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Functional characterization of the catalytic site of the tetanus toxin light chain using permeabilized adrenal chromaffin cells

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The molecular events underlying the inhibition of exocytosis by tetanus toxin were investigated in permeabilized adrenal chromaffin cells. We found that replacement of amino acid residues within the putative zinc binding domain of the tetanus toxin light chain such as of histidine (position 233) by cysteine or valine, or of glutamate (position 234) by glutamine completely abolished the effect of the light chains on Ca^{2+} induced catecholamine release. Dipicolinic acid, a strong chelating agent for zinc, also prevented the effect of the tetanus toxin light chain. Zn^{2+} and, less potently Cu^{2+} and Ni^{2+}, but not Cd^{2+} and Co^{2+}, restored the activity of the neurotoxin. These data show that zinc and the putative zinc binding domain constitute the active site of the tetanus toxin light chain. Neither captopril, an inhibitor of synaptobrevin cleavage nor peptides spanning the site of synaptobrevins cleaved by the tetanus toxin in neurons, prevented the inhibition of Ca^{2+} induced catecholamine release by the tetanus toxin light chain. This suggests that synaptobrevins are not a major target of tetanus toxin in adrenal chromaffin cells.

Exocytosis; Catecholamine; Calcium; Tetanus toxin; Light chain; Zinc; Adrenal medulla

1. INTRODUCTION

Synapses are sites of neuronal communication specialized on the regulated release of transmitters that excite or inhibit the target cells. Within the nerve terminals, transmitters are stored in synaptic vesicles which fuse with the plasma membrane and release their contents in response to an increase of intracellular calcium triggered by an invading action potential. Synaptic vesicle membranes contain a number of proteins which have been cloned and characterized in the recent years [1,2]. Some of these proteins interact with proteins at the plasma membrane and have been implicated in docking and fusion of synaptic vesicles. For example synaptophysin, which like its congener synaptoporin [3], is thought to be involved in the formation of the initial pore [4] and interacts with physophilin at the presynaptic membrane [5]. Similarly synaptotagmin, which is thought to act as a calcium receptor during exocytosis [6], binds to neurexins and syntaxins, recently detected new families of plasma membrane proteins [7–9]. Synaptobrevin, the most conserved synaptic vesicle membrane protein [10], apparenly attaches to syntaxins via a complex that includes cytoplasmic proteins [11].

Recent functional investigations support the idea that the synaptic vesicle membrane proteins mentioned above participate directly in the exocytotic process: antibodies to synaptophysin and peptides corresponding to the cytoplasmic domain of synaptotagmin inhibit exocytosis [12–15]. Blockade of transmitter release by tetanus toxin is paralleled by the cleavage of synaptobrevin [16–18]. Tetanus toxin, similar to botulinum A toxin and related neurotoxins, consist of a heavy and a light chain linked by a disulfide bond [19]. After entry of the neurotoxins into the nerve terminals the light chain blocks transmitter release in *Aplysia* neurons and mammalian nerve terminals [20–25].

The key observation that the light chains of tetanus and botulinum A toxin harbor the active domain has initially been made with permeabilized adrenal chromaffin cells [26–32] which, unlike exocrine cells [33], have the intracellular target of the neurotoxins. The light chains of the neurotoxins bind zinc and contain the HELIH motif shared with a variety of zinc peptidases [19,34–38] suggesting that zinc and the metal binding motif are essential for the inhibition of exocytosis by the neurotoxins.

In the present study we directly addressed this question and analyzed whether mutation of the zinc binding motif of the tetanus toxin light chain affects its function in adrenal chromaffin cells. We also investigated the role of zinc and other ions as components of the active site of the tetanus toxin light chain. Finally we examined whether substances, which prevent cleavage of synaptobrevin and blockade of neurotransmission by tetanus toxin in neurons, abolish also the effect of the neurotoxin in the endocrine adrenal chomaffin cell.

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2. EXPERIMENTAL

2.1. *Materials*

Tetanus toxin light chain, prepared as described recently [39–41] was a generous gift from U. Weller (Institut für Medizinische Mikrobiologie, Universität Mainz). The expression of recombinant light chains of tetanus toxin in *E. coli* strain M15 using a newly synthesized gene [42] encoding the entire light chain with six C-terminally added H-residues will be published elsewhere (H. Niemann et al., unpublished). The protein was purified from the bacterial lysates on a Ni-agarose column. Mutants were generated by replacement of a *Sna*BI-*Fph*I restriction fragment [42] with suitable synthetic oligonucleotides. Recombinant light chain (wild-type; WT), recombinants in which histidine (position 233) was replaced by cysteine or valine (mutants $H^{233}C$, or $H^{233}V$) and the recombinant $E^{234}Q$, in which glutamate (position 234) was substituted by glutamine, were kindly provided by H. Niemann, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany.

