Different VAMP/Synaptobrevin Complexes for Spontaneous and Evoked Transmitter Release at the Crayfish Neuromuscular Junction

SHAO-YING HUA, ^1^ DOROTA A. RACIBORSKA, ^1^ WILLIAM S. TRIMBLE, ^2^ AND MILTON P. CHARLTON ^1^  
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Hua, Shao-Ying, Dorota A. Raciborska, William S. Trimble, and Milton P. Charlton. Different VAMP/synaptobrevin complexes for spontaneous and evoked transmitter release at the crayfish neuromuscular junction. J. Neurophysiol. 80: 3233–3246, 1998. Although vesicle-associated membrane protein (VAMP/synaptobrevin) is essential for evoked neurotransmitter release, its role in spontaneous transmitter release remains uncertain. For instance, many studies show that tetanus toxin (TeNT), which cleaves VAMP, blocks evoked transmitter release but leaves some spontaneous transmitter release. We used recombinant tetanus and botulinum neurotoxin catalytic light chains (TeNT-LC, BoNT/B-LC, and BoNT/D-LC) to examine the role of VAMP in spontaneous transmitter release at neuromuscular junctions (nmj) of crayfish. Injection of TeNT-LC into presynaptic axons removed most of the VAMP immunoreactivity and blocked evoked transmitter release without affecting nerve action potentials or Ca^{2+} influx. The frequency of spontaneous transmitter release was little affected by the TeNT-LC when the evoked transmitter release had been blocked by >95%. The spontaneous transmitter release left after TeNT-LC treatment was insensitive to increases in intracellular Ca^{2+}. BoNT/B-LC, which cleaves VAMP at the same site as TeNT-LC but uses a different binding site, also blocked evoked release but had minimal effect on spontaneous release. However, BoNT/D-LC, which cleaves VAMP at a different site from the other two toxins but binds to the same position on VAMP as TeNT, blocked both evoked and spontaneous transmitter release at similar rates. The data indicate that different VAMP complexes are employed for evoked and spontaneous transmitter release; the VAMP used in spontaneous release is not readily cleaved by TeNT or BoNT/B. Because the exocytosis that occurs after the action of TeNT cannot be increased by increased intracellular Ca^{2+}, the final steps in neurotransmitter release are Ca^{2+} independent.

INTRODUCTION

Proteolysis of the vesicle-associated membrane protein (VAMP)/synaptobrevin (Baumert et al. 1989; Trimble et al. 1988) by tetanus toxin (TeNT) blocks evoked neurotransmitter release at synapses (for review, see Monte- cucco and Schiavo 1994), and this is strong evidence of a requirement for VAMP in calcium (Ca^{2+})-triggered exocytosis. However, there is a curious contradiction in the effects of TeNT on spontaneous transmitter release; most studies show that while the frequency of spontaneous quantal transmitter release is reduced greatly, unlike evoked release, it is not eliminated altogether (Capogna et al. 1997; Dreyer 1989; Dreyer and Schmitt 1981; Duchen and Tonge 1973; Habermann et al. 1980; Herreros et al. 1995; Kryzhavskii et al. 1971; Mellanby and Thompson 1972; Sweeney et al. 1995; Weller et al. 1991).

Evoked transmitter release consists of the synchronous occurrence of the same quantal events as spontaneous release (Katz 1969). Because evoked release is simply a gross acceleration of spontaneous release, it is surprising that both events are not affected in the same way by proteolysis of a key protein. Such a discrepancy could arise if the spontaneous release process does not require VAMP or uses a VAMP isoform that lacks the cleavage site for TeNT or if some VAMP is protected from TeNT by interactions with other proteins. In any case, these observations lead to the general hypothesis that evoked and spontaneous transmitter release use somewhat different molecular mechanisms.

We investigated this discrepancy using new knowledge about the mechanisms by which various clostridial toxins attack VAMP; these toxins require a binding site on VAMP in addition to the site where enzymatic cleavage occurs. Although TeNT and botulinum toxin-D (BoNT/D) cleave VAMP at different sites, their binding sites are similar. On the other hand, TeNT and botulinum toxin-B (BoNT/B) cleave VAMP at the same site but their binding sites are different (Pellizzari et al. 1996, 1997) (Fig. 1).

If spontaneous release does not require VAMP, then none of the clostridial toxins known to cleave VAMP should block spontaneous release. If the VAMP used for spontaneous release lacks the TeNT cleavage or binding sites or if these sites are occluded by protein interactions, then other toxins that use different sites may be effective in blocking spontaneous release. Indeed VAMP does form complexes with other SNAP receptors (SNAREs) that protect it from cleavage by TeNT but this protection is lost if the complex is dissociated by the action of other synaptic proteins, soluble NSF attachment protein (α-SNAP) and N-ethylmaleimide-sensitive factor (NSF) (Hayashi et al. 1995; Otto et al. 1997; Pellegrini et al. 1994, 1995). Thus investigation of the role of VAMP in spontaneous and evoked transmitter release with multiple clostridial toxins may reveal functional attributes of these complexes or their intermediate states.

After injection of recombinant catalytic light chain of TeNT or BoNT/B (TeNT-LC, BoNT/B-LC) into presynaptic...
Electrophysiology

Experiments were conducted on the opener muscle of the first walking leg of small (~5 cm) crayfish, Procambarus clarkii, at room temperature (Wojtowicz and Atwood 1984). This muscle is innervated by a single excitatory axon and a single inhibitory axon, one of which was separated from the rest of the nerve bundle at the meropodite segment while all the other nerves were cut to ensure that only the selected axon would be stimulated. The proximal end of the axon was stimulated electrically by a platinum electrode. The crayfish saline contained (in mM) 205 NaCl, 5.4 KCl, 13.5 CaCl₂, 2.7 MgCl₂, 10 d-glucose, and 10 HEPES. In Ca²⁺-free saline, equinormal NaCl was substituted for CaCl₂ and 1 mM of ethylene glycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA, Sigma, St. Louis, MO) was added to chelate free Ca²⁺. The pH of each saline used was adjusted to 7.4 with NaOH. When Ca²⁺-free saline was applied, the bathing saline was exchanged several times to eliminate Ca²⁺ as completely as possible.

