Amino Acid Residues Involved in Membrane Insertion and Pore Formation of *Clostridium botulinum* C2 Toxin[†]

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ABSTRACT: The actin-ADP-ribosylating *Clostridium botulinum* C2 toxin consists of the enzymatic component C2I and the binding component C2II. C2II forms heptameric channels involved in translocation of the enzymatic component into the target cell. On the basis of the heptameric toxin channel, we studied functional consequences of mutagenesis of amino acid residues probably lining the lumen of the toxin channel. Substitution of glutamate-399 of C2II with alanine blocked channel formation and cytotoxicity of the holotoxin. Although cytotoxicity and rounding up of cells by C2I were completely blocked by exchange of phenylalanine-428 with alanine, the mutation increased potassium conductance caused by C2II in artificial membranes by about 2−3-fold over that of wild-type toxin. In contrast to its effects on single-channel potassium conductance in artificial membranes, the F428A mutation delayed the kinetics of pore formation in lipid vesicles and inhibited the activity of C2II in promoting $^{86}Rb^+$ release from preloaded intact cells after pH shift of the medium. Moreover, F428A C2II exhibited delayed and diminished formation of C2II aggregates at low pH, indicating major changes of the biophysical properties of the toxin. The data indicate that phenylalanine-428 of C2II plays a major role in conformational changes occurring during pore formation of the binding component of C2II.

Clostridium botulinum C2 toxin is a member of the family of binary actin-ADP-ribosylating toxins that include *Clostridium perfringens* iota toxin (*1*), *Clostridium spiroforme* toxin (*2*), *Clostridium difficile* ADP-ribosyltransferase (*3, 4*), and the vegetative insecticidal peptide (VIP) from *Bacillus cereus* (*5*). All of these toxins are composed of an enzymatic component and a binding/translocation component, which are expressed as separate proteins (*6*). *C. botulinum* C2 toxin consists of an ∼50 kDa enzymatic component C2I harboring the ADP-ribosyltransferase activity and an ∼80 kDa binding and translocation component C2II (*7*). The binding component is activated by proteolytic cleavage of a 20 kDa N-terminal fragment. The active 60 kDa part of C2II then forms SDS-stable heptamers that bind to the surface of target cells through receptors (*8*), which were identified as asparagine-linked complex/hybrid carbohydrates (*9*). The enzymatic component C2I binds to the C2II heptamers followed by endocytosis of the toxin-receptor complex. At low endosomal pH of endosomes C2II heptamers insert into membranes and form channels, allowing translocation of the enzymatic component C2I into the cytosol (*8, 10*). Once in the cytosol, C2I ADP-ribosylates nonmuscle G-actin (*11*). ADP-ribosylated actin is unable to polymerize (G-actin trapping) but can bind to the barbed ends of actin filaments to act as a capping protein (*12*). Trapping and capping causes depolymerization of the actin cytoskeleton and cell rounding.

The C2II binding component is structurally similar to the PA binding component of anthrax toxin (*13–15*). Both binding components, C2II and PA, are comprised of four domains. In PA, domain 1 is involved in interaction with the enzymatic components (lethal factor or edema factor), domain 2 in pore formation, domain 3 in oligomerization, and domain 4 in receptor binding. PA also forms ring-shaped heptamers, which are responsible for the delivery of the enzymatic components of anthrax toxin into the cytosol (*14–17*).

Based on a large array of biochemical data (*8, 18*), the crystal structures of C2II (*19*) and PA (*17*), and models of the heptameric toxin components, amino acid residues putatively lining the channel lumen have been identified. Here we studied functional properties of residues glutamate-399, aspartate-426, and phenylalanine-428 on the ion conductance of C2II in artificial membranes, on pore formation in vesicles and intact cells, on aggregate formation after acidification, and on cytotoxicity in CaCo-2 cells.

EXPERIMENTAL PROCEDURES

Materials. Dulbecco's minimum Eagle's medium (MEM) and Dulbecco's MEM cell culture medium were from Biochrom (Berlin, Germany). McCoy's 5A medium and fetal

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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. Diphytanoylphosphatidylcholine (DiphPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Trypsin and thrombin were purchased from Sigma (Deisenhofen, Germany), and trypsin inhibitor from soybean was from Roche Diagnostics (Mannheim, Germany). The QuikChange site-directed mutagenesis kit and the Pfu polymerase were purchased from Stratagene (Heidelberg, Germany). Oligonucleotides were obtained from Sigma Genosys. The pGEX-2T vector and glutathione−Sepharose 4B were included in the glutathione *S*-transferase (GST) gene fusion system from Pharmacia Biotech (Uppsala, Sweden). Polymerase chain reactions were performed with a Mastercycler gradient from Eppendorf (Hamburg, Germany).

