Clostridium botulinum C2 Toxin

IDENTIFICATION OF THE BINDING SITE FOR CHLOROQUINE AND RELATED COMPOUNDS AND INFLUENCE OF THE BINDING SITE ON PROPERTIES OF THE C2II CHANNEL

Received for publication, November 30, 2007. Published, JBC Papers in Press, December 11, 2007, DOI 10.1074/jbc.M709807200

Tobias Neumeyer†, Bettina Schiffer‡, Elke Maier§, Alexander E. Lang‡, Klaus Aktories§, and Roland Benz¶†

From the †Lehrstuhl für Biotechnologie, Theodor-Boveri-Institut (Biozentrum) der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany and §Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität Freiburg, D-79104 Freiburg, Germany

Clostridium botulinum C2 toxin belongs to the family of binary AB type toxins that are structurally organized into distinct enzyme (A, C2I) and binding (B, C2II) components. The proteolytically activated 60-kDa C2II binding component is essential for C2I transport into target cells. It oligomerizes into heptamers and forms channels in lipid bilayer membranes. The C2II channel is cation-selective and can be blocked by chloroquine and related compounds. Residues 303–330 of C2II contain a conserved pattern of alternating hydrophobic and hydrophilic residues, which has been implicated in the formation of two amphipathic β-strands involved in membrane insertion and channel formation. In the present study, C2II mutants created by substitution of different negatively charged amino acids by alanine-scanning mutagenesis were analyzed in artificial lipid bilayer membranes. The results suggested that most of the C2II mutants formed SDS-resistant oligomers (heptamers) similar to wild type. The mutated negatively charged amino acids did not influence channel properties with the exception of Glu399 and Asp426, which are probably localized in the vestibule near the channel entrance. These mutants show a dramatic decrease in their affinity for binding of chloroquine and its analogues. Similarly, F428A, which represents the Φ-clamp in anthrax protective antigen, was mutated in C2II in several other amino acids. The C2II mutants F428A, F428D, F428Y, and F428W not only showed altered chloroquine binding but also had drastically changed single channel properties. The results suggest that amino acids Glu399, Asp426, and Phe428 have a major impact on the function of C2II as a binding protein for C2I delivery into target cells.

Clostridium botulinum C2 toxin belongs to the family of binary toxins of the AB type. The C2 toxin consists of two distinct components: component A, which is the enzymatically active subunit C2I, and component B (C2II), which is involved in the transport of the enzymatic component into the cytosol across the target cell membrane. There C2I exerts its catalytic activity (1–3). Other members of the family of binary toxins are the iota toxin of Clostridium perfringens (4, 5), ADP-ribosyltransferase of Clostridium difficile (6–8), Clostridium sordellii toxin (9, 10), the vegetative insecticidal proteins (VIPs) of Bacillus cereus and Bacillus thuringiensis (11, 12), and the anthrax toxin of Bacillus anthracis (13, 14).

C2I and C2II are released separately into the extracellular media (2, 15). C2I (~49 kDa) ADP-ribosylates monomeric G-actin but not oligomeric F-actin at position arginine 177, which inhibits polymerization of G-actin (16, 17) and largely affects ATP binding and ATPase activity (18). Through the action of C2I, the actin cytoskeleton breaks down, leading to cell rounding and cell death (19, 20). C2II (~80 kDa), the B subunit, needs to be cleaved by trypsin to obtain its biological activity (21). This cleavage generates a ~60-kDa fragment, which forms a ring-shaped heptamer (22), and a ~20-kDa fragment, which dissociates from C2II. The binding of the C2II heptamer depends on presence of asparagine-linked carbohydrates on the surface of target cells (23) and is also able to bind the enzymatic component C2I (24–26). The complex is endocytosed into the cell. Acidification of the endosome triggers C2I translocation into the cytosol (22, 27).

