The Role of Transglutaminase in the Mechanism of Action of Tetanus Toxin*

(Received for publication, June 3, 1994)

Julie A. Coffield, Robert V. Considine, Janet Jeyapaul, Andrew B. Maksymowycz, Ten-de Zhang, and Lance L. Simpson

From the Departments of Medicine and Pharmacology, Jefferson Medical College, Philadelphia, Pennsylvania 19107

Tetanus toxin is a zinc-dependent metalloendopeptidase that cleaves synaptobrevin, a polypeptide found in the membranes of synaptic vesicles. This action is thought to account for toxin-induced blockade of transmitter release. However, Facchiano and Luini (Facchiano, F., and Luini, A. (1992) J. Biol Chem. 267, 13267-13271) have proposed that tetanus toxin can stimulate transglutaminase, and Facchiano et al. (Facchiano, F., Benfenati, F., Valtorta, F., and Luini, A. (1993) J. Biol Chem. 268, 4588-4591) have further proposed that the stimulated enzyme produces cross-linking of synapsin. These actions might also account for toxin-induced blockade of exocytosis. Therefore, a series of experiments were performed to evaluate the possibility that tetanus toxin exerts its effects via transglutaminase. The results indicated that clostridial neurotoxins were poor substrates for the cross-linking effects of transglutaminase, and transglutaminase was a poor substrate for the proteolytic actions of tetanus toxin. In addition, at concentrations relevant to blockade of exocytosis, clostridial neurotoxins did not act on intact cells to stimulate transglutaminase, nor did they act on the isolated enzyme to stimulate cross-linking of putrescine and dimethylcasein. When used as competitive inhibitors of endogenous transglutaminase substrates, glycine methyl ester and monodansylcadaverine did not block toxin action. Furthermore, concentrations of calcium that were too low to support transglutaminase activity did not prevent toxin action. The data suggest that stimulation of transglutaminase is not the principal mechanism by which tetanus toxin blocks exocytosis in nerve cells.

Tetanus toxin is an unusually potent substance that acts inside vulnerable cells to block mediator release (1, 2). In the recent past, two hypotheses have been advanced to explain the intracellular actions of the toxin. According to one hypothesis, tetanus toxin is a zinc-dependent metalloendopeptidase that cleaves synaptobrevin (3, 4). This peptide is believed to participate in the fusion process that regulates exocytosis, and thus cleavage of the substance might plausibly lead to blockade of neurotransmitter and hormone release.

According to a second hypothesis, tetanus toxin interacts with the enzyme transglutaminase (5). The toxin itself can serve as a substrate for transglutaminase, but more importantly, the toxin can stimulate transglutaminase to act on other substrates. Thus, tetanus toxin has been shown to induce transglutaminase to cross-link synapsin, a polypeptide that has been implicated in neurotransmitter release (6). Cross-linking of this peptide might lead to immobilization of vesicles and blockade of exocytosis.

Although the synaptobrevin hypothesis and the transglutaminase hypothesis are seemingly quite different, there is one possible area of commonality. Facchiano et al. (6) have speculated that tetanus toxin could activate transglutaminase by proteolytic cleavage that converts an inactive precursor to an active product. If this were true, the endoproteolytic activity of tetanus toxin would be directed against two substrates (viz., synaptobrevin, transglutaminase), and in both cases proteolysis would contribute to blockade of exocytosis.

Tetanus toxin-induced proteolytic cleavage of synaptobrevin has now been well documented, and this phenomenon almost certainly has a role in blockade of mediator release. By contrast, the purported role of transglutaminase has not been established. Therefore, experiments were done to address two related issues: (i) to determine whether transglutaminase plays a pathophysiological role in tetanus toxin action, and (ii) to determine whether transglutaminase plays a physiological role in the normal process of transmitter release.

