Botulinum Neurotoxin A Blocks Synaptic Vesicle Exocytosis but Not Endocytosis at the Nerve Terminal

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Abstract. The supply of synaptic vesicles in the nerve terminal is maintained by a temporally linked balance of exo- and endocytosis. Tetanus and botulinum neurotoxins block neurotransmitter release by the enzymatic cleavage of proteins identified as critical for synaptic vesicle exocytosis. We show here that botulinum neurotoxin A is unique in that the toxin-induced block in exocytosis does not arrest vesicle membrane endocytosis. In the murine spinal cord, cell cultures exposed to botulinum neurotoxin A, neither K^+-evoked neurotransmitter release nor synaptic currents can be detected, twice the ordinary number of synaptic vesicles are docked at the synaptic active zone, and its protein substrate is cleaved, which is similar to observations with tetanus and other botulinal neurotoxins. In marked contrast, K^+ depolarization, in the presence of Ca^{2+}, triggers the endocytosis of the vesicle membrane in botulinum neurotoxin A–blocked cultures as evidenced by FM1-43 staining of synaptic terminals and uptake of HRP into synaptic vesicles. These experiments are the first demonstration that botulinum neurotoxin A uncouples vesicle exo- from endocytosis, and provide evidence that Ca^{2+} is required for synaptic vesicle membrane retrieval.

Key words: botulinum toxin type A • presynaptic terminals • synaptic vesicles • endocytosis • exocytosis

The dynamic balance between exocytosis and endocytosis at the nerve terminal is critical for maintaining neuronal function during physiologic activity. The vesicular release of chemical neurotransmitters is an exocytic process that adds synaptic vesicle membrane to the plasmalemma of the nerve terminal (Ceccarelli et al., 1973; Heuser and Reese, 1973; Valtorta et al., 1988). Neuronal activity also involves a compensatory endocytosis such that the pool of synaptic vesicles is replenished and the size of the terminal is maintained. The internalization of HRP during a stimulation interval and electron microscopy during subsequent rest labels the membrane organelles and defines the temporal sequence of the synaptic vesicle retrieval process (Heuser and Reese, 1973).

That the amount of membrane retrieved matches that inserted into the plasmalemma during regulated secretion, along with a strong temporal link (Ryan, 1996; Zucker, 1996), form the basis for the belief that vesicle exo- and endocytosis are tightly coupled. This coupling between synaptic vesicle exocytosis, in response to stimulation, and the retrieval of synaptic vesicle membrane by endocytosis has been exploited in the development of an experimental approach using the styryl dye FM1-43 for the optical detection of synaptic activity in living preparations (Betz and Bewick, 1992). Vesicles recycled in concert with synaptic activity become labeled with FM1-43, and nerve terminals in which the exocytosis/endocytosis of synaptic vesicles has occurred appear fluorescent.

Tetanus toxin (TeNT)[1] (Bergey et al., 1983, 1987) and botulinum neurotoxin A (BoNT A) (Bigalke et al., 1985) abolish excitatory and inhibitory postsynaptic potentials in spinal cord neuronal cultures, and the fine structure of the TeNT-blocked synaptic terminal reflects this (Neale et al., 1989; Neale, E.A., L.M. Bowers, V. Dunlap, and L.C. Williamson. *Amer. Soc. Cell Biol.* 1995, 1899). In TeNT-blocked terminals, synaptic vesicles are aligned at the active zone in increased numbers, suggesting that TeNT does not impair the directional movement of vesicles to the active zone but instead blocks transmitter release at a later step, but before vesicle fusion with the presynaptic membrane.

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1. *Abbreviations used in this paper:* BoNT, botulinum neurotoxin; NMJ, neuromuscular junction; SNAP-25, synaptosomal-associated protein of 25 kD; TeNT, tetanus neurotoxin; TTX, tetrodotoxin; VAMP, vesicle-associated membrane protein.

In this study, we compare the effects of TeNT and BoNT A on synaptic function in dissociated cell cultures of fetal mouse spinal cord. These neurotoxins block the exocytosis of neurotransmitter by cleaving specific proteins proposed to mediate the docking and/or fusion of synaptic vesicles with the presynaptic membrane (Montecucco and Schiavo, 1995). We correlate blockade of neurotransmitter release with proteolysis of toxin substrates and with atypical accumulation of synaptic vesicles along the presynaptic active zone. Both of these observations are consistent with the toxins' interference with the formation of a functional SNARE complex (Söllner et al., 1993) leading to synaptic vesicle exocytosis. We applied FM1-43 to neuronal cultures rendered electrically quiescent by exposure to TeNT and several serotypes of the BoNTs, anticipating that in the absence of synaptic vesicle exocytosis there would be no driving force for endocytosis and no uptake of FM1-43. Our data demonstrate that when neuronal activity is blocked by TeNT or most BoNTs, K^+ depolarization triggers neither synaptic vesicle exocytosis nor endocytosis. In marked contrast, BoNT A, while appearing to block vesicle exocytosis, does not prevent K^+ depolarization-induced retrieval of synaptic vesicle membrane, as evidenced by uptake of FM1-43 into synaptic terminals and HRP into synaptic vesicles. The uncoupling of endocytosis from exocytosis by BoNT A allows a characterization of the vesicle retrieval process, and further strengthens the importance of the clostridial neurotoxins as probes for the study of intracellular membrane trafficking events.

Materials and Methods

Materials

Purified TeNT (2×10^7 mouse lethal doses/mg of protein) was a gift from Dr. William Habig (Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD). Except as noted, purified BoNT A was purchased from List Biological Laboratories, Inc. and BoNT C was from the Centre for Applied Microbiology and Research (5.2×10^7 and 1.0×10^7 mouse LD_{50}/mg protein, respectively). BoNT A (9.4×10^6 mouse LD_{50}/mg protein) from Calbiochem-Novabiochem Corp. was used for the experiments presented in Fig. 5, a–c, and Fig. 8. BoNT A provided by Drs. Eric Johnson and Michael Goodenough (University of Wisconsin, Madison, WI) was used for the experiments in Figs. 4 and 5. Concentration of BoNT A was adjusted based on the IC_{50} for blockade of glycine release; the effects of each preparation on exo- and endocytosis were tested and found to be identical. BoNT B (1.26×10^6 LD_{50}/mg protein) was purchased from Calbiochem-Novabiochem Corp. [^{3}H]Glycine (sp act 12.2 Ci/mmol) was purchased from Amersham Corp. Affinity-purified rabbit polyclonal antisynapsin 1a was obtained from Chemicon International, Inc. Affinity-purified rabbit polyclonal antibodies against amino acids 1–32 of the variable domain of vesicle-associated membrane protein (VAMP) 1 and against the COOH-terminal 12 amino acids of synaptosomal-associated protein of 25 kD (SNAP-25) (Rossetto et al., 1996) were a gift of Dr. Cesare Montecucco (University of Padova, Padova, Italy). FM1-43 was obtained from Molecular Probes and HRP Type VI from Sigma Chemical Co.

Cultures

Timed pregnant C57Bl/6NCR mice were obtained from the Frederick Cancer Research and Development Center. Neuronal cell cultures were prepared from mouse fetuses, 13 d in gestation as described previously (Ransom et al., 1977; Fitzgerald, 1989). Dissociated spinal cord cells were plated at 1×10^6 cells per 35-mm plastic culture dish coated with Vitrogen-100® (Collagen Corp.). For immunohistochemistry and FM1-43 uptake, spinal cord cells were plated at 2.5×10^5 cells per dish onto a confluent glial feeder layer (Neale et al., 1991). Cultures were maintained at 35°C in a humidified atmosphere containing 10% CO_2, fed twice weekly by partial medium replacement, and were used between the third and fifth week after plating.

TeNT (10–100 ng/ml) or BoNT A (10–300 ng/ml) was added to the cultures in serum-free medium for 16–26 h before experiments (unless noted otherwise). Immunohistochemistry for synapsin, VAMP, and SNAP-25 was performed as described in detail (Williamson et al., 1996), using anti-synapsin at 1:1,000, anti-VAMP at 1:400, and anti–SNAP-25 at 1:400.

Neurotransmitter Release

K^+-evoked, Ca^{2+}-dependent release of glycine was assayed as described previously (Williamson et al., 1996). In brief, cultures were labeled with [^{3}H]glycine, rinsed, and incubated in Ca^{2+}-free solutions containing 3 mM K^+ with 0.5 mM EGTA, followed by 56 mM K^+ (no EGTA), and finally in depolarizing medium containing 2 mM Ca^{2+} and 56 mM K^+. For this study, 20 μl of incubation medium was withdrawn either at 1-min or 20-s intervals as noted (see Fig. 2) for scintillation spectrometry.

