Ca^{2+} or Sr^{2+} Partially Rescues Synaptic Transmission in Hippocampal Cultures Treated with Botulinum Toxin A and C, But Not Tetanus Toxin

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Botulinum (BoNT/A–G) and tetanus toxins (TeNT) are zinc endopeptidases that cleave proteins associated with presynaptic terminals (SNAP-25, syntaxin, or VAMP/synaptobrevin) and block neurotransmitter release. Treatment of hippocampal slice cultures with BoNT/A, BoNT/C, BoNT/E, or TeNT prevented the occurrence of spontaneous or miniature EPSCs (sEPSCs or mEPSCs) as well as the [Ca^{2+}]$_o$-independent increase in their frequency induced by phorbol ester, 0.5 nM α-latrotoxin, or sucrose. [Ca^{2+}]$_o$-independent and -dependent release thus requires that the target proteins of clostridial neurotoxins be uncleaved. In contrast, significant increases in mEPSC frequency were produced in BoNT-treated, but not TeNT-treated, cultures by application of the Ca^{2+} ionophore ionomycin in the presence of 10 mM [Ca^{2+}]$_o$. The frequency of sEPSCs was increased in BoNT-treated, but not TeNT-treated, cultures by increasing [Ca^{2+}]$_o$ from 2.8 to 5–10 mM or by applying 5 mM Sr^{2+}. Large Ca^{2+} and Sr^{2+} influxes thus can rescue release after BoNT treatment, albeit less than in control cultures. The nature of the toxin-induced modification of Ca^{2+}-dependent release was assessed by recordings from monosynaptically coupled CA3 cell pairs. The paired-pulse ratio of unitary EPSCs evoked by two presynaptic action potentials in close succession was 0.5 in control cultures, but it was 1.4 and 1.2 in BoNT/A- or BoNT/C-treated cultures when recorded in 10 mM [Ca^{2+}]$_o$. Log–log plots of unitary EPSC amplitude versus [Ca^{2+}]$_o$ were shifted toward higher [Ca^{2+}]$_o$ in BoNT/A- or BoNT/C-treated cultures, but their slope was unchanged and the maximal EPSC amplitudes were reduced. We conclude that BoNTs reduce the Ca^{2+} sensitivity of the exocytotic machinery and the number of quanta released.

Key words: Ca^{2+}; clostridial neurotoxins; exocytosis; α-latrotoxin; protein kinase; transmitter release

Synaptic vesicle exocytosis is accomplished by a series of protein interactions in the axon terminal (for review, see Schweizer et al., 1995; Südhof, 1995). The so-called synaptosomal-associated receptor (SNARE) proteins include the vesicle-associated membrane proteins (v-SNAREs), vesicle-associated membrane protein (VAMP)/synaptobrevin and synaptotagmin, and the plasma-associated target proteins (t-SNAREs), synaptosomal-associated protein (SNAP-25) and syntaxin. VAMP/synaptobrevin, syntaxin, and SNAP-25 form a tight heterotrimeric complex *in vitro* that has been proposed to be essential for exocytosis (Söllner et al., 1993a,b). Evidence that the SNARE proteins are required for neurotransmitter release comes from studies showing that tetanus toxin (TeNT) and the seven serotypes of botulinum neurotoxin (BoNT) block transmitter release by acting as zinc-dependent endopeptidases that proteolyse several SNARE proteins (for review, see Niemann et al., 1994; Schiavo et al., 1995). For example, TeNT cleaves the v-SNARE VAMP/synaptobrevin (Link et al., 1992; Schiavo et al., 1992), BoNT/A and BoNT/E cleave the t-SNARE SNAP-25 (Blasi et al., 1993a; Schiavo et al., 1993), and BoNT/C cleaves the t-SNARE syntaxin alone (Blasi et al., 1993b)

or together with SNAP-25 (Foran et al., 1996; Osen-Sand et al., 1996; Williamson et al., 1996).

Clostridial neurotoxins are powerful inhibitors of exocytosis from various non-neuronal cells and nerve terminals (for review, see Dolly et al., 1994; Poulain et al., 1995). How the cleavage of SNARE proteins by clostridial toxins causes this inhibition of release is not known. Large numbers of synaptic vesicles remain associated with presynaptic release sites after clostridial toxin treatment (Harris and Miledi, 1971; Hunt et al., 1994; Broadie et al., 1995). In addition, SNARE proteins cleaved by clostridial toxins remain capable of forming heterotrimeric complexes, and these can be dissociated by ATP, much like complexes formed by intact proteins (Hayashi et al., 1994; Pellegrini et al., 1995). If the docking of vesicles to the plasma membrane and the assembly of fusion complexes are not inhibited, some other physiological mechanism(s) must underlie the actions of clostridial toxins.

The fusion of neurotransmitter-containing vesicles with the plasma membrane can occur spontaneously or in response to action potential-induced Ca^{2+} influx (Katz, 1969). Spontaneous exocytosis occurs at a low rate in the absence of Ca^{2+} influx, and this rate is increased by elevations of [Ca^{2+}]$_i$ (Kita and Van der Kloot, 1974). A number of agents promote exocytosis by mechanisms that may not involve an increase in [Ca^{2+}]$_i$, however, including phorbol ester activators of protein kinase C (PKC) (Malenka et al., 1987; Gillis et al., 1996); the black widow spider venom component, α-latrotoxin (α-LTx) (Longenecker et al., 1970) (for review, see Rosenthal and Meldolesi, 1989); and hyperosmotic, sucrose-containing saline (Fatt and Katz, 1952) (for review, see Van der Kloot and Molgó, 1994). At the *Drosophila*

neuromuscular junction, syntaxin and VAMP/synaptobrevin must be present and intact to support α-LTx-induced, but not sucrose-induced, release (Broadie et al., 1995; Sweeney et al., 1995). In contrast, α-LTx may remain effective at clostridial toxin-treated mammalian neuromuscular junctions, but not sucrose-containing saline (Dreyer et al., 1987; Gansel et al., 1987). Unfortunately, the actions of secretagogues have not been studied extensively at toxin-treated vertebrate CNS synapses.

To address these issues, we incubated hippocampal slice cultures with clostridial neurotoxins and then examined the effects of this treatment on spontaneous glutamate release, on $[Ca^{2+}]_o$-dependent-evoked release, and on $[Ca^{2+}]_o$-independent release elicited by phorbol ester, sucrose, or α-LTx.

MATERIALS AND METHODS

Slice cultures, electrophysiology, and drug application. Organotypic hippocampal slice cultures were prepared from 6-d-old rat pups as described in detail elsewhere (Gähwiler, 1981). After 2–4 weeks *in vitro*, cultures were placed in a recording chamber on an inverted microscope and superfused with control saline containing (in mM): 137 NaCl, 2.7 KCl, 2.8 $CaCl_2$, 2.5 $MgCl_2$, 11.6 $NaHCO_3$, 0.4 NaH_2PO_4, and 5.6 glucose, pH 7.4, at 24°C. $SrCl_2$ was applied in Ca^{2+}-free saline. CA3 pyramidal cells were whole-cell voltage-clamped to -70 mV, using pipettes containing (in mM): 140 K-gluconate, 10 KCl, 5 HEPES, 2 $MgCl_2$, and 1.1 EGTA, pH 7.3. Cell capacitance and series resistance (10.0 ± 0.5 MΩ; $n = 50$, randomly selected) were monitored routinely. Miniature EPSCs (mEPSCs) and spontaneous EPSCs (sEPSCs) were acquired and analyzed as described previously (Capogna et al., 1996). Unitary EPSCs between pairs of monosynaptically connected CA3 pyramidal neurons were recorded as described previously (Debanne et al., 1996). The presynaptic cell was recorded in current-clamp mode with a 1 M $KMeSO_4$-filled sharp microelectrode, and action potentials were elicited with 20 msec depolarizing current pulses (0.1–0.2 Hz). The postsynaptic cell was whole-cell voltage-clamped to -70 mV. All statistical comparisons were performed with two-tailed paired or two sample *t* tests; numerical values are given as mean $\pm$ SEM.

Serum-free medium containing TeNT or BoNTs was applied in the test tube containing a slice culture under sterile conditions after >14 d *in vitro*. Then the culture was returned to the incubator for ~48 hr at 36°C before immunocytochemistry, immunoblotting, or electrophysiological recording was performed. α-LTx (Alomone Labs, Jerusalem, Israel) was applied focally by drop application (20 nl, 0.5 or 3 nM) after saline perfusion was stopped, as described elsewhere (Capogna et al., 1996). Tetrodotoxin (TTX, Sankyo, Tokyo, Japan), bicuculline methochloride (Fluka AG, Buchs, Switzerland), AMPA (Tocris Cookson, Bristol, UK), and phorbol 12,13-diacetate (PDAc) and ionomycin (LC Laboratories Europe, Läufelfingen, Switzerland) were bath-applied. BoNT/C was purchased from WAKO Chemicals (Neuss, Germany).

