Growth Cone Collapse and Inhibition of Neurite Growth by *Botulinum* Neurotoxin C1: A t-SNARE Is Involved in Axonal Growth

Michihiro Igarashi,* Shunji Kozaki,‡ Susumu Terakawa,§ Shigeru Kawano,‖ Chizuka Ide,‖ and Yoshiaki Komiya*

*Department of Molecular and Cellular Neurobiology, Gunma University School of Medicine, Maebashi, Gunma 371, Japan; ‡Department of Veterinary Science, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 593, Japan; §Photon Medical Research Center, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-31, Japan; and ‖Department of Anatomy, Faculty of Medicine, University of Kyoto, Sakyo-ku, Kyoto 606-01, Japan

Abstract. The growth cone is responsible for axonal growth, where membrane expansion is most likely to occur. Several recent reports have suggested that presynaptic proteins are involved in this process; however, the molecular mechanism details are unclear. We suggest that by cleaving a presynaptic protein syntaxin, which is essential in targeting synaptic vesicles as a target SNAP receptor (t-SNARE), neurotoxin C1 of *Clostridium botulinum* causes growth cone collapse and inhibits axonal growth. Video-enhanced microscopic studies showed (*a*) that neurotoxin C1 selectively blocked the activity of the central domain (the vesicle-rich region) at the initial stage, but not the lamellipodia in the growth cone; and (*b*) that large vacuole formation occurred probably through the fusion of smaller vesicles from the central domain to the most distal segments of the neurite. The total surface area of the accumulated vacuoles could explain the membrane expansion of normal neurite growth. The gradual disappearance of the surface labeling by FITC-WGA on the normal growth cone, suggesting membrane addition, was inhibited by neurotoxin C1. The experiments using the peptides derived from syntaxin, essential for interaction with VAMP or α-SNAP, supported the results using neurotoxin C1. Our results demonstrate that syntaxin is involved in axonal growth and indicate that syntaxin may participate directly in the membrane expansion that occurs in the central domain of the growth cone, probably through association with VAMP and SNAPs, in a SNARE-like way.

THE nerve growth cone is a special structure at the leading edge of the extending axon and is responsible for axonal guidance and elongation. The growth cone consists of two distinct domains: the peripheral (P-)[1] and the central (C-) domains. P-domain, a microfilament-rich region including filopodia and lamellipodia, is responsible for motility (Dailey and Bridgman, 1993); C-domain, a vesicle-rich region (Forscher et al., 1987; Dailey and Bridgman, 1993), is the site where membrane expansion for axonal growth is suggested to occur (Goldberg and Burmeister, 1986; Forscher et al., 1987; Pfenninger and Friedman, 1993). The growth cone alters its shape between active (advancing) and inactive (ceasing) forms in response to an appropriate (and inappropriate) stimulus for axonal growth (Kater and Mills, 1991; Schwab et al., 1993). An active growth cone is large with several filopodia, while an inactive one, known as a collapsed growth cone, is very small with few or no filopodia. Thus, selective blocking of C- or P-domain activity probably induces growth cone collapse. If any functional blocker specific to a component in the growth cone causes growth cone collapse, this would be strong evidence that the component is essential to the growth cone functions of either C- or P-domain (e.g., Fan et al., 1993). Precise experiments using blockers (after excluding the possibility that such blockers affect the general health of the neurons) will provide valuable information about the physiological activity of growth cones.

The molecular mechanisms of membrane expansion in the growth cone are not presently understood. The most likely explanation for this phenomenon is that the vesicles in the C-domain are added to the growth cone plasma-

Address correspondence to Michihiro Igarashi, Department of Molecular and Cellular Neurobiology, Gunma University School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371, Japan. Tel: (81) 272-20-7111 ext. 7952. Fax: (81) 272-20-7955. e-mail: bunshiby@news.sb.gunma-u.ac.jp

Portions of this work have appeared in abstract form (1995. *Cell Struct. Funct.* 20[Suppl.]:566*a*).

1. *Abbreviations used in this paper*: C-domain, central domain; CNS, central nervous system; DPD, 2,2′-dipyridyl; DRG, dorsal root ganglion; P-domain, peripheral domain; SLO, streptolysin O; SNARE, SNAP receptor; VEC-DIC, video-enhanced contrast–differential interference contrast.

lemma through exocytosis (Lockerbie et al., 1991; Pfenninger and Friedman, 1993; De Camilli, 1993), although controversial views have been raised (Dailey and Bridgman, 1991; Popov et al., 1993). A molecular model for vesicular targeting in exocytosis was recently proposed and referred to as the SNAP receptor (SNARE) hypothesis (Söllner et al., 1993; Rothman, 1994). Although there is a vesicular targeting mechanism independent of the SNARE mechanism (Ikonen et al., 1995), the SNARE hypothesis is presently applicable to other vesicular trafficking events as well as neurotransmitter release. The hypothesis indicates that vesicular targeting depends upon the protein complex formation between proteins on vesicular membrane, known as vesicular SNARE (v-SNARE), and proteins on the target membrane, known as target SNARE (t-SNARE), and that α/β/γ-SNAP recruitment and sequential dissociation causes fusion of the predocked vesicles. Three proteins have been identified as the SNARE components in the mature presynaptic terminal: the vesicular membrane protein synaptobrevin/VAMP is a v-SNARE, and both SNAP-25 and syntaxin/HPC-1 in the presynaptic plasma membrane are t-SNAREs (Söllner et al., 1993). Based on this information, an axonal growth hypothesis has emerged, suggesting that the SNARE mechanism works for membrane expansion occurring in the growth cone (De Camilli, 1993; Catsicas et al., 1994; Bark and Wilson, 1994; Futerman and Banker, 1996). Since inhibition of SNAP-25 expression by antisense oligonucleotides prevents neurite elongation (Osen-Sand et al., 1993), this hypothesis bears examination. We have not yet investigated whether the SNARE mechanism works directly in the growth cone for axonal growth. Thus, for us to demonstrate this hypothesis, many problems still remain to be solved.

Clostridial neurotoxins are useful in understanding membrane trafficking events concerning the SNARE mechanism because they inhibit neural transmission through each metalloprotease activity specific to each SNARE component (Niemann et al., 1994; Montecucco and Schiavo, 1994). Among them, neurotoxin C1 of *Clostridium botulinum* cleaves syntaxin (Blasi et al., 1993; Schiavo et al., 1995), and as a result, syntaxin is released from the plasmalemma, and the neurotransmitter release is blocked (Blasi et al., 1993; Mochida et al., 1995). Therefore, neurotoxin C1 can deplete membrane-bound syntaxin in situ.

In this study, we investigated whether the SNARE hypothesis can be applied to axonal growth and whether syntaxin is necessary to growth cone functions by assaying growth cone collapse–inducing activity of neurotoxin C1 (Blasi et al., 1993). A video-enhanced microscopic study revealed that neurotoxin C1 selectively blocked the activity of C-domain, but not P-domain, and caused extremely large vesicle formation, probably through fusion among smaller vesicles. The syntaxin-derived peptides with amino acids critical for interaction with VAMP or α-SNAP caused morphological changes similar to neurotoxin C1, suggesting that syntaxin acts in the growth cone through a SNARE mechanism. These observations provide unique evidence that membrane expansion for axonal growth occurs through the exocytotic behavior of the vesicles in the growth cone's C-domain in a SNARE-like way and involves syntaxin.

Materials and Methods

Cell Culture and Collapse Assay

Explants of chick dorsal root ganglion (DRG) and retina at embryonic day 7 (E7) were cultured on laminin-coated slide chambers as previously described (Igarashi et al., 1993). Neurotoxin C1 purified from *C. botulinum* type C (Kurazono et al., 1985) was added to the culture medium and incubated at 37°C at a final concentration of 200 nM. After 120 min, the cultured explant was fixed, and all of the growth cones were scored (80–150 per explant), followed by the criteria previously described (Igarashi et al., 1993; Strittmatter et al., 1994) with slight modification: the growth cone with a maximal diameter <1.5 times that of the distal neuritic shaft was considered to be collapsed. In the time frame experiments, the incubation time with neurotoxin C1 was changed to 30, 60, or 90 min. In some experiments, 2 mM of 2,2'-dipyridyl (DPD) dissolved in DMSO, or DMSO alone, was added to the culture medium 30 min before the addition of neurotoxin C1. For heat treatment, neurotoxin C1 was heated at 80°C for 20 min and then added. For immunoblotting, we removed 50 DRGs and incubated with 200 nM neurotoxin C1 for 120 min, and then analyzed the DRG proteins using antibodies against syntaxin or VAMP (gifts of M. Takahashi, Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo, Japan). To examine the recovery of the toxin-induced collapse, we removed the medium containing neurotoxin C1 after 1-h incubation and rinsed the explant with prewarmed fresh medium three times. We cultured the explant again in the medium containing 0.5 mM DPD. To investigate the effect of neurotoxin C1 on neurite outgrowth, we cultured the dissociated DRG neurons in the presence of 200 nM neurotoxin C1. After 8 h, the cultured neurons were fixed, and the number of neurons with neurites whose length was > 20 μm was counted.