Electrophysiology

Nystatin-perforated whole cell patch clamp experiments were performed on control and toxin-treated neurons. Patch electrodes were fire-polished; electrode resistance was ~2 MΩ. Resting membrane potentials were measured and spontaneous postsynaptic currents (PSCs) were continually recorded with an Axopatch-1B amplifier, and analyzed with an ITC-16 computer interface, a Macintosh computer, and Synapse software developed by Instrutech Corporation and Synergistic Research Systems. For recording, the cultures were bathed in 1 ml of a Hepes-buffered salt solution (HBSS) containing 136 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, and 0.1% BSA at pH 7.25 with the osmolality adjusted with sucrose to 325 ± 5 mmol/kg (Westbrook and Brenneman, 1984). The pipette medium contained 145 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 5 mM Hepes, 1 mM EGTA, and 100 nM free Ca^{2+}, pH 7.3. After baseline recordings were obtained, 1 ml of HBSS containing 56–90 mM KCl (with NaCl adjusted) was applied over the course of 8–18 s to the surface of the patched neuron under direct microscopic observation. With addition of the high K^+ solution, resting membrane potentials were depolarized (average −60.7 ± 4.1 mV to −16.7 ± 1.7 mV; $n = 9$). PSC amplitude and duration, beginning within 1 s of the full application of K^+, were integrated as PSC area with Synapse software.

Uptake of FM1-43 and of HRP

For FM1-43 loading, control and toxin-exposed cultures were rinsed in HBSS and depolarized for 5 min at 35°C with 56 mM KCl in isosmotic HBSS containing 2 mM $CaCl_2$ and 2 μM FM1-43 (Betz and Bewick, 1992). Cultures were rinsed several times over 20–30 min in HBSS either containing 1 μM tetrodotoxin, or without Ca^{2+} and containing 0.5 mM EGTA, to clear surface membranes of the dye while preventing spontaneous network activity and consequent loss of FM1-43 from within labeled terminals. Living cultures were photographed in this rinse solution; some cultures underwent a second round of depolarization (in the absence of FM1-43) to destain synaptic terminals. FM1-43 labeled cultures were photographed using a Zeiss Photomicroscope II with a 40× plan-neofluar lens and T-MAX 3200 film. For uptake of HRP, incubations were identical to the above except that the stimulation medium contained 8–10 mg/ml HRP and the cultures were depolarized for 2–5 min. HRP was visualized after glutaraldehyde fixation (see below) by a 15-min incubation in 3,3′-diaminobenzidine tetrahydrochloride (0.75 mg/ml) in 0.05 M Tris-HCl, pH 7.6, containing 0.01% hydrogen peroxide.

Electron Microscopy

Cultures were prepared for electron microscopy as described in detail in Neale et al. (1978). In brief, fixation in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.3, was followed by postfixation in 1% osmium tetroxide, en bloc staining with uranyl acetate, rapid dehydration in an ethanol series and embedding in the culture dish. After curing, the Epon disc was separated from the plastic dish and fields of interest were selected, removed, and mounted on Epon blanks for ultrathin sectioning. Stained ultrathin sections were examined in a JEM-1010 (JEOL USA Inc.) electron microscope fitted with a goniometer stage. Synaptic terminals were located and the specimens were tilted if necessary to obtain a

cross-sectional view of the apposing synaptic membranes. Terminals were photographed at 40,000×.

Morphometry

Electron micrographs were printed at 100,000×. The distance between individual synaptic vesicles and the presynaptic membrane was measured manually, and those vesicles lying within 10 nm were scored as morphologically docked. The distance of 10 nm appears reasonable in light of the estimated size of the coiled-coiled core complex proteins linking the synaptic vesicle to the presynaptic membrane (Hanson et al., 1997; Lin and Scheller, 1997). Micrographs were digitized and NIH Image software was used to measure the length of active zones. Vesicle counts were obtained directly from micrographs. Unpaired *t* tests were used to determine differences between samples.

Results

Toxin Effects on Vesicle Exocytosis

Spinal cord cultures attain electrophysiologic maturity 3 wk after plating when the frequency of spontaneous postsynaptic potentials approaches a maximum (Jackson et al., 1982). Morphologic development also proceeds rapidly after plating. Fig. 1 illustrates the morphology of neurons (a) and the number and distribution of synaptic terminals (b) in a 3-wk-old culture. Synaptic boutons contain one or more active zones and a complement of either round or flattened small electron lucent vesicles, and occasional larger dense core vesicles (Fig. 1 c).

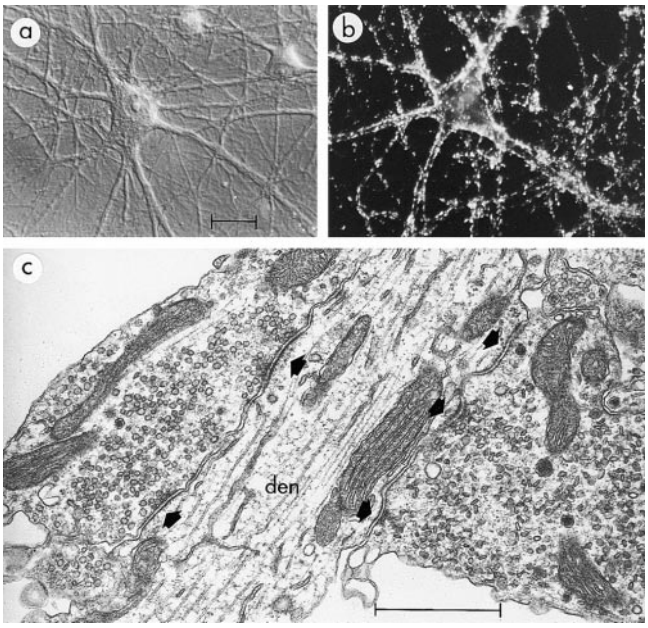

Figure 1. Spinal cord neurons in low density cell culture for 3 wk. The neurons settle on top of flattened glial cells, extend axonal and dendritic processes, and establish functional synaptic connections. (a) Soma and dendrites of a large spinal cord neuron, several smaller neurons, and a meshwork of axonal and dendritic processes are seen in this field. Hoffman modulation contrast optics. (b) Immunohistochemistry for synapsin, an integral membrane protein of synaptic vesicles. Focal fluorescence marks aggregations of synaptic vesicles within synaptic terminals, which encrust neuronal somata and dendrites. (c) Electron microscopy confirms that somatic and dendritic (den) surfaces are virtually covered with synaptic terminals. The terminal on the left contains predominantly round clear synaptic vesicles and two synaptic active zones (arrows) whereas the terminal on the right contains flattened clear vesicles and three active zones. Bars: (a and b) 25 μm; (c) 1 μm.

Neurotransmitter release has been assayed by preloading control and toxin-exposed cultures with [^{3}H]glycine or [^{3}H]glutamine and determining the level of radioactivity released into the culture medium after 5 min of depolarization with 56 mM KCl and 2 mM $CaCl_2$ (Williamson et al., 1992). In cultures exposed for 20 h to either TeNT or BoNT A (0.06 nM or 10 ng/ml), K^+-evoked release of both glycine and glutamate appears completely arrested (Williamson et al., 1996).

High extracellular concentrations of Ca^{2+} (5–10 mM) have been shown to partially overcome a BoNT A–induced block in exocytosis (Capogna et al., 1997; Trudeau et al., 1998). K^+ depolarization sustained for several minutes might induce an influx of Ca^{2+} sufficient to trigger rapid (Klingauf et al., 1998) and/or transient synaptic vesicle exocytosis that is not obvious in a 5-min assay. To rule out the possibility that, in BoNT A–treated cultures, there is a bolus of neurotransmitter released within seconds of depolarization that might not be detected by this assay, we examined cultures for glycine release at 20-s intervals after the addition of depolarizing medium (Fig. 2). Radioactivity released from cultures in 56 mM K^+ without Ca^{2+} is taken as the baseline; values above this are considered a result of Ca^{2+}-dependent K^+-induced release. The rate of release from control cultures increases with age in vitro; variability among controls is due most likely to differences in number and maturity of synaptic terminals. In six experiments from three dissections assayed between 23 and 30 d after plating, loss of radiolabeled glycine from TeNT-treated cells is similar in high K^+ medium with or without Ca^{2+}, even at times shortly after exposure to Ca^{2+}. The same absence of Ca^{2+}-dependent glycine release is seen in sister cultures treated with BoNT A. Assays terminated after 20 s in high K^+ plus Ca^{2+} similarly showed no significant difference between counts released from TeNT- and BoNT A–treated cultures. Experiments repeated on cultures of spinal cord neurons grown on a feeder layer of cortical astrocytes yielded similar results (data not shown). We attempted to induce transmitter release by a medium change either from 0 to 2 mM Ca^{2+} (both with 56 mM K^+, as shown) or from 3 mM to 56 mM K^+ (both with Ca^{2+}, not shown). In all cases (eight experiments), results are the same; radioactivity lost from BoNT A–treated cultures is very nearly identical to that lost from sister cultures treated with TeNT, and does not exceed that lost in high K^+ medium without Ca^{2+}.

