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The thioredoxin reductase-thioredoxin system is involved in the entry of tetanus and botulinum neurotoxins in the cytosol of nerve terminals

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ABSTRACT

Tetanus and botulinum neurotoxins cause paralysis by cleaving SNARE proteins within the cytosol of nerve terminals. They are endocytosed inside acidic vesicles and the pH gradient across the membrane drives the translocation of their metalloprotease L domain in the cytosol. This domain is linked to the rest of the molecule by a single interchain disulfide bridge that has to be reduced on the cytosolic side of the membrane to free its enzymatic activity. By using specific inhibitors of the various cytosolic protein disulfides reducing systems, we show here that the NADPH-thioredoxin reductase-thioredoxin redox system is the main responsible for this disulfide reduction. In addition, we indicate auranofin, as a possible basis for the design of novel inhibitors of these neurotoxins.

1. Introduction

Bacteria of the genus *Clostridium* produce one tetanus neurotoxin (TeNT) which causes the spastic paralysis of tetanus and seven different botulinum neurotoxins (BoNT/A, /B, /C, /D, /E, /F, and /G) which cause the flaccid paralysis of botulism [1]. They differ for antigenic properties and for their intracellular targets [2,3]. They have a similar four-domains structure [4–7] which makes them capable of entering, via a four step mechanism, the cytosol of nerve terminals [7–9]. Only the N-terminal domain of 50 kDa, termed L chain, reaches the cytosol where it cleaves the three SNARE proteins which form the core of the neuroexocytosis apparatus thus blocking neurotransmitter release causing paralysis [10,11]. Tetanus has been a major killer in the past, but nowadays it is prevented by a very effective vaccine except for those countries not provided with an operative system of preventive medicine [12]. Botulinum neurotoxins are so toxic to humans (MLD_{50} is in the range of 0.2–1 ng/kg) as to be considered for potential use in bioterrorism [13], but, at the same time, they are currently used as therapeutics to treat a number of human syndromes characterized by hyperfunction of peripheral nerve terminals [14–16]. Human botulism is rare, and it follows the ingestion of BoNT contaminated food or of spores of neurotoxigenic Clostridia, contamination of wounds or excessive dosage during cosmetic treatments [1,17]. The duration of human botulism depends on the amount and type of BoNT, with BoNT/A and /C having the longest duration and BoNT/E the shortest [1,18,19]. This situation calls for the development of novel drugs that can counteract TeNT and BoNT action by affecting any of the four steps of the mechanism of intoxication: (a) binding; (b) endocytosis; (c) membrane translocation and (d) metalloprotease cleavage of SNAREs. Before their binding to the presynaptic membrane of neurons, the toxins are best neutralized by specific anti-toxin antibodies [20,21] which prevent the first step of the entry of TeNT and BoNT into their target neurons. Currently, several groups are attempting to block any of the three subsequent steps [22–30].

The present knowledge on the mode of membrane translocation of these neurotoxins is summarized in a model which envisages the insertion of the H_N domain into the membrane of the acidic toxin-containing vesicle to form a ion channel which acts as a chaperone assisting the unfolding and translocation of the L chain, which remains linked to the H_N domain via a single interchain

Abbreviations: AF, auranofin; ATO, arsenic trioxide; BoNT, botulinum neurotoxin; BSO, buthionine sulfoximine; CGN, cerebellar granule neurons; TeNT, tetanus neurotoxin

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disulfide bridge [7,31,32]. The L chain then refolds at the neutral pH of the cytosol and detaches following reduction of the interchain SS bond by an unknown reducing agent of the cytosol [32]. An intact SS bond is essential for neurotoxicity [33,34] and for translocation of the L chain and it must be reduced on the cytosolic side [32].

There are several disulphide bond reducing systems in the cytosol of cells [35,36] and, here, we have used a pharmacological approach to identify which of them is involved in the reduction of the interchain SS bond of TeNT and BoNTs. At the same time, this study has identified a molecule, which may lead to novel drugs effective for the treatment of tetanus and botulism patients.

