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# Bacterial protein toxins and cell vesicle trafficking

C. Montecucco[a,*], E. Papini[a] and G. Schiavo[b]

[a]*Centro CNR Biomembrane and Dipartimento di Scienze Biomediche, Università di Padova, Via Trieste 75, I-35121 Padova (Italy), Fax +39 49 8276049, e-mail: toxin@cribi1.bio.unipd.it*
[b]*Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York (New York 10021, USA)*

**Abstract.** A group of bacterial protein toxins interfere with vesicular trafficking inside cells. Clostridial neurotoxins affect mainly the highly regulated fusion of neurotransmitter- and hormone-containing vesicles with the plasma membrane. They cleave the three SNARE proteins: VAMP, SNAP-25 and syntaxin, and this selective proteolysis results in a blockade of exocytosis. The *Helicobacter pylori* cytotoxin is implicated in the pathogenesis of gastroduodenal ulcers. It causes a progressive and extensive vacuolation of cells followed by necrosis, after a cytotoxin-induced alteration of membrane trafficking by late endosomes. Vacuoles originate from this compartment in a rab7-dependent process and swell because they are acidic and accumulate membrane-permeant amines.
**Key words.** Tetanus; botulism; neurotoxins; proteases; exocytosis; endosomes; membrane fusion.

## Introduction

During the course of evolution a limited number of bacteria have 'explored' all animal tissues as environments potentially able to support their growth, proliferation and spread. Some of these bacteria have adopted aggressive strategies which include the modification of the host physiology to their advantage, i.e. to increase their proliferation and/or spread [1]. Such objectives are pursued by a variety of different means. A very popular one is that of producing protein toxins. Hundreds of different toxins have been identified so far. They display a variety of activities: (1) they may alter cells of the host in such a way as to improve bacterial multiplication and/or diffusion; (2) they act against inflammatory or lymphoid cells; or (3) they may fool the immune system (i.e. superantigens) [2, 3].

There are several reasons for studying the mechanism of action of bacterial protein toxins: (1) toxins are the products of the longterm coevolution of the toxin-producing bacteria with the animal host. Hence, each toxic activity has been 'shaped' around key physiological aspects of cells. By studying the mechanism of action of these toxins, we may also learn about fundamental features of cells. (2) The molecular understanding of toxic cell damage leads to a deeper comprehension of the pathogenesis of the disease in which toxins are implicated. (3) In the case of toxins playing a major role as virulence factors, the study of their mode of action provides information for the design of preventive or therapeutic vaccines against the disease.

Our studies have centred around some toxins belonging to the first group, and in particular protein toxins with intracellular targets. All of them have 'chosen' to affect fundamental aspects of cell physiology [2, 3]. Recently we have concentrated on toxins that interfere with different aspects of vesicle traffic inside cells.

* Corresponding author.

## Tetanus and botulinum neurotoxins and exocytosis

These toxins are produced by toxigenic strains of bacteria of the genus *Clostridium* and are released upon bacterial lysis. They are the most poisonous substances known: the mouse LD50 is between 0.1 and 1 ng/kg body weight. Tetanus toxin (TeNT) and botulinum neurotoxins (abbreviated BoNT, seven different types indicated with letters from A to G) are respectively the sole cause of the neuroparalytic syndromes of tetanus and botulism [4–6]. These toxins spread in the organism from the site of entry and damage nerve terminals, which become incapable of releasing neurotransmitters. BoNTs specifically affect the release of acetylcholine, particularly at neuromuscular junctions. TeNT acts on central synapses, particularly on the inhibitory synapses of the Renshaw cells of the spinal cord.

These neurotoxins bind with high specificity to the presynaptic membrane. Notwithstanding a long search begun in the previous century, the receptors of these neurotoxins are not yet known. Available experimental results can be rationalized by implicating both a protein and polysialogangliosides, which are particularly enriched at presynaptic terminals, in the neurospecific binding. Recently, Nishiki et al. [7] have reported evidence that the amino-terminal portion of synaptotagmin, a transmembrane protein of small synaptic vesicles, together with gangliosides binds BoNT/B with high affinity. The receptors of the other neurotoxins are not

