Channel Formation by the Binding Component of *Clostridium botulinum* C2 Toxin: Glutamate 307 of C2II Affects Channel Properties *in Vitro* and pH-Dependent C2I Translocation *in Vivo*†

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ABSTRACT: The binding component (C2II) of the binary *Clostridium botulinum* C2 toxin mediates transport of the actin ADP-ribosylating enzyme component (C2I) into the cytosol of target cells. C2II (80 kDa) is activated by trypsin cleavage, and proteolytically activated C2II (60 kDa) oligomerizes to heptamers in solution. Activated C2II forms channels in lipid bilayer membranes which are highly cation selective and voltage-gated. A role for this channel in C2I translocation across the cell membrane into the cytosol is discussed. Amino acid residues 303−331 of C2II contain a conserved pattern of alternating hydrophobic and hydrophilic residues, which likely facilitates membrane insertion and channel formation by creating two antiparallel β-strands. Some of the residues are in strategic positions within the putative C2II channel, in particular, glutamate 307 (E307) localized in its center and glycine 316 (G316) localized on the trans side of the membrane. Here, single-lysine substitutions of these amino acids and the double mutant E307K/G316K of C2II were analyzed *in vivo* and in artificial lipid bilayer experiments. The pH dependence of C2I transport across cellular membranes was altered, and a pH of $\leq$5.2 was needed for C2I translocation into target cells; otherwise, no change in C2II-promoted entry of C2I into Vero cells was observed. The channel properties of C2II were substantially changed by the mutations, as evidenced by reduced cation selectivity. Interestingly, the voltage dependence of wild-type C2II was completely lost for the E307K mutant, which means that E307 is responsible for voltage gating. Chloroquine blocked the E307K mutant channel and intoxication of Vero cells by mutant C2II and C2I, indicating that chloroquine binding does not involve E307. Overall, the voltage gating and cation selectivity of the C2II channel do not play an important role in translocation of C2I into the cytosol.

Clostridium botulinum C2 toxin belongs to the family of binary toxins that are structurally organized into distinct enzyme and binding components. Other members of this toxin family are iota toxin from *Clostridium perfringens* (*1*), *Clostridium difficile* ADP ribosyltransferase (*2*), *Clostridium spiroforme* toxin (*3*), anthrax toxin from *Bacillus anthracis*, and the vegetative insecticidal proteins (VIPs) produced by *Bacillus cereus* (*4*). C2I, the enzyme component of C2 toxin, ADP ribosylates G-actin at Arg177 (*5*). ADP ribosylation of actin causes the complete breakdown of the actin cytoskeleton, cell rounding, and death (*6*). The 80 kDa binding component C2II of C2 toxin requires proteolytic cleavage in order to obtain biological activity (*7*). Proteolytic activation generates an N-terminal 20 kDa fragment, which dissociates from C2II, and the 60 kDa fragment forms ring-shaped heptamers in solution (*8*). The C2II heptamers bind to N-linked complex carbohydrates on the surface of target cells and mediate translocation of C2I into the cytosol (*9*). After receptor-mediated endocytosis, the C2I−C2II complex is transported to endosomes where the acidic pH triggers translocation of C2I into the cytosol (*8*). Like that of other binary toxins, the mechanism by which the enzyme component crosses the endosomal membrane to access its cytosolic substrate is poorly understood. Presumably, low pH causes insertion of C2II into membranes, creating a transmembrane channel, which may serve as a translocation pathway. Addition of trypsin-nicked C2II to artificial lipid bilayer membranes results in ion conductive channels (*10*). The C2II channels have an average single-channel conductance of 55 pS in 0.1 M KCl and are cation selective and voltage-gated (*10*). Furthermore, C2II channels reconstitute in full orientation into the membrane when added to one side of the membrane. They are blocked by chloroquine and some related compounds in a dose-dependent manner *in vitro* and *in vivo* (*11*).

The sequence of C2II is significantly homologous with the sequences of the binding components of other binary toxins, e.g., iota b of iota toxin (*12*) and the protective antigen

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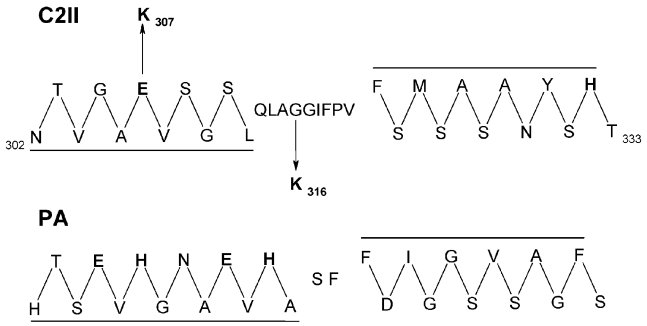

FIGURE 1: Comparison of the putative channel-forming domains of PA and C2II. The multiple-sequence alignment results were obtained using the BCM Search Launcher. The amino acids are given in the one-letter code. Charged residues are in bold. The putative amphipathic β-strands are similar to those proposed previously (*16*). The hydrophobic residues are marked with horizontal lines.

(PA)[1] of anthrax toxin (*13*). Like C2II, iota b and PA require proteolytic activation to form heptamers and channels in artificial membranes (*14*, *15*). In PA, a large flexible loop (residues 302−325) has been identified which inserts into membranes after acidification and forms a 14-stranded β-barrel that spans the membrane (*16*). The loop shows a pattern of alternating hydrophobic and hydrophilic residues, which is conserved in C2II and iota b (*16*). Therefore, it was proposed that residues 303−331 of C2II are involved in membrane insertion and channel formation (*17*). However, residues lining the channel differ in C2II and iota b, resulting in different channel properties (*10*, *18*). Unlike C2II, iota b forms pores in artificial lipid bilayer membranes which are not voltage-dependent, show a linear dependence on the bulk aqueous KCl concentration, and have only a weak affinity for chloroquine and related compounds (*18*).

