

# Noradrenaline release from permeabilized synaptosomes is inhibited by the light chain of tetanus toxin

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Noradrenaline release from rat brain cortical synaptosomes permeabilized with streptolysin O can be triggered by  $\mu\text{M}$  concentrations of free  $\text{Ca}^{2+}$ . This process was inhibited within minutes by tetanus toxin and its isolated light chain, but not by its heavy chain. The data demonstrate that the effect of tetanus toxin on NA release from purified synaptosomes is caused by the intraterminal action of its light chain.

Tetanus toxin; Streptolysin O; Permeabilization; Exocytosis; Synaptosome

## 1. INTRODUCTION

Tetanus toxin (TeTx), is a highly neurotoxic protein, which preferentially blocks neurotransmitter release from neurons of the central nervous system (cf. [1–3]). It is synthesized by *Clostridium tetani* and consists of disulphide-linked heavy (100 kDa) and light chains (50 kDa) (cf. [3]) [4,5]. In contrast to the intact toxin, the individual chains alone lack toxicity in vivo [6,7].

When directly injected into the cytosol, TeTx inhibits exocytosis from adrenal chromaffin cells as revealed by cell capacitance measurements [8]. Also, in permeabilized adrenal chromaffin cells, pheochromocytoma cells (PC12 cells) [9–11] and neurosecretosomes from the posterior pituitary gland [12], TeTx turned out to be a potent inhibitor of noradrenaline (NA) and vasopressin release. In addition it has been shown that TeTx is more effective in these cells in the presence of a reducing agent, which causes chain separation [9,10,12]. Indeed, the ability of TeTx to inhibit release of catecholamines [10,13] and vasopressin [12] was found to be confined to the light chain subunit of this protein. Microinjection of TeTx and its isolated chains or of mRNA encoding for TeTx light chain in ganglia of *Aplysia californica*,

allowed to extend these findings to invertebrate neurons [14,15]. In contrast, amylase release from streptolysin O (SLO)-permeabilized pancreatic acinar cells is insensitive to TeTx and its light chains [16].

Taken together the available data suggest that exocytosis from endocrine cells, endocrine nerve endings and invertebrate neurons but not from exocrine cells is blocked by the light chain of TeTx. However, the intracellular effect of TeTx on neurotransmitter release from neurons of the mammalian central nervous system, the targets in TeTx-induced neuromuscular diseases, was not studied to date.

SLO-permeabilized synaptosomes release NA upon addition of  $\text{Ca}^{2+}$  in  $\mu\text{M}$  concentrations [17–19]. This preparation is suitable to introduce macromolecules into the presynaptic terminal, since antibodies against the intrasynaptosomal protein kinase C substrate B-50 inhibit  $\text{Ca}^{2+}$ -induced NA release as well as B-50 phosphorylation [18,19]. In the present study we used this preparation to study the intraterminal effect of TeTx and its isolated chains on  $\text{Ca}^{2+}$ -induced NA release.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Tetanus toxin was separated into its heavy and light chains by isoelectric focusing in a sucrose gradient with ampholyte under reducing conditions in 2 mol/l urea [20]. Before use, all toxin preparations were dialyzed against the following buffer: 139 mM potassium glutamate, 20 mM PIPES, 5 mM EGTA, 1 mM  $\text{MgCl}_2$ , pH 6.5.

L-[7,8- $^3\text{H}$ ]noradrenaline (specific activity 34 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Percoll was obtained from Pharmacia (Uppsala, Sweden), ATP from Boehringer (Mannheim, Germany), SLO from Wellcome (Weesp, The Netherlands), EGTA and BSA from Sigma (St. Louis, USA).

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Abbreviations: BoNT/A, botulinum A toxin; H, heavy chain; L, light chain; NA, noradrenaline; SLO, streptolysin O; TeTx, tetanus toxin.

## 2.2. Methods

### 2.2.1. [ $^3\text{H}$ ]noradrenaline release from SLO-permeabilized synaptosomes

Synaptosomes were prepared from cerebrocortex of male Wistar rats (100–120 g) as described [21]. They were incubated in oxygenated buffer A (mM: 123 NaCl, 5 KCl, 2  $\text{CaCl}_2$ , 2  $\text{MgCl}_2$ , 1.15  $\text{NaH}_2\text{PO}_4$ , 20 PIPES, 5.6 D-(+)-glucose, pH 6.8), labelled with [ $^3\text{H}$ ]NA (2.5  $\mu\text{Ci}/\text{mg}$  synaptosomal protein) and washed as described earlier [18].

