Specific Antibodies against the Zn\textsuperscript{2+}-binding Domain of Clostridial Neurotoxins Restore Exocytosis in Chromaffin Cells Treated with Tetanus or Botulinum A Neurotoxin\textsuperscript{*}

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Although tetanus and botulinum A neurotoxins are ineffective in cultured chromaffin cells, they will inhibit carbachol-induced release of noradrenaline provided they gain access to the cytosol either through artificial pores generated in the plasma membrane or by binding to incorporated exogenous gangliosides. The block of exocytosis persists for weeks followed by a slow recovery of cell function. When specific anti-botulinum A toxin antibodies are introduced into cells through pores after manifestation of the block by botulinum A neurotoxin, restoration of exocytotic function is accelerated and fully reestablished within 4 days. The same time course of restoration is seen with anti-tetanus toxin antibodies in cells poisoned by tetanus toxin. Since the light chains of the toxins are enzymatically active, we have introduced polyclonal and monoclonal anti-light chain antibodies into the cytosol. Of all light chain antibodies tested, only those directed against the peptide homologous to the zinc-binding sequence, which is present in both neurotoxins, restored exocytosis regardless of which toxin caused the block. These results indicate that the zinc-binding domain is directly involved in the interaction of the light chains with their substrates and that the toxins have to be present continuously to maintain the block.

Tetanus (Tetx)\textsuperscript{1} and botulinum A neurotoxins (BoNtx) belong to a group of neurotoxic proteins that are Zn\textsuperscript{2+}-binding proteins (Wright et al., 1992; Sanders and Habermann, 1992; Schiavo et al., 1992a). The toxins in this group have a common motif in the amino acid sequence of their light chains (HELIH), similar to motifs found in other metalloproteases. Tetx loses its toxicity when incubated with chelators (EDTA), indicating a pivotal role of its capability to bind zinc (Sanders and Habermann, 1992; Schiavo et al., 1992c). The neurotoxins degrade with high specificity synaptic proteins that are involved in the fusion of transmitter-containing vesicles with the plasma membrane, thus inhibiting transmitter release (Schiavo et al., 1992b; Blasi et al., 1993). Whereas Tetx acts preferentially in spinal inhibitory neurons by blocking the release of glycine and \(\gamma\)-aminobutyric acid (Curtis and de Groat, 1968), BoNtx inhibits synaptic transmission at the peripheral neuromuscular junction (Ambache, 1948). After intoxication, recovery of physiological functions is a slow process. It has been claimed that, in the case of botulism, nerve sprouting and a simultaneous appearance of newly synthesized peripheral acetylcholine receptors correlate with relief of clinical symptoms (Pestronk and Drachman, 1978; Duchen and Stritch, 1968). In the case of tetanus, hints about the mechanisms of physiological function recovery came from spinal cord neurons in culture. The toxin produced a state of neuronal hyperexcitability that faded eventually into synaptic quiescence (Bergey et al., 1987). The latter effect is due to an additional block of excitatory transmitter release that can be evoked at higher concentrations of toxin than those necessary to block glycine release. A partial restoration of excitatory activity occurred concomitantly with the intracellular degradation of Tetx. It was not clear whether these neurons resume synaptic transmission by sprouting or by complete degradation of the toxin, such that resynthesis of its target would no longer be counteracted by the proteolytic activity of the toxin (Habig et al., 1986).

The intracellular targets of Tetx and BoNtx are present not only in nerve cells but also in certain other secretory cells, such as chromaffin cells (Ahnert-Hilger et al., 1989a; Marxen et al., 1989). However, since chromaffin cells lack gangliosides in their plasma membrane (Marxen and Bigalke 1989; Marxen et al., 1991), they cannot take up the toxins unless artificial manipulations are performed. Nevertheless, chromaffin cells represent useful tools for studying the interference of toxins with exocytosis because they constitute a homologous cell population and offer an excellent access to structures controlling hormone release. There are several ways to introduce the toxins into the cytosol. By applying a strong electric field to the cells, pores are formed in the plasma membrane through which the toxins can diffuse into the cytosol (Bartels and Bigalke, 1992). Openings

