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Time course and temperature dependence of the membrane translocation of tetanus and botulinum neurotoxins C and D in neurons

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ABSTRACT

Tetanus and botulinum neurotoxins act inside nerve terminals and, therefore, they have to translocate across a membrane to reach their targets. This translocation is driven by a pH gradient, acidic on the cis side and neutral on the cytosol. Recently, a protocol to induce translocation from the plasma membrane was established. Here, we have used this approach to study the temperature dependence and time course of the entry of the L chain of tetanus neurotoxin and of botulinum neurotoxins type C and D across the plasma membrane of cerebellar granular neurons. The time course of translocation of the L chain varies for the three neurotoxins, but it remains in the range of minutes at 37 °C, whilst it takes much longer at 20 °C. BoNT/C does not enter neurons at 20 °C. Translocation also depends on the dimension of the pH gradient. These data are discussed with respect to the contribution of the membrane translocation step to the total time to paralysis and to the low toxicity of these neurotoxins in cold-blood vertebrates.

1. Introduction

Tetanus is caused by the tetanus neurotoxin (TeNT) which is produced by *Clostridium tetani* [1]. TeNT binds to peripheral motoneurons and migrates retroaxonally to enter inhibitory interneurons of the spinal cord where it blocks neurotransmitter release causing a spastic paralysis [2]. At variance, the botulinum neurotoxins (seven serotypes with many variants: BoNT/A to /G) act mainly on peripheral cholinergic nerve terminals [3,4]. TeNT and BoNTs are made of two chains linked by a single inter-chain disulfide bond: the L chain (one domain of 50 kDa) and the H chain (100 kDa, three domains) [2,5–9]. The C-terminal half of the H chain (H_C) is responsible for neurospecific binding [10] and consists of two 25 kDa domains: the N-terminal one is highly conserved, and has been proposed to assist the correct positioning of the toxin on the membrane for the subsequent membrane insertion via binding PIPs [11,12]. The C-terminal 25 kDa domain of H_C of TeNT, BoNT/C and /D were recently shown to harbor two polysialoganglioside binding sites [13–19]. This is a unique feature among the clostridial neurotoxins [10,20] and it allows them to bind to the neuronal membrane in such a way that exposure to low pH is sufficient to induce their entry into the membrane with translocation of the L chain into the cytosol [21]. This is not the case of the other BoNTs which require the presence of their proteins receptors [10].

After binding, the BoNTs are endocytosed into an intracellular compartment whose luminal pH is acidified by a v-ATPase proton pump, which is specifically inhibited by bafilomycin A1 [22,23].

Low pH induces the N-terminal half of the H chain (H_N) to insert into the membrane with formation of a transmembrane ion channel [24–28]. There is evidence that H_N acts as a chaperone that assists the translocation of the L chain into the cytosol in a process dependent on the transmembrane pH gradient [9,29–33].

In the cytosol of nerve terminals, the L chains cleave one of the three SNARE proteins: VAMP/synaptobrevin (by TeNT, BoNT/B, /D, /F and /G), SNAP-25 (by BoNT/A, /C and /E) and syntaxin (by BoNT/C) [34–36].

The intoxication of nerve terminals by TeNT and BoNTs can be assayed in neurons [37–43], but the process of membrane translocation from the low pH intracellular compartment is not accessible to investigation. Recently, methods to bypass this entry process and induce the translocation of the L chain from the plasma membrane have been devised [21,28]. Using this approach, we have made an extensive analysis of the time course of the membrane translocation of the L chains of TeNT, BoNT/C and /D and have found that at 37 °C the translocation of the L chain from the cell exterior to the cytosol is very rapid, i.e. it occurs in minutes. Translocation is strongly temperature dependent and virtually no translocation takes place at 20 °C.

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2. Materials and methods

2.1. Toxins

TeNT was isolated and purified from supernatant of *C. tetani* as previously described [44]. BoNT/C was isolated and purified from supernatants of *Clostridium botulinum* strain NCTC 8264 as previously described [45], whilst BoNT/D (derived from strain 1873) was produced in *Escherichia coli* and purified as described [46].

