Biochemical Pharmacology 98 (2015) 522–530

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism

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ARTICLE INFO

Article history:
Received 5 August 2015
Accepted 29 September 2015
Available online 9 October 2015

Chemical compounds studied in this article:
Myricetin (PubChem CID: 5281672)
Curcumin (PubChem CID: 969516)
Ebselen (PubChem CID: 3194)
PX12 (PubChem CID: 219104)

Keywords:
Botulism
Botulinum neurotoxins
Thioredoxin system
Ebselen
Inhibitors
Peripheral neuroparalysis

ABSTRACT

Botulinum neurotoxins (BoNTs) form a growing family of metalloproteases with a unique specificity either for VAMP, SNAP25 or syntaxin. The BoNTs are grouped in seven different serotypes indicated by letters from A to G. These neurotoxins enter the cytosol of nerve terminals *via* a 100 kDa chain which binds to the presynaptic membrane and assists the translocation of a 50 kDa metalloprotease chain. These two chains are linked by a single disulfide bridge which plays an essential role during the entry of the metalloprotease chain in the cytosol, but thereafter it has to be reduced to free the proteolytic activity. Its reduction is mediated by thioredoxin which is continuously regenerated by its reductase. Here we show that inhibitors of thioredoxin reductase or of thioredoxin prevent the specific proteolysis of VAMP by the four VAMP-specific BoNTs: type B, D, F and G. These compounds are effective not only in primary cultures of neurons, but also in preventing the *in vivo* mouse limb neuroparalysis. In addition, one of these inhibitors, Ebselen, largely protects mice from the death caused by a systemic injection. Together with recent results obtained with BoNTs specific for SNAP25 and syntaxin, the present data demonstrate the essential role of the thioredoxin–thioredoxin reductase system in reducing the interchain disulfide during the nerve intoxication mechanism of all serotypes. Therefore its inhibitors should be considered for a possible use to prevent botulism and for treating infant botulism.

1. Introduction

Several species of anaerobic bacteria of the genus *Clostridium* produce botulinum neurotoxins which belong to seven different serotypes (BoNT/A-/G) [1,2]. Their number is rapidly growing and many different sub-serotypes are presently known. The biological and toxicological properties of these novel BoNTs are poorly understood, but the limited amount of experimental data indicate that they act predominantly at peripheral cholinergic nerve terminals, causing a long lasting blockade of acetylcholine release with ensuing paralysis of skeletal and autonomic nerve terminals, characteristic of botulism [3]. Apart from BoNT/D [4–6], BoNTs are the most toxic poisons for humans and are classified as potential bioterrorist weapons [7,8]. This extremely high toxicity results from their neurospecificity and from their catalytic activity, which leads to knock-out of proteins essential to the neurotransmitter release apparatus [2,9]. All BoNTs consist of a metalloprotease light chain (L, 50 kDa) and a heavy chain (H, 100 kDa) linked by a strictly conserved interchain disulfide bond. This molecular structure has been shaped during evolution in order to exploit essential physiological features of the vertebrate nervous system. Indeed BoNTs bind specifically to peripheral nerve terminals presynaptic membrane [10] *via* the C-terminus of the H chain which interacts with polysialogangliosides leading to toxin accumulation. The subsequent binding to a protein receptor, transiently exposed on the membrane, is harnessed for their endocytosis [11,12]. In the case of BoNT/A the endocytic organelles were identified as synaptic vesicles [13,14]. Similar data are not available for the other BoNTs, but several experiments performed with vacuolar ATPase proton pump inhibitors clearly indicate that all these neurotoxins enter the lumen of an acidic compartment [15,16]. Indeed it is established that all serotypes have to undergo a low-pH driven

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http://dx.doi.org/10.1016/j.bcp.2015.09.023
0006-2952/

membrane translocation of the L chain, mediated by the N-terminal part of H chain [9,17,18]. Once on the cytosolic side, the L metalloprotease remains attached to the H chain *via* the interchain disulfide bridge. This bond is strictly conserved among serotypes, sub-serotypes and also tetanus neurotoxin, which is structurally and functionally related to BoNTs. Remarkably, the premature reduction of this disulfide completely abrogates the toxicity of all clostridial neurotoxins, underscoring its fundamental role in the intoxication process [19–23]. The reduction of this bond is essential to release the catalytic activity of the L metalloprotease within the cytosol versus the three SNARE proteins [20]. Indeed, also in the test-tube BoNTs cannot cleave their recombinant substrates unless this linkage is reduced [24,25]. Once enabled through reduction, the L chain of BoNT/B, /D, /F and /G cleave VAMP at different peptide bonds, BoNT/A and /E cleave SNAP25, while BoNT/C is particular because it is the only one capable to cleave two substrates, SNAP25 and syntaxin [26,27].

We recently reported that the thioredoxin reductase (TrxR)–thioredoxin (Trx) redox system is present on the cytosolic surface of synaptic vesicles and that its inhibition with specific drugs very effectively prevented the neuroparalysis induced by the three SNAP25 specific BoNTs (A, C and E) [28,29]. Here, we extended the study to the four VAMP-specific BoNTs (B, D, F and G) [16,30] using the four chemicals whose structures are shown in Fig. 1 and which are well characterized inhibitors of TrxR–Trx system. Myricetin is a flavonoid which reacts with the selenium atom present in the active site of the reduced TrxR, providing its irreversible inhibition [31]. Curcumin is a polyphenol of vegetal origin that irreversibly inhibits TrxR forming a 1:2 adduct [32]. In both cases, the direct consequence of inhibition is the loss of Trx reducing potential. PX12 acts mainly on thioredoxin by alkylating a non-catalytic cysteine residue, generating a steric hindrance that prevents the interaction with its reductase. As a result, Trx remains permanently in the oxidized, inactive, form [33,34]. Ebselen acts on both members of the redox couple, as it is an excellent substrate for the mammalian TrxR and a highly efficient oxidant of reduced Trx. Thus, Ebselen prevents the normal function of both enzymes [35].

