Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc

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Tetanus and botulinum neurotoxins are the most potent toxins known. They bind to nerve cells, penetrate the cytosol and block neurotransmitter release. Comparison of their predicted amino acid sequences reveals a highly conserved segment that contains the HExxH zinc binding motif of metalloendopeptidases. The metal content of tetanus toxin was then measured and it was found that one atom of zinc is bound to the light chain of tetanus toxin. Zinc could be reversibly removed by incubation with heavy metal chelators. Zn²⁺ is coordinated by two histidines with no involvement in cysteines, suggesting that it plays a catalytic rather than a structural role. Bound Zn²⁺ was found to be essential for the tetanus toxin inhibition of neurotransmitter release in *Aplysia* neurons injected with the light chain. The intracellular activity of the toxin was blocked by phosphoramidon, a very specific inhibitor of zinc endopeptidases. Purified preparations of light chain showed a highly specific proteolytic activity against synaptobrevin, an integral membrane protein of small synaptic vesicles. The present findings indicate that tetanus toxin, and possibly also the botulinum neurotoxins, are metalloproteases and that they block neurotransmitter release via this protease activity.

Key words: metalloprotease/neurotransmitter release/synaptobrevin/tetanus toxin/zinc

Introduction

Tetanus toxin (TeTx) is the agent responsible for the development of tetanus, an often fatal spastic paralysis due to the block of neurotransmitter release of the spinal cord inhibitory interneurons (Simpson, 1989). It is produced by toxigenic strains of *Clostridium tetani* as a single chain, which is readily cleaved by proteases at an exposed loop to generate the active two-chain protein (H chain, 100 kDa and L, 50 kDa), depicted in Figure 1A (Krieglstein *et al.*, 1990). The H chain is involved in neurospecific cell binding and penetration while the L chain is responsible for the inactivation of a key component of the neuroexocytosis machinery (Montecucco, 1986; Simpson, 1989; Niemann, 1991; Schiavo *et al.*, 1991). Structurally and functionally closely related to TeTx are the botulinum neurotoxins

(BoNT, seven different serotypes) which cause the flaccid paralysis of botulism by inhibiting ACh release at the neuromuscular junction (Simpson, 1989).

Recently the cDNA-deduced amino acid sequences of five BoNT serotypes have been determined and aligned with that of TeTx (Niemann, 1991). Despite the very similar mechanism of action, these neurotoxins show an overall low degree of homology, having a few segments of close similarity. The most conserved segment is located in the central region of the L chain and the common elements of this peptide are reported in Figure 1B. We noticed that this segment includes the HExxH zinc binding motif of metalloendopeptidases. In these enzymes the zinc ion is tetra-coordinated and it is essential for their catalytic activity (Matthews, 1988; Vallee and Auld, 1990). The two histidines of the motif interact with the zinc ion while the glutamic residues play a catalytic role. The third ligand is another glutamic residue whose position in the sequence has been identified in bacterial neutral zinc endopeptidases (Vallee and Auld, 1990), but it is still unidentified in matrix metalloproteases and it is also not evident in sequences of TeTx and BoNTs. The fourth ligand is water.

In this work we demonstrate that TeTx does indeed contain a zinc atom coordinated by two histidines and that the zinc ion is essential for TeTx inhibition of neurotransmitter release in *Aplysia* neurons. We also report that highly purified preparations of the L chain of TeTx show a proteolytic activity specific for synaptobrevin, an integral membrane protein of small synaptic vesicles, and that its effect on neurotransmitter release is inhibited by phosphoramidon, a highly specific inhibitor of zinc endopeptidases.

Fig. 1. Schematic structure and zinc binding motifs of tetanus and botulinum neurotoxins and of matrix metalloendopeptidases. (A) Schematic structure of the clostridial neurotoxins of tetanus and botulism. (B) The segment of closest similarity between the six clostridial neurotoxins (TeTx, BoNT serotypes A, B, C1, D and E) whose sequences are known (Niemann, 1991) is located in the central portion of the L chain. The features common to the six clostridial neurotoxins are reported in the amino acid one letter code and with h for hydrophobic, u for uncharged and x for any residue; the zinc binding motif of matrix zinc endopeptidases (Vallee and Auld, 1990) is aligned just below.