2. Materials and methods

2.1. Reagents

Auranofin, (1-thio-β-D-glucopyranosatotriethylphosphine gold-2,3,4,6-tetraacetate) BSO (buthionine-sulfoximine), ATO (arsenic trioxide), $KAuCL_4$ (potassium tetrachloroaurate(III) hydrate), cDDP (cisplatin), cytosine arabinoside, DNAse I, poly-L-lysine, were purchased from Sigma Aldrich. Anti-VAMP2 Mab, anti-Syntaxin Mab were from Synaptic System. TeNT, BoNT/B, and /C were purified as previously described [37,38] whilst BoNT/D was produced in *Escherichia coli* via recombinant methods as described [39].

2.2. Cell cultures

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats [40]. Briefly, rat cerebella were firstly mechanically disrupted and then trypsinated in the presence of DNase I. Cells were collected and plated into 24 well plates, coated with poly-L-lysine (50 μg/ml), at cell density of 3×10^5 cells per well. Cultures were maintained at 37 °C, 5% CO_2, 95% humidity in BME supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine an 50 μg/ml gentamycin. To arrest growth of non-neuronal cells, cytosine arabinoside (10 μM) was added to the medium 18–24 h after plating.

2.3. Assay of inhibitors of the intoxication of CGNs with tetanus and botulinum neurotoxins

CGNs were used at 6–8 div. Cells were pre-incubated with AF or BSO or ATO or cisplatin or $KAuCl_4$ at increasing concentrations in BME 10% FBS, 25 mM KCl and left for 6 h at 37 °C and 5% CO_2. Then the indicated toxin was added and left for 15 min at 37 °C. Thereafter, the cells were washed with BME in order to remove the toxin and the normal culture medium was restored with the indicated concentration of inhibitor for 12 h at 37 °C. The translocation of the L chains of the various neurotoxins was evaluated following their specific proteolytic activity by immunoblotting with anti-SNAREs antibodies.

2.4. Immunoblotting

Cells were lysed with 100 mM Tris–HCl, 1% SDS, pH 6.8, containing protease inhibitors (complete Mini EDTA-free, Roche). Protein concentration was determined with the BCA test (Pierce BCA protein assay, Thermo Scientific), and equal amounts were loaded onto a 4–12% NuPage gel (Invitrogen) and separated by electrophoresis in 1× MES buffer (Invitrogen). Proteins were then transferred onto Protran nitrocellulose membranes (Whatman) and then saturated for 1 h in PBS-T (PBS 0.1% Tween 20) containing 5% non-fat milk. Incubation with primary antibodies (specific for VAMP2 and Syntaxin 1A) was performed overnight at 4 °C. The membranes were washed three times with PBS-T and incubated with secondary antibodies HRP-conjugated. Finally membranes were washed several times with PBS-T and visualization was carried out using the Luminata Crescendo (Merck Millipore). The amount of cleaved VAMP (TeNT, BoNT/B and /D) was determined as a ratio with respect to Syntaxin 1A, whilst the opposite was done in the case of BoNT/C.

2.5. In vitro proteolytic activity

TeNT and BoNT/D (2 μg) were treated with reducing buffer (150 mM NaCl, 10 mM NaH_2PO_4, 15 mM DTT pH 7.4) for 30 min at 37 °C. The activated toxins were split into different tubes and in half of them AF was added (1 μM final concentration). Five micrograms of recombinant GST-VAMP2 was added in each tube and the reaction was left for 2 h at 37 °C. SNARE cleavage was assessed by Comassie staining in SDS–PAGE.

2.6. Viability test

CGNs were seeded in a 96 wells plate at a cell density of 10^5 cells per well. After 6 div, different concentration of AF, ranging from 0 to 10 μM, were added and left for 24 h. Neurons were then washed and MTS assay (Promega) performed according to supplier indication. Absorbance was recorded at 490 nm using a Spectra Count™ plate reader (Canberra Industries, Meridien, USA) and cell viability has been reported as percentage vs. non-treated neurons.

