The large clostridial cytotoxins toxin A and toxin B from _Clostridium difficile_ are major virulence factors known to cause antibiotic-associated diarrhea and pseudomembranous colitis. Both toxins mono-glycosylate and thereby inactivate small GTPases of the Rho family. Recently, it was reported that toxin B, but not toxin A, induces pore formation in membranes of target cells under acidic conditions. Here, we reassessed data on pore formation of toxin A in cells derived from human colon carcinoma. Treatment of 86Rb

- The efficacy of pore formation was dependent on membrane cholesterol, since cholesterol depletion of membranes with methyl-
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- C. difficile toxins A and B are large single-chained proteins of 308 and 270 kDa. Recent studies indicate a tripartite structure (15, 16). The catalytic domain is located at the N terminus (17). A hydrophobic stretch in the middle of the protein is thought to mediate membrane insertion and pore formation. The C terminus, which mainly consists of clostridial repetitive oligopeptides, takes part in membrane binding (18, 19). Accordingly, the actions of toxins A and B on mammalian target cells is suggested to follow a sequence of steps including membrane binding, endocytosis, translocation, (20) and finally, catalytic modification of target GTPases (8, 21, 22). Whereas the nature of the membrane receptor of toxin B is totally unclear, toxin A binding to various types of carbohydrate structures has been reported (23, 24). However, the precise type of receptor is still a matter of debate.

Once taken up by endocytosis, two major trafficking pathways are known for bacterial toxins by which the catalytic activity reaches the cytosol. Cholera and Shiga toxin utilize the retrograde pathway; the toxins are transported from the endosomal compartment via the Golgi apparatus to the endoplasmic reticulum from where the catalytic domain is released to the cytoplasm (25–27). Another group of toxins, including diphtheria, anthrax, or _Clostridium botulinum_ C2-toxins, are translocated from acidic endosomes (“short trip model”) (28–30). Here, the acidification of the endosomal lumen induces a conformational change of the corresponding polypeptides, resulting in an insertion of hydrophobic regions into the endosomal membrane. This is accompanied by pore formation and allows translocation of the catalytic domain. Consistently, baflomycin, an inhibitor of the vacuolar H+-ATPase pump, inhibits intoxication of the corresponding toxins (31).

In the case of large clostridial cytotoxins, the uptake mechanism is best understood for toxin B. In agreement with the short trip model, a pH-dependent conformational change and the inhibition of intoxication by baflomycin have already been shown (13, 20). The catalytic domain could be detected in the cytosol (32, 33). The binding and trans location domains are thought to remain in the endosomes and to be transported to the lysosomes for consecutive degradation. Furthermore, toxin B was shown to form pores in cell membranes as well as in artificial membranes under acidic conditions. Although toxins A and B display 48% identity at amino acid sequence (15), no pore formation at cellular membranes could be detected for toxin A under the same conditions (31).

To clarify recent discrepancies in pore formation obtained with toxins A and B, we compared the interaction of both toxins with cells derived from colon carcinoma. When utilizing highly toxin A-sensitive HT-29 cells, we found a comparable formation of rubidium-permeable pores in target cell membranes under acidic conditions for both toxins.
Moreover, we show that the pore formation of the toxins as well as the intoxication efficiency of cells is dependent on the presence of cholesterol. Regardless of the role of cholesterol, we excluded glycosylphosphatidylinositol (GPI)2-anchored structures as possible receptors for both toxins. Consistent with the rubidium release assays, toxin A was able to induce pores in artificial membranes only in the presence of cholesterol.

EXPERIMENTAL PROCEDURES

Materials and Proteins—Ham’s F-12/Dulbecco’s minimum Eagle’s medium (MEM) (1:1) and Dulbecco’s MEM cell culture medium were from Biochrom (Berlin, Germany). McCoy’s 5A medium and fetal calf serum were obtained from PAN Biotech GmbH (Aidenbach, Germany). Cell culture materials were purchased from Falcon (Heidelberg, Germany). Rubidium-86 (specific activity 2 mCi/mg) was from PerkinElmer Life Sciences. Cholesterol and water-soluble cholesterol were obtained from Sigma-Aldrich. Diphytanoyl phosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL).

Toxins A and B from C. difficile VPI 10463 were purified as described (34). Recombinant His-tagged toxin A (rToxA) and an enzyme-deficient mutant of toxin A (rToxAed) were cloned and expressed in Bacillus megaterium and purified as described (35). Polyclonal antibodies directed against rToxA (α-rToxA) or rToxAed (α-rToxAed) were produced in rabbit.

Cell Culture and Determination of Transepithelial Resistance—CHO-K1 cells were cultivated in Ham’s F-12/Dulbecco’s minimum Eagle’s medium (1:1), CaCo-2 cells were cultivated in Dulbecco’s minimum Eagle’s medium, and HT-29 cells were cultivated in McCoy’s 5A medium. All cells were routinely kept in tissue culture flasks at 37 °C and 5% CO2 with medium containing 10% heat-inactivated (30 min, 56 °C) fetal calf serum, 2 mM l-glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin and were trypsinized and reseeded 2 or 3 times a week.