Results

Metal content of TeTx

To determine the nature and amount of metals bound to TeTx, the toxin and its L chain were isolated by chromatographic methods followed by high performance size exclusion liquid chromatography (Lin *et al.*, 1985; Ozutsumi *et al.*, 1985; Schiavo *et al.*, 1990). Toxicity was determined in mice ($MLD_{50} < 1$ ng/kg) and purity was estimated to be $>99\%$ by densitometric scanning of SDS-PAGE gels stained with Coomassie blue. As shown in Figure 2, atomic absorption analysis of different preparations of TeTx gave a range of zinc content between 0.70 and 0.85 atoms per TeTx molecule and between 0.9 and 1.0 zinc atoms per molecule of L chain, while the H chain contains no zinc (not shown). A metal content slightly below an integer value is a common finding with metalloproteins due to metal loss during protein purification. These values hence indicate the presence of one atom of zinc per TeTx molecule and that all the zinc content of TeTx is associated with the L chain. Preliminary experiments indicate that BoNT serotypes A, B and E also contain one zinc atom per toxin molecule (G.Schiavo *et al.*, unpublished data). Co, Cu, Fe, Mn and Ni were also measured and found to be below the limits of detection.

Zinc removal with heavy metal chelators

Upon incubation with EDTA, the toxin released most of its Zn^{2+} content (filled bars in Figure 2). This occurred without protein denaturation because Zn^{2+} could be reassociated with the toxin upon incubation in a Zn^{2+} -containing medium (hatched bars in Figures 2 and 4). Zn^{2+} could also be partially removed by treatment with other heavy metal chelating agents such as *o*-phenanthroline (OP), dipicolinic acid (DPA), tetrakis-(2-pyridylmethyl)ethylenediamine anhydride (TPEN) and diethylenetriaminepentaacetic anhydride (DTPA).

Chemical modification of histidines and cysteines

In the zinc endopeptidases, two histidines are involved in zinc coordination (Matthews *et al.*, 1972; Pauptit *et al.*,

Fig. 2. Zinc content, measured by atomic absorption, of TeTx and of its L chain treated with various chelators. Amount of zinc, in atoms per protein molecule $\pm$ SD of four different toxin preparations, bound to TeTx and to its L chain before (N, open bars) or after EDTA treatment (EDTA, filled bars) or after EDTA incubation, dialysis and further incubation with $50 \mu M$ $ZnCl_2$ (R, hatched bars). The effect of the following heavy metal chelators is also shown: *o*-phenanthroline (OP), dipicolinic acid (DPA), tetrakis-(2-pyridylmethyl)ethylenediamine anhydride (TPEN) and diethylenetriaminepentaacetic anhydride (DTPA). Co, Cu, Fe, Mn and Ni were also assayed and were found to be below the limits of detection. Experimental details are in Materials and methods.

1988; Vallee and Auld, 1990; Thayer *et al.*, 1991). Chemical modification of TeTx with diethylpyrocarbonate (DEPC), a histidine modifying reagent (Ovadi *et al.*, 1967; Miles, 1977; Papini *et al.*, 1989), indicates that also in TeTx histidines are involved in zinc coordination. In fact an average of 1.8 histidine residues, out of the six present, were modified with DEPC in the native L chain, while this number varied in the Zn^{2+} -depleted L chain of TeTx according to the level of residual Zn^{2+} . An average of 1.5 additional histidines were modified by DEPC in L chain preparations

Fig. 3. Histidine modification with DEPC of native and zinc-depleted L chain of TeTx. (A) The increase in absorbance at 243 nm is indicative of the formation of *N*-carboxyhistidine. (B) The number of histidines modified by DEPC, as determined by absorbance change at 243 nm; bars are the $\pm$ SD of three different experiments on L chain preparations with an average residual zinc content of 19%. No absorbance change was detected at 278 nm indicative of no modification of tyrosine residues.