These findings prompted us to examine whether glutamate 307, which is missing in the iota b channel, confers specific properties on the C2II channel. In addition, we analyzed the effect of a lysine substitution of glycine 316, which is presumably located on the trans side of the C2II channel (see Figure 1). Here, we show that two residues, E307 and G316, are important for specific properties of the C2II channel. Glutamate 307 influences distinctive features of the C2II channel like voltage gating and selectivity, but is definitely not involved in chloroquine binding. Furthermore, we show that substitution of glutamate 307 has no effect on the biological activity of C2II but influences the pH of external acidification, which is required for translocation of C2I across cell membranes. Our findings suggest that glutamate 307 confers specific biophysical properties on the channel formed by C2II but does not strongly influence channel behavior *in vivo*, e.g., translocation of C2I into target cells.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). The pGEX-2T vector was included in the glutathione *S*-transferase Gene Fusion System from Pharmacia Biotech (Uppsala, Sweden). Polymerase chain reactions were performed with a T1 Thermocycler from Biometra (Göttingen, Germany), and DNA sequencing was done with an Abi prism 310 Genetic analyzer from Perkin-Elmer (Langen, Germany). Taq polymerase was purchased from Roche Molecular Diagnostics. Bafilomycin A1 was from Calbiochem (Bad Soden, Germany). Glutathione−Sepharose 4B beads were obtained from Pharmacia Biotech. Cell culture medium was purchased from Biochrom (Berlin, Germany), and fetal calf serum was obtained from PAN Systems (Aidenbach, Germany). Thrombin and chloroquine were obtained from Sigma (Deisenhofen, Germany). Trypsin and trypsin inhibitor were from Roche Diagnostics (Mannheim, Germany). Rubidium ($^{86}Rb^+$) was from NEN (Köln, Germany).

Construction of C2II Mutants. Mutations in the C2II gene were constructed by site-directed mutagenesis using the Quickchange kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions with the plasmid pGEX-2T-C2II (*17*) as a template. For each mutant, two complementary primers were needed (only one of two complementary oligonucleotides is indicated): E307K, 5′-ACT GTT GGC GCA AAA GTT TCA GGT AGT TTA-3′; G316K, 5′-TTA CAA CTT GCT AAA GGT ATA TTC CCT-3′. Plasmids encoding altered C2II were transformed into *Escherichia coli* BL21 cells, and the constructs were confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins. Various recombinant proteins were expressed as GST fusion proteins in *E. coli* BL21 cells harboring the respective plasmid. Proteins were purified as described previously (*19*) and incubated with thrombin (3.25 NIH units/mL bead suspension) for cleavage of the fusion proteins from GST. Thereafter, the suspension was centrifuged, and an aliquot of the resulting supernatant was subjected to SDS−PAGE. C2II proteins were activated with 0.2 μg of trypsin/μg of protein for 30 min at 37 °C.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the methods of Laemmli (*20*). Gels were stained with Coomassie Brilliant Blue R-250.

Cell Culture and Cytotoxicity Assay. Vero cells were cultivated in tissue culture flasks at 37 °C and 5% CO_2 in Dulbecco's MEM supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were routinely trypsinized and reseeded three times per week. For cytotoxicity assays, cells were grown as subconfluent monolayers and treated with different concentrations of C2II and its mutants and 100 ng/mL C2I. Cells were incubated with the toxin components for 30 min on ice to allow toxin binding. Subsequently, cells were incubated for 15 min at 37 °C to start uptake. Fresh medium (pH 7.5) was added; cells were further incubated at 37 °C, and the percentage of cell rounding was determined from microscopic pictures.

$^{86}Rb^+$ Efflux Measurements. For $^{86}Rb^+$ efflux experiments, CHO cells were plated in 24-well culture plates. Six hours after plating had been carried out, fresh medium containing 1 μCi of $^{86}Rb^+$/mL was added, and cells were incubated for a further 20 h. C2II proteins were added in serum-free medium, and cells were incubated on ice for 1 h to allow toxin binding. Subsequently, the cells were washed twice

[1] Abbreviations: CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; Ia, iota a; Ib, iota b; PA, protective antigen; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SD, standard deviation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VIP, vegetative insecticidal protein.

with cold medium to remove unbound toxin. To initiate membrane insertion of the toxins, cells were treated with serum-free medium (pH 4.5−7.5) for 5 min at 37 °C. Cells were further incubated at 4 °C, and after 30 min, the medium was removed and the rate of $^{86}Rb^+$ release was determined by liquid scintillation counting in a 1209 Rackbeta β-counter from LKB Wallac (Gräfeling, Germany).

Black Lipid Bilayer Experiments. Black lipid bilayer membranes were formed as described previously (*21*). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of ~0.5 mm^2. Membranes were formed across the hole by painting onto a 1% solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane. The single-channel recordings were performed using Ag/AgCl electrodes (with salt bridges) connected in series to a voltage source and a current amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart or tape recorder. Zero-current membrane potential measurements were performed by establishing salt gradients across membranes containing 100−1000 C2II channels as described previously (*21*).

RESULTS

Characterization of C2II Mutants E307K, G316K, and E307K/G316K. Sequence comparison of C2II protein with the binding components of iota and anthrax toxins suggests that residues 303−331 of C2II are involved in membrane insertion and channel formation of C2II (Figure 1). The C2II channel is cation selective. This channel property is presumably based on negative point charges at the channel mouth or within the channel (*10*). E307 is the only negative charge within the prospective strands that build the β-barrel cylinder of the C2II heptamer. To test whether channel properties such as cation selectivity are important for C2I translocation into the cytosol, positively charged residues were introduced into the C2II channel. We constructed C2II mutant proteins, in which glutamate 307 and/or glycine 316 was replaced with a lysine.

The mutant proteins were expressed in *E. coli* BL21 cells and purified as described previously (*19*). Like the C2II wild-type protein, C2II mutants formed SDS-stable oligomers after tryptic activation, which could be detected when the proteins were subjected to SDS−PAGE without heating (Figure 2). The C2II mutants were then assayed for toxicity *in vivo* with Vero cells. The cells were incubated with 100 ng/mL C2I and increasing concentrations of wild-type and mutant C2II proteins, respectively. After incubation for 3 h at 37 °C, pictures were taken and the percentage of cell rounding was determined. As shown in Figure 3, the cell rounding activity of the double mutant E307K/G316K was similar to that of the wild-type protein. When compared to wild-type C2II, the mutant E307K exhibited slightly higher, and G316K slightly lower, activity.