Construction of C2II Mutants. Point mutations within C2II were engineered using the QuikChange mutagenesis method (Stratagene). Based on the plasmid sequence of pGEX-2T-C2II (*18*), C2II-specific primers were designed, resulting in following amino acid substitutions: F428A, F428W, F428Y, F428D, D426A, and E399A. Following PCR (performed according to the manufacturer's instructions of the QuikChange mutagenesis kit), the methylated DNA was digested by the addition of 1 μL of *Dpn*I (10 units/μL) for 3 h at 37 °C and used to transform chemical competent *Escherichia coli* TG1 cells. Plasmid DNA was prepared using standard procedures and checked by DNA sequencing.

Cell Culture and Cytotoxicity Assay. CaCo-2 and embryonic bovine lung (EBL) cells were cultivated in Dulbecco's minimum Eagle's medium, HT-29 cells were cultivated in McCoy's 5A, and HeLa cells were cultivated in Dulbecco's modified Eagle's medium. All media contained 2 mM L-glutamate, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were routinely kept in tissue culture flasks at 37 °C and 5% CO_2 and were trypsinized and replated twice per week. For cytotoxicity experiments, cells were replated in 12-well dishes. After washing with phosphate-buffered saline the medium was changed to serum-free Dulbecco's MEM. The cells were incubated with 250 ng/mL C2I and with 500 ng/mL wild-type and/or mutant C2II for up to 3 h at 37 °C. Pictures were taken with an AxioCam HRC camera and Axio Vision software (Carl Zeiss AG, Oberkochen, Germany).

Expression, Purification, and Activation of Recombinant Proteins. C2I and mutant and wild-type C2II were expressed in *E. coli* BL21(DE3) as recombinant glutathione *S*-transferase fusion proteins and purified by affinity chromatography with glutathione−Sepharose 4B according to the manufacturer's instructions (*20*). Glutathione *S*-transferase was cleaved off by thrombin (3.25 NIH units/mL of bead suspension) followed by inactivation of thrombin utilizing benzamidine beads. C2II was activated with 0.2 μg of trypsin/μg of protein for 30 min at 37 °C (*8*). The active form of C2II was used in all experiments shown. Only for production of wild-type and mutant heptamer mixtures full-length wild-type and F428A C2II (each 100 ng/mL) were mixed in a ratio of 1:6, 2:5, 3:4, 4:3, 5:2, or 6:1. After mixing proteins were activated with trypsin. Proteins were analyzed by SDS−PAGE. Wild-type and F428A C2II exhibited the same susceptibility to trypsin treatment, indicating no major changes in the overall structure of the proteins (see Supporting Information Figure S1).

Transepithelial Resistance (TER) Assay. For the TER assay, CaCo-2 cells were plated on Falcon cell culture inserts and incubated 5−7 days with medium exchange every 3 days. Assays were performed when TER values reached ~1800−2000 ohms·cm^2. TER was determined using a resistance system for electrophysiological readings of filter cups (Endohm-12; World Precision Instruments, Sarasota, FL). To avoid a drastic drop in starting TER, all washing and incubation steps were carried out using medium supplemented with 10% fetal calf serum. Wild-type and/or mutant activated C2II (500−200 ng/mL) and C2I (250−100 ng/mL) were applied to the upper compartment of the FalconTM cell culture inserts, and the resulting decrease in TER was monitored for up to 10 h.

In Vivo Pore-Forming Assay. $^{86}Rb^+$ release experiments were performed as described recently (*21*). HT-29 cells were plated in complete medium containing $^{86}Rb^+$ (1 μCi/mL) at a density of ~2 × 10^5 cells/well in 24-well cell culture plates. After 48−72 h cells were washed twice with phosphate-buffered saline and cooled to 4 °C. Afterwards, fresh medium (4 °C, without serum) containing wild-type or mutant activated C2II was added. Proteins were allowed to bind for 60 min at 4 °C, followed by washing twice with cold medium to remove unbound toxin. To initiate membrane insertion of the toxins, warm medium (37 °C; without serum, pH 5.0−7.5) was applied, and cells were incubated for 5 min at 37 °C. After a shift back to 4 °C, incubation was continued for 40 min at low temperature to allow $^{86}Rb^+$ efflux. Aliquots of the medium were removed, and $^{86}Rb^+$ release was determined by liquid scintillation counting.

Liposome Preparation and K^+ Release Assay. Liposomes were prepared as described (*28*). Briefly, POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) in chloroform (20 mg/mL) was dried under N_2 gas to form a lipid film, followed by vacuum for 3 h to remove residual solvent. The dried lipid film was rehydrated with K^+ buffer (150 mM KCl and 10 mM HEPES, pH 7.4) to form multilamellar vesicles and then subjected to three freeze−thaw cycles and extrusion through a polycarbonate filter of 200 nm pore size (Nucleopore Inc., Pleasanton, CA) in a miniextruder (Avanti Polar Lipids, Alabaster, AL). Size-exclusion chromatography and cryoelectron microscopy were used to check the quality of the prepared large unilamellar vesicles. The majority of liposomes were unilamellar, with diameter ~150−200 nm (not shown). Afterward liposomes were transferred into 150 mM NaCl and 20 mM Tris-HCl, pH 8.5, by buffer exchange, as described (*19*). The liposomes were added to K^+-release buffer (50 mM NaOAc and 150 mM NaCl, pH 5.0) and after 1 min wild-type and/or mutant C2II (3 μg/mL) was added. K^+ release was monitored under constant stirring by using a K^+-selective electrode (Orion Research, Scottsdale, AZ).