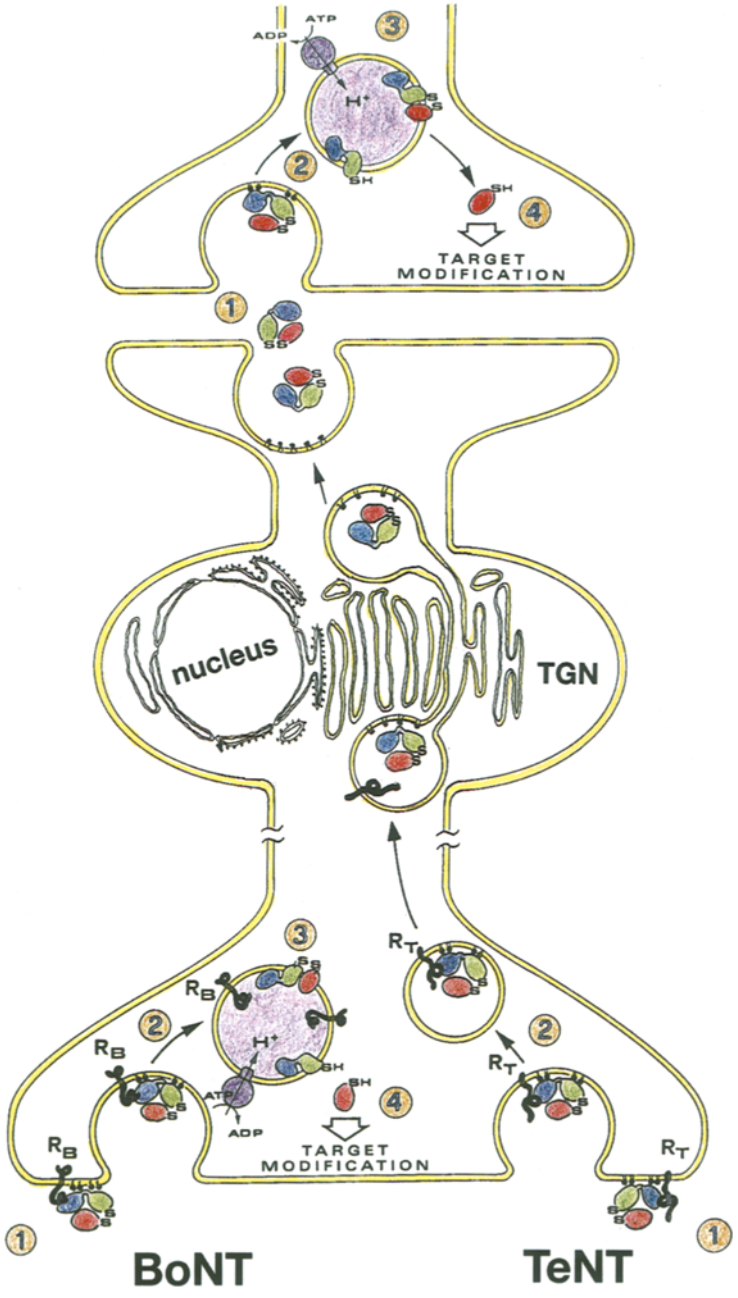


Figure 1. Tetanus and botulinum neurotoxin entry at nerve terminals and cytosolic acitivity. This figure depicts a possible scenario for toxin entry into neurons that is in agreement with available experimental data. (1) BoNTs bind to the presynaptic membrane at the neuromuscular junction and are subsequently internalized inside vesicles (2). The lumen of these vesicles is acidified by a vacuolar ATPase proton pump. At acid pH, BoNTs change conformation, penetrate the lipid bilayer of the vesicle and translocate the L chain into the cytosol (3). Inside the cytosol, L displays a metalloproteolytic activity specific for three protein components of the neuroexocytosis apparatus, termed SNARE proteins (4). BoNT/B, /D, /F and /G attack VAMP, a protein of synaptic vesicles; BoNT/A and /E cut SNAP-25 and BoNT/C cleaves SNAP-25 and syntaxin, two proteins of the cytosolic face of the presynaptic membrane. Unlike BoNT, TeNT binds to the presynaptic membrane and is internalized inside a vesicle, which transports TeNT all along the peripheral motor neuron axon to the cellular body and then releases it into the synapse with Renshaw cells of the spinal cord. Here it is taken up by these inhibitory interneurons, presumably just as BoNT is taken up at the periphery. The result is inhibition of the neuroexocytosis of glycine and GABA. (Reprinted with the permission of Cambridge University, see ref. 6.)

yet known, but it can be anticipated that they have to be essential proteins that cannot be altered by a nerve cell without affecting its viability. Toxin or virus receptors so far identified are proteins acting as growth factors or cytokine receptors, transporters and ion channels, i.e. proteins playing essential roles in cell physiology.