2.7. BoNT/D LD_{50} assay

For in vivo experiments Swiss-Webster adult male CD1 mice weighing 25–30 g were used. Mice were housed under controlled light/dark conditions and food and water was provided ad libitum. All experiments were performed in accordance with the Italian guidelines, law n. 116/1992 and were approved by the Animal Ethical Committee of our University. AF was dissolved in a stock solution with ethanol. Mice were i.p. injected (injection volume 250 μl) with 12 mg of AF per kg of body weight or with the vehicle (0.9% NaCl). Animals were incubated for 6 h before the injection of the indicated dose of BoNT/D diluted in 0.9% NaCl (injection volume 250 μl). The animals were monitored every 3 h for 96 h, after which the experiment was considered ended.

2.8. Estimation of thioredoxins reductase activity

Thioredoxin reductase activity was assayed as previously described [41]. Briefly, 0.1 mg of cell extract was tested at room temperature in 0.2 M Na,K-phosphate buffer (pH 7.4), supplemented with 2 mM EDTA and 0.4 mM NADPH. Reactions were started adding 2 mM DTNB and its reduction was followed spectrophotometrically at 412 nm. Values after 1 min after start had been reported.

3. Results

The major protein disulfide reducing systems of the cell cytosol, in addition to the glutathione pool, are the NADPH-thioredoxin reductase-thioredoxin (Trx) system [35] and the glutathione-glutaredoxin system [36]. They are implicated in controlling a variety of cell functions altered in a number of human diseases [42]. Accordingly a large number of drugs have been developed to be evaluated as candidate drugs for clinical use [43,44]. We have taken here a pharmacological approach and used a set of inhibitors of these cell disulphide reducing system to identify which of them is implicated in the reduction of the interchain disulphide bond of clostridial neurotoxins, a reduction that takes place in the cytosol [32–34].

Buthionine sulfoximine (BSO) and arsenic trioxide (ATO) are well characterized for the depletion of the cytosolic pool of glutathione [43,44]. Under conditions in which residual GSH of cerebellar granular neurons was lowered by these drugs below 20% of the normal content, no effect on the VAMP2 cleavage induced by BoNT/D and TeNT was observed (Fig. S1 A and B), indicating that neither GSH nor the glutathione-glutaredoxin system play a relevant role in the process. This negative result left open the possibility that the Trx system might be implicated.

Several inhibitors of the Trx system are available, including curcumin and other flavonoids, cisplatin, $KAuCl_4$, but the most specific one is auranofin (AF) [45]. An attractive feature of AF is that it is currently used in the treatment of human rheumatoid arthritis [46] in addition to being considered for the treatment of different forms of cancers and HIV infection [45,47].

Fig. 1B shows that AF protects cerebellar granular neurons from the action of different doses of tetanus neurotoxin (TeNT) and that about 1 μM AF confers a full protection against TeNT intoxication (Fig. 1A), measured as cleavage of VAMP-2, while it does not affect cell vitality (Fig. S2).

The inhibitory effect of AF is not limited to TeNT as it protects CGN from the cleavage of VAMP-2 induced by BoNT/B (panel A of Fig. 2) and the cleavage of syntaxin 1A induced by BoNT/C (Fig. 2B). It is noteworthy that AF protects the intoxication of CGN from different neurotoxins in the same range of concentrations. This is consistent with an action of AF on the same step of the intoxication process. Auranofin is reported to be very specific for Trx [41,48,49], however we tested its ability to inhibit the Trx of CGN. Fig. S3 shows that the same holds true for the neurons used here. The different range of concentration values found for the inhibition of the activity of the neurotoxins and for the Trx inhibition could be attributed to the different permeability of the plasma membrane of AF in different cell lines (or to the different activity of Trx in different cell types) [41].