Immunocytochemistry. Hippocampal cultures were fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, washed in incubation medium (PB, 1.5% horse serum, 1.5% goat serum, and bovine serum albumin), and then left overnight in incubation medium containing Triton X-100 (0.4% v/v) at 4°C. A mouse anti-synaptophysin monoclonal antibody (SY38, 1:20 dilution; Boehringer Mannheim, Mannheim, Germany) was applied (84 hr at 4°C) in incubation medium containing 0.4% Triton X-100, together with anti-VAMP/synaptobrevin-2 (1:100 dilution), anti-SNAP-25 (1:200 dilution), or anti-syntaxin (1:200 dilution) affinity-purified antibodies from rabbit [provided by C. Montecucco, University of Padua, Italy; for detailed description of the epitopes recognized by these antibodies, see Rossetto et al. (1996) and Williamson et al. (1996)]. Cultures were washed in incubation medium, followed by application of a biotinylated anti-rabbit antibody (1:200 dilution; 3 hr at 22°C), and then washed again before using an anti-mouse antibody (1:150 dilution; 20 min at 22°C) directly conjugated to FITC to reveal the anti-synaptophysin antibodies. Cultures were washed again before avidin–Texas Red was applied to reveal the anti-SNARE antibodies. Cultures were mounted in Slow Fade (Molecular Probes, Portland, OR) and imaged on a laser scanning confocal microscope (Zeiss LSM-410, Oberkochen, Germany), using lasers tuned to 488 nm (FITC) and 543 nm (Texas Red) and a 100× objective (1.4 numerical aperture). Ten optical sections were collected from area CA3 at 0.2-μm-depth intervals, and three-dimensional reconstruction was made with IMARIS software (Bit-

plane AG, Zurich, Switzerland). Immunocytochemistry was performed "blind," and treated and untreated cultures were processed simultaneously. The mean pixel intensity and SD for each of the SNARE proteins were calculated from the original unprocessed optical section by Voxelshop software (Bitplane AG).

Immunoblotting. Four control or clostridial neurotoxin-treated cultures were pooled and washed three times in PBS containing a protease inhibitor cocktail (Complete, Boehringer Mannheim). The samples were solubilized in the same buffer containing Triton X-100 (1% v/v) for 1 hr before centrifugation at $80,000 \times g$ for 1 hr. Equal volumes of the supernatants were diluted into SDS-PAGE sample buffer, boiled for 5 min at 95°C, and analyzed on 12.5% polyacrylamide gels. After blotting, a protein stain of the nitrocellulose revealed no obvious differences in the amount or pattern of proteins loaded. The blots were probed by incubation with antibodies against synaptophysin (SY38, Boehringer Mannheim), SNAP-25 (SMI 81, Affiniti Research Products, Nottingham, UK), syntaxin (HPC1, Sigma, Deisenhofen, Germany), and VAMP/synaptobrevin-2 and synaptotagmin (kindly provided by M. Takahashi, Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan; for details, see Oho et al., 1995). Note that these antibodies recognize different epitopes of the SNARE proteins from those used for immunocytochemistry. Bound immunoreactivity was detected by ECL (Amersham, Little Chalfont, UK), as described previously (Pellegrini et al., 1995). The analysis was performed blind on three separate sets of cultures.

RESULTS

Clostridial neurotoxins cleave SNARE proteins in hippocampal slice cultures

Rat hippocampal slice cultures were incubated with TeNT (50–100 ng/ml, 45 ± 4 hr, $n = 23$), BoNT/A (50–100 ng/ml, 55 ± 2 hr, $n = 44$), BoNT/C (50–100 ng/ml, 50 ± 2 hr, $n = 34$), or BoNT/E (1 μg/ml, 59 ± 5 hr, $n = 6$). The effectiveness of the neurotoxin treatment was examined with immunocytochemistry and with Western blots (Fig. 1). VAMP/synaptobrevin-2 immunoreactivity was virtually absent in TeNT-treated cultures. In BoNT/A-treated cultures SNAP-25 was immunocytochemically undetectable with an antibody directed against the C-terminal portion of the molecule (Williamson et al., 1996), and Western blot analysis revealed a decrease in the molecular weight of essentially all SNAP-25. BoNT/C treatment abolished SNAP-25 and syntaxin immunocytochemical staining, strongly reduced the amount of uncleaved syntaxin in the immunoblots, and decreased the molecular weight of the majority of SNAP-25, as detected by using another antibody that recognizes BoNT-cleaved SNAP-25. In contrast, in toxin-treated cultures, synaptic terminals remained densely stained with the anti-synaptophysin antibody, suggesting that synaptic vesicles were retained in the terminals of treated cultures. The amount of synaptophysin immunoreactivity appeared to be reduced slightly in TeNT-treated cultures; however, none of the neurotoxins affected the amount of the synaptic vesicle protein synaptotagmin in immunoblots (data not shown). TeNT thus cleaved VAMP/synaptobrevin-2, BoNT/A cleaved SNAP-25, and BoNT/C cleaved syntaxin and SNAP-25 in hippocampal slice cultures, consistent with previous reports in isolated synaptic vesicles, synaptosomes, and cultured cells (Foran et al., 1996; Osen-Sand et al., 1996; Williamson et al., 1996) (for review, see Schiavo et al., 1995).

Clostridial neurotoxins block basal and $[Ca^{2+}]_o$-independent release

Incubation of cultures with clostridial neurotoxins invariably blocked glutamate release in control saline. The frequency of mEPSCs recorded from CA3 pyramidal cells in the presence of 0.5 μM TTX and 40 μM bicuculline methochloride (to block action potential-dependent release and $GABA_A$ receptors, respectively) was reduced significantly by all toxins relative to
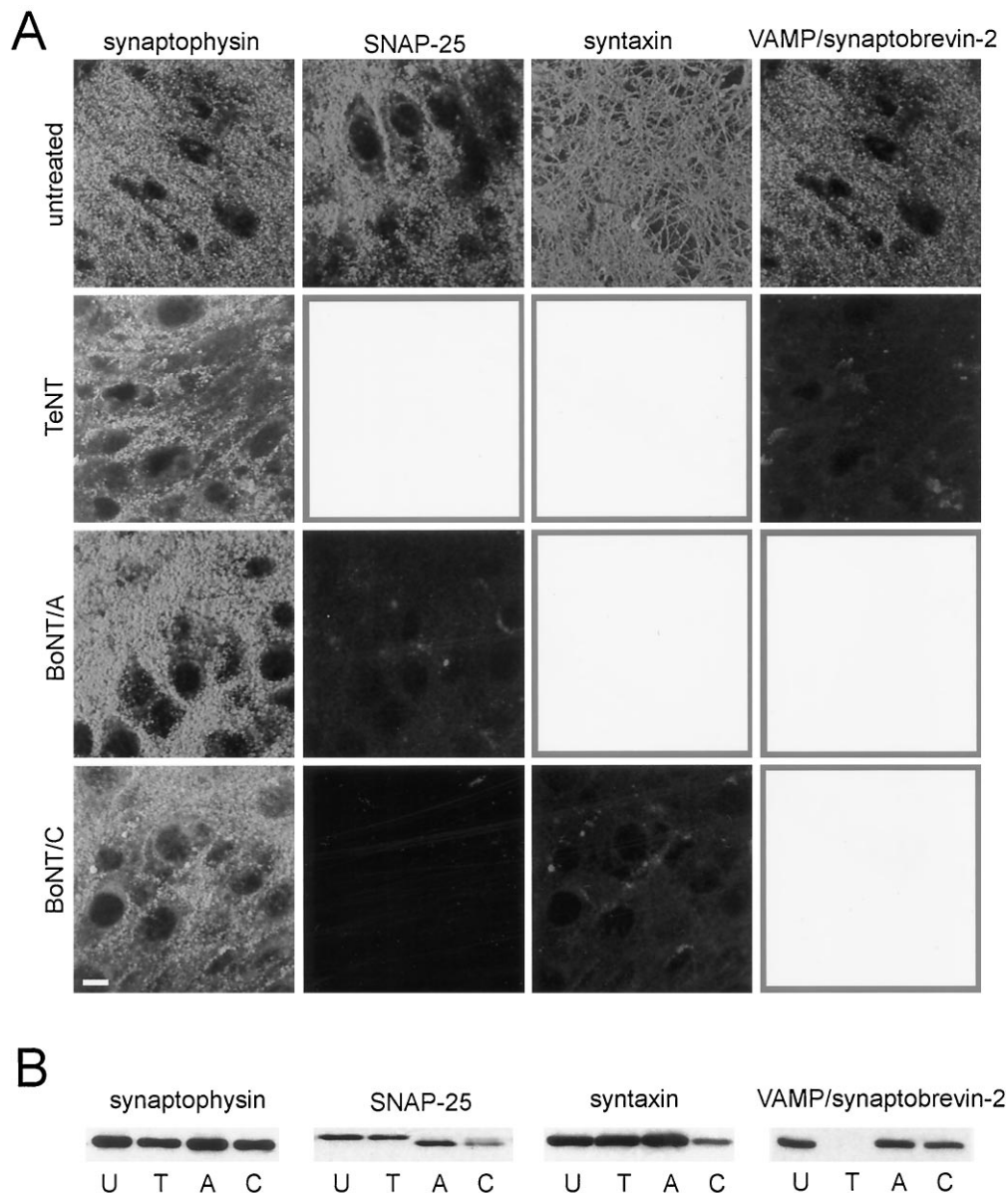
Figure 1. Clostridial neurotoxins effectively cleave SNAREs. Control and clostridial toxin-treated hippocampal slice cultures were labeled with antibodies directed against SNAREs and the synaptic vesicle marker synaptophysin, as indicated, or subjected to immunoblot analysis. *A,* Immunocytochemical staining for SNAREs was abolished in large part in toxin-treated cultures, whereas staining for the synaptic vesicle-associated protein synaptophysin remained. Cytotoxic effects of toxin treatment were not apparent (cf. Osen-Sand et al., 1996). Scale bar, 10 μm. Autofluorescence of macrophages can be seen in toxin-treated cultures stained for SNAREs. Mean pixel intensity and SD were calculated for each of the SNARE proteins from the unprocessed original data sets. The mean pixel intensities for both the treated and untreated cultures for the different SNARE proteins are as follows: *SNAP-25*—untreated, 59.23 ± 34.33; BoNT/A, 18.86 ± 11.68; BoNT/C, 11.83 ± 6.65; *syntaxin*—untreated, 60.09 ± 29.03; BoNT/C, 19.53 ± 13.81; *VAMP/synaptobrevin-2*—untreated, 56.19 ± 29.79; TeNT, 16.66 ± 12.39. *B,* VAMP/synaptobrevin-2 and syntaxin immunoreactivity was absent or strongly reduced in Western blots of TeNT toxin-treated (*T*) and BoNT/C-treated (*C*) cultures, respectively, as compared with untreated control cultures (*U*), whereas synaptophysin immunoreactivity was present. BoNT/A (*A*) and BoNT/C treatment reduced the molecular weight of SNAP-25.

