the FEBS Journal
REVIEW ARTICLE

Epsilon toxin: a fascinating pore-forming toxin

Michel R. Popoff

Institut Pasteur, Paris, France

Keywords
aerolysin; *Clostridium perfringens*; *Clostridium septicum* alpha toxin; enterotoxemia; epsilon toxin; glutamate; lipid bilayer; necrosis; pore; pore-forming toxin

Correspondence
M. R. Popoff, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France
Fax: +33 1 40613123
Tel: +33 1 45688307
E-mail: mpopoff@pasteur.fr

(Received 18 February 2011, revised 11 April 2011, accepted 18 April 2011)

doi:10.1111/j.1742-4658.2011.08145.x

Epsilon toxin (ETX) is produced by strains of *Clostridium perfringens* classified as type B or type D. ETX belongs to the heptameric β-pore-forming toxins including aerolysin and *Clostridium septicum* alpha toxin, which are characterized by the formation of a pore through the plasma membrane of eukaryotic cells consisting in a β-barrel of 14 amphipatic β strands. By contrast to aerolysin and *C. septicum* alpha toxin, ETX is a much more potent toxin and is responsible for enterotoxemia in animals, mainly sheep. ETX induces perivascular edema in various tissues and accumulates in particular in the kidneys and brain, where it causes edema and necrotic lesions. ETX is able to pass through the blood–brain barrier and stimulate the release of glutamate, which accounts for the symptoms of nervous excitation observed in animal enterotoxemia. At the cellular level, ETX causes rapid swelling followed by cell death involving necrosis. The precise mode of action of ETX remains to be determined. ETX is a powerful toxin, however, it also represents a unique tool with which to vehicle drugs into the central nervous system or target glutamatergic neurons.

Introduction

Clostridium perfringens is a Gram-positive, rod-shaped, anaerobic and sporulating bacterium, which produces the largest number of toxins of any bacteria. It is a proteolytic and glucidolytic *Clostridium* that grows rapidly in complex medium. According to the main lethal toxins (alpha, beta, epsilon and iota), *C. perfringens* is divided into five toxinotypes (A–E). Epsilon toxin (ETX) is synthesized by toxinotypes B and D. However, the large diversity of toxin combinations that can be produced by *C. perfringens* strains makes classification into five toxinotypes more complex [1].

Based on the toxins produced, *C. perfringens* is responsible for diverse pathologies in humans and animals, resulting from gastrointestinal or wound contamination and including food poisoning, enteritis, necrotic enteritis, enterotoxemia, gangrene and puerperal septicemia. Toxinotype B is the causative agent of lamb dysenteria, which is found only in some countries, for example the UK, whereas toxinotype D is responsible for enterotoxemia, a fatal, economically important disease of sheep found worldwide. ETX contributes with beta toxin to the pathogenesis of toxinotype B, and it is the causative virulence factor of all symptoms and lesions caused by toxinotype D. ETX is one of the most potent toxins known. Its lethal activity ranges just below the botulinum neurotoxins. Indeed, the lethal dose by intraperitoneal injection in mice is 1.2 $ng{\cdot}kg^{-1}$ for botulinum neurotoxin A and 70 $ng{\cdot}kg^{-1}$ for ETX [2,3]. For this reason, ETX is considered to be a potential biological weapon, classified as a category B biological agent, although very few ETX-mediated natural diseases have been reported in humans [4]. ETX belongs to the family of aerolysin pore-forming toxins, however, its

Abbreviations
ETX, epsilon toxin; MDCK, Madin–Darby canine kidney; PFT, pore-forming toxin.

precise mode of action, accounting for its high potency, remains to be defined [5]. This review focuses on recent advances in the study of ETX, a fascinating compund that shares basic pore-forming activity with other toxins, but which develops a much more potent lethal activity.

Enterotoxemia

Enterotoxemia is characterized by high levels of toxin production in the intestine, this toxin then passes through the intestinal barrier and disseminates via the circulation (toxemia) to several organs, causing toxic shock and death. The natural habitat of *C. perfringens* type D, like the other toxinotypes, is the environment: soil, dust, sediment, cadavers, litter and also the digestive tract of healthy animals. *Clostridium perfringens* is not a usual inhabitant of the digestive tract, however, it can be found in low numbers ($< 10^3$ bacteria·g^{-1}) in the intestines of animals without associated pathology [6,7].

High production of ETX in the intestine and subsequent disease are conditioned by an overgrowth of ETX-producing *C. perfringens* ($> 10^6$ bacteria·g^{-1}, usually 10^8–10^9 bacteria·g^{-1}) in the intestinal content, essentially in the small intestine. Rapid multiplication of *C. perfringens* can occur in the digestive tract of very young animals in which the resident intestinal microflora, which is inhibitory of *C. perfringens* colonization, is not yet developed or is not yet functional. This is the case in lamb dysenteria due to *C. perfringens* type B, which occurs only during the first days of life. Overeating a highly concentrated ration or a rapid change to a rich diet such as one high in cereal, young cereal crops or abundant and luxuriant pasture is a common cause of enterotoxemia in older lambs and sheep. Such alimentary conditions induce a perturbation in the microbial balance in the gut and massive passage into the small intestine of undigested fermentable carbohydrates, like starch, which are normally metabolized in the rumen and are an excellent substrate for *C. perfringens* growth. In addition, any cause of intestinal stasis contributes to the accumulation of *C. perfringens* and ETX in the intestinal loops.

Clostridium perfringens type D enterotoxemia is very common in lambs, less frequent in sheep and goats, and occasional in other animal species. Rapidly growing lambs are most susceptible. This raises the question, what are the host intestinal conditions permitting selective *C. perfringens* type D overgrowth in the digestive tract of susceptible animals compared with more resistant animal species?

Clostridium perfringens type D enterotoxemia, also called pulpy kidney disease in lambs, is rapidly fatal. The peracute clinical form is characterized by sudden death without premonitory signs. In the acute form, which is very rapid (few minutes to several hours, no more than 12 h), excitatory type neurological symptoms are predominant and include violent convulsions, opisthotonos, struggling, nystagmus, bruxism, ataxia and then lateral recumbency, violent movements of paddling, ptyalism, hyperthermia and coma. Sheep usually develop a more chronic form, also called focal symmetrical encephalomalacia, Diarrhea might be observed in addition to neurological signs in animals surviving for a few days. By contrast to sheep, fibrotic and hemorrhagic enterocolitis in the absence of cerebral lesions is more common in goats [8–10].

In the peracute form, only a few lesions can be observed, for example, microscopic brain lesions, edema and petechia in various organs including pericardial effusions, subendothelial ecchymoses and occasionally pulmonary and pleural effusions. Macroscopic brain lesions are more evident in animals with a longer duration of the disease and consist of symmetrical foci of hemorrhagic or gelatinous softening in the corpus striatum, thalamus and midbrain cerebellar peduncles. Lambs dying from enterotoxemia show characteristic modifications of the kidneys. Just after death, the kidneys are swollen or congestive, but they autolyse more rapidly than normal, with the cortical parenchyma being totally liquefied. In addition, hyperglycemia and glucosuria are frequently found [11–15].

ETX and human disease

The dramatic diseases induced by *C. perfringens* ETX in certain animal species raise the question of whether humans might also be a target of this toxin? Primary human renal tubular epithelial cells and the human kidney cell line G-402 are sensitive to ETX, albeit to a lesser extent than the highly sensitive dog kidney cell line, Madin–Darby canine kidney (MDCK) cells [16,17], suggesting that humans might be susceptible to ETX. However, *C. perfringens* type D disease is extremely rare in humans, even in farmers or others who come into contact with diseased animals or their environment. Two reports mention a *C. perfringens* type D infection in humans. One concerned a person with acute intestinal obstruction and subsequent development of *C. perfringens* type D and production of ETX in the intestine. In this case, a portion of the ileum was gangrenous and blood-stained fluid was present in the peritoneal cavity [18]. A second case, hospitalized for treatment of ankylosing spondylitis, developed abundant diarrhea and

abdominal pain. *Clostridium perfringens* type D was isolated from stool and antibodies against ETX were evidenced in the serum [19].