In addition, whole cell patch clamp recordings were obtained from neurons before and after depolarization. In untreated control cultures, application of 56–90 mM K^+ with 2 mM Ca^{2+} produces synaptic currents (Fig. 3 a). When baseline recordings are obtained from similar cultures maintained in 1 μM tetrodotoxin (TTX), K^+-evoked synaptic currents are observed at levels similar to controls (Fig. 3 b). Synaptic currents are not detected in cultures exposed for 16–20 h to either TeNT or BoNT A (Fig. 3, c and d), at toxin concentrations 35–50% as high as those used for the morphologic studies. The observations in cultures treated with BoNT A are indistinguishable from

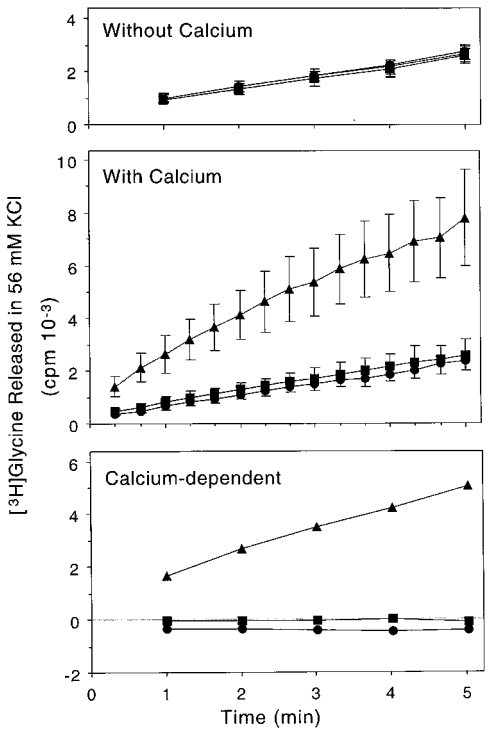

Figure 2. Time course of release of [^{3}H]glycine in response to K^+ depolarization. (top) Control and toxin-treated spinal cord cultures were incubated with [^{3}H]glycine, rinsed as described in Materials and Methods, and changed into HBSS containing 56 mM K^+ and no calcium. Samples were withdrawn at 1-min intervals and counted. Levels of radioactivity released from control and toxin-blocked cultures are essentially identical. (middle) The medium was changed to one containing 56 mM K^+ and 2 mm Ca^{2+} and samples were withdrawn every 20 s. Radioactivity released from control cultures in the presence of calcium is increased, whereas that released from toxin-treated cultures is virtually unchanged from the zero-Ca^{2+} samples. (bottom) Difference in radioactivity released in the presence and absence of Ca^{2+}. Values for TeNT and BoNT A are very similar and show no Ca^{2+} dependence. Experiments were performed on cultures from three dissections, assayed between 23 and 30 d after plating. Each data point is the mean ± SD. Triangles, controls, $n = 7$; circles, TeNT (100 ng/ml for 24–26 h), $n = 6$; and squares, BoNT A (200 ng/ml for 24–26 h), $n = 6$.

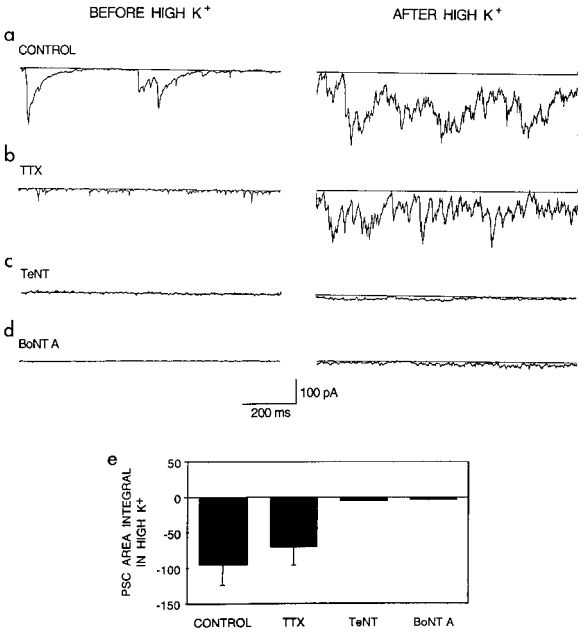

Figure 3. Representative membrane currents recorded from control, TTX-, TeNT-, and BoNT A–treated spinal cord neurons showing spontaneous network and K^+-evoked activity. Membrane potentials were clamped at a holding potential of − 60 mV. (a) Spontaneous synaptic currents are recorded in untreated neurons and the frequency of these currents is increased by the application of K^+. (b) Spontaneous synaptic currents, presumably related to ongoing neuronal spike activity, are eliminated by 1 μM TTX, although small TTX-resistant events are observed. Substantial synaptic currents are recorded, however, after depolarization with K^+. (c) Neurons treated with TeNT display neither spontaneous nor K^+-evoked synaptic currents. (d) Neurons treated with BoNT A show no spontaneous synaptic currents, and K^+ depolarization (56–90 mM) is unable to evoke synaptic activity. The recording shown was obtained within 1 s of a 14-s application of high K^+ medium. (e) Histogram (in arbitrary units) of average integrated synaptic currents in high K^+ medium. Control, $n = 4$; TTX, $n = 3$; TeNT (100 ng/ml for 24–26 h), $n = 2$; and BoNT A (200 ng/ml for 24–26 h), $n = 4$. Bars denote SEM; BoNT A is significantly different by the *t* test from control ($P < 0.02$) and from TTX ($P < 0.03$) and not different from TeNT ($P = 0.66$).

those in TeNT, with levels of activity at least 20-fold less than in controls (Fig. 3 e). These data were obtained within 1 s after application of high K^+ medium; manual analysis of records during the application of high K^+ provided no evidence of synaptic events in either TeNT- or BoNT A–treated cultures.

Control and toxin-blocked cultures were stained immunohistochemically for the synaptic proteins known to be substrates for proteolysis by these toxins. In control cultures, antibodies against VAMP (Fig. 4 a) yield a staining pattern almost identical to that seen with antisynapsin (Fig. 1 b). However, VAMP is not detectable in cultures when neurotransmitter release is arrested by TeNT (Fig. 4 b). Similarly, the COOH terminus of SNAP-25 is evident in untreated cultures (Fig. 4 c), and is not observed in BoNT A–blocked cultures (Fig. 4 d).

Synaptic fine structure was examined in sister cultures at the time of synaptic blockade, with and without K^+ depolarization (Fig. 5). The length of individual active zones was measured and docked synaptic vesicles (within 10 nm of the presynaptic membrane) were counted (Table I). In toxin-blocked cultures, the average length of the active zones is not significantly different from controls (i.e., 0.45–0.50 μm). In nonstimulated but spontaneously active control preparations (Fig. 5 a), ~7.8 ± 4.7 (SD) vesicles/μm of active zone appear docked. In toxin-blocked cultures, the number of docked synaptic vesicles is almost twice as high as in control cultures (Fig. 5, b and c, and Table I). In controls, ~25% of active zones have >10 docked vesicles/μm, whereas in cultures exposed to TeNT or BoNT A, ~80% of active zones have >10 vesicles/μm. Experiments with BoNT B (not shown) show identical results. However, when control cultures are stimulated with 56 mM KCl and 2 mM $CaCl_2$ before fixation (Fig. 5 d), there is a reduction

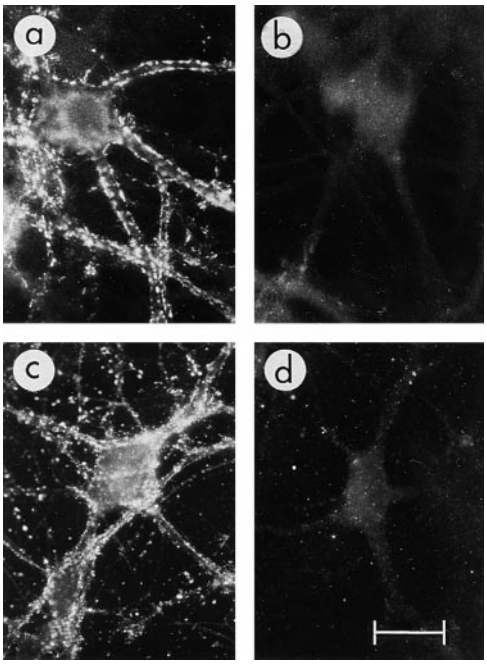

Figure 4. Immunohistochemistry for VAMP and SNAP-25. (a) VAMP, like synapsin, is an integral protein of the synaptic vesicle membrane and shows a similar immunohistochemical localization (compare with Fig. 1 b). (b) VAMP is the substrate for proteolytic cleavage by TeNT. In cultures exposed to TeNT (10 ng/ml for 16 h), VAMP cannot be detected immunohistochemically when neurotransmitter release is blocked. (c) SNAP-25, another of the proteins implicated in the vesicle docking/fusion complex, is associated with the axonal membrane by palmitoyl groups. Its immunohistochemical localization suggests concentration at synapses. (d) BoNT A cleaves nine amino acids from the COOH terminus of SNAP-25. When neurotransmitter release is blocked by BoNT A (200 ng/ml for 16 h), antibody against the COOH terminus no longer detects this portion of SNAP-25. Bar, 25 μm.