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\textsuperscript{1} The abbreviations used are: Tetx, tetanus neurotoxin; BoNtx, botulinum A neurotoxin; LC, light chain; HC, heavy chain; DC, dichain; poly, polyclonal; mono, monoclonal; GM1, \(1\beta\)NeuAc-G\(\alpha\)Gos\(\alpha\)Cer; G\(\alpha\)1,4,6; NeuAc, \(1\beta\)NeuAc-G\(\alpha\)Gos\(\alpha\)Cer; G\(\alpha\)1,4,6, NeuAcy, \(1\beta\)NeuAcy-G\(\alpha\)Gos\(\alpha\)Cer; G\(\alpha\)1,4,6, IV\(\beta\) NeuAcy, \(1\beta\)NeuAcy-G\(\alpha\)Gos\(\alpha\)Cer; G\(\alpha\)1,4,6, IV\(\beta\) NeuAcy-\(1\beta\) NeuAcy-G\(\alpha\)Gos\(\alpha\)Cer.}
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Table 1

| Antibody       | DC-Tetx | DC-BoNtx | LC-Tetx | LC-BoNtx | HC-Tetx | HC-BoNtx | Albumin-HELIH |
|----------------|---------|----------|---------|----------|---------|----------|---------------|
| Poly-DC-BoNtx  | ND      | ND       | ND      | ND       | ND      | ND       |               |
| Poly-DC-Tetx   | +       | ND       | +       | ND       | -       | -        |               |
| Mono-LC-Tetx   | +       | ND       | +       | ND       | -       | -        |               |
| Poly-LC-BoNtx  | ND      | ND       | ND      | ND       | -       | -        |               |
| Poly-P2        | (+)     | +        | +       | +        | -       | -        |               |
| Poly-P3        | ND      | -        | -       | -        | -       | -        |               |

Exp. 1: Summary of enzyme-linked immunosorbent assay tests

Wells were coated with the respective antigen, and antibody-containing solutions were added after thorough washing. Wells were then loaded with peroxidase-conjugated anti-IgG antibodies followed by the color reaction. Pairwise difference in OD was statistically significant (*p < 0.05, Student's t-test). Positive and negative reactions are marked by (+) and (−), respectively. Reactions just above the detection limit are marked by (±).

1. Neutralization of Clostridial Neurotoxins

2. Localization of Neurotoxin Receptors

3. Inhibition of Exocytosis

4. Modulation of Cell Function

5. Implications for Neurotransmission

6. Future Directions

7. Conclusion
FIG. 1. Time-dependent restoration of exocytosis. Chromaffin cells were electroporated in the presence of 66 nM (A) and 6.6 nM (○) Tetx and 6.6 nM (●) and 0.6 nM (○) BoNtx, respectively. Cells were seeded on collagen-coated multwell plates and maintained in culture. Growth medium was changed twice a week. Exocytosis was determined after different periods of time (abscissa). Inhibition of exocytosis expressed as a percentage of $[^{3}H]$noradrenaline release from electroporated cells in the absence of toxins (mean ± S.D., n = 3). Among the experiments, release from unpoisoned cells fluctuated by 20–30%. This apart, release remained stable for the first 3 weeks. From the 4th week on, however, release decreased continuously to arrive at 12–16% after 6 weeks.

FIG. 2. Intracellular anti-DC-BoNtx antibodies prevent the inhibitory action of BoNtx. Chromaffin cells were exposed to an electric field in the presence of either electroporation solution alone (a, c) or electroporation solution containing poly-DC-BoNtx (b, d). Cells were then seeded onto culture plates. After 24 h, the solution in all dishes was replaced with a ganglioside-containing growth medium. After another 24 h, cells were exposed to low ionic strength solution with (c, d) and without (a, b) BoNtx (6.6 nM) for 24 h, followed by a 24-h incubation in toxin-free growth medium. $[^{3}H]$Noradrenaline release (ordinate), given as a percentage of total radioactivity, was initiated by stimulation with carbachol. Values are the mean of three determinations ± S.D. The differences between bars a and c as well as c and d were statistically significant (p < 0.001).

RESULTS

When chromaffin cells were exposed to an electric field in the presence of two concentrations of BoNtx or Tetx, carbachol-stimulated release of $[^{3}H]$noradrenaline decreased in a concentration-dependent fashion (Fig. 1). The toxins' concentrations determined not only the extent of the block of exocytosis but also its duration. When cells were exposed to lower concentrations of toxin, the restoration of hormone release was somewhat faster, but it was still a slow process. It took weeks before noradrenaline release was fully re-established (Fig. 1). During this time the toxins did not affect the viability of the cells.