control cultures ($p < 0.001$) (Table 1; compare Fig. 2*A* vs Figs. 3 and 5*A–C*). Furthermore, the frequency of action potential-dependent sEPSCs recorded in control saline (without TTX) also was reduced significantly by all neurotoxins ($p < 0.001$) (Table 1; compare Fig. 2*B–D* vs Figs. 4 and 5*D–F*).

The toxins had no effect on postsynaptic glutamate sensitivity, as indicated by their lack of effect on mean mEPSC amplitudes (control, 13.3 ± 0.7 pA, $n = 32$; TeNT, 16 ± 1.3 pA, $n = 12$; BoNT/A, 14 ± 1.2 pA, $n = 24$; BoNT/C, 12.6 ± 1.3 pA, $n = 14$; $p > 0.05$ vs control for all toxins), or on the amplitude of inward

currents elicited by application of AMPA (30 sec, 1 μM) (control, 147 ± 12 pA, $n = 5$; TeNT, 167 ± 31 pA, $n = 5$; $p > 0.05$). In untreated cultures the mean amplitude of sEPSCs was greater than the mean amplitude of mEPSCs (sEPSCs 18.6 ± 1.8 pA, $n = 16$; mEPSCs 13.3 ± 0.7 pA, $n = 32$; $p < 0.001$), consistent with a significant contribution of action potential-induced multiquantal events in the absence of TTX. In contrast, there was no significant difference in the amplitude of spontaneous events before and after application of TTX to clostridial neurotoxin-treated cultures, consistent with an inhibition of action potential-evoked

Table 1. Effects of clostridial neurotoxins on hippocampal synaptic transmission

	Control		TeNT		BoNT/A		BoNT/C	
	Before	After	Before	After	Before	After	Before	After
mEPSC frequency (Hz)								
Control	0.92 ± 0.10 (45)°	–	0.11 ± 0.02 (14)[#]	–	0.07 ± 0.01 (24)[#]	–	0.04 ± 0.01 (15)‖	–
PDAc	1.50 ± 0.22	7.40 ± 0.90 (8)‡	0.11 ± 0.03	0.18 ± 0.06 (9)*	0.05 ± 0.01	0.06 ± 0.02 (7)*	0.07 ± 0.04	0.08 ± 0.04 (6)*
α-LTx (0.5 nM)	0.79 ± 0.14	11.16 ± 1.67 (26)‡	0.13 ± 0.04	0.11 ± 0.05 (6)*	0.10 ± 0.04	0.11 ± 0.05 (7)*	0.05 ± 0.02	0.07 ± 0.02 (8)*
α-LTx (3 nM/10 mM Ca)	–	–	0.15 ± 0.09	0.14 ± 0.10 (3)*	0.06 ± 0.02	6.70 ± 1.20 (4)§§	0.09 ± 0.03	8.07 ± 3.50 (3)*
Ionomycin (2.8 mM Ca)	1.07 ± 0.15	7.03 ± 1.96 (15)§§	0.13 ± 0.05	0.16 ± 0.08 (6)*	0.07 ± 0.03	0.14 ± 0.10 (5)*	0.09 ± 0.02	0.18 ± 0.06 (5)*
Ionomycin (10 mM Ca)	–	–	0.11 ± 0.04	0.64 ± 0.33 (4)*	0.05 ± 0.02	3.11 ± 0.79 (8)§§	0.09 ± 0.02	2.65 ± 0.74 (5)§
sEPSC frequency (Hz)								
Control	1.68 ± 0.25 (18)°	–	0.09 ± 0.01 (32)[#]	–	0.09 ± 0.01 (41)[#]	–	0.09 ± 0.02 (22)‖	–
5 mM Ca/0.5 mM Mg	1.17 ± 0.17	4.71 ± 0.84 (7)†	0.10 ± 0.02	0.10 ± 0.04 (9)*	0.09 ± 0.02	0.91 ± 0.23 (15)†	0.09 ± 0.03	1.25 ± 0.32 (9)§§
10 mM Ca/0.5 mM Mg	2.12 ± 0.33	19.12 ± 4.03 (4)§	0.10 ± 0.02	0.69 ± 0.29 (8)*	0.11 ± 0.02	4.24 ± 1.36 (10)§§	0.08 ± 0.02	2.54 ± 0.37 (10)‡
2.8 mM Sr/2.5 mM Mg	1.45 ± 0.25	3.45 ± 0.72 (6)§	0.10 ± 0.03	0.05 ± 0.01 (3)*	0.09 ± 0.05	0.62 ± 0.12 (7)§§	0.14 ± 0.06	1.04 ± 0.28 (5)§
5 mM Sr/0.5 mM Mg	1.45 ± 0.25	7.30 ± 1.70 (6)†	0.09 ± 0.02	0.05 ± 0.01 (6)*	0.09 ± 0.04	1.13 ± 0.33 (5)§	0.16 ± 0.06	1.83 ± 0.58 (6)§
7.5 mM Sr/0.5 mM Mg	–	–	0.12 ± 0.03	0.15 ± 0.01 (4)*	–	–	–	–
Sucrose	1.80 ± 0.20	11.07 ± 1.07 (3)§§	0.07 ± 0.03	0.05 ± 0.01 (5)*	0.08 ± 0.01	0.09 ± 0.06 (3)*	0.03 ± 0.01	0.03 ± 0.02 (3)*

Significance assessed relative to values in same cells before treatment: *not significantly different ($p > 0.05$); §$p < 0.05$; §§$p < 0.01$; †$p < 0.005$; ‡$p < 0.001$; –, not determined. Significance of sEPSC vs mEPSC frequency: [#]not significantly different ($p > 0.1$); ‖$p < 0.04$; °$p > 0.001$.

The number of observations is given in parentheses. The effects of α-LTx and ionomycin on mEPSC frequency in control cultures include data from Capogna et al. (1996).

transmitter release (TeNT—sEPSCs 17.7 ± 1.1 pA, $n = 32$; mEPSCs 16.0 ± 1.3 pA, $n = 12$; $p > 0.1$; BoNT/A—sEPSCs 16.4 ± 1.0 pA, $n = 41$; mEPSCs 14.0 ± 1.2 pA, $n = 24$; $p > 0.1$; BoNT/C—sEPSCs 13.9 ± 1.5 pA, $n = 22$; mEPSCs 12.6 ± 1.3 pA, $n = 14$; $p > 0.5$). Further support for this conclusion is that the frequency of spontaneous synaptic currents in toxin-treated cultures in large part was unaffected by TTX (Table 1).

α-LTx was found previously to be able to trigger glutamate release from hippocampal slice cultures in a $[Ca^{2+}]_o$-independent manner when applied at <1 nM (Capogna et al., 1996). In addition, PDAc (3 μM) produced an increase in mEPSC frequency in untreated cultures that also was unaffected by removal of Ca^{2+} from the extracellular saline (1.7 ± 0.4 Hz before and 9.2 ± 0.9 Hz 5 min after application of 3 μM PDAc, and 8.9 ± 1.3 Hz 3 min after subsequent application of Ca^{2+}-free/1 mM EGTA-containing saline, $n = 3$; Fig. 2A). After treatment with BoNT/A, BoNT/C, or TeNT, however, the frequency of mEPSCs was not increased by application of either 3 μM PDAc (Figs. 3A, 5A) or 0.5 nM α-LTx (Figs. 3C, 5C).