In experiments recording spontaneous miniature excitatory post-synaptic potentials (mEPSPs), 10 μM picrotoxin (Sigma) was added to the saline to block γ-aminobutyric acid (GABA) receptors responsible for spontaneous miniature inhibitory post-synaptic potentials (mIPSPs) (Takeuchi and Takeuchi 1969).

The preparation was mounted on an upright epifluorescence microscope (Nikon Optiphot-2, Nikon, Tokyo, Japan), and either the excitatory or the inhibitory axon was impaled with a glass microelectrode filled with 3 M sodium nitrate and human VAMP2.

M ETH ODS

Expression and purification of His₆-tagged recombinant light chain of tetanus toxin and botulinum toxin type D

Recombinant toxin light chain DNA, containing a carboxyl terminal His₆ tag in Qiangen Express plasmid pQE3, was generously provided by Dr. H. Niemann (Eisel et al. 1993). Bacteria harboring the DNA were grown to log phase (OD₆₀₀ = 0.6–0.7) and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich, Oakville, ON, Canada). The culture then was grown for an additional 2.5 h to allow expression of the recombinant protein.

The harvested cells were frozen overnight at −20°C, disrupted by pulse sonication, and centrifuged (20 min at 30,000 g, 4°C). and the supernatant containing the His₆-ToNT-LC was circulated through nickel-agarose beads for 3 h, allowing the binding of the histidine tag to nickel on the beads. After removing weakly bound proteins by washing the beads with 30 mM imidazole, the beads then were incubated with 100 mM imidazole added to the buffer to elute the His₆-ToNT-LC protein off the beads. The buffer containing the recombinant protein was collected and dialyzed against reaction buffer [20 mM Na₂-Hydroxylpiperazine-N'-2-ethane-sulfonic acid (HEPES) and 100 mM KCl, pH 7.5; 6 h at 4°C]. The dialyzed protein was immediately made into aliquots and frozen at −80°C under nitrogen gas to prevent degradation.

FIG. 1. Binding and cleavage sites of tetanus toxin (TeNT) and botulinum neurotoxin catalytic light chains (BoNT/B and BoNT/D) on vesicle-associated membrane protein (VAMP) (modified from Pelizzari et al. 1996, 1997). ▢, binding sites V₁ and V₂ [SNARE (SNAP receptor) motif] for each toxin. TeNT and BoNT/D use the same binding site (V₁), whereas BoNT/B uses a different site (V₂). →, TeNT and BoNT/B cleave at the same site, but BoNT/D cleaves at a different site. Binding and cleavage sites are found within the domain that participates in protein-protein interaction. Antibody used for Western blots and immunocytochemistry was made using a fragment of VAMP (aa 33–94) encompassing most of the protein interaction domain and all of the cleavage sites. Amino acid numbers pertain to rat and human VAMP2.
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anti-guinea pig IgG (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA). In some experiments, the preparation was incubated further with rabbit antisynaptotagmin (1:500) (Littleton et al. 1993) and revealed with Texas red-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR). The preparation then was scanned with a confocal laser scanning microscope (Bio-Rad MRC-600, Watford, UK) using a 40 water immersion lens (Nikon, 0.55 NA). The FITC was excited at 488 nm, and emission light longer than 515 nm was collected. For Texas red, excitation was 544 nm and emission light >630 nm was collected.

**Calcium imaging**

To examine Ca\(^{2+}\) influx into the nerve terminals, the excitatory axon was first penetrated with an electrode filled with 50 mM KCl and 200 \(\mu\)M Calcium Green-1 hexapotassium salt (Molecular Probes). The dye was iontophoresed into the axon by applying negative current (about −5 nA) continuously to the electrode. After the dye had diffused to nerve terminals, the preparation was scanned by the Biorad 600 confocal microscope using a \(\times20\) Nikon objective (0.35 NA, 20-mm working distance), which is suitable for simultaneous electrophysiological recordings. The Calcium Green-1 was excited at 488 nm, and the emission light >515 nm was collected. To study Ca\(^{2+}\) influx, a series of 20 scans of the synaptic boutons on the same muscle fiber penetrated by a recording electrode were taken at a rate of 1 image / 2.1 s, starting shortly before a 5- or 10-s train of stimulation (30 Hz). After control images of stimulus induced Ca\(^{2+}\) signals were obtained, TeNT-LC was injected into the presynaptic axon. When the toxin effect was developed fully, another series of stimulus-dependent Ca\(^{2+}\) signals was obtained. The fluorescence intensity of the boutons was averaged for the area of the boutons of interest and Ca\(^{2+}\) entry was expressed in relative change in the intensity as

\[
\Delta F/F = (F_{\text{stimulation}} - F_{\text{rest}})/F_{\text{rest}}
\]

**RESULTS**

We wanted to examine the molecular mechanisms of exocytosis at a mature synapse in which we could measure and modify presynaptic membrane potential and \([\text{Ca}^{2+}]_i\), in which we could inject macromolecules into the presynaptic terminal, and in which we could clearly distinguish and measure evoked and spontaneous quantal transmitter release from a single identified presynaptic cell. Neuromuscular junctions of some arthropods fulfill these criteria, and we used the claw-opener muscle of the crayfish leg, in which the presynaptic axons are large enough to permit insertion of microelectrodes for recording (Wojtowicz and Atwood 1984) and injection of exogenous molecules (Dixon and Atwood 1989).