Table I. Counts of Docked Synaptic Vesicles*

	Resting	KCl-stimulated
Control	7.8 ± 4.7‡	5.7 ± 4.7
	(24%§, n = 34)	(16%; n = 31)
TeNT	14.1 ± 5.7	14.6 ± 6.3
	(81%; n = 53)	(78%; n = 42)
BoNT A	14.0 ± 5.6	14.3 ± 3.0
	(77%; n = 35)	(89%; n = 26)

Resting cultures were fixed and processed for electron microscopy after toxin exposure for 16 h (TeNT at 100 ng/ml or BoNT A at 200 ng/ml). Sister cultures were fixed after 5 min of depolarization in 56 mM KCl and 2 mM $CaCl_2$. Ultrathin sections were tilted to observed the presynaptic membrane in cross-section, and docked synaptic vesicles were counted. Differences between control and toxin-blocked cultures are significant; $P < 0.001$ (t test).
*Within 10 nm of the presynaptic membrane.
‡Number of docked vesicles/μm active zone ± SD.
§Percentage of active zones with >10 docked vesicles/μm.

in the number of vesicles at the active zone with a lower percentage (16%) of active zones showing >10 vesicles/μm. In both TeNT- (Fig. 5 e) and BoNT A–blocked (Fig. 5 f) cultures, K^+ depolarization fails to elicit neurotransmitter release and does not reduce the number of docked vesicles, with 78% of the active zones having >10 docked vesicles/μm for TeNT, and 89% for BoNT A. Thus, relative to nonstimulated controls, TeNT- and BoNT A–blocked terminals have more synaptic vesicles apposed to the presynaptic membrane of the active zone, and K^+ stimulation does not reduce this number, as it does in controls. Thus, functional assays of toxin action are corroborated by morphologic evidence for proteolysis of toxin substrates and for impedance of synaptic vesicle exocytosis.

Toxin Effects on Vesicle Recycling

To demonstrate further the toxin-induced electrical quiescence, we used the styryl dye FM1-43 for the optical detection of synaptic activity (Betz and Bewick, 1992). Depolarization of control cultures in the presence of FM1-43 results in a pattern of fluorescence consonant with the labeling of synaptic terminals (Fig. 6 a). Uptake of FM1-43 is not observed with depolarization in the absence of Ca^{2+} (Fig. 6 b) and is markedly reduced without depolarization (Fig. 6 c). As expected, cultures treated for 22 h with 10 ng/ml TeNT (Fig. 6 d), 10–100 ng/ml BoNT C (Fig. 6 e) (Williamson et al., 1996), or BoNTs B or D (10 ng/ml; not shown), fail to release neurotransmitter with K^+ depolarization, and also fail to take up FM1-43. In marked contrast, cultures blocked with 10 ng/ml of BoNT A, which exhibit no K^+-evoked release of neurotransmitter (Williamson et al., 1996) show intense K^+-stimulated uptake of FM1-43. Increasing the concentration of BoNT A to 200 ng/ml (Fig. 6 f) or 300 ng/ml (not shown) failed to abolish FM1-43 uptake. As in controls, FM1-43 loading of BoNT A–treated cultures requires both extracellular Ca^{2+} (Fig. 6 g) and elevated K^+ (Fig. 6 h). These results indicate that BoNT A blockade of neurotransmitter release does not prevent vesicle membrane reuptake by endocytosis, and that membrane retrieval is dependent on Ca^{2+}.

Evidence that FM1-43 labeling is associated with recycled synaptic vesicles is provided by the ability to destain a labeled preparation by further stimulation in the absence of dye. Indeed, K^+ stimulation of control cultures preloaded with FM1-43 results in dye loss (Fig. 6 i). When dye-loaded cultures are stimulated in the absence of Ca^{2+}, exocytosis of labeled vesicles is prevented and fluorescence is retained (Fig. 6 j). However, in BoNT A–blocked cultures that have been loaded with FM1-43, subsequent depolarization (in the presence of Ca^{2+}) does not induce destaining (Fig. 6 k). This provides further evidence that synaptic vesicles in BoNT A–blocked cultures are unable to undergo exocytosis.

To strengthen the hypothesis that FM1-43 staining of BoNT A–blocked terminals is related to vesicle membrane endocytosis, we examined the fine structure of synaptic terminals. In spontaneously active control cultures (Fig. 7 a), clathrin-coated pits and vesicles are seen only occasionally, although empty clathrin baskets (arrowheads; Kanaseki and Kadota, 1969) are not uncommon. When control cultures are stimulated (56 mM K^+ and 2 mM Ca^{2+} for 5 min; Fig. 7 b), small coated pits and vesicles (arrows) are observed with increased frequency and empty clathrin baskets with decreased frequency. In cultures blocked with TeNT (Fig. 7 c) or with BoNT A (Fig. 7 e), synaptic vesicles accumulate at the presynaptic membrane,

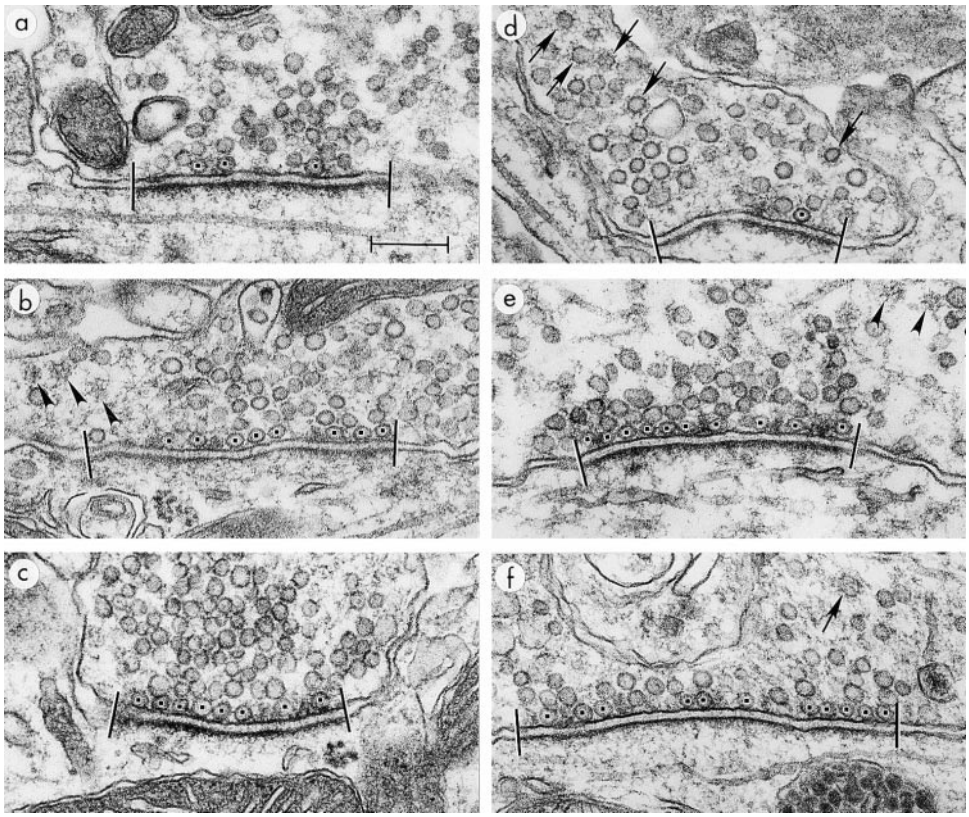

Figure 5. Fine structure of the active zone. Active zones are marked by vertical bars, and docked synaptic vesicles (within 10 nm of the presynaptic membrane), with black squares. (a–c) Resting cultures. a is from a spontaneously active, control culture; b is from a culture in which neurotransmitter release was blocked by TeNT; and c is from a culture blocked by BoNT A. The number of synaptic vesicles docked at the active zone is increased in toxin-treated terminals. Note clathrin organized into baskets (arrowheads) in b. In this experiment, TeNT (100 ng/ml) and BoNT A (1.0 μg/ml) were added for 16 h. (d–f) K^+-stimulated active zones. In a stimulated control culture (d), fewer synaptic vesicles appear docked (marked with squares) than in spontaneously active cultures. Note the increased occurrence of clathrin-coated vesicles (arrows). In contrast, when TeNT (100 ng/ml)– or BoNT A (List, 200 ng/ml)–blocked cultures are stimulated with K^+ (e and f), the number of docked vesicles remains high. Arrowheads mark empty clathrin baskets. Bar, 0.2 nm.

empty clathrin baskets are typical, and coated pits and vesicles are extremely rare. When TeNT-blocked cultures are stimulated with K^+ (Fig. 7 d), these synaptic features remain unchanged, suggesting a lack of membrane movement. When BoNT A–blocked cultures are depolarized (Fig. 7 f), the number of docked synaptic vesicles remains elevated, although clathrin-coated pits are found with increased frequency and empty clathrin baskets have disappeared. Thus, stimulated BoNT A cultures take on those features which suggest active retrieval of synaptic vesicle membrane.