The effects of clostridial toxins on neurotransmitter release were dependent on the concentration and time of toxin exposure. In cultures treated with 50 ng/ml BoNT/C for only 18–21 hr, for example, the frequency of mEPSCs was 0.6 ± 0.25 Hz, and subsequent application of 3 μM PDAc increased the frequency of mEPSCs to 3.43 ± 0.29 Hz ($n = 3$; compare Table 1). Positive correlations between the extent of reduction of the frequency of sEPSCs or mEPSCs and the amount of immunocytochemically detectable uncleaved SNAREs also were found (data not shown).

TeNT, BoNT/A, or BoNT/C thus were equally effective in blocking glutamate release as well as in the ability of phorbol esters and α-LTx to induce $[Ca^{2+}]_o$-independent exocytosis in hippocampal cultures. All of these forms of exocytosis thus depend on the integrity of VAMP/synaptobrevin, SNAP-25, and syntaxin. Finally, these results provided a positive control that the

toxins had impaired release from virtually all of the excitatory synaptic terminals within the treated cultures.

Large influxes of Ca²⁺ or Sr²⁺ rescues transmitter release in BoNT/A- or BoNT/C-treated cultures

The block of transmitter release in control saline could be overcome by inducing large increases in intraterminal $[Ca^{2+}]$ via several means in BoNT/A- or BoNT/C-treated, but not in TeNT-treated, cultures (Table 1).

Application of the Ca^{2+} ionophore ionomycin (2.5 μM) in control saline increased mEPSC frequency in untreated (Capogna et al., 1996), but not in clostridial neurotoxin-treated, cultures. If the concentration of Ca^{2+} in the extracellular saline was increased from 2.8 to 10 mM during ionomycin application, however, then significant increases in mEPSC frequency were produced in BoNT/A- or BoNT/C-treated cultures, but not in TeNT-treated cultures (Figs. 3B, 5B). The frequency of mEPSCs under these conditions nevertheless was lower than the frequency of mEPSCs in control cultures after application of ionomycin in control saline. In saline containing 10 mM Ca^{2+}, increases in mEPSC frequency also could be produced in BoNT/A- or BoNT/C-treated, but not TeNT-treated, cultures by applying a high concentration of α-LTx (3 nM, Figs. 3C, 5C), which triggers release from hippocampal cultures in a $[Ca^{2+}]_o$-dependent manner (Capogna et al., 1996).

We next examined whether increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels also could restore action potential-mediated sEPSCs in the absence of TTX. In control cultures a significant elevation of the sEPSC frequency (Table 1; Fig. 2B) was produced by increasing release probability with saline containing 5 or 10 mM Ca^{2+}/0.5 mM Mg^{2+}. In BoNT/A- or BoNT/C-treated cultures, sEPSC frequency also was increased by saline containing 5 or 10 mM Ca^{2+}/0.5 mM Mg^{2+} (Table 1; Figs. 4A2, 5D). In TeNT-treated cultures, in contrast, no significant
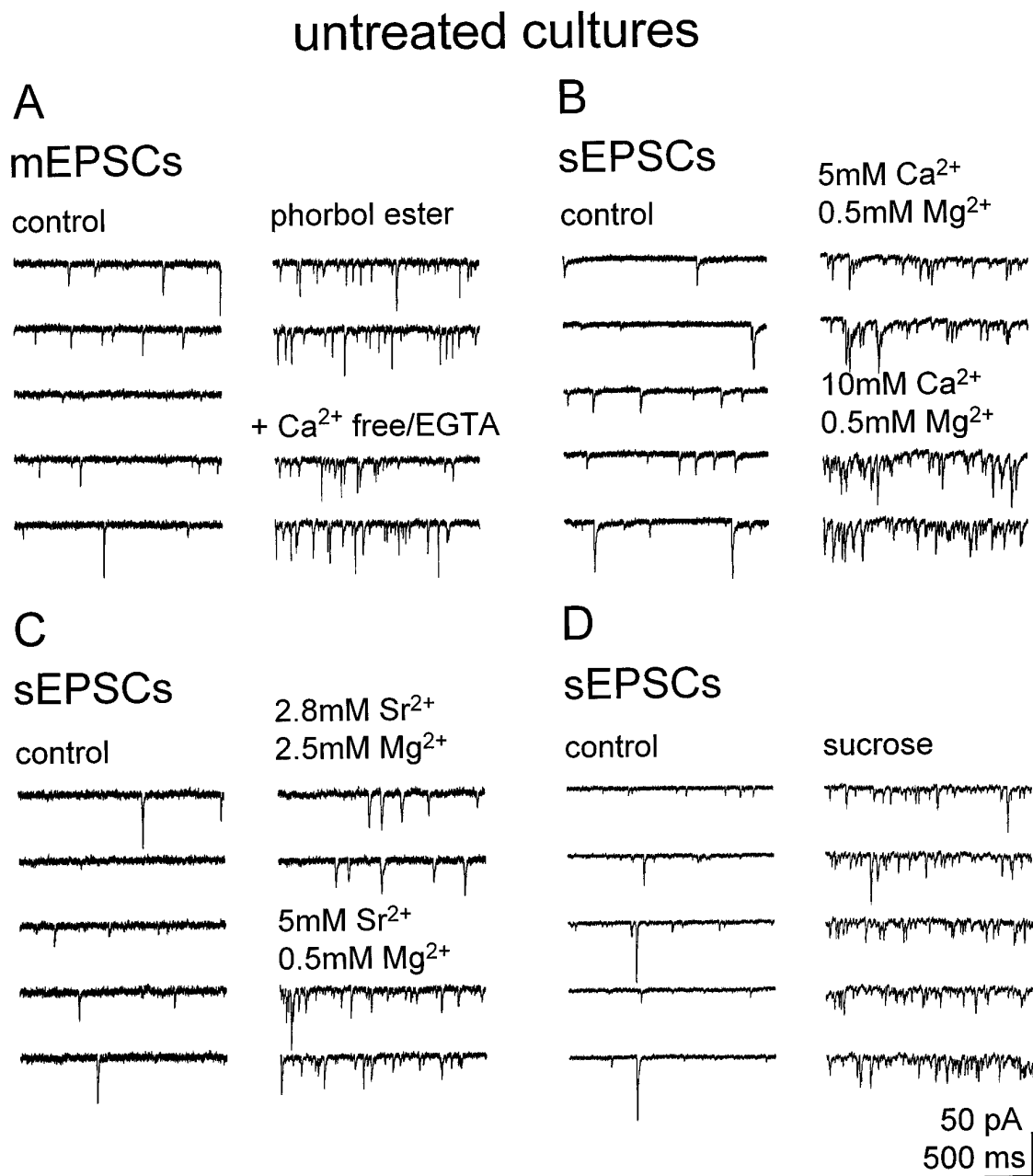
untreated cultures

Figure 2. In untreated cultures the frequency of mEPSCs is enhanced by phorbol ester, and the frequency of sEPSCs is increased by saline containing 5 or 10 mM Ca^{2+}/0.5 mM Mg^{2+}, 2.8 or 5 mM Sr^{2+}/0.5 mM Mg^{2+}, or sucrose. Each sweep shows a continuous recording before and after application of 3 µM PDAc (*A, phorbol ester*), 5 or 10 mM Ca^{2+}/0.5 mM Mg^{2+}, 2.8 or 5 mM Sr^{2+}/0.5 mM Mg^{2+}, or sucrose (100 mM). *A,* PDAc increased mEPSC frequency in a $[Ca^{2+}]_o$-independent manner in untreated cultures (2.2 Hz before PDAc, 8.1 Hz after PDAc, and 8.5 Hz after subsequent application of Ca^{2+}-free/1 mM EGTA-containing saline). *B, C,* Both 5 or 10 mM Ca^{2+}/0.5 mM Mg^{2+} and 2.8 or 5 mM Sr^{2+}/0.5 mM Mg^{2+} increased sEPSC frequency in untreated cultures (1.6 Hz before, and 8.7 and 21.2 Hz after 5 and 10 mM Ca^{2+}, respectively; 1.8 Hz before, and 2.6 and 7.9 Hz after 2.8 and 5 mM Sr^{2+}, respectively). *D,* Application of 100 mM sucrose enhanced sEPSC frequency in control cultures (1.6 Hz before and 13.2 Hz after sucrose). Data on the increases in mEPSC frequency induced by ionomycin and α-LTx in control cultures are reported in Capogna et al. (1996).

increases in sEPSC frequency were observed after raising $[Ca^{2+}]_o$ (Table 1; Fig. 4*A1*). We conclude that the rescue of release can occur regardless of the means of Ca^{2+} entry.

Removal of extracellular Ca^{2+} and application of saline containing 5 mM Sr^{2+}/0.5 mM Mg^{2+} induce desynchronized release (Table 1; Fig. 2*C*), perhaps by activating a distinct Ca^{2+} sensor protein (Goda and Stevens, 1994; Li et al., 1995). We therefore tested the ability of Sr^{2+} to rescue release in toxin-treated cul-

tures. Indeed, replacement of extracellular Ca^{2+} with Sr^{2+} resulted in an increase in sEPSC frequency in BoNT/A- or BoNT/C-treated cultures, but not in TeNT-treated cultures (Table 1; Figs. 4*B1,B2*, 5*E*). Both Ca^{2+} and Sr^{2+} thus were able to rescue release after cleavage of SNAP-25 alone or together with SNAP-25.