Wild-Type C2II and C2II-E307K Translocate C2I at Different pH Values across Cellular Membranes. It has been shown previously that C2 toxin enters the cytosol in a pH-dependent manner from an endosomal compartment (*8*). Translocation of C2I into target cells can also occur when the extracellular compartment is acidified (pH ≤5.4) (*8*). It

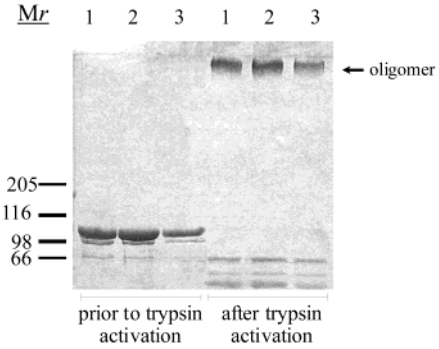

FIGURE 2: Analysis of mutant C2II proteins. Proteins were expressed as GST fusion proteins in *E. coli* BL21 cells and cleaved with thrombin from glutathione−Sepharose beads. Proteins were subjected to 3−12.5% SDS−PAGE and stained with Coomassie blue: lane 1, E307K/G316K; lane 2, G316K; and lane 3, E307K. Proteins, which were activated with trypsin, were subjected to SDS−PAGE without prior heating of the samples.

is possible that glutamate 307 may be involved in the pH dependence because the corresponding residue is missing in the membrane insertion sequence of iota b (*15*). Therefore, we compared C2I transport by wild-type C2II and E307K in experiments where the extracellular compartment was acidified to pH 4.8, 5.0, 5.2, or 5.4. Vero cells were pretreated with the specific V-ATPase inhibitor bafilomycin A1 to block toxin uptake by its normal route. Cells incubated with C2I and E307K only exhibited C2 toxin morphology after exposure to a pH of <5.2 (Figure 4). In contrast, cells which were incubated with wild-type toxin rounded up, when they were exposed to medium at pH 5.4.

Pore Formation of C2II-E307K in Vivo. Wild-type C2II forms pores in cellular membranes at acidic pH. Wild-type C2II-dependent C2I translocation and pore formation both occur at pH 5.4. Therefore, we were interested in whether glutamate 307 also influences the pH value at which pore formation occurs *in vivo*. This was analyzed by measuring the rate of $^{86}Rb^+$ release from CHO cells mediated by C2II-E307K. $^{86}Rb^+$-preloaded CHO cells were incubated for 1 h on ice with C2II-E307K to allow toxin binding. The cells were washed and incubated for 5 min with acidified medium. Scintillation counting of released $^{86}Rb^+$ revealed that C2II-E307K permeabilized CHO cells in a manner similar to that of wild-type C2II at pH 5.4, indicating that glutamate 307 influences pH-dependent transport across cellular membranes but not pore formation after acidification (Figure 5).

Influence of Chloroquine on the Activity of C2II Mutant Proteins. Chloroquine and related compounds block the C2II channel *in vitro* and intoxication of cells *in vivo*, possibly by binding to glutamate 307 (*11*). We therefore analyzed the effect of chloroquine on intoxication of Vero cells with E307K. Pretreatment of Vero cells with chloroquine for 30 min prior to toxin addition completely blocked cytotoxic effects of wild-type C2II and mutants (data not shown). To exclude the possibility that the inhibitory effect of chloroquine was due to its ability to inhibit endosomal acidification, we performed extracellular acidification experiments in the presence of 1 mM chloroquine. Vero cells were pretreated with bafilomycin A1 for 30 min at 37 °C. Chloroquine and the toxin components were added to cells incubated at 4 °C for 30 min. Cells were washed, and bafilomycin-containing medium at pH 7.5 or 4.8, with or without chloroquine (1 mM), was added to the cells. After incubation for 10 min at

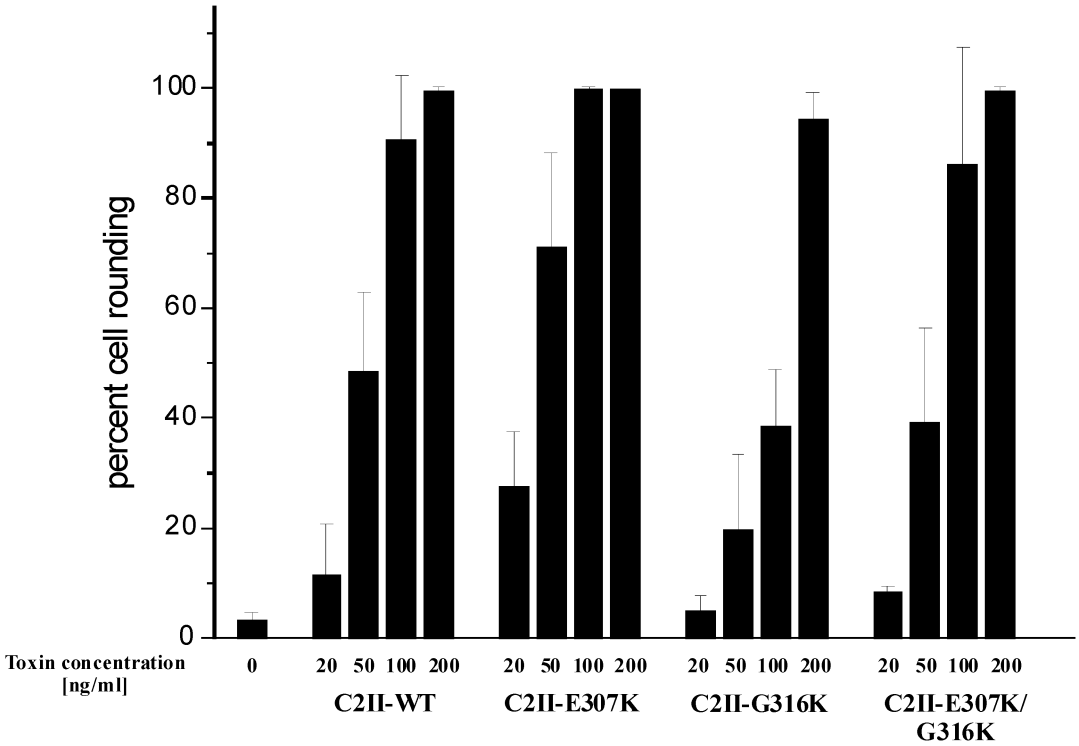

FIGURE 3: Toxicity of mutant C2II proteins toward Vero cells. Vero cells were incubated at 37 °C in serum-free medium with 100 ng/mL C2I and the indicated concentrations of activated wild-type C2II and mutant proteins. Three hours after toxin addition, cells were photographed and the number of rounded cells was determined.

37 °C, fresh medium (37 °C, pH 7.5) was added and cells were further incubated at 37 °C. As shown by phase contrast microscopy in Figure 6, acidic pH induced translocation of C2I and subsequent cell rounding in the presence of bafilomycin, which blocked cytotoxic C2 effects at neutral pH. The addition of chloroquine to the medium inhibited cytotoxic effects induced by wild-type C2II and E307K (shown for E307K in Figure 6), indicating that glutamate 307 does not play an essential role in chloroquine binding to C2II.