To confirm that synaptic terminals incapable of vesicle exocytosis can be stimulated to retrieve vesicle membrane, the vesicle-loading experiments were repeated with HRP substituted for FM1-43 in the stimulation medium. Control and toxin-blocked cultures were depolarized by K^+ with Ca^{2+} for 5 min in the presence of 10 mg/ml HRP, rinsed for 30 min in medium containing TTX, and then fixed. A large proportion of synaptic vesicles in control cultures (Fig. 8 a) contain HRP reaction product. As expected, HRP-labeled vesicles are rare in TeNT-blocked cultures (Fig. 8 b). Depolarization of BoNT A–blocked cultures, however, results in the labeling of a much greater proportion of small vesicles (Fig. 8 c). When BoNT A–blocked cultures are fixed almost immediately after depolarization (i.e., without an extended rinse), clathrin-coated vesicles of the appropriate size are labeled with HRP (insets). These experiments lend further credence to the proposal that, in BoNT A–blocked neurons, synaptic vesicles can be endocytosed in the absence of temporally associated vesicle exocytosis, and indicate that the recycling process involves the clathrin-coated membrane organelles observed in control preparations.

Table II. Calcium Dependence of Synaptic Vesicle Exo- and Endocytosis

		$+Ca^{2+}$*	$-Ca^{2+}$‡			$+Ca^{2+}$	$-Ca^{2+}$
Control	Load	10.5§	0	BoNT A	Load	4.8	0
	Unload	2.5‖	10.6		Unload	5.2¶	ND

Control and BoNT A- (200 ng/ml for 18 h) exposed cultures were rinsed in normal or Ca^{2+}-free‡ medium and incubated with HRP (8 mg/ml) for 2 min in medium containing 56 mM KCl with or without Ca^{2+}. Cultures were rinsed for 20 min in a Ca^{2+}-free buffered salts solution. Cultures loaded with HRP in the presence of Ca^{2+} were rinsed in normal or Ca^{2+}-free salt solution and incubated for 5 min in 56 mM KCl with or without Ca^{2+} followed by a 10-min wash in Ca^{2+}-free salt solution. Cultures were fixed for electron microscopy both after loading and after unloading. Vesicles were counted in micrographs at 100,000×; at least 2,000 vesicles were counted for each control sample and 5,500 for each BoNT A sample.

*2 mM $CaCl_2$.

‡0 mM $CaCl_2$ and 0.5 mM EGTA.

§Percent HRP-labeled vesicles after KCl-induced loading or unloading.

‖Significantly lower than Control, Load, plus Ca^{2+} ($P < 0.05$).

¶Not significantly different from BoNT A, Load, plus Ca^{2+}.

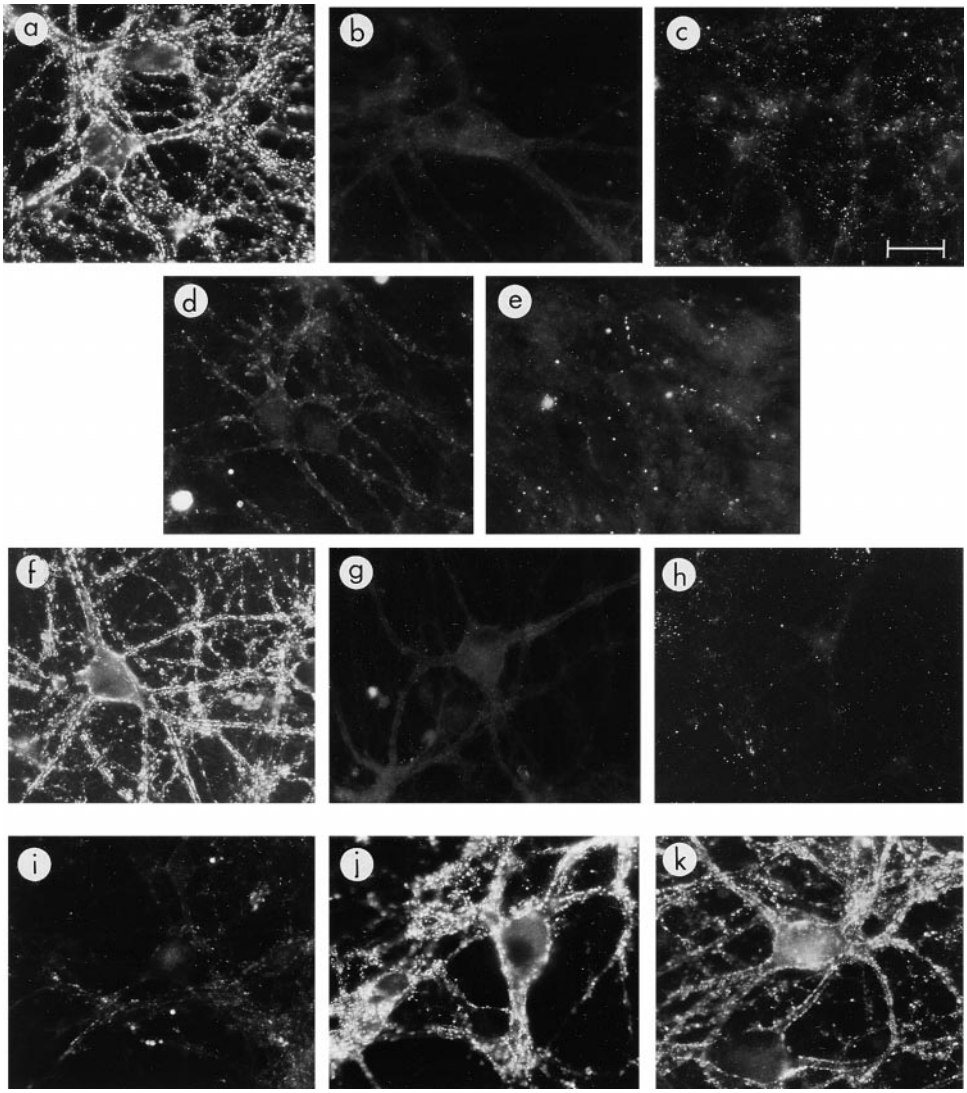

Figure 6. FM1-43 staining (a–h) and destaining (i–k) with K^+ depolarization. (a–c) Control cultures. (a) 56 mM KCl; 2 mM $CaCl_2$. The fluorescent image is strikingly similar to Figs. 1b and 4a. The labeled structures are individual active synaptic terminals containing a number of synaptic vesicles whose membranes are labeled with the fluorescent dye as a result of vesicle membrane recycling during the stimulation interval. (b) 56 mM KCl, 0 mM $CaCl_2$, and 0.5 mM EGTA. Uptake of FM1-43 is not detectable. (c) 3 mM KCl and 2 mM $CaCl_2$. Some uptake of FM1-43 occurs as a result of spontaneous network activity. (d) TeNT (10 ng/ml for 22 h), 56 mM KCl, and 2 mM $CaCl_2$. (e) BoNT C (100 ng/ml for 22 h), 56 mM KCl, and 2 mM $CaCl_2$. When synaptic vesicle exocytosis is blocked by TeNT or BoNT C, uptake of FM1-43 is decreased substantially. (f–h) BoNT A (200 ng/ml; i.e., >20 times that required to block neurotransmitter release). (f) 56 mM KCl and 2 mM $CaCl_2$. Although intensity is reduced from controls, FM1-43 labeling is unequivocal in cultures blocked by BoNT A. (g) 56 mM KCl, 0 mM $CaCl_2$, and 0.5 mM EGTA. As in control cultures, uptake of FM1-43 requires Ca^{2+}. (h) 3 mM KCl and 2 mM $CaCl_2$. There is essentially no labeling of toxin-blocked cultures without K^+ depolarization. (i and j) Control cultures loaded with FM1-43 in 56 mM KCl and 2 mM $CaCl_2$, as in a. (i) Loaded culture subsequently depolarized with 56 mM KCl and 2 mM $CaCl_2$. There is substantial loss of FM1-43 from synaptic terminals as a result of exocytosis of labeled vesicles (compare with a). (j) Loaded culture subsequently depolarized with 56 mM KCl, 0 mM $CaCl_2$, and 0.5 mM EGTA. The absence of Ca^{2+} prevents synaptic vesicle exocytosis and the destaining of labeled terminals. (k) BoNT A (200 ng/ml for 22 h)–blocked culture loaded with FM1-43 in 56 mM KCl and 2 mM $CaCl_2$, as in f, and subsequently incubated with 56 mM KCl and 2 mM $CaCl_2$ in an attempt to destain. In contrast to control cultures, it is not possible to destain BoNT A–blocked cultures, providing further evidence that vesicle exocytosis cannot be stimulated. Bar, 25 μm.