Together with our previous reports [28,36], the present results provide a strong indication that the reduction of the single interchain disulfide bond is a newly identified key event in nerve intoxication of all BoNTs. We therefore propose that TrxR–Trx inhibitors can be considered as a novel and general class of anti-BoNTs drugs and discuss their possible use in humans.

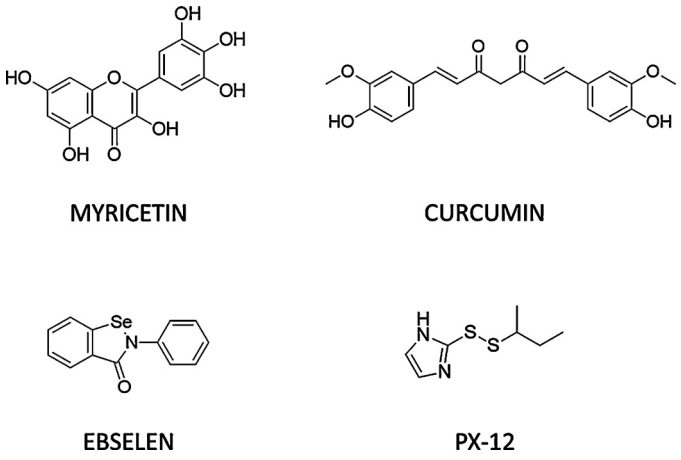

Fig. 1. Thioredoxin–thioredoxin reductase inhibitors used in this study. 3,3′,4′,5,5′,7-Hexahydroxyflavone (Myricetin) and (E,E)-1,7-bis(4-hydroxy-3 methoxyphenyl)-1,6 heptadiene-3,5-dione (Curcumin) preferentially inhibit thioredoxin reductase, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen) both thioredoxin and thioredoxin reductase and 2-[(1-methylpropyl) dithio]-1H-imidazole (PX12) inhibits thioredoxin.

2. Materials and methods

2.1. Reagents

3,3′,4′,5,5′,7-Hexahydroxyflavone (Myricetin), (E,E)-1,7-bis(4-hydroxy-3 methoxyphenyl)-1,6 heptadiene-3,5-dione (Curcumin), cytosine β-D-arabinoside, DNAse I and poly-L-lysine were purchased from Sigma– Aldrich. 2-[(1-Methylpropyl) dithio]-1H-imidazole (PX12) was purchased from Santa Cruz Biotechnology and 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen) was purchased from Cayman Chemical. Antibodies: VAMP2 (104 211) and Syntaxin-1A (110 111) were from Synaptic System, SNAP25 (SMI81, ab24737) was from Abcam. Botulinum neurotoxins B, D and G were produced in *Escherichia coli via* recombinant methods [37–39] whilst BoNT/F was purified as previously described [40].

2.2. Neuronal cultures

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats as previously described [41]. Briefly, cerebella were isolated, mechanically disrupted and then trypsinized in the presence of DNase I. Cells were then collected and plated into 24 well plates, pre-coated with poly-L-lysine (50 μg/ml), at a cell density of 4×10^5 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 gentamicin (hereafter indicated as complete culture medium). To arrest growth of non-neuronal cells, cytosine arabinoside (10 μM) was added to the medium 18–24 h after plating.

2.3. Botulinum neurotoxins inhibition assay on CGNs

CGNs at 6–8 days *in vitro* (DIV) were incubated with increasing concentrations of the indicated inhibitor in complete culture medium for 30 min at 37 °C. Thereafter, the indicated toxin was diluted in complete culture medium and added to CGNs in order to obtain the following final concentrations: BoNT/B (2 nM) or BoNT/F (4 nM) or BoNT/G (4 nM). Incubation was prolonged for 12 h at 37 °C. In the case of BoNT/D, owing to its potency, the toxin was added at a final concentration of 0.025 nM and incubated for 15 min at 37 °C. The neuronal culture was then washed and the culture medium with the same concentration of inhibitor was restored for 12 h. Toxicity was evaluated following the specific proteolytic activity of BoNTs *via* immunoblotting with antibodies specific for VAMP2, SNAP25 and syntaxin. All inhibitors were dissolved in DMSO and stored at −80 °C.

2.4. Immunoblotting

Cells were directly lysed with Laemmli sample buffer containing protease inhibitors (complete Mini EDTA-free, Roche). Cell lysates were loaded onto a 4–12% NuPage gel (Life technologies) and separated by electrophoresis in 1X MES buffer (Life technologies). Proteins were transferred onto Protran nitrocellulose membranes (Whatman) and saturated for 1 h in PBST (PBS, 0.1% Tween 20) supplemented with 5% non-fatty milk. Incubation with primary antibodies was performed overnight at 4 °C. The membranes were then washed three times with PBST and incubated with secondary HRP-conjugated antibodies for 1 h. Finally, membranes were washed twice with PBST and once with PBS; visualization was carried out using Luminata Crescendo (Merck Millipore).