ETX genetics

Clostridium perfringens contains a single circular chromosome, which shows some degree of diversity between strains, with those of gastrointestinal origin harboring a large number of mobile elements probably acquired by horizontal transfer in the digestive ecosystem [20]. The ETX gene is located on large plasmids in *C. perfringens*, like the other main toxin genes (beta and iota), which are used for *C. perfringens* typing. This accounts for the large genetic diversity in *C. perfringens* strains, because plasmids can be acquired, rearranged or lost. Thus, a *C. perfringens* strain can change from one toxinotype to another by acquisition or loss of a toxigenic plasmid. The ETX gene is harbored by diverse plasmids [21]; at least five (48–110 kbp) in *C. perfringens* type D [22] and a 65-kbp plasmid in *C. perfringens* type B have been described [23]. The same strain can contain several toxin plasmids. Indeed, most *C. perfringens* type B strains carry a 65-kbp ETX plasmid, a 90-kbp beta toxin plasmid and a third plasmid with the lambda protease gene; some *C. perfringens* type D strains contain a ETX plasmid and a smaller one with a Beta2 toxin gene [22,23]. By contrast, a single plasmid can harbor several toxin genes. For example, some plasmids from *C. perfringens* type D can encode three toxins, ETX, *C. perfringens* enterotoxin and Beta2 toxin [22]. Additional genes located on these large plasmids encoding for specific metabolic pathways or potential virulence factors such as collagen adhesin or sortase might be responsible for the adaptation of distinct *C. perfringens* toxinotypes to specific ecological niches, for example, *C. perfringens* type D in the digestive tract of ruminants and more specifically sheep. Moreover, plasmids in *C. perfringens* types B and D contain insertion sequences that can mobilize toxin genes between different plasmids, between plasmid and chromosome or *vice versa*, as for the *C. perfringens* enterotoxin gene [22–25]. Plasmids carrying the ETX gene in *C. perfringens* types B and D have probably evolved from a common ancestor by insertion of mobile genetic elements [26]. In addition, plasmids with an ETX gene from *C. perfringens* type D contain the *tcp* conjugative locus, are conjugative and can be transferred into other *C. perfringens* strains such as *C. perfringens* type A [21]. Thus, the horizontal transfer of toxin plasmids contributes to the genetic complexity and plasticity of *C. perfringens* toxinotypes.

ETX production

ETX is synthesized during the exponential growth phase of *C. perfringens* as a single protein containing a signal peptide (32 N-terminal amino acids). The secreted protein (32 981 Da) is poorly active and is called a prototoxin [27]. The prototoxin is activated by proteases such as trypsin, α-chymotrypsin and λ-protease, which are produced by *C. perfringens*. Activation by λ-protease is comparable with that obtained with trypsin plus α-chymotrypsin. The λ-protease removes 11 N-terminal and 29 C-terminal residues, whereas trypsin plus α-chymotrypsin cleaves 13 N-terminal residues and the same 29 C-terminal amino acids. This results in a reduction in size (28.6 kDa) and an important decrease in the p*I* value from 8.02 to 5.36, probably accompanied by a conformational change. The charged C-terminal residues might prevent interaction of the protein with its substrate or receptor [3].

Intestinal absorption of ETX and dissemination through the blood circulation

Sheep can support ETX accumulation (10^2–10^3 mouse lethal dose·mL^{-1}) in the intestine without associated symptoms for a few hours. The high ETX concentration then induces an increase in the permeability of the intestinal mucosa, mediating the passage of toxin into the blood (Fig. 1) [7,28–31]. In experimental mice and rat intestinal loops, ETX at a concentration of 10^3 mouse lethal dose·mL^{-1} and higher causes an accumulation of fluid in the intestinal lumen, a decrease in transepithelial electrical resistance and an increase in the passage of macromolecules across the intestinal barrier [32–34]. The absence of histological and ultrastructural changes in the intestinal epithelium suggests increased passage through the paracellular pathway. The only lesions observed are paravascular edema and apoptotic cells in the lamina propia [33]. Subsequent ETX absorption into the general circulation occurs from the small and large intestine, but not from the stomach in mice [35]. However, the precise mechanism of ETX-dependent increased permeability of the intestinal barrier remains to be defined.

ETX also influences gastrointestinal motility. Contradictory results have been obtained in experimental animal models. ETX was found to cause contraction of isolated rat ileum as a consequence of an indirect ETX action via the nervous system [36]. However, ETX administered orally or intravenously in mice reduces gastrointestinal transit via an as yet undefined mechanism [37]. Inhibition of gastrointestinal motility

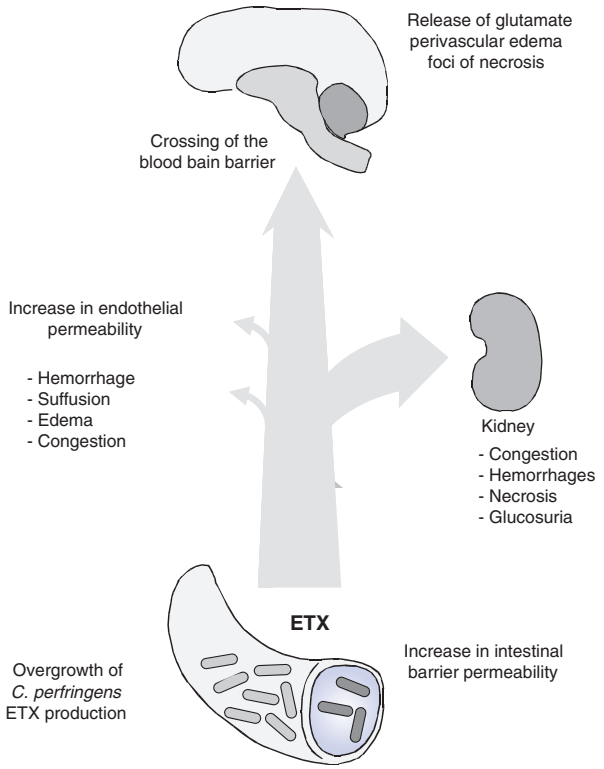

Fig. 1. Schematic representation of epsilon toxin (ETX) distribution in the organism during a *Clostridium perfringens* enterotoxemia and main pathological effects.

is a risk factor for bacterial overgrowth and toxin accumulation in the intestinal lumen.

Edema and petechias, which have been observed in various tissues from naturally or experimentally intoxicated animals, indicate that ETX targets endothelial cells and alters the integrity of the vascular barrier. It has been evidenced that ETX efficiently increases the vascular permeability of rat mesentery microvessels [38] or skin vessels after intradermal ETX injection [39]. These effects seem to result from a direct interaction of ETX with endothelial cells and not from an indirect signaling cascade, such as an inflammatory response induced by ETX [38]. Fluorescent ETX injected intravenously in mice binds to the luminal surface of the endothelium of most blood vessels [39,40]. The observation of necrotic cells and gaps in endothelium indicates that ETX modifies the integrity of the endothelial barrier via the destruction of cells rather than disassembly of the intercellular junctions [38]. However, endothelial cell lines from various animal species responsive to *C. perfringens* type D enterotoxemia are not sensitive to ETX [41]. It is possible that cultured cell lines have lost the specific ETX receptor of primary endothelial cells. Moreover, ETX has been reported to increase blood pressure subsequent to vessel contraction in skin via an as yet undefined mechanism, possibly including increased membrane permeability to Na^+ and the release of noradrenaline from adrenergic nerve terminals [42,43]. Targeting of endothelial cells and increased endothelial barrier permeability seem to be among the major ETX effects in susceptible animal species. The mechanism and importance of hemodynamic alteration remain to be defined.

ETX and kidney disorders

Rapid post-mortem autolysis of kidneys is characteristic of lamb enterotoxemia (pulpy kidney disease) and is less evident in sheep and other animal species. At the time of death in lambs intoxicated with ETX, only a variable degree of congestion is observable in the kidneys. At 2 h, and to a more pronounced extent at 4 h post mortem, kidneys show interstitial hemorrhage between tubules and degeneration of the proximal tubule epithelium [11]. Similar findings are observed in mice, which show congestion and hemorrhage in the medulla, as well as severe degeneration of the distal tubule epithelium [40]. ETX binds specifically to the basolateral side of distal tubule epithelial cells, in agreement with the degenerative effects in this epithelium, and also to the luminal surface of proximal tubules, although in a nonspecific manner, indicating filtration of the toxin by the glomerules [40,44]. Interestingly, nephrectomy shortens the time to death in mice injected intravenously with ETX, suggesting that the kidneys play a protective role by trapping the toxin from the circulation and eliminating it from the organism [44]. In addition, only a few cultured cell lines are sensitive to ETX *in vitro*, and these are from kidneys, for example MDCK, the murine renal cortical collecting duct principal cell line ($mpkCCD_{c14}$), and to a lesser extent the renal cell line from human kidney, G-402 [16,45–47], indicating that the kidney is one of the main target organs for ETX. In addition, kidney alteration is also involved in glucosuria, which is observed in lamb enterotoxemia, However, glucosuria results mostly from an hyperglycemic response which is probably mediated by the mobilization of hepatic glycogen subsequent to ETX-dependent vascular endothelial damage [48].