**TeNT-LC cleaves crayfish VAMP**

The proteolytic activity of TeNT-LC was assayed on central nervous tissues of crayfish and rat. Samples of homogenized crayfish abdominal ganglia and rat brain were incubated with or without recombinant TeNT-LC, BoNT/B-LC or BoNT/D-LC at 37°C for 1 h and subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blotting. Figure 2 shows that an antibody against VAMP that recognizes an epitope spanning the toxin cleavage sites (see Fig. 1) (Boyd et al. 1995; Li et al. 1994; Shone et al. 1993) recognized a protein extracted from crayfish ganglia with apparent molecular mass similar to that...
detected in the rat brain homogenate. Immunoreactivity of this band was reduced greatly in extracts from both species after incubation with recombinant TeNT-LC, indicating that proteolytic cleavage had occurred. Similar results were obtained with another antibody against rat brain VAMP-1 and VAMP-2 (Cain et al. 1992). BoNT/B-LC and BoNT/D-LC also cleaved crayfish VAMP (see Figs. 7 and 8).

We next asked whether recombinant TeNT-LC could cleave VAMP in a live presynaptic cell. Proteolytic activity was verified after electrophysiological experiments in which TeNT-LC was injected into one of the two main branches of the excitatory axon in the crayfish claw-opener muscle (Fig. 3A). The preparation then was fixed and incubated with anti-VAMP. In the claw-opener muscle, the excitatory and inhibitory nerves usually run parallel and close to each other, appearing as two strands of boutons. Figure 3B shows VAMP immunoreactivity in presynaptic boutons on a muscle fiber the nerve branch of which was far from the toxin injection site. The anti-VAMP distribution at this distal site revealed a double strand of nerve terminal boutons corresponding to the closely parallel excitatory and inhibitory boutons. There was little if any VAMP immunoreactivity in axons. Figure 3C shows that, near the injection site, only a single strand of boutons corresponding to the uninjected inhibitory boutons contained much VAMP immunoreactivity. There was a parallel strand of faint staining in the poisoned excitatory terminals. These results show that VAMP exists in both the excitatory and the inhibitory nerve terminals (Fig. 3B) and that the VAMP in the excitatory terminals near the injection site had been cleaved. We presume that at the distal site, insufficient TeNT-LC had arrived to cleave VAMP noticeably. All postsynaptic electrophysiological recordings of transmitter release were made at muscle sites very close to the presynaptic toxin injection sites.

In some experiments, nerve terminals also were stained for synaptotagmin immunoreactivity (Cooper et al. 1995a) after the muscle had been incubated first with anti-VAMP. Figure 3D shows the double-staining image of a muscle in which the excitatory nerve had been injected with the TeNT-LC (same muscle as Fig. 3, B and C). The anti-synaptotagmin revealed both the excitatory and inhibitory boutons, whereas the anti-VAMP revealed only the uninjected inhibitory boutons. The muscle was scanned twice with the confocal microscope, once for anti-VAMP and once for anti-synaptotagmin distributions. The merged image shows the inhibitory boutons that contained both synaptotagmin and VAMP in yellow. The results indicate that the VAMP in the poisoned nerve terminals had been cleaved by TeNT-LC, but distribution of the synaptotagmin was unaffected. Similar results were obtained with BoNT/B-LC and BoNT/D-LC (Figs. 7 and 8).

TeNT-LC inhibits both glutamate and GABA release

If VAMP is required for transmitter release in crayfish synapses, then destruction of VAMP should block release. The excitatory axon was penetrated with a microelectrode containing the TeNT-LC and a fluorescent Ca$^{2+}$ indicator dye, Calcium Green-1. The electrode solution then was injected into the axon with brief pulses of pressure, which caused the appearance of tiny puffs of fluorescence in the axoplasm as seen with fluorescence microscopy. Presynaptic action potentials and EPSPs evoked by stimulating the excitatory axon, and spontaneous mEPSPs were recorded continuously. In all the experiments performed on 14 preparations, pressure injection of TeNT-LC caused a large decrease in EPSP amplitude and, in most cases, totally blocked EPSPs evoked by trains of three to six stimuli at 100 Hz. A typical result is presented in Fig. 4A. Similar results were obtained with natural isolated TeNT-LC (not shown). All data presented in this paper were obtained with recombinant toxin catalytic light chains.

Although the EPSPs were blocked completely after TeNT-LC injection, the presynaptic action potentials were affected very little (Fig. 4B), confirming that the toxin does not block action potential generation or propagation. In three experiments, injection of boiled TeNT-LC solution with Calcium Green-1 (Fig. 4D) or Calcium Green-1 in electrode solution with no toxin (n = 1) did not change EPSP amplitude, thus indicating that the toxin effect is specific and requires active enzyme.

The time required for the EPSP amplitude to decrease by 50% varied from 19 to 96 min and averaged 49 min (n = 9). This latency is due partially to diffusion and transport of the enzyme to the most distal boutons on the impaled muscle fiber and to the rate of VAMP cleavage by the enzyme. Because when FITC-dextran (3 or 70 kDa) was injected we observed that fluorescence reached the most distal boutons on the impaled muscle fiber in this time, it is likely that all boutons received toxin. The results from immunocytochemistry showed that VAMP was cleaved at sites distal to the injection and recording sites during these experiments (Fig. 3). Therefore the toxin LC should have reached all the boutons on the penetrated muscle fiber that was near the presynaptic injection site. Some reduction in toxin activity occurred after a sample was thawed, and to minimize variability from this source, the toxin was used at most for 3 days after thawing.

We also tested whether TeNT-LC could block inhibitory transmitter release. Taking advantage of the dual excitatory and inhibitory innervation of the crayfish claw-opener muscle, we injected TeNT-LC into the inhibitory axon in another two experiments to see if the toxin could block GABA secretion. As illustrated in Fig. 4E, the toxin reduced the amplitude of IPSPs produced by stimulation of the inhibitory axon. The remainder of this paper addresses excitatory synapses exclusively.

The total block of EPSPs could not be ameliorated by stimulation at 30 Hz for 10 s, a procedure that normally facilitates release several-fold (Atwood and Wojtowicz 1986; Zucker 1989). However, facilitation still could be demonstrated as long as EPSPs still were produced. To ask whether TeNT-LC affected facilitation, we compared the toxin’s effect on the first and later EPSPs caused by a train of three pulses at 100 Hz. After the toxin was injected and partial blockade obtained, all three EPSPs were inhibited to a similar extent, indicating that the toxin has little effect on short-term facilitation. Other types of plasticity, such as long-term facilitation, could not be studied owing to the relatively rapid decline of transmitter release after TeNT-LC injection.

