Low pH-Induced Formation of Ion Channels by *Clostridium difficile*

Toxin B in Target Cells*

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Running title: Ion channel of *Clostridium difficile* toxin B
SUMMARY

_Clostridium difficile_ toxin B (269 kDa) which is one of the causative agents of the antibiotic-associated diarrhea and the pseudomembranous colitis, inactivates Rho GTPases by glucosylation. Here we studied the uptake and the membrane interaction of the toxin with eukaryotic target cells. Bafilomycin A1, which prevents acidification of endosomal compartments, blocked the cellular uptake of toxin B in Chinese Hamster Ovary cells (CHO) cells. Extracellular acidification (pH ≤ 5.2) induced uptake of toxin B into the cytosol even in the presence of bafilomycin A1. Toxin B increased $^{86}$Rb$^+$ release when preloaded CHO cells were exposed to low pH (pH ≤ 5.6) for 5 min. Release of $^{86}$Rb$^+$ depended on the concentration of toxin B and on the pH of the extracellular medium. An antibody directed against the holotoxin prevented channel formation, whereas an antibody against the N-terminal enzyme domain was without effect. The N-terminally truncated toxin B fragment consisting of amino acids 547-2366 increased $^{86}$Rb$^+$ efflux when cells were exposed to low pH. Toxin B induced also pH-dependent channel formation in artificial lipid bilayer membranes. Also _Clostridium sordellii_ lethal toxin, another member of the family of large clostridial cytotoxins, induced increased $^{86}$Rb$^+$ release at low pH. The results suggest that large clostridial cytotoxins including _Clostridium difficile_ toxin B and _Clostridium sordellii_ lethal toxin undergo structural changes at low pH of endosomes, which is accompanied by membrane insertion and channel formation.
INTRODUCTION

The large clostridial cytotoxins from *Clostridium difficile* toxin A (308 kDa) and toxin B (269 kDa) are major virulence factors of the antibiotic-associated diarrhea and the causative agents of the pseudomembranous colitis (1-4). Both toxins are O-glucosyltransferases which modify the small GTPases of the Rho family Rho, Rac and Cdc42 by mono-glucosylation at threonine37 and threonine35, respectively (5;6). The glucosylation blocks the biological functions of Rho GTPases, which are molecular switches in a large array of signal processes including regulation of the actin cytoskeleton (7;8). Accordingly, some of the best documented effects of large clostridial cytotoxins are the toxin-induced redistribution of actin filaments, morphological changes and rounding-up of cells.

Structure-function analyses of large clostridial cytotoxins suggest a tripartite organization of the toxins similar as known for diphtheria toxin. According to this model, the catalytic domain is located at the N-terminus (9), while the C-terminal part of the toxins is believed to bind to the cellular receptor (10-12). In the middle of the molecule is a rather small hydrophobic region possibly involved in the translocation of the toxin into the cytoplasm. Because the substrates of the toxins are intracellularly located, translocation of the toxins across the cell membrane is a prerequisite for their actions on Rho GTPases. However, little is known about the site and mechanism of translocation of the toxins. Two major traffic pathways for bacterial exotoxins have been described. One group of toxins including cholera toxin (13), shiga toxins (14) and *Pseudomonas aeruginosa* exotoxin A (15) appear to be internalized after binding to their specific receptors and then follow a retrograde pathway back to the ER where the membrane translocation occurs. Another group of toxins, appears to enter the cytosol from the low pH compartment of endosomes. Prototypes of this group are diphtheria toxin (16), anthrax...
toxin (17) and \textit{C. botulinum} \textsuperscript{1} C2 toxin (18). It is believed that endosomal acidification leads to a conformational change of these toxins thereby allowing insertion into the endosomal membrane and eventually translocation across the endosomal membrane into the cytosol. Some previous reports suggested that also toxin B escapes from the endosome into the cytoplasm (19). Toxins which enter the cytosol from endosomes, were shown to induce ion permeable channels in artificial membranes and in cell membranes when exposed to acidic pH (20;21). Therefore, we investigated whether acidification also triggers formation of channels by large clostridial cytotoxins. Here we report that under acidic conditions \textit{C. difficile} toxin B and \textit{C. sordellii} lethal toxin induce membrane permeabilization in eukaryotic cells as well as channel formation in artificial lipid bilayer membranes.