ETX and brain disorders

The brain is the second organ, after the kidneys, where ETX accumulates massively (Fig. 1). By contrast to kidneys, ETX binds to brain in an exclusively specific

manner and with high affinity (nM range) [49,50]. This indicates that ETX passes the blood–brain barrier and recognizes specific cells or sites in the brain. Indeed, ETX has been shown to alter the integrity of the blood–brain barrier, permitting not only its own passage, but also that of macromolecules such as horseradish peroxidase or serum albumin [49,51–55]. Marked extravasation of serum albumin into the brain has been evidenced in lambs or rats intoxicated with ETX [52,56], and extravasation of horseradish peroxidase, as measured in mouse brain after intravenous injection of less than one lethal dose, is extremely rapid ($\sim$ 20 min) [53,55]. Such a rapid decrease in blood–brain barrier permeability facilitates rapid ETX accumulation in the brain. However, the ETX mechanism of blood–brain barrier perturbation is not yet fully understood. In its prototoxin form, ETX binds to brain endothelial cells and induces decreased expression of the endothelial barrier antigen, which is a specific marker of central nervous system barrier vessels. The ETX-dependent reduction in endothelial barrier antigen production in brain endothelial cells by an as yet undefined pathway is accompanied by a rapid, but mild, increase in blood–brain barrier permeability [54]. Impairment of endothelial barrier antigen expression might be an early effect of ETX on the blood–brain barrier. Endothelial cells then show macroscopic alterations including swelling, abundance of clear vacuoles and loss of intracellular organelles, as well as protrusions or blebbing of the luminal surface. Later, their cytoplasm is very thin and the nuclei pyknotic, leading to a very attenuated capillary endothelium [57–59].

In mice, ETX causes bilaterally symmetrical lesions in several brain areas including cerebral cortex, corpus striatum, vestibular area, corpus callosum, lateral ventricles and cerebellum, whereas in lambs or sheep, more restricted areas are invloved, such as basal ganglia, thalamus, subcortical white matter, substantia nigra, hippocampus and cerebellar peduncles [57,58]. The most early and prominent lesions consist of perivascular edema, and these lesions have been described in various animal species including mice [51,55,60], rats [56,61,62], sheep [39,63–65] and calves [66]. Widening of the perivascular space is the main early change and is observed as soon as 1 h after intraperitoneal injection of a sublethal dose in mice. Perivascular edema then progresses, leading to stenosis of the capillary lumen [60]. Perivascular edema is mainly distributed in white matter and is accompanied by swelling of the perivascular astrocytic cells [54,57], predominantly in the cerebellum [11,57]. Swelling is also observed in axon terminals and dendrites, and the myelin sheath is distended by edema [59]. Some neuronal damage occurs, and consists of swelling, vacuolation and necrosis, mainly in neurons from certain brainstem nuclei, or of cell shrinkage with hyperchromatosis and nuclear pyknosis, most commonly in the cerebral cortex, hippocampus and thalamus [56]. A consequence of brain vasogenic edema is an overexpression of aquaporin-4 (the most abundant water channel in the central nervous system involved in water homeostasis), mainly in astrocytic cells. Upregulation of aquaporin-4 represents a host response in an attempt to resolve the ETX-induced edema [62].

In the subacute and chronic forms of the disease, the brain lesions ultimately change in foci of necrosis and hemorrhage. Two pathways might account for the generation of necrotic lesions. (a) Impairment of the blood–brain vessels leads to vasogenic edema and reduced perfusion of the tissues and therefore to tissue hypoxia and necrosis. (b) Alternatively, ETX, which diffuses in the brain parenchyma, can directly damage neurons and other cell types. This does not preclude that a combination of both processes might be involved. However, the cells directly targeted by ETX remain to be determined.

Using fluorescent ETX injected intravenously, it has been confirmed that both activated and precursor toxin forms bind to the luminal surface of brain vascular endothelial cells [67,68]. In addition, active fluorescent ETX, but not the prototoxin, passes through the blood–brain barrier and accumulates in brain tissue, preferentially in cerebellum, cerebellar peduncles, cerebral white matter, hippocampus, thalamus, corpus striatum, olfactive bulb and colliculi [67,68]. These areas of ETX diffusion correspond to regions of the brain that have been already identified as the main sites of ETX-induced histological changes, supporting a direct action of the toxin on neuronal cells and/or other brain cells. Fluorescent ETX binds *in vitro* to the myelin structure of the central and peripheral nervous systems from mice, sheep, cattle and even humans [68,69]. However, myelin does not seem to be the primary target of ETX because intravenously injected toxin in mice does not show a correlation between the ETX staining pattern and myelin-containing structures [69]. Moreover, it has been found that fluorescent ETX binds to only a subset of astrocytes and microglia cells and is cytotoxic for these cells [67]. However, the pathological significance of ETX on astrocytes and microglia cells is not known. A more detailed analysis of ETX binding to mouse cerebellum has identified granule cells and oligodendrocytes, but not Purkinje cells and astrocytes, as ETX target cells [68]. ETX binding to myelin probably accounts for ETX staining of oligo-

dendrocytes, which are involved in myelin synthesis in contrast to astrocytes, which participate in blood–brain barrier function, regulation of local pH and electrolytes, and probably in the recapture of neurotransmitter. ETX stains brain white matter, which is enriched in myelinated axons and oligodendrocyte cell bodies. It is noteworthy that ETX binds to the cell body of granule cells or other target cells, but not to cell axons or nerve terminals [68,69], suggesting a specific ETX interaction with a cell body membrane receptor.

ETX stimulates glutamate release

A direct and rapid ETX effect in brain concerns the stimulation of glutamate release. First, it was found that ETX injected at a low dose in rats rapidly (4 h) induces neuronal damage, characterized by cell shrinking, vacuolation and nucleus pyknosis, mainly in the hippocampus and cortex. These effects were not accompanied by perivascular edema or a reduction in blood flow in the hippocampus and were specifically inhibited by glutamate receptor inhibitors, indicating that ETX interferes directly with glutamatergic neurons [70]. It was then shown that ETX increases glutamate release from mouse hippocampus and not from other brain areas [71]. Thereby, ETX seems to target specifically glutamatergic neurons stimulating the release of glutamate and then inducing cell alteration (shrinkage, pyknosis).

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system. Its excessive release is probably the main cause of the neurological symptoms of excitation, which are observed in ETX-dependent enterotoxemia. The precise mechanism by which ETX stimulates glutamate release is not yet fully understood. Because ETX incubation with a synaptosomal fraction containing nerve terminals did not elicit glutamate release, ETX probably interacts directly with the cell bodies of neurons or other cell types in the nervous tissue to induce its effects on neurotransmitter release [69]. Indeed, it has been confirmed that in mouse cerebellum, in addition to oligodendrocytes, ETX binds only to the somata of granule cells, which are glutamatergic neurons, and not to nerve terminals, neuronal extensions or other neuronal cell types such as GABAergic Purkinje cells [68]. Alterations in blood circulation in the brain, such as ischemia, are among the factors that stimulate the glutamate release. However, no modification of cerebral blood flow was observed during the period of ETX-dependent stimulation of glutamate release [71], again indicating direct activity of ETX on neuronal or glial cells. This is further supported by the fact that ETX induces glutamate release from primary and cultured cerebellar granule cells, although it can not be ruled out that oligodendrocytes, which are targeted by ETX, are not also involved. Moreover, as measured by patch clamp, ETX triggers membrane depolarization leading to a decrease in membrane electrical resistance and an increase in intracellular Ca^{2+}. Does ETX mediate glutamate efflux by pore formation through the membrane, plasma membrane disruption or stimulation of the neuroexocytosis machinery? The observation that the absence of extracellular Ca^{2+} or methyl β-cyclodextrine, which sequesters membrane cholesterol and impairs ETX pore activity, prevents the ETX-dependent intracellular Ca^{2+} increase and glutamate release, argues for ETX activity on the release machinery of glutamate [68]. Although the most direct and prominent effect of ETX on brain is the stimulation of glutamate release, the toxin also induces the release of other neurotransmitters such as dopamine [72].