Since the duration of exocytosis block depended on the toxin dose, it might be feasible to shorten the recovery period by neutralizing intracellular toxin. Since antibodies have a molecular weight similar to that of the toxins, they can also pass through the artificial pores (Bartels and Bigalke, 1992). Alternatively, clostridial toxins may enter chromaffin cells whose plasma membrane had been left intact but enriched with exogenous gangliosides. Carbachol-induced release of $[^{3}H]$noradrenaline decreased by 50% when ganglioside-treated cells were exposed to 6.6 nM BoNtx (Fig. 2). This block was prevented when specific polyclonal anti-dichain BoNtx antibodies (160 IU/ml) (poly-DC-BoNtx) were introduced into the cytosol by electroporation 2 days before exposure to BoNtx (Fig. 2). The block was not prevented when the antibodies were applied without electroporation under otherwise identical conditions (not shown). This indicates that BoNtx reached the same cytosolic compartment as the antibodies, although toxin and anti-body crossed the plasma membrane by different routes.

Antibodies could reverse a block previously established by ganglioside-mediated BoNtx entry, although recovery was slow (Fig. 3). Chromaffin cells electroporated in the absence of antibodies did not recover from the BoNtx treatment within 6 days after BoNtx application (Fig. 3). The degree of recovery 48 h after antibody treatment, when restoration is still incomplete (also see Fig. 1), was not enhanced by higher concentrations of the antibodies (Fig. 4, inset), though the restoration of catecholamine release depended on the concentration of specific antibodies present during the electroporation procedure (Fig. 4).

Since the proteolytic activity of both toxins is located in their light chains, we used the following light chain-specific antibodies in release experiments: 1) guinea pig polyclonal antibodies raised against the light chain of BoNtx (poly-LC-BoNtx), 2) mouse monoclonal antibodies against the light chain of Tetx (mono-LC-Tetx), 3) rabbit (poly-P1 and poly-P2) or mouse (poly-P3) polyclonal antibodies raised against a synthetic peptide identical to the zinc-binding motif (HELIIH) found in the light chains of both toxins. Controls consisted of a mouse monoclonal antibody raised against Tetx heavy chain (mono-HC-Tetx) and guinea pig polyclonal antibody raised against BoNtx heavy chain (poly-HC-BoNtx). Each antibody was initially tested in enzyme-linked immunosorbent assay for immunoreactivity against its antigen. For the detection of anti-peptide antibodies, a different conjugate of HELIIH was used. Poly-P1 and poly-P2 had identical titers to the synthetic peptide (Fig. 5). Poly-P1 recognized DC-Tetx, DC-BoNtx, LC-Tetx, and LC-BoNtx (Fig. 5) while poly-P2 only recognized DC-Tetx and DC-BoNtx (Table 1). Poly-P3 did not recognize LC- and DC-BoNtx nor LC-Tetx. Poly-LC-BoNtx did not react with DC-BoNtx. All other antibodies reacted as expected (Table 1).

The capability of these antibodies to neutralize toxin was tested using three different experimental designs. In one set of experiments antibodies and toxins were mixed prior to addition to intact chromaffin cells incubated with gangliosides. With
this paradigm, only homologous antibodies against intact toxins or heavy chains prevented poisoning (Fig. 6). Antibodies against the light chains did not inhibit poisoning by toxin using the preincubation paradigm (Table II, Fig. 6), although they did bind their respective antigens (Table I). In a second set of experiments, the same mixtures were incubated with chromaffin cells during electroporation. With this protocol, mono-HC-Tetx and poly-HC-BoNtx lost their ability to prevent poisoning, suggesting that these antibodies inhibited binding or uptake of the toxin rather than its proteolytic activity (Table II, Fig. 6). Mono-LC-Tetx and poly-LC-BoNtx did not inhibit the proteolytic activity of Tetx or BoNtx despite their ability to bind to their respective antigens although not to HELIH (Tables I and II). In contrast, poly-P1 and -P2 were able to prevent poisoning by both toxins, and poly-P3 was able to prevent poisoning by


**Table II**

**Summary of the neutralization tests in release experiments**

Release experiments followed three experimental designs. 1) Intact cells, preloaded with gangliosides, were exposed to toxin-antitoxin mixtures (extracellular). 2) Electroporated cells (intracellular) were exposed to toxin-antitoxin mixtures (preventing). 3) Electroporated cells were exposed to antitoxin alone (restoring) after ganglioside and toxin exposure. In the second set, antitoxin neutralized both light chain toxins and DC toxins. Pairs marked with ND were not tested for neutralization. Positive neutralization is marked by + and the failure to neutralize by –, respectively.