Toxin binding at unmyelinated nerve terminals is followed by endocytosis inside vesicles whose nature has not been characterized [8–10] (fig. 1). However, it is clear that this endocytosis is linked to the electrical activity of the synapse, because exercising animals show toxic damage more rapidly (reviewed in ref. 11). After internalization at the neuromuscular junction of peripheral motor neurons, BoNT and TeNT have different destinies. The former enters the cytosol of these nerve terminals; the latter is transported inside vesicles along the axons up to the spinal cord (see fig. 1) and migrates transsynaptically into inhibitory interneurons [12, 13]. It then enters the cytosol of these cells, as BoNTs do at the periphery. Hence, TeNT and BoNTs follow different pathways of vesicular trafficking inside peripheral motor neurons that lead them to act at different synapses. However, their mode of cell entry appears to be essentially similar.

The third step of toxic neuron damage is the movement of the catalytic light chain of the toxin (chain L, 50 kDa) across the membrane of the vesicle into the cytosol. There is considerable evidence that the heavy chain (chain H, 100 kDa) is mainly responsible for such a process, which is driven by acidification of the vesicular lumen by a vacuolar ATP(adenosine triphosphate)ase proton pump [14, 15]. It is also well documented that these neurotoxins exhibit a low pH-driven conformational change from a 'neutral' water-soluble conformation to a hydrophobic 'acid' conformation [16–27]. This partitions into the lipid bilayer and somehow assists the translocation of the L chain. Several studies have documented the ability of the H chain to form ion channels [16, 19, 21, 24, 25, 27]. While most researchers agree on the possibility that this channel is related to the process of translocation of the L polypeptide chain into the cytosol, there are different views on the mode of translocation: (1) via a pore inside H subunit(s), (2) via a cleft formed by H subunit(s) at the lipid-protein boundary and (3) via disruption of the membrane vesicle (reviewed in ref. 6). On the basis of the results of membrane photolabelling experiments [20, 22], we proposed model (2), and this view is now supported by the finding that the protein translocating pores of the endoplasmic reticulum [28, 29] and of the plasma membrane of prokaryotes [30] are also open laterally to lipids.

Hence, to enter cells these neurotoxins parasitize the endocytic pathway of cellular membrane trafficking. Inside the vesicle, they exploit the physiological acidification of the lumen to change conformation and move across the membrane into the cytosol, where the catalytic chain displays its enzymic activity. The L chains of TeNT and BoNTs are highly specific metalloproteinases.

Among the thousands of neuronal proteins, they appear to recognize solely three proteins that are key members of the neuroexocytosis squadron. Biochemical and genetic evidence shows that the release of neurotransmitters that takes place at the active zones of nerve terminals is mediated by (1) proteins on the membrane of neurotransmitter-containing vesicles (termed v-SNAREs), (2) proteins on the presynaptic membrane (termed t-SNARES) and (3) a set of soluble proteins that are recruited from the cytosol when the vesicle binds to the cytosolic face of the active zone of the plasmalemma (reviewed in refs 31–34). Such multiprotein binding gives rise to a 20S complex that is energized by ATP hydrolysis and triggers the release of neurotransmitters within hundreds of microseconds of the rise in local calcium concentration following the opening of calcium channels governed by membrane potential.

The eight clostridial neurotoxins are very specific for VAMP, a 13-kDa vesicle protein and for two presynaptic membrane proteins, SNAP-25 and syntaxin. These proteins are the only known neurotoxin proteolytic substrates and are cleaved at different peptide bonds by the different neurotoxins. TeNT and BoNT/B, /D, /F and /G recognize and cleave VAMP, thus releasing a large portion of the VAMP molecule into the cytosol [35–40]. The residual membrane-bound VAMP fragment is presumably unable to carry out the function(s) of VAMP, and this leads to inhibition of exocytosis. The role(s) of VAMP is not yet known, but its fundamental importance is apparent from the fact that VAMP is not specific to the small synaptic vesicles of nerve terminals, but is present on a variety of vesicles in almost every cell which has been looked at ([41] and references cited therein). VAMP is proposed to be responsible for addressing the vesicle to the target membrane [31, 34], but this suggestion is not supported by the morphology of TeNT-intoxicated synapses, which show an apparently normal docking of small synaptic vesicles to active zones of the presynaptic membrane [42–44].