For the transepithelial resistance (TER) assay, CaCo-2 cells were seeded on FalconTM cell culture inserts and incubated 6–9 days with medium exchange every 3 days. Assays were performed when TER values reached ~2000–3000 ohm·cm2. TER was determined using a resistance system for electrophysiological readings of filter cups (Endohm-12; World Precision Instruments, Sarasota, FL). To deplete cholesterol, cells were incubated with methyl-β-cyclodextrin for 30 min at 37 °C before toxin treatment. To avoid a drastic drop in starting-TER due to treatment with methyl-β-cyclodextrin, all washing and incubation steps were carried out using medium supplemented with 10% fetal calf serum. Toxin A (100 ng/ml; ~300 pM) and methyl-β-cyclodextrin (0.5–2 mM) were applied to the upper compartment of the FalconTM cell culture inserts, and decrease of TER was monitored for up to 9 h.

86Rb+ Efflux Measurements—86Rb+ efflux experiments were conducted essentially as described recently (31). HT-29 or CHO-K1 cells were plated in adequate complete medium containing 86Rb+ (1 μCi/ml) at a density of ~2 × 105 cells/well in 24-well cell culture plates. After 48–72 h cells were washed with phosphate-buffered saline 3 times and cooled down to 4 °C. Afterward, fresh medium (4 °C, without serum) containing toxin A, recombinant toxin A variants, or toxin B in varying concentrations was added. Toxins were allowed to bind for 30–60 min at 4 °C, and subsequently cells were washed 2 times with cold medium to remove unbound toxin. To initiate membrane insertion of the toxins, warm medium (37 °C; without serum, pH 4.5–7.5) was applied, and cells were incubated for 5 min at 37 °C. After a shift back to 4 °C, incubation was continued for 30 to 40 min at low temperature to allow 86Rb+ efflux. Aliquots of the medium were removed, and 86Rb+ release was determined by liquid scintillation counting.

For inhibition of pore formation with anti-toxin A polyclonal antibody, native and recombinant toxins were preincubated with 1–30 μl of the corresponding antisera for 1 h at 4 °C. Afterward, the toxin-antibody mixture was used in 86Rb+ efflux assays. Alternatively, the antisera was applied after toxin A binding at 4 °C for 1 h.

Methyl-β-cyclodextrin Treatment and Repletion of Cholesterol—To deplete cholesterol, monolayers of 86Rb+-loaded HT-29 cells in 24-well cell culture plates were treated with 0.5–10 mM methyl-β-cyclodextrin in buffer A (10 mM HEPES, pH 7.5, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) for 20–40 min at 37 °C. After washing two times with buffer A, cells were incubated with toxin A and used for rubidium release assay as described above.

Cholesterol (water-soluble) was loaded to cells by incubating control and cholesterol-depleted cells with cholesterol (0.1/0.25 mM cholesterol and 1.25 mM methyl-β-cyclodextrin, respectively) for 30 min at 37 °C. The unincorporated cholesterol was removed by washing the cells with buffer A before cells were used in the 86Rb+ release assay as detailed above.

Black Lipid Bilayer Experiments—The methods used for black lipid bilayer experiments have been described previously in detail (36). Membranes were formed either from a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) or from a 1% solution of a mixture of diphytanoyl phosphatidylcholine and cholesterol (molar ratio 1:1) or from a 1% solution of oxidized cholesterol (36) in n-decane. Reduction of pH was achieved by the addition of predetermined amounts of 0.1% HCl to the volume on one or both sides bathing lipid bilayer membranes (37).

Phosphatidylinositol-specific Phospholipase C (PI-PLC) Treatment of HT-29 Cells—Subconfluent HT-29 were incubated with 0.1 unit of PI-PLC/ml for 60 min at 37 °C (PI-PLC, Roche Applied Science). PI-PLC was removed, and the cells were incubated with toxin A (100 ng/ml; ~300 pM) and toxin B (200 ng/ml; ~740 pM), respectively, for 30 min on ice to allow cell binding. Unbound toxins were removed, and cell morphology was examined at 37 °C. The efficacy of PI-PLC treatment was checked by determination of the enzyme activity of alkaline phosphatase, which is a GPI-anchored membrane protein (38).