The Single Mutants E307K and G316K and the Double Mutant E307K/G316K Form Channels in Artificial Membranes. C2II forms channels in artificial lipid bilayer membranes made of different pure lipids and increases membrane conductance by many orders of magnitude (*10*). Membrane experiments were performed with the single mutants E307K and G316K and the double mutant E307K/G316K to check whether the mutants also formed channels in artificial membranes. The C2II mutants were added at a defined concentration (~100 ng/mL) to one side of black lipid bilayer membranes made of diphytanoylphosphatidylcholine and *n*-decane. The subsequent increase in the membrane current was steep for ~10−20 min. Only a small further increase (as compared with the initial one) occurred after that time. The membrane conductance measured 30 min after addition of C2II mutants at defined concentrations was taken as a measure of the membrane activity of the C2II mutants. The results suggest that the mutants also had a high membrane activity. The highest activity similar to that of the wild type was observed for the E307K mutant. Membrane activity for G316K and E307K/G316K was lower than that of the wild type by a factor of approximately 2−3. These results suggested that the three C2 mutants also formed channels in lipid bilayer membranes.

Analysis of the Single-Channel Conductance of the C2II Mutants. Wild-type C2II forms defined channels with a single-channel conductance of ~150 pS in a 1 M KCl solution (*10*). We performed single-channel experiments to check whether the somewhat altered membrane activity of the mutants was caused by a change in channel structure. Figure 7 shows single-channel recordings of wild-type C2II and C2II mutants E307K, G316K, and E307K/G316K taken from diphytanoylphosphatidylcholine/*n*-decane membranes in a 0.1 M KCl solution. The channels formed by these mutants had on average single-channel conductances of ~15, ~40, and ~15 pS, respectively. This result suggested indeed that the mutation had some influence on the conductance of the C2II channel because the mutant C2II had a smaller conductance than wild-type C2II (55 pS; see also Table 1). This result indicated that the mutations influenced both the channel forming activity and the single-channel conductance.

Single-channel experiments were also performed with salts containing ions other than K^+ and Cl^- and with other KCl concentrations. These experiments were performed to gain some insight into the biophysical properties of the mutant C2II channels. The results are summarized in Table 1 together with data which have been derived previously for wild-type C2II (*10*). The results in Table 1 show that the influence of cations on single-channel conductance was more substantial than that of anions such as acetate. This result is consistent with the assumption that the mutant C2II channels are cation selective similar to wild-type C2II, although the mutant channels contain one (G316K), two (E307K), or three (E307K/G316K) net positive charges more per monomer than wild-type C2II (in total 7, 14, or 21 per heptamer, respectively). Table 1 also shows the average single-channel conductance, *G*, as a function of the KCl concentration in the aqueous phase for all three mutants. Interestingly, we

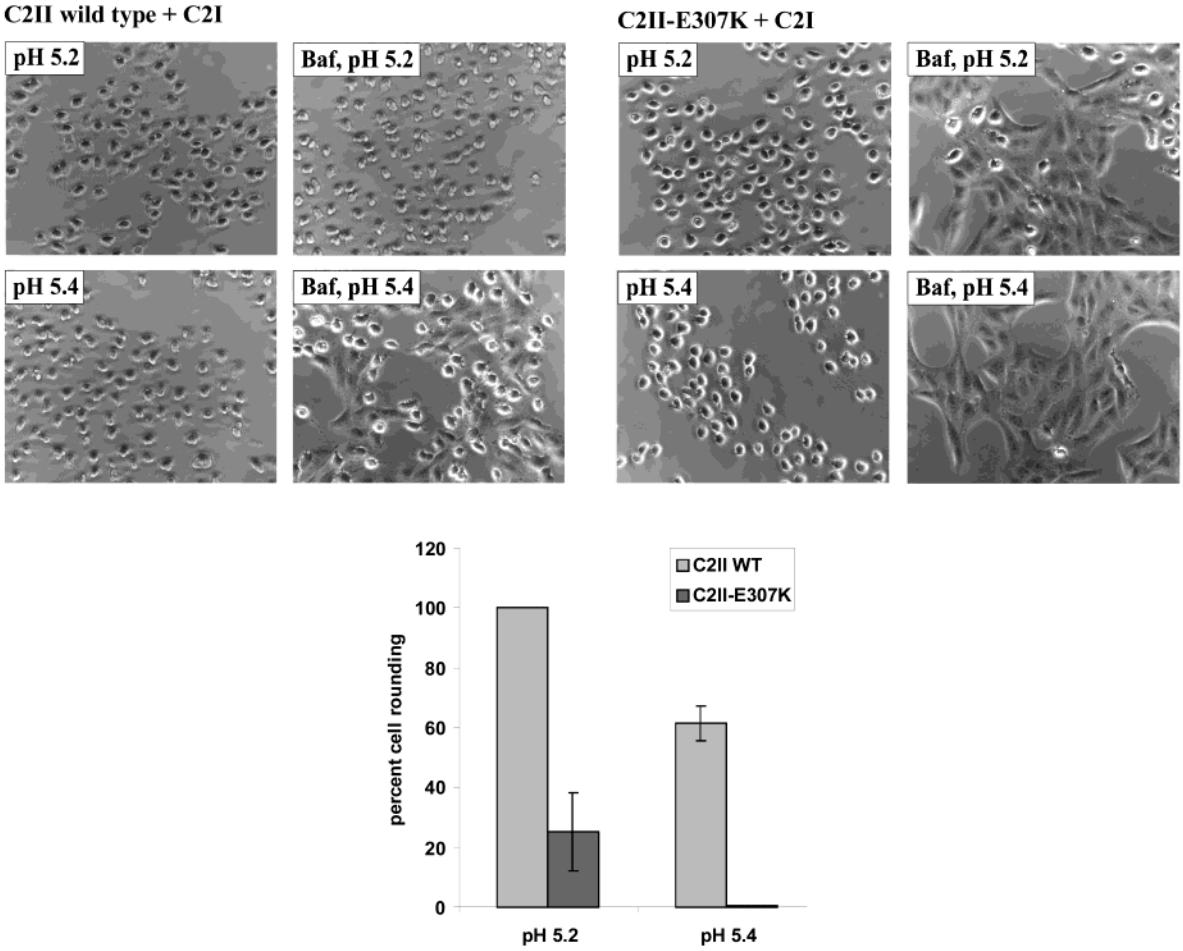

FIGURE 4: Different pH requirements of wild-type C2II and E307K for C2I delivery into the cytosol after extracellular acidification. Vero cells, which were pretreated with 100 nM bafilomycin A1 for 30 min, were incubated for 30 min at 4 °C with 100 ng/mL C2I and 200 ng/mL wild-type C2II and C2II-E307K, respectively. Thereafter, the cells were exposed to serum-free medium at different pH values for 15 min. Cells were further incubated in complete medium at 37 °C for 2 h; pictures were taken, and the percent cell rounding was determined.

