Tetanus Toxin Inhibits Neuroexocytosis Even When Its Zn²⁺-dependent Protease Activity Is Removed*

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Tetanus toxin (TeTX) is a dichain protein that blocks neuroexocytosis, an action attributed previously to Zn²⁺-dependent proteolysis of synaptobrevin (Sbr) by its light chain (LC). Herein, its cleavage of Sbr in rat cerebrocortical synaptosomes was shown to be minimized by captoportil, an inhibitor of certain metallo-endoproteases, whereas this agent only marginally antagonized the inhibition of noradrenaline release, implicating a second action of the toxin. This hypothesis was proven by preparing three mutants (H233A, E234A, H237A) of the LC lacking the ability to cleave Sbr and reconstituting them with native heavy chain. The resultant dichains were found to block synaptosomal transmitter release, albeit with lower potency than that made from wild type LC; as expected, captoportil attenuated only the inhibition caused by the protease-active wild type toxin. Moreover, these protease-inactive toxins or their LCs blocked evoked quantal release of transmitter when micro-injected inside Aplysia neurons. TeTX was known to stimulate in vitro a Ca²⁺-dependent transglutaminase (TGase) (Facchiano, F., and Luini, A. (1992) J. Biol. Chem. 267, 13267–13271), an affect found here to be reduced by an inhibitor of this enzyme, monodansylcadaverine. Accordingly, treatment of synaptosomes with the latter antagonized the inhibition of noradrenaline release by TeTX while not affecting Sbr cleavage. This drug also attenuated the inhibitory action of all the mutants. Hence, it is concluded that TeTX inhibits neurotransmitter release by proteolysis of Sbr and a protease-independent activation of a neuronal TGase.

Tetanus toxin (TeTX) is a 150-kDa protein produced by Clostridium tetani that causes tetanus by specifically and irreversibly blocking transmitter release (I). It consists of a heavy chain (HC) disulfide-linked to a light chain (LC) that possesses a Zn²⁺ binding motif (HEXXH) characteristic of neutral metallo-endoproteases (2). Binding of TeTX via the HC to neuronal exo-acceptors mediates internalization, while the LC inhibits exocytosis, at least in part, by Zn²⁺-dependent proteolytic cleavage of synaptobrevin (Sbr) (also called vesicle-associated membrane protein) at position Gln⁷⁶-Phe⁷⁷. Sbr I isosform of rat is not degraded due to a substitution of a Val; for Phe⁷⁷ (2). Certain aspects of TeTX action cannot be satisfactorily explained by Sbr cleavage alone, including the requirement of an intact cytoskeleton for the toxin to exert its full activity in synaptosomes (3), and the inhibition of transmitter release observed when mRNAs for nonproteolytic mutants (cf. Ref. 4) of its LC were expressed in Aplysia neurons (5–6). In fact, TeTX activates liver and brain transglutaminase (TGase) in vitro (7). Different TGase isoforms have been detected in synaptosomal cytosol and on synaptic vesicles (8). The nerve terminal-specific protein, synapsin I, is cross-linked by the vesicular TGase; furthermore, ability of the latter to incorporate spermidine into synapsin I is stimulated by TeTX in vitro (8). These findings may help to explain why inhibition of TGase potentiates neurotransmitter release (9) and insulin secretion (10); moreover, they implicate a role for this enzyme in exocytosis. However, the involvement of TGases in the TeTX-induced blockade of neuroexocytosis has been challenged (11). In the present study, it is demonstrated that TeTX can inhibit neurotransmitter release in the absence of Sbr cleavage, and this protease-independent action is antagonized by a TGase inhibitor.

EXPERIMENTAL PROCEDURES

Rat cerebrocortical synaptosomes were prepared and release of triated noradrenaline (NA) measured as outlined previously (12). Electrophysiological monitoring of acetylcholine (ACh) release from neurons of Aplysia californica is described elsewhere (13). All samples to be tested in this latter preparation were mixed with a solution of dye (10%, w/v, fast green FCF (Sigma), at near saturation in 100 m NaCl). Toxin samples were air pressure intracellularly injected (~1% of the cell body volume) under visual and electrophysiological monitoring by means of a third microelectrode impaled into the presynaptic cell body (13). Intracellular administration of the buffer used to prepare the toxins was found to cause no change in ACh release.