\textsuperscript{1} Abbreviations: C., Clostridium; CDB\textsuperscript{1-546}, catalytic domain of \textit{C. difficile} toxin B (amino acids 1-546); CDB\textsuperscript{547-2366}, enzymatic inactive fragment of \textit{C. difficile} toxin B (amino acids 547-2366); CHO, Chinese hamster ovary; LT, lethal toxin of \textit{C. sordellii}. 
EXPERIMENTAL PROCEDURES

Materials - Cell culture medium was from Biochrom (Berlin, Germany) and fetal calf serum from PAN Systems (Aidenbach, Germany). Cell culture materials were obtained from Falcon (Heidelberg, Germany). Thrombin was from Sigma (Deisenhofen, Germany). Rubidium-86 (specific activity 2 mCi/mg) was from NEN Life Science Products (Boston, USA). Bafilomycin A1 was from Calbiochem (Bad Soden, Germany). Toxin B from C. difficile VPI 10463 and C. sordellii lethal toxin were purified as described elsewhere (22). A polyclonal antibody against toxin B (anti-Tox B) was produced in a rabbit. The toxin B fragment CDB1-546 was cloned, expressed and purified as described (22) and a monoclonal antibody (anti-CDB1-546) was produced against this fragment.

Cloning and expression of toxin B fragment CDB547-2366 - CDB547-2366 was amplified from genomic DNA from C. difficile VPI 10463 by PCR with Taq polymerase (Roche Diagnostics, Mannheim, Germany) and the oligonucleotide primers CDB547-BamH1 (5’-ggggatccgataatcttgatttttctcaaaat-3’) and CDB3N-EcoR1 (5’-gaattcctattcactaatcactaattg-3’). Amplification was done by denaturing at 94°C for 10 sec, primer anealing at 55°C for 30 sec, extension at 68°C for 6 min and repeated for 25 cycles. The resulting PCR product (2 µl) was cloned into pCR2.1 vector (Invitrogen, NV Leek, The Netherlands) according to manufacturer’s instructions. For expression of CDB547-2366, the gene was excised with BamH1/EcoR1 (NEB Biolabs, Schwabach, Germany) and cloned in the BamH1/EcoR1 digested pGEX2T plasmid (Pharmacia Biotech, Uppsala, Sweden) containing a double glycine linker. Proteins were expressed in E. coli-TG1 as recombinant GST-fusion proteins and purified by affinity chromatography with glutathion Sepharose 4B according to the
manufacturer’s instructions. GST was cleaved off by thrombin and proteins were analyzed by SDS-PAGE according to the method of Laemmli (23).

Cell culture and toxin translocation assay - CHO-K1, HT-29, Caco-2 and Vero cells were cultivated in tissue culture flasks at 37°C and 5% CO₂ in Ham’s F12/Dulbecco’s MEM (1:1), containing 5% heat-inactivated (30 min, 56°C) fetal calf serum, 2 mM L-glutamate, 100 U/ml penicillin and 100 µg/ml streptomycin. Rat Basophilic Leukemia (RBL) cells were cultivated in Eagle’s minimal essential medium plus Earle’s salts containing 15% (v/v) heat inactivated fetal calf serum, 4 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were routinely trypsinized and reseeded twice a week. For toxin B translocation assay, CHO cells were preincubated for 15 min at 37°C with 100 nM bafilomycin A1 in serum free medium (Ham’s F12/DMEM) and subsequently toxin B (100 ng/ml) was added and cells were incubated for further 2 h at 4°C in serum-free medium. Cells were washed with cold medium and incubated for 5 min in serum-free medium (Ham’s F12/DMEM) at pH 7.5, 5.2, or 4.5, respectively. Subsequently, cells were incubated in complete medium at 37°C, pH 7.5 in the presence of bafilomycin A1 and after 1.5 h phase contrast pictures were taken.