MATERIALS AND METHODS

Toxins and Drugs—Tetanus toxin was purchased from Calbiochem. Botulinum neurotoxin type B in the unactivated form was kindly provided by Dr. Y. Kamata (University of Osaka Prefecture). The neurotoxin was activated by adding it to N-tosyl-phenylalanine chloromethylketone-treated trypsin that was coupled to agarose beads (trypsin: toxin, 1:40 (w/w)). The mixture was incubated at 37 °C for 15 min in 0.02 M sodium phosphate buffer, pH 7.0. The reaction was terminated by centrifugation and aspiration of activated toxin. The homogeneity and molecular structure of the toxins were confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (see below), and the biological activity of the toxins was measured on mouse phrenic nerve-hemidiaphragm preparations (see below). Guinea pig liver transglutaminase, glycine methyl ester, and monodansylcadaverine were purchased from Sigma.

Enzyme Assay—Transglutaminase activity was assayed as described by Facchiano and Luini (5), with two exceptions. First, enzyme and toxin were preincubated at the same concentrations at which they were assayed, rather than preincubated at a high concentration and then diluted. Second, tritiated putrescine (30 Ci/mmol) was used in place of tritiated spermidine. The assay buffer was composed of 12 mM Tris-HCl, pH 7.8, 14 mM dithiothreitol, 2 mM MgCl2, 2 mM CaCl2, and other ingredients as listed under "Results." Depending on experimental protocol, either dimethylcasein (20 μM), endogenous protein, or a clostridial neurotoxin was used as a substrate.

Neuromuscular Preparations—Mouse phrenic nerve-hemidiaphragm preparations were excised and suspended in physiological buffer that was bubbled with 95% O2, 5% CO2. The physiological solution had the following composition (mM): NaCl, 137; KCl, 5; CaCl2, 1.8; MgSO4, 1.0; NaHCO3, 24; Na2HPO4, 1.0; and 1-glucose, 11. Gelatin (0.01%) was added as an auxiliary protein to diminish nonspecific inactivation of toxin.

* This work was supported in part by NINCDS Grant NS-22153 and by United States Department of Army Contract DAMD17-90-C-0048, and National Research Service Award Fellowships 1-F32-NS0472-01 and 1-F32-DK08888-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Rm. 314-JAH, Jefferson Medical College, 1020 Locust St., Philadelphia, PA 19107. Tel.: 215-855-8531; Fax: 215-855-2169.
Tissues were used to monitor stimulus-evoked muscle twitch or spontaneous miniature end plate potentials. For experiments on evoked twitch, phrenic nerve were stimulated at 0.1 Hz, and muscle responses were recorded by using a strain gauge transducer and a physiological recorder. Toxin-induced paralysis was measured as a 90% reduction in twitch response to nerve stimulation. For experiments on end plate responses, preparations were pinned in a small Petri dish coated with agar and superfused (34 °C) with fresh physiological saline. Standard intracellular recordings were obtained using glass microelectrodes filled with 3 M KCl. Tip resistances ranged between 20 to 40 MΩ. Resting membrane potentials ranged between -60 and -90 mV.

Sporneurons end plate potentials and evoked responses were monitored for a baseline period of 30-60 min before addition of toxin or drug. When toxin alone was added after the baseline period, evoked responses were monitored until onset of paralysis (see above). Spontaneous miniature and end plate potentials were then recorded for an additional 60-90 min. When drug alone was added after the baseline period, spontaneous potentials were recorded for an additional 90-120 min. When toxin and drug were studied together there was a progression of events, as follows: 30-min exposure to drug, recording of spontaneous miniature end plate potentials for 30 to 60 min, addition of toxin and monitoring of responses until onset of paralysis, recording of spontaneous end plate potentials for 30-60 min. The average number of end plates studied during baseline periods was five. The average number of end plates studied during drug or toxin exposure was nine. A minimum of three experiments was done for each paradigm.