In addition to syntaxin, BoNT/C cleaves SNAP-25 [50]. However, we could not quantify the inhibitory activity of AF on the action of BoNT/C on its second substrate because AF interferes with the immunoblotting assay of SNAP-25 cleavage used here. As AF interacts with selenium and sulfur containing compounds, it might as well interact with the cysteine quartet of SNAP-25 after cell solubilization. In vivo, this does not occur as AF is not cytotoxic to neurons, and no AF neurological side effects in humans have been reported. This difference is likely to be due to the fact that the SNAP-25 cysteine quartet is inserted in the membrane and is therefore protected from the interaction with AF. However, this effect of AF prevented us to test its activity on BoNT/A and BoNT/E. We attempted to use two other Trx inhibitors that may overcome this problem: cisplatin and $KAuCl_4$. However, they were found to be very toxic to CGN in culture at concentrations close to their reported IC_{50} (not shown) [48].

In terms of toxicity in mice, the most powerful clostridial neurotoxin is BoNT/D (this preparation gave a LD_{50} value <0.4 nanogram/kg). Fig. 3 shows that AF also protects CGN from the cleavage of VAMP-2 induced by this neurotoxin type and again in the same range of concentrations.

The inhibitory activity of AF on the intoxication of neurons by TeNT and BoNT/B, /C and /D is not due to an effect on the fourth step of the process, i.e. their metalloprotease activity as control experiments performed in vitro with toxins and substrates alone did not reveal in any case an inhibition by AF (not shown).

These results prompted us to test the possible protective activity of AF in vivo in mice injected with BoNT/D, chosen because it is the most powerful clostridial neurotoxin in mice. However, AF used at the dose of 12 mg/kg, corresponding to about 175 μM considering the total body fluids as 10% of the mouse weight, did not protect mice from the flaccid paralysis induced by BoNT/D (Fig. 4). This dose was recently used to show that AF inhibits the activation of the Nlrp1b inflammasome [51].

4. Discussion

The main finding reported here is that auranofin (AF), a specific active site inhibitor of thioredoxin reductase, a member of one of the major cell protein disulfide reducing systems, inhibits the intracellular activity of TeNT and of BoNT/B, /C and /D. At the same time, AF does not inhibit the metalloproteolytic activity of the L