ETX structure

At the amino acid sequence level, ETX shows some homology with the *Bacillus sphaericus* mosquitocidal toxins Mtx2 and Mtx3, with 26% and 23% sequence identity, respectively [27]. Mtx2 and Mtx3 are specific for mosquito larvae, which are activated by proteolytic cleavage and probably act by pore formation [73]. In addition, a hypothetical protein encoded by a gene located in the vicinity of the C2 toxin genes on a large plasmid in *Clostridium botulinum* type D shows a sequence similarity with that of ETX [74].

ETX retains an elongated form and contains three domains that are mainly composed of β sheets [75] (Fig. 2). Despite poor sequence identity (14%), the overall structure of ETX is significantly related to that of the pore-forming toxin aerolysin produced by *Aeromonas* species [76,77], and to the model of alpha toxin from *C. septicum*, an agent of gangrene [78]. However, ETX is a much more potent toxin with 100 × more lethal activity in mouse than aerolysin and *C. septicum* alpha toxin [3,76,79]. The main difference between the toxins is that the aerolysin domain I, which is involved in the initial interaction of the toxin with cells, is missing in ETX. Domain 1 of ETX consists of a large α helix followed by a loop and three short α helices, and is similar to domain 2 of aerolysin, which interacts with the glucosyl phosphatidylinositol anchors of proteins. This domain of ETX might have a similar function of binding to receptor. A cluster of aromatic residues (Tyr49, Tyr43, Tyr42, Tyr209 and Phe212) in ETX domain 1 could be involved in receptor binding

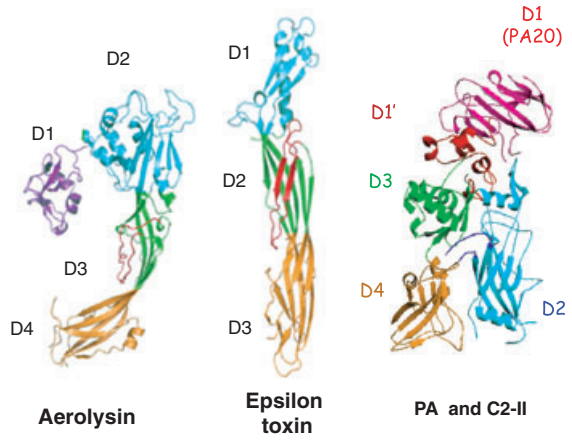

Fig. 2. Structural comparison of aerolysin, epsilon toxin and binding components of anthrax toxins (PA) or *Clostridium botulinum* C2 toxin (C2-II). The pore-forming domain β strands in aerolysin and epsilon toxin are in red.

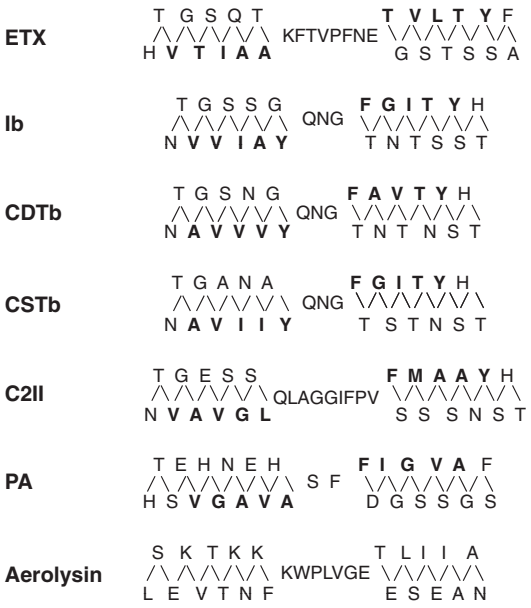

Fig. 3. Sequence alignments of the pore forming domains of epsilon toxin (ETX), binding components of the clostridial binary toxins [*Clostridium perfringens* Iota toxin (Ib), *Clostridium difficile* transferase (CDTb), *Clostridium spiroforme* toxin (CSTb), *Clostridium botulinum* C2 toxin (C2-II)] and aerolysin.

[75]. Domain 2 is a β-sandwich structurally related to domain 3 of aerolysin. This domain contains a two-stranded sheet with an amphipatic sequence predicted to be the channel-forming domain (see below). In contrast to the cholesterol-dependent cytolysins, only one amphipathic β-hairpin from each monomer is involved in the pore structure of ETX and other heptameric β-pore-forming toxins like aerolysin. Domain 3 is also a β-sandwich analogous to domain 4 of aerolysin and contains the cleavage site for toxin activation. After removal of the C-terminus, domain 3 is likely involved in the monomer–monomer interaction required for oligomerization [5,75].

The pore-forming domain has been identified in domain 2. The segment His151–Ala181 contains alternate hydrophobic–hydrophilic residues, which are characteristic of membrane-spanning β-hairpins, and forms two amphipatic β strands on ETX structure. Site-directed mutagenesis confirmed that this segment is involved in ETX channel activity in lipid bilayers [80]. Interestingly, the ETX pore-forming domain shows higher sequence similarity to those of the binding components (Ib, C2-II, CDTb, CSTb) of clostridial binary toxins (Iota toxin, C2 toxin, *C. difficile* transferase, *C. spiroforme* toxin, respectively), and to a lesser extent to *Bacillus anthracis* protective antigen (the binding component of anthrax toxins), than with that of aerolysin (Fig. 3). However, the ETX segment Lys162–Glu169, which is exposed to the transmembrane side of the channel and forms the loop linking the two β strands forming the transmembrane β-hairpin, is unrelated at the amino acid sequence level to those of other beta-pore-forming toxins (β-PFTs). The ETX loop is flanked by two charged residues, Lys162 and Glu169, and contains a proline in the central part, similar to the sequence of the corresponding aerolysin loop. Binding components share a similar structure organization with that of β-PFTs and notably contain an amphipathic flexible loop that forms a β-hairpin, playing a central role in pore formation [81,82]. This suggests that binding components and β-PFTs have evolved from a common ancestor. However, β-PFTs have acquired a specific function consisting in the translocation of the corresponding enzymatic components of binary toxins through the membrane of endosomes at acidic pH. By contrast, β-PFTs such as ETX and aerolysin can form pores in plasma membrane at neutral pH, which are responsible for cytotoxicity.

Essential amino acids for the lethal activity have been identified using biochemistry and mutagenesis. A previous study with chemical modifications shows that His residues are required for the active site, and Trp and Tyr residues are necessary for the binding to target cells [83]. The molecule contains a unique Trp and two His. Amino acid substitutions showed that His106 is important for the biological activity, whereas His149 and Trp190 probably are involved to maintain the structure of ETX, but they are not essential for the activity [84].

Molecular and cellular mechanism of action

Specific activity of ETX is also observed in cultured cells. Only very few cell lines including renal cell lines from various species such as MDCK, mpkCCD$_{cl4}$ and to a lesser extent the human leiomyoblastoma (G-402) cells, are sensitive to ETX [16,47]. Surprisingly, kidney cell lines from ETX-susceptible animal species like lamb and cattle are ETX resistant, suggesting that the ETX receptor in primary cells is lost in cultured cell lines ([47] and unpublished).

A marked swelling is observed in the first phase of intoxication, followed by mitochondrial disappearance, blebbing and membrane disruption. The cytotoxicity can be monitored using an indicator of lysosomal integrity (neutral red) or mitochondrial integrity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [16,46,47,85–87].

ETX binds to the MDCK cell surface, preferentially to the apical site, and recognizes a specific membrane receptor, which is not present in insensitive cells. Binding of the toxin to its receptor leads to the formation of large membrane complexes which are very stable when incubation is performed at 37 °C. By contrast, the complexes formed at 4 °C are dissociated by SDS and heating. This suggests a maturation process like a prepore and then a functional pore formation. Endocytosis and internalization of the toxin into the cell was not observed, and the toxin remains associated with the cell membrane throughout the intoxication process [86]. The ETX large membrane complex in MDCK cells and synaptosomes corresponds to the heptamerization of toxin molecules within the membrane and pore formation [86,88,89]. ETX prototoxin is able to bind to sensitive cells but does not oligomerize, in contrast to activated ETX. Thus, the 23 C-terminal residues of the prototoxin control the toxin activity by preventing heptamerization. These amino acids are removed in the active toxin molecule [88].