86Rb⁺ efflux measurements - For 86Rb⁺ efflux experiments, cells were plated in complete medium (Ham’s F12/DMEM containing 5% fetal calf serum) at a density of approximately 2 x 10⁵ cells/well in 24-well culture plates. At 8 h after plating, fresh medium containing 86Rb⁺ (1µ Ci/ml) was added and cells were incubated for further 14 h. Cells were chilled at 4°C and fresh medium (4°C, Ham’s F12/DMEM without serum) containing toxin A or toxin B was added. Toxins were allowed to bind for 2 h at 4°C and subsequently washed 2 times with cold medium to remove unbound toxin. To initiate membrane insertion of the
toxins, cells were treated with warm medium (37°C; Ham’s F12/DMEM without serum, pH 4.0 - 7.5) for 5 min at 37°C. Cells were further incubated at 4°C and after various incubation times aliquots of the medium were removed and $^{86}$Rb$^+$ release was determined by liquid scintillation counting in a 1209 Rackbeta β-counter from LKB Wallac (Gräfeling, Germany).

*Black lipid bilayer experiments* – The methods used for black lipid bilayer experiments have been described previously in detail (24). Membranes were formed from a 1 % solution of asolectin or diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster AL) in n-decane.
RESULTS

Translocation of cell bound toxin B into the cytoplasm – Treatment of CHO cells with bafilomycin A1, which blocks the vacuolar H⁺-ATPase pump, prevented the cytotoxic effect of toxin B. To test the effect of pH on toxin uptake, toxin B was allowed to bind to cells at 4°C. After 2 h, cells were incubated for 5 min at 37°C with bafilomycin containing medium of pH 7.5, 5.2 or 4.5, respectively. Thereafter, the acidified media were replaced by neutral medium containing bafilomycin and cells were incubated for additional 1.5 h at 37°C. Then cells were analyzed for cytopathic effects induced by toxin B by phase contrast microscopy. As shown in Fig. 1, cells rounded up when the pH was shifted for 5 min to 5.2 or 4.5 but not at pH 7.5. The results indicate that at low pH the toxin directly crosses the cell membrane even in the presence of bafilomycin. The data are in line with the recent report that toxin B escapes from an acidic endosomal compartment into the cytoplasm (19).

Toxin B-induced ⁸⁶Rb⁺-release from CHO cells - The conditions allowing the direct uptake of toxin B across the cytoplasmic membrane were used to test whether extracellular acidification leads to channel formation and membrane permeabilization by toxin B. To this end the efflux of ⁸⁶Rb⁺ from preloaded cells were studied. At first, ⁸⁶Rb⁺-preloaded CHO cells were incubated without and with toxin B and heat-inactivated toxin B, respectively. An increase in ⁸⁶Rb⁺ release was measured when toxin B-treated cells were incubated in an acidified medium of pH 5.2 (Fig. 2A). The increased ⁸⁶Rb⁺ release depended not only on low pH but additionally required short term (5 min) incubation at 37°C. It is noteworthy that under these conditions, cells did not round up, indicating no intoxication by toxin B.
Acidification of medium in the absence of toxin B and in the presence of heat-inactivated toxin, or binding of toxin B to cells without subsequent acidification did not increase $^{86}$Rb$^+$ release. This result indicates that toxin B inserted into the membrane of eukaryotic cells and increased membrane permeability after exposure of cells to acidic pH. In Vero cells, toxin B caused a similar increase in efflux of $^{86}$Rb$^+$ at low pH (Fig. 2B). This finding indicates that the channel formation by toxin is not restricted to CHO cells. However, we were not able to detect an increase in $^{86}$Rb$^+$ release in HT-29 and Caco-2 cells. Further studies on toxin B-induced $^{86}$Rb$^+$-release were performed with the CHO-K1 cell line.

