The light chain but not the heavy chain of botulinum A toxin inhibits exocytosis from permeabilized adrenal chromaffin cells

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The heavy and light chains of botulinum A toxin were separated by anion exchange chromatography. Their intracellular actions were studied using bovine adrenal chromaffin cells permeabilized with streptolysin O. Purified light chain inhibited the Ca^{2+}-stimulated [^{3}H]noradrenaline release with a half-maximal effect at about 1.8 nM. The inhibition was incomplete. Heavy chain up to 28 nM was neither effective by itself nor did it enhance the inhibitory effect of light chain. It is concluded that the light chain of botulinum A toxin contains the functional domain responsible for the inhibition of exocytosis.

Botulinum A toxin; Chain, light; Chain, heavy; Chromaffin cell, permeabilized; Exocytosis

1. INTRODUCTION

Botulinum A toxin (Botx A) belongs to the extremely poisonous clostridial neurotoxins [1,2]. The 150 kDa protein consists of a heavy chain (100 kDa) and a light chain (50 kDa) covalently linked by a disulfide bond. It inhibits transmitter release from neurons [1,2] as well as catecholamine release from adrenal chromaffin cells [3,4]. Direct application into the cytosol of adrenal chromaffin cells either by injection [5] or through large pores in the plasma membrane [6] confirmed that Botx A attacks a step during exocytosis common to neurons and chromaffin cells.

The inhibition of catecholamine release by both tetanus toxin and Botx A is increased in the presence of a reducing agent [6,7]. This observation indicates that the disulfide bond linking the two chains in the clostridial neurotoxins has to be cleaved during their activation. Indeed the action of tetanus toxin is due to its light chain whereas its heavy chain has no effect [8]. In the present study we prepared and used the heavy and light chains of Botx A to find out which part of the molecule affects exocytosis and whether Botx A is activated in the cell by a mechanism similar to that proposed for tetanus toxin [8].

2. MATERIALS AND METHODS

2.1. Materials

Botx A (two chain form) as obtained from Batelle Institute Frankfurt, FRG, was homogeneous in SDS gel electrophoresis. It was separated into its heavy and light chain by HPLC anion exchange chromatography using a modification of an LPLC procedure as described earlier [9]. All steps were performed at 18°C at pH 8.4 (adjusted with NaOH). Typically 600 μg (1 ml) Botx A were first desalted on an Econo-Pac 10GD (Bio-Rad) column and then applied to a Mono Q 5/5 (Pharmacia-LKB) column both equilibrated with buffer A containing (mM): $Na_2B_4O_7$ 7.5 and NaH_2PO_4 15. The Mono Q column was washed with 6 ml of buffer A. For chain separation 50 mM dithiothreitol (DTT) dissolved in 1.5 ml of buffer A was applied to the column and the flow stopped for 2 h. Then the light chain was eluted at a rate of 0.25 ml/min with 7.5 ml of a mixture of 9 parts buffer B (buffer A supplemented with 2 M urea and 50 mM DTT) and one part of buffer C containing (mM): $Na_2B_4O_7$ 75, NaH_2PO_4 150, urea 2000, DTT 50. The absorbed heavy chain was finally eluted from the column with 7.5 ml of

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buffer C. Fractions were collected and the chains were localized using a protein assay [10]. Immediately after separation the chains were dialyzed against KG buffer I containing (mM): K^+ glutamate 150, Pipes 10, EGTA 0.5, NTA 5, pH 7.2 supplemented with 1 or 5 mM DTT. Toxicity (LD_{50}, s.c. in mice) was 1 ng/kg for the two chain form and between 1 and 5 μg/kg for the separated heavy or light chains, respectively. Botulinum A antitoxin, an immune serum from horse containing 750 IU/ml was obtained from Behring Werke Marburg, FRG, and dialyzed against KG-buffer I before use. Streptolysin O (SLO) was purified [11]; it was kindly provided by S. Bhakdi (Giessen). The activity of SLO, routinely checked using 2.5% rabbit erythrocytes, is given in hemolytic units (HU/ml) [12].
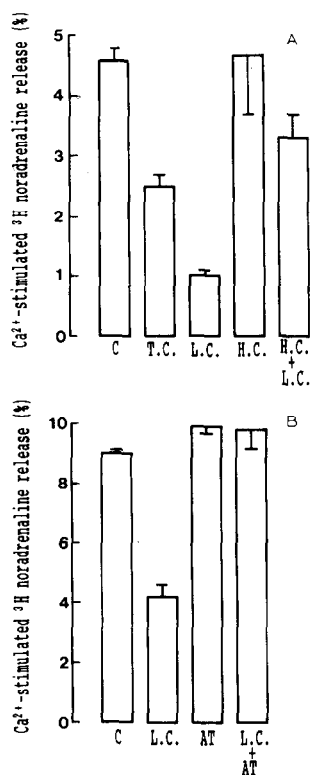
2.2. Methods

