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Minireview

Bacterial protein toxins penetrate cells via a four-step mechanism

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Abstract

Bacteria produce several protein toxins that act inside cells. These toxins bind with high affinity to glycolipid or glycoprotein receptors present on the cell surface. Binding is followed by endocytosis and intracellular trafficking inside vesicles. Different toxins enter different intracellular routes, but have the common remarkable property of being able to translocate their catalytic subunit across a membrane into the cytosol. Here, a toxin modifies a specific target with ensuing cell alterations, necessary for the survival and diffusion strategies of the toxin producing bacterium.

Key words: Bacterial toxin; Mechanism; Internalization, Membrane translocation; Target

1. Introduction

Many pathogenic bacteria produce protein toxins that are important or essential virulent factors [1–3]. Bacterial protein toxins can be divided in two groups: (a) toxins acting at the plasma membrane level, where they interfere with transmembrane signalling pathways or alter membrane permeability; (b) toxins acting inside cells where they enzymatically modify a specific cytosolic target. This short review will focus on the mechanism of cell penetration of this latter group of toxins.

In several cases the toxin is solely responsible for all clinical symptoms of the corresponding disease. Hence, understanding the mechanism of cell intoxication leads both to the knowledge of the molecular pathogenesis and to the discovery of new aspects of cell physiology. In the case of bacterial toxins, this basic knowledge has resulted in many applications: (a) toxins are powerful tools to study cellular functions [1–3]; (b) several toxins are now used in the treatment of various tumors after coupling to suitable tumor-specific vectors [4]; (c) the botulinum neurotoxins are used in the therapy of a series of dystonias [5].

These toxins are characterized by an overall similar structural and functional architecture. They consist of two disulfide-linked parts: protomer A is endowed with an enzymic activity which is displayed in the cell cytosol, while protomer B is responsible for cell binding and penetration (Fig. 1).

The process of cell intoxication has been traditionally divided into three steps: binding, penetration and target modification. Recent evidence based mainly on diphtheria toxin (DT) and *Pseudomonas aeruginosa* exotoxin A (ETA) and on new developments in the study of cell membrane trafficking indicate that a three-step model is no longer adequate to describe the process. Here, cell intoxication is discussed in terms of a four-step process composed of binding, internalization, membrane translocation and enzymic modification of a cytosolic target.

2. Binding to cells

The clinically active concentrations of toxins are very low [6]. Moreover, some of them are released in a rapidly cleared environment such as the gastrointestinal tract. Hence, they have to bind rapidly and firmly to the cell surface. They do so via two different modes of binding and this corresponds to two different toxin structural organizations (Fig. 1).

Oligomeric B toxins are composed of a pentameric disc-shaped binding protomer with a small central cavity [7–9]. Each B subunit of heat-labile *Escherichia coli* toxin (LT), cholera toxin (CLT) and Shiga toxin (ST), contain a low affinity binding site for the oligosaccharide of a glycolipid molecule [1–3]. These toxins are thought to bind to cells via a first interaction with one glycolipid molecule, rapidly followed, in the two-dimensional plane of the plasma membrane, by encounters with other glyocolipid molecules. The final result is a high-affinity cell association due to a pentavalent binding. Pertussis toxin (PT) acts in the upper respiratory tract and its B protomer has the same oligosaccharide binding fold with the addition of two lateral projections, that are believed to be involved in binding glycoproteins [9].

The catalytic domain A has little protein–protein contacts with B to which is linked via a long α-helical seg-

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Abbreviations: BoNT, botulinum neurotoxin; CLT, cholera toxin; DT, diphtheria toxin; EF, anthrax edema factor; LT, heat-labile *E. coli* toxin; PT, pertussis toxin; ST, Shiga toxin; TeNT, tetanus neurotoxin.