Table 1: Average Single-Channel Conductances, *G*, of the Channel Formed by C2II Mutants E307K, G316K, and E307K/G316K in Different Salt Solutions[a]

		G (pS)			
salt	*c* (M)	wild type	E307K	G316K	E307K/ G316K
KCl	0.01	12	nd	nd	nd
	0.03	25	nd	nd	nd
	0.1	55	15	40	15
	0.3	80	35	65	40
	1.0	150	130	150	130
	3.0	380	430	450	450
LiCl	1.0	60	60	65	60
K^+ acetate (pH 7.0)	1.0	120	85	120	85
NH_4Cl	1.0	250	230	250	230

[a] The single-channel conductances of wild-type C2II are given for comparison. The membranes were formed of diphytanoylphosphatidylcholine dissolved in *n*-decane. The aqueous solutions were unbuffered and had a pH of 6 unless otherwise indicated. The applied voltage was 50 mV, and the temperature was 20 °C. The average single-channel conductance was calculated from at least 100 single events. *c* represents the concentration of the aqueous salt solution. nd means not detectable. The data for wild-type C2II were taken from Schmid et al. (*10*).

observed a 1:1 relationship between conductance and KCl concentration for the single mutant E307K and the double mutant E307K/G316K. This is in contrast to wild-type C2II and the G316K mutant where the single-channel conductance, *G*, was dependent on the square root of the concentration, which indicates point charge effects on the channel properties (*10*) (see also the Discussion).

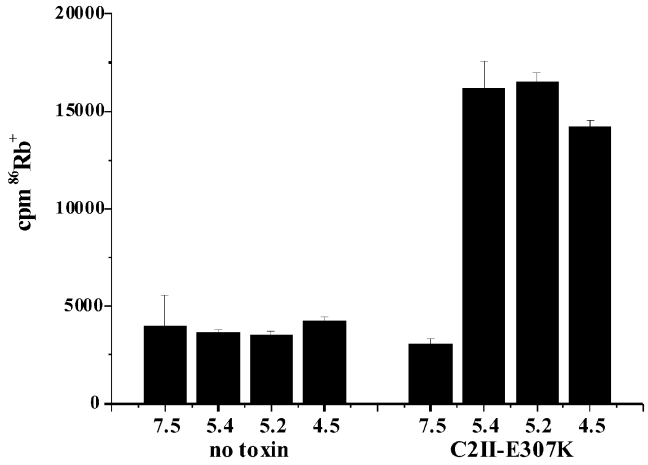

FIGURE 5: *In vivo* pore formation by C2II-E307K. CHO cells were grown in a 24-well plate (~2×10^5 cells/well) and loaded for 20 h with $^{86}Rb^+$ (1 μCi/mL). The medium was removed, and cells were incubated for 1 h at 4 °C in serum-free medium with 500 ng/mL trypsin-activated C2II-E307K. The medium was removed and replaced with prewarmed (37 °C) medium at pH 7.5, 5.4, 5.2, or 4.5. After a 5 min incubation at 37 °C, cells were cooled to 4 °C for an additional 30 min. The complete medium was removed, and radioactivity was determined by scintillation counting. Values are given as the mean ± SD.

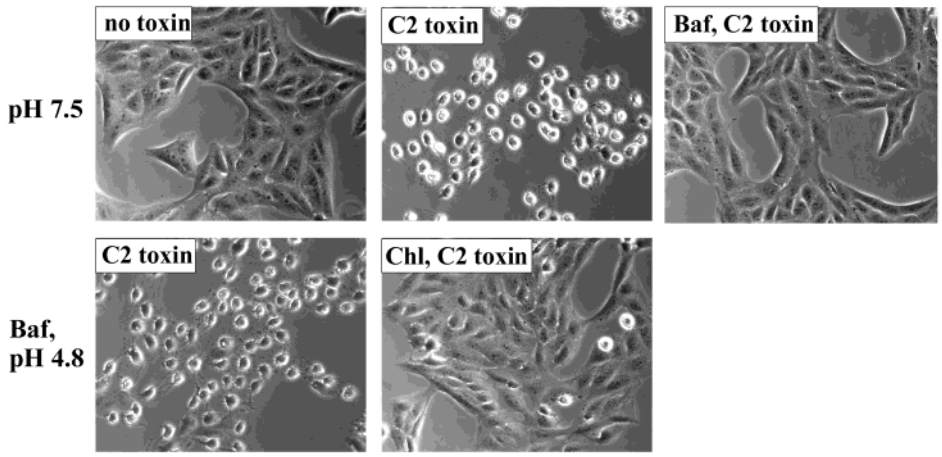

FIGURE 6: Influence of chloroquine on C2I translocation across cellular membranes. Vero cells were pretreated with bafilomycin A1 (Baf) for 30 min at 37 °C and subsequently incubated for 30 min at 4 °C in serum-free medium containing 1 mM chloroquine (Chl), 100 ng/mL C2I, and 200 ng/mL C2II-E307K. Cells were washed and incubated for 15 min with chloroquine-containing serum-free medium at pH 7.5 or 4.8. Thereafter, cells were further incubated at 37 °C in complete medium, and pictures were taken after 2 h.