Video-enhanced Microscopic Observation

We cultured E7 chick DRG explants on the laminin-coated glass-bottom culture dish (MatTek Corp., Ashland, MA). A video microscopy analysis using video-enhanced contrast–differential interference contrast (VEC-DIC) was performed (Mannivannan and Terakawa, 1994). We examined the growth cones under an inverted Nomarski microscope equipped with a ×100 DIC objective lens and a ×2.5 insertion lens (Axiovert 35; Carl Zeiss Inc., Thornwood, NY). We used petroleum jelly to fix the explant-plated coverslip to a square hole made in the center of a plastic slide. A coverglass was plated onto the culture when we added PBS or neurotoxin C1. The temperature was held at 36°C. We performed the morphometrical analysis of the data from video images using a Micro computer imaging device (MCID) image analyzer (Imaging Research Inc., St. Catherines, Ontario, Canada).

Immunohistochemistry and Electron Microscopy

For immunohistochemistry, we fixed the cultured DRG neurons by 4% paraformaldehyde and permeabilized them with 0.25% Triton X-100 for 5 min. We incubated the samples with an mAb against syntaxin 1 (Yoshida et al., 1992) (1:250; gift of M. Takahashi) at 37°C for 120 min, and then incubated them with FITC secondary antibody. The culture was also double labeled with YL1/2 mAb (1:50) (Fan et al., 1993), specific to tyrosinated α-tubulin to visualize microtubules, and with rhodamine-phalloidin to visualize F-actin before and after incubation with neurotoxin C1. To label the growth cone, we added 2 μg/ml of FITC-WGA (Seikagaku Kogyo Co., Tokyo, Japan) to the medium and incubated it for 10 min. We then removed the medium, rinsed the explant with prewarmed fresh medium three times and cultured the explant again in the fresh medium. In some experiments, neurotoxin C1 (200 nM) was added to the medium for 1 h after FITC-WGA labeling, and the medium containing the toxin was exchanged for the fresh medium. At a predetermined time, we fixed the explant and scored the number of the total growth cones and that of the FITC-labeled ones. We determined the labeling of the proximal portion of the neurite by scoring the number of the labeled neurites 25 μm from the margin of the DRG explants. For electron micrographs, we incubated chick DRG growth cones with 200 nM neurotoxin C1 for 120 min, fixed them with 1.5% glutaraldehyde–2.5% paraformaldehyde, and then embedded and thin sectioned them.

Introduction of Peptides to the Neurons and the Growth Cones

DRG explants from E17 rats were cultured for 1 d. We incubated the explants with streptolysin O (SLO) (Sigma Chemical Co., St. Louis, MO) as described by Galli et al. (1994) with slight modification: a rat DRG explant was incubated with 2.5 U/ml of SLO (final concentration) for 15 min. After incubation, we rinsed the explant with prewarmed medium. Each peptide was then added to the culture medium at a final concentration of 10^{-4} M, and incubated for 1 h. We added PBS as the control. SLO alone caused no significant change of the growth cone collapse percentage up to 25 U/ml for 15-min incubation. The peptide conjugation with FITC was done as described by Bloch-Gallego et al. (1993) with a slight modification: we incubated FITC peptide with the explant for 10 min after permeabilization treatment. The trituration method for introducing each peptide into the dissociated neurons was done as described previously (Bloch-Gallego et al., 1993; Strittmatter et al., 1994), and we scored neurons as before. Peptide 1 (AVDYVERAVS) and peptide 2 (VESQGEMIDR) are decapeptides derived from positions 240 to 249 and 223 to 232 of the rat syntaxin 1a. Peptide 1 contains one of the critical portions for its association with VAMP (Kee et al., 1995); peptide 2 is involved in the interaction both with VAMP and with α-SNAP (Kee et al., 1995). Peptide 1m (*VV*DA*E*RAVS) and peptide 2m (*AES*A*G*EMIDR) are mutated peptides incapable of peptide–protein interactions (Kee et al., 1995; the underlining indicates the substituted amino acids).

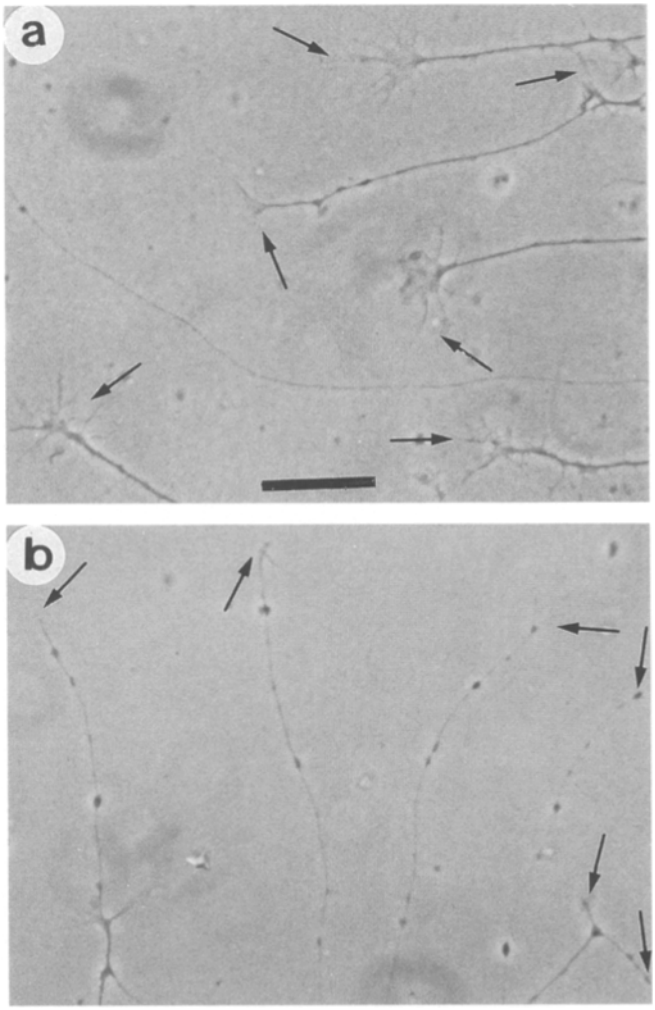

Figure 1. Phase-contrast micrographs of chick DRG growth cones in the absence (*a*) or the presence (*b*) of 200 nM *botulinum* neurotoxin C1 (120 min). Arrows indicate the growth cones. Note the distinct shapes between *a* and *b*: the growth cones in *a* and *b* correspond to the normal fan-shaped growth cones and the collapsed growth cones with reduced areas. Bar, 20 μm.

Results

Neurotoxin C1 Causes Growth Cone Collapse by Its Proteolytic Activity against Syntaxin

Neurotoxin C1 induced a shape change of growth cones from the normal fan-shaped view (Fig. 1 *a*) to a clublike view with reduced area (Fig. 1 *b*). The toxin caused severe growth cone collapse of chick DRG (Fig. 1), chick retinal (Fig. 2 *a*), and rat DRG neurons (>80%; data not shown).

