed at the ends of the transected axons ( $n = 7/14$ ). Under these experimental conditions, the spectrin immunofluorescent pattern is not altered, even though an increase in proteolytic activity is imaged upon the removal of calpeptin [ $n = 4$ ; Fig. 4(A–D)]. It is therefore evident that the timing of the expression of calpain activity is crucial and that it has to take place immediately after axotomy to fully transform the axonal structure into that of a GC.

### Spectrin Is Proteolyzed by the Elevation of $[Ca^{2+}]_i$ in Intact Axons

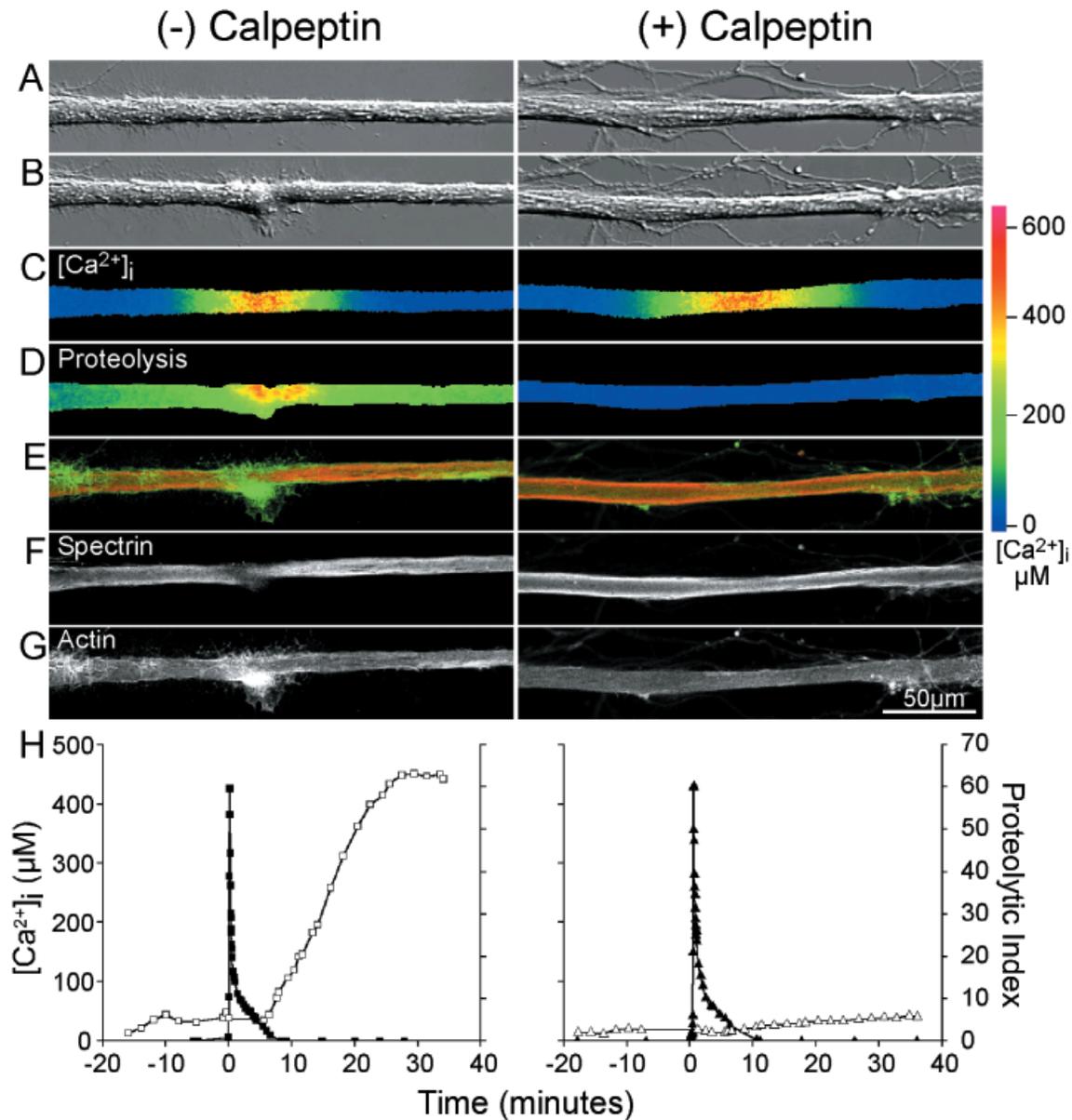
In previous studies from our laboratory we demonstrated that the transient and localized elevation of the  $[Ca^{2+}]_i$  in the axon, rather than other injury related processes, is a sufficient trigger to induce GC formation. This was done by inducing GC formation in response to a transient and local application of ionomycin to an axonal segment of an intact *Aplysia* neuron. Thus, locally elevating the intra-axonal  $[Ca^{2+}]_i$  of an intact neuron to 300–500  $\mu M$  results in the local activation of calpain and in the formation of a GC (Gitler and Spira, 1998; Ziv and Spira, 1997). It was therefore of interest to observe whether under this experimental paradigm spectrin proteolysis would occur in a manner similar to that observed after axotomy. If the formation of the GC necessitates spectrin proteolysis, as we hypothesize, then spectrin should be proteolyzed in the axonal segment in which the  $[Ca^{2+}]_i$  was transiently elevated, even in the absence of mechanical injury. Indeed, the application of ionomycin caused a reduction in the intensity of the spectrin signal at points where the  $[Ca^{2+}]_i$  was elevated ( $n = 4$ ; Fig. 5, left panel). The focal increase in F-actin concentration at that spot illustrates that a GC was beginning to extend from the ionomycin application area. In the presence of calpeptin, spectrin was not proteolyzed and actin did not polymerize, indicating a failure to form a GC ( $n = 3$ ; Fig. 5, right panel). We