Fig. 4. Dot blot assay of $[^{65}Zn^{2+}]$ binding to various samples of L chain and of the Leu230-Gly244 peptide of TeTx. The samples were applied onto nitrocellulose paper and overlaid with 50 nM $^{65}ZnCl_2$ for 60 min. After washings and drying, the nitrocellulose paper strips were exposed at $-80^\circ C$ to a Kodak X-OMAT film. Samples are as follows: L + Zn, native L chain; L + Zn + DEPC, native L chain treated with a 30-fold molar excess of DEPC; L - Zn, L chain depleted with 10 mM EDTA and dialysed; L - Zn + DEPC, L chain depleted of Zn^{2+} by incubation with EDTA and treated with a 30-fold molar excess of DEPC; DT, diphtheria toxin; Peptide, Leu230-Gly244 peptide; Peptide + DEPC, Leu230-Gly244 peptide treated with a 30-fold molar excess of DEPC. Other experimental details are in Materials and methods.

with an average residual zinc content of 19%, with no concomitant formation of *o*-carboxytyrosine (not shown). Taken together, the results of Figure 3 indicate that two histidines are involved in Zn^{2+} coordination and that bound Zn^{2+} protects them from reacting with DEPC.

In many zinc proteins, cysteine residues are involved in zinc coordination (Vallee and Auld, 1990). However, the same number of sulfhydryl groups (1.3 ± 0.2 per L chain molecule) were titrated with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) on the L chain before and after Zn^{2+} depletion. Hence cysteines are not involved in Zn^{2+} coordination of TeTx unlike in alcohol dehydrogenase, aspartate transcarbamoylase or in the zinc finger of DNA-binding proteins (Vallee and Auld, 1990). The absence of cysteine coordination emphasizes the similarity of TeTx to zinc endopeptinases and suggests that the Zn^{2+} ion of TeTx plays a catalytic, rather than a structural role, as in all zinc enzymes with histidine coordination (Vallee and Auld, 1990).

$[^{65}\text{Zn}^{2+}]$ binding to TeTx light chain and to Leu230–Gly244 fragment

To gain further evidence for the involvement of the conserved segment shown in Figure 1B in zinc coordination, the peptide Leu230–Gly244 of TeTx was chemically synthesized. Figure 4 shows that this peptide binds $[^{65}\text{Zn}^{2+}]$ in a dot blot test and that this binding is abolished by DEPC treatment. It would be expected that the peptide binds less $[^{65}\text{Zn}^{2+}]$ than the TeTx L chain because the peptide lacks the third zinc ligand present in the native protein. Figure 4 also shows that the zinc atom of the L chain of TeTx is exchangeable and that, after DEPC modification, the Zn^{2+} -depleted L chain is unable to reincorporate the zinc ion, while a parallel DEPC treatment of the native L chain does not affect its ability to exchange its zinc atom. This result provides further support for the involvement of histidines in Zn^{2+} coordination. The dot blot assay used here is very specific since diphtheria toxin, which also has three histidines interspaced by three residues

Fig. 5. Inhibition of acetylcholine release induced by intracellular application of the L chain of TeTx. Identified couples of neurons making identified cholinergic synapses in the buccal ganglion of *Aplysia californica* were used for the assay of chemically modified L chain of the TeTx as detailed in Materials and methods. The L chain samples, injected at time zero (arrows) were: **panel A**, native L chain and its Zn^{2+} -depleted form (19% residual Zn^{2+}); **panel B**, DEPC-treated native chain (1.8 histidines modified) and Zn-depleted DEPC-treated L chain (18% residual Zn^{2+} in this sample, 3.3 histidines modified). In order to remove Zn^{2+} ions from both extracellular and intracellular medium, in one series of experiments (panel A, filled triangles), the buccal ganglion was pre-incubated for 100 min in the presence of 50 μM DTPA and 30 μM TPEN; DTPA was also mixed into the Zn^{2+} -depleted L chain before injection to a final intracellular concentration of 200 μM . Control experiments showed that, while DTPA and TPEN are inefficient in zinc removal as shown in Figure 2, they prevent zinc re-uptake by the L chain.

(His484xxxHisxxxHis492), does not show any sign of [$^{65}\text{Zn}^{2+}$] binding.