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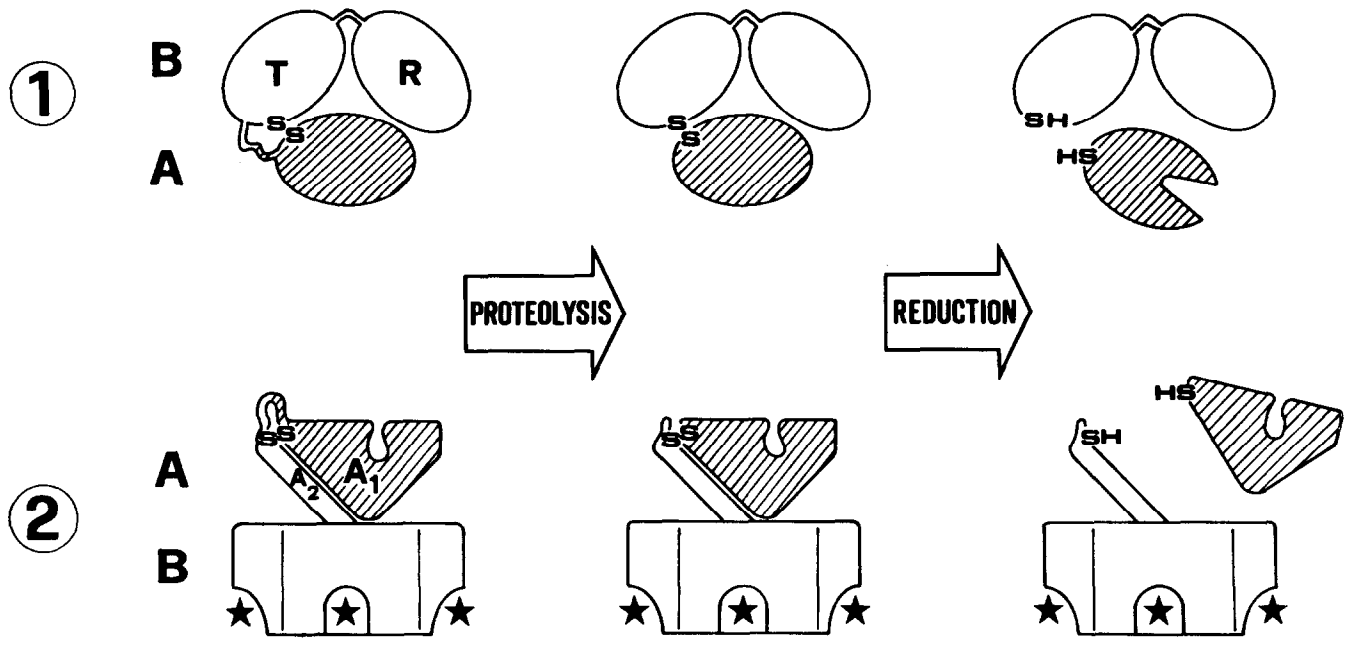

Fig. 1. Schematic structure of bacterial protein toxins with intracellular targets. These toxins are organized in an active protomer A, endowed with catalytic activity, linked via a disulfide bond to a B protomer, responsible for cell binding and penetration. The toxin is synthesized as a single polypeptide chain and cleaved at a single site by proteases. Reduction is required to freed the enzymic activity of A. (1) A group of toxins including diphtheria toxin, exotoxin A, tetanus and botulinum neurotoxins and the anthrax toxic complex are organized in three domains: A is the enzyme part, R is responsible for cell binding and T is involved in the membrane translocation of A (see Fig. 2). (2) Another group of toxins which includes cholera toxin, heat-labile toxin, pertussis toxin and the Shiga toxins are characterized by an oligomeric saccharide binding protomer B. The catalytic subunit A1 is linked via a linker peptide (A2) that extends inside a central pore of oligomer B. There is very little protein–protein contact between A and B. The saccharide binding sites (indicated by a star) are located in the lower part of the B oligomer. At variance from the three-domain toxins, here it is impossible to identify a membrane translocating domain.

ment and a segment which penetrates into the central hole of the B oligomer. Hence, upon cell binding, the A moiety points toward the extracellular medium, dozens of Ångströms away from the membrane surface.