To determine whether a major effect of TeNT-LC is a
FIG. 3. TeNT-LC reduced VAMP immunoreactivity in synaptic boutons. A: diagram of the muscle and innervation from which pictures B–D were taken. TeNT-LC was injected into the excitor axon through electrode “pre,” and excitatory postsynaptic potentials (EPSPs) were recorded from a nearby muscle fiber by electrode “post.” B: anti-VAMP staining of boutons located distal to the injection site. As shown in A, this muscle fiber was innervated by the right branch of the axon. TeNT-LC would have had to move from the injection site down one branch and up the other to reach this site. VAMP immunoreactivity shows a double string of boutons corresponding to both excitatory and inhibitory boutons. Insufficient toxin had reached these excitatory boutons to cleave much VAMP. C: VAMP immunoreactivity near the injection site. Here only the uninjected inhibitory boutons retained normal VAMP immunoreactivity and a single string of boutons is visible. Scale is the same as for B. D: double staining with anti-VAMP (a) and anti-synaptotagmin (b). After the pictures in B–D were made, anti-synaptotagmin was applied. Color picture (c) is the merged image of a and b. Red, anti-synaptotagmin; green, anti-VAMP; yellow, both antibodies. VAMP immunoreactivity is not affected in the uninjected inhibitory boutons, but synaptotagmin immunoreactivity shows the location of all boutons including those depleted of VAMP. Arrow heads in C and Da indicate the faint staining of the poisoned boutons. The preparation was fixed after an electrophysiological experiment had determined that evoked EPSPs had been blocked near the injection site. Note that VAMP immunoreactivity in the injected excitatory boutons near the injection site was reduced greatly, but at distal site B, immunoreactivity was not diminished. Similar results were obtained in another 3 experiments. It is reduction of Ca$^{2+}$ entry, we performed Ca$^{2+}$ imaging experiments in injected boutons. Calcium Green-1 was injected into the excitatory axon to monitor changes in $[Ca^{2+}]_i$, and the boutons on muscle fibers from which EPSPs were recorded were scanned shortly before, during, and soon after the nerve stimulation to create Ca$^{2+}$ images at different times. Nerve firing and postsynaptic responses were monitored during 5- or 10-s stimulus trains at 30 Hz to ensure effective stimulation. The fluorescence intensity of several boutons was averaged for each scan and the relative change in the fluorescence ($\Delta F/F$) was plotted. After TeNT-LC was injected and EPSPs blocked, Ca$^{2+}$ signals were similar to those obtained before TeNT-LC treatment (Fig. 4C). Similar results were obtained in another three experiments. It is
FIG. 4. TeNT-LC blocks both EPSPs and inhibitory postsynaptic potentials (IPSPs). A: TeNT-LC completely blocked the EPSPs. Amplitude of the 6th EPSP shown in B was plotted. B: examples of action potentials of the excitatory axon (top) and corresponding EPSPs from a muscle fiber (bottom), taken at different times as indicated in A. Small changes in action potentials are due to changes in electrode resistance. C: TeNT-LC had minimal effects on Ca$^{2+}$ influx. Fluorescence of Calcium Green-1 in a single bouton was scanned before TeNT-LC injection and after EPSPs had been blocked by the TeNT-LC. During the time indicated, the excitatory axon was stimulated for 5 s at 30 Hz. Similar results were obtained in another 3 experiments. D: injection of boiled toxin solution in another 2 preparations did not change the amplitude of EPSP. Stimulation was stopped for a while for miniature potential observation, resulting in the discontinuity of the recording before the injection. E: IPSP also was inhibited by TeNT-LC injection. r, beginning of injection, which was stopped when small branches of the presynaptic neuron and its boutons were filled. A shorter period of injection does not necessarily mean that less toxin was injected.

thus clear that TeNT-LC has little effect on Ca$^{2+}$ influx but uncouples Ca$^{2+}$ influx from transmitter release (Hunt et al. 1994; Llinás et al. 1994).

Spontaneous mEPSP frequency is relatively insensitive to TeNT-LC

During these electrophysiological experiments, mEPSPs were recorded simultaneously with evoked EPSPs. We measured the amplitude and frequency of mEPSPs to determine if they were affected by TeNT-LC. Figure 5, A and B, shows a typical result from a group of 14 experiments. The mEPSP frequency was not significantly reduced, even long after the EPSP was blocked. The mean mEPSP frequency of 14 fibers in 14 different muscles was 0.41 ± 0.07 (SE) Hz when measured for 10 or 15 min before TeNT-LC injection. After toxin injection, mean mEPSP frequency in the same fibers was 0.53 ± 0.10 Hz, not significantly different from the pretreatment control frequency ($P > 0.05$, t-test). The average change in mEPSP frequency after TeNT-LC injection was +0.13 ± 0.07 Hz ($P > 0.05$, t-test) and the average percent change was +39 ± 22% ($P > 0.05$, t-test). Thus TeNT-LC injection has no significant effect on spontaneous release frequency even though EPSP amplitude evoked by three to six stimuli at 100 Hz had been reduced by >95%.