Synaptosomes (final concentration: 0.17 mg/ml) were permeabilized with 0.3 IU/ml SLO in buffer A (without  $\text{CaCl}_2$  and  $\text{MgCl}_2$ ) in the presence of 1 mg/ml BSA, 2 mM ATP, 10 mM EGTA, 2 mM free  $\text{Mg}^{2+}$ , the indicated concentration of  $\text{Ca}^{2+}$  and the TeTx preparation to be tested for 5 minutes at  $37^\circ\text{C}$  (water bath).  $\text{Ca}^{2+}/\text{EGTA}$  buffers were calculated and prepared as described [22].

Incubations were stopped by centrifugation for 25 s at  $10,000 \times g$ . [ $^3\text{H}$ ]NA release was determined by liquid scintillation counting of the radioactivity in the supernatant. NA release is expressed as percentage of total [ $^3\text{H}$ ]NA incorporated.

### 2.2.2. Other determinations

Protein concentrations were determined according to [23] using BSA as a standard. Statistical analysis was performed with a two-tailed Student's *t*-test. A value of  $P < 0.05$  was considered to be significant.

## 3. RESULTS

Increasing the free  $\text{Ca}^{2+}$  concentration from  $10^{-8}$  M to  $10^{-5}$  M induced the release of 12% of total [ $^3\text{H}$ ]NA incorporated by SLO-permeabilized synaptosomes. This  $\text{Ca}^{2+}$ -induced [ $^3\text{H}$ ]NA release was inhibited by addition of TeTx light chain within 5 minutes of incubation in a dose-dependent manner (Fig. 1,  $\text{IC}_{50}$  about 300 nM). Incubation with 100, 300 and 1000 nM light chain led to 9%, 30% and 48% inhibition of  $\text{Ca}^{2+}$ -induced NA release, respectively. The basal [ $^3\text{H}$ ]NA release (in the presence of  $10^{-8}$  M  $\text{Ca}^{2+}$ ) was not significantly affected up to 300 nM LC. 1000 nM light chain caused a slight reduction of basal release (Fig. 1). Intact TeTx (two-

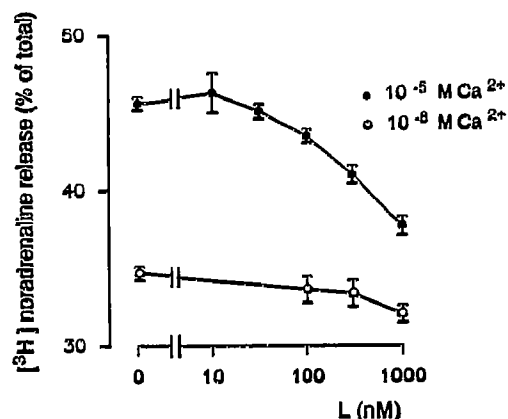


Fig. 1. Effect of TeTx light chain on [ $^3\text{H}$ ]NA release from SLO-permeabilized synaptosomes. Synaptosomes were permeabilized with 0.3 IU/ml SLO in the presence of  $10^{-8}$  M  $\text{Ca}^{2+}$  (open circles) or  $10^{-5}$  M  $\text{Ca}^{2+}$  (filled circles) for 5 minutes at  $37^\circ\text{C}$ . The indicated concentrations of TeTx light chain were present throughout the experiment (abscissa). [ $^3\text{H}$ ]NA release was measured as described (see Methods) and is expressed as percentage of total [ $^3\text{H}$ ]NA incorporated (ordinate). Data are means  $\pm$  SEM of 12–24 determinations obtained from four independent experiments.