Cell Culture—NG-108 neuroblastoma cells, which were kindly provided by Drs. M. Nirenberg (National Institutes of Health), were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 µg hygromycin, and 1 µg aminopterin. Cells were differentiated by diminishing the serum content to 5%, then adding 1 mM N2-O-dibutyryladenosine 3',5'-cyclic monophosphate. Cells were incubated in differentiation medium for 6-10 days prior to experiments.

Acetylcholine release was measured by a modification of the method of McGee et al. (7). NG-108 cells were grown in 90-mm tissue culture dishes and differentiated as described above. Prior to release studies, cells were labeled for 24-36 h with 1.5 µCi/mmol 1-Choline chloride (50 mCi/mmol) in differentiation medium containing dibutyryl cyclic adenosine monophosphate. Following the labeling period, the radioactive medium was removed, and cells were washed with Dulbecco's modified Eagle's medium for 30 min. The wash was accomplished by removing the culture medium and adding fresh medium every 5 min during the 30-min wash period. Following the washes the medium was changed to Dulbecco's modified Eagle's medium with 0.1 mM eserine sulfate, plus 0.1 mM NaHCO3, (control cells) or 44 mM KCl (high K+-treated cells). The cells were incubated in release medium for 10 min. Radioactive acetylcholine was separated from precursor choline by organic extraction as described previously (7).

In Vitro Translation of Rat VAMP-2 (Synaptobrevin)—Rat VAMP-2 cDNA (kindly provided by Dr. Scheller, Stanford University) inserted into BlueScript KSII (Stratagene) was transcribed and translated using an in vitro system (TNT, Promega). Synaptobrevin was synthesized at 37 °C for 90 min in a 50-µl reaction mixture containing 2 µg of DNA, 25 µl of TNT reticulocyte lysate, 40 µCi of [35S]methionine, and 1 µl of TNT T3 RNA polymerase. Following synthesis, the reaction mixture was portioned into 5-µl aliquots and stored at -20 °C.

Digestion of Synaptobrevin with Tetanus Toxin—Tetanus toxin 1 µl (1 µg/ml) was diluted to 10 µl of phosphate-buffered saline supplemented with 10 µM diithiothreitol. Toxin was reduced at 37 °C for 30 min, after which various concentrations of reduced toxin (see "Results") were added to 5 µl of synaptobrevin translation mix and incubated at 37 °C for 30 min. All digestion reactions were treated with 50 µg/ml RNase A at 37 °C for an additional 15 min to remove RNA background. Reduced products (7 µl) were mixed with 20 µl of SDS-sample buffer and resolved on 15% SDS-polyacrylamide gel electrophoresis gels. Gels were fixed, stained with Coomassie, and destained, with the final change of destain being from 7.5% to 4% methanol.

The molecular weight 100,000 flow-through, which represented toxin-free transglutaminase, was concentrated on the molecular weight 30,000 membrane. Transglutaminase fractions were then mixed with sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis in 7.5% gels (see below).

Polyacrylamide Gel Electrophoresis—The cross-linking of endogenous proteins in NG-108 cells was monitored by using polyacrylamide gel electrophoresis in 3% presence of sodium dodecyl sulfate as described by Laemmli (8). The underlying concept was that transglutaminase-induced cross-linking would produce high molecular weight proteins that should be retained in the stacking gel (9).

NG-108 cells were homogenized in assay buffer (12.5 mM Tris, pH 7.4, 20 mM dithiothreitol) at 37 °C, then incubated for 180 min in homogenization buffer that contained different concentrations of calcium ranging from 0 to 10 mM. Cell lysates (50 µg of protein/tube) were subjected to electrophoresis using a 4% stacking gel and a 10% separating gel. Gels were stained with Coomassie Brilliant Blue and destained with 10% acetic acid and 50% methanol.

Data Analysis—The data in the figures and tables are presented as the mean ± S.E. For experiments on tissue preparations, each data point reflects an n of 3 or more. For enzyme assays, each experiment was done at least twelve times, and within each experiment samples were done in triplicate.