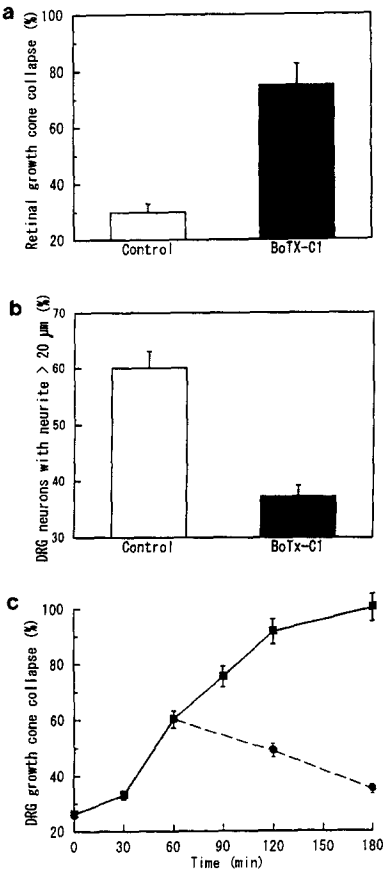

Figure 2. (*a*) Growth cone collapse of chick retinal neurons induced by neurotoxin C1 (*outlined bar*, control; *filled bar*, C1-treated neurotoxin). Each explant was incubated with 200 nM neurotoxin C1 for 120 min. This data is shown as mean ± SEM (six independent experiments). (*b*) The inhibitory effect of neurotoxin C1 to neurite outgrowth of chick DRG neurons. The dissociated DRG neurons were cultured and incubated with 200 nM neurotoxin C1 for 8 h, and then fixed and scored. The data are shown as mean ± SEM (four independent experiments). (*c*) The time course of neurotoxin C1 (200 nM)–induced DRG growth cone collapse (*squares*). To examine the recovery, the toxin-containing medium was removed after 1-h incubation and exchanged for the medium containing 0.5 mM DPD (a metalloprotease inhibitor); the culture was then continued. The percentage of the collapsed growth cone after medium change is represented as circles. The data are shown as mean ± SEM (four to eight independent experiments).

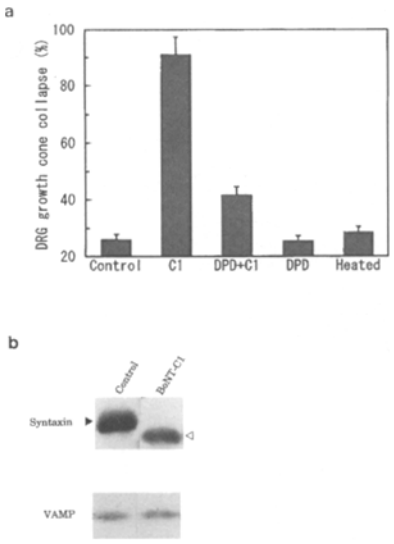

Figure 3. The growth cone collapse induced by neurotoxin C1 is through its protease activity. (*a*) Blockade of neurotoxin C1-induced DRG growth cone collapse by DPD or heat treatment of C1. The data are shown as mean ± SEM (six independent experiments). (*b*) The immunoblotting of chick DRG proteins without or with neurotoxin C1 treatment. Note that the intact syntaxin (*filled arrowhead*) is remarkably reduced by the neurotoxin C1 treatment and that the slightly smaller fragment (*empty arrowhead*) is newly generated; VAMP is not changed by the same treatment.

Neurotoxin C1 inhibited the neurite outgrowth of dissociated DRG neurons (Fig. 2 *b*). This was not due to neuronal cell death since >90% of the neurons excluded trypan blue in the presence of neurotoxin C1. The delayed neurite growth was due to the inhibition of neurite growth and not to a delayed initiation of outgrowth; the addition of neurotoxin C1 to the DRG neurons with neurites inhibited further neurite growth (data not shown). The growth cone collapse induced by neurotoxin C1 was blocked by DPD, a hydrophobic inhibitor of metalloproteases (Fig. 3 *a*). The amount of intact syntaxin was reduced, and a fragment slightly smaller than the intact syntaxin was generated after we treated chick DRG with neurotoxin C1 (Fig. 3 *b*). Thus, we concluded that the growth cone collapse by neurotoxin C1 is due to its proteolytic activity against syntaxin. The amino acid sequences revealed that chicken syntaxin 1a and 1b also have a site susceptible to neurotoxin C1, at a position similar to rat syntaxin 1a/b (Schiavo et al., 1995; Akagawa, K., personal communication). Neurotoxin C1 did not appear to cause significant growth cone collapse until 30 min after the application; however, the collapsed growth cone percentage gradually increased and reached ~100% after 2 h (Fig. 2 *c*). This action time lag is reasonable, since neurotoxin C1 must be taken into cells and activated by intracellular proteases (Niemann et al., 1994; Montecucco and Schiavo, 1994). To examine the growth cone collapse recovery, we removed the medium containing neurotoxin C1 and added the new medium containing DPD. As a result, the collapsed growth cone percentage gradually decreased (Fig. 2 *c*), indicating recovery from the collapse.

Neurotoxin C1 Inhibits the Activity of C-Domain in the Growth Cone and Induces Accumulation of Large Vacuoles

Video-microscopic observation of growth cone collapse by neurotoxin C1 revealed two important points: (*a*) C-domain, where various vesicles are enriched normally (Dailey and Bridgman, 1993), has gradually shrunk after 40 min (Figs. 4, *a–f*, and 8, *a* and *b*); activity of P-domain, i.e., filopodial or lamellipodial extension, was relatively preserved until 80 min after neurotoxin application (Fig. 4, *a–f*); and (*b*) smaller vacuoles (<0.3 μm^2) probably necessary for membrane expansion (Lockerbie et al., 1991; Pfenninger and Friedman, 1993; Dailey and Bridgman, 1993) were observed in normal growth cones (Fig. 4, *g–i*), and unusually large vacuoles (>0.5 μm^2) had formed and accumulated from C-domain in the growth cone to the distal axon by C1 treatment (Fig. 4).

The outgrowth rate was constant until 30 min after we administrated neurotoxin C1; after 30 min it began to fluctuate, and we observed a transient retraction of the growth cone (negative outgrowth rate). After 80 min the neurite outgrowth totally ceased (Figs. 4 and 5 *c*).

We clearly observed these large vacuoles not only in VEC-DIC views but also in electron micrographs (Fig. 6) and in phase-contrast micrographs (data not shown). Some of the larger vacuoles were formed by the fusion of smaller vacuoles (Fig. 7 *a*).

We calculated the ratio to examine whether the surface area of the vacuoles accumulated by C1 treatment corresponded to the expanded membrane area in the normal neurite growth (Fig. 7 *b*). The ratio gradually increased and finally approached 1.0 (Fig. 7 *b*) when the neurite growth ceased (Fig. 8 *c*). In normal neurite growth, the ratios were within the range of 0.03–0.15 (six independent experiments).

Cytoskeletal Components Are Preserved by the Neurotoxin C1 Treatment

The syntaxin immunoreactivity in the growth cone was distributed mainly in C-domain, near the distal portion of the axon (Fig. 8, *a* and *c*); the filopodia were not intensely stained. This data suggests that syntaxin is normally localized in the vesicle-rich region of the growth cone. In a growth cone collapsed by neurotoxin C1, C-domain and the most distal portion of the axonal shaft were still faintly stained; however, P-domain was barely stained by the anti-syntaxin mAb (Fig. 8, *b* and *d*). The localization of microtubules in growth cones did not change before or after the neurotoxin C1 treatment (Fig. 8, *f* and *h*). Although reduction of the C-domain area had begun (60 min), filopodial F-actin was relatively preserved in comparison with the control (Fig. 8, *e* and *g*). The distances between the distal ends of F-actin and that of microtubules are 7.9 ± 0.9 μm (control, $n = 15$) and 7.5 ± 0.9 μm (C1-treated, $n = 15$), indicating no statistically significant difference. We concluded that the growth cone collapse induced by neuro-