Increasing the osmolarity of the extracellular saline by adding 100 mM sucrose also increased sEPSC frequency in control cul-
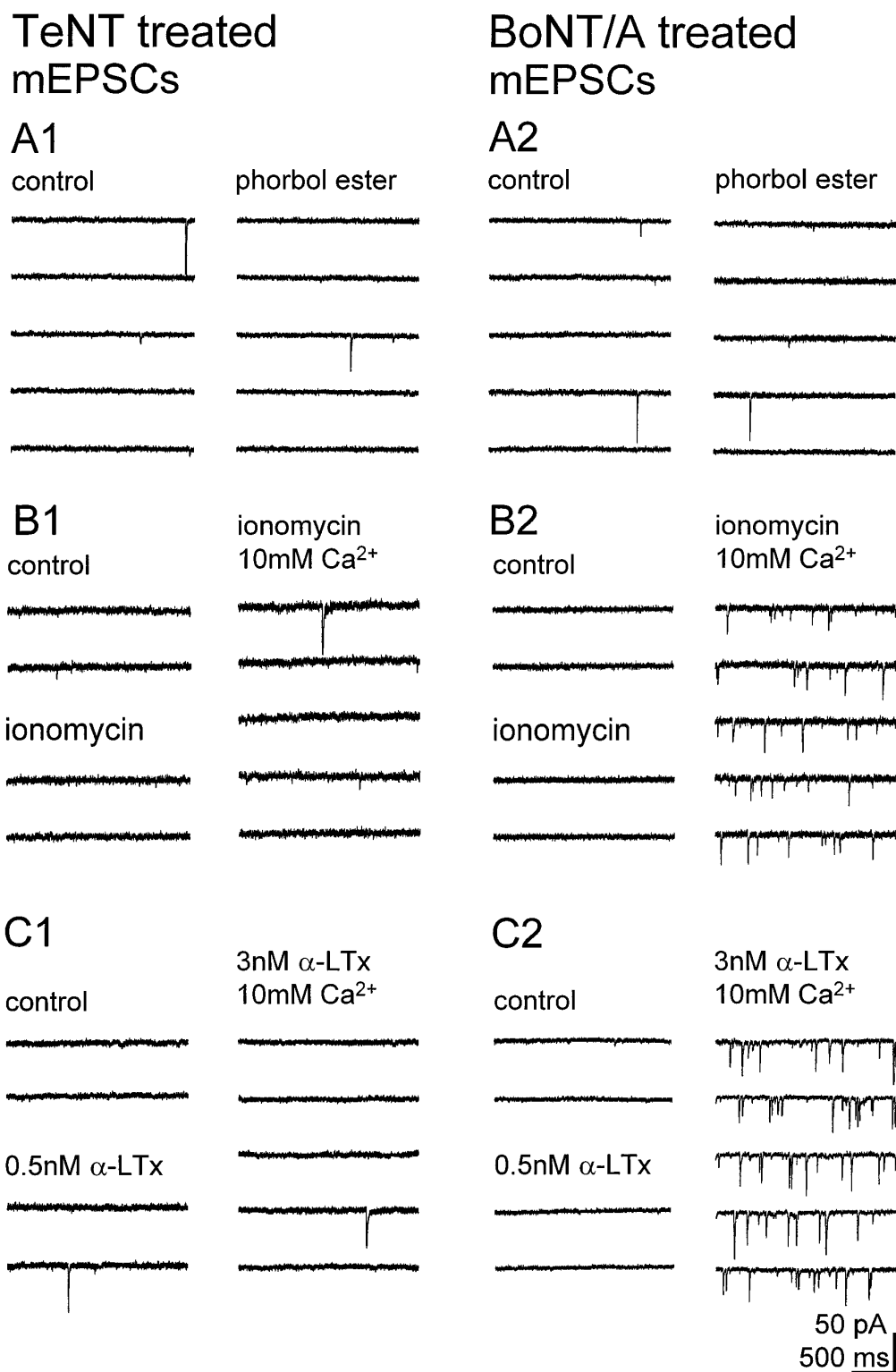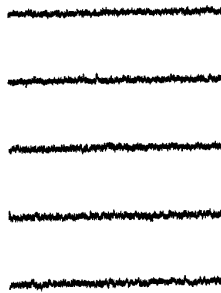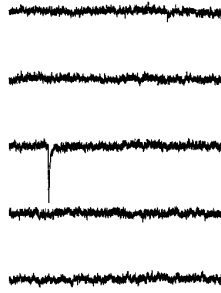
Figure 3. The frequency of mEPSCs is very low in clostridial toxin-treated cultures but is increased by large Ca^{2+} influx into the terminal in BoNT/A-treated, but not TeNT-treated, cultures. Each sweep shows a continuous recording before and after application of 3 μM PDAc (*A, phorbol ester*), the Ca^{2+} ionophore ionomycin (*B*), and α-LTx (*C*). *A*, PDAc did not increase mEPSC frequency in either TeNT- or BoNT/A-treated cultures (0.1 Hz before and 5 min after PDAc; 0.07 Hz before and 0.06 Hz 5 min after PDAc, respectively). *B1, C1,* Neither 2.5 μM ionomycin applied in control or 10 mM Ca^{2+} containing saline nor 0.5 nM α-LTx applied in control saline or 3 nM α-LTx applied in 10 mM Ca^{2+} increased mEPSC frequency in TeNT-treated cultures (0.06 Hz in control, 0.07 Hz 5 min after ionomycin in 2.8 mM Ca^{2+}, and 0.11 Hz 5 min after ionomycin in 10 mM Ca^{2+}; 0.06 Hz in control, 0.05 Hz 5 min after 0.5 nM α-LTx in 2.8 mM Ca^{2+}, and 0.05 Hz 5 min after 3 nM α-LTx in 10 mM Ca^{2+}). *B2, C2,* Ionomycin and α-LTx increased mEPSC frequency only after $[Ca^{2+}]_o$ was raised from 2.8 (control) to 10 mM in BoNT/A-treated cultures (0.04 Hz in control, 0.07 Hz 5 min after ionomycin in 2.8 mM Ca^{2+}, and 3.6 Hz 5 min after ionomycin in 10 mM Ca^{2+}; 0.06 Hz in control, 0.05 Hz 5 min after 0.5 nM α-LTx in 2.8 mM Ca^{2+}, and 5.2 Hz 5 min after 3 nM α-LTx in 10 mM Ca^{2+}).

TeNT treated
sEPSCs

BoNT/A treated
sEPSCs

A1
control   10mM Ca^{2+}
                       0.5mM Mg^{2+}

A2
control   10mM Ca^{2+}
                       0.5mM Mg^{2+}

B1
control   5mM Sr^{2+}
                       0.5mM Mg^{2+}
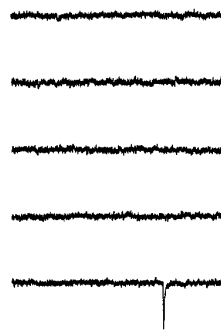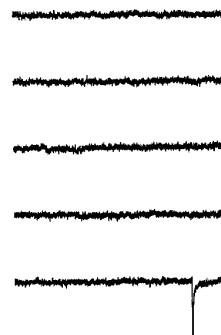
B2
control   5mM Sr^{2+}
                       0.5mM Mg^{2+}

C1
control   sucrose
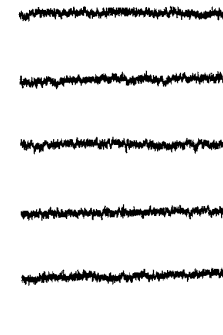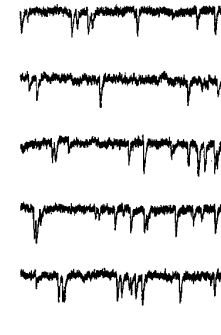
C2
control   sucrose
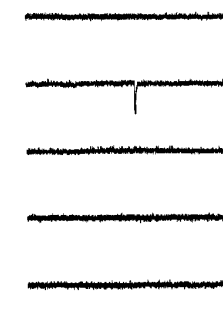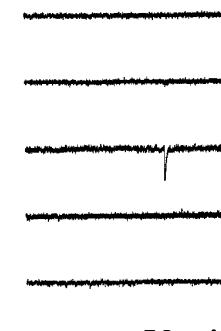
50 pA
500 ms

Figure 4. The frequency of sEPSCs is very low in clostridial neurotoxin-treated cultures and is increased by large Ca^{2+} or Sr^{2+} influxes in BoNT/A-treated, but not TeNT-treated, cultures. Each sweep shows a continuous recording before and after application of saline containing 10 mM Ca^{2+}/0.5 mM Mg^{2+} (*A*, high [Ca^{2+}]$_o$), saline containing 5 mM Sr^{2+}/0.5 mM Mg^{2+} (*B*, high [Sr^{2+}]$_o$), or sucrose (100 mM). *A1, B1,* Neither high [Ca^{2+}]$_o$ nor high [Sr^{2+}]$_o$ increased sEPSC frequency in TeNT-treated cultures (0.09 Hz before and 5 min after high [Ca^{2+}]$_o$; 0.14 Hz before and 0.09 Hz 5 min after high [Sr^{2+}]$_o$). *A2, B2,* Both high [Ca^{2+}]$_o$ and high [Sr^{2+}]$_o$ increased sEPSC frequency in BoNT/A-treated cultures (0.12 Hz before and 4.6 Hz 5 min after high [Ca^{2+}]$_o$; 0.04 Hz before and 2.4 Hz 5 min after high [Sr^{2+}]$_o$). *C1, C2,* Application of 100 mM sucrose did not increase sEPSC frequency in TeNT- or BoNT/A-treated cultures (0.06 Hz before and 5 min after sucrose, and 0.05 Hz before and 5 min after sucrose, respectively).