Black Lipid Bilayer Experiments. The method used for preparation of black lipid bilayers has been described previously (*22*). The experimental setup consisted of a Teflon chamber divided into two compartments by a thin wall and connected by a small circular hole with a surface area of about 0.4 mm^2. The aqueous solutions on both sides of the membrane were buffered with 10 mM MES−KOH to pH 6. Membranes were formed by spreading across the hole a 1%

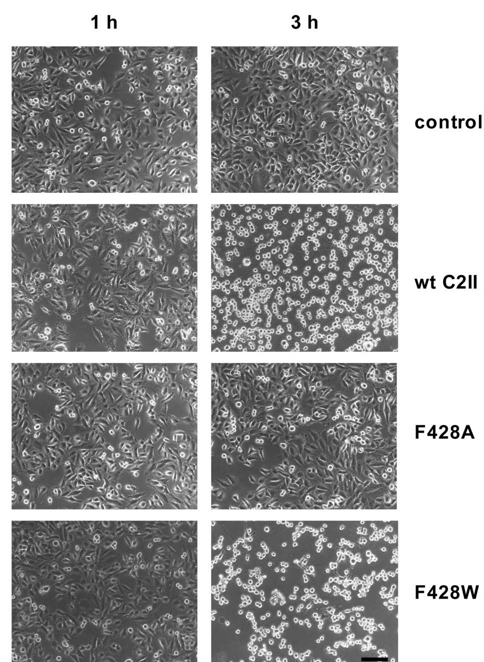

FIGURE 1: Effect of C2II F428 mutants on cytotoxicity. HeLa cell monolayers were washed with PBS and thereafter incubated with 250 ng/mL C2I and with 500 ng/mL wild-type or mutant C2II in serum-free medium at 37 °C. After 1 and 3 h of toxin treatment, pictures were taken. Scale bar represents 200 μm.

solution of diphytanoylphosphatidylcholine dissolved in *n*-decane. After the membranes had turned black, wild-type or mutant activated C2II were added to the cis side of the membrane. The temperature was maintained at 20 °C during experiments. Membrane conductance was measured after application of a fixed membrane potential by using a pair of Ag/AgCl electrodes with salt bridges inserted into the aqueous solutions on both sides of the membrane. Electrodes were connected in series to a voltage source and a homemade current amplifier made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart recorder (Rikadenki, Freiburg, Germany).

Aggregation Studies. Activated wild-type C2II or F428A (each 3 μg, in a volume of 50 μL) was added to 500 μL "pH 8.5 buffer" (20 mM Tris and 150 mM NaCl) or to "pH 5.0 buffer" (20 mM MES and 150 mM NaCl) at room temperature with continuous stirring. Formation of aggregates was monitored by measurement of the increasing absorbance (light scattering) at 600 nm over time with a PerkinElmer LS 50 B luminescence spectrometer.

RESULTS

Effects of C2II Phe-428 Mutants on Cytotoxicity. It has been suggested that Phe-427 of PA, which lines the lumen of the PA channel, has a phi-clamp function that is involved in translocation of the enzymatic components EF and LF of anthrax toxin (*23*). Here, we studied the role of the analogous residue Phe-428 in C2II, which is also proposed to be located in the channel formed by the heptameric C2II. Treatment of epithelial cell monolayers with C2 toxin causes rounding up of cells and destruction of the monolayer. Figure 1 shows that wild-type C2II caused significant rounding up of cells within 3 h at a concentration of 250 ng/mL C2I and 500 ng/mL C2II. By contrast no effect was observed with C2I plus the F428A mutant of C2II. We also made several other conservative mutations in position 428 of C2II. The F428W mutant induced cell rounding up in the presence of C2I after 3 h of treatment, while intoxication by the F428Y mutant was slightly reduced (data not shown).