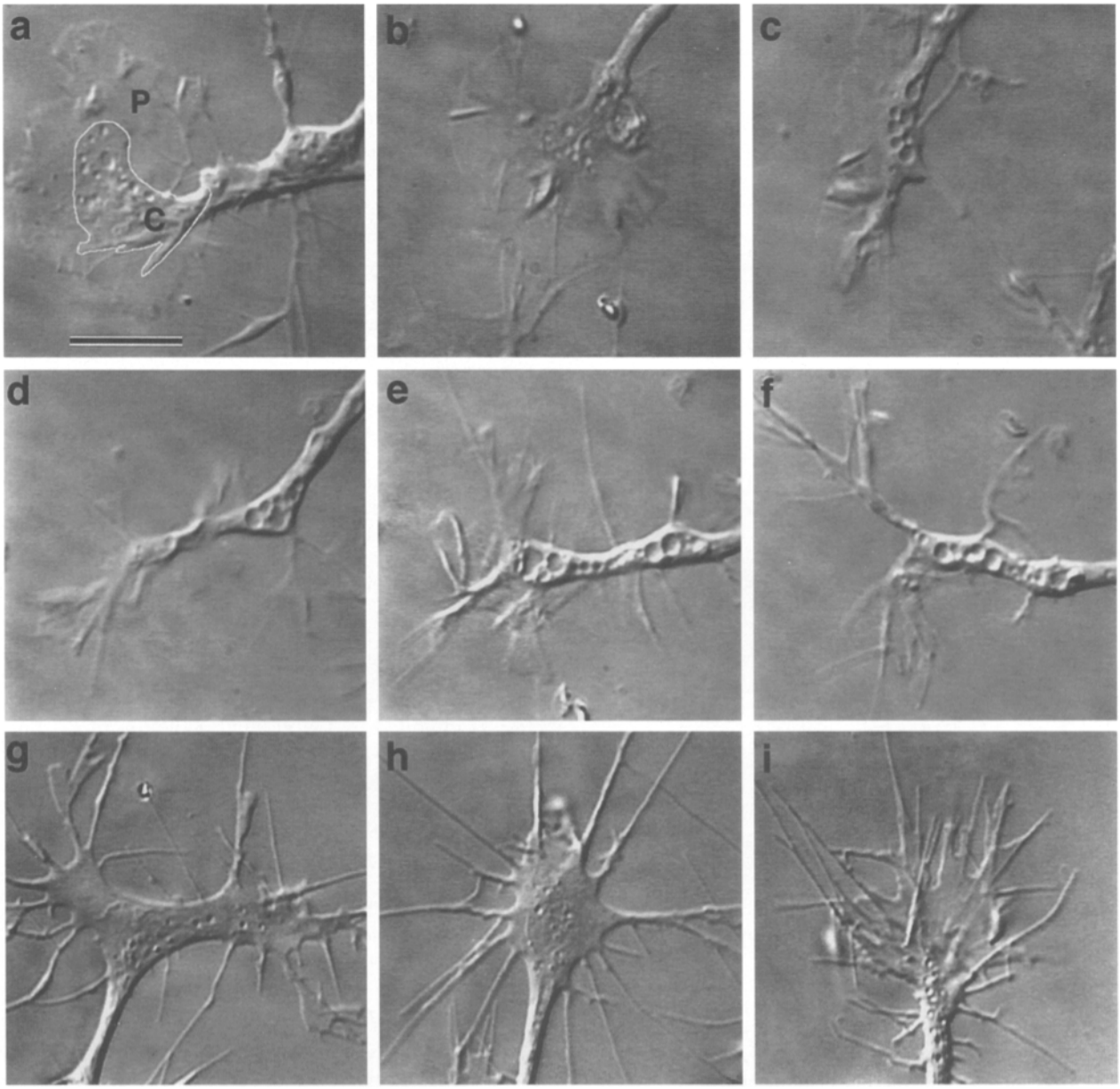

Figure 4. Real-time process of growth cone collapse by neurotoxin C1 treatment (*a–f*) using VEC-DIC. The analysis of growth cone collapse induced by *botulinum* neurotoxin C1. The views of a growth cone at every 20 min, after addition of 200 nM neurotoxin C1 into the culture medium. Bar, 10 μm. Note that marked shrinkage of the C-domain and large vacuoles are observed after 40 min or more. The boundary between the C-domain (*C*) and the P-domain (*P*) is shown in *a*. The domain boundary is determined by the definition described by Dailey and Bridgman (1993). The views of a growth cone at 0, 40, or 80 min (*g–i*) after addition of PBS as control.

toxin C1 was not due to filopodial or lamellipodial retraction, but rather due to inhibition of maintaining C-domain.

Loss of FITC-WGA Labeling in the Growth Cone Is Selectively Delayed by Neurotoxin C1

The labeling of growth cone by WGA is eventually lost, and new membrane insertion may occur there (Pfenninger and Maylié-Pfenninger, 1981). If membrane expansion selectively occurs in the growth cone, then neurotoxin C1 should affect the disappearance of labeled WGA there. Using FITC-WGA, we labeled all of the growth cones immediately (Fig. 9). In the control experiment, the growth cone labeling gradually disappeared over 2 h; all of the proximal neurites were still labeled at that time (Fig. 9). Significantly, more growth cones in the C1-treated group were still labeled than in the control (Fig. 9), indicating that C1 toxin delays the disappearance of the growth cone labeling. The proximal neurite labeling was not affected by C1 treatment (Fig. 9).

Peptides Derived from Syntaxin and Critical to Interaction with VAMP or α-SNAP Cause Growth Cone Collapse and Inhibition of Neurite Outgrowth

We performed growth cone collapse assays, using the pep-

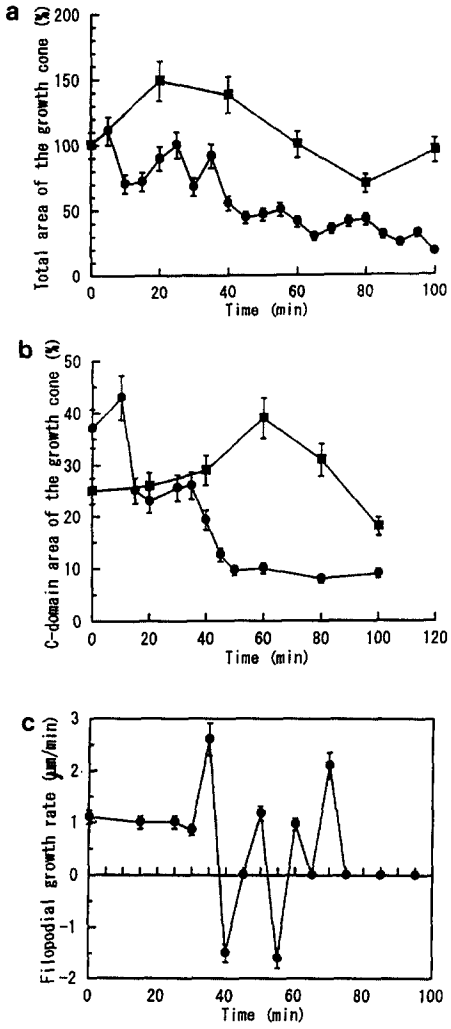

Figure 5. The analysis of data obtained from the VEC-DIC views on a DRG growth cone collapse by neurotoxin C1. (*a*) Time course of the whole area change in the normal (*squares*) and in the neurotoxin C1-treated growth cones (*circles*). (*b*) Time course of the area change of central (C-) domain in the normal (*squares*) and in the neurotoxin C1-treated growth cones (*circles*). (*c*) The outgrowth rate of neurites in the presence of neurotoxin C1. The negative rate values represent retraction. The filopodia of the normal growth cone grew at an approximately constant rate of 1.5 μm/s (average). The morphometric analyses for each real-time view were done using the MCID system. Data on each graph are shown as the mean of three independent experiments. The data are shown as mean ± SEM (three independent experiments).

tides derived from syntaxin, to examine whether syntaxin involvement in growth cone functions is through the interaction between syntaxin and VAMP. We confirmed by fluorescent labeling of the peptides used here that all peptides entered the growth cones by SLO treatment and the neurons by trituration, and that the entrance efficacy of each peptide was not significantly different (data not shown). The peptide 1 (amino acid numbers from positions 240 to 249 of rat syntaxin 1a) contains a critical portion for its association with VAMP (Kee et al., 1995). This peptide caused growth cone collapse of rat DRG neurons (Fig. 10 *a*). The peptide having amino acids at positions 240 and 244 of peptide 1, which are substituted to others (peptide 1m), loses its association with VAMP (Kee et al., 1995). Peptide 1m did not cause significant growth cone collapse (Fig. 10 *a*). Peptide 2 (amino acid numbers from 223 to 232 of the syntaxin 1a), involved in interaction with both VAMP and α-SNAP (Kee et al., 1995), induced more growth cone collapse than peptide 1 ($P < 0.05$, *t* test); its weak substitute, peptide 2m, was ineffective in inducing growth cone collapse (Fig. 10 *a*). 10^{-6} M of the peptide 1 with SLO also caused significant collapse (45.0 ± 4.5%, four independent experiments; $P < 0.05$, vs the control; *t* test). Without SLO permeabilization, however, peptides 1 and 2 would not have affected the growth cone morphology (Fig. 10 *a*).