RESULTS

Pore Formation by Toxin A in HT-29 Cells—In the case of large clostridial cytotoxins, pore formation under acidic conditions was shown only for toxin B and Clostridium sordellii lethal toxin (31). In all cells tested (e.g. CHO, HeLa, Vero), toxin A failed to induce pores under the same conditions. Because toxin A has been described as an enterotoxin with much less cytotoxic activity than toxin B on non-enterocytic cells, the susceptibility of cells is one possible explanation. To investigate pore formation by toxin A, highly susceptible enterocytic HT-29 and control CHO-K1 cells were preloaded with 86Rb+ before the toxins were applied at low temperature to allow binding but not internalization. To mimic the acidification in early endosomes, extracellular pH was decreased to pH values ranging from 4.5 to 5.6, and the cells were concomitantly shifted to 37 °C for 5 min. These conditions should enable the toxins to undergo the conformational change necessary for membrane insertion and pore formation. Upon membrane insertion, 86Rb+ was released from the cells through the pores, and therefore, the presence of 86Rb+ in the medium indicates pore formation. Cells that were

2 The abbreviations used are: GPI, glycosylphosphatidylinositol; Tox, toxin; rToxA, recombinant His-tagged ToxA; rToxAed, enzyme-deficient mutant of toxin A; CHO, Chinese hamster ovary; TER, transepithelial resistance; PI-PLC, phosphatidylinositol-specific phospholipase C; BLM, artificial black lipid membrane.
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FIGURE 1. Comparison of pore formation by toxins A and B in CHO and HT-29 cells induced by low pH. CHO and HT-29 cells were preloaded with 86Rb+ and cooled to 4 °C, and then toxin A or toxin B was added at different concentrations for binding. A and B served as negative controls. C, variations in the acidic shift concerning pH value (pH 5.6 instead of pH 5) or temperature (4 °C instead of 37 °C) as indicated. A–C, mean values of three independent experiments ± S.D. are shown as x-fold induction of 86Rb+ release as compared with controls without toxin.

kept at constant neutral pH and cells that were treated without toxins served as controls (pH values are as indicated in Fig. 1).

As reported recently (31), when CHO cells were tested with 500 ng/ml toxin A (~1.6 nM) and toxin B (~1.8 nM), toxin A caused only an insignificant rubidium release compared with toxin B, which was used as positive control (Fig. 1A). In contrast, when HT-29 cells were used under the same conditions, toxins A and B produced comparable rubidium release (Fig. 1B). Therefore, toxin A has the same potential to induce pores in the cell membrane as toxin B in HT-29 cells. A 3-fold increase in toxin concentrations was not paralleled by a strong signal increase, most likely indicating the formation of a limited number of pores caused by saturation of toxin binding. As expected, denaturation of the toxins at 95 °C for 5 min before application completely inhibited toxin-dependent channel formation (Fig. 1B).

The dynamics of 86Rb+ release were quite rapid. About 80% were released within 5 min after the acidic shift, and maximum values were reached after 30 min. An increase of the pH above pH 5.5 abolished pore formation (e.g. pH 5.6 in Fig. 1C). x-fold induction of rubidium release as compared with corresponding controls without toxin is shown; see also Fig. 2). Moreover, if the acidic shift was not paralleled by a temperature shift to 37 °C, no 86Rb+ release was detectable (Fig. 1C). All measurements were conducted at pH 5, and efflux times were between 30 and 40 min if not otherwise mentioned. Note that the cells did not round up during the whole experimental procedure, indicating that no intoxication occurs under the chosen conditions (4 °C).

Determination of EC50 and pH Optimum—To further characterize the toxin A-induced pore formation, a wide range of toxin A concentrations from 1 to 7500 pM were tested at pH 5 (see Fig. 2A). The x-fold induction of toxin A-induced 86Rb+ efflux is shown in Fig. 2A, and the corresponding normalized values against maximum value are shown in Fig. 2B. The EC50 for toxin A-induced pore formation is in the range of 240 pM, and concentrations as low as 100 pM induced significant rubidium release from the cells. The concentration of toxin A (500 ng/ml; ~1.6 nM), which was used in subsequent rubidium release assays, caused ~90% of the maximal effect observed.

Different pH values ranging from pH 6 to 4.5 were tested for the induction of 86Rb+ release, and the x-fold induction of pore formation as compared with the corresponding control at pH 7.5 is shown in Fig. 2B. Maximal effect in pore formation was detectable at pH 5. Further reduction of the pH did not increase signal strength. Above pH 5, rubidium release decreased with increase in pH values. At pH 5.5, 86Rb+ release was reduced to one-third of maximum values, and at and above pH 5.6 no pore formation was detectable (see also Fig. 1C). Here, 500 pM toxin A was used, but comparable results were achieved with 100 and 1000 pM toxin A (data not shown).

Altogether, these data indicated that toxin A inserted into membranes of eukaryotic cells and increased the membrane permeability after exposure to low pH. Similar results were obtained with CaCo-2 cells (although signal strength was less pronounced; data not shown), demonstrating that pore formation was not restricted to HT-29 cells but was also true for other colonic cell lines susceptible to toxin A.

Specificity of Toxin A-induced Pore Formation—To verify that the 86Rb+ release is toxin A-specific, different preparations of the toxin
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were used. Moreover, the inhibitory effect of toxin A-specific antisera on toxin binding and subsequent induction of $^{86}$Rb$^{+}$ efflux was tested. HT-29 cells were preloaded with $^{86}$Rb$^{+}$ and treated either with ToxA, a recombinant toxin A produced in B. megaterium (rToxA), or an enzyme-deficient mutant of toxin A (rToxA$_{ed}$). The mutation of the aspartic acid residues at positions 286 and 288 to alanine resulted in complete inhibition of $^{86}$Rb$^{+}$ efflux. Control cells remained untreated or were treated with toxin A alone (as indicated). Inhibition was monitored by light microscopy and pictures were taken after 2 h.