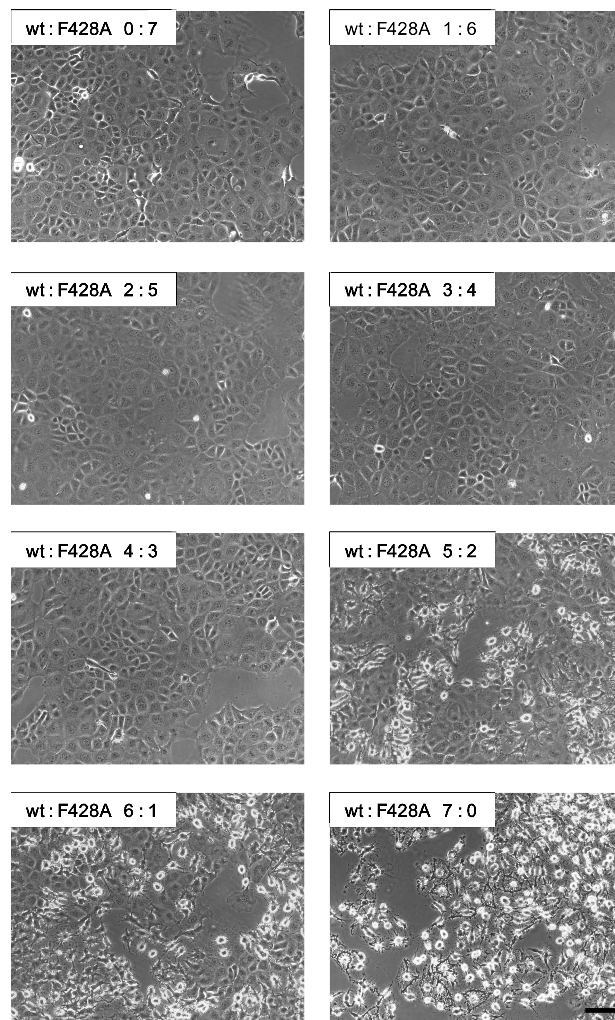

FIGURE 2: Cytotoxicity of C2II heteroheptamers. Monomeric wild-type C2II was mixed with C2II F428A in a ratio of 0:7, 1:6, 2:5, 3:4, 4:3, 5:2, 6:1, and 7:0. After mixing proteins were activated with trypsin. EBL cell monolayers were incubated with 250 ng/mL C2I and 500 ng/mL wild-type C2II or C2II F428A in serum-free medium at 37 °C. After 3 h of toxin treatment, pictures were taken. Scale bar represents 200 μm.

Next we studied intoxication of cells with mixtures of wild-type C2II and the F428A mutant. Because activated C2II forms heptamers, we stepwise increased the ratio of the mutant F428A protein to the wild-type binding component in the heptamers from zero (only wild-type C2II) to seven (only mutant C2II). This was done by mixing the respective amounts of full-length wild-type and mutant proteins followed by activation of the protein mixture in the presence of trypsin, which immediately induces formation of the ring-shaped heptamers (*8*). When 1 or 2 parts of the mutant F428A were mixed with 6 or 5 parts of wild-type toxin component, cells were intoxicated in the presence of the enzymatic component C2I to the same degree as observed with the fully wild-type C2II (Figure 2). However, when 3 or more parts of the mutant protein were mixed with wild-

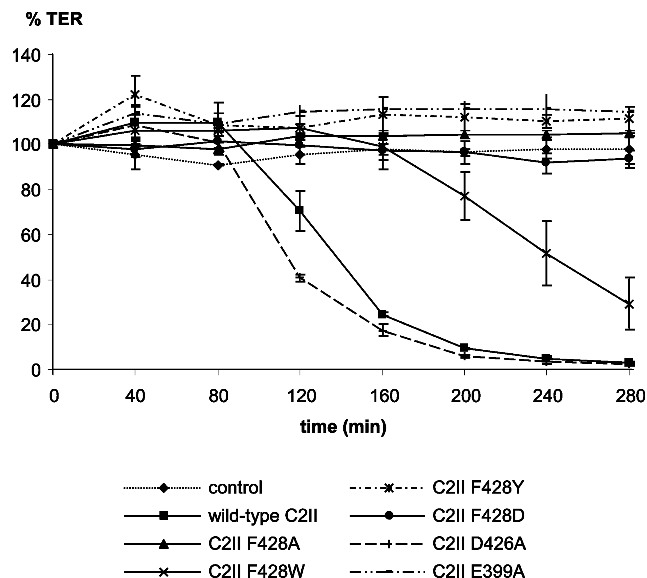

FIGURE 3: Intoxication of human colon carcinoma (CaCo-2) cells with wild-type and mutant C2II. CaCo-2 cells were grown to confluency on a semipermeable membrane until TER reached about 1800−2000 ohms·cm^2. C2I (100 ng/mL) and activated wild-type or mutant C2II (200 ng/mL) were applied to the apical reservoir. Reduction of TER due to cell rounding over time (min) served as a parameter for intoxication. Results are from mean values of at least three independent experiments ± SD and are shown as percent of starting TER (% TER).

type C2II, the resultant heptameric complex was inactive and was unable to elicit intoxication in the presence of C2I.

Decrease in Transepithelial Electrical Resistance Caused by C2 Toxin. Because rounding up of cells is a relatively late event for determination of cell toxicity, we studied the effects of C2 toxin on the transepithelial electrical resistance in CaCo-2 cell monolayers. In the monolayer assay, a decrease in TER is a readily quantifiable early event that is highly sensitive for the activity of the toxin. Wild-type C2II (200 ng/mL) and C2I (100 ng/mL) essentially abolished TER within 240 min with a half-time of 140 min (Figure 3). We compared the effects of various Phe-428 mutants with that of the wild-type toxin. F428A and F428D induced no change in TER even after 5 h of incubation. F428W exhibited a reduced effect, with a prolonged half-time of 240 min. F428Y showed an even slower rate of intoxication, a decrease in TER after 7 h (data not shown). Additional mutations were made in sequences adjacent to Phe-428. D426A reduced TER to a similar extent as the wild-type toxin, while the double mutant (D426A, F428A) was not able to intoxicate cells or reduce TER. In addition, another mutant of C2II, E399A, was not able to reduce TER.