2.2. Cell cultures

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats. Cerebella were mechanically disrupted and then treated with trypsin (Sigma, 800 μg/ml) in the presence of DNase I (Sigma, 100 μg/ml). Cells were collected and plated into 24 well plates coated with poly-L-lysine (50 μg/mL) at a 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 and 50 μg/mL gentamycin. To arrest growth of non-neuronal cells, cytosine arabinoside (Sigma, 10 μM) was added to the medium 18–24 h after plating.

2.3. Assay of SNARE cleavage in CGNs by tetanus and botulinum neurotoxins after transient low pH exposure

Cells were incubated with toxin in ice-cooled MEM 10% FBS pH 7.4 and left at 4 °C for 15 min. After washing twice with the same cold medium, pre-warmed medium A (123 mM NaCl, 6 mM KCl, 0.8 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM NaP_i, 5 mM citric acid, 5.6 mM glucose, 10 mM NH_4Cl), adjusted at pH 7.4 (positive control, PC), 5.5 or 4.5 with 1 M TRIS-base, was added and left for indicated times at the indicated temperature. Cells where then washed twice with MEM and further incubated at 37 °C in MEM 10% FBS pH 7.4 containing 100 nM Bafilomycin A1 (Sigma) for 12 h. 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 1X 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 (monoclonal, Synaptic System, Germany), for SNAP25 [21], and for Syntaxin 1A [47] 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 Luminata Crescendo (Merck Millipore). The amount of cleaved VAMP (TeNT, BoNT/D) was determined as a ratio with respect to SNAP25, whilst in the case of BoNT/C, SNAP25 and Syntaxin cleavage was reported as a ratio vs. VAMP.

3. Results

3.1. Translocation of the TeNT L chain into neurons

It is well established that TeNT and BoNTs enter into nerve terminals via endocytosis inside acidic compartments, but the molecular mechanism of membrane translocation of the L chain remains ill known. Recently, the L chain was induced to enter into nerve terminals from the plasma membrane with a protocol that bypasses endocytosis and involves the exposure of the cell surface bound toxin to a low pH medium [21,28]. In such a way several parameters of the process can be determined. Here, we have used this approach to study the temperature and time dependence of the membrane translocation of the TeNT, BoNT/C and /D into primary cultures of cerebellar granular neurons (CGN) which are highly sensitive to neurotoxins. These three neurotoxins were chosen because they bind to two polysialogangliosides and therefore can enter into cells at low pH without the requirement of cell culture manipulation to expose the synaptic vesicle receptor [28].

Fig. 1 shows that the L chain of TeNT enters the cytosol of CGN within minutes of exposure at 37 °C, as deduced by the fact that its metalloprotease L chain cleaves the same amount of VAMP as that cleaved by controls where the toxin was allowed to enter via its normal endocytic route which involves synaptic vesicles in neurons of the central nervous system [39,48]. In preliminary experiments we had established that an incubation time of 12 h at 37 °C, in the presence of bafilomycin A1 to block the normal endocytic route of entry, was sufficient to achieve maximal VAMP cleavage and therefore the read-out time was not limiting. The number of L chains which are translocated in the cytosol is slightly higher when the external acid pH was 4.5 than when it was 5.5. This is due to the fact that TeNT must be protonated to insert into the membrane and at the lower pH value a higher proportion of the protonatable groups are titrated.

Membrane translocation is much less efficient at 28 °C and it is prevented at 20 °C. This latter finding is important because it contributes to explain why cold-blood animals like reptiles and amphibians are poorly sensitive to TeNT [49–51].