The addition of activated C2I to artificial lipid bilayer membranes results in the formation of ion-permeable channels that are formed by C2II heptamers (22, 28, 29). These channels could serve as translocation pathway for C2I through the endosomal membrane (22). Evidence has been presented that the protective antigen (PA)2 of anthrax toxin homologous to C2II provides such a pathway to carry the anthrax enzymatic components (EF, LF) into the cytosol of the target cells (30, 31). The C2II channel is cation-selective and voltage-gated (28). The C2II heptamer inserts oriented in the membrane when added to one side of the membrane (29). Chloroquine and other 4-aminoquinolines are able to block reconstituted C2II channels in vivo and in vitro (29). The half-saturation constant, Ks, of the binding of chloroquine is in the micromolar range (32). The exact mechanism of intoxication as well as inhibition of channel function with 4-aminoquinolines is not well understood.

The C2II heptamer is not known in its membrane-spanning structure; this applies to PA too. However, in the case of the PA, a water-soluble precursor (prepropeptide) has been crystallized (33),

*This work was supported by Deutsche Forschungsgemeinschaft Grants SFB 487, project A5, and SFB 388, project A13, and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Lehrstuhl für Biotechnologie, Theodor-Boveri-Institut (Biozentrum) der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany. Tel.: 49-931-8894501; Fax: 49-931-8884509; E-mail: roland.benz@mail.uni-wuerzburg.de.

‡The abbreviations used are: PA, protective antigen; MES, 4-morpholineethanesulfonic acid; pS, picosiemens.
which was also used to model the C2II prepro (34). Using the structure of the PA prepro and sequence comparisons mentioned above, we identified negatively charged amino acids as putative candidates involved in C2II channel function. Some of these amino acids are presumably located in the vestibule of the channel. Furthermore, residue Phe427 of PA (also known as F-clamp) plays a crucial role for translocation of EF or LF into the cytosol and also influences binding of quinacrine (35). C2II also has a phenylalanine in a relevant position (Phe428). To check its role in the interaction of C2II with 4-aminoquinolones, Phe428 was mutated to several amino acids with different or similar properties (F428A, F428D, F428Y, and F428W). Here, we show that residues Glu272, Glu280, Asp341, or Glu346, are important for binding of 4-aminoquinolones, which block the C2II channels. Mutation of these residues leads to altered channel properties, such as single channel conductance, ion selectivity, and inhibitor binding, including its ionic strength dependence. Asp342 is also important for assembly of the channel, membrane insertion, and/or channel function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Diphytanoyl phosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL). All salts were analytical grade (Merck). Ultrapure water was prepared by passing deionized water through Milli-Q equipment (Millipore, Bedford, MA). The QuikChange™ site-directed mutagenesis kit was bought from Stratagene (Heidelberg, Germany). Oligonucleotides were obtained from Sigma. The pGEX-2T vector was derived from the glutathione S-transferase fusion protein of *Escherichia coli* BL21 harboring the separate DNA fragments in plasmid pGEX-2T. Proteins were purified as described previously (36) and eluted with 10 mM glutathione, 100 mM NaCl, and 50 mM Tris (pH 8.0) or incubated with thrombin (3.25 National Institutes of Health units/ml of bead suspension) for cleavage of the fusion proteins from glutathione S-transferase. Thereafter, the suspension was centrifuged at 500 × g for 10 min at room temperature, 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.

**Lipid Bilayer Experiments**—Black lipid bilayer experiments were performed as described previously (37). 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 0.3–0.5 mm². The aqueous solutions on both sides of the membrane were buffered with 10 mM MES-KOH to pH 6. Membranes were formed by spreading a 1% solution of diphytanoyl phosphatidylcholine dissolved in n-decane across the hole. After the membranes turned black, activated C2II or activated C2II mutants were added to one side of the membrane, the cis side. The potentials applied to the membranes throughout the study always refer to those applied to the cis side, the side of the addition of C2II and its mutants. Electrical measurements were performed using Ag/AgCl electrodes (with salt bridges) 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). Zero-current membrane potential measurements were performed by establishing salt gradients across membranes containing 100–1000 channels as has been described earlier (38).