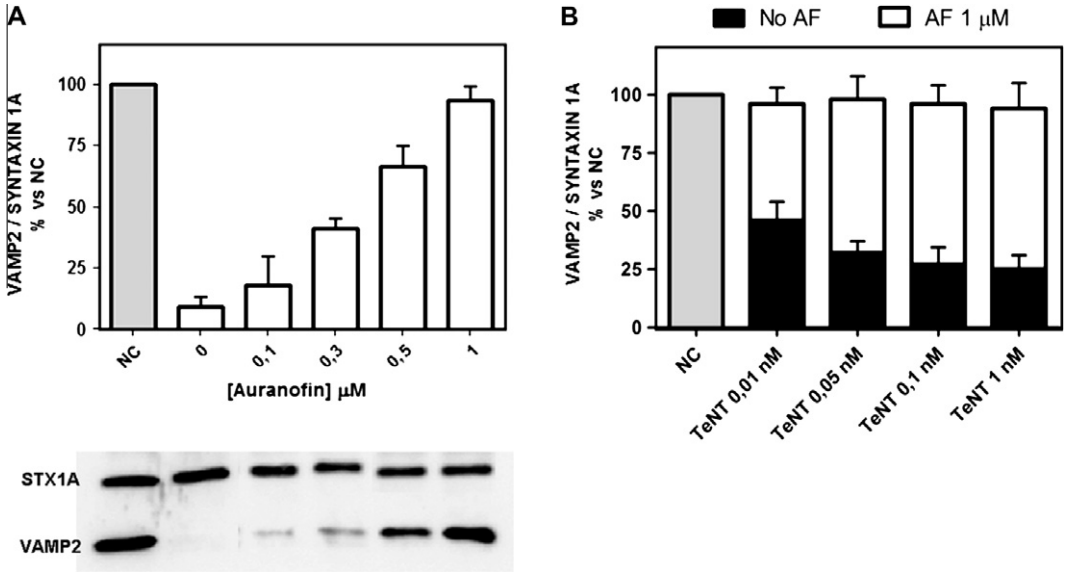

Fig. 1. TeNT cleavage of VAMP is inhibited by auranofin in cerebellar granular neurons. The neurons were pre-incubated for 6 h with the indicated concentrations (A) or with 1 μM of auranofin (B, empty bars) at 37 °C, washed with culture medium and incubated with 1 nM (A) or the indicated concentrations of TeNT (B) at 37 °C for 15 min in culture medium, washed and incubated for 12 h at 37 °C in the presence of the reported inhibitor concentration. In panel B, black bars represent the samples incubated without auranofin. Cells were lysed and the SNARE proteins were estimated by immunoblotting with specific antibodies (lower panel); Residual VAMP2 was quantified by densitometry and its percentage ratio with syntaxin 1A plotted, taking the value in non-treated cells (grey bar) as 100%. S.D. values derive from three independent experiments performed in triplicates. The lower panel shows a representative immunoblotting.

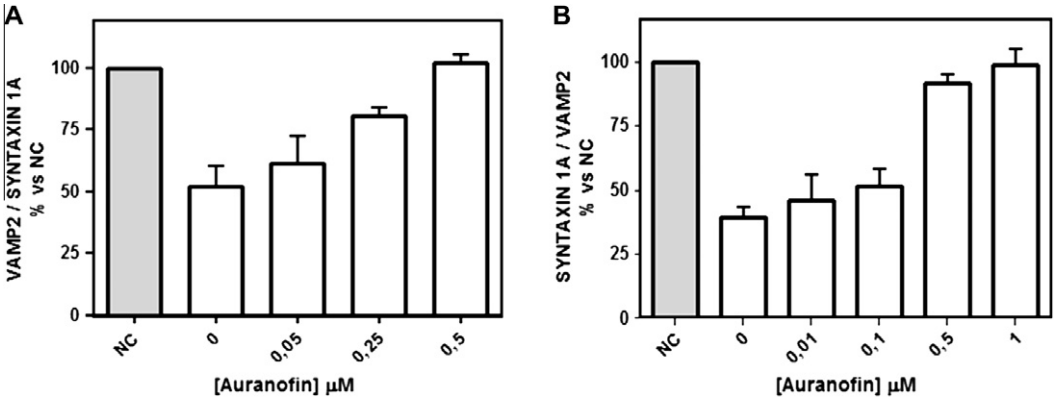

Fig. 2. BoNT/B and C cleavage of VAMP in cerebellar granular neurons is inhibited by auranofin. The neurons were pre-incubated for 6 h with the indicated concentrations of auranofin at 37 °C, washed with BME and incubated with 10 nM BoNT/B (A) or 1 nM BoNT/C (B) at 37 °C for 15 min in culture medium, washed and incubated for 12 h at 37 °C in the presence of the reported inhibitor concentration. Cells were lysed and the SNARE proteins were estimated by immunoblotting with specific antibodies; Residual VAMP2 (A) or residual syntaxin 1A was quantified by densitometric analysis and its percentage ratio with the respective non-substrate SNARE plotted, taking the value in non-treated cells (grey bar) as 100%. S.D. values derive from three independent experiments performed in triplicates.