When we used trituration as another permeabilization method (Bloch-Gallego et al., 1993), peptide 1 inhibited neurite outgrowth, and peptide 2 had a stronger effect than peptide 1 ($P < 0.05$, *t* test), while neither 1m nor 2m demonstrated any effect (Fig. 10 *b*). These results suggest that the interaction between syntaxin and VAMP or α-SNAP is necessary for growth cone activity, and that inhibition of these interactions causes growth cone collapse and inhibition of axonal growth.

Discussion

Our results clearly show that the normal growth cone function is inhibited by the gradual depletion of existing membrane-bound syntaxin by *botulinum* neurotoxin C1, probably by blocking membrane expansion there. In addition, the syntaxin-derived peptides containing sites critical to the formation of SNARE complex caused growth cone collapse and inhibited neurite outgrowth in a similar fashion to neurotoxin C1. Therefore, we concluded that syntaxin is essential to axonal growth. Possible mechanisms involving syntaxin for new membrane addition in the growth cone are discussed below.

Neurotoxin C1 First Acts at the Growth Cone

Using trypan blue, we verified that the neurons treated with neurotoxin C1 were shown alive. Under our usual experimental conditions (200 nM of neurotoxin C1 for 2-h incubation), DRG neurite degeneration rarely occurred. Long-term incubation of DRG explants (>4 h) induced neurite degeneration with abnormal varicosities along the neurite, but growth cone collapse always preceded the neurite degeneration; a neurite with varicosities always had a collapsed growth cone, but a neurite with a collapsed growth cone did not necessarily have varicosities along it. This is strong evidence that neurotoxin C1 initially acts on the growth cone, and that growth cone collapse by neurotoxin C1 is due to neuronal cell death or axonal degeneration. Similar results were observed in a central nervous system (CNS) neuron (Kurokawa et al., 1987; Osen-Sand et al., 1996; Catsicas, S., personal communication). Since removal of the neurotoxin and the presence of a membrane-permeable protease inhibitor completely inhibited new growth cone collapse (Fig. 2 *c*), this treatment does not, at this point, induce a fatal effect on the neurons. This partial recovery is probably due to the newly transported syntaxin from the cell body.

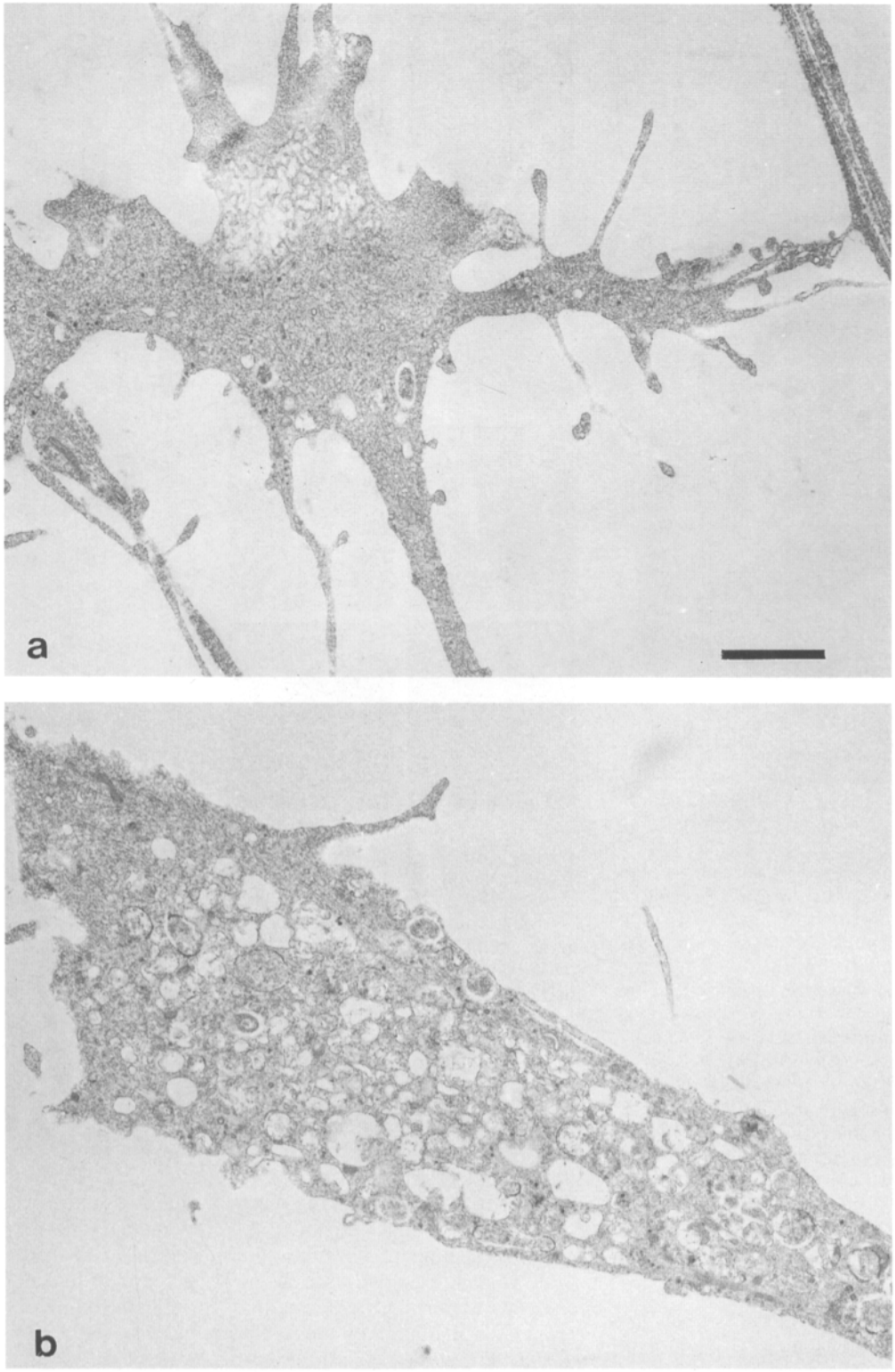

Figure 6. EM views of the growth cones before (*a*) and after (*b*) incubation with neurotoxin C1 of *C. botulinum*. Note that the growth cone in *b* has many vacuoles with the reduced C-domain and few and short filopodia. Bar, 2 μm.

Neurotoxin C1 Selectively Blocks the Activity of C-domain

The immunohistochemical results revealed that the first event induced by neurotoxin C1 does not involve a change in cytoskeletal organization (Figs. 5 and 8). This is a unique property of growth cone collapse, distinct from the collapse induced by collapsin that initiates F-actin depolymerization (Fan et al., 1993). Since filopodial activity continues when the growth cone collapses and C-domain begins shrinking (Fig. 4), we conclude that the growth cone collapse by neurotoxin C1 is due to C-domain dependence and P-domain independence at the initial stage.