did not examine the distribution of spectrin immediately after the application of ionomycin, because it is not possible to assess whether GC formation has been induced prior to the detection of morphological changes.

### Calpeptin Slows Down the Extension of the Lamellipodium of Nascent GCs

Proteolysis in the axonal tip and the GC's center is imaged for a period of approximately an hour after axotomy (Gitler and Spira, 1998). Therefore, the question arises whether sustained calpain activity has a role in the further development of the nascent GC besides its role in the triggering of the formation of the GC. We investigated this question by comparing the extension rate of the GC's lamellipodium in the presence ( $n = 5$ ) and absence ( $n = 7$ ) of calpeptin as well as by examining the effect of calpeptin on the morphology of the lamellipodia. The experiments were carried out by adding 100  $\mu M$  calpeptin to the bath 2–15 min after the axons were transected, always before a lamellipodium extended from the transected axonal tip. The area of the GC's lamellipodium was measured for the following 3 h at fixed intervals. We found that calpeptin substantially slowed down the extension rate of the lamellipodium [Fig. 6(A)]. Examination of the morphology of both groups revealed that lamellipodia that extended in the absence of calpeptin started splitting into small neurites after approximately 2 h [Fig. 6(B)]. In comparison, lamellipodia that extended in the presence of calpeptin did not develop any thin processes, and consisted mainly of a few nonbifurcating lamellae [Fig. 6(C)]. The difference in the extension rates cannot be attributed to a failure in the formation of a microtubular cytoskeleton in the GC, because microtubules were observed in GCs that extended in the presence of calpeptin (not shown).

To examine whether calpeptin has a role in later stages of the development of the GC, we examined whether calpeptin has an effect on the rate of extension of the lamellipodia when it is added 1.5 to 2 h after axotomy, prior to the splitting of the lamella into small neurites. Before calpeptin was added, we examined whether changes in the proteolytic index could be measured in the GC by transiently adding bCAA-R110 to the bath. We found that the product of the proteolytic activity was not detectable by our imaging system at this time ( $n = 4$ ). Nevertheless, we found that the addition of calpeptin slowed down the rate of extension of the lamellipodia immediately (not shown), whereas the application of the carrier solution did not. This result implies that a calpeptin-sensitive



**Figure 5** Transient elevation of the  $[Ca^{2+}]_i$  in intact axons induces calpeptin-sensitive proteolysis of spectrin. Ionomycin was focally applied to the membrane of intact axons while  $[Ca^{2+}]_i$ , proteolytic activity and morphological alterations were monitored. Afterwards, the neurons were fixed and stained for the presence of spectrin and F-actin. The left panel of images portrays an experiment conducted in the absence of calpeptin, while the right panel portrays an experiment conducted in the presence of 100  $\mu M$  calpeptin. (A) DIC images of both axons prior to the localized application of ionomycin. (B) DIC images acquired 35 min after the application of ionomycin, prior to fixation. Notice that a GC was formed only in the absence of calpeptin. (C) Pseudocolor images depicting the maximal levels of  $[Ca^{2+}]_i$  attained during the ionomycin application. Notice that the levels of  $[Ca^{2+}]_i$  in both axons are comparable. A  $[Ca^{2+}]_i$  scale bar is provided to the right. (D) Pseudocolor images illustrating that proteolytic activity was induced only in the absence of calpeptin. (E) Combined image of the distribution of spectrin (red) and F-actin (green) within the axon. (F) Images showing solely the distribution of spectrin within the axons. Notice that in the axon to which ionomycin was applied in the absence of calpeptin, the spectrin signal is markedly reduced at the site of application. In contrast, no such effect is seen in the right panel. (G) Images showing

enzymatic activity continues to exert its effect on the rate of the extension of the GC in the advanced stages of its extension.