For measurements of Sbr content (12), synaptosomes were subjected to SDS-PAGE followed by Western blotting, using an antibody raised against a 62-mer synthetic Sbr peptide (see Ref. 14). Blots were quantified, using the Amersham ECL system, under conditions in which the signal intensities were in the linear range of the film, and scanned using a Hirschmann computerized densitometer; the results were normalized against similarly measured values (12) for synaptophysin, a protein known to be unaffected by TeTX. Native or recombinant LC were incubated with synaptosomal vesicles (0.5 mg/ml; purified by differential centrifugation from bovine cortex (see Ref. 14)) for 90 min at 37 °C in 50 mM Hepes, 400 mM NaCl, 5 mM dithiothreitol, 2 μM ZnSO₄ (pH 7.4) and subjected to SDS-PAGE/Western blotting, as above. TGase was assayed at 37 °C in 50 mM Tris-HCl (pH 8.2) containing...
10 mM dithiothreitol, 0.1 mM CaCl₂, 4 mg/ml casein, and 5 milliunits of the guinea pig liver type II TGase (Sigma). The enzyme was preincubated for 30 min at 37 °C in the absence or presence of various TeTX concentrations prior to its addition to the reaction mixture [1,4-3H]putrescine (7.5 μCi/assay sample; 0.12 mM final concentration) was then added. Aliquots of the reaction mixture, at appropriate times, were added to cold 10% (w/v) final trichloroacetic acid and the precipitates washed and prepared for scintillation counting. Monodansylcadaverine (MDC) was used in the enzyme preincubation and reaction mixtures at a final concentration of 0.1 mM.

DNAs encoding wild type (WT) and mutated LC (H233A, E234A, and H237A) were constructed and amplified (14), using polymerase chain reaction technology. Each construct was subcloned into pMAL-C2 vector containing an engineered thrombin cleavage site and expressed in Escherichia coli (pM109) as a fusion with maltose-binding protein (MBP). The soluble fusion proteins were extracted in the presence of protease inhibitors (14) from sonicated cells and purified by affinity chromatography on amylose resin. Purity of the LC preparations was determined by SDS-PAGE and by Western blotting (14). Dichain species were reconstituted by mixing equimolar amounts of HC, purified from TeTx, with recombinant WT or each mutant LC-MBP fusion followed by dialysis (see Ref. 14 for details). The re-assembled MBP-LC-HC complexes were then absorbed to amylose resin; nonreconstituted HC and any contaminants were washed away, and the bound MBP-LC-HC complexes then eluted with 10 mM maltose. MBP was cleaved from each fusion protein using thrombin (Sigma) at 4 °C for 16 h (at an enzyme:protein ratio of 4 units to 300 μg). Free maltose was removed by extensive dialysis, and the dichain preparations were separated from the released MBP by a further chromatographic step on amylose resin. The exact concentrations of the various proteins were determined by the Bradford protein assay using bovine serum albumin as a standard. For each individual dichain species, at least three different preparations behaved similarly toward inhibition of synaptosomal NA release (Fig. 2A), indicating the reproducibility between batches.

Zn²⁺ content of the various fusion proteins was measured using a Perkin-Elmer 1100B atomic absorption spectrometer after the samples had been extensively dialyzed at 5°C against metal-free 10 mM Hepes buffer (pH 7.4). The amount of Zn²⁺ associated with each LC was calculated by subtracting the content for MBP alone, treated similarly, from that of MBP-LC fusion proteins.

RESULTS

TeTx-induced Blockade of Synaptosomal Neurotransmitter Release Persists after the Toxin’s Cleavage of Sbr Is Minimized by Captopril—Treatment of synaptosomes with TeTX gave a concentration-dependent reduction of depolarization-evoked Ca²⁺-dependent NA release, but this was only marginally attenuated by captopril (Fig. 1A), an inhibitor of certain metallopeptases that gives partial antagonism of the neuromyelitic action of TeTX at the mammalian neuromuscular junction (12). Yet, Western blotting of these same synaptosomes poisoned with TeTX revealed that captopril virtually prevented its dose-dependent cleavage of Sbr (Fig. 1B). The incomplete proteolysis of Sbr seen (33 ± 5.8% of total remaining) is in accordance with the known amount of toxin-insensitive Sbr isoform 1 in rat (2). A similar pattern of results was obtained with mouse synaptosomes in which both Sbr isoforms are cleaved by TeTX. These findings suggest that blockade of secretion by TeTX involves an additional, captopril-insensitive mechanism.

Protease-deficient Recombinant LCs when Recombined with HC Block Synaptosomal NA Release—Native LC and rec WT MBP fusion cleaved Sbr, but even high concentrations of the mutants (up to 5 μM) failed to yield any such proteolysis (Fig. 2A, inset). This confirms earlier data with the E234A mutant (14) and extends the results to H233A and H237A; the two histidines mutated are thought to be important in Zn²⁺ binding by TeTX (5, 15). Consistently, the fusion proteins of H233A and H237A contained a much reduced Zn²⁺ content (0.23 ± 0.02 and 0.25 ± 0.02 mol of Zn²⁺/mol of toxin, respectively; average