RESULTS

Sequence Homology and Substrate Activity—There are two domains in tetanus toxin that reportedly have sequence homology with transglutaminase substrates and thus are presumably sites of transglutaminase-induced cross-linking (5). It is interesting that these two domains do not have significant homology with another (Fig. 1).

One of the domains that reportedly has substrate homology is found in the light chain of the toxin. It should be noted that only the light chain is essential for the intracellular actions that culminate in blockade of exocytosis (10, 11).

The primary sequence of the light chain of tetanus toxin was aligned with the primary sequences of the light chains of botulinum neurotoxins A to E. This was done to determine whether there was homology in the putative transglutaminase substrate domain (Fig. 1). Of the five botulinum neurotoxins, only type B possessed both sequence homology with tetanus toxin and an essential glutamine. Serotype A possessed weak homology and a misaligned glutamine. The other three serotypes possessed variable homology and no reactive glutamine.

The light chains of tetanus toxin and botulinum neurotoxins type A to E were searched to determine whether there were other domains in which the group shared sequence homology and a reactive glutamine. The results, which are shown in Fig. 1, indicate that there was a region in the carboxyl terminus of the molecules that possessed an aligned glutamine. However, recent evidence indicates that this portion of the toxin molecule is not essential for blockade of exocytosis (12).

The alignment data indicate that only tetanus toxin and botulinum neurotoxin type B have domains that could account for substrate activity. Therefore, these two toxins were assayed as substrates for transglutaminase at concentrations that are relevant to blockade of transmitter release at the neuromuscular junction and in NG-108 cells (10-12 to 10-5 M; see below). The results of these experiments were negative. Even when tested at a high concentration (1 × 10-8 M), the toxins possessed little if any ability to serve as substrates for transglutaminase-induced cross-linking. This indicates that: (a) tetanus toxin and botulinum neurotoxin type B are not important substrates for transglutaminase, and (b) to the extent that the toxins are substrates, this is not an important part of the process of blocking transmitter release.

Stimulation of Transglutaminase Activity—Experiments were done on intact cells and on isolated enzyme preparations.
to determine whether tetanus toxin or botulinum neurotoxin type B would stimulate transglutaminase at meaningful concentrations. In the initial experiment various concentrations of tetanus toxin were incubated with NG-108 cells for 180 min, after which the extent of toxin-induced blockade of acetylcholine release was measured. The results (Fig. 2) indicated that concentrations in the range of $10^{-12}$ to $10^{-10}$ M produced partial to complete blockade of transmitter release.

In the next experiment, NG-108 cells were incubated for 180 min with $1 \times 10^{-8}$ M tetanus toxin. Cells were then ruptured by sonication and exposed to varying concentrations of exogenous calcium (55 °C; 180 min). Transglutaminase activity was assayed by quantifying the amount of cross-linked protein in the stacking gel, as described by Barsigian et al. (9). The results indicated that, even at a concentration that totally blocks exocytosis, tetanus toxin did not alter the pattern or amount of cross-linked protein (Fig. 3). It did not induce the appearance of cross-linked protein at low calcium concentrations (e.g. 100 µM calcium) nor did it increase the amount at high calcium concentrations (e.g. 10 mM).

In the final experiment, tetanus toxin and botulinum neurotoxin type B were examined for their ability to stimulate transglutaminase-mediated incorporation of tritiated putrescine into dimethylcasein. Toxins and transglutaminase were tested at various ratios (0.1 to 1.0; 1.0 to 1.0; 1.0 to 0.1), at various concentrations (maximum, $1 \times 10^{-5}$ M), and for various lengths of time (30, 60, and 120 min). There was no paradigm in which the toxins produced a statistically significant increase in the amount of tritiated putrescine incorporated into dimethylcasein.