FIGURE 3. Pore formation by toxin A, recombinant toxin A, and enzyme-deficient toxin A is inhibited by anti-toxin A antibodies. A, toxin A prepared from supernatant of C. difficile VPI 10463 (ToxA), recombinant wild-type toxin (rToxA), and enzyme deficient mutant (rToxA$_{ed}$) were tested in the $^{86}$Rb$^{+}$ release assay as detailed before. Two different anti-toxin A antisera were used to verify the specificity of the assay (a-rToxA, polyclonal antibody raised against UDP-dialedehyde-inactivated rToxA; a-rToxA$_{ed}$, polyclonal antibody raised against rToxA$_{ed}$). These were incubated with the toxin before the $^{86}$Rb$^{+}$ release assay for 60 min at 4 °C, and the toxin-antibody mixture was then applied to the cells. B, left panels, instead of toxin A, toxin B was preincubated with a-rToxA. Right panels, a-rToxA was either preincubated with toxin A like above (pre) or applied to the cells after toxin binding but before acidic shift (post). C, titration of the antibody amount (here: a-rToxA) necessary for inhibition of pore formation in rubidium release assay with 0, 1, 5, and 30 μM. A-C, the x-fold induction of $^{86}$Rb$^{+}$ release as compared with corresponding controls without toxin is shown (mean values of at least three experiments ± S.D.). D, inhibitory effect of the antisera on toxin A binding/intoxication. Native toxin A (final concentration 500 ng/ml; ~1.6 μM) was preincubated with the corresponding sera (see above), and afterward, HT-29 cells were treated with the toxin-antibody mixture. Control cells remained untreated or were treated with toxin A alone (as indicated). Inhibition was monitored by light microscopy and pictures were taken after 2 h.

were treated. Moreover, the inhibitory effect of toxin A-specific antisera on toxin binding and subsequent induction of $^{86}$Rb$^{+}$ efflux was tested. HT-29 cells were preloaded with $^{86}$Rb$^{+}$ and treated either with ToxA, a recombinant toxin A produced in B. megaterium (rToxA), or an enzyme-deficient mutant of toxin A (DXXD mutant, produced in B. megaterium, rToxA$_{ed}$). The mutation of the aspartic acid residues at positions 286 and 288 to alanine in toxin B was formerly shown to reduce the enzymatic activity by about 1000-fold (39). The corresponding residues in toxin A are at position 285 and 287, and their change to alanine also inhibited glucosyltransferase activity.3 All three toxin variants induced the release of $^{86}$Rb$^{+}$ after an acidic pulse from HT-29 cells in a comparable manner (Fig. 3A). Because the toxins were from different sources (B. megaterium), these findings indicated the specificity of pore formation. Similarly, toxin A highly purified by thyreoglobulin affinity chromatography had the same effect (data not shown).

When the toxins were preincubated with toxin A-specific antisera before application to HT-29 cells, inhibition of pore formation was observed. An antibody raised against UDP-dialedehyde-inactivated recombinant protein (a-rToxA) reduced efflux of $^{86}$Rb$^{+}$ to ~25%, and an antibody raised against enzyme-deficient toxin A (a-rToxA$_{ed}$) reduced efflux of $^{86}$Rb$^{+}$ to ~15% of the maximum values (Fig. 3A) when the same amounts of the antibodies were used. These effects were comparable for all three toxin variants used. In contrast, when toxin B was preincubated with a-rToxA under the same conditions, no reduction of pore formation was detectable, indicating the specificity of anti-toxin A serum (Fig. 3B). A slight difference was observed when the antibody was either preincubated with the toxin before application (see above, Fig. 3B, pre) or applied to the cell suspension after toxin binding but before the acidic shift (Fig. 3B, post). Therefore, the antisera is either able to partially extract toxin A already bound to the cell surface or it interferes with the conformational changes of the toxin responsible for membrane insertion and pore formation. The titration of the optimal amount of antibody for preincubation with toxin A is shown in Fig. 3C.

The inhibitory effect of the antisera is also demonstrated by the prevention of cytotoxic effects of toxin A in cell culture. HT-29 cells were treated with 50 ng/ml (~160 pm) toxin A or a toxin A-antibody mixture as indicated in Fig. 3D. Both antibodies completely abolished cell rounding.