3.2. Translocation of the BoNT/C L chain into neurons

BoNT/C is the only BoNT, which cleaves two SNARE proteins at the same time: it cleaves both SNAP-25 within its C-terminus and syntaxin close to the transmembrane segment [9,34,36]. The low pH driven translocation of BoNT/C into CGNs is different from that of TeNT (Fig. 2). In fact this neurotoxin shows a similar time course at 37 °C, but it is ineffective at 28 °C even when it was exposed to a medium of pH 4.5. This holds true for the assay of syntaxin cleavage as well as for that of SNAP-25 (left panels of Fig. 2). As the cleavage of a SNARE protein depends on both the number of L chain that reach the cytosol and on their metalloproteolytic turnover number, a strict comparison among different neurotoxins is not warranted. However, the extent of difference is remarkable and data obtained with the same toxin type remain comparable. BoNT/C has been associated with outbreaks of botulism in birds [3,4,52] and it is tempting to associate this finding to the fact that birds have a higher body temperature than mammals. BoNT/C could have been modified through evolution to act in animals endowed with a high body temperature.

3.3. Translocation of the BoNT/D L chain into neurons

BoNT/D is the most toxic of the BoNTs and this high toxicity is paralleled by its activity of neurons in culture. Fig. 3 shows that already at a concentration of 0.05 nM the large majority of VAMP2 is cleaved by the L chains that have translocated across the plasma membrane within one minute of exposure at 37 °C. Even at 20 °C half of the cell content of VAMP2 is proteolysed in the samples exposed to an external pH of 4.5 for 10 min. These results indicate that the membrane translocation of the L chain of BoNT/D is very rapid and has an apparent lower temperature dependence than those of TeNT and BoNT/C.

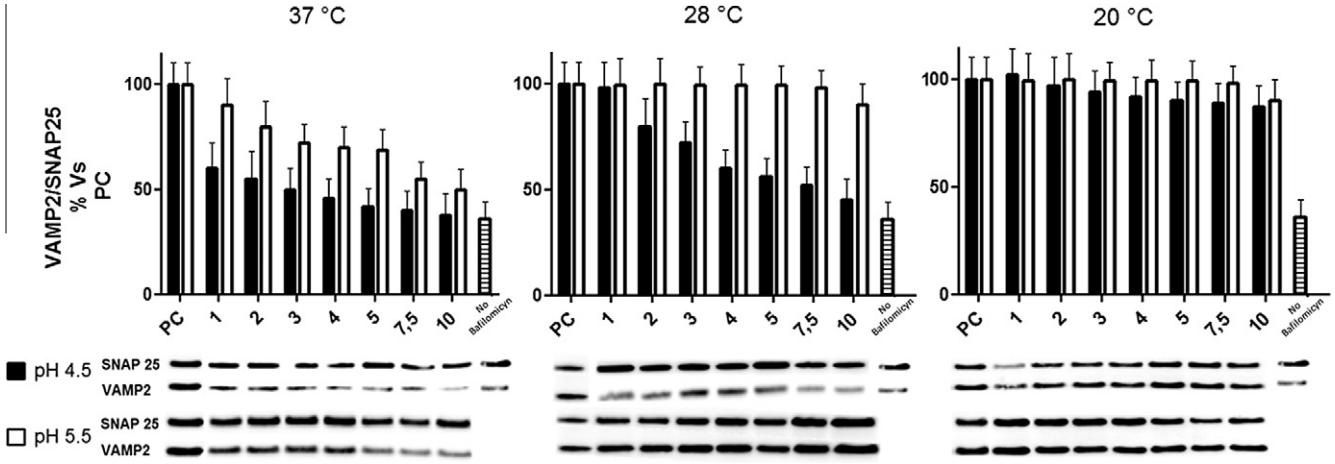

Fig. 1. Temperature and time dependence of TeNT mediated cleavage of VAMP/synaptobrevin in cerebellar neurons in culture. Cells were incubated with TeNT (1 nM) at 4 °C for 15 min, washed and incubated at the indicated temperature with buffers at different pH value (4.5, filled columns; 5.5 empty columns) for the indicated time in minutes (abscissa); after washing, the samples were incubated for 12 h with cell culture medium in the presence of bafilomycin A1 (100 nM), and their content of SNARE proteins was estimated by immunoblotting with specific antibodies. Values are reported as the ratio between the staining with the antibody specific for VAMP/synaptobrevin2 and the staining with the antibody specific for SNAP25, and normalized vs. the value obtained by incubating the neurons with the toxin for 10 min at pH 7.4 (PC), 37 °C, taken as 100%. Striped columns at the right of each panel are values obtained by treating neurons as PC without adding bafilomycin A1 during the 12 h incubation and gives the maximum SNARE cleavage that can be obtained with this toxin. SD values refer to three different experiments performed in triplicates. The lower part shows immunoblotting patterns obtained in a representative experiment.