Vesicle Exocytosis Requires Ca^{2+}

To confirm that HRP-labeled vesicles in control cultures are competent for exocytosis, we loaded terminals by stimulating for 2 min in the presence of 8 mg/ml HRP. HRP-loaded terminals were subjected to a second interval of K^+ depolarization and the change in percentage of labeled vesicles was determined (Fig. 9 and Table II). After HRP-loading of the control cultures, ~10% of total synaptic vesicles contain HRP (Fig. 9 a). In cultures stimulated after loading, labeled vesicles decrease to 2.5% of total vesicles (Fig. 9 c). Without Ca^{2+} in the stimulation medium, the percentage of labeled vesicles remains high (10%; not shown). Thus, the loss of HRP is consistent with synaptic vesicle exocytosis.

Vesicle Endocytosis Requires Ca^{2+}

In BoNT A–blocked cultures, HRP loading (as above) labels ~5% of the synaptic vesicles (Fig. 9 b). This percentage is not decreased by subsequent depolarization (Fig. 9 d and Table II), confirming that exocytosis is not stimulated in the presence of BoNT A. When loading of either BoNT A–blocked or control cultures is attempted in the absence of extracellular Ca^{2+}, after a rinse in Ca^{2+}-free buffer, there is no HRP in synaptic vesicles (Table II), and there are no HRP-labeled cisternae. In control cultures, failure to load can be taken as a consequence of the zero Ca^{2+}-induced block in exocytosis. The absence of vesicle recycling in BoNT A–blocked terminals under these conditions is not due to a failure of vesicle exocytosis since the

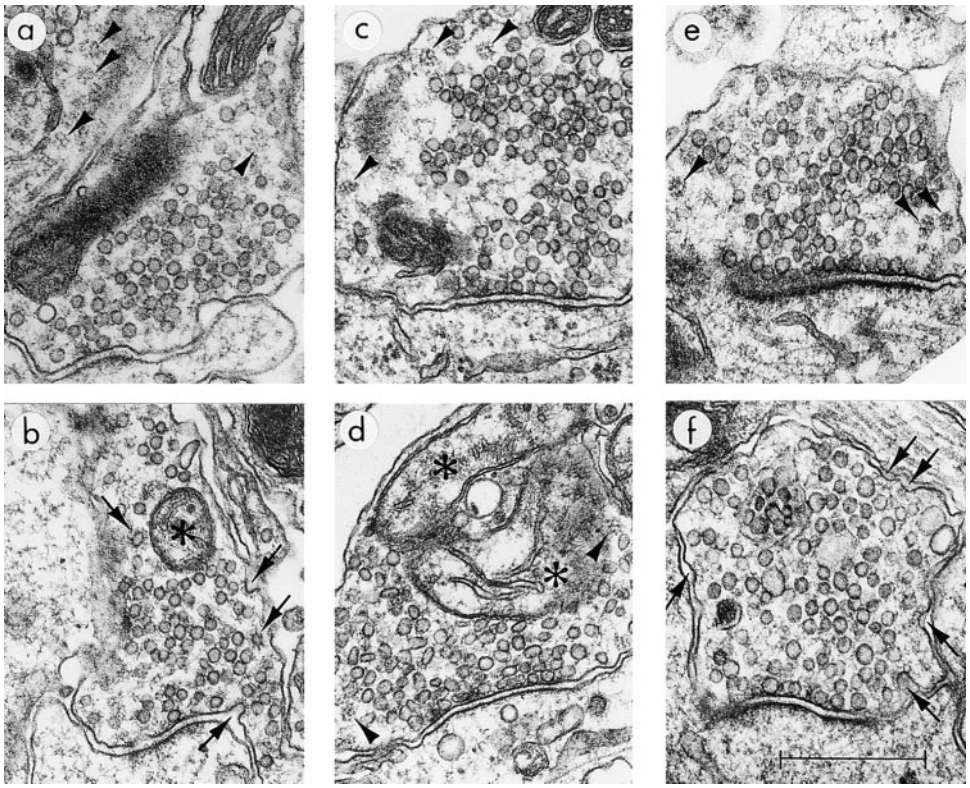

Figure 7. Clathrin and synaptic vesicle recycling. (a) Resting control culture. Clathrin-coated pits and vesicles are seen only occasionally, whereas empty clathrin baskets (arrowheads) are more numerous. (b) K^+-depolarized control culture. Clathrin-coated pits and vesicles (arrows) are seen commonly; empty clathrin baskets are rare. Note the electron lucent matrix of mitochondria (asterisks) in cultures fixed during stimulation. (c) Resting TeNT-blocked culture. Clathrin-coated pits and vesicles are almost never seen although empty clathrin baskets are found easily. (d) K^+-depolarized TeNT-blocked culture. K^+ stimulation causes a change in the density of the mitochondrial matrix, but no noteworthy change in the distribution of synaptic vesicles at the active zone or frequency of clathrin baskets or clathrin-coated membranes. (e) Resting BoNT A–blocked culture. This culture appears similar to the TeNT-blocked culture in the absence of clathrin-coated membranes and the persistence of empty clathrin baskets. (f) K^+-depolarized BoNT A–blocked culture. This culture appears more similar to the stimulated control than to the stimulated TeNT-blocked culture. Empty clathrin baskets have all but disappeared and clathrin-coated membranes can be found. Toxin concentrations as in Fig. 5. Bar, 0.5 μm.

endocytosis of HRP in blocked cultures occurs in the absence of measurable exocytosis. Therefore, it must be concluded that the endocytic retrieval of synaptic vesicle membrane requires Ca^{2+}.

Membrane Available for Endocytosis Is Limited

In another set of cultures (data not shown), we mock-loaded BoNT A–blocked cultures (2 min in high K^+, with Ca^{2+} but no HRP) from one to four times before adding HRP to the stimulation medium. The number of vesicles labeled with HRP decreases with each stimulation interval. When HRP is added for a fifth stimulation, labeled vesicles are rare. Thus, there appears to be a finite source of surface membrane that can be retrieved and recycled as synaptic vesicles.

Discussion

In spinal cord cell cultures, biochemical, electrophysiologic, and morphologic assays provide evidence for ample exo- and endocytosis of synaptic vesicles. Treatment of cultures with TeNT results in the cessation of both neurotransmitter release by synaptic vesicle exocytosis and retrieval of synaptic vesicle membrane by endocytosis. In contrast, in BoNT A–blocked cultures, substantial endocytosis occurs in the absence of evidence for exocytosis as measured by these assays. The data as a whole imply that a reservoir of synaptic vesicle membrane exists on the plasma membrane of the nerve terminal, that calcium is required to initiate the uptake of this membrane for recycling, and that BoNT A, unlike TeNT, does not interfere with the process of synaptic vesicle membrane retrieval.

Toxin Substrates and Vesicle Docking

Atypical accumulation of docked synaptic vesicles is associated with tetanus intoxication (Hunt et al., 1994; Mellanby et al., 1988; Neale et al., 1989; Broadie et al., 1995) and with disruption of the function of the *N*-ethylmaleimide–sensitive factor (Kawasaki et al., 1998; Schweizer et al., 1998). This morphology suggests an inability of synaptic vesicles to fuse with the presynaptic membrane and is entirely compatible with the demonstration that TeNT is a zinc endopeptidase that cleaves VAMP (Schiavo et al., 1992), a homologue of a protein critical for transport vesicle docking and fusion at the yeast (Protopopov et al., 1993) and mammalian (McMahon et al., 1993) Golgi apparatus. TeNT and BoNTs A and C, each acting on a different protein of the ternary core complex (Montecucco and Schiavo, 1995), induce blockade of neurotransmitter release, which correlates with toxin cleavage of its specific substrate (Williamson et al., 1996). We report here that BoNTs A and B also cause an accumulation of synaptic vesicles at the active zone at a time when neurotransmitter release is arrested. These observations support the requirement of the ternary complex (VAMP, syntaxin, and SNAP-25) for vesicle fusion but not for vesicle docking.