The peptides QFET and ASQFETS were kindly prepared by Mrs. J. Neckermann and Dr. T. Ruppert (Ulm) by solid phase synthesis using a automatic synthesizer (431A, Applied Biosystems, Foster City, CA, USA) employing F-moc chemistry. The product was detached from the resin with TFA, and analyzed by HPLC on a Pep-S 5 μm column (Pharmacia/LKB, Freiburg, Germany). The peptides were characterized by Edman sequencing on a model 473A sequencer (Applied Biosystems, Foster City, CA, USA). Peptides 1 (DALQAGASVFESSAAKLKRK) and 2 (DALQAGASQFETSAAKLKRK) were generous gifts from H. Niemann, Tübingen, Germany.

3-Mercapto-2-methylpropionyl-L-proline (captopril) was kindly provided by Bristol-Myers Squibb, München. Fungizone, penicillin, streptomycin, DMEM, FCS were purchased from Seromed, Berlin; collagenase (cl. histolyticum) from Serva, Heidelberg; cytosine arabinosid from Sigma, München, and [^{3}H]noradrenaline from Amersham, Braunschweig. All the other chemicals were of analytical grade.

2.2. *Methods*

2.2.1. Cell cultures

Fresh bovine adrenals were rinsed free of blood by vascular perfusion with ice-cold medium I (in mM: 150 NaCl, 5 KCl, 10 glucose, 10 HEPES pH 7.4) supplemented with 0.25 μg/ml fungizone, 50 IU penicillin and 50 μg/ml streptomycin. Subsequently medium I was supplemented with 0.05% collagenase (cl. histolyticum) and 0.5% BSA followed by incubation of the adrenals for one hour at 37°C. Then the medullae were removed, cut into small pieces and incubated with collagenase for a further hour. The cell suspension was filtered through a nylon mesh (250 μm) and washed 3 times with medium I supplemented with 2% BSA. The cells were resuspended in culture medium (DMEM) containing 10% fetal calf serum and 5 μm cytosine arabinoside. They were plated on precoated (20 μg/ml rat tail collagen) wells (diameter 11.3 mm) at a density of 3×10^5 cells/well. The cell cultures were maintained in culture for 2–10 days at 37°C with 5% CO_2.

2.2.2. Assay for exocytosis

Adrenal chromaffin cells were incubated for one hour with [^{3}H]noradrenaline (1.3 μCi/ml) in serum-free culture medium (DMEM) supplemented with 1 mM ascorbic acid. Ca^{2+} was removed from the cultures by 3 washes with medium II (in mM: 140 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 0.5 ascorbic acid, 15 PIPES pH 7.2) supplemented with 11 mM glucose, followed by washes with medium III (in mM: 150 NaCl, 1 EGTA, 10 PIPES pH 7.2) and medium IV (in mM: 150 potassium glutamate, 0.5 EGTA, 5 NTA, 10 PIPES, pH 7.2). The osmolarity of the media was maintained between 300 and 310 mOs-mol/kg.

The cells were permeabilized with SLO (63 HU/150 μl) as described previously [33,43] in potassium glutamate medium (in mM: 150 potassium glutamate, 0.5 EGTA, 5 EDTA, 2 Mg^{2+}-ATP, 7.67 magnesium acetate to obtain a free Mg^{2+} concentration of 1, 10 PIPES, pH 7.2), supplemented with 0.1% BSA and 1 mM dithiothreitol as a reducing agent for 2 min at 30°C. The medium was then replaced by a fresh one containing the neurotoxins and/or the chemicals to be tested and incubated for 25 min at 30°C. Then the cells were stimulated for 15 min at 30°C with potassium glutamate medium containing 30 μM free Ca^{2+}. Basal release was determined in the same medium with no Ca^{2+} added. Free Ca^{2+} concentrations were calculated and controlled with a Ca^{2+} specific electrode as described [44].

[^{3}H]Noradrenaline released into the supernatant and remaining in the cells (after lysis with 0.2% SDS) was determined. Percentage of [^{3}H]noradrenaline released was calculated using catecholamine content of the lysate plus released catecholamines as 100%. The values given are means ± S.D. of 3 wells.