Characterization of toxin B-induced $^{86}$Rb$^+$-release from CHO cells - At first, the influence of pH on the kinetics of the $^{86}$Rb$^+$-release from toxin B-treated CHO cells was studied. When the medium of toxin B-treated cells were shifted to pH 5.2 for 5 min at 37°C and, subsequently, cells were incubated on ice at pH 5.2, the increase in $^{86}$Rb$^+$-release mainly occurred within 20 to 30 min. A low additional increase in $^{86}$Rb$^+$-release occurred in a second phase within 90 min (Fig. 3). A similar release of $^{86}$Rb$^+$ was observed, when toxin B-treated cells were kept for 5 min in a medium of pH 5.2 and were then incubated at 4°C at pH 7.5 (not shown). This finding suggests that membrane permeabilization by toxin B is achieved by a brief acidic pulse. Once formation of channels has occurred, channels remained open to release $^{86}$Rb$^+$ even at physiological pH. To further characterize the formation of channels, we studied the pH dependency of the toxin effects in more detail. $^{86}$Rb$^+$ preloaded CHO cells were incubated with toxin B and shifted to pH 7.5, 5.6, 5.2 or 5.0, respectively. At the lowest pH (5.0), the largest increase in release of $^{86}$Rb$^+$ occurred. At pH 5.6, $^{86}$Rb$^+$
efflux was much less pronounced than at pH 5.2 or 5.0, and at pH 7.5 no increased release was measured (Fig. 4A). Next, $^{86}\text{Rb}^+$ preloaded CHO cells were incubated with increasing concentrations of toxin B (25, 100 and 500 ng/ml, respectively). Subsequently, cells were shifted to pH 5.2 and released $^{86}\text{Rb}^+$ was determined. $^{86}\text{Rb}^+$ efflux depended on the concentration of the toxin. Treatment of cells with 100 and 500 ng/ml of toxin B, respectively did not result in significant differences in $^{86}\text{Rb}^+$ release (Fig. 4B), most likely indicating saturation of the toxin binding capacity of CHO cells. This finding suggests that the specific binding of toxin B to its cell membrane receptor is a prerequisite for subsequent channel formation. Moreover, the data indicate that channel formation is not caused by an unspecific integration of toxin B into cell membranes.

Influence of anti-toxin B antibodies on toxin B induced $^{86}\text{Rb}^+$-release - To confirm that receptor binding of toxin B is necessary for subsequent membrane permeabilization, toxin B was incubated with an antibody directed towards the complete toxin, which mainly recognizes the receptor binding domain of toxin B (25), or with an antibody against its N-terminal catalytic domain, respectively. Then CHO cells were incubated with the respective toxin/antibody mixture. The antibody against the holotoxin inhibited binding of toxin B to CHO cells and prevented the cytotoxic effects (Fig. 5A). In contrast, the antibody against the catalytic domain did not inhibit toxin B induced rounding of the cells (Fig. 5A). Accordingly, the latter antibody did not affect toxin B induced $^{86}\text{Rb}^+$-release from CHO cells (Fig. 5B). Incubation of toxin B with the antibody against the holotoxin prevented the toxin-induced membrane permeabilization. However, this antibody was without effect when added to cells after binding of toxin B (Fig. 5B). This observation suggests that only toxin B which was
already bound to the cell membrane receptor formed channels at low pH.