## DISCUSSION

In this study we found that calpain activity is critical for the initiation of GC formation, a process that occurs within a period of less than 5 min after axotomy. We define this period as the window of opportunity for the transformation of an axonal segment into a GC (Fig. 1). Once the cascade of cellular events leading to GC formation is set into motion, calpain activity is not absolutely necessary for the emergence of the GC, in the sense that the GC does emerge even if calpain is inhibited only a few minutes after axotomy. Although calpeptin does not cause GC formation to fail when added after the window of opportunity, it does slow down the extension rate of the lamellipodia (Fig. 6), arguing that calpain has other roles in addition to triggering GC formation.

The physiological function of calpain is not yet known (Sorimachi et al., 1997). Most studies refer to calpain as an enzyme involved in pathological or degenerative processes (Chan and Mattson, 1999; Stracher, 1999). Our observations are consistent with a growing number of reports attributing to calpain roles in remodeling and regeneration (for example see Barnoy et al., 1996; Faddis et al., 1997; Gitler and Spira, 1998; Huttenlocher et al., 1997; Lynch and Seubert, 1989; Potter et al., 1998). Other proteolytic processes have been shown to exert fundamental effects on GC function under nonpathological conditions. For example, proteasome-mediated proteolysis participates in the chemotropic responses of retinal GCs (Campbell and Holt, 2001).

### Calpain's Role in the Initiation of GC Formation

Our results suggest that the proteolysis of spectrin by calpain is one of the processes that underlie the initi-

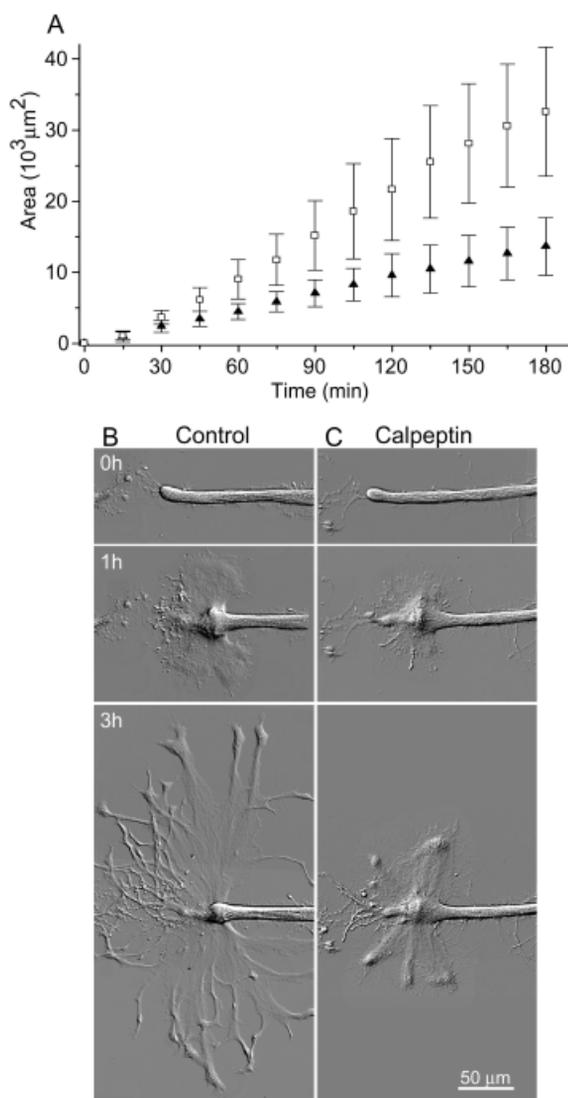
ation of GC formation. First, spectrin is proteolyzed within the window of opportunity for the initiation of GC formation (Fig. 3). We observed that calpeptin application prior to axotomy inhibits both the formation of the GC and the proteolysis of spectrin (Figs. 3 and 5). If calpain activity is blocked after spectrin is proteolyzed, the blockage does not stop the ongoing extension of the GC's lamellipodium (Fig. 1). On the other hand, if calpain activity is deferred, so that it becomes temporally dissociated from axotomy and from the transient elevation of the  $[Ca^{2+}]_i$ , spectrin is not visibly proteolyzed in the transected tip of the axon, and GC formation is compromised (Fig. 4). To conclude, GC formation is preceded by spectrin proteolysis. In situations in which spectrin proteolysis does not take place, GC formation is inhibited.