| Antibody          | Extracellular Preventing | Intracellular Preventing | Restoring |
|-------------------|--------------------------|--------------------------|-----------|
|                   | BoNTx                  | Ttx                      | BoNTx     | Ttx          | BoNTx | Ttx          |
| Poly-DC-BoNTx     | +                       | ND                       | +         | ND           | +     | ND           |
| Poly-DC-Ttx       | ND                      | +                        | ND        | +            | ND    | ND           |
| Mono-HC-Ttx       | ND                      | +                        | ND        | –            | ND    | –            |
| Poly-HC-BoNTx     | +                       | ND                       | ND        | ND           | ND    | ND           |
| Mono-LC-Ttx       | ND                      | –                        | ND        | ND           | ND    | ND           |
| Poly-LC-BoNTx     | –                       | ND                       | –         | ND           | –     | ND           |
| Poly-P            | +                       | –                        | ND        | ND           | –     | ND           |
| Poly-P2           | –                       | +                        | +         | +            | +     | +            |
| Poly-P3           | –                       | –                        | –         | ND           | ND    | ND           |

Ttx (Table II). The concentration dependence of the protection is shown in Fig. 7a. The amount of poly-P1 contained in approximately 10 mg of IgG was equivalent to 40 IU of poly-DC-Ttx in preventing the block of exocytosis.

Like the polyclonal DC-toxin antibodies, polyclonal antibodies P1 and P2 were also able to reverse a block that had been established in the paradigm where ganglioside-treated cells were exposed to toxin followed by antibody electroporation 1 day later (Table II). Fig. 7b shows the concentration dependence for reversal of BoNTx poisoning by poly-P1. 10 mg of IgG was as effective in restoring exocytosis as 120 IU of poly-BoNTx.

Table II summarizes the effects of the different antibodies with respect to the three experimental designs and toxins used.

**DISCUSSION**

Electroporation produces transient pores in the plasma membrane (Bartels and Bigalke, 1992) large enough to allow proteins such as Ttx and BoNTx and immunoglobulins to diffuse through these openings. When the pores are closed by membrane fusion a short time later, the proteins remain trapped inside the cells. Since chromaffin cells lack binding sites for clostridial neurotoxins (Marxen et al., 1989), the toxins cannot have gained access to the cytosol by any other route than by diffusion through the electroporated pores. Exocytosis is blocked by either toxin in a concentration-dependent fashion. Toxicity indicates that the disulfide bonds connecting the heavy and light chains have been cleaved within the cells because only the reduced light chains are capable of proteolytic activity (Ahnert-Hilger et al., 1989a, 1989b) and that the light chains of both toxins are able to reach their intracellular substrates and degrade them. Exocytosis is blocked by Ttx and BoNTx over a long period of time. The recovery of exocytotic function is a slow process, and the rate of substrate resynthesis may be decisive for the progress to restoration. Since the toxins will presumably degrade substrate proteins as long as they are active, a restoration of noradrenaline release will not occur until the amount of intracellular active toxin declines to a level at which the degradation of the substrate is supersed by its synthesis. The decline in the activity of the toxins can be accelerated by neutralizing them with specific anti-toxin antibodies that, like the toxins, are able to pass through the plasma membrane by way of the electroporated pores. Thus, the duration of the block of exocytosis can be shortened. Despite the presence of antibodies, the recovery of exocytotic function proceeds at a slow pace and reaches an upper rate that cannot be accelerated by higher antitoxin concentrations. Thus, the rate of resynthesis of the substrates following neutralization may govern restoration. Neutralization itself occurs rapidly because exocytosis is not blocked if antibodies are present in the cytosol before the toxins (for Ttx, see Bartels and Bigalke (1992)). This can be achieved by electroporating the cells in the presence of antibodies and subsequently incubating them with gangliosides that will be incorporated into the plasma membrane and serve as binding sites and vehicle of entry for the toxins. After the neutralization with specific antibodies, BoNTx-poisoned cells showed a time course of recovery nearly identical to those treated with Ttx (Bartels and Bigalke, 1992), although the substrates acted on by the toxins differ from each other (Schiavo et al., 1992a, 1992b, 1992c; McMahon et al., 1993). This could mean either that the rates of resynthesis are similar for different substrates or that a cellular structure must be resynthesized that contains both substrates. The substrates for Ttx are synaptobrevin II and cellubrevin (Schiavo et al., 1992; Link et al., 1992; McMahon et al., 1993), and that for BoNTx is...
Antibodies to neutralize intracellular light chains may be due to the fact that specific polyclonal anti-Tetx and anti-BoNtx antibodies do not recognize an identical binding site (234 HELIH) were capable of neutralizing Tetx. The antibodies (poly-P1–P3) raised against a synthetic peptide resembling the zinc binding site (234 HELIH) were neutralizing Tetx, in which zinc cannot bind due to replacement of histidine 234 or glutamic acid 235 with alanine, lose their efficacy to block exocytosis with chromaffin cells. The antibodies (poly-P1–P3) raised against a synthetic peptide resembling the zinc binding site (234 HELIH) were capable of neutralizing Tetz. The antibodies cannot prevent the action of either toxin when preincubated with toxins followed by addition to ganglioside-enriched chromaffin cells (this paper) or to the phrenic hemidiaphragm preparation. The differences in binding affinities of poly-P1–P3 to the holotoxins or their light chains may indicate that these anti-HELIH antibodies do not recognize an identical structure within the peptide. The zinc-binding domain is probably hidden within the intact molecule, a concept supported by the fact that specific polyclonal anti-Tetx and anti-BoNtx antibodies, respectively, are not active against the HELIH peptide in an enzyme-linked immunosorbent assay. Physiologically, anti-HELIH antibodies will probably not be present within the pool of polyclonal anti-toxin antibodies. The latter are capable of neutralizing extra- and intracellular toxins by forming complexes with the toxins. Apparently, these complexes are not taken further up into intact cells or, if formed in the cytosol, prevent the active site of the enzymes from recognizing their substrates. The inability of either mono- or polyclonal anti-BoNtx antibodies to neutralize intracellular light chains may be due to a formation of less stable complexes or a lack of complex formation. Alternatively these antibodies may bind to epitopes on the light chain that are irrelevant to their proteolytic activity. However, upon exposure of the zinc-binding domain of either Tetx or BoNtx within the cell, anti-HELIH antibody can bind to the proteolytic site and prevent proteolysis of the natural substrate.