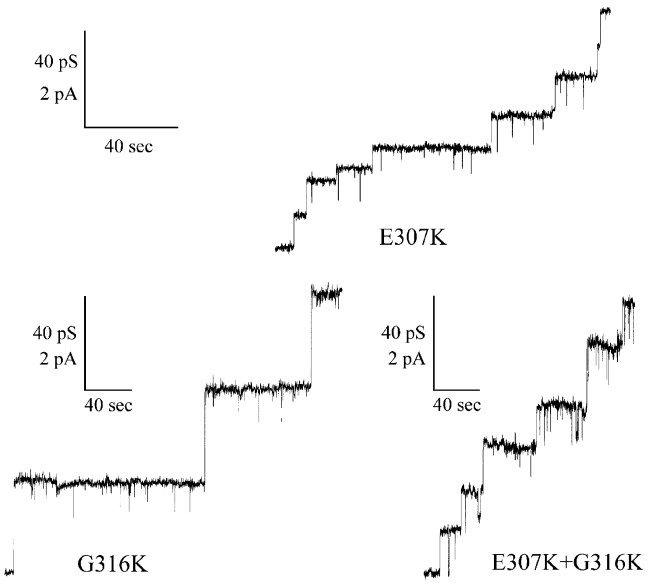

FIGURE 7: Single-channel recordings of diphytanoylphosphatidylcholine/*n*-decane membranes in the presence of C2II mutants E307K, G316K, and E307K/G316K. The aqueous phase contained 0.1 M KCl (pH 6) and a designated C2II mutant (40 ng/mL). The applied membrane potential was 50 mV; $T = 20$ °C.

Zero-Current Membrane Potentials. We performed zero-current membrane potential measurements to obtain further information about the channel properties of the C2II mutant channels. After incorporation of 100−1000 mutant channels into the diphytanoylphosphatidylcholine/*n*-decane membranes, the salt concentration on one side of the membranes was increased 5-fold from 100 to 500 mM and the zero-current potential was measured 5 min after the gradient had been established. For all three mutants (E307K, G316K, and E307K/G316K) employed in these experiments, the more dilute side (100 mM) was always positive, which indicated preferential movement of K^+ through the C2II mutant channel; i.e., the channels were cation selective as was already suggested from the single-channel recordings (see Table 1). It is noteworthy that this result was unexpected because the creation of 7, 14, or 21 net positively charged groups in or near the channel should create an anion selective C2II channel, which we did not observe (see the Discussion).

Table 2: Zero-Current Membrane Potentials, V_m, of Diphytanoylphosphatidylcholine/*n*-Decane Membranes in the Presence of Wild-Type C2II and Different C2II Mutants Measured for 5-Fold Gradients of KCl[a]

C2II	zero-current membrane potential V_m (mV)	cation selectivity P_{cation}/P_{anion}
wild type	−	11
E307K	22	4.0
G316K	26	5.6
E307K/G316K	25	5.5

[a] V_m is defined as the difference between the potential at the dilute side (100 mM) and the potential at the concentrated side (500 mM). The pH of the aqueous salt solutions was 6; $T = 20$ °C. The permeability ratio P_{cation}/P_{anion} was calculated with the Goldman−Hodgkin−Katz equation (*21*) on the basis of at least four individual experiments. The permeability ratio for wild-type C2II was as previously described by Schmid et al. (*10*).

The zero-current membrane potentials for KCl are given in Table 2 together with the ratios of the permeabilities P_{cation} and P_{anion} calculated from the Goldman−Hodgkin−Katz equation.

The Mutation of Glutamic Acid 307 Does Not Influence Chloroquine-Mediated Blockage of C2II. As seen before, chloroquine blocks the C2II channel *in vitro* (*10*, *11*). Titration experiments were performed with E307K to check whether this mutation has any influence on the binding of chloroquine. The measurements were performed in the following way. After the insertion of a large number of mutant channels in the membrane, chloroquine was added at increasing concentrations to the aqueous phase on the cis side of the membrane. Subsequently, the current decreased in a dose-dependent manner, which means that chloroquine indeed blocked the pore function of the mutant and decreased the single-channel conductance. The half-saturation constant for this process was ~32 μM, which means that the stability constant for chloroquine binding to the C2II mutant channel E307K is ~31000 L/mol (1 M KCl). This has to be compared with a half-saturation constant for chloroquine binding to the C2II wild-type channel of 44 μM (*11*), which means that the E307K mutation minimally influenced chloroquine binding to C2II.

Glutamic Acid 307 Is Responsible for Voltage-Dependent Gating of the C2II Channel. In previous studies, we could

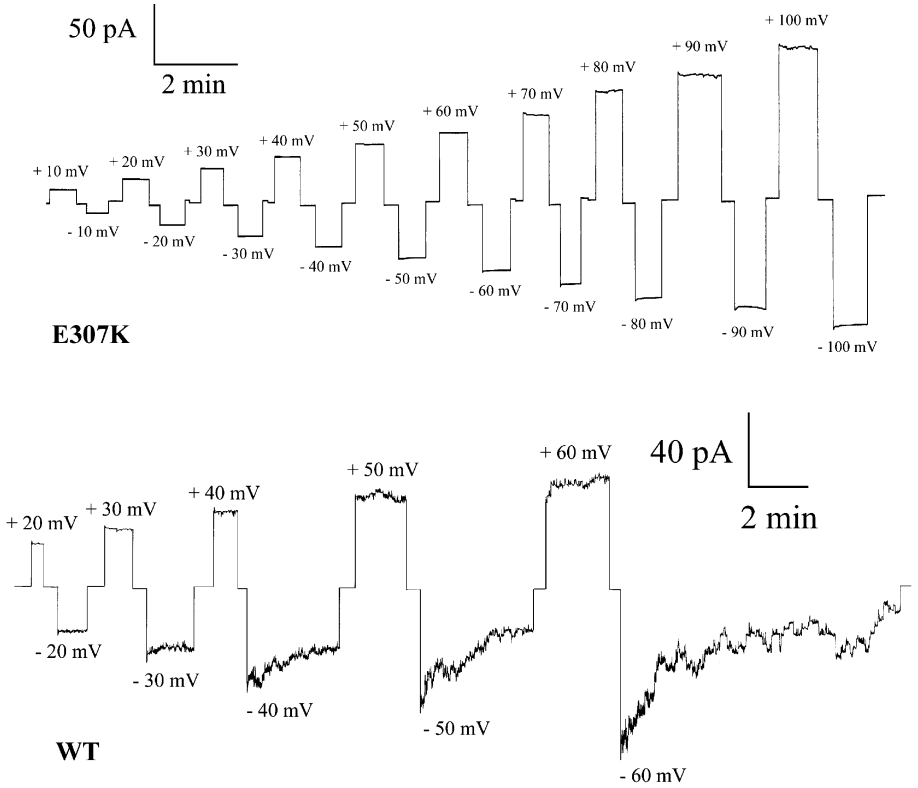

FIGURE 8: Current responses of the C2II-E307K mutant and wild-type C2II upon application of positive and negative potentials to the cis side of a membrane made of diphytanoylphosphatidylcholine and *n*-decane bathed in 1 M KCl. (A) The cis side contained 100 ng/mL E307K; $T = 20$ °C. (B) The cis side contained 100 ng/mL wild-type C2II. Note that the membrane current decreased only when the cis side, containing C2II, was negative.