The proteolysis of spectrin is detected by retrospective immunolabeling within less than one minute after axotomy. Imaging of the fluorescent signal produced by the cleaved proteolytic substrate is detected, on the other hand, only minutes after axotomy [compare time course of proteolytic activity in Fig. 2 to spectrin immunolabeling in Fig. 3(A)]. This apparent inconsistency derives most likely from differences in the sensitivity of the two techniques, so that proteolytic activity, which is sufficient to rapidly proteolyze submembrane spectrin, is insufficient to produce enough free R110 to be detected by the imaging system. The spatial correlation between proteolytic activity imaging and spectrin immunolabeling [Fig. 3(E)] is consistent with these events being derived from the same occurrence.

Spectrin is a constituent of the membrane skeleton, a structure that confers stability to the plasma membrane and which anchors it to the underlying cytoskeleton (Bennett, 1990). It is therefore to be expected that proteolysis of spectrin should weaken the attachment of the plasma membrane to the underlying axonal structures. Indeed, it has been reported that upon transection, the plasma membrane detaches from the central axonal core at the tip of the axon (Spira et al., 1993; Ziv and Spira, 1997), suggesting the severance of its links with the cytoskeleton. Consistent with this

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solely the distribution of F-actin within the axons. Notice that actin has polymerized extensively at the site of application, marking the location of the emerging GC. In the right panel, only a modest increase in F-actin density can be observed at the application site. (H) Time course of the  $[Ca^{2+}]_i$  transient measured at the site of the application of ionomycin (filled symbols) and the proteolysis index measured at that same location (open symbols). Notice that although the  $[Ca^{2+}]_i$  was raised to the same value and for a similar length of time in both axons, proteolytic activity did not increase in the presence of calpeptin (triangles), whereas it increased significantly in its absence (squares). The time of the application of ionomycin is designated as  $t = 0$ .



**Figure 6** Calpeptin slows down the long-term extension of the GC's lamellipodia. Neurons were axotomized, and the rate of increase of the visible surface area of the GC's lamellipodia was measured. Calpeptin was added before lamellipodia emerged, between 2 to 15 min after axotomy was performed. (A) Comparison of the extension rate of lamellipodia in the presence (filled triangles) and absence (open squares) of calpeptin. Shown are average values ( $\pm$ S.D.) of the surface areas observed in  $n = 5$  (calpeptin) and  $n = 7$  (control) experiments. The difference between the surface areas becomes highly significant ( $p < .01$ , Student's  $t$  test) 45 min after axotomy. (B) Example of a GC extending in control conditions. Notice the extensive branching of the lamella and the formation of thin neurites. (C) Example of a GC extending in the presence of calpeptin. In this instance, calpeptin was added to the bathing medium 12.5 min after axotomy. Time is given in hours from axotomy.

view, evaginations of plasma membrane can be observed in transected lamprey axons *in vivo* (McHale et al., 1995). The proteolysis of spectrin and the possible weakening of the membrane skeleton may facilitate the access of intra-axonal vesicles to the plasma membrane (Aunis and Bader, 1988; Perrin et al., 1987; Sikorski et al., 2000). This is of importance, because the GC has been shown to be a site of membrane insertion by exocytotic processes (Craig et al., 1995; Dai and Sheetz, 1995). Therefore, weakening of the membrane skeleton as a barrier to membrane fusion may be important in the formation of a GC. The reduction in the submembrane spectrin immunofluorescence intensity at the axon's tip following transection could be the result of partial or total spectrin proteolysis. This aspect, as well as the fate and redistribution of spectrin fragments within the axoplasm, was not determined. However, a reduction in the intensity of the spectrin immunofluorescent signal under the plasma membrane was clearly documented.