Pore Formation in Artificial Membranes and Intact Cells. It has been reported that the C2II binding component causes pore formation in artificial membranes and intact cells (*8, 24*). To study the effects of the exchange of Phe-428 to alanine on pore formation in black lipid membranes, we applied activated binding component consisting of different ratios of wild-type and mutant proteins (Table 1). The heptameric binding component with seven wild-type proteins caused a conductance of 40 pS. Fractional increase of the F428A mutant in C2II increased the conductance in a stepwise manner until 140 pS for the full F428A mutant

Table 1: Single-Channel Conductance of Pores Formed by Mixtures of C2II WT and C2II F428A[a]

C2II/C2II F428A ratio	*G* (pS)
C2II F428A	140 ± 18
C2II WT/C2II F428, 1/6	118 ± 16
C2II WT/C2II F428, 2/5	101 ± 20
C2II WT/C2II F428, 3/4	98 ± 20
C2II WT/C2II F428, 4/3	73 ± 17
C2II WT/C2II F428, 5/2	72 ± 20
C2II WT/C2II F428, 6/1	58 ± 12
C2II WT	40 ± 3

[a] Single-channel conductance was determined in 150 mM KCl buffered to pH 6 (10 mM MES−KOH). The membranes were formed of diphytanoylphosphatidylcholine dissolved in *n*-decane. The applied voltage was 20 mV, and temperature was kept at 20 °C. The average single-channel conductance was calculated from at least 100 single events, and the standard deviation is indicated.

heptamer. This increase in conductance is in line with that found in the respective studies with the F427A mutant in PA (*23*).

Next, we studied the effects of the exchange of Phe-428 of C2II on pore formation induced by the binding component in HT-29 cells. To this end, the cells were preloaded overnight with $^{86}Rb^+$ ions prior to treatment with the activated binding component on ice. Subsequently, a pH shift of the culture medium mimicking the acidic pH of endosomes allowed the insertion of the heptameric binding component into the membrane, followed by pore formation and $^{86}Rb^+$ ion release. As expected, wild-type C2II caused an increase in the release of $^{86}Rb^+$ ions (Figure 4A). F428W, which behaved similarly to the wild-type toxin in the TER assay, also induced similar $^{86}Rb^+$ ion release. F428Y, which was less efficient than wild-type toxin in inducing the rounding up of cells and reduction in TER, was able to cause $^{86}Rb^+$ release. However, the mutant F428A did not induce $^{86}Rb^+$ release under similar conditions. This was also true for the double mutant D426A, F428A, although the single mutant D426A still caused $^{86}Rb^+$ release. E399A, which was not able to intoxicate cells, likewise did not cause $^{86}Rb^+$ release. Although these results were in disagreement with pore formation observed in artificial membranes, where the F428A mutant was found to actually increase conductance as compared to wild-type C2II, we were able to detect a small $^{86}Rb^+$ release induced by the F428A mutant when the incubation time at 37 °C was extended from 5 to 15 min (Figure 4B).

As data with black lipid membranes were obtained with K^+ ions as charge carrier and pore formation in intact cells was done with Rb^+ ions, we repeated the studies in artificial membranes in the presence of Rb^+ ions. The results demonstrated that C2II mutants formed stable channels with the recordings of both wild-type and mutant proteins showing the typical step-like characteristics (see Figure 5) caused by the superposition of the long-lived C2II channels forming stable heptamers in the membranes (*24, 25*). Some of the mutants had the same single-channel conductance as wild-type C2II (see Table 2). For others, it differed considerably from that of wild-type C2II (50 pS), ranging from 10 to 130 pS in 150 mM RbCl and 10 mM MES−KOH, pH 6 (20 mV applied voltage). The changes appeared to be dependent on the size, properties, and position of the amino acid introduced in exchange of the original one. As was observed with KCl, the F428A mutant caused a considerable increase