3. RESULTS AND DISCUSSION

In order to find out whether the HELIH motif (residues 233–237 in the tetanus toxin light chain; TetxL), shared by clostridial neurotoxins and zinc peptidases [19,38] is involved in the function of tetanus toxin, we first analyzed whether mutational modification of the motif affects the activity of TeTxL. We found that the recombinant wild type TeTxL (WT) at 100 nM almost completely inhibited Ca^{2+} induced catecholamine release from permeabilized chromaffin cells (Fig. 1). By contrast, using the same concentrations of recominant light chains in which histidine^{233} was replaced by cysteine or valine (mutants $H^{233}C$ and $H^{233}V$) or recombinant $E^{234}Q$ in which glutamate^{234} was substituted by glutamine, no inhibition of calcium induced catecholamine release was observed (Fig. 1). Recently it was found that also chemical modification of histidines of the tetanus toxin abolishes the effect of tetanus toxin on transmitter release in *Aplysia* neurons [16]. In metalloendoproteases

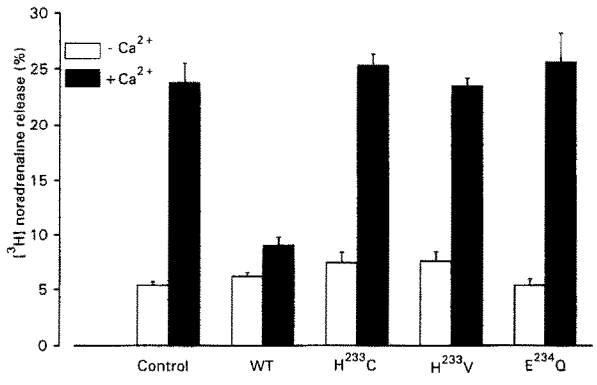


Fig. 1. Wild-type TetxL but not mutants ($H^{233}C$, $H^{233}V$, $E^{234}Q$) of TetxL inhibit catecholamine release. After permeabilization the cells were incubated for 25 min at 30°C with potassium glutamate medium (control), 100 nM recombinant tetanus toxin light chain (wild-type; WT) or 100 nM mutated recombinant tetanus toxin light chains ($H^{233}C$, $H^{233}V$, $E^{234}Q$) in potassium glutamate medium (see section 2.2). Then the cells were incubated for 15 min at 30°C with potassium glutamate medium containing 30 μM free Ca^{2+} (filled bars) or no Ca^{2+} (open bars) and the percentage of [^{3}H]noradrenaline released into the supernatant was determined. Note the inhibition of Ca^{2+} induced catecholamine release by wild-type TetxL whereas mutants bearing point mutations in the HELIH motif ($H^{233}C$, $H^{233}V$, $E^{234}Q$) were inactive. Data are means ± S.D. of 3 wells. One out of 7 experiments with similar results.

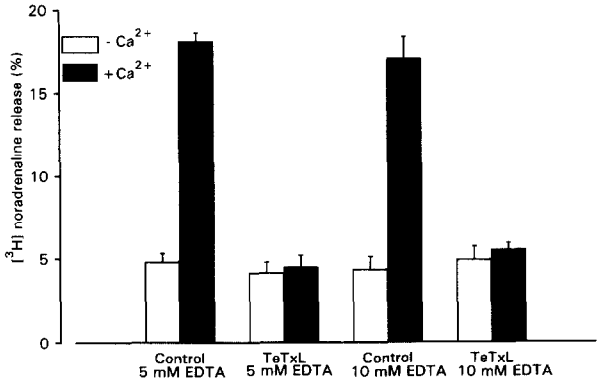


Fig. 2. EDTA has no effect on the activity of the tetanus toxin light chain. Natural TetxL was first preincubated for 30 min at 30°C in potassium glutamate medium containing 5 or 10 mM EDTA and then added to the permeabilized chromaffin cells. After a further incubation period of 25 min (see section 2.2 and Fig. 1) the release of [^{3}H]noradrenaline was determined in the absence or presence of 30 μM free Ca^{2+}. Compared to the control (presence of EDTA, no TetxL) release of [^{3}H]noradrenaline was almost completely inhibited by TextL and not affected by the different EDTA concentrations. Data are means ± S.D. of 3 wells. Three similar experiments with different cell preparations.