ETX binding to susceptible cells or synaptosomes and subsequent complex formation are prevented by protease treatment, but not or weakly by phospholipase C, glycosidases or neuraminidase, indicating a protein nature for the ETX receptor [50,69,86,90]. The ETX receptor might be related to a 34 or 46 kDa protein or glycoprotein in MDCK cells [86,90] and to a 26-kDa sialyglycoprotein in rat brain [50]. Hepatitis A virus cellular receptor 1 has been shown to facilitate ETX cytotoxicity in MDCK cells and the human kidney cell line ACHN. ETX binds to hepatitis A virus cellular receptor 1 *in vitro* [91]. However, it is not yet clear whether hepatitis A virus cellular receptor 1 is a functional ETX receptor. Moreover, although ETX does not directly interact with a lipid, the lipid environment of the ETX receptor is critical for the binding of ETX to a cell surface, because it is prevented by detergent treatment [86,90]. It is noteworthy that ETX can interact with artificial lipid bilayers and form functional channels, without the requirement for a specific receptor, in contrast to cell membrane, albeit less efficiently than in MDCK cells. Lipid bilayers have smooth surfaces without any surface structure including the surface-exposed carbohydrates and proteins of biological membranes, which means that the toxins can interact with the hydrocarbon core of the lipid bilayer and insert without the help of receptors, whereas receptors are required to promote such an interaction in the cell membrane [92].

In synaptosomes and MDCK cells, the ETX receptor has been localized in lipid raft microdomains, which are enriched in certain lipids such as cholesterol and sphingolipids and in certain proteins like glucosyl phosphatidylinositol-anchored proteins, suggesting that such a protein could be an ETX receptor [45,89]. However, in contrast to aerolysin and *C. septicum* Alpha toxin, ETX does not interact with a glucosyl phosphatidylinositol-anchored protein as receptor, because phosphatidylinositol-specific phospholipase C did not impair binding or ETX complex formation [45]. Localization of the ETX receptor in lipid microdomains is further supported by the fact that ETX prototoxin and active form bind preferentially to detergent-resistant membrane fractions and only activated ETX forms heptamers in detergent-resistant membrane [89]. In addition, membrane cholesterol removal with MβCD impairs ETX binding and pore formation [45,68,89]. The composition of lipid rafts in sphingomyelin and gangliosides as well as membrane fluidity influences ETX binding to sensitive cells, heptamerization and cytotoxicity [93,94]. Thus, inhibitors of sphingolipid or glycosphingolipid synthesis increase cell susceptibility to ETX, whereas inhibitor of sphingomyelin synthesis or addition of GM1 dramatically decreases ETX binding and subsequent heptamerization [93]. Moreover, phosphatidylcholine molecules which increase membrane fluidity, facilitate ETX binding and assembly [94]. ETX bound to its receptor shows a confined mobility on cell membrane probably permitting interaction between ETX monomers and subsequent oligomerization [95]. Local lipid composition and membrane fluidity likely control ETX bound to receptor in cell membrane. In addition, lipids such as diacylglycerol and phosphatidyl ethanolamine, which induce

a negative membrane curvature, increase ETX pore formation in liposome, whereas lipids having an opposite effect like lyso-phosphatidylcholine, impair ETX activity [94]. This is consistent with the model of a ETX prepore formation and subsequent insertion into the membrane to form a functional channel. The structure of ETX pore has been defined as a cone shape [96], and thus its insertion in lipid bilayer might be favored by a specific lipid membrane organization. Therefore, although ETX does not directly bind to a lipid receptor, the lipid composition and physical properties of membrane influence ETX access to the receptor, ETX monomer assembly, and insertion of ETX pore in membrane.

The cytotoxicity is associated with a rapid loss of intracellular K^+, and an increase in Cl^- and Na^+, whereas the increase in Ca^{2+} occurs later. In addition, the loss of viability also correlates with the entry of propidium iodide, indicating that ETX forms large pores in the cell membrane. Pore formation is evident in an artificial lipid bilayer. ETX induces water-filled channels permeable to hydrophilic solutes up to a molecular mass of 1 kDa, which represent general diffusion pores slightly selective for anions [92]. In polarized MDCK cells, ETX induces a rapid and dramatic increase in permeability. Pore formation in the cell membrane is likely responsible for the permeability change of cell monolayers. Actin cytoskeleton and organization of tight and adherens junctions are not altered, and the paracellular permeability to macromolecules is not significantly increased upon ETX treatment [45,97]. ETX causes rapid cell death by necrosis characterized by a marked reduction in nucleus size without DNA fragmentation. Toxin-dependent cell signaling leading to cell necrosis is not yet fully understood and includes ATP depletion, AMP-activated protein kinase stimulation, mitochondrial membrane permeabilization and mitochondrial–nuclear translocation of apoptosis-inducing factor, which is a potent caspase-independent cell death factor (Fig. 4) [45]. The early and rapid loss of intracellular K^+ induced by ETX, and also by *C. septicum* Alpha toxin, seems to be the early event leading to cell necrosis [98]. It is intriguing that ETX, which has a pore-forming activity related to that of aerolysin and *C. septicum* Alpha toxin, is much more active. Does ETX induce a specific intracellular signal responsible for a rapid cell death? MβCD, which prevents ETX pore formation in lipid rafts, does not inhibit the sudden decrease in cellular ATP and cell necrosis [45]. A subset of ETX channels unaffected by MβCD might be sufficient to trigger an intracellular signal leading to cell necrosis, excluding the requirement for a large diffusion pore to induce the intracellular toxic program. Therefore, ETX is a very potent toxin, which alters the permeability of cell monolayers such as epithelium and endothelium causing edema and cell death, however, its precise mode of action remains unclear.

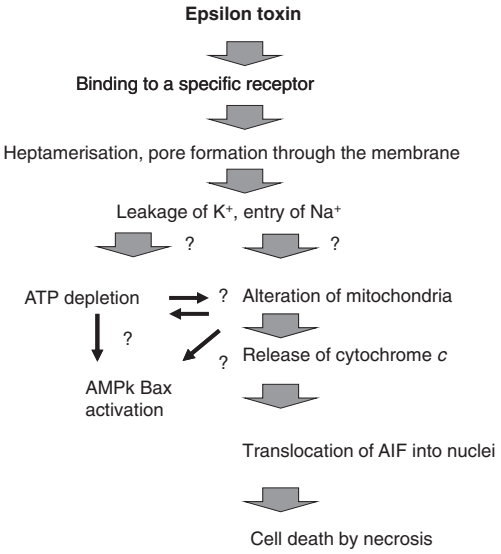

Fig. 4. Main steps of epsilon toxin mode of action.

Prevention

Vaccines against toxins which disseminate through the general circulation and which target organs or tissues at a distance from the gastrointestinal tract are among the most efficient vaccines. Indeed, vaccines against enterotoxemia due to *C. perfringens* ETX are used extensively in veterinary medicine [99]. Classically, toxin-based vaccines derive from chemically detoxified toxins. New approaches consist of genetically detoxified toxins such as toxin mutants or toxin subunits, which are non-biologically active but retain the toxin immunogenicity. Recombinant ETX vaccines are under investigation [84,99–102]. For example, cysteine substitutions at Ile51–Ala114 and Val56–Phe118 yield a non-cytotoxic ETX mutant, which could be a vaccine candidate [103].

To prevent the toxic effects of ETX, polyclonal and monoclonal antibodies have been developed. For example, monoclonal antibodies targeting an epitope close of the pore-forming domain have been found to be efficiently neutralizing [104]. Chemical inhibitors of ETX have been investigated by screening a large compound library. Three compounds, *N*-cycloalkylbenzamide, a furo(2,3-*b*)quinoline and a 6*H*-anthra(1,9-*cd*)isoxazol, inhibit ETX channel activity and cell

death, but not ETX binding to cell or ETX oligomerization. These inhibitors possibly block the ETX pore or interfere with an unidentified host factor involved in ETX-dependent cytotoxicity [105]. Interestingly, these inhibitors are specific of ETX and do not prevent aerolysin cytotoxicity, arguing again for a differential mode of action between both pore-forming toxins although they are structurally related and form similar functional pores.