demonstrate that the C2II channel shows asymmetric voltage gating when the protein is added to only one side of the membrane (*10*, *11*). Here we studied the voltage dependence of C2II mutants E307K, G316K, and E307K/G316K. Activated C2II mutant E307K (500 ng/mL) was added to the cis side of a black diphytanoylphosphatidylcholine/*n*-decane membrane. After 30 min, the conductance increased considerably, and the experiment shown in Figure 8A was started. Increasing voltages of both polarities were applied to the C2II mutant channels, and the current was measured as a function of time. Surprisingly, the mutant channel was not voltage-dependent. Figure 8B shows a control experiment with wild-type C2II, which was performed in a manner similar to that described for the C2II mutant E307K. For positive voltage at the trans side, the membrane current decreased in an exponential fashion. For negative potentials at the trans side, current did not decrease as observed previously (*10*). The data were analyzed in the following way. Membrane conductance (G) as a function of voltage, V_m, was measured for wild-type C2II when the opening and closing of channels reached an equilibrium, i.e., after the exponential decay of the membrane current following the voltage step V_m (see Figure 9). G was divided by the initial value of the conductance (G_o, which was a linear function of the voltage) obtained immediately after the onset of the voltage. The data of Figure 9 (▲) correspond to the asymmetric voltage dependence of wild-type C2II (mean of four membranes) when the protein is added to the cis side. The empty squares show means of similar experiments with the C2II mutant E307K where no voltage dependence was observed. This result not only indicated asymmetric insertion of the C2II component into the membranes but also that the

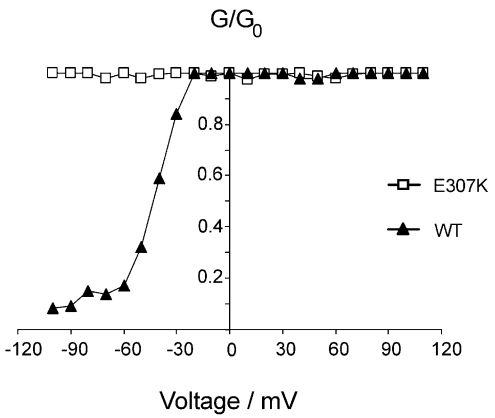

FIGURE 9: Ratio of the conductance G at a given membrane potential (V_m) to the conductance G_o at 10 mV as a function of the membrane potential V_m. The empty squares show measurements, in which 100 ng/mL E307K was added to the cis side of membranes. The filled triangles refer to control experiments where wild-type C2II was added to the cis side. The voltage refers always to that on the cis side of the membrane. The aqueous phase contained 1 M KCl. $T = 20$ °C. Means of four experiments are shown.

voltage dependence is clearly caused by glutamic acid 307. Similar experiments were also performed with the C2II mutants G316K and E307K/G316K. G316K possessed a voltage dependence similar to that of wild-type C2II, whereas E307K/G316K was not voltage-dependent, which is in agreement with the results of experiments with E307K.

DISCUSSION

In Vivo Activities of C2II Mutants. The C2II mutants used in this study were designed in such a way that important

amino acids were altered within the putative channel-forming domain. E307 is the only negatively charged amino acid among residues 303−331 of C2II which form the two putative antiparallel β-strands crossing the lipid bilayer membrane when we assume for the C2II channel a similar structure as for PA of anthrax toxin (*16*, *22*). It is clear that the seven glutamic acid residues within the channel formed by a C2II heptamer should have a major impact on the channel properties. Furthermore, we exchanged glycine 316 with lysine (G316K). According to our model, glycine 316 is localized within the channel opening on the trans side of the C2II channel. Again, the replacement of seven glycines with lysines on one side of the channel should strongly influence its properties.

We first examined the effects of E307K and G316K on the biological activity of C2II. The mutants could be activated by trypsin as wild-type C2II and were also unimpaired in oligomerization. Intoxication experiments showed that the double mutant was as active as wild-type C2II, whereas the activities of E307K and G316K were slightly increased and reduced, respectively. C2I is translocated from an endosomal compartment into the cytosol following acidification (*8*). Experiments in which endosomal conditions were mimicked by extracellular acidification revealed that lysine substitution of glutamate 307 influenced the pH, which enables C2I transport into the cytosol. In the case of wild-type C2II, an extracellular pH of 5.4 was sufficient to allow translocation of C2I into the cytosol; E307K required a pH of <5.2. Interestingly, we could not detect any difference between pore formation by wild-type C2II and E307K. Both proteins induced $^{86}Rb^+$ efflux from preloaded cells at pH 5.4. To date, we have no explanation of why E307K pore formation and C2I transport occur under different pH conditions. However, it is noteworthy that iota b, which is missing the corresponding glutamic acid residue, likewise requires lower pH values than wild-type C2II to deliver the enzyme component into the cytosol (*23*).

C2II, iota b of iota toxin, and PA of anthrax toxin are significantly structurally and functionally homologous. They all form pores in membranes and mediate translocation of the respective enzyme components in an acidic pH-dependent manner from an endosomal compartment into the cytosol (*8*, *15*, *23*). However, the precise mechanism of membrane translocation has not yet been described for any of these toxins. Several models are currently discussed. Either the channel formed under acidic conditions transports several enzyme components into the cytosol, or the process of membrane insertion and subsequent channel formation is the driving force for membrane translocation of the enzyme components. Our experiments show that with E307K, $^{86}Rb^+$ efflux and C2I transport into the cytosol do not occur at the same pH. This finding demonstrates that the C2IIa pore must be involved in translocation of C2I: when the pore properties were changed, translocation of C2I required another pH. The changed C2IIa pore behaves like the iota pore because translocation of Ia via Ib across cell membranes required a more acidic pH than C2 toxin. One possible explanation for our observation might be that pore formation of the binding component C2IIa and translocation of the enzyme component C2I are independent steps. Therefore, membrane insertion and pore formation of C2IIa may require a pH different from that which drives C2I translocation. Channel formation is, however, an essential prerequisite but not sufficient to allow C2I translocation across cellular membranes.