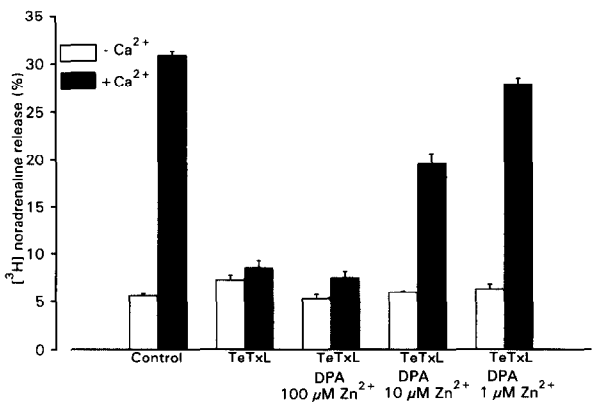


Fig. 4. Effect of different concentrations of Zn^{2+} on the activity of TetxL. Natural TetxL (100 nM) were preincubated with 100 μM DPA followed by 1, 10 or 100 μM Zn^{2+} and then added to the permeabilized chromaffin cells. After a further incubation period of 25 min (see section 2.2) catecholamine release was determined in the absence or presence of 30 μM free Ca^{2+} (see section 2.2). While 100 μM Zn^{2+} completely restored the activity of TetxL, 10 μM Zn^{2+} was only partly effective. Data are means ± S.D. of 3 wells (3 similar experiments).

the first histidine within the HELIH motif interacts with the zinc ion whereas the adjacent glutamate plays a catalytic role [45,46]. Our mutational analysis directly shows the functional importance of these aminoacids in the HELIH motif for the inhibition of exocytosis by the tetanus toxin light chain in adrenal chromaffin cells.

Zinc is bound to the histidine rich metal binding site within the TeTxL [16,35]. Removal of zinc from tetanus toxin at neutral pH by chelating agents such as EDTA requires long incubation and elevated temperature (e.g. one hour at 37°C) [16,47]. In our attempts to remove zinc with the aim to inhibit the activity of the TeTxL we used a variety of chelators. Treatment of light chains with different concentrations of EDTA, which binds zinc with high affinity, at room temperature, at 30°C and different periods of incubation did not result in an inactivation of TeTxL (Fig. 2). Similarly, NTA, 2,2'-dipyridil, 1,10-phenanthroline, diethyldithiocarbamate and phosphoramidon, when tested at concentrations not affecting calcium induced catecholamine release from the cells, were without effect (not shown). Only

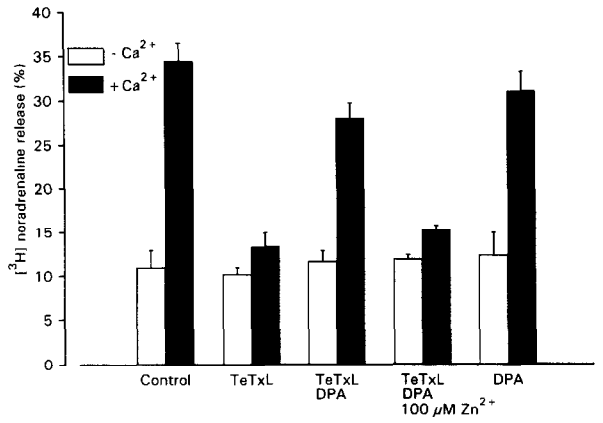


Fig. 3. Effect of Zn^{2+} on the activity of TetxL. Natural TetxL was first preincubated at 30°C in potassium glutamate medium with or without 100 μM dipicolinic acid (DPA) followed by 100 μM Zn^{2+} and then added to the permeabilized chromaffin cells. After a further incubation period of 25 min (see section 2.2) the release of [^{3}H]noradrenaline was determined in the absence or presence of 30 μM free Ca^{2+}. DPA abolished the effect of TetxL and 100 μM Zn^{2+} restored the activity of TetxL. Data are means ± S.D. of 3 wells (16 similar experiments). The release determined in the presence of DPA alone was not significantly different from the controls (Scheffe test).