Chromaffin cells from bovine adrenal medulla were prepared and kept in short-time cultures as described ([6], for review see [13]). After preloading the cells with tritiated noradrenaline they were permeabilized with SLO (60 HU/ml) for 2 min at 37°C [12] and incubated between 5 and 25 min with the respective toxin preparation dissolved in KG buffer II (KG-buffer I containing additional 2 mM Mg^{2+}/ATP complex and 1 mM free Mg^{2+}) supplemented with 0.1% BSA and 1 mM DTT. Finally the cells were stimulated with fresh KG buffer II containing micromolar amounts of free Ca^{2+} for 10 min [6,12]. Free Ca^{2+} concentrations were calculated using the stability constants given [14] and were routinely monitored by a Ca^{2+}-sensitive electrode [15].

3. RESULTS

Ca^{2+}-induced catecholamine release from SLO-permeabilized adrenal medullary chromaffin cells in primary culture is affected by the two chain form of Botx A (5 nM) in the presence of 1 mM DTT (fig.1A and [6]). When given alone the light chain (5 nM) inhibited Ca^{2+} induced exocytosis from permeabilized adrenal chromaffin cells, whereas the same amount of heavy chain was ineffective (fig.1A). The equimolar mixture of the
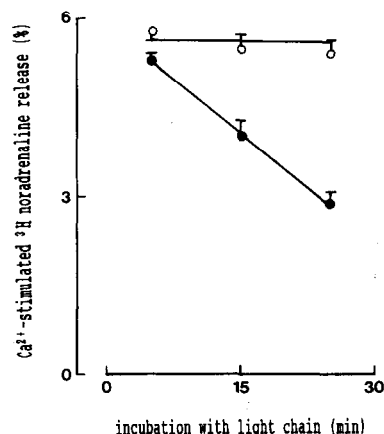

Fig.1. Specificity of the inhibitory effect of Botx A light chain on Ca^{2+} stimulated [³H]noradrenaline release from permeabilized chromaffin cells. (A) Permeabilized adrenal chromaffin cells were incubated with KG-buffer (see section 2), 5 nM Botx A two chain form (T.C.), 5 nM Botx A light chain (L.C.), 5 nM Botx A heavy chain (H.C.) or a combination of 5 nM heavy plus 5 nM light chain for 25 min at 37°C. Then they were stimulated for 10 min with fresh KG-buffer containing 30 μM free Ca^{2+}. Release in the absence of Ca^{2+} (3.9 ± 0.2) was subtracted. (B) Prior to the addition of Ca^{2+} (30 μM free) the permeabilized cells were incubated for 25 min at 37°C with KG-buffer, 5 nM light chain, or 7.5 IU/ml botulinum A antitoxin (AT) or 5 nM light chain plus 7.5 IU/ml AT. All additives were kept for 2 h at room temperature before use. Release in the absence of Ca^{2+} (3.6 ± 0.3) was subtracted. Values are the mean of 3 samples ± SD.

Fig.2. Time course of the inhibitory action of Botx A light chain on Ca^{2+}-stimulated [³H]noradrenaline release. Permeabilized adrenal chromaffin cells were incubated for 25 min in KG-buffer II (see section 2.2) supplemented with 0.1% BSA and 1 mM DTT. Botx A light chain (5 nM) (closed symbols) was present either from the beginning or added 15 min or 5 min before the buffer was replaced by a fresh one containing either no or 30 μM free Ca^{2+}. Controls (with no light chain added, open symbols) were treated similarly. Values represent the Ca^{2+} stimulated release of 3 samples ± SD. Release in the absence of Ca^{2+} (4.0 ± 0.7 for controls and 4.1 ± 0.2 for light chain present for 25 min) was subtracted. Values are the mean of 3 samples ± SD.

heavy and the light chain was rather less effective than the separated light chain alone (fig.1A, see also fig.3). The inhibitory action of the light chain was prevented by preincubation with antitoxin indicating its specific action. The antitoxin itself did not modify exocytosis from the permeabilized chromaffin cells (fig.1B).