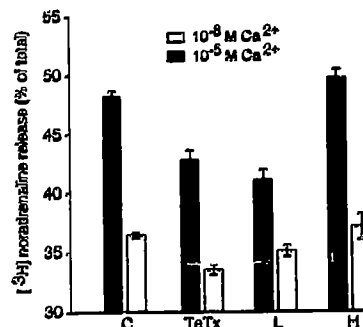


Fig. 2. Effect of TeTx and its isolated light and heavy chain on [ $^3\text{H}$ ]NA release from SLO-permeabilized synaptosomes. Synaptosomes were permeabilized with 0.3 IU/ml SLO in the presence of  $10^{-8}$  M  $\text{Ca}^{2+}$  (open bars) or  $10^{-5}$  M  $\text{Ca}^{2+}$  (filled bars) for 5 minutes at  $37^\circ\text{C}$ . Throughout the experiment, either no toxin (C) or TeTx, its light (L) or heavy chains (H) were present each at 1000 nM. [ $^3\text{H}$ ]NA release is expressed as percentage of total [ $^3\text{H}$ ]NA incorporated (ordinate). Data are means  $\pm$  SEM of 6–18 determinations obtained from three independent experiments.

chain form) was less active when compared to the effect of its light chain (Fig. 2). The inhibitory effect of TeTx and its light chain was abolished by boiling (not shown). Isolated TeTx heavy chains lacked any effect (Fig. 2).

## 4. DISCUSSION

A three step-model for the action of clostridial neurotoxins such as TeTx and botulinum A toxin (BoNT/A) has been suggested (cf. [3,24,25]). The three steps involve (i) binding of the toxins to the cell surface via the heavy chain, (ii) internalization and transfer to the cytoplasm and (iii) inhibition of a  $\text{Ca}^{2+}$ -dependent step in exocytosis by the light chain. Using intact cell preparations, the intracellular action of clostridial neurotoxins could not directly be investigated. The direct intracellular application of the toxins and their fragments in normal or neoplastic chromaffin cells via a patch clamp pipette [8] or by permeabilization of the plasma membrane [9,10,13,26–29] allowed to demonstrate that toxicity is confined to the light chains of both, TeTx and BoNT/A and that the toxins act at a step downstream of the action of  $\text{Ca}^{2+}$  during exocytosis.

In this study we investigated for the first time the intraterminal effects of TeTx in endings from the mammalian central nervous system. We used a SLO-permeabilized synaptosome preparation, which has been shown to release NA in a  $\text{Ca}^{2+}$ -dependent manner [17–19]. With this preparation, which is suitable to introduce macromolecules without affecting the release machinery [19,30], we show that the TeTx light chain inhibits  $\text{Ca}^{2+}$ -induced NA release. The inhibitory effect of the light chain is observed within 5 minutes. Since significant permeabilization with SLO can be observed after about 1 minute [18] and NA release is complete in about 3 minutes (Hens and De Graan, unpublished), the effect of the light chain probably occurs within 3 minutes.

The results are consistent with the observed short time required for the action of TeTx light chain in permeabilized neurosecretosomes from the posterior pituitary gland [12] and are indicative for a fast intracellular action of the TeTx light chain. Intact TeTx was less effective in permeabilized synaptosomes than the isolated light chain. This may indicate that chain separation, which presumably is an important process during TeTx and BoNT/A activation [9,10,12,26,31], by endogenous systems [32], is probably not completed within the 5 minutes of incubation.

In contrast to the light chain, the heavy chain alone did not inhibit [<sup>3</sup>H]NA release from SLO-permeabilized synaptosomes. Similarly, intracellular application of TeTx light chains but not of heavy chains was reported to inhibit vasopressin release from neurosecretosomes of the posterior pituitary gland [12], acetylcholine from ganglia of *Aplysia californica* [14] and NA from adrenal chromaffin cells [10,13].

The above-mentioned results show similarities with those reported for BoNT/A, which is in many respects very similar to TeTx (cf. [1–3]). In normal or neoplastic chromaffin cells as well as in neurohypophyseal nerve endings, only the light chain, but not the heavy chain of BoNT/A inhibits exocytosis [27–29,31]. However, when injected into *Aplysia* neurons, the isolated light chain of BoNT/A, has no effect. In these neurons both the heavy and the light chains must be simultaneously present to inhibit transmitter release [33,34]. The intracellular effect of BoNT/A and its isolated chains on synapses of the central nervous system has yet not been investigated. It will be interesting to see, whether the light chain alone will have any effect in this system or whether, in analogy to invertebrate neurons, the presence of the heavy chain is required.

In summary, our data show that the light chain of tetanus toxin alone inhibits transmitter release from permeabilized rat brain synaptosomes. They support previous investigations carried out with adrenal chromaffin cells, nerve endings from the posterior pituitary gland and invertebrate ganglia and extend them to neurons of the central nervous system being structures directly involved in tetanus toxin-induced disease.

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