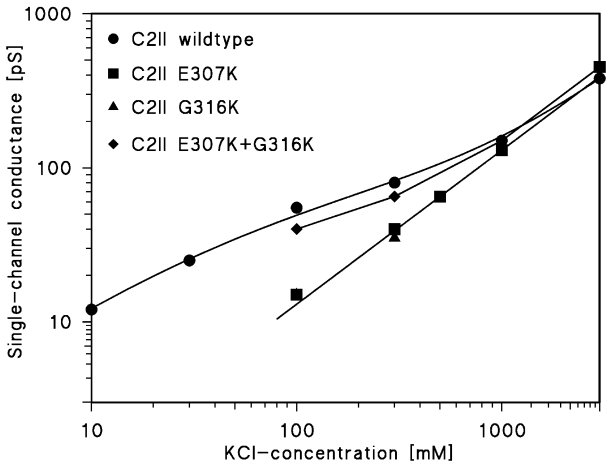

FIGURE 10: Single-channel conductance of the three C2II mutants as a function the KCl concentration in the aqueous phase. The top solid line represents the fit of the single-channel conductance data of wild-type C2II with a combination of eqs 1−4 of Schmid et al. (*10*), assuming the presence of negative point charges (1.5 negative charges; $q = -2.4 \times 10^{-19}$ A) within the channel and assuming a channel diameter of 0.7 nm. *c* is the concentration of the KCl solution in molar; *G* is the average single-channel conductance in picosiemens (10^{-12} S). The straight line explains the single-channel conductance of the E307K and E307K/G316K mutants and corresponds to a linear function between channel conductance and bulk aqueous concentration. Note that the single-channel conductance of the G316K mutant is not a linear function of the KCl concentration.

The C2II Mutants Have Altered Single-Channel Properties. One interesting result of the lipid bilayer studies was that the C2II mutants formed defined channels with lower membrane activity, which had, like wild-type C2II, a long lifetime and approximately the same single-channel conductance at high ionic strengths (1 and 3 M KCl). The channel properties differed considerably from those of the C2II wild-type channel at low ionic strengths. Whereas the single-channel conductance of wild-type C2II exhibited a dependence on the square root of salt concentration (see Table 1 and Figure 10), E307K and the double mutant E307K/G316K exhibited an almost linear dependence on the bulk aqueous salt concentration (Figure 10). This result suggests that ion transport through these mutant channels is not influenced by negative point charges, which is characteristic of the wild-type C2II channel (*10*). This is likely due to the special structure of the C2II channel, which may contain on the cis side vestibule of the channel many negatively charged groups that lead together with the positively charged channel interior to a channel containing both types of charges with an excess of negative charges. These channels show linear relationships between salt concentration and conductance (*24*).

Similar considerations apply to ion transport through the G316K mutant channel. Its single-channel conductance deviates from linearity at low KCl concentrations (see Figure 10) in a manner similar to that of wild-type C2II. Although this mutant has seven probably mostly positively charged lysines on the trans side of the channel, there is no indication from selectivity measurements and single-channel data that it is anion selective. Furthermore, the single-channel conductances shown in Table 1 and Figure 10 are consistent with the assumption of point negative net charges for the

G316K mutant channel despite the many lysines at its opening on the trans side. This means that the vestibule at the cis side of the channel contains many negatively charged amino acids (like in PA, VIP1, and iota b) (*16*, *22*), which results from image charges at the membrane surface on the other side in a channel that is cation selective (*10*, *25*). This effect is so strong that the positively charged groups on the other side are not only counterbalanced but also converted into negative point charges. Otherwise, the properties of the G316K mutant channel cannot be understood.

Glutamate 307 Is Responsible for Voltage-Dependent Gating of the C2II Channel. Wild-type C2II possesses voltage-dependent gating when the protein is added to the cis side of the membrane, and the trans side is set to a positive potential (*10*, *11*). With a starting point of ~30−40 mV, the channels close in an exponential fashion and become almost completely closed when 60 mV is applied to the trans side. In this study, we could demonstrate that the exchange of glutamic acid 307 with a lysine completely abolished the voltage dependence of C2II. This means that an electric field pulling the seven glutamic acids inside the C2II channel to the trans side leads to channel closure presumably by collapse of the β-barrel cylinder within the membrane formed by the 14 β-strands of the heptamer. Replacement of the seven negatively charged groups with positive lysines obviously stabilizes the β-barrel cylinder, suggesting that the mushroom-like structure on the membrane surface plays an important role in stabilizing the membrane channel, as has been discussed for α-hemolysin of *Staphylococcus aureus* (*26*). The possibility that the β-barrel cylinder is directly involved in channel gating is very interesting, because the voltage gating of bacterial and mitochondrial porins as well as certain toxins, which contain mostly β-strands, is still a matter of debate (*24*, *27*−*29*). The possibility that the surface-exposed loops play an important role in gating, in particular, because a pH decrease resulted in channel closure and a much higher voltage dependence has been discussed (*30*, *31*). The results presented here suggest that charged groups within a β-strand may also contribute to the voltage dependence of a porin-like channel, i.e., a channel formed by a β-barrel cylinder.

Chloroquine Binding Is Not Dependent on E307. Chloroquine and related compounds are able to inhibit intoxication of target cells at micromolar concentrations (*11*). This effect is probably related to chloroquine-mediated channel block *in vitro*, because chloroquine could bind to C2II and inhibit not only ion conductance through the C2II heptamer but also C2I binding, which is a prerequisite for C2I transport into target cells (*8*, *17*). Previously, it has been suggested that E307 could be involved in this process because the closely related binding component of iota toxin (Ib) does not contain a negatively charged amino acid within the channel-forming domain and possesses a much lower level of chloroquine binding *in vivo* and *in vitro* (*11*, *18*). If these considerations were be correct, then the E307K mutant would also exhibit a much smaller chloroquine effect *in vivo* and *in vitro*, which we definitely did not observe. This means that E307 is not directly involved in chloroquine binding. It seems moreover that the highly negatively charged vestibule is the binding place of chloroquine, as previously discussed for the electrophysiological measurements. This is presumably also the place for C2I recognition and binding, which suggests a major impact of chloroquine on C2I transport.

The binding components of C2 toxin, anthrax toxin, and iota toxin form channels in biological and artificial membranes (*14*). The formation of channels is an important prerequisite for toxin translocation into the target cells. However, the results presented here suggest that the structure of the channel, which limits its biophysical properties such as voltage dependence, point charges, and selectivity, has only a minor effect on toxin translocation. Further studies of C2II mutations are necessary for elucidation of structural requirements that are necessary to promote toxin transport in target cells.

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