The inhibitory action of the light chain increased with the incubation time as previously shown for the two chain toxin [6]. The light chain was present either for 5, 15, or 25 min prior to the stimulation with Ca^{2+}. A slight inhibition of exocytosis was observed during 5 min presence of light chain, which increased to about 50% after 25 min. Even when present during the whole incubation period the light chain did not alter the basal release (fig.2).

The inhibitory action of Botx A light chain was always incomplete. It started already below 1 nM, was maximal with doses over 5 nM and half maximal at 1.8 ± 1.2 nM (SD; $n = 4$). The presence of 28 nM Botx A heavy chain did not add to the inhibitory action caused by the light chain. Indeed
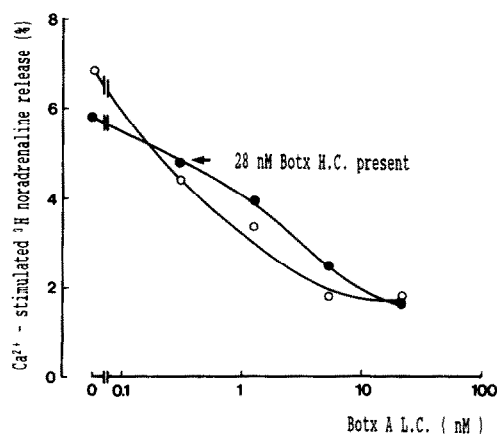

Fig.3. Dose–response curve of the inhibitory action of Botx A light chain on exocytosis in the absence or presence of its heavy chain. Permeabilized adrenal chromaffin cells were incubated for 25 min at 37°C in KG buffer II with increasing amounts of light chain (abscissa). The buffer was replaced with fresh buffer either with or without 30 μM free Ca^{2+} and cells were further incubated for 10 min. The values represent the Ca^{2+} stimulated release. The effects of Botx A light chain in the absence (open symbols) or presence (closed symbols) of 28 nM Botx A heavy chain are shown. Controls were also run in the absence or presence of heavy chain. Values (mean of two samples) in the absence of Ca^{2+} (3.4/3.8 for controls and 3.6/3.7 for 28 nM heavy chain) were subtracted. Note that Botx A heavy chain did not substantially alter the Ca^{2+} stimulated release.

rather a slight reduction of the efficacy of the light chain was observed (fig.3, see also fig.1A).

4. DISCUSSION

The results reported here indicate that the light chain of Botx A contains the functional domain responsible for the intracellular inhibition of exocytosis. Addition of heavy chain rather inhibits than promotes the inhibitory effect. As already shown for tetanus toxin in *Aplysia* neurons [16] and chromaffin cells [8], inhibition of exocytosis due to Botx A can be mimicked by its light chain. The fact that only part of their molecules is required to elicit the intracellular effects adds the clostridial neurotoxins to a group comprising cholera toxin [17], diphtheria toxin [18], *pertussis* toxin [19] and the botulinum toxin C_2 [20]. In these toxins a larger subunit mediates binding and promotes internalization whereas a smaller component is an enzyme which affects various intracellular processes. However, any enzymatic activity of the light chain of either Botx A or tetanus toxin is still to be found.

The action of the Botx A light chain in chromaffin cells differs from that in *Aplysia* neurons. Here both the heavy and the light chain of Botx A must be present to inhibit transmitter release [21] whereas in chromaffin cells the action of Botx A light chain is independent of the heavy chain (this study). On the other hand the light chain of tetanus toxin alone is fully active in both *Aplysia* neurons [16] and adrenal chromaffin cells [8]. The differences may be caused by the chemical instability of the Botx A light chain. When given alone it might quickly lose its activity in the high osmolar medium required for the *Aplysia* neurons. Addition of the heavy chain may stabilize the light chain which then inhibits exocytosis.

The modes of action of tetanus toxin and botulinum A toxin are not strictly identical. This is true for the neuromuscular junction of the mouse hemidiaphragm [22] and for adrenal chromaffin cells [6,8]. Thus Botx A but not tetanus toxin may attack a step during exocytosis which is different in neurons as compared to chromaffin cells.

Further studies using endocrine cells and mammalian neurons will be necessary to shed light on the pharmacokinetics and pharmacodynamics of clostridial neurotoxins at the cellular level.

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