Cholesterol Dependence of Toxin-induced $^{86}$Rb$^{+}$ Efflux—An increasing number of reports indicate a requirement for membrane cholesterol in membrane binding, insertion, and pore formation of bacterial protein toxins (40). Therefore, we investigated the effect of cholesterol depletion on membrane permeabilization by toxin A. HT-29 cells loaded with $^{86}$Rb$^{+}$ were incubated with methyl-$β$-cyclodextrin at increasing concentrations (as indicated in Fig. 4A) for 30 min at 37 °C. Methyl-$β$-cyclodextrin is known to deplete cholesterol from cellular membranes (41, 42). After washing, cells were cooled to 4 °C, and $^{86}$Rb$^{+}$ release assay was performed as described above. Fig. 4A shows that methyl-$β$-cyclodextrin impaired pore formation in a dose-dependent manner. Reduction of $^{86}$Rb$^{+}$ efflux was already detectable at concentrations of 0.5 mM methyl-$β$-cyclodextrin. At 1 mM methyl-$β$-cyclodextrin, the compound inhibited membrane permeabilization by about 50%. 2.5 mM methyl-$β$-cyclodextrin resulted in complete inhibition. Toxin A and toxin B are supposed to accomplish their own uptake in the same way. Accordingly, when toxin B was tested in the same experimental setup, cholesterol depletion had the same inhibitory effect on toxin B-induced $^{86}$Rb$^{+}$ release (Fig. 4A).

To verify a specific cholesterol-dependent effect, cholesterol was repleted after methyl-$β$-cyclodextrin treatment. HT-29 cells treated either with 1 or 2.5 mM methyl-$β$-cyclodextrin were subsequently incubated with 0.1 or 0.25 mM water-soluble cholesterol, respectively, for 20 min at 37 °C before the $^{86}$Rb$^{+}$ release assay was performed. The reduction in $^{86}$Rb$^{+}$ release observed by cholesterol depletion was completely reversed after the re-addition of cholesterol in the case of the lower concentrations (1 mM/0.1 mM; Fig. 4B). The reversion was statistically significant with a p value of 0.0035. Higher concentrations of methyl-$β$-cyclodextrin (2.5 mM) and cholesterol (0.25 mM) had the same effect, but under this condition the recovery of the $^{86}$Rb$^{+}$ efflux was not complete in comparison to the untreated control. This was due to loss of cells during the assay verified by the cholesterol control (see Fig. 4B, right panels, cells lost) and by light microscopy (data not shown). Regardless of these technical difficulties, a p value of about 0.015 cor-
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Incubation with methyl-
cholesterol (0.1 mM / 0.25 mM) for 20 min at 37 °C (cholesterol) and pore formation for large clostridial cytotoxins is likely. Therefore, a general requirement for cholesterol during membrane insertion and pore formation for large clostridial cytotoxins is likely.

Inhibitory Effect of Methyl-β-cyclodextrin on Cell Intoxication by Toxin A—The data regarding pore formation under artificial conditions (i.e. acidic pulse) implied a role of cholesterol in the uptake mechanism of toxin A. Therefore, we investigated a possible effect of methyl-β-cyclodextrin on the intoxication efficiency of toxin A in cell culture. A sensitive method for monitoring cell intoxication by clostridial toxins is the measurement of the TER of confluent cell monolayers grown on semipermeable filters. For that, enterocytic CaCo-2 cells were grown on filters until starting resistance reached around 2000–3000 ohms cm². Cells were pretreated with methyl-β-cyclodextrin at concentrations of 0.5, 1, and 2 mM (37 °C, 30 min) preceded toxin application (toxin A, 100 ng/ml; ~300 pm). All compounds were added to the apical reservoir, since for toxin A treatment apical addition is more effective than basolateral addition (data not shown). Cell intoxication (TER decrease) was monitored for up to 9 h. Fig. 5A shows the TER values as percent of starting resistance, which was defined as TER measured directly after incubation with methyl-β-cyclodextrin and toxin application. In Fig. 5B the TER values were normalized against the corresponding controls for the purpose of clarity. Mean values of at least three independent experiments ± S.D. are shown (S.D. indications are skipped in controls of Fig. 5A). The data indicate that the onset of intoxication is delayed, and the intoxication rate is slowed down in a methyl-β-cyclodextrin concentration-dependent manner. These experiments were thoroughly repeated with different cell densities. The inhibitory effect of methyl-β-cyclodextrin on intoxication efficiency was best detectable at high starting resistances. At low cell densities the effect was less pronounced (data not shown). The effect of cholesterol on the efficiency of intoxication is in line with a role of cholesterol in clostridial toxin uptake, demonstrating that this effect is not restricted to artificial experimental setups.

Cholesterol-dependent Pore Formation of Toxin A in Black Lipid Membranes—According to the rubidium release assays, it was formerly shown for toxin B that it is able to induce ion-permeable channels in artificial black lipid membranes (BLMs) (31). Again, toxin A failed to form comparable pores in this assay, when bilayers from diphytanoyl phosphatidylcholine/n-decane were used at neutral pH. Based on the finding of cholesterol dependence for toxin A action on target membranes, we investigated the pore-forming capacity of highly purified
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**FIGURE 6.** Pore formation by toxin A in cholesterol-free and cholesterol-containing black lipid membranes. 