Several control experiments were performed to verify that events counted were mEPSPs and therefore represented spontaneous quantal excitatory transmitter release. To exclude the spontaneous mEPSPs, 10 μM picrotoxin routinely was included in saline (Takeuchi and Takeuchi 1969) and during these electrophysiological experiments, mEPSPs were recorded simultaneously with evoked EPSPs. We measured the amplitude and frequency of mEPSPs to determine if they were affected by TeNT-LC. Figure 5, A and B, shows a typical result from a group of 14 experiments. The mEPSP frequency was not significantly reduced, even long after the EPSP was blocked. The mean mEPSP frequency of 14 fibers in 14 different muscles was 0.41 ± 0.07 (SE) Hz when measured for 10 or 15 min before TeNT-LC injection. After toxin injection, mean mEPSP frequency in the same fibers was 0.53 ± 0.10 Hz, not significantly different from the pretreatment control frequency ($P > 0.05$, t-test). The average change in mEPSP frequency after TeNT-LC injection was +0.13 ± 0.07 Hz ($P > 0.05$, t-test) and the average percent change was +39 ± 22% ($P > 0.05$, t-test). Thus TeNT-LC injection has no significant effect on spontaneous release frequency even though EPSP amplitude evoked by three to six stimuli at 100 Hz had been reduced by >95%.

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FIG. 5. TeNT-LC blocked EPSPs but had minimal effect on mEPSPs. A: plots of EPSP amplitude, mEPSP frequency and mEPSP amplitude simultaneously recorded in an experiment. Pressure injection (bar, TeNT-LC) of the electrode solution sometimes caused a transient increase in mEPSP frequency as shown here. Graph of mEPSP amplitude is a continuous record of the measured amplitude of all the mEPSPs in a 2-h experiment. B: example of mEPSP recordings before and after TeNT-LC injection from same experiment as in A. Right sweeps taken 47 min after starting the injection when the evoked EPSPs had been blocked. C: trypsin eliminated spontaneous transmitter release. After evoked transmitter release had been blocked by TeNT-LC injection, trypsin (2% by weight in 150 mM KCl and 50 μM FITC-dextran) was injected into the excitatory axon at the time indicated (→).

A

B

C

to destroy its synapses. Figure 5C shows an example in which evoked transmitter release had been blocked by injection of TeNT-LC, at which time trypsin injection abolished the spontaneous transmitter release and caused loss of FITC-dextran (3 kDa), a fluorescent dye contained in the injection buffer. Interestingly, trypsin first caused a gross increase in the frequency of spontaneous mEPSPs. Because all synaptic activity was eventually eliminated by trypsin injection into the excitor axon, all the mEPSPs must have originated from transmitter released from the excitor boutons.

Spontaneous release is independent of increased intracellular Ca²⁺ after TeNT-LC

The preceding experiments enable us to separate spontaneous transmitter release from evoked release and suggest that the spontaneous transmitter release may not use exactly the same mechanism as evoked release. Because action potentials failed to recruit the vesicles responsible for miniature potentials after TeNT-LC injection, it is possible that the remaining spontaneous transmitter release is not sensitive to Ca²⁺. To test this hypothesis, we first examined Ca²⁺ dependency of miniature potentials before the toxin treatment and found that in intact synapses, mEPSP frequency was not sensitive to changes in [Ca²⁺]. In five experiments, when normal saline was exchanged for Ca²⁺-free saline containing 1 mM EGTA, mEPSP frequencies were not significantly different (0.85 and 0.80 Hz, respectively, P > 0.4, n = 5); the average change in frequency was −0.05 ± 0.06 Hz (P > 0.2, t-test) and the average percent change was −4 ± 6% (P > 0.2, t-test) (cf. Zucker and Lando 1986). Next we asked whether increases in [Ca²⁺], caused by Ca²⁺ entry though Ca²⁺ channels could affect mEPSP frequency. When depolarizing current was applied through an electrode impaling the axon, there was a large increase in mEPSP frequency (Wojtowicz and Atwood 1984). The increase in mEPSP frequency caused by depolarization was blocked in Ca²⁺-free saline with 1 mM EGTA, indicating that the effect of the depolarizing current was mediated by Ca²⁺ influx (Fig. 6A). However, after EPSPs had been blocked with TeNT-LC injection, depolarizing current in normal [Ca²⁺]₀ saline failed to induce any change in mEPSP frequency.
Figure 6B is typical of three experiments. It also was observed with Ca$^{2+}$ imaging that, after TeNT-LC injection, depolarizing current still caused increases in [Ca$^{2+}$], whereas mEPSP frequencies were not changed (2 experiments, not shown here). Therefore, depolarization of the terminal membrane had elevated [Ca$^{2+}$], but after TeNT-LC treatment this Ca$^{2+}$ could not increase mEPSP frequency.

**Botulinum toxins B and D affect neurotransmitter release differently**

Because spontaneous release was little affected by TeNT, it is possible that VAMP is not required by spontaneous transmitter release or that VAMP used for the spontaneous transmitter release is for some reason relatively resistant to TeNT-LC. As reviewed in Fig. 1, VAMP cleavage by TeNT-LC requires a specific TeNT-LC binding sequence (activation site) as well as a precise cleavage site (Pellizzari et al. 1996; Rossetto et al. 1994). If the VAMP used for spontaneous transmitter release lacks either the binding or cleavage site or if one of the sites is not exposed to the toxin, then the toxin would have little effect on mEPSP frequency. We next tested whether the VAMP responsible for spontaneous release could be cleaved if a toxin was used that requires a binding site on VAMP different from that used by TeNT. We chose BoNT/B, which shares the VAMP cleavage site with TeNT but not the binding site (Pellizzari et al. 1996), and asked whether this toxin could affect the spontaneous transmitter release. When we injected into the axon a solution of BoNT/B-LC that contained 1 mM EDTA in the buffer, neither EPSP amplitude nor mEPSP frequency was changed, probably because the EDTA chelated Zn$^{2+}$ necessary for toxin activity (Schiavo et al. 1992). This showed that the toxin has no nonspecific effects. When the EDTA concentration was lowered to 80 and 200 $\mu$M of ZnSO$_4$ was added, injection of BoNT/B-LC blocked the evoked release but mEPSP frequency was not significantly different; the average frequency was 0.48 ± 0.12 Hz before injection and 0.61 ± 0.05 Hz, after injection (P > 0.05, n = 5) (Fig. 7, A and B). The average change in frequency was 0.12 ± 0.12 Hz (P > 0.05, t-test) and the average percent change in frequency was +56 ± 33% (P > 0.05, t-test). Injection of boiled toxin solution did not change EPSP amplitude (n = 1). BoNT/B-LC cleaved crayfish VAMP in vitro (Fig. 7D), and immunostaining of the injected preparations showed greatly reduced VAMP immunoreactivity in the presynaptic boutons (n = 5) when evoked release had been blocked, thus confirming the cleavage of VAMP in cells (Fig. 7C).