Calpain may also target additional cytoskeletal proteins. For example, calpain degrades MAPs, which affect microtubule stability (Johnson et al., 1991), and homologues of protein 4.1 (Croall et al., 1986), which affect the interaction of actin with spectrin (Kontogianni-Konstantopoulos et al., 2001). Because actin dynamics play such a central role in GC behavior (Gallo and Letourneau, 1999), the modulation of its interaction with spectrin may exert additional control on the emergence of the GC. Overexpression of calpastatin, calpain's endogenous inhibitor, drastically reduces the ability of fibroblasts to extend actin-based protrusions (Potter et al., 1998). The possibility exists that the inhibition of calpain in these cells stabilizes the existing interactions of actin with other structural components, including spectrin, reducing the dynamic properties of actin. In this context it is interesting to note that spectrin has been shown to fragment and crosslink actin filaments *in vitro* (Stromqvist, 1987), suggesting that the proteolysis of spectrin is capable of changing actin dynamics.

The activation of additional enzymes, such as  $\text{Ca}^{2+}$ -dependent actin modulators (Welch et al., 1997) and kinases/phosphatases (Goldberg and Wu, 1995; Lautermilch and Spitzer, 2000), may be necessary for the formation of the GC. In addition, direct destabilization of the cytoskeleton by calcium ions may take place (Weisenberg and Deery, 1981). The convergence of the various pathways may be necessary for the successful transformation of the axonal structure into that of a GC. It is notable that phosphorylation can modulate calpain activity (Greenwood et al., 1993; Salamino et al., 1997), illustrating the potential complexity of the modulation of GC initiation. The

existence of parallel enzymatic processes can also explain our results showing that deferral of calpain activity compromises GC formation. The temporary inhibition of calpain may dissociate calpain from the effect of other factors (enzymes or elevated  $[Ca^{2+}]_i$  levels) necessary for the successful initiation of the transformation process. Another possible explanation stems from the observation that calpain is translocated to the membrane during its activation (Kawasaki and Kawashima, 1996). At this location, calpain is ideally located to proteolyze spectrin. When the  $Ca^{2+}$  levels decline, calpain may change its location, distancing it from its intended substrates, so that upon its deferred activation by the removal of calpeptin, it no longer effectively affects spectrin distribution.

### Calpain's Role in the Extension of the GC's Lamellipodia

We found that addition of calpeptin after the termination of the critical period in which spectrin is proteolyzed does not stop the emergence of the GC's lamellipodium, but does slow down its extension rate (Fig. 6). Calpeptin also inhibits the splitting of the lamellipodium into individual neurites when added at later stages. These observations demonstrate that calpain activity participates in the process of GC extension and in its restructuring. Even though we could not measure calpain activity at advanced times, calpain may maintain a low level activity for extended periods after its activation (Faddis et al., 1997), or it may be constitutively active at low levels under control conditions (Castejon et al., 1999), exerting long-term control on the rate of the extension of the GC's lamellipodia.

Whether processing of spectrin is important during the extension of the GC's lamellipodium could not be determined, because spectrin levels were minimal at the transected tip for long periods of time after axotomy [Fig. 3(C)]. Other cytoskeletal proteins, such as microtubule associated proteins, neurofilaments and NCAM, have been reported to be sensitive to proteolysis by calpain (Chan and Mattson, 1999), and actin dynamics have been shown to be affected by calpain activity (Potter et al., 1998). All of this may prove to be relevant to the effect of calpeptin on lamellipodial extension.

Our results suggest that the process of GC formation that takes place after either axotomy or the local elevation of the  $[Ca^{2+}]_i$  in intact axons is composed of at least two stages: triggering and extension. The first stage involves the transformation of the well-differentiated axonal structure into that of a GC. As an indispensable part of this process, the axon's mem-

brane skeleton is locally remodeled by calpain. Calpain proteolyzes the submembrane spectrin network and probably additional substrates, finally leading to the local polymerization of actin filaments, a central feature of GC structure. This defines the location of GC formation and sets the extension stage into motion. Because the blockage of proteolytic activity by preapplication of calpeptin totally blocks GC formation, we believe that the activation of calpain is one of the critical initial processes of the cascade of events that culminates in the formation of the GC. After GC formation has been initiated, calpain activity is no longer absolutely necessary for the extension of the GC's lamellipodia, as evidenced by the continuation of lamellipodial extension in the presence of calpeptin. Nevertheless, calpain activity modulates this process, so that calpeptin slows down the rate of expansion of the GC and affects its subsequent morphology.

We would like to thank Drs. Ruth Oren and Nili Iluz for their assistance in the completion of this study. D.G. received a fellowship from the Clore Foundation. M.E.S. is the Levi DeVialli Prof. in neurobiology.

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