Concluding remarks

ETX belongs to the heptameric β-PFTs family including aerolysin and *C. septicum* alpha toxin, which are characterized by the formation of a pore consisting in a β-barrel resulting from the arrangement of 14 amphipatic β strands [5]. Although these toxins share a similar mechanism of pore formation, ETX is much more potent than aerolysin and *C. septicum* alpha toxin. A main difference is that aerolysin and *C. septicum* alpha toxin recognize glucosyl phosphatidylinositol-anchored proteins as receptors, whereas ETX receptor, although localized in lipid rafts, is distinct from glucosyl phosphatidylinositol-anchored proteins and is distributed in a limited number of cell types. The specific ETX receptor possibly accounts for the high potency of ETX, which might also be dependent on a specific intracellular signaling induced by the toxin. Another particularity of ETX, compared with the other β-PFTs, is its ability to cross the blood–brain barrier, likely mediated by the interaction with its specific receptor. ETX can be considered as a neurotoxin because it targets specific neurons, which are glutamatergic neurons. By contrast to the other bacterial neurotoxins which inhibit the release of neurotransmitter, ETX has an opposite effect by stimulating the release of glutamate and also acts on other non-neuronal cells. This opens the door to design ETX molecules as a delivery system to address compounds into the central nervous system. Thereby, ETX has been used to facilitate the transport of the drug, bleomycin, through the blood–brain barrier for the treatment of experimental malignant brain tumor in mice [106]. Whether ETX is a powerful toxin, which requires a medical vigilance for the prevention of animals, this toxin also represents a unique tool to vehicle drugs in he central nervous system and/or to target glutamatergic neurons.

References

1 Petit L, Gibert M & Popoff MR (1999) *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiol* **7**, 104–110.

2 Gill DM (1987) Bacterial toxins: lethal amounts. In *Toxins and Enzymes* (Laskin AI & Lechevalier HA eds), pp. 127–135. CRC Press, Cleveland.

3 Minami J, Katayama S, Matsushita O, Matsushita C & Okabe A (1997) Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. *Microbiol Immunol* **41**, 527–535.

4 Mantis NJ (2005) Vaccines against the category B toxins: staphylococcal enterotoxin B, epsilon toxin and ricin. *Adv Drug Deliv Rev* **57**, 1424–1439.

5 Knapp O, Stiles BG & Popoff MR (2010) The aerolysin-like toxin family of cytolytic, pore-forming toxins. *Open Toxicol J* **3**, 53–68.

6 Bullen JJ (1952) Enterotoxaemia of sheep: *Clostridium welchii* type D in the alimentary tract of normal animals. *J Pathol Bacteriol* **64**, 201–206.

7 Bullen JJ & Battey I (1957) Enterotoxaemia of sheep. *Vet Rec* **69**, 1268–1273.

8 Finnie JW (2004) Neurological disorders produced by *Clostridium perfringens* type D epsilon toxin. *Anaerobe* **10**, 145–150.

9 Uzal FA (2004) Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. *Anaerobe* **10**, 135–143.

10 Uzal FA & Kelly WR (1998) Experimental *Clostridium perfringens* type D enterotoxemia in goats. *Vet Pathol* **35**, 132–140.

11 Gardner DE (1973) Pathology of *Clostridium welchii* type D enterotoxaemia. II. Structural and ultrastructural alterations in the tissues of lambs and mice. *J Comp Pathol* **83**, 509–524.

12 Gardner DE (1973) Pathology of *Clostridium welchii* type D enterotoxaemia. I. Biochemical and haematological alterations in lambs. *J Comp Pathol* **83**, 499–507.

13 Filho EJ, Carvalho AU, Assis RA, Lobato FF, Rachid MA, Carvalho AA, Ferreira PM, Nascimento RA, Fernandes AA, Vidal JE *et al.* (2009) Clinicopathologic features of experimental *Clostridium perfringens* type D enterotoxemia in cattle. *Vet Pathol* **46**, 1213–1220.

14 Uzal FA & Kelly WR (1996) Enterotoxaemia in goats. *Vet Res Commun* **20**, 481–492.

15 Uzal FA & Songer JG (2008) Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. *J Vet Diagn Invest* **20**, 253–265.

16 Shortt SJ, Titball RW & Lindsay CD (2000) An assessment of the *in vitro* toxicology of *Clostridium perfringens* type D epsilon-toxin in human and animal cells. *Hum Exp Toxicol* **19**, 108–116.

17 Fernandez Miyakawa ME, Zabal O & Silberstein C (2011) *Clostridium perfringens* epsilon toxin is cytotoxic for human renal tubular epithelial cells. *Hum Exp Toxicol* **30**, 275–282.

18 Gleeson-White MH & Bullen JJ (1955) *Clostridium welchii* epsilon toxin in the intestinal contents of man. *Lancet* **268**, 384–385.
19 Kohn J & Warrack GH (1955) Recovery of *Clostridium welchii* type D from man. *Lancet* **268**, 385.
20 Myers GS, Rasko DA, Cheung JK, Ravel J, Seshadri R, DeBoy RT, Ren Q, Varga J, Awad MM, Brinkac LM *et al*. (2006) Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Res* **16**, 1031–1040.
21 Hughes ML, Poon R, Adams V, Sayeed S, Saputo J, Uzal FA, McClane BA & Rood JI (2007) Epsilon-toxin plasmids of *Clostridium perfringens* type D are conjugative. *J Bacteriol* **189**, 7531–7538.
22 Sayeed S, Li J & McClane BA (2007) Virulence plasmid diversity in *Clostridium perfringens* type D isolates. *Infect Immun* **75**, 2391–2398.
23 Sayeed S, Li J & McClane BA (2010) Characterization of virulence plasmid diversity among *Clostridium perfringens* type B isolates. *Infect Immun* **78**, 495–504.
24 Deguchi A, Miyamoto K, Kuwahara T, Miki Y, Kaneko I, Li J, McClane BA & Akimoto S (2009) Genetic characterization of type A enterotoxigenic *Clostridium perfringens* strains. *PLoS ONE* **4**, e5598.
25 Kobayashi S, Wada A, Shibasaki S, Annaka M, Higuchi H, Adachi K, Mori N, Ishikawa T, Masuda Y, Watanabe H *et al*. (2009) Spread of a large plasmid carrying the *cpe* gene and the *tcp* locus amongst *Clostridium perfringens* isolates from nosocomial outbreaks and sporadic cases of gastroenteritis in a geriatric hospital. *Epidemiol Infect* **137**, 108–113.
26 Miyamoto K, Li J, Sayeed S, Akimoto S & McClane BA (2008) Sequencing and diversity analyses reveal extensive similarities between some epsilon-toxin-encoding plasmids and the pCPF5603 *Clostridium perfringens* enterotoxin plasmid. *J Bacteriol* **190**, 7178–7188.
27 Hunter SE, Clarke IN, Kelly DC & Titball RW (1992) Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. *Infect Immun* **60**, 102–110.
28 Bullen JJ & Batty I (1957) Experimental enterotoxaemia of sheep: the effect on the permeability of the intestine and the stimulation of antitoxin production in immune animals. *J Pathol Bacteriol* **73**, 511–518.
29 Bullen JJ & Scarisbrick R (1957) Enterotoxaemia of sheep: experimental reproduction of the disease. *J Pathol Bacteriol* **73**, 495–509.
30 Fernandez Miyakawa ME, Ibarra CA & Uzal FA (2003) *In vitro* effects of *Clostridium perfringens* type D epsilon toxin on water and ion transport in ovine and caprine intestine. *Anaerobe* **9**, 145–149.
31 Fernandez Miyakawa ME & Uzal FA (2003) The early effects of *Clostridium perfringens* type D epsilon toxin in ligated intestinal loops of goats and sheep. *Vet Res Commun* **27**, 231–241.
32 Fernandez-Miyakawa ME, Jost BH, Billington SJ & Uzal FA (2008) Lethal effects of *Clostridium perfringens* epsilon toxin are potentiated by alpha and perfringolysin-O toxins in a mouse model. *Vet Microbiol* **127**, 379–385.
33 Goldstein J, Morris WE, Loidl CF, Tironi-Farinatti C, McClane BA, Uzal FA & Fernandez Miyakawa ME (2009) *Clostridium perfringens* epsilon toxin increases the small intestinal permeability in mice and rats. *PLoS ONE* **4**, e7065.
34 Batty I & Bullen JJ (1956) The effect of *Clostridium welchii* type D culture filtrates on the permeability of the mouse intestine. *J Pathol Bacteriol* **71**, 311–323.
35 Losada-Eaton DM, Uzal FA & Fernandez Miyakawa ME (2008) *Clostridium perfringens* epsilon toxin is absorbed from different intestinal segments of mice. *Toxicon* **51**, 1207–1213.
36 Sakurai J, Nagahama M & Takahashi T (1989) Contraction induced by *Clostridium perfringens* epsilon toxin in the isolated rat ileum. *FEMS Microbiol Lett* **49**, 269–272.
37 Losada-Eaton DM & Fernandez-Miyakawa ME (2010) *Clostridium perfringens* epsilon toxin inhibits the gastrointestinal transit in mice. *Res Vet Sci* **89**, 404–408.
38 Adamson RH, Ly JC, Fernandez-Miyakawa M, Ochi S, Sakurai J, Uzal F & Curry FE (2005) *Clostridium perfringens* epsilon-toxin increases permeability of single perfused microvessels of rat mesentery. *Infect Immun* **73**, 4879–4887.
39 Buxton D (1978) Further studies on the mode of action of *Clostridium welchii* type-D epsilon toxin. *J Med Microbiol* **11**, 293–298.
40 Soler-Jover A, Blasi J, Gomez de Aranda I, Navarro P, Gibert M, Popoff MR & Martin-Satue M (2004) Effect of epsilon toxin–GFP on MDCK cells and renal tubules *in vivo*. *J Histochem Cytochem* **52**, 931–942.
41 Uzal FA, Rolfe BE, Smith NJ, Thomas AC & Kelly WR (1999) Resistance of ovine, caprine and bovine endothelial cells to *Clostridium perfringens* type D epsilon toxin *in vitro*. *Vet Res Commun* **23**, 275–284.
42 Nagahama M, Iida H & Sakurai J (1993) Effect of *Clostridium perfringens* epsilon toxin on rat isolated aorta. *Microbiol Immunol* **37**, 447–450.
43 Sakurai J, Nagahama M & Fujii Y (1983) Effect of *Clostridium perfringens* epsilon toxin on the cardiovascular system of rats. *Infect Immun* **42**, 1183–1186.
44 Tamai E, Ishida T, Miyata S, Matsushita O, Suda H, Kobayashi S, Sonobe H & Okabe A (2003) Accumulation of *Clostridium perfringens* epsilon-toxin in the mouse kidney and its possible biological significance. *Infect Immun* **71**, 5371–5375.
45 Chassin C, Bens M, de Barry J, Courjaret R, Bossu JL, Cluzeaud F, Ben Mkaddem S, Gibert M, Poulain B, Popoff MR *et al*. (2007) Pore-forming epsilon toxin