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REFERENCES

Alderson, K., Holds, J. B., and Anderson, R. L. (1991) Neurology 41, 1800–1805
Ahert-Hilger, G., Weller, U., Dausseneroth, M. E., and Habermann, E. (1989a) FEBS Lett. 242, 245–249
Ahert-Hilger, G., Bader, M. F., Bhakdi, S., and Gratel, M. (1989b) J. Neurochem. 52, 1751–1758
Ambache, N. (1948) Nature 161, 482–483
Bartels, F., and Bigalke, H. (1992) Infect. Immun. 60, 302–307
Bergey, G., Bigalke, H., and Nelson, P. J. (1967) Neurophysiology (Kiev) 57, 121–131
Bittner, M. A., DasGupta, B. R., and Holtz, R. W. (1989) J. Biol. Chem. 264, 10354–10360
Blasi, J., Chapman, F. R., Link, E. P., Bina, T., Yamassaki, S., De Camill, P., Sudhof, T. C., Niemann, H., and Jahn, R. (1993) Nature 365, 160–163
Curtis, D. R., and de Groat, W. C. (1968) Brain Res. 10, 208–212
DasGupta, B. R., and Sutjamorphy, V. (1984) Toxicon 22, 415–424
Duchen, L. W., and Stritch, S. J. (1968) J. Exp. Physiol. 53, 84–89
Habermann, E., Muller, H., and Hudeil, M. (1988) J. Neurochem. 51, 522–527
Habig, W., Bigalke, H., Bergey, G., Neale, E. A., Hardegree, M. C., and Nelson, P. G. (1986) J. Neurochem. 47, 930–937
Jahn, R., and Sudhof, T. C. (1993) J. Neurochem. 61, 12–21
Kenimer, J. G., Habig, W. H., and Hardegree, M. C. (1983) Infect. Immun. 42, 942–948
Kosaki, S., Togashi, S., and Sakaguchi, G. (1983) Jpn. J. Med. Sci. Biol. 34, 61–68
Lambert, H., Pankov, R., Gauthier, J., and Hancock, R. (1990) Biochem. Cell Biol. 68, 729–734
Link, E., Edelmann, L., Cheu, J. H., Bina, T., Yamassaki, S., Eisel, U., Baumert, M., Sudhof, T. C., Niemann, H., and Jahn, R. (1992) Biochem. Biophys. Res. Commun. 199, 1017–1023
Livett, B. G. (1984) Physiol. Rev. 64, 1103–1161
Marxen, P., and Bigalke, H. (1989) Neurosci. Lett. 107, 261–266
Marxen, P., Fuhrmann, U., and Bigalke, H. (1989) Toxicon 27, 649–658
Marxen, P., Ahert-Hilger, G., Wellhoener, H. H., and Bigalke, H. (1990) Toxicon 28, 1077–1082
Marxen, P., Erdmann, G., and Bigalke, H. (1991) Toxicon 29, 181–189
McMahon, H., Ushkarov, V. A., Link, E., Edelmann, L., Niemann, H., Jahn, R., and Sudhof, T. C. (1993) Nature 364, 346–349
Olsen, J., and Hiller, F. (1987) Clin. Pharmacol. 6, 570–574
Penner, R., Neher, E., and Dreyer, F. (1986) Nature 324, 76–78
Pestronk, A., and Drachman, D. (1978) Science 199, 1223–1225
Sanders, D., and Habermann, E. (1992) Naunyn-Schmiedeberg's Arch. Pharmacol. 346, 356–361
Schiavo, G., Rossetto, O., Santucci, A., DasGupta, B. R., and Montecucco, C. (1992a) J. Biol. Chem. 267, 33479–33483
Schiavo, G., Benfenati, F., Poulin, B., Polverino de Laureto, P., DasGupta, B., and Montecucco, C. (1992b) Nature 359, 832–835
Schiavo, G., Poulin, B., Rossetto, O., Benfenati, F., Tauc, L., and Montecucco, C. (1992c) EMBO J. 11, 3577–3581
Spaun, J., and Ignjat, J. (1970) Bull. W. H. O. 43, 523–534
Sudhof, T. C., and Jahn, R. (1991) Neuron 6, 665–677
Wright, J. F., Pernollet, M., Reboul, A., Aude, C., and Colomb, M. G. (1992) J. Biol. Chem. 267, 9053–9058