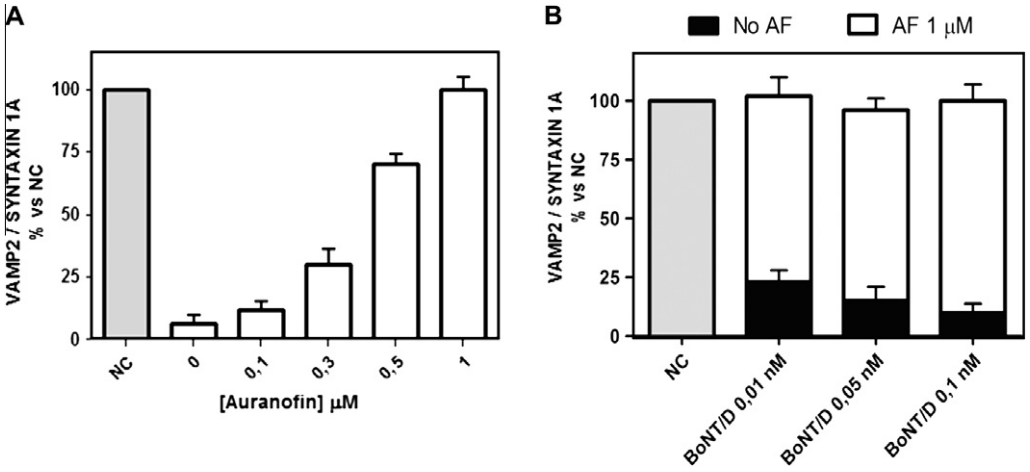

Fig. 3. Auranofin inhibits the VAMP cleavage by BoNT/D in cerebellar granular neurons. The neurons were pre-incubated for 6 h with the indicated concentrations (A) or with 1 µM auranofin (B, empty bars) at 37 °C, washed with medium and incubated for 15 min with 0.1 nM (A) or the indicated concentration (B) of BoNT/D at 37 °C in culture medium, washed again and incubated for 12 h at 37 °C in the presence of the reported inhibitor concentration. (B) black bars represent the samples incubated without auranofin. Cells were lysed and the SNARE proteins were estimated by immunoblotting with specific antibodies; residual VAMP was quantified as in Fig. 1. S.D. values derive from three independent experiments performed in triplicates.

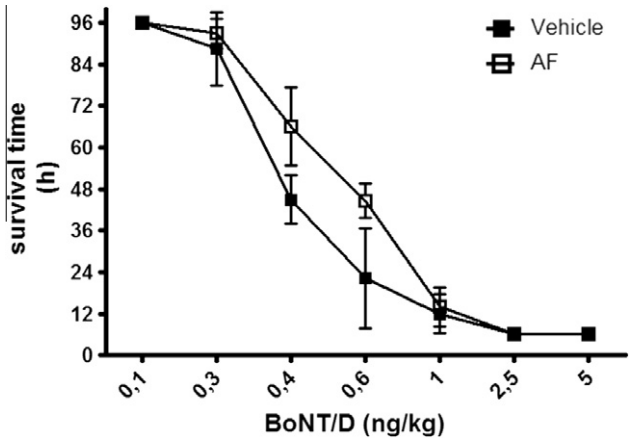

Fig. 4. Effect of auranofin on the BoNT/D MLD_{50} assay. Adult male CD1 mice were i.p. injected with 12 mg/kg of AF or with the vehicle 6 h before the i.p. injection of the indicated dose of BoNT/D. The animals were monitored every 3 h for 96 h. S.D. values derive from three different experiments performed with groups of three animals each.

chains of these neurotoxins. The NADPH-thioredoxin reductase-thioredoxin system very efficiently reduces in vitro the single interchain disulfide bond of TeNT [33] and of BoNTs (our unpublished results). The reduction of this SS bond in the cytosol is essential to free the SNARE cleaving activity of the L chain [31,32]. Taken together, these findings provide a strong indication that the thioredoxin system plays a major role, if not a unique one, in releasing the L chain of TeNT and BoNTs after their translocation across the membrane of the endocytic vesicle assisted by their respective H_N domains.

An attractive feature of thioredoxins is that they share structural similarities with group-I and group-II chaperonins [52,53] and indeed thioredoxins promote the folding of proteins in redox-independent reactions [53–55]. Thus, it is tempting to speculate that the thioredoxin system not only detaches the L chain from the H_N domain by severing the remaining disulfide link, but also acts before by assisting the refolding of the L chain as it emerges from H_N transmembrane channel.

The disappointing lack of protective activity of AF on the in vivo intoxication of mice with BoNT/D can be explained by the fact that AF does not cross the blood–brain barrier and it may as well be not capable of reaching the cytosol of the motor axon terminals

protected by the terminal Schwann cells on one side and the muscle fiber on the other side. However, the present data indicate that it is possible to use auranofin as a lead to design auranofin-based novel inhibitor of the clostridial neurotoxins capable of acting in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.11.007.

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