**Effect of toxin B fragment CDB\textsuperscript{547-2366} on \textsuperscript{86}Rb\textsuperscript{+}-release from CHO cells** - To determine the part of toxin B which mediates membrane permeabilization of cells after acidification, we cloned and expressed a toxin B fragment. CDB\textsuperscript{547-2366} consists of residues 547-2366, i.e. of the putative receptor binding and membrane insertion domains, and lacks the catalytic domain (Fig. 6A). The proteins were allowed to bind to \textsuperscript{86}Rb\textsuperscript{+} preloaded CHO cells and cells were subsequently exposed to pH 7.5 or 4.5, respectively. The release of \textsuperscript{86}Rb\textsuperscript{+} was determined. Fig. 6 shows that the release of \textsuperscript{86}Rb\textsuperscript{+} from preloaded CHO cells was increased, when cells were treated with CDB\textsuperscript{547-2366} and, subsequently, exposed to acidic medium. It is noteworthy that the concentration of the toxin B fragment CDB\textsuperscript{547-2366} (4 µg/ml) was higher than that of the full-length toxin B (200 ng/ml), which was applied in parallel.

**Effect of C. sordellii lethal toxin on \textsuperscript{86}Rb\textsuperscript{+}-release from cells** - Lethal toxin (LT) was tested for an influence on \textsuperscript{86}Rb\textsuperscript{+} release from cells after exposure to low pH medium. Therefore, the toxin was allowed to bind to preloaded CHO cells and cells were subsequently shifted to pH 7.5 or 4.5, respectively for 5 min at 37°C and incubated for further 55 min at 4°C. As shown in Fig. 7, treatment of cells with LT and subsequent exposure to low pH medium increased the efflux of \textsuperscript{86}Rb\textsuperscript{+} from cells. Similar results were obtained when Rat Basophilic Leukemia (RBL) cells were used.
conditions of low pH - The toxin B mediated efflux of $^{86}$Rb$^+$ from cells indicated that toxin B formed channels, which were at least permeable to ions. Therefore, we studied whether ion-permeable channels are also formed in lipid bilayer membranes. In a first set of experiments, we studied the effect of toxin B on membranes formed from different lipids such as phosphatidylcholine, and asolectin at pH 6. Under these conditions we observed only rare current fluctuations in single channel recordings (see Fig. 8 A). The fluctuations were very rapid as Fig. 8 A demonstrates and had only a short lifetime on the order of seconds. Their single-channel conductance was approximately 0.2 to 1.5 nS in 1 M KCl. Enhanced single channel activity was observed, however, when the pH was lowered to pH 5 and many more channels were recorded under the conditions of the lowered pH (see Fig. 8 B). Again the current fluctuations were very rapid and it was difficult to provide a precise value for the single-channel conductance. Histograms of the fluctuations that could be resolved suggest that the pH did not influence the size of the channels as compared to pH 6. The low pH seemed to increase simply the channel-forming activity. Measurement with other salts, such as LiCl suggested that a variety of ions were permeable through the toxin B channel, but it seemed to have generally a higher permeability for cations than for anions.
DISCUSSION

Bacterial toxins which modify intracellular substrates must translocate across a lipid bilayer membrane to reach their targets (26). Following endocytosis, two pathways are described which are used by bacterial toxins to reach their cytosolic substrates. One possible route for toxin uptake (e.g., cholera toxin (13)) is the retrograde transport from endosomal compartments via the Golgi apparatus to the ER from where the toxins translocate into the cytoplasm. Other toxins (e.g., diphtheria toxin (16) or the binary anthrax toxin (17)) translocate directly from the acidic endosomal compartment into the cytosol. Inhibition of the cytotoxic action of toxin B by bafilomycin, as reported recently and as shown in this report, indicate that also toxin B requires an acidic endosome for cellular uptake (19). In line with this notion is the finding that the lowering of the extracellular pH allows uptake of the toxin even in the presence of bafilomycin. So far, our knowledge of the events that cause translocation of toxin B are very limited. In the present communication we report that toxin B is capable of inducing ion permeable channels, a process which might reflect molecular mechanisms closely related to translocation of the toxin into the cytosol. For detection of channel formation, the release of \(^{86}\text{Rb}^+\) from preloaded cells was measured and conditions were chosen that mimic the endosomal compartment, e.g., cell bound toxin B was exposed to an acidic buffer (pH < 5.6) for a short time (5 min) at 37°C to induce channel formation by the toxin. Similarly, we observed a strong induction of channel formation in lipid bilayer membranes when the pH was lowered to pH 5 (see Fig. 8). At pH 6 only a very small channel-forming activity was observed. It is noteworthy, that pH-dependent channel formation has been reported for several protein toxins which are taken up from endosomes (18;21). It is believed that the low pH present in endosomal compartments causes conformational changes of the toxin, which are
accompanied by surface exposition of otherwise intramolecularly located hydrophobic region of the toxins. Then, the hydrophobic regions insert into the bilayer membrane to form channels and/or to allow protein translocation. Recently, Qa’Dan et al. (19) analyzed the pH-induced conformational changes occurring in toxin B. Using various fluorescence methods it was shown that the hydrophobicity of toxin B was increased at pH ≤5.0 but not at pH ≥5.5 concluding that toxin B undergoes pH-induced structural changes finally allowing membrane insertion and translocation of the toxin from the acidic endosome into the cytosol.