TeTx inhibition of neurotransmitter release in *Aplysia* neurons is zinc dependent

The best characterized system for testing the intracellular activity of clostridial neurotoxins is the identified couples of neurons making synapses in the buccal ganglion of *Aplysia californica* (Poulain *et al.*, 1988, 1990, 1991; Mochida *et al.*, 1990). In this cellular system it could be directly demonstrated that the L chain of TeTx is the intracellularly active portion of the toxin (Mochida *et al.*, 1990; Poulain *et al.*, 1990, 1991). Figure 5A shows that both the native L chain and the Zn^{2+} -depleted L chain of TeTx were effective in inhibiting the evoked neurotransmitter release eventually reaching the same level of inhibition. However, the kinetics of inhibition of the Zn^{2+} -depleted L chain were much slower. On the contrary, if the Zn^{2+} -depleted L chain was injected into a presynaptic neuron pre-treated with the heavy metal chelators (Arslan *et al.*, 1985), no inhibitory activity was recorded for at least 3 h (filled triangles in Figure 5A). Figure 5B shows that DEPC treatment of the native L chain does not modify its inhibitory activity with respect to the untreated control (Figure 5A). On the contrary, the L chain treated with DEPC after Zn^{2+} depletion inhibited neurotransmitter release very poorly; residual activity could be accounted for by the residual Zn^{2+} -containing L chain.

These experiments suggest that the Zn^{2+} -depleted L chain is slowly able to regain its zinc ion from the cytoplasmic pool, while a Zn^{2+} -depleted and DEPC modified one is not, and that bound Zn^{2+} is necessary for the L chain inhibition of neurotransmitter release.

Phosphoramidon blocks the TeTx inhibition of neurotransmitter release in *Aplysia* neurons

Phosphoramidon [(N- α -L-rhamnopyranosyl-hydroxyphosphinyl)-L-leucyl-L-tryptophan] is a very specific inhibitor of

zinc endopeptidases (Komiya *et al.*, 1975; Weaver *et al.*, 1977; Lennarz and Strittmatter, 1991). When phosphoramidon was injected into *Aplysia* neurons, as shown in Figure 6, it completely blocked the L chain effect on neurotransmitter release, while by itself the drug was ineffective. The small inhibition of neurotransmitter release detectable long after toxin injection is expected because of diffusion of phosphoramidon out of the nerve cell and dissociation of the toxin-inhibitor complex. Given the high specificity of phosphoramidon inhibition, this result strongly supports the proposal that TeTx is a zinc endopeptidase.

Protease activity of the light chain of TeTx

The zinc ion plays a central role in the peptide bond hydrolysis catalysed by zinc proteinases (Matthews, 1988; Vallee and Auld, 1990). The experiments described above indicate that two histidines are involved in Zn^{2+} coordination in TeTx. The putative zinc binding site of TeTx is suggested by secondary structure prediction methods to be α -helical with the two histidines on the same face of the α -helix as is the case for the three zinc proteases whose three-dimensional structure has been resolved (Matthews *et al.*, 1972; Pauptit *et al.*, 1988; Thayer *et al.*, 1991). Together with the phosphoramidon inhibition of the intracellular toxin activity, these similarities suggest that TeTx may be a zinc-dependent protease.

As a first approach, we incubated TeTx with a large number of chromophoric protease model substrates with no positive findings except for a weak protease activity on the general protease substrate casein. As a possible candidate for the toxin action we next assayed small synaptic vesicles (SSVs), purified from rat cerebral cortex (Huttner *et al.*, 1983; Benfenati *et al.*, 1989). Figure 7 shows that the L chain of TeTx does not change the SDS-PAGE protein pattern of highly purified SSVs, except for the disappearance of a single band of 19 kDa with the concomitant appearance of a 7 kDa and a 12 kDa fragment; the latter peptide overlaps with an SSV component of the same apparent molecular