2 A. C. Ashton, Y. Li, and J. O. Dolly, unpublished observation.
captopril, a feature obviously not observed with any of the mutants which appeared nearly equally effective. Importantly, despite the partial blockade of ACh release seen 2 h after injection, cleavage of the MBP fusion protein did not lead to an increase in potency (not shown). Note that intracellular injection of MBP alone (50 nM final intrasomatic concentration) did not alter ACh release (Fig. 3A). Typically, at 10 nM, ~70% inhibition of ACh release was seen 2 h after injection. Cleavage of the MBP fusion protein did not lead to an increase in potency (not shown). Note that intracellular injection of MBP alone (50 nM final intrasomatic concentration) did not alter ACh release (Fig. 3A). All three of the mutant LC-fusion proteins exhibited a clear inhibitory activity; however, the extent of blockade was not as great as that induced by the rec WT material (Fig. 3, A and B). H233A was less active (activity detected only at 50 nM) than the other two mutants which appeared nearly equally effective. Importantly, at variance with the observed effect of rec WT LC preparation, a total blockade of ACh release could not be induced by increasing the intracellular concentration (>100 nM) of the protease-deficient LC-fusion proteins. A similar pattern of inhibition was obtained for the reconstituted dichains, with the toxin formed from the rec WT LC being more potent than that derived from LC E234A or H237A (Fig. 3C). However, no inhibitory activity was detected with 50 nM of the H233A dichain, in keeping with this mutant LC fusion having yielded the lowest level of blockade (see above).

Inhibition of Synaptosomal TGase Activity with MDC Reduces the Potency of TeTX in Blocking NA Release—The protease-independent action observed above was suspected to implicate TGase because of enhancement of its activity by TeTX in vitro (7) and the ability of the brain enzyme to cross-link synapsin I (8). Hence, it was verified herein that TeTX causes a significant potentiation of the liver TGase II, when the ratio of toxin:enzyme was varied between 0.2 and 1, using a similar assay to that described (7) except for a different donor substrate. These conditions yielded a maximum increase in the enzymic activity of 35.5 ± 18.3% (n = 9 independent experiments). Moreover, this enhancement was reduced by MDC, an inhibitor of TGases (9). Using the highest MDC concentration (0.1 mM) that avoids interference with NA release from synaptosomes, the TGase activity was lowered in the absence and presence of MDC (Fig. 3B).
presence of TeTX to 50.7 ± 7.5% and 45.5 ± 8.4% (n = 5) of the non-drug-treated control value. To circumvent the lower activity of the recombinant dichains and to establish the non-drug-treated control value. To circumvent the lower activity of these being expressed at too low a level or that difficulties arise when comparing the neuromuscular paralyzing activity of native and rec WT or mutant TeTX, because dichain reconstituted using rec WT LC is much less potent (14), despite exhibiting only a 2.5-fold lower activity in cleaving Sbr. It should be noted, however, that this preparation. A likely possibility is that motor nerve terminals do not contain the TeTX-sensitive isofrom of TGase and so in this particular peripheral nerve type the toxin may act solely by proteolyzing Sbr. It should be noted, however, that difficulties arise when comparing the neuromuscular paralyzing activity of native and rec WT or mutant TeTX, because dichain reconstituted using rec WT LC is much less potent (14), despite exhibiting only a 2.5-fold lower activity in cleaving Sbr. Data not shown). In contrast to the neuromuscular junction, the cerebrocortical synaptosomes used herein contain a variety of catecholaminergic nerve terminals; at least some of these were shown to contain the toxin-susceptible TGase, as is also the case for the cholinergic Aplysia neurons studied. Such a difference between nerve types does not detract from the important role implicated for TGase(s) in exocytosis from the results presented herein and as determined previously (7–10).

### DISCUSSION

The ability of protease-deficient TeTX mutants to inhibit exocytosis was shown for synaptosomal NA secretion and ACh release from Aplysia neurons (see Table I for summary of results). Furthermore, the same rank order of efficacies of the mutant and WT preparations was found in both models, with H233A being the least active. In accordance with the neurotoxicity observed herein with H233A, inhibition of ACh release has been observed in Aplysia neurons following injection of mRNA for LC mutants in which His233 was replaced with Leu, Phe, Cys, or Val (6). However, substitution of His233 by Gly or Pro abolished the said activity, presumably because these residues are helix breakers. However, E234Q or E234K mutants did not prove toxic in this system; this apparent discrepancy may be due to these being expressed at too low a level or that different amino acids substituted at the same position may alter the protein folding and, thereby, remove its neuromuscular activity.