**A.** Single-channel recording of a black lipid bilayer membrane made of diphytanoyl phosphatidylcholine in the presence of toxin A from *C*. difficile, purified by thyroglobulin affinity chromatography. 

**B.** Single-channel recording of a black lipid bilayer membrane made of a mixture of diphytanoyl phosphatidylcholine/cholesterol (molar ratio 1:1) in the presence of toxin A. The conductance spikes indicate formation of short-lived channels by toxin A. C. Single-channel recording of a black lipid bilayer membrane made of a mixture of diphytanoyl phosphatidylcholine/cholesterol (molar ratio 1:1) in the presence of toxin A, inactivated by heating to 100 °C. A–C, the aqueous phase contained 1 M KCl, pH 5, at T = 20 °C. The applied voltage was 50 mV. The concentration of toxin A was 1 μg/ml (≈3.2 nM). D, current recording of black lipid bilayer membrane made of oxidized cholesterol in the presence of 0.02 μg/ml (≈65 pm) toxin A. The left side of the recording shows the channel-forming activity of toxin A at pH 6. After about 7.5 min the sensitivity of the current recording was increased by a factor of 10. Two minutes later pH was lowered to pH 5 (right side arrow), which resulted shortly after the pH change in a rapid increase of membrane current by more than a factor of 10. The aqueous phase contained 1 M KCl at T = 20 °C. The applied voltage was 50 mV. Note that oxidized cholesterol facilitated channel formation by toxin A, nS, nanosiemens.

**FIGURE 7.** Effect of PI-PLC treatment of cells on toxin A/B-induced morphological changes. Subconfluent HT-29 cells were treated with PI-PLC (0.1 units/ml) for 60 min at 37 °C followed by washing and cell binding of toxin A (100 ng/ml; ≈300 pm) or toxin B (200 ng/ml; ≈740 pm) on ice for 20 min. After extensive washing, cells were incubated at 37 °C, and cells showing morphological changes were counted at the indicated time points (dotted line, PI-PLC pretreated; solid line, without PI-PLC treatment). Effect of PI-PLC itself on morphological changes after 240 min is also shown ○. The percentage of cells with changed morphology (n = 4, ±S.D.) was determined from 1000 cells. The efficacy of PI-PLC treatment is shown by the inserted bars; treatment of Caco-2 cells with 0.1 units/ml for 60 min (gray bar) led to cleavage of almost 90% of GPI-anchored alkaline phosphatase compared with total surface alkaline phosphatase activity (open bar). AU, absorbance units.

Toxin A in BLMs formed from pure diphytanoyl phosphatidylcholine, from a diphytanoyl phosphatidylcholine/cholesterol mixture, and from oxidized cholesterol. Similarly as described above for pure diphytanoyl phosphatidylcholine/n-decane membranes at neutral pH, no channel formation could be observed in membranes without cholesterol when the pH was lowered to pH 5 and below (data not shown). Fig. 6D shows the absence of pore formation when 1 μg of toxin A (≈3.2 nM) was added to membranes made of pure diphytanoyl phosphatidylcholine at pH 5. However, channel formation was observed at pH 5, when the membrane-forming lipid contained cholesterol. Fig. 6B shows an experiment of this type. 1 μg/ml toxin A (≈3.2 nM) was added to the aqueous phase (1 M KCl, pH 5), bathing a black lipid bilayer membrane made from a diphytanoyl phosphatidylcholine/cholesterol mixture (molar ratio 1:1). The addition of the toxin resulted in the formation of transient channels in the cholesterol-containing membrane, with a maximum single-channel conductance of about 2 nanosiemens. The life time of the channels was about 10–20 ms under these conditions. It is noteworthy that toxin A inactivated by heating to 100 °C did not increase membrane conductance in the lipid bilayer assay when membranes made of diphytanoyl phosphatidylcholine/cholesterol mixture (molar ratio 1:1, see Fig. 6C) or membranes made of oxidized cholesterol were used (data not shown). This result suggested that the conductance increase was caused by active toxin A and was absent when toxin A was inactive.

Formation of transient channels was also observed in membranes formed of oxidized cholesterol/n-decane when 20 ng/ml toxin A (≈65 pm) was added to the aqueous phase at pH 6 (see Fig. 6D), presumably because these membranes facilitate insertion of membrane active components as compared with pure phospholipid membranes (43). However, channel formation was enhanced significantly under these conditions when the pH was lowered to pH 5 by the addition of HCl to the aqueous phase (see Fig. 6D). The conductance of the membranes increased under these conditions at least by a factor of 10. Very often the conductance increase after the lowering of the pH was so high that the membranes made from oxidized cholesterol collapsed. These data demonstrate that similar to toxin B, toxin A is also able to initiate ion-permeable channels in artificial membranes. In contrast to toxin B, toxin A is strictly dependent on the presence of cholesterol in the lipid bilayer assay. These results are in parallel to the results of methyl-β-cyclodextrin treatment in rubidium release assays.