Because the insensitivity of spontaneous release to TeNT and BoNT/B contradicts results obtained in several other preparations (see introduction and discussion), we wanted to confirm that a reduction in mEPSP frequency could have been detected. To demonstrate this, we measured the temperature dependence of spontaneous release frequency (Barrett et al. 1978). In three experiments, cold saline of 8–12°C was applied after EPSPs had been blocked at room temperature by BoNT/B-LC injection. This treatment caused an average reduction in mEPSP frequency of 68% (Fig. 7E). This showed that large changes in mEPSP frequency easily could have been detected in experiments with TeNT-LC and BoNT/B-LC.

In both vertebrates and invertebrates, more than one type of VAMP has been detected (Chin et al. 1993; Elfendink et al. 1989), some of which do not have the cleavage or binding sites for TeNT and BoNT/B and are resistant to these toxins (Pellizzari et al. 1996; Schiavo et al. 1992; Yamasaki et al. 1994a,b). We therefore examined effects of BoNT/D-LC, which cleaves at a site found on most of the known VAMPs (Schiavo et al. 1993); this is different from the site cleaved by TeNT and BoNT/B. BoNT/D-LC injected into the excitatory axon gradually decreased the amplitude of evoked

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<th>0.2 mV</th>
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*FIG. 6. TeNT-LC blocked mEPSPs induced by Ca$^{2+}$ influx. A: mEPSPs induced by presynaptic depolarizing currents were blocked in Ca$^{2+}$-free saline. Left: recordings in normal saline ([Ca$^{2+}$]$_o$ = 13.5 mM). Right: from the same muscle fiber but in Ca$^{2+}$-free saline with 1 mM EGTA. The 2nd and the 3rd sets of sweeps from the top were recorded when the axon was depolarized by passing currents through the electrode inserted into the excitatory axon. Intensity of the currents is indicated for each set of sweeps. B: presynaptic depolarization failed to induce any increase in mEPSP frequency after TeNT-LC injection in normal [Ca$^{2+}$]$_o$ saline. Procedure was similar to A, and the currents depolarizing the axon were as indicated. A and B are from different experiments.*
FIG. 7. BoNT/B-LC affects evoked and spontaneous transmitter release differently. A and B: simultaneous recordings of evoked and spontaneous transmitter release at 1 muscle fiber. Toxin was injected during the time indicated by bars. C: immunoreactivity of VAMP (top) after injection of BoNT/B-LC had blocked evoked transmitter release is reduced in excitor boutons but normal in uninjected inhibitory boutons. Bottom: both boutons are stained with antibody to synaptotagmin. Scale bar (25 μm) is the same for both panels. D: Western blot of crayfish CNS extract before (−) and after (+) proteolysis by BoNT/B-LC. Methods were the same as in Fig. 2. E: in another experiment, cold saline (8–12°C) was applied after EPSPs had been blocked by BoNT/B-LC (injected from time 0) at room temperature (∼21°C). Frequency of mEPSPs decreased at the lower temperature.

transmitter release (Fig. 8A), which is similar to the effect of TeNT-LC. Unlike the effects of TeNT or BoNT/B, the frequency of spontaneous transmitter release was reduced with a similar time course (Fig. 8B). In the five preparations tested, average mEPSP frequency decreased from a control value of 0.32 ± 0.09 Hz to 0.04 ± 0.02 Hz (P < 0.001) when EPSP amplitude had been reduced to 5% of the control (3–6 stimuli at 100 Hz). The average change in mEPSP frequency was −0.28 ± 0.10 Hz (P < 0.05, t-test) and the average percent change in frequency was −81 ± 8.9% (P < 0.05, t-test). In two of these experiments, spontaneous release was blocked totally. When BoNT/D-LC solution was injected with 1 mM EDTA (n = 2), there was no effect on either evoked or spontaneous transmitter release, thus
BoNT/D-LC inhibits both evoked and spontaneous transmitter release. A and B: from simultaneous recordings of evoked (A) and spontaneous (B) EPSPs in 1 muscle fiber. Toxin was injected during the time indicated by bars. C: immunoreactivity of VAMP (left), after injection of BoNT/D-LC had blocked evoked transmitter release, is reduced in excitatory boutons but normal in uninjected inhibitory boutons. Right: both boutons are stained with antibody to synaptotagmin. Scale bar (50 μm) is the same for both panels. D: Western blot of crayfish CNS extract before (−) and after (+) proteolysis by BoNT/D-LC. Methods were the same as in Fig. 2.

indicating that the effect was specific and required active enzyme. BoNT/D-LC cleaved crayfish VAMP in vitro (Fig. 8D) and immunostaining of the injected preparations showed greatly reduced VAMP immunoreactivity in the presynaptic boutons, thus confirming that blockade of transmitter release was accompanied by the cleavage of VAMP in presynaptic terminals (Fig. 8C).

**Discussion**

The results show that VAMP is concentrated in presynaptic boutons of both excitatory and inhibitory synapses in crayfish and that TeNT-LC cleaves crayfish VAMP in vitro. When injected into presynaptic axons, TeNT-LC blocked evoked transmitter release without affecting presynaptic action potentials or Ca++ influx, and VAMP immunoreactivity was reduced greatly. Thus the physiological effect of TeNT is most likely due to cleavage of VAMP. This is consistent with results for isolated natural TeNT-LC injected into the squid giant synapse (Hunt et al. 1994; Llinás et al. 1994) and *Aplysia* synapses (Mochida et al. 1989) and for TeNT-LC expressed in *Drosophila* (Sweeney et al. 1995). TeNT also cleaved VAMP in mouse spinal cord cells (Williamson et al. 1996), rat hippocampal slice cultures (Capogna et al. 1997), and leech neurons (Bruns et al. 1997). Our data show that in addition to blocking excitatory release, TeNT-LC also blocked inhibitory transmitter release.