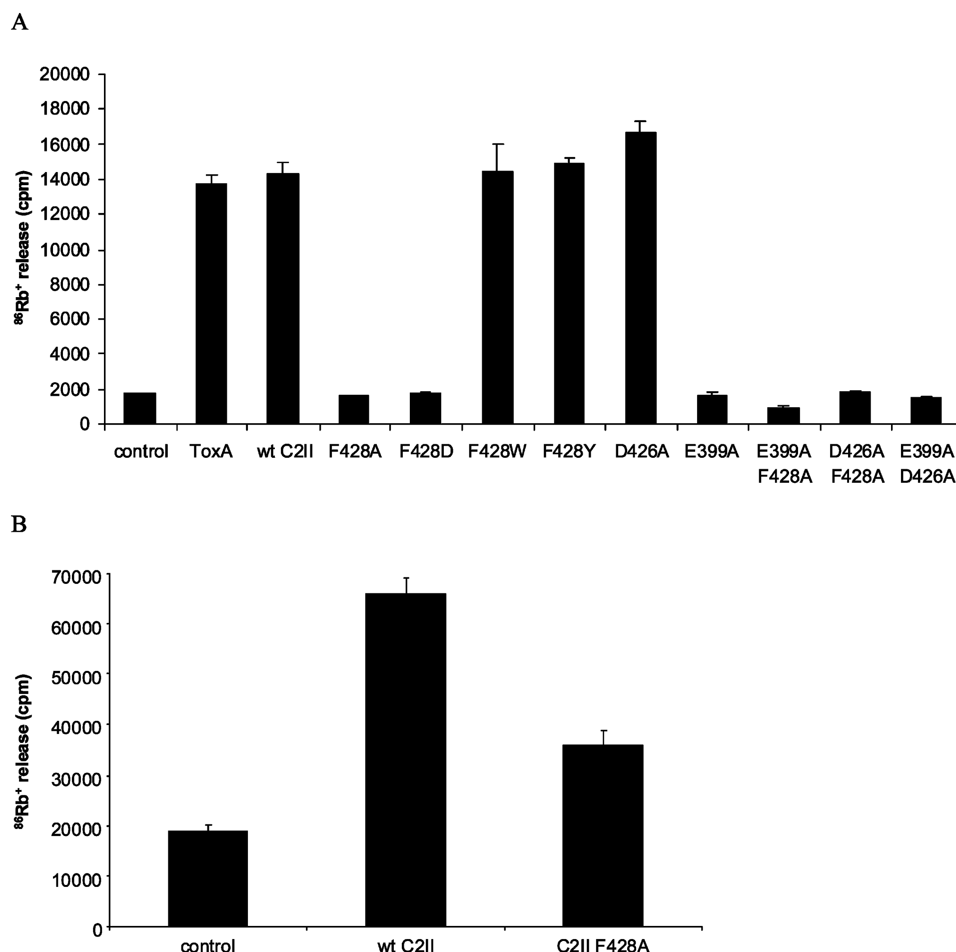

FIGURE 4: Comparison of pore formation by wild-type and mutant C2II in HT-29 cells. Cell monolayers in 24-well culture plates were preloaded with $^{86}Rb^+$ (1 μCi/mL) for 48−72 h and thereafter washed twice with PBS. Subsequently, wild-type and mutant C2II (1 μg/mL) was allowed to bind at 4 °C for 1 h (no protein as control; ToxA from *C. difficile* as positive control). Cells were exposed for 5 min (A) or 15 min (B) to an acidic shift (pH 5.0) at 37 °C to initiate membrane insertion of C2II. After an additional incubation of 40 min at 4 °C, the radioactivity in the medium was measured by using liquid scintillation counting.

in conductance in the presence of Rb^+ ions from 50 to 130 pS in black lipid membranes. Alanine substitution mutagenesis of the negatively charged amino acids E399 and D426 also had a substantial impact on the structure of the C2II heptamers and resulted in a strong decrease the single-channel conductance. However, whereas the E399A mutant still formed defined channels, the D426A mutant exhibited a broad distribution of single events probably indicating a disturbed structure of the heptamers (*26*).

Potassium Ions Release from Lipid Vesicles. To study the kinetics of pore formation in more detail, we chose the model of K^+ release from lipid vesicles. To this end, lipid vesicles were preloaded with potassium chloride, and release of K^+ ions from vesicles was determined by using a potassium electrode. A time course of this experiment is shown in Figure 6. Wild-type C2II and the F428W and F428Y mutants exhibited almost the same initial rates of K^+ ions release, although the mutants did not reach the same maximal level. In contrast, release of K^+ ions induced by F428A was significantly delayed, and the maximal level was much lower than that observed with wild-type C2II or the other mutants. These findings are in agreement with the observed reduced release of Rb^+ ions after prolonged incubation with the F428A mutant.

Aggregation of C2 Toxin at Low pH. One of the crucial steps in the translocation of binary toxins is the low pH-dependent insertion of the binding component into membranes. It is suggested that in analogy to PA this process depends on major changes in the overall structure of the binding component C2II with rearrangement of the $2\beta2-2\beta3$ loop of each monomer to form the 14-stranded β-barrel structure of the heptamers, which then insert into the membrane (*19*). To analyze whether the exchange of Phe-428 to alanine has any major consequences for the extent and dynamics of the acid-induced structural rearrangements of C2II, we studied the aggregation of the binding component upon acidification. Figure 7 shows that substitution of Phe-428 with alanine reduced the acid-induced aggregation of C2II, indicating major physicochemical changes in the properties of the toxin.

DISCUSSION

Here, we studied the role of Phe-428 in C2II, which is proposed to be located in the channel formed by the heptameric C2II (*26, 27*). We found that exchange of Phe-428 with alanine in C2II blocked the cytotoxicity of the C2 toxin in the cell morphology assay as well as in the TER assay. The exchange of Phe-428 with tryptophan slightly delayed the intoxication process, but full intoxication was eventually observed. Exchange of phenylalanine with tyrosine further reduced cytotoxicity with cell intoxication

FIGURE 5: Current recordings of diphytanoylphosphatidylcholine/*n*-decane membranes of channels formed by wild-type C2II and its mutants. The proteins were added to the cis side of different membranes in a concentration of about 10 ng/mL about 5 min before the start of the recordings. The aqueous phase contained 0.15 M RbCl and 10 mM MES−KOH (pH 6). The applied membrane potential was 20 mV; T = 20 °C. The traces show individual single-channel recordings of the C2II mutants as indicated. Please note that the current/conductance scales are different for wt C2II and the mutants.