So far the structural basis of the pH-dependent channels is not clear. Our studies show that the N terminal catalytic domain (toxin B fragment consisting of amino acids 1-546) is not necessary for channel formation. By contrast a N-terminal truncated toxin B fragment that consisted of amino acid residues 547 through 2366 was capable of channel formation. However, because the C-terminal fragment was only active at higher concentrations than the holotoxin, it is suggested that also the N-terminus might play a minor role in binding/insertion or stabilization of the holotoxin. Nevertheless, the results are in line with the view that the C-terminus and the middle part of large clostridial toxins are involved in receptor binding and translocation. This hypothesis is supported by the findings that an antibody directed against the holotoxin, which mainly recognizes the receptor binding domain of toxin B (25), inhibited channel formation and intoxication, whereas an antibody against the N-terminal catalytic domain of toxin B was not able to prevent the increase in Rb efflux. In experiments with artificial black lipid bilayer membranes, membrane permeabilization by toxin B was detected at pH 6.0 and increased under more acidic conditions (pH 4.5). Here, the antibody against the holotoxin had no effect on toxin B induced membrane permeabilization. This indicates that the antibody did not affect membrane insertion of toxin B but might inhibit the toxin binding to the cell surface. However, it has to be clarified whether insertion into
artificial membranes and into cell membranes follow the identical mechanisms. Moreover, the functional dissociation of channel formation and cytopathic effects induced by the toxin is corroborated by the finding that in spite of increased $^{86}\text{Rb}^+$ efflux, the cells still showed the control morphology when experiments were performed at 4° C and only the acidic shift was done at 37° C.

An increased $^{86}\text{Rb}^+$ efflux was measured when CHO, Vero or RBL cells were studied. These cell lines show high sensitivity towards toxin B and appear to have a sufficient amount of cell membrane receptor molecules. By contrast, we were not able to detect toxin B induced channel formation in some other cell lines (HT-29, Caco-2). In agreement with the possible destruction of the barrier function of the cytoplasmic membrane of CHO, Vero or RBL cells, we were able to identify channel formation in artificial lipid bilayer membranes. Channels were formed with the two lipids tested. This result seems to represent a contradiction to the obvious receptor-mediated permeabilization of cells. However, it has to be kept in mind that other toxins, such as C2 toxin (20) or the RTX toxins (27-29) form channels in lipid bilayer membranes without the need of receptors, whereas they all need a receptor for biological activity. An important role of the toxin receptor in events finally resulting in channel formation is supported by the concentration effect studies at high concentration of toxin, showing limitation of channel formation most likely by reaching the binding capacity of cells. In contrast to toxin B, toxin A from C. difficile showed no detectable channel forming activity in the $^{86}\text{Rb}^+$ release assay with CHO cells. This might be due to the relative insensitivity of CHO cells to toxin A and to their low content of toxin A receptor molecules. Toxin A which is also termed enterotoxin is known to exhibit much less cytotoxic effects on many cultured cells than toxin B. Similar to toxin B, C. sordellii lethal toxin caused channel formation in CHO and RBL cells which are especially sensitive towards the lethal toxin, suggesting that
channel formation is a general activity of large clostridial cytotoxins.