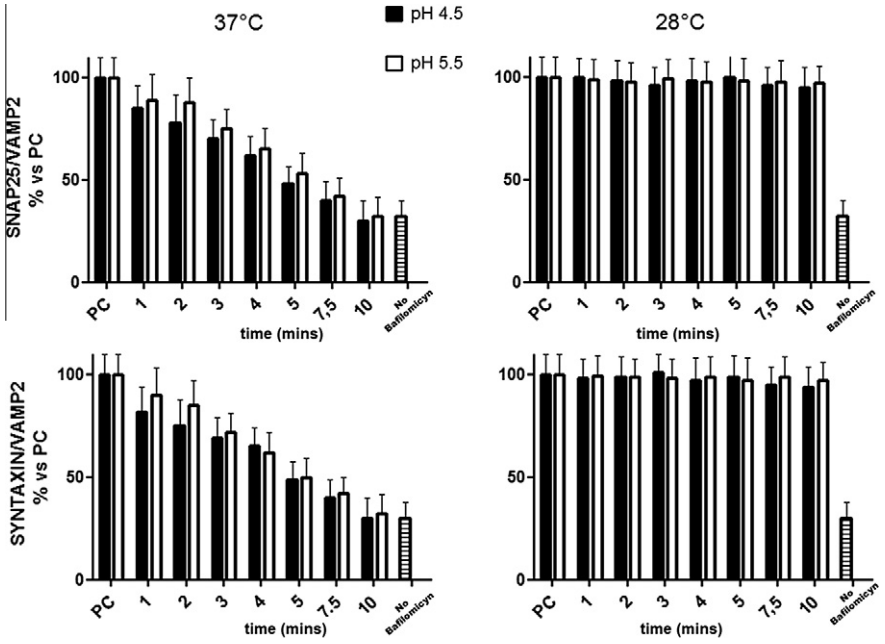

Fig. 2. Temperature and time dependence of BoNT/C mediated cleavage of SNAP25 and syntaxin A1 in cerebellar granular neurons in culture. Cells were incubated with BoNT/C (1 nM) at 4 °C for 15 min and treated as described in Fig. 1 legend. Values are reported as the ratio between the staining with the antibody specific for SNAP25 (upper panels) or syntaxin (lower panels) and the staining with the antibody specific for VAMP/synaptobrevin2, as described in Fig. 1. White striped columns are as in Fig. 1 legend and give the maximum of SNARE cleavage that can be obtained with this toxin. SD values refer to three different experiments performed in triplicates.

4. Discussion

The main general result presented here is that the membrane translocation of the L chain driven by a transmembrane pH gradient, acidic on the toxin side, is very rapid and it is completed within minutes at 37 °C. Given the large structural similarity among the known clostridial neurotoxins [7], this conclusion may be extended to the other neurotoxins, but this remains to be investigated. Tetanus and botulism develop over many hours/days from intoxication [1–4]. Also intoxication of the hemidiaphragm preparation or of neurons in culture require at least half an hour or two hours, respectively. The present results indicate that the long period of time between toxin application and blockade of neurotransmitter release caused by SNARE cleavage cannot be attributed to the membrane translocation step. Most likely, the time required to cleave a substantial fraction of the SNARE proteins takes the large part of the time to intoxication *in vitro* and *in vivo*.