**Persistence of spontaneous release after TeNT and BoNT/B**

TeNT and BoNT/B caused no net reduction in the frequency of spontaneous quantal transmitter release (Figs. 5 and 7) when evoked release was blocked by >95%. This contrasts with previous reports in other synapses in which TeNT holotoxin blocked evoked release and also caused large decreases in the frequency of miniature potentials (Kryzhanovskii et al. 1971, 95% reduction of frequency; Melianby and Thompson 1972, total block; Duchen and Tonge 1973, −64%, −93%; Habermann et al. 1980, −97%; Weller et al. 1991, −92%; Herreros et al. 1995, −72%; Capogna et al. 1997, −88%). *Drosophila* lacking neural synaptobrevin also have reduced mEPSP frequency.
spontaneous transmitter release still occurred after TeNT instance, elevation of \( \text{Ca}^{2+} \) because both are reduced greatly by TeNT. However, in all studies except that of Mellanby and Thompson (1972), some spontaneous transmitter release still occurred after TeNT poisoning.

To reconcile the resistance of spontaneous release to TeNT and BoNT/B in the crayfish claw-opener neuromuscular junction (nmj) with the large reductions in frequency seen in other preparations, it is instructive to consider how the rate of spontaneous transmitter release is controlled. We propose that, in all intact synapses, there is a basal rate of spontaneous transmitter release that is \( \text{Ca}^{2+} \) independent. In addition there is a \( \text{Ca}^{2+} \)-dependent rate of spontaneous release. Thus the total frequency of spontaneous release in the intact synapse depends on the resting \( [\text{Ca}^{2+}]_o \), changes of which can occur if \( \text{Ca}^{2+} \) influx, efflux, or release are altered: i.e., spontaneous release = basal \( \text{Ca}^{2+} \)-independent release + \( \text{Ca}^{2+} \)-dependent spontaneous release.

Evoked transmitter release is simply the synchronization of \( \text{Ca}^{2+} \)-dependent asynchronous release due to a rapid increase in local \( [\text{Ca}^{2+}]_o \) caused by \( \text{Ca}^{2+} \) channel opening during an action potential. Under resting conditions, the \( \text{Ca}^{2+} \) permeability of nerve terminals may differ in different synapses: this will depend on the details of \( \text{Ca}^{2+} \)-channel voltage dependence, channel modulators and the presynaptic resting potential, a variable that is affected easily by many experimental conditions. When presynaptic resting \( \text{Ca}^{2+} \) conductance is high, there will be a chronic \( \text{Ca}^{2+} \) influx that will accelerate the rate of \( \text{Ca}^{2+} \)-dependent spontaneous release. In these preparations, the frequency of spontaneous release will be somewhat dependent on extracellular \( \text{Ca}^{2+} \) concentration. If for any reason \( [\text{Ca}^{2+}]_o \) is allowed to increase sufficiently, then \( \text{Ca}^{2+} \) stimulation of release occurs and the spontaneous release rate will be increased. For instance, at the frog nmj, Erulkar et al. (1978) showed that stimulation in \( [\text{Ca}^{2+}]_o \), saline caused a reduction in spontaneous release frequency, thus indicating that under normal conditions, some of the spontaneous release requires intracellular \( \text{Ca}^{2+} \) and that under a reversed \( \text{Ca}^{2+} \) gradient some of the \( \text{Ca}^{2+} \) left the presynaptic terminal. Furthermore, in some experiments the frequency of spontaneous release is reduced by presynaptic \( \text{Ca}^{2+} \) channel blockers (Grinnell and Pawson 1989; Losavio and Muchnik 1997), thus indicating that chronic \( \text{Ca}^{2+} \) influx can keep spontaneous release frequency elevated. In other preparations such as the crayfish nmj, resting \( \text{Ca}^{2+} \) entry may be negligible, and spontaneous frequency would have little dependence on \( [\text{Ca}^{2+}]_o \) or \( [\text{Ca}^{2+}]_m \), may be regulated to such a low level that it does not normally accelerate the \( \text{Ca}^{2+} \) sensitive component and therefore does not contribute to the total spontaneous release frequency.

Cleavage of VAMP by TeNT and BoNT/B evidently blocks evoked transmitter release by removing \( \text{Ca}^{2+} \) sensitive release and thus reveals the basal \( \text{Ca}^{2+} \)-insensitive release rate. In those synapses in which the spontaneous release frequency has been accelerated by high resting \( [\text{Ca}^{2+}]_o \), cleavage of VAMP by these toxins will reduce the mEPSP frequency. We simulated this condition by elevating \( [\text{Ca}^{2+}]_o \), by depolarization; the acceleration of spontaneous transmitter release caused by depolarization induced \( \text{Ca}^{2+} \) entry was eliminated after TeNT-LC action (Fig. 6). This type of result is typical of many studies on effects of TeNT (except for TeNT-expressing \( Drosophila \)) (Sweeney et al. 1995). For instance, elevation of \( [\text{Ca}^{2+}]_o \), by \( \text{Ca}^{2+} \) ionophore could not rescue transmitter release in TeNT-poisoned cultured hippocampal neurons from rat. This contrasts with the observation that the consequences of SNAP-25 cleavage by BoNT/A can be reversed partially by high-frequency stimulation or other manoeuvres that increase \( [\text{Ca}^{2+}]_o \), (Capogna et al. 1997 and references therein). The implication here is that TeNT and BoNT/B cleave the VAMP, which is responsible for both evoked transmitter release and for any \( \text{Ca}^{2+} \)-stimulated spontaneous release. If resting \( [\text{Ca}^{2+}]_o \), is too low to have accelerated spontaneous release, then the frequency will not be affected by cleavage of VAMP by TeNT or BoNT/B. It is not clear why the contribution of \( \text{Ca}^{2+} \)-stimulated spontaneous release is extremely small relative to \( \text{Ca}^{2+} \)-insensitive spontaneous release in crayfish claw-opener nmjs, but this could be due to an unusual relationship between \( [\text{Ca}^{2+}]_o \), and transmitter release or could be due to the relative ease with which metabolic requirements can be met in physiological experiments. Crustacean synapses can be classified as tonic or phasic; the former include the crayfish claw-opener synapses and are characterized by low probability of release and great facilitation. Phasic synapses have a high probability of release and low facilitation (Atwood and Wojtowicz 1986; Msghina et al. 1998). It will be interesting to determine whether different types of synapses in \( Crustacea \) all react similarly to these toxins. Finally, the data indicate that the final steps in the exocytosis of synaptic vesicles do not require elevation of \( [\text{Ca}^{2+}]_o \), (Quastel et al. 1971); spontaneous release is independent of \( [\text{Ca}^{2+}]_o \), increases after TeNT or BoNT/B.