Table 2: Average Single-Channel Conductance, G, of Pores Formed by C2II WT and Mutants in Different Aqueous Salt Solutions[a]

wild-type and mutant C2II	G (pS) in 150 mM KCl	G (pS) in 150 mM RbCl
E399A	13 ± 2	18 ± 2
D426A	20 ± 8	
F428A	140 ± 18	130 ± 9
F428D	110 ± 22	110 ± 7
F428Y	60 ± 5	75 ± 5
F428W	5 ± 1	10 ± 1
E399A, D426A	14 ± 2	30 ± 2
E399A, F428A	60 ± 4	80 ± 5
D426A, F428A	40 ± 2	40 ± 2
E399A, D426A, F428A	30 ± 4	30 ± 1

[a] The aqueous salt solution had a concentration of 150 mM and was buffered to pH 6 with 10 mM MES−KOH. The membranes were formed of diphytanoylphosphatidylcholine dissolved in *n*-decane. The applied voltage was 20 mV, and temperature was kept at 20 °C. The average single-channel conductance was calculated from at least 100 single events, and the standard deviation is indicated. Note that the channels formed by the mutant D426A displayed such a broad distribution of conductance that it was impossible to provide a reasonable average value.

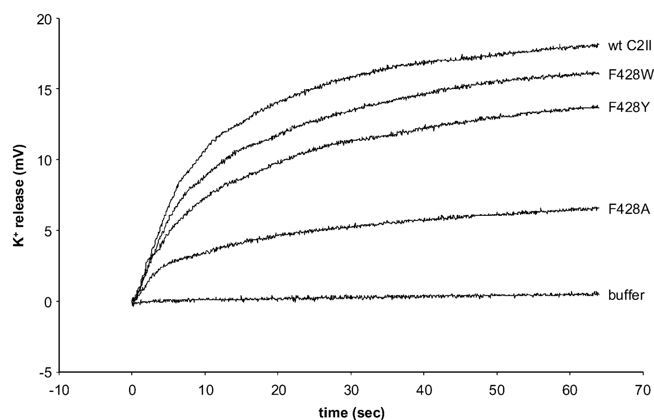

FIGURE 6: Effects of F428 mutations on the permeabilization of liposomal membranes by C2II. Liposomes containing 150 mM KCl and 10 mM HEPES, pH 7.4, were added to K^+ release buffer (50 mM NaOAc and 150 mM NaCl, pH 5.0), and after 1 min wild-type or mutant C2II (3 μg/mL) was added. The solution was stirred continuously with a magnetic stirrer, and K^+ release was monitored with a K^+-selective electrode. Buffer was used as control.

observed only after prolonged incubation. These data indicated that Phe-428 has a pivotal role in C2II intoxication similar to that found for the analogous residue Phe-427 in PA of anthrax toxin. This was further supported by findings obtained by using varying ratios of F428A present in the C2II complex. When more than two subunits, on average, in the C2II heptamer contained the F428A mutation, cyto-

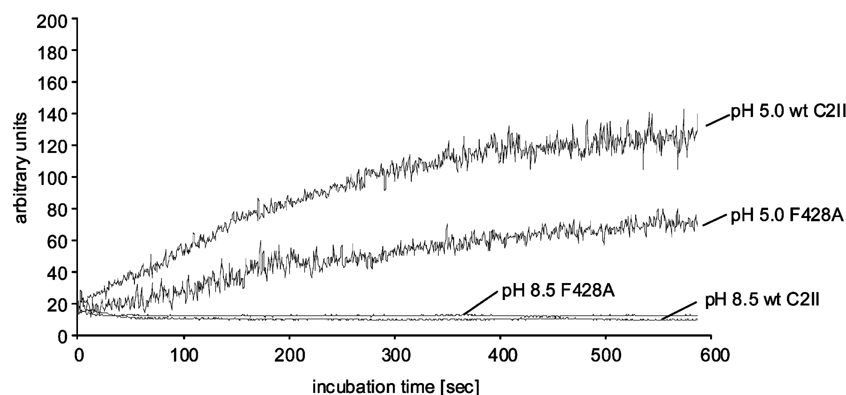

FIGURE 7: Aggregation properties of C2II induced at low pH. Activated wild-type or F428A C2II (3 μg each) was added to pH 8.5 buffer (20 mM Tris and 150 mM NaCl) or to pH 5.0 buffer (20 mM MES and 150 mM NaCl) at room temperature with continuous stirring. The intensity of scattered light at 600 nm was monitored in a time course with a LS 50 B luminescence spectrometer and was expressed as arbitrary units.

toxicity was significantly reduced, demonstrating a strong dominant-negative effect of this mutation.

