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The tetanus toxin light chain inhibits exocytosis

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The intracellular action on exocytosis of various forms of tetanus toxin was studied using adrenal medullary chromaffin cells, the membrane barrier of which has been removed by permeabilization with streptolysin O. Such cells still release catecholamines on stimulation with calcium. The two-chain form of tetanus toxin (67 nmol/l) strongly inhibited exocytosis, but only if dithiothreitol was present as a reducing agent. Purified light chain completely prevented [^{3}H]noradrenaline release with a half-maximal effect at about 5 nmol/l. Heavy chain (up to 11 nmol/l) and unprocessed single-chain toxin (up to 133 nmol/l) were without effect. It is concluded that the original single-chain form of tetanus toxin has to be processed by proteolysis and reduction to yield a light chain which inhibits transmitter release.

Tetanus toxin; Light chain; Streptolysin O; Exocytosis; (Chromaffin cell)

1. INTRODUCTION

Tetanus toxin is one of the most poisonous substances known. It is first produced by *Clostridium tetani* as a single-chain protein [1,2]. Subsequent proteolytic processing leads to a pharmacologically more active [3], two-chain form consisting of disulfide-linked heavy (98 kDa) and light (53 kDa) chains. The individual chains alone produced by reduction are not neurotoxic [4]. Intoxication requires hours before transmitter release is blocked. This delay reflects the time required for the binding of the toxin to the plasma membrane and its subsequent internalization before the toxin can interact with its as yet unknown intracellular target (cf. [2]). Adrenal medullary chromaffin cells, closely related to neurones, lack the capacity to bind tetanus toxin [2] and therefore are insensitive to externally applied toxin [5,6]. Sensitivity may be introduced by pretreatment of such cells with a ganglioside mixture containing tri- and tetrasialogangliosides as putative receptors (Marxen, Fuhrmann and Bigalke, personal communication). When directly injected into these cells tetanus toxin inhibits Ca^{2+}-induced exocytosis as revealed by cell capacitance measurement [7].

Binding and internalization can be by-passed using permeabilized cell preparations. With these methods the cytoplasm becomes fully accessible to extrinsic substances including Ca^{2+}, drugs and toxins. Pheochromocytoma (PC 12) or adrenal medullary chromaffin cells in culture, when permeabilized with pore-forming proteins (cf. [8]) still release catecholamines in response to micromolar concentrations of Ca^{2+} [9–13]. Cells can be made permeable to low molecular mass solutes with staphylococcal α-toxin [9–14] and to large proteins such as immunoglobulins with streptolysin O (SLO) [8,13,15]. We have recently looked for an interference of various forms of tetanus toxin or its chains with the Ca^{2+}-stimulated [^{3}H]noradrenaline release from SLO-permeabilized chromaffin cells.

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2. EXPERIMENTAL

2.1. *Materials*

The two-chain form of 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. The isoelectric points were 5.9, 4.8, and 7.2 for the two-chain form, light chain and heavy chain, respectively. Single-chain toxin and its two-chain form were prepared and characterized as described [3]. Toxicity (LD_{50}, s.c. in mice) was 4 ng/kg mouse for the single-chain tetanus toxin, 2 ng/kg for the two-chain form, 100–300 μg/kg for the heavy chain and 30–40 μg/kg for the light chain. B-fragment of tetanus toxin was obtained by papain cleavage as given in [16] with a toxicity of approx. 100 μg/kg mouse [17]. Before use all toxin preparations were dialyzed against KG-buffer.

2.2. *[^{3}H]Noradrenaline release from SLO-permeabilized adrenal medullary chromaffin cells*

Chromaffin cells from bovine adrenal medulla [18] were kept in short-time cultures (2–7 days) and preloaded with 0.1 μmol/l [^{3}H]noradrenaline (1.3 μCi/ml) for 1 h at 37°C. After several washes with Ca^{2+}-free salt solutions (mmol/l): 150 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 0.5 ascorbic acid, 11 glucose, 15 Pipes (pH 7.2), the cells (3×10^5) were permeabilized for 2 min at 37°C with 30 HU/ml streptolysin O (SLO) in KG-buffer (mmol/l): 150 K^+ glutamate, 5 NTA, 0.5 EGTA, 2 ATP, 5.5 Mg^{2+} acetate, 10 Pipes (pH 7.2), supplemented with 0.1% bovine serum albumin. The buffer was exchanged for a fresh one containing the tetanus toxin preparations to be tested. After incubation for 25 min at 37°C the cells were challenged for 10 min with KG-buffer containing the indicated amounts of free Ca^{2+} in the presence (fig.1a, fig.2 open circles) or absence (fig.1b, fig.2 closed circles) of toxin. [^{3}H]Noradrenaline was estimated in the supernatant and in the cells after lysis in 0.2% SDS [13]. The values give the percentage of radioactivity released by calcium within 10 min. The release in the absence of calcium (0.36 ± 0.03%/min and 0.34 ± 0.06%/min in the presence of 8.4 nmol/l light chain, SD, $n = 4$) was subtracted. Free Ca^{2+} concentrations were calculated using the stability constants [19] and controlled by a Ca^{2+}-sensitive electrode [20].