BoNT/C treated

mEPSCs

A
control   phorbol ester

B
control   ionomycin

ionomycin,
10mM Ca²⁺

C
control   0.5nM α-LTx

3nM α-LTx,
10mM Ca²⁺

sEPSCs

D
control   5mM Ca²⁺
0.5mM Mg²⁺

10mM Ca²⁺
0.5mM Mg²⁺

E
control   2.8mM Sr²⁺ /
2.5mM Mg²⁺

5mM Sr²⁺
0.5mM Mg²⁺

F
control   sucrose

50 pA
500 ms

Figure 5. In BoNT/C-treated cultures the low frequency of sEPSCs or mEPSCs is increased only by large Ca^{2+} or Sr^{2+} influx into the axon terminal. *A*, PDAc (3 μM) did not increase mEPSC frequency in BoNT/C-treated cultures (0.05 Hz before and 0.03 Hz 5 min after PDAc). *B, C,* Ionomycin and α-LTx each increased mEPSC frequency only after $[Ca^{2+}]_o$ was raised from 2.8 (control) to 10 mM in BoNT/C-treated cultures (0.13 Hz in control, 0.36 Hz 5 min after ionomycin in 2.8 mM Ca^{2+}, and 5.3 Hz 5 min after ionomycin in 10 mM Ca^{2+}; 0.08 Hz in control, 0.05 Hz 5 min after 0.5 nM α-LTx in 2.8 mM Ca^{2+}, and 3.3 Hz 5 min after 3 nM α-LTx in 10 mM Ca^{2+}). *D,* Raising $[Ca^{2+}]_o$ from 2.8 mM (control) to 5 or 10 mM and decreasing $[Mg^{2+}]_o$ from 2.5 mM to 0.5 mM increased sEPSC frequency in BoNT/C-treated cultures (0.05 Hz control, 0.5 Hz 5 min after 5 mM Ca^{2+}, and 3.9 Hz 5 min after 10 mM Ca^{2+}). *E,* Application of either 2.8 mM Sr^{2+}/2.5 mM Mg^{2+} or 5 mM Sr^{2+}/0.5 mM Mg^{2+} containing saline increased sEPSC frequency in BoNT/C-treated cultures (0.04 Hz control, 0.85 Hz 5 min after 2.8 mM Sr^{2+}, and 1.73 Hz 5 min after 5 mM Sr^{2+}). *F,* Application of 100 mM sucrose did not increase sEPSC frequency in BoNT/C-treated cultures (0.06 Hz before and 5 min after sucrose).

tures (Table 1; Fig. 2*D*). This effect was independent of $[Ca]_o$, because the frequency of sEPSCs in 50 mM sucrose was not reduced by applying Ca^{2+}-free/1 mM EGTA-containing saline (5.7 ± 0.9 Hz before and 5.4 ± 1.4 Hz after; *n* = 3), in agreement with Rosenmund and Stevens (1996). No increase in sEPSC frequency was produced by 100 mM sucrose in cultures treated with any clostridial toxin (Table 1; Figs. 4*C*, 5*F*).

Effects of BoNT/E treatment

BoNT/E cleaves more of SNAP-25 than BoNT/A (26 vs 9 amino acids) (Binz et al., 1994). We therefore asked whether rescue of release could be obtained after BoNT/E treatment. Cleavage of SNAP-25 with BoNT/E resulted in the same electrophysiological phenotype as BoNT/A or BoNT/C treatment. In BoNT/E-treated cultures the mean frequency of mEPSCs was very low (0.07 ± 0.05 Hz, *n* = 6) and was not increased by application of phorbol ester (0.08 ± 0.06 Hz and 0.07 ± 0.03 Hz, *n* = 5; *p* > 0.05; before and after 3 µM PDAc). In contrast, significant increases in mEPSC frequency were produced by ionomycin when it was applied in saline containing 10 mM Ca^{2+} (0.07 ± 0.06 Hz and 1.2 ± 0.06 Hz, *n* = 4; *p* < 0.01; before and after ionomycin). Similarly, the mean frequency of sEPSCs was very low in control saline (0.06 ± 0.02 Hz, *n* = 12) and was significantly increased by 10 mM Ca^{2+}/0.5 mM Mg^{2+}-containing saline (0.1 ± 0.02 Hz and 1.2 ± 0.3 Hz, *n* = 6; *p* < 0.05; before and after 10 mM Ca^{2+}/0.5 mM Mg^{2+}). Enhanced influx of Ca^{2+} also was able to rescue release after a large portion had been cleaved from the C terminus of SNAP-25.

Actions of BoNT/A and BoNT/C on evoked unitary EPSCs

When two action potentials are elicited in close succession in a single cell, the second action potential triggers the release of more vesicles than the first, because the release probability remains transiently elevated because of the residual Ca^{2+} that remains in the nerve terminal after the first action potential (Katz and Miledi, 1968). We therefore examined the effects of BoNT/A and BoNT/C treatment on unitary EPSCs elicited by two action potentials at 50–80 msec intervals with dual recordings of monosynaptically coupled CA3 pyramidal cells. The paired-pulse ratio (PPR = amplitude of all EPSC2s/amplitude of all EPSC1s) of unitary EPSCs was inversely dependent on release probability (Fig. 6), as previously reported (Debanne et al., 1996). In untreated cultures in control saline (Fig. 6) (*n* = 5), the amplitude of EPSC1 was 41 ± 14 pA, the probability that the first presynaptic action potential failed to trigger an EPSC was 0.05 ± 0.03, and the mean PPR was 1.4 ± 0.5. Application of saline containing 10 mM Ca^{2+}/0.5 mM Mg^{2+} increased the mean amplitude of EPSC1 to 63 ± 5 pA, decreased the failure probability to 0, and decreased the PPR to 0.5 ± 0.1 (*n* = 3), consistent with an increase in release probability. Lowering release probability with saline containing 1 mM Ca^{2+}/2.5 mM Mg^{2+} saline produced opposite changes (*n* = 3; data not shown). In BoNT/A-treated (*n* = 5) or BoNT/C-treated (*n* = 3) cultures (Fig. 6) in the presence of 10 mM Ca^{2+}/0.5 mM Mg^{2+} saline, in contrast, the mean amplitude of EPSC1 was very small (BoNT/A, 7 ± 3 pA; BoNT/C, 13 ± 6 pA pA), the failure probability was very high (BoNT/A, 0.56 ± 0.15; BoNT/C, 0.38 ± 0.24), and the PPR was much greater than in control cultures (BoNT/A, 1.4 ± 0.2; BoNT/C, 1.2 ± 0.2). We conclude that the basal release probability in BoNT/A- or BoNT/C-treated cultures was much lower than in control cultures but that it could be increased moderately by elevating $[Ca^{2+}]_o$. Cleavage of either SNAP-25 with BoNT/A or SNAP-25 and syntaxin with BoNT/C
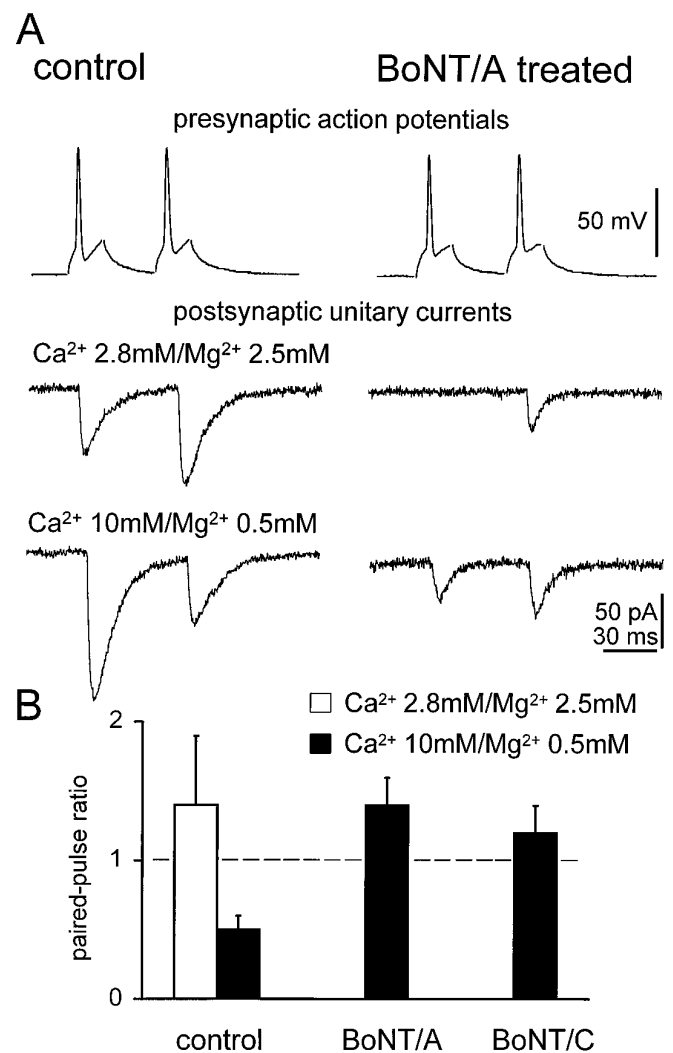