**PI-PLC Treatment of HT-29 Cells**—The involvement of cholesterol in the cellular uptake of toxin A and toxin B is a hint that the toxin receptors may reside in rafts. To test whether the receptors are GPI-anchored structures (carbohydrates or proteins), HT-29 cells were treated with PI-PLC to release GPI-anchored structures from the cell surface. After incubation with 0.1 units of PI-PLC/ml for 60 min at 37 °C, PI-PLC was removed, and the cells were incubated with toxin A (100 ng/ml; ≈300 pm) and toxin B (200 ng/ml; ≈740 pm), respectively, for 30 min on ice to allow binding of the toxins to cells. Unbound toxins were removed by washing, and cell morphology was examined at 37 °C. PI-PLC-treated cells exhibited the identical time course of intoxication and, thus, the same sensitivity to the toxins as non-treated cells (Fig. 7). The efficacy of PI-PLC treatment was checked by determination of the enzyme activity of alkaline phosphatase, which is a GPI-anchored membrane protein (38). Because the expression of alkaline phosphatase on HT-29 cell surface is very poor, cleavage of alkaline phosphatase from Caco-2 cells was tested. After PI-PLC treatment, up to 90% of total alkaline phosphatase activity was found in the soluble fraction. This
control verifies the functionality of PI-PLC treatment under the chosen conditions. These data indicate that GPI-anchored proteins/carbohydrate structures are to be excluded as potential receptors for toxin A and B.

**DISCUSSION**

*Clostridium difficile* toxins A and B are both glucosyltransferases that modify Rh subfamily proteins. To reach their targets, clostridial cytotoxins bind to receptors at the cell surface and enter the cells via receptor-mediated endocytosis (20, 44, 45). Once taken up, two major pathways are known for intracellular transport and translocation of the catalytic moiety into the cytosol of target cells; that is, the retrograde transport and the uptake from acidic endosomes (27, 46).

The retrograde transport from endosomal compartments via the Golgi apparatus to the endoplasmic reticulum is described, e.g. for cholera toxin (25, 26). In this case, the catalytic domain enters the cytosol from the endoplasmic reticulum. The translocation directly from acidic endosomes is described for diphtheria toxin and many other toxins (28–30, 47). The acidification of the endosomal lumen initiates a conformational change of the toxins by which the hydrophobic region is believed to become surface-exposed. This allows membrane insertion and subsequent toxin translocation. In the case of toxin B, an increase in hydrophobicity induced by acidic pH was described (48).

It was recently reported that toxin B, but not toxin A, causes pore formation in CHO, HeLa, and Vero cells (31). Considering the primary action of toxin A as an enterotoxin, we focused on human cell lines derived from colon carcinoma and compared the effects of toxin A and toxin B in HT-29 and CaCo-2 cells. A well established method to measure pore-forming capacity of bacterial cytotoxins is the mimicry of the endosomal environment by lowering the extracellular pH after toxin binding to the cells at low temperature. The pore formation in plasma membranes upon acidification, measured by $^{86}$Rb$^+$ efflux, may reflect processes related to molecular mechanisms during toxin translocation. In fact, there are several reports on pH-dependent channel formation of toxins taken up from acidic endosomes (49–54). As mentioned above, induction of a conformational change by low pH is believed to expose hydrophobic regions, which are located intramolecularly at neutral pH, allowing binding on and insertion into lipid bilayers.

Here we report for the first time that cell membrane-bound toxin A is capable of forming ion-permeable channels upon acidification. Using a highly susceptible cell line (HT-29 cells) derived from human colon carcinoma, $^{86}$Rb$^+$ ions are released from preloaded cells only under acidic conditions. The EC$_{50}$ value was in the range of ~240 pm toxin A. A significant induction of membrane pores is detectable at 100 pm toxin A. This is a concentration (around 30 ng/ml) commonly used for toxin A intoxication assays. These relatively low concentrations are in line with a high affinity binding of toxin A to the corresponding cells and argue against specific effects emerging from over-saturation with the toxin under *in vitro* conditions. The optimal pH for most efficient pore formation was at pH 5. No rubidium release could be observed at pH ≥ 5.6. This pH optimum is consistent with observations for toxin B, where pH 5 but not pH 5.5 induces the increase in hydrophobicity, as described by Qa’dan et al. (48). Toxin B also forms pores at a pH ranging from pH 4.5 to 5.2 (31). The application of recombinant and highly purified native toxin A obtained from a thyroglobulin affinity column in the $^{86}$Rb$^+$ release assay indicates that the ion efflux is exclusively due to toxin A action. Therefore, effects caused by contaminating proteins can be largely excluded (55). In line with a specific action of toxin A is the finding that anti-toxin A antibodies inhibited pore formation.

A tripartite structure of clostridial glycosylating toxins has been proposed (15, 56). The C-terminal binding and translocation domains of toxins A and B are functionally separated from the N-terminal catalytic domain (17). Therefore, catalytic activity should play no role in pore formation. This was corroborated by the use of an enzyme deficient mutant (DXD mutant) of recombinant toxin A (39). Increase in rubidium release by this mutant protein indicates that pore formation is functionally independent of glucosyltransferase activity. Furthermore, it demonstrates the functional dissociation of channel formation and typical cytotoxic effects based on actin depolymerization.