3. RESULTS

Ca^{2+}-induced catecholamine release from SLO-permeabilized adrenal medullary chromaffin cells in primary culture was not affected by the two-chain form of tetanus toxin (67 nmol/l) (fig.1), although it is highly toxic in vivo. This molecule consists of two S-S-linked moieties, the heavy (98 kDa) and light (53 kDa) chains. Recently a simple and rapid method for their separation which yields highly purified chains with minimal toxicity (see section 2) has been developed. When given alone light chain was found to inhibit the Ca^{2+}-induced exocytosis from permeabilized cells, whereas the heavy chain was ineffective (fig.1a).

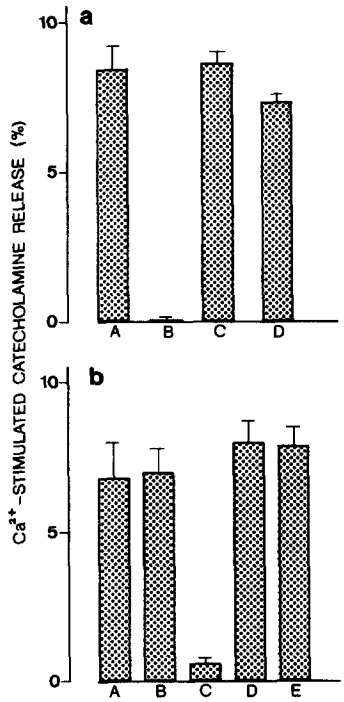

Fig.1. Specificity of the inhibitory effect of tetanus toxin light chain on Ca^{2+}-stimulated catecholamine release from permeabilized chromaffin cells. (a) Permeabilized bovine adrenal medullary chromaffin cells were incubated with (A) KG-buffer (see section 2), (B) 8.4 nmol/l light chain, (C) 4.6 nmol/l heavy chain and (D) 67 nmol/l two chain form of tetanus toxin for 25 min at 37°C. Then they were stimulated for 10 min with fresh KG medium containing 16 μmol/l free Ca^{2+} and the released [^{3}H]catecholamines were determined (see section 2). (b) Prior to the addition of Ca^{2+} (13 μmol/l final concentration) the permeabilized cells were incubated for 25 min at 37°C with (A) KG-buffer, (B) 50 IU/ml tetanus antitoxin, an immunoserum from horse, Behring Werke, Marburg, FRG containing 5000 IU/ml, (C) 8.4 nmol/l light chain, (D) the same amount of light chain but previously heated for 15 min at 95°C or (E) 8.4 nmol/l light chain plus 50 IU/ml antitoxin. As in a, the percentage of [^{3}H]noradrenaline released within 10 min is given (see section 2). Values are the mean of three samples ± SD.

The effects of light chain were abolished by boiling or preincubating with antitoxin (fig.1b). Half-maximal effects on Ca^{2+}-induced exocytosis were obtained at a concentration of about 5 nmol/l and complete inhibition at 20 nmol/l light chain (fig.2). The interchain disulfide bridge of tetanus toxin can be cleaved with DTT. When pretreated in this way the two-chain form of tetanus toxin became effective in the permeabilized cells