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LEGENDS TO FIGURES

Fig. 1. **Influence of extracellular pH on toxin B uptake into bafilomycin A1-treated CHO cells.** CHO cells were preincubated for 15 min at 37°C with 100 nM bafilomycin A1 followed by a 2 h incubation with toxin B (100 ng/ml) in serum-free medium. After washing, cells were incubated for 5 min in serum-free medium (37°C + Baf) at pH 7.5, 5.2, or 4.5, respectively. Cells were further incubated at 37°C in complete neutral medium (pH 7.5) containing Bafilomycin A1. After 1.5 h, phase contrast pictures were taken. In parallel, cells were incubated with 100 ng/ml toxin B (Tox B) or without any drug (con).

Fig. 2. **Toxin B-mediated release of $^{86}\text{Rb}^+$ from CHO and Vero cells after acidification.** A. CHO cells were preloaded with $^{86}\text{Rb}^+$ (14 h, 1 µCi/ml) and subsequently incubated for 2 h at 4°C with toxin B (100 ng/ml) or with heat-inactivated toxin B (100 ng/ml) in fresh medium without serum. For control, cells were incubated without toxin. Cells were washed to remove unbound toxin and prewarmed medium (37°C) pH 7.5 or 5.2, respectively, was added. Cells were incubated at 37°C for 5 min and subsequently at 4°C for further 55 min. The complete medium (500 µl) was removed and $^{86}\text{Rb}^+$ release was determined by liquid scintillation counting. Values are given as mean ± S. D. (n = 3). B. Vero cells preloaded with $^{86}\text{Rb}^+$ were incubated with toxin B (100 ng/ml) or for control without toxin B. Cells were shifted for 5 min to pH 5.2 and further incubated at 4°C for 25 min. The medium was assayed for $^{86}\text{Rb}^+$. Values are given as mean ± S. D. (n = 3).

Fig. 3. **Effect of pH on toxin B-mediated $^{86}\text{Rb}^+$-efflux.** $^{86}\text{Rb}^+$ preloaded CHO cells were
incubated for 2 h at 4°C with toxin B (100 ng/ml) or for control without toxin. Cells were washed to remove unbound toxin and prewarmed medium pH 5.2 was added. Cells were incubated at 37°C for 5 min and subsequently at 4°C at pH 5.2 (control, n; toxin B, s) or 7.5 (control, l; toxin B, t), respectively. After further 5, 10, 15, 25, 55 and 85 min, 100 µl of the medium were removed and $^{86}$Rb$^+$ release was determined by scintillation counting.

Fig. 4. **pH- and concentration-dependence of toxin B-mediated $^{86}$Rb$^+$-efflux.** $^{86}$Rb$^+$ preloaded CHO cells were incubated for 2 h at 4°C with toxin B (100 ng/ml) or for control without toxin. Cells were washed to remove unbound toxin and prewarmed medium (pH 7.5) (n), 5.6 (l), 5.2 (s) or 5.0 (t), respectively, was added. Cells were incubated at 37°C for 5 min and subsequently at 4°C. After 10, 20, 30 and 60 min, aliquots (100 µl) of the medium were removed and released $^{86}$Rb$^+$ was determined (A). $^{86}$Rb$^+$ preloaded CHO cells were incubated for 2 h at 4°C with toxin B (25, 100 and 500 ng/ml, respectively) or for control without toxin B. Cells were washed and prewarmed medium (pH 5.2) was added. Cells were incubated at 37°C for 5 min and subsequently at 4°C. After 10 (n), 20 (l), 30 (s) and 60 min (t), aliquots (100 µl) of the medium were removed and $^{86}$Rb$^+$ was determined (B).