Pore-forming capacity of toxin A was also observed in CaCo-2 cells, although signal strength was less pronounced (data not shown). This finding is in contrast to earlier reports by Barth and coworkers (31), who did not observe increased $^{86}$Rb$^+$ efflux in CaCo-2 or HT-29 cells in the presence of either toxin. We observed that the susceptibility of the epithelial cell lines partially depends on their growth and differentiation state, which may explain these discrepancies. In accordance with the mentioned report, toxin A failed to induce ion-permeable channels in other cell lines tested like CHO, HeLa, and Vero cells. The explanation for this may be that “physiological” toxin entry is not associated with detectable pore formation. For detection, a surplus of toxin is necessary to allow formation of a lot of pores. In the case of modestly sensitive cells, cellular toxin receptors are saturated with toxin A under physiological concentrations, and a further increase of toxin A concentration has no effect. Taken together, at least for epithelial cells derived from human intestine, which are the primary targets of the toxins, channel formation upon acidification seems to be a general mechanism induced by both toxins.

The influence of cholesterol on pore formation by toxins A and B is an important novel finding of the present report. We tested the influence of methyl-$eta$-cyclodextrin, a cholesterol-sequestering agent (41, 42), on pore formation by toxin A in HT-29 cells. A concentration-dependent reduction in channel formation by toxin A was observed with increasing amounts of methyl-$eta$-cyclodextrin. A complete inhibition of pore formation by the toxin was achieved at ≥2.5 mM methyl-$eta$-cyclodextrin. Similar results were obtained with toxin B. Repletion of cholesterol by incubation with cholesterol-saturated methyl-$eta$-cyclodextrin solution confirmed that the loss of cholesterol was responsible for reduced membrane permeabilization. In line with these findings is the inhibitory effect of methyl-$eta$-cyclodextrin on the efficiency of cell intoxication, measured by the decrease of TER.

The cholesterol dependence of toxin A/B cell entry prompted us to test the putative involvement of rafts and especially of the thereby residing GPI-anchored carbohydrates or proteins. GPI-anchored structures were released by PI-PLC treatment of cells, but this treatment had no effects on kinetics of toxin A- and B-induced cell rounding. Because the efficacy of PI-PLC treatment was validated by the release of the GPI-anchored alkaline phosphatase, GPI-anchored structures can be excluded as potential receptors for toxin A as well as for toxin B.

The *in vitro* experiments presented here demonstrate that cholesterol has a major impact on toxin A-induced channel formation in artificial bilayers. Channel formation in lipid bilayers was only observed with active toxin. Without cholesterol no channel formation was observed in lipid bilayers from pure lipids even at very high toxin concentration or low pH. The addition of cholesterol to the lipid used for membrane formation or the use of lipid bilayers from oxidized cholesterol enhanced channel formation, in particular when the pH was lowered to pH 5. Thus, we observed an absolute requirement of cholesterol for formation of ion-permeable channels in artificial membranes induced by toxin A. It is noteworthy that toxin A seems to act differently in this respect than toxin B, since toxin B inserts and forms pores in artificial bilayers without the requirement for cholesterol (31). How-
ever, low pH applied in the lipid bilayer assay accelerated channel formation for both toxins (see above). Furthermore, both toxins formed transient channels with a short lifetime of milliseconds (31). Channels formed by toxin A in lipid bilayers had a single-channel conductance of about 1 to 2 nanosiemens in 1 M KCl. This result suggests that toxin A-induced channels have approximately the same dimensions as channels formed by porins (57) or by the binding components of C2 and anthrax toxins (53, 58). It is noteworthy that toxin A and toxin B insert into artificial bilayers in the absence of any specific receptors. Also many other membrane active toxins do not need special receptors for the interaction with lipid bilayers (59, 60).

The precise molecular mechanism by which cholesterol affects insertion and pore formation is not known so far. Partial depletion of cholesterol with methyl-cyclodextrin allows no discrimination between direct or indirect effects of cholesterol. Although inhibition of toxin A-induced $^{86}$Rb$^+$ efflux was complete after cholesterol depletion by methyl-cyclodextrin, it is difficult to discriminate between a "specific" effect of cholesterol in membrane insertion from a modifying effect of cholesterol depending on increase in membrane fluidity and/or lateral segregation of lipids and formation of microdomains. Besides maintaining the membrane fluidity, cholesterol facilitates the conversion of lamellar lipid structure to inverted hexagonal structures (61), which may have a facilitating role in insertion of transmembrane peptide structures (62). Whether cholesterol directly influences C. difficile toxin activity, e.g. induction of conformational changes by direct binding has to be studied in more detail. However, the influence of methyl-cyclodextrin on $^{86}$Rb$^+$ release by both clostridial toxins is a clear indication that cholesterol dependence is a general feature of pore formation by the toxins.

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