BoNT/C cleaves syntaxin near the membrane surface and thus releases most of the molecule into the cytosol, again causing a long-lasting inhibition of neurotransmitter release [45–46]. In contrast, BoNT/A, /C and /E remove only a short COOH-terminal peptide from SNAP-25 [47–53]. This indicates that the carboxyl-terminus of SNAP-25 plays an essential role in neuroexocytosis, one that is not yet understood. The fact that an excitatory toxin such as the spider alpha-latratoxin can overcome the blockade of neuroexocytosis caused by BoNT/A [54] but not that caused by the VAMP-specific toxins indicates that this carboxyl-terminal region of SNAP-25 is not part of the fusion pore itself.

A striking feature of this novel group of zinc-endopeptidases is their specificity. Among the thousands of proteins present in the cytosol, no other substrate has been found in addition to the three SNAREs. Clearly, the segments around the cleavage sites cannot be solely responsible for this recognition, because they differ

from each other [6]. On the other hand, the L chains of these neurotoxins are very similar both in terms of their amino acid sequences [55] and their predicted secondary structures [56]. This suggests that they recognize the tertiary structure of the SNAREs and that the three substrates have a common toxin recognition motif [57]. Indeed, the SNARE proteins share multiple copies of a motif characterized by the presence of three negative charges spaced by hydrophobic residues [58]. The three negatively charged residues appear to be particularly important [59, 60]. Recent evidence indicates that the main determinant of the specific recognition of the three SNARE by the neurotoxins is a double interaction with (a) a segment that includes the peptide bond to be cleaved and (b) another segment closely similar in VAMP, SNAP-25 and syntaxin, which accounts for antibody cross-reactivity and cross-inhibition of the different neurotoxin types. The relative contribution of segments (a) and (b) to the specificity and strength of neurotoxin binding remains to be determined. It also remains to identify other segments that modulate individual interactions of each toxin. In the case of TeNT and BoNT/B it is clear that the cluster of positively charged residues present in VAMP after the cleavage site is important for maximal rate of cleavage [61]. Peptide bond hydrolysis within region (a) leaves the toxin bound to its substrate only via its interaction with (b) and other regions. This is expected to cause a large decrease in binding affinity, which in turn should lead to a rapid release of the hydrolysed substrate.

The physiological role of the SNARE motif is not yet clear. Recent studies from the laboratory of Kelly [62, 63] indicate clearly that it is involved in the regulation of the insertion of VAMP in vesicles. Further studies are necessary to determine the role in SNAP-25 and syntaxin.

In conclusion, the neurotoxins produced by clostridia cause tetanus and botulism following a very elaborate pathway of toxic neuron damage. They exploit the endocytic pathway at presynaptic terminals to enter cells. They then make use of the acidification of the vesicular lumen to change conformation and to translocate the enzymic domain of the toxin into the cytosol. Here, they display a highly specific proteolytic activity towards essential proteins of the neuroexocytosis machinery in such a way as to block the release of neurotransmitters for prolonged periods of time.

## The vacuolating cytotoxin of *Helicobacter pylori*

Fairly recently the field of gastroenterology has been revolutionized by the demonstration that gastroduodenal ulcers have an infectious aetiology. Clear evidence associates prolonged infection of the stomach mucosa with toxigenic strains of *Helicobacter pylori* with the development of atrophic gastritis, gastroduodenal ulcers and stomach adenocarcinoma in humans [64–70]. A major virulence factor produced by *H. pylori* is a cytotoxin, termed vacA, that causes vacuolar degeneration of cells in vitro [71–76]. Oral administration of purified vacA to mice is sufficient to induce degeneration of the gastric mucosa and recruitment of inflammatory cells, two key events in the process that eventually leads to gastric ulcers [77]. *H. pylori* strains with an altered vacA gene are noncytotoxic [74, 75]. This and other evidence implicate vacA in the pathogenesis of human gastroduodenal ulcers [78].