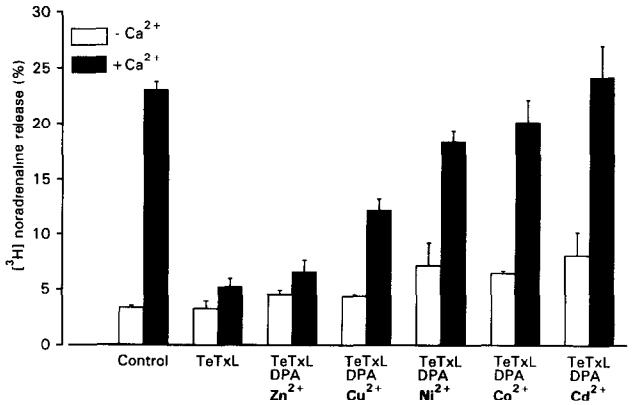


Fig. 5. Effect of different divalent cations on the activity of TetxL. Preincubation of 100 nM natural TetxL with 100 μM DPA and 100 μM Zn^{2+} yielded a fully active light chain. TetxL was only partly active upon addition of 100 μM Cu^{2+} and less activity was observed with 100 μM Ni^{2+}, Co^{2+} and Cd^{2+}. Data are means ± S.D. of 3 wells (9 similar experiments). The release observed in the presence of Zn^{2+} Cu^{2+} and Ni^{2+} was significantly different from that in the controls (Scheffe test). DPA alone does not significantly change catecholamine release (see Fig. 3).

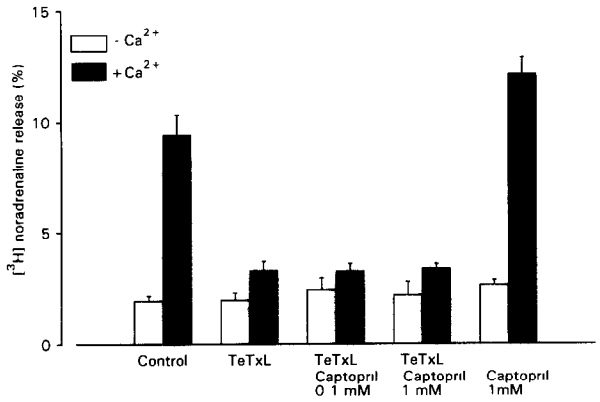
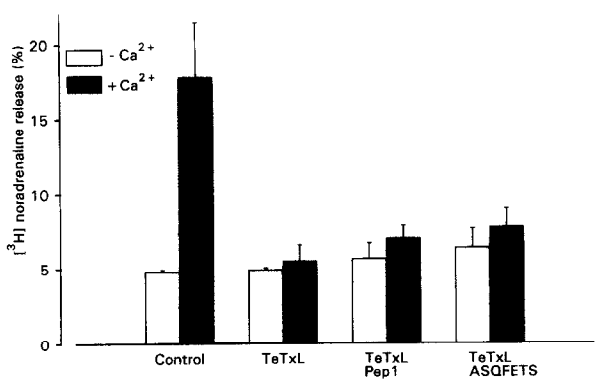


Fig. 6. Captopril has no effect on the activity of TeTxL. Natural TeTxL (100 nM) in the absence or presence of 0.1 or 1 mM captopril were added to permeabilized chromaffin cells followed by 25 min of incubation at 30°C. Then the release of catecholamines was determined in the presence and absence of 30 μM calcium. Captopril, a potent inhibitor of zinc containing peptidases and cleavage of synaptobrevin by tetanus toxin had no effect on the inhibitory action of TetxL. Captopril alone had no effect on the Ca^{2+} induced catecholamine release by permeabilized chromaffin cells. Data are means ± S.D. of 3 wells (4 similar experiments).

dipicolinic acid (DPA), a chelating agent with high affinity for zinc and low affinity for calcium and magnesium [48], effectively blocked the activity of TeTxL already within 5 min at 30°C. This blockade was reversible since subsequent addition of 100 μM zinc restored the activity of TeTxL (Fig. 3). We determined the amounts of zinc in the TeTxL preparation (Dayanithi et al., unpublished) in the permeabilization medium containing 5 mM EDTA and 0.5 mM EGTA (see section 2.2) by atomic absorption spectroscopy and found that it contained 0.68 atoms zinc per mole TeTxL. Following treatment with 100 μM DPA we measured 0.05 atoms zinc per mole TeTxL. This indicates that zinc is effectively removed from TeTxL by DPA, a chelating agent which blocks the effect of TeTxL on catecholamine release.