Figure 6. Unlike in control cultures, paired-pulse facilitation (PPF) occurs in both control and 10 mM Ca^{2+}/0.5 mM Mg^{2+} containing saline after BoNT/A or BoNT/C treatment. *A*, Dual recordings of monosynaptically connected CA3 pyramidal neurons recorded in a control (*left*) and a BoNT/A-treated culture (*right*). *Top traces* are pairs of presynaptic action potentials with an interval of 50 msec. *Bottom traces* are representative single sweeps of postsynaptic unitary currents elicited either in control saline containing 2.8 mM Ca^{2+}/2.5 mM Mg^{2+} or in saline containing 10 mM Ca^{2+}/0.5 mM Mg^{2+}. In the control cell pair, application of 10 mM Ca^{2+}/0.5 mM Mg^{2+} produced an increase in release probability, and paired-pulse depression was observed in 97% of the individual trials. In contrast, in the cell pair from a BoNT/A-treated culture, PPF was observed in 64% of the individual trials in the presence of 10 mM Ca^{2+}/0.5 mM Mg^{2+} containing saline, and failures of EPSC1 occurred in 93% of trials in control saline. *B*, In control cultures the mean paired-pulse ratio between two CA3 pyramidal cells was >1 in control saline but <1 after the release probability was increased by raising the Ca^{2+}/Mg^{2+} ratio. In BoNT/A- and BoNT/C-treated cultures, the paired-pulse ratio was >1 in saline containing 10 mM Ca^{2+}/0.5 mM Mg^{2+}. Failures of transmission were included in the calculation of the paired-pulse ratio.

thus resulted in a decrease in the ability of Ca^{2+} influx through voltage-dependent channels to trigger synaptic vesicle exocytosis.

Does BoNT/A or BoNT/C treatment impair the cooperative action of multiple Ca^{2+} ions, as previously suggested for BoNT/A treatment at the neuromuscular junction (Cull-Candy et al., 1976)? In control cultures log–log plots of mean unitary EPSC amplitude as a function of $[Ca^{2+}]_o$ were fit with a straight line for
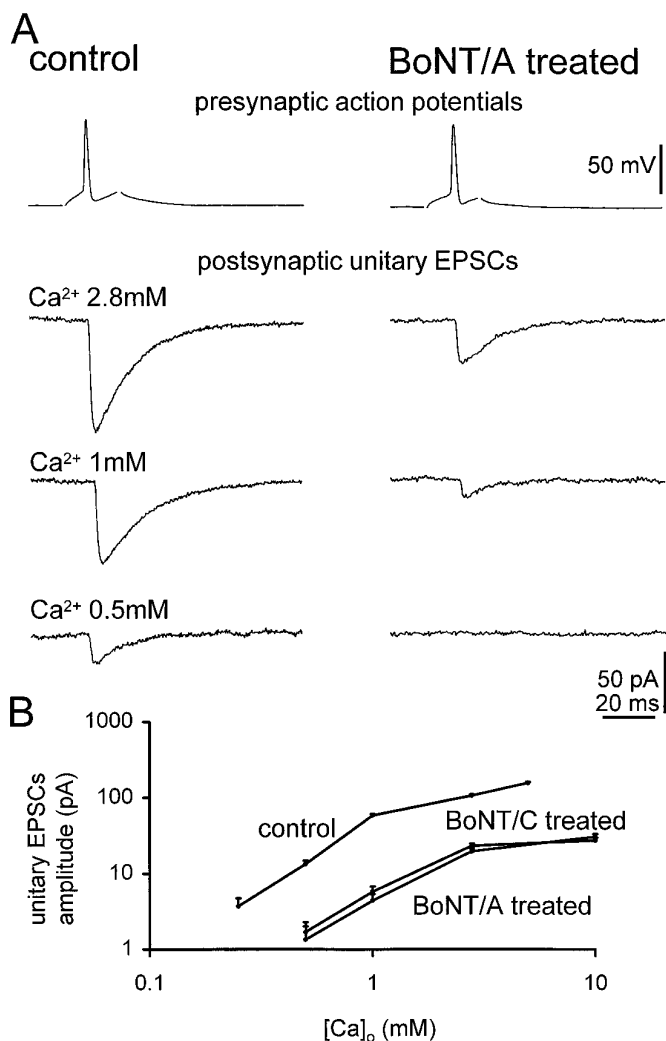
Figure 7. BoNT/A or BoNT/C treatment decreases the maximum number of quanta released and the Ca^{2+} sensitivity, but not cooperativity, of the exocytotic machinery. *A*, Action potentials elicited in a presynaptic CA3 pyramidal cell and unitary EPSCs recorded in a postsynaptic CA3 pyramidal cell in control (*left traces*) and BoNT/A-treated cultures (*right traces*). Each trace is a representative example from 60 trials for each $[Ca^{2+}]_o$, which was decreased from 2.8 to 0.5 mM. $[Mg^{2+}]_o$ was kept constant throughout the experiment at 2.5 mM. *B*, Log–log plot of mean unitary EPSC amplitudes, evoked by single action potentials, as a function of $[Ca^{2+}]_o$ in individual cell pairs in control, BoNT/A-treated, and BoNT/C-treated cultures. The control and BoNT/A data are from the cell pairs illustrated in *A*. Note that EPSC amplitudes approach a maximum, presumably corresponding to successful release at all synapses. In BoNT/A- or BoNT/C-treated cultures, the relation was shifted to the right, indicating a decrease in Ca^{2+} sensitivity, and the maximum EPSC amplitude was decreased, indicating a reduction in the maximum number of quanta released. The slope of this relation (for $[Ca^{2+}]_o = 0.25$–1 mM in the control culture; $[Ca^{2+}]_o = 0.5$–2.8 mM in the BoNT/A- or BoNT/C-treated culture) was not affected by toxin treatment, indicating that Ca^{2+} cooperativity (Dodge and Rahamimoff, 1967) was not changed. Failures of transmission were included in the calculation of mean EPSC amplitude.

$[Ca^{2+}]_o \leq 1$ mM. The slope of this line, an index of Ca^{2+} cooperativity, was 2.0 ± 0.1 ($n = 3$) (Fig. 7), consistent with observations at the neuromuscular junction for this range of $[Ca^{2+}]_o$ (Dodge and Rahamimoff, 1967). In BoNT/A-treated ($n = 3$) or BoNT/C-treated ($n = 2$) cultures, the curves were shifted toward higher $[Ca^{2+}]_o$, but the slope of the initial portion of the relationship was not affected (BoNT/A, 2.2 ± 0.6; BoNT/C,

2.3 ± 0.2) (Fig. 7). We conclude that the cooperative action of multiple Ca^{2+} ions in initiating vesicle fusion is unaffected by cleavage of SNAP-25 or both SNAP-25 and syntaxin.

DISCUSSION

Both spontaneous and evoked release require SNARE proteins in CNS

We observed that VAMP/synaptobrevin-2 was cleaved after TeNT treatment, that syntaxin and SNAP-25 were cleaved after BoNT/C treatment, and that SNAP-25 was cleaved after BoNT/A treatment, as assessed with both immunohistochemistry and Western blot analysis in hippocampal slice cultures, consistent with previous studies (Foran et al., 1996; Osen-Sand et al., 1996; Williamson et al., 1996) (for review, see Schiavo et al., 1995). In contrast, synaptic vesicles remained in toxin-treated cultures, as indicated by the presence of synaptophysin and synaptotagmin immunoreactivity.

The probability of evoking neurotransmitter release was greatly reduced in toxin-treated cultures, as indicated by the small amplitude of unitary EPSCs between pairs of CA3 pyramidal cells and paired-pulse facilitation in saline containing 10 mM Ca^{2+}/0.5 mM Mg^{2+}. In addition, BoNTs and TeNT decreased the frequency of both TTX-sensitive sEPSCs and TTX-insensitive mEPSCs to the same low level of ~0.1 Hz. We do not know what accounts for those synaptic events that are resistant to the action of the toxin. It is possible that they are released from a subpopulation of vesicles containing SNARE proteins not cleaved by BoNTs or TeNT, perhaps because they were present in heterotrimeric complexes (Hayashi et al., 1994; Pellegrini et al., 1995). This seems unlikely, however. First, the SNARE complexes probably would have turned over many times during the 48 hr of toxin treatment. Second, phorbol esters and α-LTx at concentrations <1 nM were ineffective in the toxin-treated cultures (see below). Alternatively, cleavage of SNAREs may very strongly reduce the likelihood of spontaneous vesicle fusion but not fully block it.