The second type of toxin structure is that of DT and ETA [10,11] with three distinct domains (Fig. 1). This three-domain organization is likely to be shared by tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNT) [12] and by the antrax complex [13]. The B protomer is composed of two parts, endowed with different functions: R, a COOH-terminal receptor binding domain; and T, an amino-terminal domain involved in membrane translocation. The A moiety is linked to B via an exposed peptide loop and by the interchain disulfide bond.

The cell surface receptor of DT and ETA have been recently identified [14,15] as a receptor for a heparin-like growth factor and the A_2-macroglobulin receptor, respectively. Acidic lipids are proposed to play a minor role in the binding but a significant one in membrane translocation (see below).

3. Internalization

While binding does not depend on temperature, the toxin-receptor complex is internalized inside membrane vesicles only at permissive temperatures (i.e. above 10°C) (Fig. 2). This stage is very relevant in the immunotherapy of tetanus or botulism, because, after internalization, the toxin is no longer neutralized by anti-toxin antibodies. The different temperature dependence and accessibility to external ligands operationally distinguish the two initial steps of cell intoxication.

Endocytosis may take place via coated vesicles, as it is the case for DT and ETA, or via non-coated vesicles, as found for CLT and TeNT [16–19]. Only partial information is available on the intracellular routing of these toxins. Morphological studies indicate that ST and ETA undergo a retrograde transport to the TGN, Golgi and ER [20]. The presence in ETA of a sequence similar to the ER-retention sequence may be involved in the recycling of this toxin inside ER [21]. CLT and LT bind to the apical membrane of polarized epithelial cells of the intestine and display their activity versus the adenylate cyclase localized on the basolateral membrane [22]. There is evidence that CLT is transported to the TGN [23]. The intracellular membrane compartment from where they enter the cytosol is not known for any of these toxins.

DT is the most intensively studied of these toxins and the one for which more information is available. After