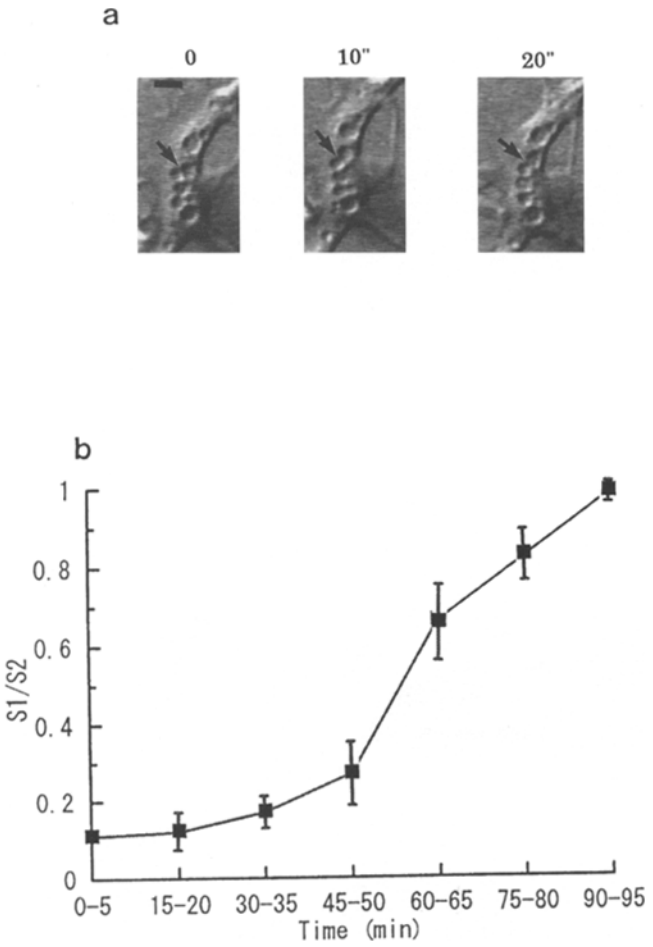

Figure 7. Formation of large vacuoles in the growth cone by neurotoxin C1 treatment. (*a*) Larger vacuole formation by fusion of smaller ones with each other. Arrows show the process of losing the wall between each vacuole. Note that the loss of the wall has been completed in 20 s. Bar, 0.5 μm. (*b*) The ratio of the increase in the surface area of large vacuoles by C1 treatment (S_1) to the expanded membrane area of the normal growing neurite (S_2) during 5-min intervals. The ratio gradually increased and finally approached 1.0. The data are shown as mean ± SEM (three independent experiments). The S_1 and the S_2 were calculated as follows: suppose that each vesicle is a sphere, and a neurite is a cylinder. The difference between the total surface areas of the vesicles was accumulated for 5 min (S_1). The membrane expansions for neurite growth occurring for 5 min under the normal conditions are calculated by the following formulas:

$S_1 = \Sigma\pi d_i^2 - \Sigma\pi d_j^2$

(d_j and d_i, diameter of each vesicle at a given time and at 5 min later)

$S_2 = (\pi D^2)/4 \times l$

(D, diameter of the distal neurite; l, elongation length of the neurite for 5 min in the normal explant).

Analysis of vesicular dynamics in the growth cone demonstrated that the membrane protrusion in P-domain is not necessary for the vesicular fusion occurring in C-domain (Dailey and Bridgman, 1993). Our results using neurotoxin C1 are consistent with this theory of segregation of the activities of both domains.

C-domain is the vesicle-rich region of the growth cone

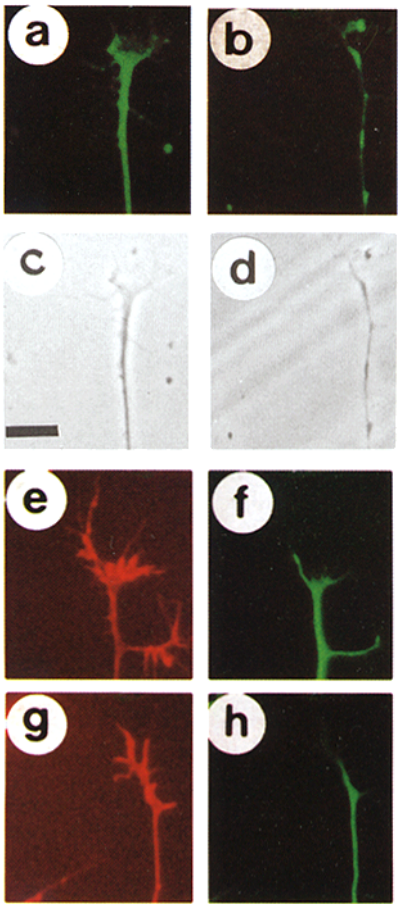

Figure 8. Typical views of immunohistochemical localization of syntaxin (*a* and *b*), F-actin (*e* and *g*), and tubulin (*f* and *h*), and in the absence (*a*, *e*, and *f*) or the presence (*b*, *g*, and *h*) of *botulinum* neurotoxin C1 (200 nM; 1-h incubation). Phase-contrast views corresponding to *a* and *b* are shown in *c* and *d*. In *e–h*, the growth cones were double labeled by rhodamine phalloidin for visualization of F-actin (*e* and *g*) and by YL1/2 mAb (rat) and FITC anti-rat antibody for visualization of tubulin (*f* and *h*). Bar, 10 μm.

(Goldberg and Burmeister, 1986; Forscher et al., 1987; Dailey and Bridgman, 1993), and these vesicles are necessary for membrane expansion for axonal growth (Pfenninger and Friedman, 1993). Thus, based on C-domain–dependent growth cone collapse by *botulinum* neurotoxin C1, we propose that syntaxin in the growth cone is directly involved in new membrane addition. Peptide 1 caused a large vacuole accumulation in the growth cone (data not shown), supporting the theory that the collapse by neurotoxin C1 is mediated by the inhibition of a SNARE mechanism.

The Implications of Membrane Expansion Occurring in the Growth Cone

There are several possible membrane dynamics of the growth cone: (*a*) new membrane addition (Pfenninger and Maylié-Pfenninger, 1981); (*b*) lateral membrane diffusion from the proximal portion of a neurite (Popov et al., 1993); and (*c*) simple exocytosis–endocytosis membrane recycling (Dailey and Bridgman, 1993). To examine which pos-

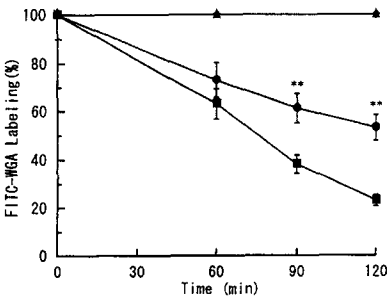

Figure 9. Time course of the remaining FITC-WGA labeling on the growth cones and the proximal portion of the neurite. Under normal conditions, the labeled growth cones (*squares*) and the labeled proximal neurites (*triangles*; at 25 μm distance from the explant margin) were scored. To examine the effect of neurotoxin C1, upon rinsing the culture after the labeling by FITC-WGA, the explant was incubated with neurotoxin C1 (200 nM) for 1 h. The culture was continued after removing the toxin-containing medium, and the labeled growth cones (*circles*) and the labeled proximal neurites (*diamonds*) were scored. The percentages of the labeled proximal portions of the neurites were ~100% even after 2 h, regardless of the toxin treatment. **, $P < 0.01$ (the C1-treated growth cone vs the normal one).

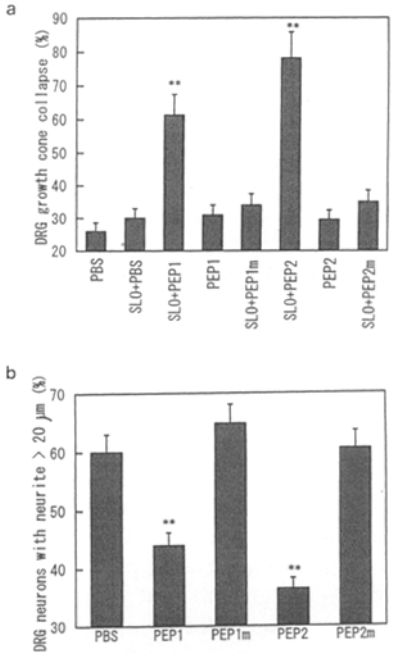

Figure 10. Growth cone collapse induced by syntaxin-derived peptides and their mutant ones. (*a*) The rat DRG growth cone collapse by the peptides after permeabilization using SLO. (*b*) The effect of these peptides on neurite outgrowth using the trituration method for introducing these peptides into the dissociated rat DRG neurons. The peptides used are as follows: peptide 1 (AVDYVERAVS, amino acid numbers from 240 to 249 of rat syntaxin 1a) (Kee et al., 1995), and peptide 1m (VVDAERAVS); peptide 2 (VESQGEMIDR; amino acid numbers from 223 to 232 of rat syntaxin 1a), and peptide 2m (AESAGEMIDR). The underline shows the amino acid substitution for the original ones. Data shown are the average and SEM of four independent experiments. **, $P < 0.01$ (vs the PBS-treated group).

sibility is most likely, we performed FITC-WGA labeling experiments with and without neurotoxin C1. FITC-WGA labeled all of the growth cone immediately, and the fluorescent intensity in the growth cone gradually decreased as the neurite grew, although its proximal neurite was still labeled (Fig. 9). In the presence of neurotoxin C1, we observed a delayed disappearance of FITC-WGA in the growth cone, but not at the proximal portion of the neurite (Fig. 9). Among the above three hypotheses, it is only the net membrane addition in the C-domain of the growth cone (Lockerbie et al., 1991; Pfenninger and Friedman, 1993; Craig et al., 1995; Dai and Sheetz, 1995) that explains the following three events without contradiction: (*a*) selective loss of the FITC-WGA on the normal growth cone surface; (*b*) its selective delay on the C1-treated growth cone; and (*c*) the reduction of the growth cone surface area. It is unlikely that the toxin blocks endocytosis rather than exocytosis; if so, the growth cone would have expanded with the C1 treatment. The maintained labeling in the proximal portion of the neurites strongly suggests that membrane addition did not occur in the proximal portion of the neurite, but rather in the growth cone. In addition, the labeling that shows delayed disappearance with neurotoxin C1 indicates that the membrane expansion process is dependent upon syntaxin.