causes membrane permeabilization and rapid ATP depletion-mediated cell death in renal collecting duct cells. *Am J Physiol Renal Physiol* **293**, F927–F937.
46 Lindsay CD, Hambrook JL & Upshall DG (1995) Examination of toxicity of *Clostridium perfringens* ε-toxin in the MDCK cell line. *Toxic In Vitro* **9**, 213–218.
47 Payne DW, Williamson ED, Havard H, Modi N & Brown J (1994) Evaluation of a new cytotoxicity assay for *Clostridium perfringens* type D epsilon toxin. *FEMS Microbiol Lett* **116**, 161–168.
48 Gardner DE (1973) Pathology of *Clostridium welchii* type D enterotoxaemia. 3. Basis of the hyperglycaemic response. *J Comp Pathol* **83**, 525–529.
49 Nagahama M & Sakurai J (1991) Distribution of labeled *Clostridium perfringens* epsilon toxin in mice. *Toxicon* **29**, 211–217.
50 Nagahama M & Sakurai J (1992) High-affinity binding of *Clostridium perfringens* epsilon-toxin to rat brain. *Infect Immun* **60**, 1237–1240.
51 Finnie JW & Hajduk P (1992) An immunohistochemical study of plasma albumin extravasation in the brain of mice after the administration of *Clostridium perfringens* type D epsilon toxin. *Aust Vet J* **69**, 261–262.
52 Griner LA & Carlson WD (1961) Enterotoxemia of sheep. II. Distribution of 131I-radioiodinated serum albumin in brain of Clostridium perfringens type D intoxicated lambs. *Am J Vet Res* **22**, 443–446.
53 Worthington RW & Mulders MS (1975) Effect of *Clostridium perfringens* epsilon toxin on the blood–brain barrier of mice. *Onderstepoort J Vet Res* **42**, 25–27.
54 Zhu C, Ghabriel MN, Blumbergs PC, Reilly PL, Manavis J, Youssef J, Hatami S & Finnie JW (2001) *Clostridium perfringens* prototoxin-induced alteration of endothelial barrier antigen (EBA) immunoreactivity at the blood–brain barrier (BBB). *Exp Neurol* **169**, 72–82.
55 Morgan KT, Kelly BG & Buxton D (1975) Vascular leakage produced in the brains of mice by *Clostridium welchii* type D toxin. *J Comp Pathol* **85**, 461–466.
56 Finnie JW, Blumbergs PC & Manavis J (1999) Neuronal damage produced in rat brains by *Clostridium perfringens* type D epsilon toxin. *J Comp Pathol* **120**, 415–420.
57 Finnie JW (1984) Ultrastructural changes in the brain of mice given *Clostridium perfringens* type D epsilon toxin. *J Comp Pathol* **94**, 445–452.
58 Finnie JW (2003) Pathogenesis of brain damage produced in sheep by *Clostridium perfringens* type D epsilon toxin: a review. *Aust Vet J* **81**, 219–221.
59 Morgan KT & Kelly BG (1974) Ultrastructural study of brain lesions produced in mice by the administration of *Clostridium welchii* type D toxin. *J Comp Pathol* **84**, 181–191.
60 Finnie JW (1984) Histopathological changes in the brain of mice given *Clostridium perfringens* type D epsilon toxin. *J Comp Pathol* **94**, 363–370.
61 Ghabriel MN, Zhu C, Reilly PL, Blumbergs PC, Manavis J & Finnie JW (2000) Toxin-induced vasogenic cerebral oedema in a rat model. *Acta Neurochir Suppl* **76**, 231–236.
62 Finnie JW, Manavis J & Blumbergs PC (2008) Aquaporin-4 in acute cerebral edema produced by *Clostridium perfringens* type D epsilon toxin. *Vet Pathol* **45**, 307–309.
63 Baldwin EM Jr & Griner LA (1956) Clostridia in diarrheal diseases of animals. *Ann NY Acad Sci* **66**, 168–175.
64 Buxton D (1978) The use of an immunoperoxidase technique to investigate by light and electron microscopy the sites of binding of *Clostridium welchii* type-D epsilon toxin in mice. *J Med Microbiol* **11**, 289–292.
65 Uzal FA, Kelly WR, Morris WE, Bermudez J & Baison M (2004) The pathology of peracute experimental *Clostridium perfringens* type D enterotoxemia in sheep. *J Vet Diagn Invest* **16**, 403–411.
66 Uzal FA, Kelly WR, Morris WE & Assis RA (2002) Effects of intravenous injection of *Clostridium perfringens* type D epsilon toxin in calves. *J Comp Pathol* **126**, 71–75.
67 Soler-Jover A, Dorca J, Popoff MR, Gibert M, Saura J, Tusell JM, Serratosa J, Blasi J & Martin-Satue M (2007) Distribution of *Clostridium perfringens* epsilon toxin in the brains of acutely intoxicated mice and its effect upon glial cells. *Toxicon* **50**, 530–540.
68 Lonchamp E, Dupont JL, Wioland L, Courjaret R, Mbebi-Liegeois C, Jover E, Doussau F, Popoff MR, Bossu JL, de Barry J *et al*. (2011) *Clostridium perfringens* epsilon toxin targets granule cells in the mouse cerebellum and stimulates glutamate release. *PLoS ONE* **5**, e13046.
69 Dorca-Arevalo J, Soler-Jover A, Gibert M, Popoff MR, Martin-Satue M & Blasi J (2008) Binding of epsilon-toxin from *Clostridium perfringens* in the nervous system. *Vet Microbiol* **131**, 14–25.
70 Miyamoto O, Minami J, Toyoshima T, Nakamura T, Masada T, Nagao S, Negi T, Itano T & Okabe A (1998) Neurotoxicity of *Clostridium perfringens* epsilon-toxin for the rat hipocampus via glutamanergic system. *Infect Immun* **66**, 2501–2508.
71 Miyamoto O, Sumitami K, Nakamura T, Yamagani S, Miyatal S, Itano T, Negi T & Okabe A (2000) *Clostridium perfringens* epsilon toxin causes excessive release of glutamate in the mouse hippocampus. *FEMS Microbiol Lett* **189**, 109–113.
72 Nagahama M & Sakurai J (1993) Effect of drugs acting on the central nervous system on the lethality in