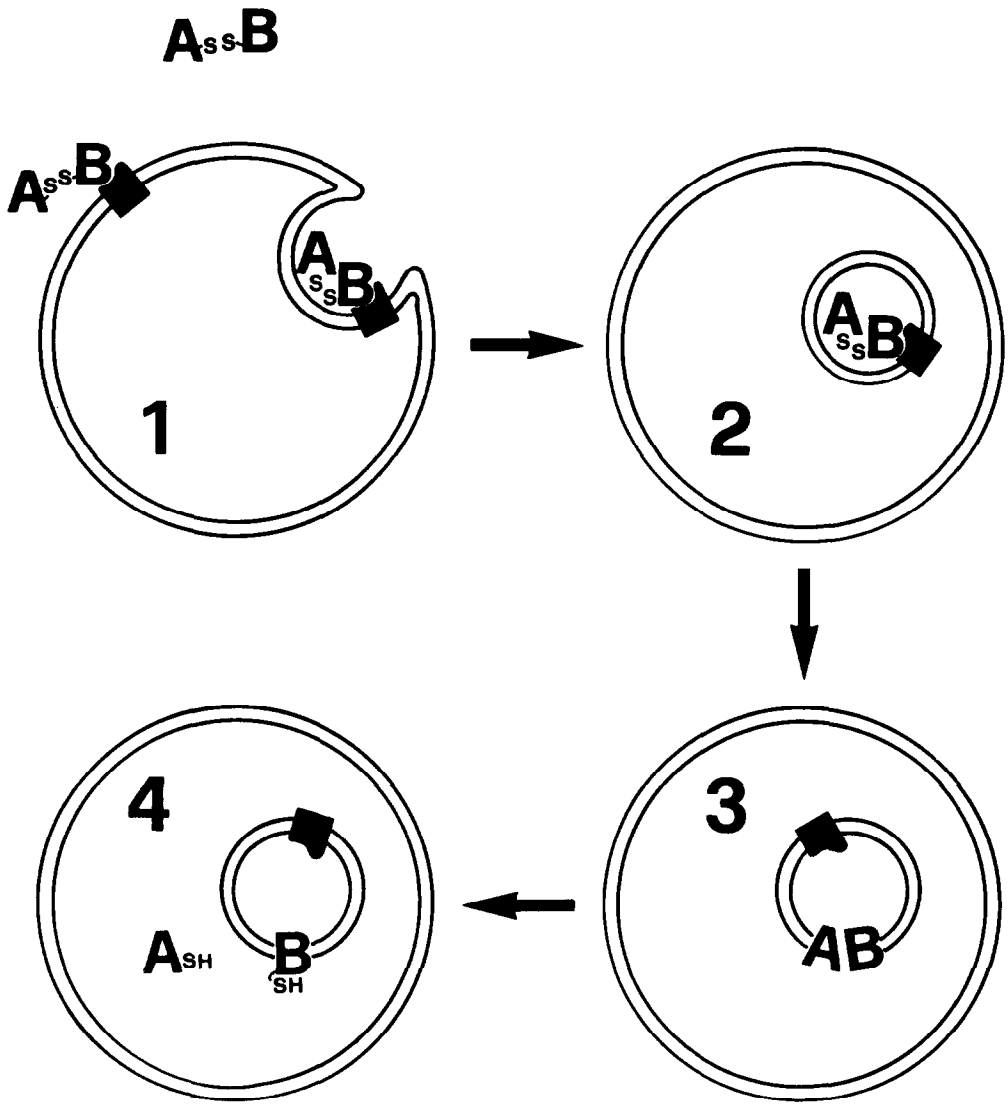

Fig. 2. Schematic picture of the four steps of the mechanism of cell intoxication with A-B type bacterial protein toxins. This process can be sub-divided into four different steps, characterized by a different topological orientation of the toxin with respect to the cell. (1) Cell binding occurs via interaction of protomer B with a lipid and/or protein component of the cell surface. (2) Binding is followed by internalization via endocytosis and intracellular routing. (3) In order to reach the cytosol, the catalytic A subunit of the toxin has to cross the vesicle membrane, a process assisted by the T domain in the three domain toxins. (4) The enzymatic A subunit performs its catalytic activity toward a specific cytosolic target with ensuing cell intoxication.

few minutes upon warming a cell with DT bound onto the surface, DT is present inside early endosomes. About one-third of the internalized DT adopts a state competent to membrane translocation and release of A in the cytosol [24]. The remaining two-thirds are unable to perform such a step (discussed in the next section) and they are degraded inside late endosomes and lysosomes in a process blocked by bafilomycin A1, which inhibits the vacuolar ATPase proton pump and the vesicular transport from early to late endosomes [24–26]. From intoxicated cells, Beaumelle et al. [27] were able to isolate endosomal fractions containing DT. There is indirect evidence that other three-domain toxins such as TeNT and BoNT and the antrax edema (EF) and lethal (LF) factors enter the cell cytosol via an acidic intracellular compartments since cell intoxication is prevented by agents that quench intracellular pH gradients [28–30].

4. Membrane translocation

4.1. Three-domain toxins

Since the targets of these toxins are located in the cytosol (or are membrane-bound and face the cytosol), at least the catalytic A subunit of the toxin has to cross the lipid bilayer (Fig. 2). This is the most obscure passage of the entire intoxication process. Membrane translocation is clearly distinct from internalization because it is

now known that only a minor proportion of the internalized toxin molecules is actually able to translocate the A moiety in the cytosol. In the case of ETA and DT, it was estimated that <5% and <35%, respectively, of internalized toxin enters the cytosol [24,31]. Moreover, after internalization, toxins appear to be able to participate in elaborated vesicular trafficking processes [20,32]. Perhaps the most striking example is provided by the intraneuronal routing of TeNT. Both TeNT and BoNT bind to the presynaptic membrane of motorneurons and are internalized inside vesicles at the neuromuscular junction (NMJ). While BoNT enter the cytosol of the NMJ synapse, TeNT remains inside vesicles that move retroaxonally to the motorneuron cell body and is discharged in the intersynaptic space with inhibitory interneurons of the spinal cord. Here, TeNT is taken up and enters the interneuron cytosol where it blocks release of neurotransmitter [12,32]. The opposite behaviour of TeNT and BoNT at the NMJ clearly indicates that internalization is not necessarily followed by membrane translocation in the cytosol. Moreover, Beaumelle et al. [27] have physically separated internalization from membrane translocation. From cells that had internalized DT, they have isolated DT-containing endosomal fractions and showed that subunit A could be induced to translocated from the endosomal lumen to the external medium upon incubation with ATP, thus providing further evidence to the fundamental role of low pH.