The Introduction of Peptides Derived from Syntaxin into Growth Cones

We used the peptides derived from syntaxin to examine whether the interaction between syntaxin and VAMP or α-SNAP is involved in axonal growth. The domain of syntaxin that is responsible for the interaction with other proteins has been determined in detail (Kee et al., 1995). Based on this data, we designed peptides 1 and 2 to include one of the critical portions for its interaction with VAMP, and with both VAMP and α-SNAP (Kee et al., 1995). According to Kee et al. (1995), mutated peptides 1m and 2m, for which two positions of specific amino acids are substituted, lose the activity to interact with such proteins. These peptides were effectively introduced into growth cones and dissociated neurons using SLO permeabilization (Galli et al., 1994) or trituration (Bloch-Gallego et al., 1993) methods. Peptide 2 was stronger in both growth cone collapse and neurite outgrowth inhibition than peptide 1 (see Results and Fig. 10). Considering the parallelism between the biological activity to inhibit the growth cone and the proposed activity to interact with VAMP or with α-SNAP (Fig. 10), we concluded that the peptides interfere with the regulation of the SNARE complex in the growth cone.

The Mechanism of Axonal Growth That Involves Syntaxin as a t-SNARE

Syntaxin/HPC-1 (Inoue et al., 1992; Bennett et al., 1992; Yoshida et al., 1992) plays an important role in the Ca^{2+}-regulated exocytosis for transmitter release because syntaxin forms the SNARE complex together with VAMP and with SNAP-25 (Söllner et al., 1993). A recent paper reports that a *Drosophila* mutant without syntaxin 1a shows subtle axonal defects and partial defects of CNS condensation (Schulze et al., 1995), indicating that syn-

taxin is significant in axonal growth in many different animals. In our experiments, not only the DRG growth cone, a type of peripheral neurons, but also the retinal one, a type of CNS neurons, was susceptible to neurotoxin C1 (Figs. 1 and 2). This also suggests that syntaxin is involved in axonal growth in many different neurons.

Neurotoxin C1 cleaves intact syntaxin (35 kD) at a site of the Lys-Arg bond near its COOH-terminal region (Blasi et al., 1993; Schiavo et al., 1995) to generate a large 31-kD fragment and a small 4-kD one from syntaxin (cf. Fig. 3 *b*), and syntaxin is consequently released from the membrane (Blasi et al., 1993). The truncated syntaxin is still able to bind SNAP-25 and VAMP (Hayashi et al., 1994) even after proteolysis by neurotoxin C1. Therefore, the most important effect of neurotoxin C1 is that syntaxin cannot function as a t-SNARE (Rothman, 1994) because it is released from the membrane. Our results suggest that the intact membrane-anchored syntaxin is essential for normal growth cone functions and axonal growth, and that removing it from the plasma membrane causes perturbation of vesicular trafficking within the growth cone. Peptides 1 and 2, derived from syntaxin, competitively inhibit the SNARE or the SNARE–SNAP complex formation against endogenous syntaxin, consequently blocking the vesicular docking for the net membrane increase in the growth cone.

Since other in vitro substrates for neurotoxin C1 (syntaxin 2, 3, or 4) (Schiavo et al., 1995) are not expressed in the nervous system (Bennett et al., 1993), we concluded that the target of C1 is syntaxin 1a/b.

The Large Vacuole Formation in the Growth Cone Induced by Neurotoxin C1

The large vacuole formation in the growth cone is a result of the collapse caused by neurotoxin C1 (Figs. 4 and 6). This large vacuole is regarded as identical to the "reverse shadowcast vacuole" in the VEC-DIC view described previously by Dailey and Bridgman (1993). Using EM, we observed large vacuoles that accumulated in the C1-treated growth cone. Based on each vacuole size, we concluded that this vacuole is a "real" vacuole (Fig. 6) (Dailey and Bridgman, 1993). The extremely large reverse shadowcast vacuoles (up to 2 μm in diameter) exist in the normal growth cones, although they are uncommon (Dailey and Bridgman, 1993). Thus, large vacuole formation observed in our experiments (Fig. 4) is not attributed to simple degeneration. Some of these vacuoles are formed from the fusion of smaller ones (Fig. 7 *a*).

As shown in Fig. 7 *b*, the total surface area of the accumulated vacuoles was eventually almost equal to the expanded membrane surface area of the normally growing neurite. We concluded that such vacuoles should have been used for membrane expansion in the growth cone, but that C1 treatment prevented the vesicles from fusing to the plasmalemma of the growth cone, and they consequently fused to each other and formed large vacuoles.

Assuming that this SNARE mechanism functions in the normal growth cone, we examined the mechanism of large vacuole formation in a growth cone collapsed by neurotoxin C1, where vesicular docking to the plasmalemma is inhibited. Since SNAP-25 without palmitoylation, distributed in the cytosolic portion, could be bound to the vesicular membrane by palmitoylation (Hess et al., 1992), SNAP-25 may act as a SNARE on the vesicle. In this case, the truncated syntaxin, released from the plasmalemma by neurotoxin C1, could form the SNARE complex with VAMP and probably with SNAP-25 on the vesicle (Hayashi et al., 1994), enabling the vesicles to fuse and form the large vacuoles. Each membranous organelle unable to dock to plasmalemma might fuse with another, through the incomplete SNARE complex. This hypothesis fully explains our observation of the large vacuole formation (Figs. 4, 5 *c*, and 6 *a*). We also suggest that syntaxin natively present on vesicular membranes, as synaptic vesicles (Walch-Solimena et al., 1995), may contribute to vesicular fusion regulation. Alternatively, a vesicle unable to be fused to plasmalemma initiates fusion, followed by a default fusion activity independent of syntaxin. Small GTP-binding proteins belonging to the *rab* family may be involved in this default vesicle fusion (Armstrong, 1995). Another possibility is that an *N*-ethylmaleimide–sensitive factor (NSF)– and *rab*-independent mechanism works in the process (Ikonen et al., 1995). Regardless if this mechanism is valid, large vacuole formation in the growth cone is significantly difficult to achieve when the membrane expansion process involving syntaxin is operating normally.

Our results indicate the importance of the SNARE mechanism for membrane expansion in the growth cone. To clarify the molecular mechanism of axonal growth indicated by our results, regulation of the SNARE complex specific to the growth cone must be understood. An investigation focused on molecular characteristics of the SNARE complex in the growth cone is therefore necessary.

We thank T. Tsujihara for his help in taking electron micrographs, and K. Akagawa and S. Catsicas for providing information on syntaxin/HPC-1 and *botulinum* neurotoxin C1, including their unpublished results. We also thank Fuji Film Co., Ltd., for help with morphometrical analyses using the MCID system. We are grateful to Mr. Richard A. Cripe, Jr., for his critical reading of this manuscript and Mr. Y. Ishida for his skillful techniques with micrographs.

This work was supported in part by grants from the Ministry of Education, Culture, Sports, and Sciences in Japan to M. Igarashi (07279208, 07680837), S. Kozaki, S. Terakawa, C. Ide, and Y. Komiya (07264207, 07458204), and from the Naito Memorial Foundation to M. Igarashi.

Received for publication 25 September 1995 and in revised form 2 April 1996.

References

Armstrong, J. 1995. Is the Golgi complex? *Curr. Biol.* 5:980–981.

Bark, I.C., and M.C. Wilson. 1994. Regulated vesicular fusion in neurons: snapping together the details. *Proc. Natl. Acad. Sci. USA.* 91:4621–4624.

Bark, I.C., K.M. Hahn, A.E. Ryabinin, and M.C. Wilson. 1995. Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neural development. *Proc. Natl. Acad. Sci. USA.* 92:1510–1514.

Bennett, M.K., N. Calakos, and R.H. Scheller. 1992. Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science (Wash. DC).* 257:255–259.