The experimental system used here offers the possibility of dissecting the low pH driven membrane translocation step, but requires that the two toxin receptors are exposed on the surface [21]. Another aspect that has to be considered is that its read out, i.e. the extent of cleavage of the SNARE protein target, de-

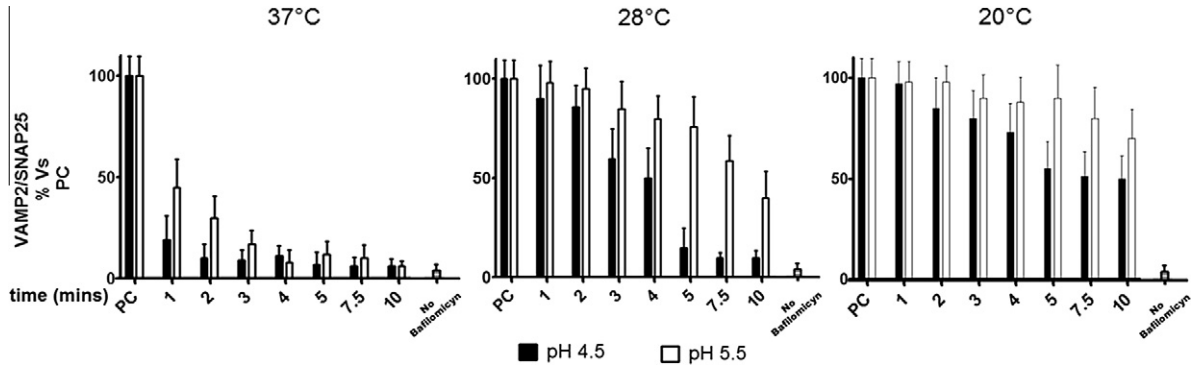

Fig. 3. Temperature and time dependence of BoNT/D mediated cleavage of VAMP/synaptobrevin2 in cerebellar granular neurons in culture. Cells were incubated with BoNT/D (50 picoMolar) at 4 °C for 15 min and treated as described in Fig. 1 legend. White striped columns are as in Fig. 1 legend and give the maximal cleavage of VAMP2 that can be obtained with this toxin. SD values refer to three different experiments performed in triplicates.

pends on both the number of L chain translocated into the cytosol and on their catalytic activity. The latter critical parameter has been analyzed in a scattered series of studies for only some BoNT types and always using artificial substrates *in vitro* [14,53–58], and never at the same time for the three toxins used here. Moreover, one does not know to what an extent the enzyme kinetics data obtained *in vitro* can be translated to the *in vivo* situation as the membrane environment and other parameters can change substantially the kinetics [59]. Therefore, the data obtained here for the same toxin at the different temperatures are homogeneous, but the comparison of those obtained with the different toxins is less stringent. Within this limitation, some differences of possible biological relevance have emerged. TeNT, BoNT/C and BoNT/D differ in the temperature dependence of the membrane translocation of their L chains. BoNT/C does not translocate at 28 °C and this property may be correlated with the fact that this BoNT serotype is associated to outbreaks of botulism in birds which have a body temperature of 42 °C. As a certain degree of L chain unfolding is believed to be involved in membrane translocation [9], it is possible that this toxin is the result of an evolution that has led to the long known higher resistance of BoNT/C to temperature [60]. On this line, the present finding that the action of TeNT is very limited if neurons are exposed to low pH at 20 °C fits in the well-documented temperature dependence of the TeNT sensitivity of amphibians and reptiles [49–51]. However, the outcome of *in vivo* toxicity experiments is the results of several steps, including tissue distribution, binding, endocytosis, membrane translocation and enzymatic cleavage of SNARE target, whilst the protocol employed here selects out only the membrane translocation step.

The extent of cleavage of VAMP2 by TeNT and BoNT/D at 28 °C (middle panels of Figs. 1 and 3) in the samples incubated for 10 min at pH 4.5 is similar to the one achieved under the optimal conditions of incubation without bafilomycin A1, whilst a significantly lower extent of cleavage was found at pH 5.5. This is consistent with the possibility that the pH of the lumen of synaptic vesicles is closer to 4.5 than to 5.5. This interesting possibility deserves to be studied with direct methods.

Further studies involving the other BoNT types are required to fully substantiate the relevant points that have emerged from the present study. But one conclusion that appears to be extendable to all the clostridial neurotoxins is that the membrane translocation step at 37 °C is very rapid and should not be considered in the attempt to explain the long lag phase existing between toxin exposure and blockade of neurotransmitter release with the development of a neuroparalysis.

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