The three-domain toxins appear to cross membranes with a mechanism different from the oligomeric B toxins, and hence they will be treated here separately. A common property of the three-domain toxins (DT, TeNT, BoNT and antrax), but not of the oligomeric B toxins, is their ability to form at low pH ion channels in planar lipid bilayers; this property is associated to the T domain [33–39]. In the case of DT and antrax protective antigen, a monovalent ion channel was shown to form also in living cells [40–43]. This toxin channel has been considered an evidence in favour of the 'tunnel model' for the membrane translocation of the catalytic subunit, first proposed by Boquet et al. [44]. It is suggested that the acid endosomal pH triggers a conformational change of the B subunit that enables it to penetrate the lipid bilayer and form a transmembrane channel large enough to accomodate the A chain in an unfolded form. The catalytic subunit crosses the membrane, protected form contact with lipids, refolds in the neutral pH of the cytosol and leaves behind the protomer B ion channel, after reduction of the A-B disulfide bond. A large number of studies have documented the conformational change of several toxins induced by low pH and the membrane insertion of the hydrophobic 'acid' form [45–47].

Although there are differences among the ion channels formed by the various toxins, the main general properties are summarized in Table 1 and compared with those of the protein conducting channels of the endoplasmic reticulum and *E. coli* bacterial membrane [48,49]. This comparison indicates that a protein with bulky hydrophobic lateral residues is unlikely to pass through such a toxin channel. The 'tunnel model' is also unable to account for the fact that at low pH the catalytic A subunit does enter in contact with the fatty acid chains of phospholipids [50–52].

An alternative view suggests that the toxin channels detected in planar lipid bilayers and cell plasma membranes are actually different from the physical entities that translocate the A chain across the membrane. In other words, the toxin channel is a left-over of step 3: it was involved in the process, but has afterwards changed its structural and electrical properties [45]. This 'cleft model' suggests that the acid endosomal pH triggers a concerted conformational change of the B and the A subunits, that become hydrophobic and penetrate the lipid bilayer. B forms a hydrophilic cleft that drives the insertion of A with its hydrophilic segments interacting with the B cleft and the hydrophobic segments exposed to lipids, thus accomodating bulky aromatic lateral chains. A proper matching of hydrophobic and hydrophilic protein–lipid and protein–protein interactions is needed to reduce the energetic cost of the translocation process. It is possible that the A protomer has a net positive charge at low pH. Due to the high energetic cost of driving charged groups across the hydrophobic core of the lipid bilayer, membrane insertion and translocation would be facilitated by formation of ionic couples with negatively-charged phospholipid head groups. This proposal emphasizes the role of non-bilayer lipidic configurations [53], though direct evidence for such structures during the membrane translocation of toxins is lacking.

Available data are insufficient to draw a picture of protomer B folding in the membrane for any of the known toxins. Most efforts are concentrated on DT and the protein segments of DT embedded in the lipid bilayer at low pH are being identified [54,55]. Parker and Pattus [47] have noticed that membrane inserting toxins possess a hairpin of hydrophobic helices shielded by the other helices of their T domain. They have suggested that acidic pH triggers the exposure of such a hydrophobic

Table 1
Comparison of the properties of toxin ion channels and protein conducting channels of endoplasmic reticulum and bacterial membrane

Propery channel	Toxin channels	Protein-conducting
Conductance	5 – 43 pS	220 pS
Ion selectivity	X^+	non-selective
Size of permanenent ion	<glucosamine	<gluconate
pH dependence	4 – 6	n.d.
Voltage gating	+	–
Voltage dependence of conductance	+	–

References in the text.

hairpin, which inserts into the membrane and forms the nucleus around which the membrane translocating devices of these toxins is organized.