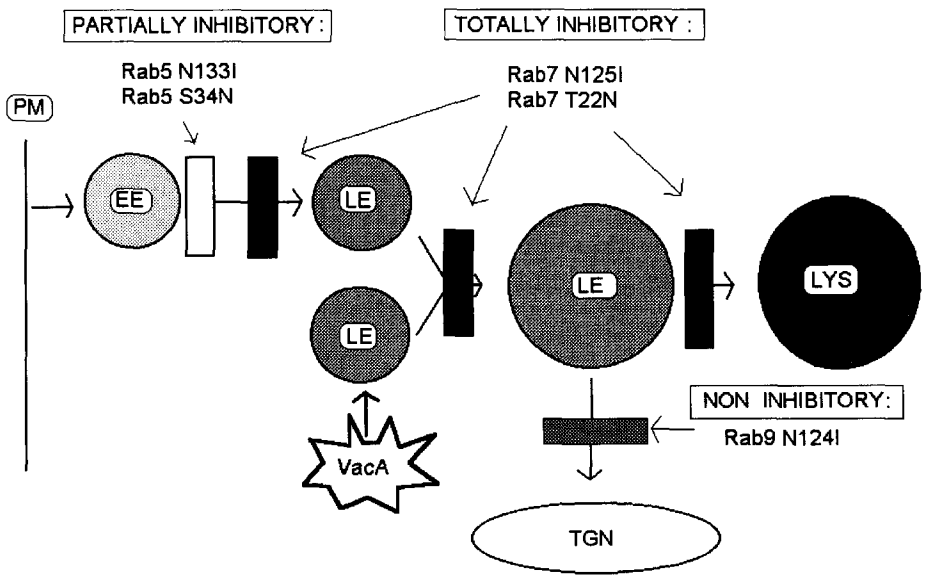


Figure 2. Schematic picture of the endocytic pathway and vacuolation with presumed site of action of the vacuolating cytotoxin of *Helicobacter pylori*. EE = early endosomes; LE = late endosomes; Lys = lysosomes; PM = plasma membrane; TGN = trans Golgi network.

VacA is released as a 95-kDa protein and can be cleaved by bacterial proteinases into two fragments, with no change in biological activity [73]. The carboxyl terminal 58-kDa fragment is able at low pH to increase the permeability of liposomes to monovalent cations [79], as seen above for the clostridial neurotoxins. VacA oligomerizes into heptamers and hexamers, whose structure has been studied by electron microscopy [80]. VacA shows the remarkable property of being activated by short exposures to very acidic pH values and being resistant to pepsin at pH 2 for prolonged periods of time: conditions mimicking the intragastric environment [81].

VacA causes the formation of large vacuoles in cultured cells as well as in vivo [71, 72, 82]. The vacuolar lumen is acidic [83] as a result of the activity of a vacuolar-type ATPase proton pump present on the vacuolar membrane [84, 85]. Vacuoles first appear in the perinuclear area and then grow in size to up to several micrometres of diameter. Such sizes can only be achieved by fusion of several smaller compartments. The toxin could promote fusion directly or it could activate a fusogenic protein. Alternatively, vacA could inhibit directly or indirectly the fission/maturation of a cell vesicular/tubular/cysternal compartment. *H. pylori*-induced vacuoles contain fluid phase markers, and their membrane is highly enriched in rab7 [86], a small GTP-binding protein previously shown to be associated with late endosomal compartments [87]. Rab proteins are known to regulate the extent and specificity of intracellular membrane traffic in eukaryotic cells [88, 89].

These proteins are anchored to specific intracellular compartments by geranyl-geranylation at their C-terminal and cycle between GDP-bound and GTP-bound forms [90]. Similar to what is found for ras protein, point mutations in highly conserved regions can stabilize rab proteins in either the GDP- or the GTP-bound conformation and cause them to act as dominant interfering mutants. Several inactive rab mutants have been generated that show a low affinity binding of both GDP and GTP or a high preference for GDP. Conversely, permanently activated rab mutants can be obtained by stabilization of the GTP-bound form via replacement of an active-site Gln residue with Ile, which blocks intrinsic GTPase activity, or by substitutions which compromise the interaction with GAP, a catalyst of GTP hydrolysis (see ref. 91 for a complete coverage of this subject). The accumulation of rab7 on the vacuole membrane could be an epiphenomenon or it could directly involve this small GTPase protein. To test these possibilities, we have transfected cells with rab7 mutants that are fixed in a permanently active or inactive form. Cells overexpressing active rab7 show a modest increase in cell vacuolization upon exposure to vacA. Conversely, cells transfected with inactive rab7 mutants do not vacuolate. In other words, inactive rab7 mutants display a dominant negative phenotype with respect to vacuolization. Comparison of the effect of overexpression of rab5 and rab9 mutants indicates that vacA affects membrane trafficking at the late endosomal stage, possibly at the level of lysosome formation (fig. 2). This possibility will be tested by studying the effect of vacA on the behaviour of various markers of the different vesicle-trafficking pathways of the cell, in the hope of identifying its target and its mechanism of action. This should contribute to the understanding of the complex events underlying vesicular trafficking in eukaryotic cells and should provide the basis for the use of vacA as a novel tool for further studies, as has been the case for the clostridial neurotoxins. At the same time, an understanding at the molecular level of the activity of vacA will provide the basis for the rational design of vacA mutants to be evaluated as candidate components of an 'antiulcer' therapeutic vaccine.

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