Bennett, M.K., J.E. Garcia-Arrarás, L.A. Elferink, K. Peterson, A.M. Fleming, C.D. Hazuka, and R.H. Scheller. 1993. The syntaxin family of vesicular transport receptors. *Cell.* 74:863–873.

Blasi, J., E.R. Chapman, S. Yamasaki, T. Binz, H. Niemann, and R. Jahn. 1993. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4821–4828.

Bloch-Gallego, E., I. Le Doux, A.H. Joliot, M. Volovitch, C.E. Henderson, and A. Prochiantz. 1993. *Antennapedia* homeobox peptide enhances growth and branching of embryonic chicken motoneurons in vitro. *J. Cell Biol.* 120:485–492.

Catsicas, S., G. Grenningloh, and E.M. Pich. 1994. Nerve-terminal proteins: to

fuse to learn. *Trends Neurosci.* 17:368–373.
Craig, A.M., R.J. Wyborski, and G. Banker. 1995. Preferential addition of newly synthesized membrane protein at axonal growth cones. *Nature (Lond.).* 375:592–594.
Dai, J., and M.P. Sheetz. 1995. Axon membrane flows from the growth cone to the cell body. *Cell.* 83:693–701.
Dailey, M.E., and P.C. Bridgman. 1991. Structure and organization of membrane organelles along distal microtubule segments in growth cones. *J. Neurosci. Res.* 30:242–258.
Dailey, M.E., and P.C. Bridgman. 1993. Vacuole dynamics in growth cones: correlated EM and video observations. *J. Neurosci.* 13:3375–3393.
De Camilli, P. 1993. Exocytosis goes with a SNAP. *Nature (Lond.).* 364:387–388.
Fan, J., S.G. Mansfield, T. Redmond, P.R. Gordon-Weeks, and J.A. Raper. 1993. The organization of F-actin and microtubules in growth cones exposed to a brain-derived collapsing factor. *J. Cell Biol.* 121:867–886.
Forscher, P., L.K. Kaczmarek, J. Buchanan, and S.J. Smith. 1987. Cyclic AMP induces changes in distribution of organelles within growth cones of *Aplysia* bag cell neurons. *J. Neurosci.* 7:3600–3611.
Futerman, A.H., and G.A. Banker. 1996. The economics of neurite outgrowth— the addition of new membrane to growing axons. *Trends Neurosci.* 19:144–149.
Galli, T., T. Chilcote, O. Mundigl, T. Binz, H. Niemann, and P. De Camilli. 1994. Tetanus toxin-mediated cleavage of cellubrevin impairs exocytosis of transferrin receptor–containing vesicles in CHO cells. *J. Cell Biol.* 125:1015–1024.
Goldberg, D.J., and D.W. Burmeister. 1986. Stages in axon formation: observations of growth of *Aplysia* axons in culture using video-enhanced contrast–differential interference contrast microscopy. *J. Cell Biol.* 103:1921–1931.
Hayashi, T., S. Yamasaki, S. Nauenberg, T. Binz, and H. Niemann. 1994. Disassembly of the reconstituted synaptic vesicle membrane fusion complex *in vitro*. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:5051–5061.
Hess, D.T., T.M. Slater, M.C. Wilson, and J.H.P. Skene. 1992. The 25 kDa synaptosomal-associated protein SNAP-25 is the methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. *J. Neurosci.* 12:4634–4641.
Igarashi, M., S.M. Strittmatter, T. Vartanian, and M.C. Fishman. 1993. Mediation by G proteins that cause collapse of growth cones. *Science (Wash. DC).* 259:77–79.
Ikonen, E., M. Tagaya, O. Ullrich, C. Montecucco, and K. Simons. 1995. Differential requirements for NSF, SNAP, and rab proteins in apical and basolateral transport in MDCK cells. *Cell.* 81:571–580.
Inoue, A., K. Obata, and K. Akagawa. 1992. Cloning and sequence analysis of cDNA for a neuronal cell membrane antigen, HPC-1. *J. Biol. Chem.* 269: 10613–10619.
Kater, S.B., and L.R. Mills. 1991. Regulation of growth cone behavior by calcium. *J. Neurosci.* 11:891–899.
Kee, Y., R.C. Lin, S.-C. Hsu, and R.H. Scheller. 1995. Distinct domains of syntaxins are required for synaptic vesicle fusion complex formation and dissociation. *Neuron.* 14:991–998.
Kurazono, H., K. Shimozawa, M. Hosokawa, and G. Sakaguchi. 1985. Procedures for large-scale production and purification of *Clostridium botulinum* C1 toxin for preparation of toxoid. *FEMS Microbiol. Lett.* 30:47–51.
Kurokawa, Y., K. Oguma, N. Yokosawa, B. Syuto, R. Fukatsu, and I. Yamashita. 1987. Binding and cytotoxic effects of *Clostridium botulinum* type A, C_1 and E toxins in primary neuron cultures from foetal mouse brains. *J. Gen. Microbiol.* 133:2647–2657.
Lockerbie, R.O., V.E. Miller, and K.H. Pfenninger. 1991. Regulated plasmalemmal expansion in nerve growth cones. *J. Cell Biol.* 112:1215–1227.
Manivannan, S., and S. Terakawa. 1994. Rapid sprouting of filopodia in nerve terminals of chromaffin cells, PC12 cells, and dorsal root neurons induced by electrical stimulation. *J. Neurosci.* 14:5917–5928.
Mochida, S., H. Saisu, H. Kobayashi, and T. Abe. 1995. Impairment of syntaxin by botulinum neurotoxin C_1 or antibodies inhibits acetylcholine release but not Ca^{2+} channel activity. *Neuroscience.* 65:905–915.
Montecucco, C., and G. Schiavo. 1994. Mechanism of action of tetanus and botulinum neurotoxins. *Mol. Microbiol.* 13:1–8.
Niemann, H., J. Blasi, and R. Jahn. 1994. Molecular mechanisms of clostridial neurotoxins. *Trends Cell Biol.* 4:179–185.
Osen-Sand, A., M. Catsicas, J.K. Staple, K.A. Jones, G. Ayala, J. Knowles, G. Grenningloh, and S. Catsicas. 1993. Inhibition of axonal growth by SNAP-25 antisense oligonucleotide. *Nature (Lond.).* 364:445–448.
Osen-Sand, A., J.K. Staple, E. Naldi, G. Schiavo, O. Rossetto, A. Petitpierre, A. Malgaroli, C. Montecucco, and S. Catsicas. 1996. Common and distinct fusion-proteins in axonal growth and transmitter release. *J. Comp. Neurol.* In press.
Pfenninger, K.H., and L.B. Friedman. 1993. Sites of plasmalemmal expansion in growth cones. *Dev. Brain Res.* 71:181–192.
Pfenninger, K.H., and M.-F. Maylié-Pfenninger 1981. Lectin labeling of sprouting neurons. II. Relative movement and appearance of glycoconjugates during plasmalemmal expansion. *J. Cell Biol.* 89:547–559.
Popov, S., A. Brown, and M-m. Poo. 1993. Forward plasma membrane flow in growing nerve processes. *Science (Wash. DC).* 259:244–246.
Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature (Lond.).* 372:55–63.
Schiavo, G., C.C. Shone, M.K. Bennett, R.H. Scheller, and C. Montecucco. 1995. Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem.* 270:10566–10570.
Schulze, K.L., K. Broadie, M.S. Perin, and H.J. Bellen. 1995. Genetic and electrophysiological studies of Drosophila syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. *Cell.* 80:311–320.
Schwab, M.E., J.P. Kapfhammer, and C.E. Bandtlow. 1993. Inhibitors of neurite growth. *Annu. Rev. Neurosci.* 16:565–595.
Söllner, T., S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, and J.E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature (Lond.).* 362:318–324.
Strittmatter, S.M., M. Igarashi, and M.C. Fishman. 1994. GAP-43 amino terminal peptides modulate growth cone morphology and neurite outgrowth. *J. Neurosci.* 14:5503–5513.
Walch-Solimena, C., J. Blasi, L. Edelmann, E.R. Chapman, G. Fischer von Mollard, and R. Jahn. 1995. The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J. Cell Biol.* 128:637–645.
Yoshida, A., C. Oho, A. Omori, R. Kuwahara, T. Ito, and M. Takahashi. 1992. HPC-1 is associated with synaptotagmin and ω-conotoxin receptor. *J. Biol. Chem.* 267:24925–24928.