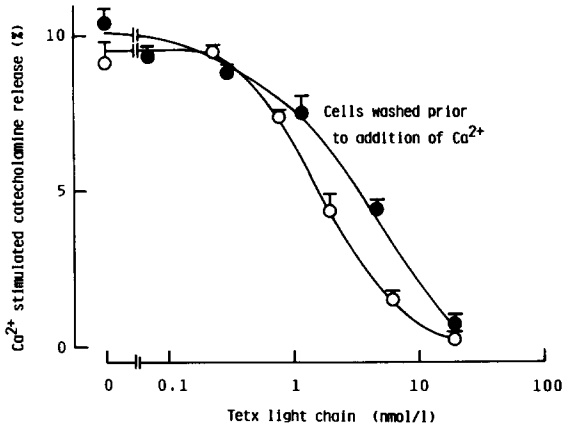

Fig.2. Dose-dependent inhibition of Ca^{2+}-stimulated catecholamine release from SLO-permeabilized chromaffin cells by tetanus toxin light chain. Permeabilized adrenal medullary chromaffin cells were treated with increasing amounts of light chain in Ca^{2+}-free KG-buffer for 25 min at 37°C. The cells were either stimulated for 10 min by adding KG-buffer supplemented with Ca^{2+} to give a free Ca^{2+} concentration of 13 μmol/l (open circles). In a parallel series the light chain was removed after 25 min by a complete exchange with fresh buffer containing 16 μmol/l free Ca^{2+} (closed circles). Values are the mean of three samples ± SD.

although the light chain was more effective (table 1). The unprocessed single-chain form of tetanus toxin is inactive by itself and cannot be activated by DTT (table 1). Similar to the two-chain form of tetanus toxin, the B-fragment was also only effective in the presence of DTT (table 2). This toxin fragment comprises the light chain covalently linked by the S-S bond to the N-terminal moiety of the heavy chain while the C-terminus of the heavy chain (C-fragment) is removed by papain cleavage [2]. These experiments show that it is not the reduction of the two [2] disulfide bridges but the appearance of the light chain that is crucial for the activation of the toxin (tables 1,2). Similar data were obtained using SLO-permeabilized PC 12 cells (not shown).

Table 1

Effects of single-chain and two-chain forms of tetanus toxin, as well as its separated heavy and light chain on exocytosis in the presence of dithiothreitol

	Ca^{2+}-stimulated catecholamine release (%)
Buffer	17.3
Two-chain form of tetanus toxin (67 nmol/l)	6
Heavy chain (11 nmol/l)	17.6
Light chain (15 nmol/l)	0.1
Single-chain tetanus toxin (133 nmol/l)	16.2

Permeabilized chromaffin cells were treated as in fig.1, except that 2 mmol/l dithiothreitol (DTT) was present during the incubation (25 min) with the various preparations of tetanus toxin. Stimulation with 26 μmol/l free calcium was performed as in fig.1b. Values are the mean of two determinations. The presence of DTT did not modify the basal or Ca^{2+} stimulated catecholamine release

Table 2

Inhibitory effect of tetanus toxin B-fragment on exocytosis in the presence of dithiothreitol

	Ca^{2+}-stimulated catecholamine release (%)
Buffer	18.9
Buffer plus 2 mmol/l DTT	17.4
B-fragment (21 nmol/l)	16.2
B-fragment (21 nmol/l) plus 2 mmol/l DTT	6.9

Permeabilized chromaffin cells were treated as in table 1. Values are the mean of two determinations

4. DISCUSSION

The results reported here indicate that the light chain contains the functional domain responsible for the intracellular inhibition of exocytosis.

Several conclusions can be drawn from the data reported here. Single-chain tetanus toxin has been termed a protoxin [3]. We assume that it is activated in two steps. Initially it is proteolytically processed into its two-chain form intermediate by enzymes from the bacteria or the host. Then it is subjected to reductive cleavage by the target cell which releases its light chain. Both events have to take place to obtain full activity. Our data agree with the results of Penner et al. [7]. They injected the two-chain form of tetanus toxin intracellularly 1 h before triggering exocytosis with Ca^{2+}. This interval should be long enough to allow at least a partial reduction. Moreover, they also found that the so-called fragment B of tetanus toxin was active under the same conditions. In our view, the heavy chain is involved in the internalization and

may protect the light chain during its transport to the intracellular target. At first sight some of our data may appear to conflict with those of Bittner and Holz [6] who reported an effect of the unreduced two-chain form of tetanus toxin. However their concentrations of toxin were 5 times higher and the model they used was closely similar to that of injected cells, since toxin was added at the same time as the permeabilizing agent. Thus a partial reduction producing the light-chain form in their preparation cannot be ruled out.

Use of permeabilized cells should permit identification of the intracellular target of tetanus toxin as well as the active domain within its light chain and afford an approach to elucidate the mechanism of the action of light chain at the molecular level.

Further clues may be obtained from comparative studies with the closely related botulinum toxins which unlike tetanus toxin inhibited catecholamine release from intact chromaffin cells in the absence of extrinsic gangliosides [5,21]. In contrast to the results described here with tetanus toxin, both light and heavy chains of botulinum A toxin must be present in *Aplysia* neurones to inhibit neurotransmitter release [22].

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