Fig. 6. Effect of phosphoramidon on the intracellular *Aplysia* neuron activity of the L chain of TeTx. L chain of TeTx (48 $\mu\text{g}/\text{ml}$) was incubated with 10.7 mM phosphoramidon (Serva) or with buffer for 30 min at room temperature. Two presynaptic *Aplysia* neurons afferent to the same postsynaptic cell were used. Neuron 1 (open circles) were made 0.37 mM in phosphoramidon and the 1 h recording before time 0 shows that the inhibitor does not by itself affect neurotransmitter release. At time 0 (arrow) neuron 1 was injected with the phosphoramidon-treated L chain while the control neuron 2 (filled circles) was injected with L chain (final concentration 10 nM). The slow recovery of activity of the toxin in neuron 1 with prolonged incubation is to be attributed to diffusion of phosphoramidon in the inhibitor free extracellular medium.

weight (lane 3 of Figure 7). The electrophoretic mobility of the 19 kDa band and its immunoblot staining with an anti-synaptobrevin monoclonal antibody (Figure 7, lane 1) indicate that this protein is synaptobrevin. The H chain did not cause any change to the SSV protein profile (not shown). The synaptobrevin specific cleavage by the L chain was completely blocked by EDTA, as expected for a zinc protease (lane 4 of Figure 7), and was only partially inhibited by phosphoramidon (not shown). This latter result is probably due to the fact that the very strict binding requirements of phosphoramidon were not met in the *in vitro* assay (Weaver *et al.*, 1977; Lennarz and Strittmatter, 1991).

Discussion

Bacteria produce a whole range of protein toxins that are essential to their pathogenicity (Alouf and Freer, 1991). Here we provide direct evidence that the toxin responsible for all the clinical symptoms of tetanus contains one atom of zinc bound to its intracellularly active L chain. No metal is bound to the H chain, the portion of the toxin responsible for binding to neuronal membranes (Schiavo *et al.*, 1991) and for cell penetration. The similarity between TeTx and metalloproteases is not confined to the amino acid sequence and to the presence of the zinc atom. Here we have also shown that zinc is coordinated via two histidines and that the TeTx peptide Leu230–Gly244, which includes the zinc binding motif of metalloendopeptidases, binds zinc. The histidine involvement in zinc binding is indicative of a catalytic role of the toxin bound metal and this is in agreement with the data obtained *in vivo*. In fact, only when zinc is bound to the toxin is the L chain able to inhibit neurotransmitter release in *Aplysia* neurons, as clearly shown by the experiments of Figure 5.

Fig. 7. Synaptobrevin is proteolytically cleaved by the L chain of TeTx. Lanes 2–4 show Coomassie blue stained SDS–PAGE of samples of purified SSV (35 µg) incubated alone (lane 2) or in the presence of TeTx L chain (lane 3) or with L chain pretreated with EDTA for 15 min at 37°C (lane 4). Lane 1 refers to a sample of SSV immunoblotted with the IgG1 anti-synaptobrevin monoclonal antibody Cl 10.1 (Baumert *et al.*, 1989).

The lack of a cell line highly sensitive to TeTx, similar to Vero cells for diphtheria toxin (Sandvig and Olsnes, 1981), represents a major obstacle to the direct demonstration of a proteolytic activity of TeTx and to the identification of its intracellular target(s). The high specificity of TeTx *in vivo* and the lack of proteolysis *in vitro* (35 different synthetic peptide substrates of various proteases assayed) suggested that the activity of TeTx should be very specific.

Possible targets of TeTx proteolytic activity are at the nerve terminal level. A protein of SSVs, the organelles that store and release neurotransmitters, appeared to be a good candidate. TeTx has been shown to act on the rat brain causing an epileptiform syndrome (Mellanby *et al.*, 1985; Bagetta *et al.*, 1991). Using SSVs isolated from rat cerebral cortex in a highly purified form (Huttner *et al.*, 1983; Benfenati *et al.*, 1989), we were able to show that TeTx is a very specific zinc endopeptidase since it cleaves only synaptobrevin, out of the many components of the vesicles. This protein is also expressed in PC12 and adrenal medulla cells (Baumert *et al.*, 1989), which are both sensitive to TeTx (Niemann, 1991).