SNAP25 (Blasi et al., 1993). All are constituents of the vesicular membrane and are involved in the fusion of the vesicles with the plasma membrane (Sudhof and Jahn, 1991; Jahn and Sudhof, 1993). Thus it is possible that recovery of exocytosis may occur after restoration of vesicles with intact fusion proteins. In nerve cells in vivo, recovery may not necessarily be restricted to the sprouting of new axons as demonstrated after injection of therapeutic doses of BoNtx (Alderson et al., 1991) but may result from restoration of intact synaptic vesicles.

The binding of zinc to the light chain of Tetz is crucial for its proteolytic activity (Sanders and Habermann, 1992; Schiavo et al., 1992c). Thus, recombinant mutants of the light chain of Tetz, in which zinc cannot bind due to replacement of histidine 234 or glutamic acid 235 with alanine, lose their efficacy to block exocytosis with chromaffin cells. The antibodies (poly-P1–P3) raised against a synthetic peptide resembling the zinc binding site (234 HELIH) were neutralizing Tetx. The antibodies cannot prevent the action of either toxin when preincubated with toxins followed by addition to ganglioside-enriched chromaffin cells (this paper) or to the phrenic hemidiaphragm preparation. The differences in binding affinities of poly-P1–P3 to the holotoxins or their light chains may indicate that these anti-HELIH antibodies do not recognize an identical structure within the peptide. The zinc-binding domain is probably hidden within the intact molecule, a concept supported by the fact that specific polyclonal anti-Tetx and anti-BoNtx antibodies, respectively, are not active against the HELIH peptide in an enzyme-linked immunosorbent assay. Physiologically, anti-HELIH antibodies will probably not be present within the pool of polyclonal anti-toxin antibodies. The latter are capable of neutralizing extra- and intracellular toxins by forming complexes with the toxins. Apparently, these complexes are not taken further up into intact cells or, if formed in the cytosol, prevent the active site of the enzymes from recognizing their substrates. The inability of either mono- or polyclonal anti-BoNtx antibodies to neutralize intracellular light chains may be due to a formation of less stable complexes or a lack of complex formation. Alternatively these antibodies may bind to epitopes on the light chain that are irrelevant to their proteolytic activity. However, upon exposure of the zinc-binding domain of either Tetx or BoNtx within the cell, anti-HELIH antibody can bind to the proteolytic site and prevent proteolysis of the natural substrate.

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