The finding that chain A can escape from liposomes once pH is returned to neutrality [51], provides experimental evidence for the reacquisition of the water-soluble 'neutral' form upon exposure to the neutral cytosolic pH. Chain A can leave the membrane only after reduction of the interchain disulfide bond. For DT, this is the rate-limiting step of the entire cell intoxication process [24]. After chain A has left the membrane, the margins of the hydrophilic cleft embedded in the lipid bilayer come closer to minimize the amount of hydrophilic protein surface exposed to the hydrocarbon chains of lipids. This leaves behind a transmembrane alignment of hydrophilic residues which constitutes a flat-shaped ion channel of reduced size and low conductance. In the 'tunnel' model, the channel that translocates chain A and the monovalent cation channel are the same entity, and formation of the tunnel is a prerequisite for membrane translocation of chain A. In the 'cleft' model, the ion channel is related to the structure that has mediated the translocation of chain A, but has no longer the same size and properties.

4.2. Oligomeric B toxins

The oligomeric B toxins also act on cytosolic targets and face the problem of the membrane translocation of chain A. Low pH does not appear to induce a conformational change and membrane penetration of these toxins. Membrane photolabelling studies [56] and the 3D structure of oligomeric B toxins [7–9,57,58] are only compatible with a mode of binding with the A1 subunit pointing out of the cell surface.

It has been suggested that, upon membrane binding, the B oligomer penetrates in the bilayer and that A1 crosses the membrane inside a tunnel made by the B oligomer [59,60]. However, membrane photolabelling experiments show no penetration of B into the bilayer [56]. In addition, the central pore of oligomer B is too small, particularly in the case of PT [7–9]. Membrane photolabelling and image reconstruction of two-dimensional crystals also showed no lipid interaction of A1. It contacts lipids only after reduction of the A1–A2 disulfide bridge [56]. On this basis, it was proposed that the trigger of the membrane insertion of A1 is the reduction of this interchain S–S bond, that releases a hydrophobic, water-insoluble A1 chain [56]. Hence, it appears that GM1 binding concentrates the toxin on the membrane in such a way that, as soon as the disulfide bridge is reduced, A1 'rolls over' oligomer B and inserts into the membrane. This is at variance with the mechanism of membrane penetration of the three-domain toxins, where protomer B plays an active role in the insertion of the catalytic subunit. The B oligomer merely places A1 near the membrane surface to maximize its insertion into the lipid bilayer. Also for these toxins, neither the chemical nature of the reducing agent nor the intracellular position at which this step takes place are known.

After membrane insertion, the surface distribution of the various amino acid residues is expected to drive the correct orientation of A1 with respect to the cytosolic substrate NAD. Vesicles carrying CLT or LT have finally to fuse with the basolateral membrane of the entherocyte cell to allow contact with their target, the adenylate cyclase complex [58]. Conversely, the A chain of ST acts on the 60 S ribosomal particle and hence has to leave the vesicle and dissolve in the cytosol similarly to the A subunits of DT and ETA.

5. Target modification

This fourth step is the final goal of the overall intoxication proces. Table 2 lists the enzymatic activities displayed inside cells by bacterial toxins. The largest group is that of the ADP-ribosyltransferases: they bind cytosolic NAD^+ and transfer the ADP-ribose moiety to a variety of cytosolic toxin-specific targets. DT and ETA modify specifically elongation factor 2 and block protein synthesis with consequent cell death [59,60]. It has been estimated that a single molecule of A is sufficient to kill a cell [62]. In contrast, cells intoxicated by other ADP-ribosyltransferases such as CLT, LT, and PT do not die, but have an altered physiology due to a large increase in c-AMP levels because these toxins specifically modify G-proteins involved in the control of adenylate cyclase [63]. Cellular effects differ as a function of the type of intoxicated cell.