Our observations suggest that TeTx acts inside neuronal cells by specifically cleaving synaptobrevin. The role of synaptobrevin in neuroexocytosis is still obscure (De Camilli and Jahn, 1990), but the present findings suggest that this protein plays a key role in the process of neurotransmitter release. In this respect, it is noteworthy that all bacterial protein toxins with intracellular targets act on key elements of cell physiology regulation such as G proteins (for cholera, pertussis and botulinum C3 toxins) or protein synthesis (for diphtheria and Shiga toxins) (Rappuoli and Pizza, 1991; Boquet and Gill, 1991).

Regulation or activation of important biological phenomena via highly selective proteolytic cleavage is not uncommon, e.g. blood coagulation or complement activation. The protease activity of TeTx, described here, is consistent with its known specificity and potency. An intracellular catalytic activity of TeTx accounts for its extreme potency because one molecule of toxin would be sufficient to inactivate a large number of synaptobrevin-containing vesicles.

Our findings provide the first explanation at the molecular level of a high incidence neuroparalytic disease such as tetanus and it opens new perspectives for the development of therapeutic protocols based on inhibitors of the zinc protease activity. Moreover, our data may become of paramount importance for the dissection of the molecular events underlying the process of neurotransmitter release.

Materials and methods

Toxin and fragments

TeTx and the L chain were isolated from culture filtrates of *Clostridium tetani*, as detailed before (Lin *et al.*, 1985; Ozutsumi *et al.*, 1985; Schiavo *et al.*, 1990), and stored at –80°C in 10 mM HEPES–Na, 50 mM sodium chloride, pH 7.2. The H chain was prepared as described by Matsuda and Yoneda (1975).

Determination of metal content

All materials were previously rinsed with Milli-Q grade water, with a conductivity > 10 MΩ. Buffers were prepared with chemicals of the highest purity available with respect to the presence of heavy metals and pre-treated with Amberlite MB-3 (Sigma). Before metal determination, the protein samples were extensively dialysed at 4°C against 150 mM Tris–Cl, pH 7.4 or 10 mM HEPES–Na, 100 mM sodium chloride, pH 7.0, using boiled dialysis tubing (Spectrum, TX). Samples were analysed for Zn, Co, Cu,

Fe, Mn and Ni with a Perkin–Elmer 4000 atomic absorption flame spectrophotometer with impact bed loading. Metal contents were measured after standardization in the linear range of concentration for each ion (0–0.5 p.p.m. for Zn, Ni and Co; 0–1 p.p.m. for Cu; 0–5 p.p.m. for Fe and Mn).

Zinc removal

TeTx or the L chain were diluted to 0.5–1.0 mg/ml with 150 mM Tris–Cl, pH 7.4, and incubated in the presence of 10 mM EDTA for 60 min at 37°C. Samples were dialysed extensively against the same buffer at 4°C and their zinc content was determined as above. Concentrations of other chelators used are as follows: 10 mM *o*-phenanthroline (Erba, Milan), 50 mM dipicolinic acid (Fluka), 30 μ M tetrakis-(2-pyridylmethyl)ethylenediamine anhydride (Molecular Probes, Eugene, OR), 200 μ M diethylenetriamine-pentaacetic anhydride (Molecular Probes).

Peptide synthesis

TeTx segment 230–244 corresponding to the sequence LLMHELIHVLHGLYG was kindly prepared by Dr A.Santucci (University of Siena) by solid phase synthesis with a SMPS 350 automatic synthesizer (Zynsler Analytic, Frankfurt) employing F-moc chemistry. The product was detached from the resin with 93% TFA, purified by HPLC on a Vydac C₁₈ column and controlled by automatic Edman degradation on an Applied Biosystems 475A microsequencer.

DEPC modification

The L chain of TeTx was diluted with 50 mM disaerated sodium phosphate 50 mM, pH 7.8 to a final concentration of 7.0 μ M. The samples were filtered through a 0.22 μ m filter (Anotec, Oxford, UK) and DEPC from a freshly prepared solution in absolute ethanol was added. The DEPC/L chain ratio never exceeded 30 mol/mol L chain. The reaction was followed at 25°C by simultaneously recording the differential absorbance at 243 nm and at 278 nm as previously described (Miles, 1977; Papini *et al.*, 1989) in a Perkin–Elmer Lambda 5 spectrophotometer. Modified residues were estimated with differential extinction coefficients of 3200 M⁻¹ cm⁻¹ at 243 nm for *N*-carboxyhistidine and 1310 M⁻¹ cm⁻¹ at 278 nm for *o*-carboxytyrosine.