The influence of the exchange of Phe-427 on pore formation in PA was previously studied in artificial vesicles, showing that a change from phenylalanine to alanine caused an increase in the conductance of K^+ ions, which is in agreement with the reduced size of the side chain of alanine as compared to phenylalanine. This increase in conductance is accompanied by a reduced translocation of the enzymatic components through the pore, because the chaperone-like phi-clamp functions might require tight interaction with the protein to be transported (*23*). A similar result was obtained in our studies with the F428A mutant of C2II in artificial black lipid membranes. The conductance of the C2II pore was increased in the mutant. The causal role of phenylalanine in conductance could be readily observed, when the proportion of F428A was stepwise increased. This also resulted in a stepwise increase in the conductance from 40 to 140 pS in the presence of K^+. In contrast, mutation of the residue Glu-399, which is putatively exposed to the water-filled vestibule of the channel and is thought to control ion flux in artificial membranes (*26*), resulted in a pronounced decrease in the single-channel conductance of C2II in KCl or RbCl, presumably because seven negatively charged residues within the vestibule of the channel were replaced with neutral residues. The ion conductance of the mutant D426A was likewise reduced, but its single-channel conductance displayed a wide range of values in contrast to the other mutations. It has been suggested that this may be caused by an undefined structure of the mutant C2II heptamer (*26*).

The suggested essential role of Glu399 was verified by studies with intact cells showing that $^{86}Rb^+$ release and cell intoxication were inhibited by the E399A mutant. Although the mutant D426A exhibited invariable or reduced ion conductance in black lipid membranes, it caused similar $^{86}Rb^+$ release and cell intoxication as the wild-type protein. However, surprisingly, when pore formation by the F428A mutant was studied with $^{86}Rb^+$-loaded intact cells after an extracellular pH shift to allow the insertion of C2II into membranes, we were not able to see any toxin-induced release of $^{86}Rb^+$ ions after incubation for 5 min. To exclude that the charge carrier K^+ might be responsible for the discrepancy observed in artificial membranes (i.e., increase of conductance by F428A as compared to inhibition of conductance in intact cells determined with Rb^+), we studied the conductance by F428A in artificial membranes in the presence of Rb^+ ions. Also, in this case we observed an increase in conductance with the F428A mutant from 50 to 130 pS, indicating that $^{86}Rb^+$ ions pass the pore formed by the F428A mutant C2II. The data suggested that Phe-428 of C2II plays an additional role in toxin uptake in C2 toxin as proposed for the analogous residue in PA.

This view was supported by the finding that the F428A mutant exhibited different insertion and pore formation kinetics than the wild-type protein. In our electrophysiological studies with black lipid membranes, properties of already formed pores were characterized, but information about the kinetics of toxin insertion was limited under the conditions used. Therefore, we applied a lipid vesicle release assay, which allowed us to study the kinetics of pore formation and gain information about the pore properties. When the effect of C2II was studied with lipid vesicles, the F428A mutant exhibited a strong reduction in the initial rate of K^+ ions release and the total amount of release was reduced. These findings can be plausibly explained by a delayed and diminished insertion of the F428A mutant into membranes as compared to wild-type C2II. In line with these data, we observed a small release of Rb^+ ions from intact cells induced by the F428A mutant after prolonged incubation at 37 °C.

Based on the crystal structure of the monomeric C2II and on the model of the heptameric binding component, Phe-428 is located near the base of the protein and is exposed to the surface, whereas after heptamerization and pore formation this residue is positioned near the middle of the pore. This change in positioning suggests that Phe-428 may participate in the rearrangements occurring during the pH-dependent conformational changes, which ultimately result in formation of the β-barrel structure and insertion into membranes. Similar to residues 303−331 forming the $2\beta2$ and $2\beta3$ strands, which are functionally equivalent to residues 303−324 of PA (*15*) and which are proposedly involved in formation of the membrane-intruding β-barrel structure of C2II (*19*), Phe-428 residue may be involved in pore formation and, thus, cannot be substituted by alanine. The hypothesis that exchange of Phe-428 with alanine has major structural consequences for the pH-dependent rearrangement of the C2II heptamers is reflected in the change of the aggregation behavior of the F428A mutant. It has been shown for PA that pH-dependent conformational changes in the structure of PA cause an SDS-resistant state and formation of

aggregates (*28*). We observed a similar behavior with C2II. Although we cannot directly deduce pore-forming properties from aggregation behavior of C2II, the reduction in aggregation of the F428A mutant indicates that the mutation of Phe-428 in C2II affects structural features of the toxin relevant for surface properties of the molecule and is not restricted to changes of the phi-clamp function of this residue. In fact, recent studies on PA (*29*) are in line with the view that Phe-427 of the binding component of anthrax toxin is not only essential as a phi-clamp but is also involved in pore formation. The pivotal role of this residue explains its conservation in C2II and in the other binding components of binary actin-ADP-ribosylating toxins.

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SUPPORTING INFORMATION AVAILABLE

One figure as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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