Fig. 5. **Influence of anti-toxin B antibodies on cytotoxic toxin B effects and pH-induced release of $^{86}$Rb$^+$ from CHO cells.** Toxin B was preincubated with an antibody against the holotoxin (anti-Tox B) or with an antibody against its catalytic domain (anti-CDB$^{1-546}$) for 30 min at 4°C. For control, toxin B was preincubated with PBS. A. CHO cells were incubated with the respective pretreated toxin B (100 ng/ml) at 37°C. The morphology of the cells after 3 h is shown. B. $^{86}$Rb$^+$ preloaded CHO cells were incubated for 2 h at 4°C in serum-free
medium with the respective pretreated toxin B (100 ng/ml) and pH shift assay (pH 7.5 versus pH 5.2) was performed. After 30 min at 4°C the medium was removed and $^{86}\text{Rb}^+$ release was measured. Values are given as mean ± S. D. (n = 3). C. Cells were treated as described above. Additionally, toxin B (100 ng/ml) was bound to CHO cells for 2 h at 4°C and, thereafter, anti-Tox B was added and cells were incubated for 1 h. After pH shift (pH 7.5 and pH 5.2, respectively) the cells were incubated for 30 min at 4°C and the medium was removed and assayed for $^{86}\text{Rb}^+$. Values are given as mean ± S. D. (n = 3).

Fig. 6. Effect of toxin B fragment CDB$^{547-2366}$ on $^{86}\text{Rb}^+$ release from CHO cells. A. SDS-PAGE of recombinant CDB$^{547-2366}$. CDB$^{547-2366}$ was expressed in E. coli and purified as described. After cleavage with thrombin, 1 µg of protein was analyzed by 7% SDS-PAGE and Coomassie staining. B. Release of $^{86}\text{Rb}^+$ from CHO cells after treatment of cells with toxin B and CDB$^{547-2366}$, respectively and exposure to low pH medium. $^{86}\text{Rb}^+$-preloaded (1µCi/ml) cells were incubated for 2 h at 4°C with toxin B (200 ng/ml) or with CDB$^{547-2366}$ (4 µg/ml) in fresh medium without serum. For control, cells were incubated without toxin. Cells were washed and prewarmed medium (37°C, pH 7.5 or 4.5), respectively, was added. Cells were incubated at 37°C for 5 min and subsequently at 4°C for further 55 min. The medium (500 µl) was removed and assayed for $^{86}\text{Rb}^+$. Values are given as mean ± S. D. (n = 3).

Fig. 7. pH-induced release of $^{86}\text{Rb}^+$ from CHO cells after treatment with lethal toxin (LT). $^{86}\text{Rb}^+$ preloaded CHO cells were incubated for 2 h at 4°C in serum-free medium with lethal
toxin (400 ng/ml). Cells were washed to remove unbound toxin and prewarmed medium
(37°C, pH 7.5 or 4.5) was added. Cells were incubated at 37°C for 5 min and subsequently at
4°C for further 55 min. The medium (500 µl) was removed to determine released 86Rb⁺.
Values are given as mean ± S. D. (n = 3).

Fig. 8. Channel formation of toxin B in artificial lipid bilayer membranes. (A) Single-channel
recording of an asolectin/n-decane membrane in the presence of toxin B from C. difficile. 10
min after the formation of the membrane, 80 ng/ml toxin B was added to the aqueous phase
on one side of the membrane. The aqueous phase contained 1 M KCl, 10 mM MES; pH 6. (B)
Similar experiment as in A. The left-hand side of the record demonstrates the small channel-
forming activity of toxin B at pH 6. About 30 min after membrane formation, the pH was
lowered to pH 5 (right-hand side arrow). Shortly after the decrease of the pH, the membrane
conductance increased caused by the formation of many channels, which had a small lifetime.
The applied membrane potential was 50 mV; T = 20°C.
Reference List


Barth et al., Fig. 7
Low pH-induced formation of ion channels by Clostridium difficile toxin B in target cells
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