Other ADP-ribosyltransferases produced by several *Clostridium* spp. direct their action on actin polimerization. The C-2 toxin specifically modifies G-actin, preventing formation of actin filaments [64], while the C-3 enzyme ADP-ribosylates Rho, a protein involved in the control of actin polymerization [65]. As a result, all phenomena depending on a functional contractile apparatus are altered and the cell may eventually die.

The edema factor of the entrax toxin complex (EF) is a calmodulin-dependent adenylate cyclase that causes a rapid rise of c-AMP and cell rounding [13]. Also *Bordetella pertussis* produces an adenylate cyclase toxin (BP-ADC) [66]. The increase of the cellular level c-AMP produced by these two toxins is only transient, because they are rapidly degraded by cellular proteases [13,66].

Yet another kind of activity is displayed by the Shiga toxins (STs). Like several plant toxins named RIPs [67], STs remove a single adenine residue from the 28S ribosomal RNA [68]. This impairs the function of the 60S ribosomal subunit and blocks protein synthesis [69]. The final result is the same as that caused by DT and ETA: cell and tissue necrosis [68].

The most recent addition to the enzyme activities of

Table 2
Enzyme activities and cytosolic targets of bacterial protein toxins with intracellular targets

Toxin	Activity	Target	Effect
DT	ADP-ribosyl-transferase	EF-2	Blockade of protein synthesis and cell death
ETA	ADP-ribosyl-transferase	EF-2	Blockade of protein synthesis and cell death
CLT	ADP-ribosyl-transferase	G_s	Increase c-AMP (alteration of permeability)
LT	ADP-ribosyl-transferase	G_s	Increase c-AMP (alteration of permeability)
PT	ADP-ribosyl-transferase	G_i, Gt	Increase c-AMP (various effects)
C2	ADP-ribosyl-transferase	Actin	Cell rounding and detachment
C3	ADP-ribosyl-transferase	Rho	Cell rounding and detachment
STs	Adenine-glycohydrolase	r-RNA 28S	Blockade of protein synthesis and cell death
EF	Adenylcyclase	None	Increase c-AMP
BP-ADC	Adenylcyclase	None	Increase c-AMP
TeNT	Zinc-proteinase	VAMP	Blockade of exocytosis
BoNT/A	Zinc-proteinase	SNAP-25	Blockade of exocytosis
BoNT/B	Zinc-proteinase	VAMP	Blockade of exocytosis
BoNT/C	Zinc-proteinase	syntaxin	Blockade of exocytosis
BoNT/D	Zinc-proteinase	VAMP	Blockade of exocytosis
BoNT/E	Zinc-proteinase	SNAP-25	Blockade of exocytosis
BoNT/F	Zinc-proteinase	VAMP	Blockade of exocytosis
BoNT/G	Zinc-proteinase	VAMP	Blockade of exocytosis

Abbreviations and references as in the text.

bacterial toxins is that of the clostridial neurotoxins responsible for tetanus and botulism. Their catalytic domains (termed L chain) are zinc-dependent proteinases that specifically attack protein components of the neuroexocytosis apparatus [12,70]. TeNT and serotypes B, D , F and G of BoNT cleave at single different peptide bonds VAMP, a membrane protein of the synaptic vesicle, implicated in the docking and fusion of the vesicles with the presynaptic membrane. Serotypes A, C and E attack SNAP-25 and syntaxin, two proteins of the presynaptic membrane. As a result, the intoxicated neurons remains alive, but the synapses loose functionality and degenerate [71]. This result indicates that VAMP, SNAP-25 and syntaxin play a fundamental role in exocytosis and, at the same time, points to their possible role in neuronal plasticity.

In conclusion, bacterial protein toxins concentrate in one molecule a variety of functions that enable them to bind and penetrate cells and to alter their physiology. The study of their mechanism of action has already revealed important aspects of cell physiology and promises to provide further information useful for the treatment of diseases, for the development of new vaccines and for the understanding of fundamental cellular processes.

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