In a further series of experiments we added different concentrations of zinc and found that 1 μM zinc was ineffective, but 10 μM partly and 100 μM completely restored the toxins ability to block exocytosis (Fig. 4). In order to find out whether other divalent cations can replace zinc at the toxins' catalytic site, we added Cd^{2+} or Co^{2+} and found that these metals were inactive while Cu^{2+} and Ni^{2+} could restore the activity of TeTxL to a certain extent (Fig. 5). Interestingly, also in recent binding studies Cu^{2+} was able to displace completely radioactive zinc from tetanus toxin [35] but Ni^{2+}, Co^{2+} and Cd^{2+} were less effective. Thus our funtional data are in accordance with the properties of zinc binding by tetanus toxin. Furthermore they show that zinc, with respect to function, can only be replaced by Cu^{2+} and Ni^{2+} as a ligand of the HELIH motif forming the catalytic site of the tetanus toxin light chain.

Cleavage of synaptobrevin, an integral membrane protein of synaptic vesicles, by tetanus toxin has been related to the inhibition of transmitter release in neurons [16–18]. In order to find out whether synaptobrevin could be a target of TeTxL in chromaffin cells we investigated the effect of substances that block synaptobrevin cleavage and prevent tetanus toxin action in neurons. Captopril, which inhibits the zinc peptidase angiotensin converting enzyme in low (nanomolar) concentrations [49] has been shown to block cleavage of synaptobrevin by tetanus toxin when applied in millimolar concentrations [17]. However, even at these high concentrations captopril did not affect the function of TeTxL in permeabilized chromaffin cells (Fig. 6). We also employed peptides, corresponding in sequence to the cleavage site of synaptobrevin 2, which were found to block the tetanus toxin light chain action within Aplysia neurons and the cleavage of synaptobrevin [17].



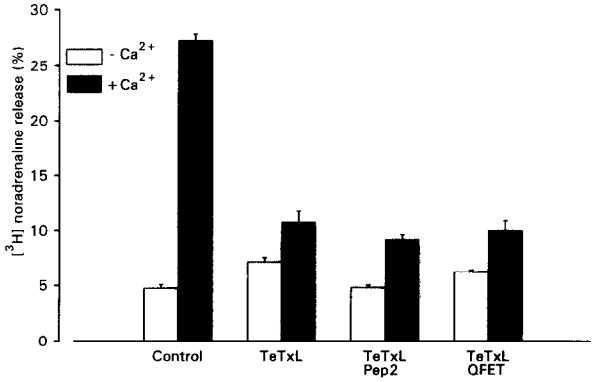


Fig. 7. (a and b) Synthetic peptides corresponding to motifs present in synaptobrevin 1 and 2 have no effect on the activity of TeTxL. Natural TeTxL (100 nM) and 100 μM peptides were added to permeabilized chromaffin cells followed by 25 min of incubation at 30°C. Then the release of catecholamines was determined in the presence and absence of 30 μM calcium. The inhibition of calcium induced catecholamine release by 100 μM TetxL was not affected by the presence of 100 μM peptide 1 (DALQAGASVFESSAAKLKRK), ASQFETS (a), peptide 2 (DALQAGASQFETSAAKLKRK) or QFET (b). The peptides alone had no effect on the Ca^{2+} induced catecholamine release (not shown). Data are means ± S.D. of 3 wells (8 similar experiments).

However, these peptides neither alone nor in the presence of TeTxL modified calcium-induced catecholamine release from chromaffin cells (Fig. 7a,b). In addition similar peptides derived from synaptobrevin 1 were inactive (Fig. 7b). The reason for the ineffectivity of captopril and the peptides is at present not clear. Posssibly the interaction of the tetanus toxin light chain with its target in chromaffin cells is less sensitive to the inhibitors used here. The absence of synaptobrevin from chromaffin vesicles, the storage organelles for catecholamines in chromaffin cells [50,51] also provides evidence for a separate target.

Recently it was reported, that tetanus toxin activates transglutaminase which then modifies synapsin 1 [52,53]. This process would impair the recruitment of synaptic vesicles for exocytosis in nerve terminals. Activation of transglutaminase by tetanus toxin depends on calcium [52] and some transglutaminases can be inhibited by zinc [54] and/or activated by proteolysis [55]. It is unclear at present whether transglutaminase(s) of chromaffin cells have similar properties and whether they may serve as targets of TeTxL in chromaffin cells.

Taken together our study shows that the HELIH motif in the tetanus toxin light chain together with bound zinc constitutes the catalytic site responsible for the inhibition of catecholamine release from adrenal chromaffin cells and that synaptobrevin cleavage does not appear to be the mechanism of tetanus toxin action in chromaffin cells.

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