**Different VAMP complexes for spontaneous and evoked release**

One hypothesis for the persistence of spontaneous release after blockade of evoked release by TeNT and BoNT/B is that there was residual VAMP that could be cleaved by these toxins but that survived intact for the lifetime of these experiments. While we cannot test this hypothesis directly, it predicts that, if VAMP is used identically for both spontaneous and evoked release, then both types of release should have declined together under attack by TeNT or BoNT/B. Instead, the rates at which spontaneous and evoked release are affected by TeNT and BoNT/B are very different.

BoNT/D, unlike TeNT and BoNT/B, blocked both evoked and spontaneous transmitter release with about the same time course. The most obvious explanation for this observation is that BoNT/D cleaves some VAMP necessary for spontaneous release that is not easily cleaved by TeNT or BoNT/B. BoNT/D holotoxin also blocks both evoked and spontaneous release at the frog nmj (Raciborska et al. 1998). There are several ways in which differential VAMP....
cleavage could produce the disparate effects of these toxins. First, there could be two vesicle populations, one that is responsible for evoked release and contains a TeNT/BoNT/B-sensitive VAMP-like protein and a second population responsible for basal spontaneous release that has a TeNT/BoNT/B-resistant VAMP species. However, it is well established that evoked release consists of one or more quantal events identical to those in spontaneous release (Cooper et al. 1995b; Dudel and Kuffler 1961; Katz 1969), and this indicates that both types of release use identical vesicles. Although it is difficult to imagine how some vesicles at the active zone could receive a different protein population than others, this possibility cannot be excluded. Alternatively each vesicle could have both types of VAMP. After the present report was prepared, Deitcher et al. (1998) showed that in Drosophila (another arthropod) with a null mutation in neuronal VAMP, spontaneous transmitter release still proceeded at 25% of the normal rate, although, unlike most results with TeNT (see review in Capogna et al. 1997), the frequency could be increased with increased Ca\(^{2+}\) entry. The existence of spontaneous release in the absence of neuronal VAMP suggests that another VAMP isoform is responsible for remaining spontaneous release or that spontaneous release does not require VAMP at all (Deitcher et al. 1998).

Drosophila has a neuronal VAMP sensitive to TeNT (DiAntonio et al. 1993; Sweeney et al. 1995) and a "nonneuronal" form resistant to TeNT (Südhof et al. 1989; Sweeney et al. 1995) in which both the TeNT and BoNT/B binding sites are different from comparable regions of mammalian VAMP (Pellizzari et al. 1996, 1997; Sweeney et al. 1995). Although the "nonneuronal" form is not abundantly expressed in the nervous system of Drosophila (Chin et al. 1993), it could be responsible for the spontaneous release that remains in the null VAMP mutant and in Drosophila that express TeNT (Sweeney et al. 1995). Although we do not yet have the sequence of any crayfish VAMPs, a molecule with some of the properties of Drosophila "nonneuronal" VAMP could cause differential toxin effects on spontaneous release if it is resistant to both TeNT and BoNT/B and susceptible to BoNT/D. If such a molecule exists in crayfish, it must perform differently from the Drosophila homolog because, unlike the situation in Drosophila, transmitter release in crayfish-opener synapses cannot be rescued by extra Ca\(^{2+}\) after action of TeNT. Our data, however, do show that because BoNT/D blocks both evoked and spontaneous release, some VAMP-like molecules participate in both forms of quantal release.

Another explanation for the differential effects of TeNT/BoNT/B and BoNT/D may be that VAMP exists in two types of complexes with other proteins, one of which functions in evoked release and the other in spontaneous release. In spontaneous release, the protein complex may shield the common site where TeNT and BoNT/B cleave VAMP (rat VAMP2 Gln76-Phe77) (Schiaivo et al. 1992; Yamasaki et al. 1994a,b) or may shield the different binding sites for both toxins (Fig. 1) (Pellizzari et al. 1996, 1997). Because BoNT/D does block spontaneous release, the cleavage site for BoNT/D (rat VAMP2 Lys59-Leu60) (Yamasaki et al. 1994a,b) must be exposed and the binding site also must be exposed. Because BoNT/D and TeNT use the same binding site (Pellizzari et al. 1996, 1997), it is reasonable to assume that the TeNT binding site is available on the VAMP complex responsible for spontaneous release, and therefore it is the TeNT/BoNT/B cleavage site that is occluded in that complex. In evoked release under our experimental conditions, the binding and cleavage sites for TeNT, BoNT/B, and BoNT/D are all exposed to the toxins because they all block evoked release. Because VAMP interacts with syntaxin and SNAP-25 in a domain that encompasses the binding and recognition sites for TeNT and BoNT/B (Fig. 1) (Hao et al. 1997; Hayashi et al. 1994), such interactions might shield VAMP from attack by TeNT or BoNT/B.

Although our data cannot distinguish between two different VAMPs responsible for spontaneous and evoked release and two different complexes of one VAMP, it is clear that the data are inconsistent with one static VAMP complex that is responsible for all transmitter release.

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