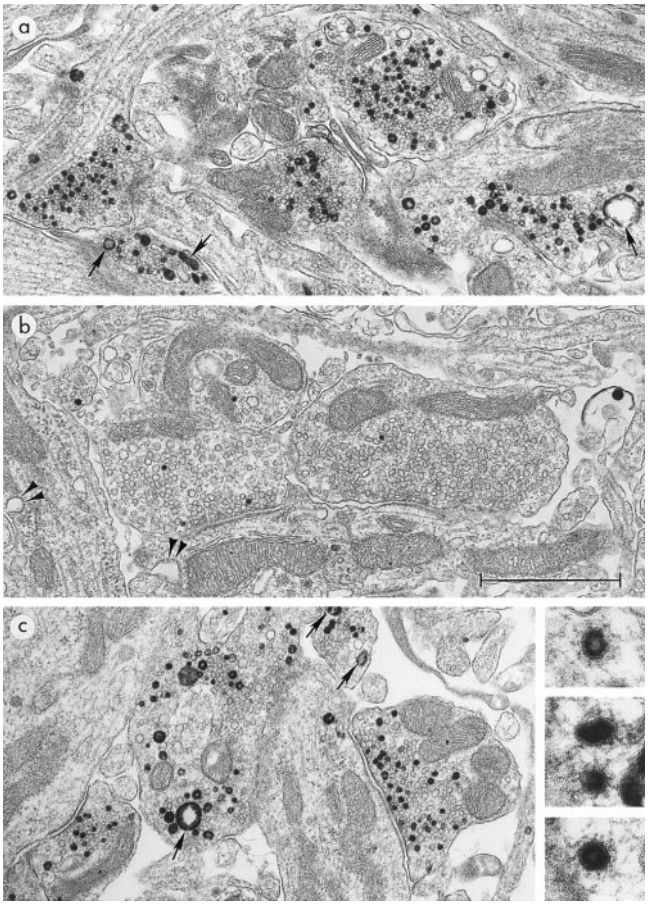

Figure 8. HRP uptake stimulated by K^+ depolarization. (a) Control. Recycled synaptic vesicles contain peroxidase reaction product. (b) TeNT (10 ng/ml for 18 h). Only occasional synaptic vesicles contain reaction product, indicating the failure of synaptic vesicles to undergo recycling. (c) BoNT A (200 ng/ml for 18 h). A large number of synaptic vesicles contain reaction product, evidence that these vesicles have been formed from surface membrane. (insets) Reaction product labeling of clathrin-coated vesicles implicates these structures in the process of vesicle recycling. Note the larger labeled structures (arrows) in a and c. That these structures are not seen in TeNT-exposed cultures suggests that they are an intermediate in the vesicle recycling pathway. Double arrowheads in b mark coated pits associated with conventional endocytosis that is unaffected by TeNT. These micrographs are the high resolution equivalents of Fig. 6, a, d, and f. Bar: (a–c) 1 μm. Insets are enlarged to 135,000×.

The occurrence of docked vesicles in toxin-treated preparations has been explained by the ability of synaptotagmin, a synaptic vesicle integral membrane protein, to bind to SNAP-25 on the presynaptic membrane (Schiavo et al., 1997). This binding is not dependent on Ca^{2+} or on the presence of syntaxin or VAMP, and occurs even with BoNT A- or E-cleaved SNAP-25. Functional docking (i.e., leading to vesicle fusion), however, requires that the proteins of the core complex be intact (Schiavo et al., 1997). This is consistent with our observation that K^+-induced depolarization of control cultures reduces the number of vesicles at the active zone, whereas depolarization of toxin-blocked cultures is not similarly effective. Thus, the clostridial neurotoxins block neurotransmitter release by preventing synaptic vesicle fusion.

Failure to Demonstrate Exocytosis

The synaptic blockade induced by BoNT A in this study is at variance with several reports of the failure of BoNT A to produce a complete block in transmitter release. The latter studies introduced toxin into normally nonreceptive PC12 or chromaffin cells by mechanical cracking (Banerjee et al., 1996) or digitonin permeabilization (Lawrence et al., 1997) of the cells, or intracellular injection of recombinant BoNT A light chain (Xu et al., 1998). It remains unclear whether this difference in the extent of blockade reflects the underlying biology of toxin internalization into intact neurons or differences in assay conditions.

It might be argued that, under the experimental conditions described here, BoNT A blocks the release of glycine and glutamate (Williamson et al., 1996), but fails to block the release of other transmitters. If so, we would expect to see some nerve terminals with, and others without, evidence of vesicle recycling. In fact, HRP-labeled vesicles are seen in every synaptic terminal. Alternatively, if BoNT A had caused the loss of stored transmitter from synaptic vesicles, depolarization would induce the exocytosis of empty vesicles. However, there is considerable evidence that BoNT A does not interfere with the synthesis and/or storage of acetylcholine (for review see Gundersen, 1980) nor TeNT with that of GABA (Collingridge and Davies, 1980).

Finally, vesicle recycling in BoNT A–blocked cultures may have occurred as a result of rapid or low level K^+-evoked exocytosis that was below the sensitivity or time resolution of our assays. Both glycine release and synaptic currents are maintained in high K^+ for several minutes in control cultures, and about half the number of vesicles are recycled in BoNT A–blocked cultures as in untreated controls. It seems unlikely that both the neurotransmitter release assays and whole cell patch clamp analysis would fail to detect exocytic responses of this magnitude. While it may not be possible to prove that absolutely no exocytosis occurs with BoNT A, there remains a striking disproportion between the level of exocytosis that could be measured and what might be expected to account for the endocytosis that is observed.

Ca^{2+} Requirement for Endocytosis

We have used the dissociation of synaptic vesicle exo- from endocytosis to show that vesicle endocytosis requires Ca^{2+}. Our results contrast with those of Ryan et al. (1993, 1996) who reported endocytosis of synaptic vesicle membrane, in cultures of hippocampal neurons, in the absence of extracellular Ca^{2+}. The time course of the decrease in intraterminal Ca^{2+} (Ryan et al., 1993), elevated by the initial membrane depolarization, suggests that superfusion with FM1-43 was begun before the free Ca^{2+} concentration had returned to the baseline. This residual elevation may have been sufficient, even in the absence of either extracellular Ca^{2+} or simultaneous depolarization, to initiate membrane retrieval.

Ca^{2+} is required to initiate vesicle endocytosis after vesicle depletion induced by α-latrotoxin at the frog neuro-

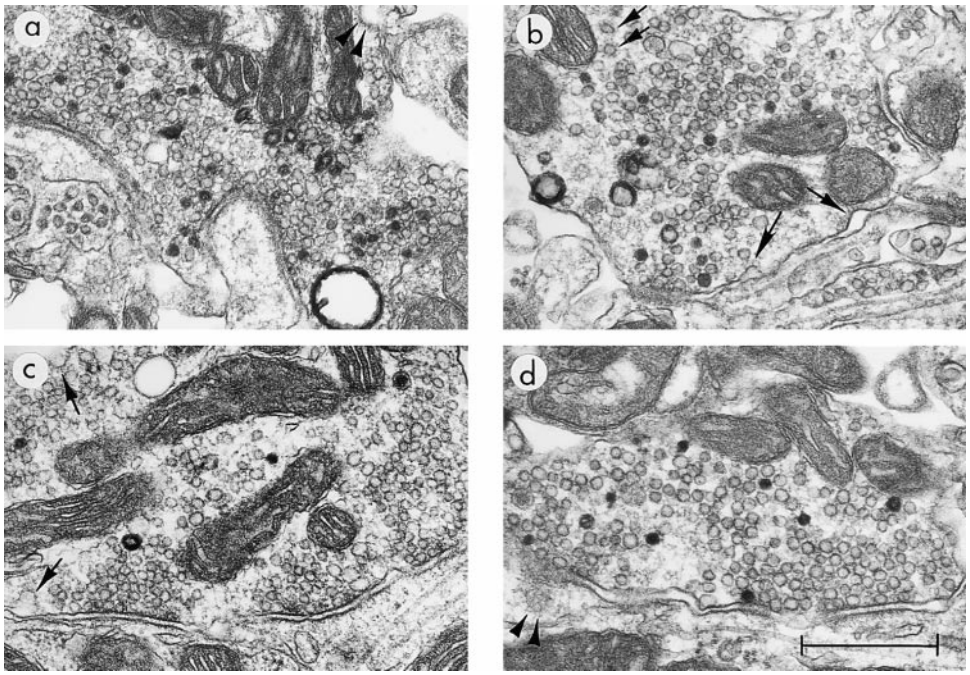

Figure 9. Exocytosis of HRP stimulated by K^+ depolarization. (a and c) Control; and (b and d) BoNT A (200 ng/ml for 18 h). Synaptic terminals are loaded with horseradish peroxidase (a, b). After subsequent stimulation in the absence of HRP, the percent labeled vesicles is lower in control (c) but unchanged in toxin-blocked (d) cultures. These micrographs are analogous to Fig. 6, a and i, and Fig. 6, f and k, providing further demonstration of the block in exocytosis induced by BoNT A. Note coated pits and coated vesicles implicated in synaptic vesicle membrane retrieval (arrows) in b and c and conventional coated pits (double arrowheads) in a and d. Bar, 0.5 μm.