Titration of free thiol groups

Native or zinc-depleted L chain samples were diluted to 1 μ M with 50 mM disaerated sodium phosphate, pH 7.8. Subsequently 10 μ l of DTNB (5 mg/ml in 50 mM sodium phosphate, pH 8.0; final concentration 0.4 mM) were added at 25°C to the samples and the absorbance at 410 nm was determined against a blank without protein. Sulfhydryl content was calculated on the basis of molar absorptivity of 13 600 M⁻¹ cm⁻¹ at 412 nm for 2-nitro-5-thio-benzoate anion and expressed as moles of free thiol groups per mole of L chain (Ellman, 1959; Schiavo *et al.*, 1990).

Determination of [⁶⁵Zn²⁺] binding

L chains in their native or zinc-depleted forms, treated or not with 1:30 molar excess of DEPC as above, or diphtheria toxin (2–32 pmol) or the putative zinc binding peptide (25–400 pmol) were dot-blotted onto nitrocellulose paper strips (0.22 μ m; Hoefer, CA). The strips were rinsed for 5 min in 25 mM Tris–Cl, 100 mM sodium chloride, pH 7.5 and successively immersed for 60 min at room temperature into 50 nM ⁶⁵ZnCl₂ (Amersham, UK; specific activity 575 mCi/mg zinc) in the same buffer. Excess [⁶⁵Zn²⁺] was removed by washing the paper strips six times with the same buffer. The amount of [⁶⁵Zn²⁺] bound was determined by exposing the dried paper strips to Kodak X-OMAT films at –80°C.

Tissue preparations and electrophysiological recordings

Experiments were performed on buccal ganglia of the sea slug *Aplysia californica* as detailed before (Poulain *et al.*, 1991). Neuronal somata (200–300 μ m diameter) were impaled with two glass microelectrodes (3 M potassium chloride; 1–5 M Ω). Postsynaptic responses due to ACh release were induced once a minute by a presynaptic action potential and recorded every 2 min as current changes by conventional two electrode voltage-clamp techniques. By taking into account the reversal potential of the response, current values were subsequently expressed as membrane conductance (nS). At this kind of synapse, ACh release per impulse remains stable for several hours, even in the absence of superfusion (Poulain *et al.*, 1986). Prior to intracellular administration, TeTx L chain samples in 10 mM Tris–HCl, pH 7.8 were mixed with a solution of Fast Green FCF (20% v/v, Sigma) to a final L chain concentration of 1 μ M. In all experiments, after control recordings were made, the L chain samples were injected in the presynaptic neuron under visual and electrophysiological monitoring. Final intrasomatic concentration of L chain was ~10 nM.

Proteolytic activity of TeTx

35 μ g of SSVs purified from rat cerebral cortex (Huttner *et al.*, 1983; Benfenati *et al.*, 1989) were incubated with 1 μ g of TeTx L chain in 270 μ l of 5 mM HEPES–Na, 0.3 M glycine, pH 7.4. Some samples contained 10 mM EDTA. After 30 min at 37°C, 30 μ l 50% TCA were added and, after 30 min at room temperature, centrifuged. Pellets were dissolved in electrophoresis sample buffer containing 5 mM EDTA and 5% 2-mercaptoethanol and boiled for 2 min. Samples were electrophoresed in 12% polyacrylamide gels (Laemmli, 1970). Immunoblotting was carried out according to Towbin *et al.* (1979), using the IgG1 anti-synaptobrevin monoclonal antibody Cl 10.1 (Baumert *et al.*, 1989) and revealed by anti-mouse alkaline phosphatase coupled secondary antibodies.

Toxicity tests

The toxicity of TeTx was determined by injecting intraperitoneally serial dilutions of the toxin with phosphate-buffered saline containing 0.1% BSA. Time before death was recorded and mouse lethal doses were calculated as described (Weller *et al.*, 1988).

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