**Cotidrical Toxins, Transglutaminase, and Neuromuscular Transmission—Transglutaminase is a calcium-dependent enzyme. As gauged by incorporation of putrescine into dimethylcasein, the EC$_{50}$ for calcium was in the range of 10–100 µM (results not illustrated). Magnesium and strontium possessed no more than 10% of the activity of calcium in supporting transglutaminase activity.

The level of cytosolic calcium in quiescent nerves is in the range of 100–300 nM (13), which is approximately 2 orders of magnitude below the EC$_{50}$ for calcium supported transglutaminase activity. Therefore, the actions of tetanus toxin and botulinum neurotoxin type B were studied on quiescent nerves. To further ensure that cytosolic calcium levels did not rise, the experiments were repeated with quiescent nerves suspended in medium in which calcium was replaced with equimolar concentrations of magnesium or strontium. Interestingly, both toxins blocked transmission when added to tissues under conditions that would not be expected to support transglutaminase activity (n = 3 or more per group). When tetanus toxin ($1 \times 10^{-9}$ M) was added to unstimulated phrenic

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**Fig. 1. Alignment of the primary structures of tetanus toxin and botulinum neurotoxin.** The upper part of the figure aligns the two domains of tetanus toxin that reportedly have sequence homology with known transglutaminase substrates (5). It is interesting that the only true homology between the two is the reactive glutamine (Q) that is characteristic of transglutaminase substrates. The middle part of the figure aligns the purported substrate domain of the light chain of tetanus toxin with the corresponding regions of the light chains of botulinum neurotoxins types A to E. As the figure illustrates, only the light chain of botulinum neurotoxin type B has significant sequence homology with the light chain of tetanus toxin. The lower part of the figure shows the only region of the light chains of the six toxins in which there is true alignment of glutamine residues. This region is in the carboxy terminus of the light chains, and it is a portion of the molecule that is not required for blockade of exocytosis (12). The primary structures for the various toxins were obtained as follows: tetanus toxin (17, 18) and botulinum neurotoxin type A (19, 20), type B (21), type C (22), type D (23), and type E (24, 25).

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**Fig. 2. Blockade of acetylcholine release from NG-108 cells.** Various concentrations of tetanus toxin were incubated with cells that had been preloaded with [methyl-14C]choline chloride. After 180 min, cells were depolarized and the medium was assayed for radioactive acetylcholine. Complete blockade of exocytosis was obtained with 180 min exposure to $10^{-12}$ M toxin.
nerve-hemidiaphragm preparations in physiological medium, the average paralysis time was 172 ± 9 min. Similarly, when botulinum neurotoxin type B (1 x 10^{-12} M) was added to preparations in physiological medium, the average paralysis time was 191 ± 12 min. The results for both toxins were not significantly different when calcium was replaced by magnesium or strontium.

As a further test of the involvement of transglutaminase in clostridial toxin action, experiments were done in the presence of glycine methyl ester and monodansylcadaverine. These agents can serve as false substrates for the enzyme, and thus they can be used to inhibit the cross-linking effects of endogenous substrates by transglutaminase (14–16).

Glycine methyl ester and monodansylcadaverine were assayed for their ability to inhibit transglutaminase-mediated incorporation of triitated putrescine into dimethylcasein. The respective IC_{50} values were: glycine methyl ester, 2 x 10^{-4} M; monodansylcadaverine, 1 x 10^{-5} M. The concentrations of the drugs had to be incremented 2-4-fold to obtain comparable effects on intact NG-108 cells.