muscular junction (NMJ) (Ceccarelli and Hurlbut, 1980) or by prolonged high frequency action potential stimulation in lamprey giant reticulospinal synapses (Gad, H., O. Shupliakov, V.A. Pieribone, and L. Brodin. *Soc. Neurosci.* 1995. 138.8; Shupliakov et al., 1997). In the latter preparation, an extracellular Ca^{2+} concentration of 11 μM was sufficient to induce clathrin-mediated synaptic vesicle endocytosis (Gad et al., 1998). Synaptic vesicle recycling studied in synaptosomes also supports a Ca^{2+} requirement for endocytosis at a concentration lower than that required for maximal exocytosis (Marks and McMahon, 1998). In that study, Ca^{2+} entry acts as the trigger by activating calcineurin which dephosphorylates a number of proteins, including amphiphysin and dynamin, implicated in clathrin-mediated endocytosis (for reviews see De Camilli and Takei, 1996; Bauerfeind et al., 1998). Low extracellular Ca^{2+} allowed near maximal endocytosis after very submaximal exocytosis, leading to the speculation that the synaptic terminal membrane might always contain a pool of recoverable membrane with "exocytosis adding to this pool and endocytosis drawing from it" (Marks and McMahon, 1998; see below). Experiments at the NMJ of *Drosophila shibire*^{ts1} mutants suggest that any Ca^{2+}-requiring step in endocytosis occurs early, i.e., before the accumulation of endocytic membrane invaginations (Ramaswami et al., 1994). Secretory vesicle membrane retrieval by rapid endocytosis in adrenal chromaffin cells and melanotrophs also appears to be Ca^{2+}-dependent although it is not mediated by clathrin-coated vesicles (Thomas et al., 1994; Artalejo et al., 1995; Mansvelder and Kits, 1998).

In this study, endocytosed FM1-43 and HRP in control cultures are associated with recycled synaptic vesicles that are exocytosis-competent. Although tracer exocytosis is not possible in BoNT A–blocked terminals, HRP-labeled vesicles are observed docked (Figs. 8 c and 9 d). Further studies are required to confirm that the recycled vesicle membrane contains the appropriate complement of proteins and that the vesicles themselves contain neurotransmitter. Nonetheless, the present work stands as a direct demonstration of a Ca^{2+} requirement for endocytosis.

Membrane Source for Vesicle Recycling

In neuroendocrine cells, endocytosis does not always strictly follow exocytosis and, under certain circumstances, exocytosed membrane may reside in the plasma membrane until specific conditions drive retrieval (Thomas et al., 1990; Engisch, K.L., and M.C. Nowycky. *Soc. Neurosci.* 1996. 494.5; Hsu and Jackson, 1996; Smith and Neher, 1997). However, immunofluorescence fails to detect synaptic vesicle proteins on the terminal plasmalemma of the NMJ under resting conditions (von Wedel et al., 1981; Valtorta et al., 1988; Torri-Tarelli et al., 1990). Similar studies in rat hippocampal cell cultures, using antibodies directed against the lumenal NH_2 terminus of synaptotagmin I (Syt_{lum}-Abs) (Matteoli et al., 1992; Kraszewski et al., 1995) also demonstrated a lack of surface membrane signal, even after depolarization for several minutes (Mundigl et al., 1995). However, radioimmunoassays using [^{125}I]Syt_{lum}-Abs provide quantitative data demonstrating a significant pool of synaptotagmin I at the cell surface even at steady state (Mundigl et al., 1995). This pool comprises about half the synaptotagmin measured after K^+ depolarization for 3 min. Given that antibody binding reflects the presence of synaptic vesicle membrane, it could be inferred that a sizable membrane reserve exists in the surface membrane of the synaptic terminal. This membrane reserve, then, could serve as the source of membrane internalized in BoNT A–treated neurons.

The present results suggest that TeNT may disrupt a component of the reuptake mechanism that is unaffected by BoNT A. VAMP-2 is recognized by AP3 for the forma-

tion of synaptic vesicles from endosomes of PC12 cells. Tetanus toxin inhibited the formation of synaptic vesicles and their coating with AP3 in vitro, whereas BoNTs C and E had no effect (Salem et al., 1998). If VAMPs were similarly a sorting signal for synaptic vesicle endocytosis in the nerve terminal, TeNT might prevent the retrieval of vesicle membrane, whereas BoNT A would not.

Pathway for Vesicle Recycling

The synaptic vesicle recycling pathway described at the NMJ involves vesicle collapse into the plasmalemma and membrane retrieval, away from the active zone, by clathrin-coated pits and vesicles, the latter fusing to form intermediate cisternal compartments that bud new synaptic vesicles (Heuser and Reese, 1973). The formation of cisternae by the coalescence of small endocytic vesicles (Heuser and Reese, 1973; but see also Takei et al., 1996), implies a homotypic fusion between recycling, but not mature, vesicles that might involve SNARE proteins (Rothman and Warren, 1994) or their equivalent that are not affected by BoNT A. Alternatively, the cisternae may be sections of tubular invaginations of the plasma membrane formed by bulk endocytosis and not the products of vesicle fusion at all (De Camilli and Takei, 1996).

Other aspects of this recycling pathway have been challenged with evidence suggesting that membrane retrieval at the NMJ by clathrin-coated vesicles occurs after only high frequency stimulation, and that the physiologic pathway for exo-endocytosis involves the formation of a fusion pore and rapid retraction of the vesicle (Ceccarelli et al., 1973; for review see Fesce et al., 1994). Electron microscopy of giant synapses in which endocytosis has been blocked by compromising dynamin function demonstrate the accumulation of clathrin-coated pits after either a low or high frequency stimulation (Shupliakov et al., 1997). Thus, it appears that clathrin plays a role in a single process that operates to recycle synaptic vesicles. Furthermore, recycling kinetics are similar after high and low frequencies and appear sufficiently slow to allow for vesicle collapse and membrane retrieval and sorting through an intermediate endosomal compartment (Ryan et al., 1997), although involvement of this intermediate compartment remains in controversy (Murthy and Stevens, 1998).

Vesicle recycling studies (Henkel and Betz, 1995) using FM1-43 at the NMJ under the influence of staurosporine have been reconciled with the fusion pore model of exocytosis. Despite the similarity of some of the staurosporine data with those obtained using BoNT A, there are a number of important differences. First, there is no evidence for neurotransmitter release; i.e., a membrane fusion event, from BoNT A–exposed motor endplates (Dreyer et al., 1987) or spinal cord cultures (Bigalke et al., 1985; Williamson et al., 1996, and this work). Second, depolarization of BoNT A–blocked neuronal terminals results in labeling of synaptic vesicles with HRP; and third, repeated intervals of stimulation before HRP incubation result in a rundown of endocytosis, suggesting a finite source of retrievable surface membrane. Our observation of HRP labeling of coated vesicles and membrane cisternae in BoNT A–treated neurons, after K^+-induced depolarization in the presence of extracellular Ca^{2+}, is entirely consistent with the collapse of synaptic vesicles into the plasma membrane and subsequent membrane retrieval by endocytic mechanisms.

That synaptic vesicle recycling occurs as unitary quantal events, which are temporally linked to single action potentials (Ryan et al., 1997), is indisputable evidence that the coupling between vesicle exo- and endocytosis is highly regulated. Membrane retrieval appears to be driven by something other than membrane incorporation (for review see Thomas et al., 1994). Further studies aimed at understanding how synaptic vesicle endocytosis occurs during BoNT A, and not during TeNT, blockade will undoubtedly provide insight into this basic mechanism and may lead to the identity of the trigger and the cascade of events it initiates.

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