These data are in agreement with the bulk of previous electrophysiological work with clostridial neurotoxins at central synapses (Bergey et al., 1987; Finch et al., 1990) and at the neuromuscular junction (Harris and Miledi, 1971; Cull-Candy et al., 1976; Dreyer and Schmitt, 1983; Dreyer et al., 1983, 1987; Gansel et al., 1987; Molgó et al., 1989). Our results are also consistent with the abolition of both constitutive and evoked glutamate release at the neuromuscular junction of *Drosophila* embryos lacking syntaxin (Broadie et al., 1995; Schulze et al., 1995). In transgenic *Drosophila* embryos expressing TeNT, evoked release is abolished, but the frequency of spontaneous glutamate release is reduced by only 50%, in contrast to our results (Broadie et al., 1995). We conclude that both constitutive and evoked release of glutamate in the mammalian CNS share a similar molecular mechanism requiring that the presynaptic proteins VAMP/synaptobrevin, SNAP-25, and syntaxin be intact.

Release induced by Ca^{2+}-independent secretagogues is blocked by treatment with clostridial neurotoxins

Exocytosis can be stimulated in the hippocampus by several agents in a manner that is independent from either $[Ca^{2+}]_o$ or $[Ca^{2+}]_i$ or both, including activators of protein kinases A or C[a] (Malenka et al., 1987; Finch and Jackson, 1990; Chavez-Noriega

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[a]Parfitt and Madison (1993) found that the enhancement of mEPSC frequency elicited by phorbol ester was partially attenuated by either Cd^{2+} or L-type Ca^{2+} channel antagonists. Differences in experimental conditions (area CA1 in acute slices vs area CA3 in slice cultures, 10 vs 3 μM PDAc; measurement of mEPSC frequency >15 vs 5 min after PDAc application) may account for the differences between their conclusion and ours.

and Stevens, 1994; Capogna et al., 1995; Trudeau et al., 1996b), α-LTx at concentrations <1 nM (Capogna et al., 1996), sucrose (Rosenmund and Stevens, 1996), ruthenium red (Trudeau et al., 1996a), and nitric oxide (Meffert et al., 1996). The effects of 0.5 nM α-LTx, phorbol ester, and sucrose[b] were prevented by all clostridial toxins. Similarly, the ability of ruthenium red (Trudeau et al., 1996b) or α-LTx at concentrations <1 nM (Capogna et al., 1996) to induce $[Ca^{2+}]_o$-independent release in hippocampus was shown previously to be prevented by TeNT or BoNT/F treatment. Finally, $[Ca^{2+}]_o$-independent nitric oxide-stimulated secretion is impaired by cleavage of any of the SNARE proteins (Meffert et al., 1996). At the mammalian neuromuscular junction, clostridial neurotoxin treatment also blocks sucrose-stimulated release fully (Dreyer et al., 1987). α-LTX, in contrast, is weakly active after TeNT treatment but unaffected by BoNT/A treatment (Dreyer et al., 1987). We thus conclude that, like normal synaptic transmission, these $[Ca^{2+}]_o$-independent forms of release depend on the integrity of VAMP/synaptobrevin, SNAP-25, and syntaxin in the mammalian CNS.

A better description of the molecular mechanism(s) by which these secretagogues act clearly is required before a mechanistic hypothesis for inhibition by clostridial toxins is suggested. Nevertheless, these results strengthen the suggestion that these $[Ca^{2+}]_o$-independent secretagogues trigger release via direct changes in the functional state of the exocytotic machinery, particularly in the final steps of exocytosis. For example, phorbol ester increases the size of the readily releasable pool of secretory granules in chromaffin cells (Gillis et al., 1996), whereas forskolin increases release probability without changing the number of vesicles available for release at hippocampal synapses (Trudeau et al., 1996b). Phosphorylation of SNAP-25 by PKC has been shown to contribute to the stimulation of release from PC12 cells (Shimazaki et al., 1996). We suggest that cleavage of SNAP-25 by clostridial toxins prevents this phosphorylation and thus accounts for the inability of phorbol esters to promote release.

Ca²⁺-dependent evoked release can be rescued after cleavage of SNAP-25 and syntaxin

The mechanism by which cleavage of SNARE proteins inhibits transmitter release is not known. The SNARE complex assembly and disassembly cycle can still occur after cleavage of SNAREs (Hayashi et al., 1994; Pellegrini et al., 1995). In addition, ultrastructural analysis of presynaptic release sites has shown that synaptic vesicles still dock, at the morphological level, in the absence of either syntaxin or intact VAMP/synaptobrevin (Harris and Miledi, 1971; Hunt et al., 1994; Broadie et al., 1995), although this may be accounted for by the binding of the vesicle protein synaptotagmin to SNAP-25 (Schiavo et al., 1997).

We found that glutamate release could be triggered by large Ca^{2+} or Sr^{2+} influxes into axon terminals after cleavage of either SNAP-25 alone or both SNAP-25 and syntaxin, but not after cleavage of VAMP/synaptobrevin-2. It is important to note that the release induced by these manipulations in toxin-treated cultures did not originate from a subpopulation of excitatory terminals not affected by the toxins, because phorbol ester, sucrose, or ionomycin and α-LTx when applied in normal saline failed to induce any release in the same BoNT-treated

cultures. Moreover, it is unlikely that the toxin-resistant release came from ectopic or abnormal release sites, because miniature and unitary postsynaptic currents in BoNT-treated cultures appeared indistinguishable in kinetics and latency from those seen in control cultures.

Enhanced Ca^{2+} influx has been shown previously to rescue acetylcholine release at the neuromuscular junction after treatment with BoNT/A or BoNT/E (Cull-Candy et al., 1976; Dreyer and Schmitt, 1983; Gansel et al., 1987; Molgó et al., 1989) (for review, see Molgó et al., 1990; Poulain et al., 1995). Consistent with our results, partial rescue of secretion from chromaffin cells also has been observed after treatment with BoNT/A, but not with TeNT (Lawrence et al., 1994, 1996; Banerjee et al., 1996). In contrast to our results, however, Ca^{2+}-mediated rescue from these cells was not obtained after BoNT/E or BoNT/C treatment (Banerjee et al., 1996; Foran et al., 1996).

In addition, we found that the mean amplitude of evoked unitary EPSCs in BoNT-treated cultures in the presence of 10 mM Ca^{2+}/0.5 mM Mg^{2+} was less than the mean amplitude of unitary EPSCs in control cultures in saline containing 2.8 mM Ca^{2+}/2.5 mM Mg^{2+}. It therefore would appear that BoNT treatment decreased the maximum number of quanta that could be released. Assuming that the number of docked vesicles is not decreased after toxin treatment (see Harris and Miledi, 1971; Hunt et al., 1994; Broadie et al., 1995), this observation suggests that cleavage of syntaxin and SNAP-25 may impair release at some point subsequent to the action of Ca^{2+} in triggering release.

In BoNT-treated cultures, plots of unitary EPSC amplitudes as a function of $[Ca^{2+}]_o$ yielded a slope similar to that seen in untreated cultures, but the relationship was shifted toward higher $[Ca^{2+}]_o$. This observation indicates that cleavage of SNAP-25 alone or together with syntaxin also decreases release by decreasing the apparent sensitivity of the release machinery for Ca^{2+}. Although the inhibition of Ca^{2+}-dependent evoked release by clostridial neurotoxins could result from an inhibition of voltage-dependent Ca^{2+} channels or an alteration in intraterminal Ca^{2+} dynamics, these possibilities have been ruled out previously (Dreyer et al., 1983; Mallart et al., 1989; Mochida et al., 1995; Stanley and Mirotznik, 1997).

There are insufficient biochemical data at present that can account for these effects of BoNT or TeNT treatment. Clostridial neurotoxins change the energetics of the assembly and disassembly of SNARE complexes, as revealed by their ability to withstand denaturation by SDS (Pellegrini et al., 1995). The SDS resistance of the SNARE complex is lower after TeNT than after BoNT/A treatment (Pellegrini et al., 1995). It is thus noteworthy that TeNT caused an inhibition of release that we could not counteract with large Ca^{2+} or Sr^{2+} influxes. One possibility is that BoNT treatment modifies the interactions between the heterotrimeric fusion complex and voltage-dependent Ca^{2+} channels (for review, see Zucker, 1996). A simple "uncoupling" of Ca^{2+} channels from fusion-competent vesicles cannot account for all of the effects of the toxins, however, because we observed that release also was inhibited by BoNT/A and BoNT/C when Ca^{2+} was transported directly into the terminal by ionomycin.

In conclusion, the rescue of release by large Ca^{2+} or Sr^{2+} influx after cleavage of SNAP-25 alone or together with syntaxin adds functional evidence for an essential postdocking role of these proteins in neurotransmitter exocytosis.

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[b]In *Drosophila*, hyperosmotic saline can induce some release after cleavage of VAMP/synaptobrevin by TeNT or in the absence of syntaxin (Broadie et al., 1995). We were unable to test the high concentrations of sucrose used in that study, however.

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