Glycine methyl ester and monodansylcadaverine were tested for their abilities to alter stimulus-evoked muscle twitch and spontaneous miniature end plate potentials (group n = 3 or more). At concentrations equal to or greater than the IC_{50} values (glycine methyl ester, 3 x 10^{-3} M; monodansylcadaverine, 1 x 10^{-5} M), the drugs produced no observable effects on muscle twitch over a period of 120 min. The drugs similarly failed to produce an effect on the frequency of spontaneous miniature end plate potentials. For example, the rate of spontaneous potentials during a base-line period of 30–60 min was 135 ± 22/min. When tissues were exposed to glycine methyl ester (3 x 10^{-3} M; 30 min), the frequency was 111 ± 12/min. In a similar experiment with monodansylcadaverine (1 x 10^{-5} M) the rate of spontaneous miniature end plate potentials during a base-line period was 99 ± 14/min, and the rate during exposure to the drug was 85 ± 6/min. These results show that transglutaminase-induced cross-linking of synaptic vesicle proteins does not play an important role in governing the normal process of neuromuscular transmission.

Experiments were done to determine whether transglutaminase inhibitors would alter clostridial neurotoxin-induced blockade of exocytosis. Both mechanical responses and electrophysiological responses were monitored. In studies on stimulus-evoked muscle twitch, neither monodansylcadaverine nor glycine methyl ester delayed the onset of toxin-induced neuromuscular blockade (Table I). In studies on electrophysiological responses, the drugs similarly failed to protect tissues against toxin-induced effects (Table II). These results are difficult to reconcile with the hypothesis that transglutaminase mediates the blocking effects of tetanus toxin.

**Sequence Homology and Proteolytic Activity—Tetanus toxin has a highly selective proteolytic action; it cleaves the Gln-Lys or Gln-Ala bonds in synaptobrevin or synaptobrevin 1.**

### Table I

<table>
<thead>
<tr>
<th>Drug pretreatment</th>
<th>Paralysis time</th>
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<tr>
<td>Tetanus toxin</td>
<td>131 ± 12</td>
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<tr>
<td>Tetanus toxin</td>
<td>124 ± 11</td>
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<tr>
<td>Tetanus toxin</td>
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<td>120 ± 14</td>
</tr>
<tr>
<td>Botulinum toxin</td>
<td>119 ± 13</td>
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</table>

* Tetanus toxin was used at a concentration of 1 x 10^{-9} M.

* The protocol for experiments involving drug pretreatment and subsequent addition of toxin is given under "Materials and Methods."

### Table II

<table>
<thead>
<tr>
<th>Toxic reaction</th>
<th>Drug pretreatment</th>
<th>Spontaneous miniature end plate potentials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine methylester</td>
<td>111 ± 12</td>
<td></td>
</tr>
<tr>
<td>Monodansylcadaverine</td>
<td>16 ± 1</td>
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* Glycine methylester was used at 3 x 10^{-3} M, and monodansylcadaverine was used at 1 x 10^{-3} M.
Tetanus toxin is a zinc-binding protein that possesses the properties of a metalloendopeptidase. The toxin cleaves a specific peptide bond in synaptobrevin 2, a vesicle-associated protein (3, 4).

An additional action for tetanus toxin has been proposed by Facchiano, Luini, and their colleagues, who reported that tetanus toxin stimulates the cross-linking enzyme transglutaminase (5). Stimulation of the enzyme may be due to proteolytic cleavage that converts an inactive precursor to an active product (6). Therefore, a series of experiments were done to evaluate the possibility that tetanus toxin, or the structurally and functionally similar botulinum neurotoxin type B, exert their effects via transglutaminase.

Sequence analysis data and enzyme-substrate experiments have been interpreted to mean that tetanus toxin is a substrate for transglutaminase (5). However, closer analysis of the sequence data reveals that evidence for substrate homology is not compelling (Fig. 1). In addition, enzyme-substrate experiments at meaningful concentrations of toxin demonstrated that the latter is not an important substrate.

Tetanus toxin reportedly stimulates transglutaminase to cross-link endogenous proteins, and this stimulation may be due to proteolytic activation of the cross-linking enzyme (6). It is noteworthy that toxin-induced cross-linking was not shown on intact cells, and toxin-induced cleavage of transglutaminase was not reported (6).