A notable observation made with all the mutants is that they never completely inhibited ACh release, irrespective of concentration used; this argues in favor of an intracellular mechanism that differs qualitatively from the action of rec WT toxin which displays both activities. Further evidence that TeTX acts by two independent reactivities was obtained by the fact that treatment with both captopril and MDC produced a greater antagonism of TeTX action on NA release than either alone, although the combination yielded no further attenuation of the toxin-induced Sbr cleavage relative to captopril alone (data not shown). Much of the previous evidence for TeTX inhibiting neuroexocytosis exclusively by proteolyzing Sbr is not conclusive. For example, the reported antagonism of TeTX action in Aplysia neurons by the peptide ASQFETS (containing the Sbr cleavage site) is difficult to reconcile with the fact that this heptapeptide neither acts as a substrate nor a competitive inhibitor of the toxin (17), a large portion of the Sbr being required for cleavage. Anti-rat Sbr IgG injected into Aplysia attenuates the neuroparalytic action of TeTX during a 3-h time course, but by then only 50% inhibition of release had occurred in the absence of IgG (18); longer incubations times or higher toxin concentrations may have shown a Sbr-insensitive component. A more unusual observation is that the E234A LC mutant does not block neuromuscular transmission in phrenic nerve diaphragm (14) and H233A or H237A are also inactive in this preparation. A likely possibility is that motor nerve terminals do not contain the TeTX-sensitive isofrom of TGase and so in this particular peripheral nerve type the toxin may act solely by proteolyzing Sbr. It should be noted, however, that difficulties arise when comparing the neuromuscular paralyzing activity of native and rec WT or mutant TeTX, because dichain reconstituted using rec WT LC is much less potent (14), despite exhibiting only a 2.5-fold lower activity in cleaving Sbr (data not shown). In contrast to the neuromuscular junction, the cerebrocortical synaptosomes used herein contain a variety of catecholaminergic nerve terminals; at least some of these were shown to contain the toxin-susceptible TGase, as is also the case for the cholinergic Aplysia neurons studied. Such a difference between nerve types does not detract from the important role implicated for TGase(s) in exocytosis from the results presented herein and as determined previously (7–10).

### Table I

Summary of the biological activities of the protease-containing and -deficient toxin preparations

| Preparation            | Cleavage of VAMP | Inhibition of synaptosomal NA release | Antagonistic action of captopril on synaptosomal NA release | Antagonistic action of MDC on synaptosomal NA release | Inhibition of ACh release in Aplysia neurons |
|------------------------|------------------|--------------------------------------|-----------------------------------------------------------|------------------------------------------------------|---------------------------------------------|
| Native LC+/TeTX+       | +++              | +++                                 | ++                                                       | +                                                    | +++                                         |
| recWT LC-MBP+/recWT LC-HC+ | +     | +++                                 | +                                                       | +                                                    | +++                                         |
| H237A LC-MBP+/H237A LC-HC+ | –      | +                                   | –                                                       | +                                                    | ++                                          |
| E234A LC-MBP+/E234A LC-HC+ | –      | +                                   | –                                                       | +                                                    | ++                                          |
| H233A LC-MBP+/H233A LC-HC+ | –      | +                                   | –                                                       | +                                                    | ++                                          |

3 B. Poulain, unpublished observations.
must be a minor, but very important component, of nerve terminals. Equivalent blotting experiments to those in Fig. 1, using various specific antibodies, failed to show toxin-induced cross-linking (i.e. a shift in relative Mr) for the kinase-regulated-synapsin 1 (a postulated target (8)), syntaxin, SNAP-25, synaptotagmin, Rab3a, or GAP-43. However, there is some evidence that TeTX exhibits an activity involving the cytoskeleton (3). Indeed, when TeTX LC was expressed in transgenic animals (19), the actin filament pattern was changed dramatically in tissues expressing LC; although the effects of the toxin’s protease activity may be involved, a contribution from TeTX enhanced TGase, leading to cross-linking of these cytoskeletal elements, seems likely. Although neither actin nor tubulin appeared to be cross-linked in TeTX-poisoned nerve terminals, drug-induced disassembly of microtubules (3) or actin microfilaments2 antagonizes the inhibition by TeTX of NA release. In view of these preliminary findings, it is intriguing to propose that TeTX could promote TGase-mediated cross-linking of unknown proteins that interact with these cytoskeletal elements. The actual specific TGase that the toxin acts upon is still unknown. The discovery that Gp is also a TGase (20) is interesting; however, the antagonism by MDC of TeTX action on release suggests that the toxin acts to stimulate the cross-linking activity of the enzyme rather than any GTP binding property.

In conclusion, we have shown that TeTX blocks release by the well characterized Sbr cleavage and by separately stimulating a TGase (see Table 1). Important consequences of our study include (a) the prospect of using TeTX to identify a novel protein that interacts with the cytoskeleton and is concerned with exocytosis and (b) a highlighted need for researchers probing the function of Sbr in cells with TeTX to take account of its protease-independent activity.

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