mice of *Clostridium perfringens* epsilon toxin. *Toxicon* **31**, 427–435.
73 Phannachet K, Raksat P, Limvuttegrijeerat T & Promdonkoy B (2011) Production and characterization of N- and C-terminally truncated Mtx2: a mosquitocidal toxin from *Bacillus sphaericus*. *Curr Microbiol* **61**, 549–553.
74 Sakaguchi Y, Hayashi T, Yamamoto Y, Nakayama K, Zhang K, Ma S, Arimitsu H & Oguma K (2009) Molecular analysis of an extrachromosomal element containing the C2 toxin gene discovered in *Clostridium* botulinum type C. *J Bacteriol* **191**, 3282–3291.
75 Cole A (2003) Structural studies on epsilon toxin from *Clostridium perfringens*. In *Protein Toxins of the Genus Clostridium and Vaccination* (Duchesnes C, Mainil J, Popoff MR & Titball R eds), p. 95. Presses de la Faculté de Médecine Vétérinaire, Liège.
76 Gurcel L, Iacovache I & van der Goot FG (2006) Aerolysin and related *Aeromonas* toxins. In *The Source Book of Bacterial Protein Toxins* (Alouf JE & Popoff MR eds), pp. 606–620. Elsevier, Amsterdam.
77 Parker MW, Buckley JT, Postma JP, Tucker AD, Leonard K, Pattus F & Tsernoglou D (1994) Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367**, 292–295.
78 Melton JA, Parker MW, Rossjohn J, Buckley JT & Tweten RK (2004) The identification and structure of the membrane-spanning domain of the *Clostridium septicum* alpha toxin. *J Biol Chem* **279**, 14315–14322.
79 Tweten RK (2001) *Clostridium perfringens* beta toxin and *Clostridium septicum* alpha toxin: their mechanisms and possible role in pathogenesis. *Vet Microbiol* **82**, 1–9.
80 Knapp O, Maier E, Benz R, Geny B & Popoff MR (2009) Identification of the channel-forming domain of *Clostridium perfringens* epsilon-toxin (ETX). *Biochim Biophys Acta* **1788**, 2584–2593.
81 Geny B & Popoff MR (2006) Bacterial protein toxins and lipids: pore formation or toxin entry into cells. *Biol Cell* **98**, 667–678.
82 Schleberger C, Hochmann H, Barth H, Aktories K & Schulz GE (2006) Structure and action of the binary C2 toxin from *Clostridium botulinum*. *J Mol Biol* **364**, 705–715.
83 Sakurai J (1995) Toxins of *Clostridium perfringens*. *Rev Med Microbiol* **6**, 175–185.
84 Oyston PCF, Payne DW, Havard HL, Williamson ED & Titball RW (1998) Production of a non-toxic site-directed mutant of *Clostridium perfringens* ε-toxin which induces protective immunity in mice. *Microbiology* **144**, 333–341.
85 Heine K, Pust S, Enzenmuller S & Barth H (2008) ADP-ribosylation of actin by the *Clostridium botulinum* C2 toxin in mammalian cells results in delayed caspase-dependent apoptotic cell death. *Infect Immun* **76**, 4600–4608.
86 Petit L, Gibert M, Gillet D, Laurent-Winter C, Boquet P & Popoff MR (1997) *Clostridium perfringens* epsilon-toxin acts on MDCK cells by forming a large membrane complex. *J Bacteriol* **179**, 6480–6487.
87 Borrmann E, Günther H & Köhler H (2001) Effect of *Clostridium perfringens* epsilon toxin on MDCK cells. *FEMS Immunol Med Microbiol* **31**, 85–92.
88 Miyata S, Matsushita O, Minami J, Katayama S, Shimamoto S & Okabe A (2001) Cleavage of C-terminal peptide is essential for heptamerization of *Clostridium perfringens* epsilon-toxin in the synaptosomal membrane. *J Biol Chem* **276**, 13778–13783.
89 Miyata S, Minami J, Tamai E, Matsushita O, Shimamoto S & Okabe A (2002) *Clostridium perfringens* epsilon-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin–Darby canine kidney cells and rat synaptosomes. *J Biol Chem* **277**, 39463–39468.
90 Payne D, Williamson ED & Titball RW (1997) The *Clostridium perfringens* epsilon-toxin. *Rev Med Microbiol* **8**, S28–S30.
91 Ivie SE, Fennessey CM, Sheng J, Rubin DH & McClain MS (2011) Gene-trap mutagenesis identifies mammalian genes contributing to intoxication by *Clostridium perfringens* epsilon-toxin. *PLoS ONE* **6**, e17787.
92 Petit L, Maier E, Gibert M, Popoff MR & Benz R (2001) *Clostridium perfringens* epsilon-toxin induces a rapid change in cell membrane permeability to ions and forms channels in artificial lipid bilayers. *J Biol Chem* **276**, 15736–15740.
93 Shimamoto S, Tamai E, Matsushita O, Minami J, Okabe A & Miyata S (2005) Changes in ganglioside content affect the binding of *Clostridium perfringens* epsilon-toxin to detergent-resistant membranes of Madin–Darby canine kidney cells. *Microbiol Immunol* **49**, 245–253.
94 Nagahama M, Hara H, Fernandez-Miyakawa M, Itohayashi Y & Sakurai J (2006) Oligomerization of *Clostridium perfringens* epsilon-toxin is dependent upon membrane fluidity in liposomes. *Biochemistry* **45**, 296–302.
95 Masson JB, Casanova D, Turkcan S, Voisinne G, Popoff MR, Vergassola M & Alexandrou A (2009) Inferring maps of forces inside cell membrane microdomains. *Phys Rev Lett* **102**, 048103-1-4.
96 Nestorovich EM, Karginov VA & Bezrukov SM (2011) Polymer partitioning and ion selectivity suggest asymmetrical shape for the membrane pore formed by epsilon toxin. *Biophys J* **99**, 782–789.
97 Petit L, Gibert M, Gourch A, Bens M, Vandewalle A & Popoff MR (2003) *Clostridium perfringens* epsilon toxin rapidly decreases membrane barrier permeability of polarized MDCK cells. *Cell Microbiol* **5**, 155–164.

98 Knapp O, Maier E, Mkaddem SB, Benz R, Bens M, Chenal A, Geny B, Vandewalle A & Popoff MR (2010) *Clostridium* septicum alpha-toxin forms pores and induces rapid cell necrosis. *Toxicon* **55**, 61–72.
99 Titball RW (2009) *Clostridium perfringens* vaccines. *Vaccine* **27**(Suppl 4), D44–D47.
100 Lobato FC, Lima CG, Assis RA, Pires PS, Silva RO, Salvarani FM, Carmo AO, Contigli C & Kalapothakis E (2010) Potency against enterotoxemia of a recombinant *Clostridium perfringens* type D epsilon toxoid in ruminants. *Vaccine* **28**, 6125–6127.
101 Mathur DD, Deshmukh S, Kaushik H & Garg LC (2010) Functional and structural characterization of soluble recombinant epsilon toxin of *Clostridium perfringens* D, causative agent of enterotoxaemia. *Appl Microbiol Biotechnol* **88**, 877–884.
102 Souza AM, Reis JK, Assis RA, Horta CC, Siqueira FF, Facchin S, Alvarenga ER, Castro CS, Salvarani FM, Silva RO *et al*. (2010) Molecular cloning and expression of epsilon toxin from *Clostridium perfringens* type D and tests of animal immunization. *Genet Mol Res* **9**, 266–276.
103 Pelish TM & McClain MS (2009) Dominant-negative inhibitors of the *Clostridium perfringens* epsilon-toxin. *J Biol Chem* **284**, 29446–29453.
104 McClain MS & Cover TL (2007) Functional analysis of neutralizing antibodies against *Clostridium perfringens* epsilon-toxin. *Infect Immun* **75**, 1785–1793.
105 Lewis M, Weaver CD & McClain MS (2011) Identification of small molecule inhibitors of *Clostridium perfringens* epsilon-toxin cytotoxicity using a cell-based high-throughput screen. *Toxins (Basel)* **2**, 1825–1847.
106 Hirschberg H, Zhang MJ, Gach HM, Uzal FA, Peng Q, Sun CH, Chighvinadze D & Madsen SJ (2009) Targeted delivery of bleomycin to the brain using photo-chemical internalization of *Clostridium perfringens* epsilon prototoxin. *J Neurooncol* **95**, 317–329.