**Titration Experiments with Chloroquine and Related Compounds**—Chloroquine and other 4-aminoquinolones block the channels formed by the binding component C2II of C2 toxin (29, 32). To investigate the binding properties of chloroquine and its analogues, titration experiments were carried out similar to those used previously to study the binding of carbohydrates to the LamB channel of *E. coli* or binding of chloroquine to C2II and PA₆₃ channels (32, 39, 40). C2II and mutants were added to black lipid bilayer membranes at concentrations of about 50 ng/ml. About 30 min after the addition of the pore-forming proteins, the rate of their reconstitution in the membranes became very low. At that time, concentrated solutions of chloroquine, primaquine, or quinacrine were added to one or both sides while stirring to allow equilibration. This resulted in the blockage of the C2II channels. The data of the channel block were analyzed in a similar way as performed previously (39). The conductance, G(c), of a C2II channel in the presence of chloroquine or related compounds with the stability constant, K, and the ligand concentration, c, is given by the

**Binding of Chloroquine to C2II Mutants**

The plasmid pGEX-2T-C2II was used as a template (36). Mutated plasmids were transformed into competent *Escherichia coli* BL21 cells, and the constructs were checked by DNA sequencing.

C2II and its mutants were expressed as glutathione S-transferase fusion proteins in *Escherichia coli* BL21 harboring the separate DNA fragments in plasmid pGEX-2T. Proteins were purified as described previously (36) and eluted with 10 mM glutathione, 100 mM NaCl, and 50 mM Tris (pH 8.0) or incubated with thrombin (3.25 National Institutes of Health units/ml of bead suspension) for cleavage of the fusion proteins from glutathione S-transferase. Thereafter, the suspension was centrifuged at 500 × g for 10 min at room temperature, 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.
maximum conductance (without ligand), $G_{\text{max}}$, times the probability that the binding site is free.

$$G(c) = \frac{G_{\text{max}}}{1 + K' \cdot c}$$  \hspace{1cm} (Eq. 1)

Equation 1 may also be written as follows,

$$\frac{(G_{\text{max}} - G(c))}{G_{\text{max}}} = \frac{K' \cdot c}{1 + K' \cdot c}$$  \hspace{1cm} (Eq. 2)

which means that the conductance as a function of the ligand concentration can be analyzed using Lineweaver-Burk plots. $K$ is the stability constant for chloroquine, primaquine, or quinacrine binding to the C2II channel. The half-saturation constant, $K_S$, of its binding is given by the inverse stability constant $K_S = K^{-1}$.

RESULTS

Generation of C2II Mutants—The C2II channel is cation-selective and is able to bind chloroquine and related compounds in the micromolar concentration range. Binding of chloroquine and its analogues is dependent on ionic strength and leads to blockage of the C2II channels (28, 29). This means presumably that ion-ion interactions are involved in binding of the positively charged chloroquine to negatively charged amino acids at the channel mouth or within the channel interior. To localize the binding site of chloroquine, which is presumably the same as for C2I, sequence comparisons of binding components C2II, PA, iota b, and VIP1 (Ac, B. thuringiensis; Aa, B. cereus) of the binary toxins C2, anthrax, iota, and the vegetative insecticidal proteins, respectively, were performed. The sequences of these binding components are significantly homologous, but they differ in binding affinity for chloroquine and related compounds (12, 29, 40, 41). Chloroquine binds to the PA channel with much higher affinity than to C2II, but it has a low affinity for binding to iota b and does not bind to VIP1.

To restrict the number of putative amino acids that could be responsible for ion-ion interactions, only some of the many negatively charged residues within the C2II sequence were chosen (see Fig. 1A) and subjected to alanine-scanning mutagenesis. The selected amino acids were different in several binding
components and localized within the vicinity of the channel forming domain of C2II (amino acids 264–490 of the mature protein) (36) that is homologous to the corresponding domain of PA (amino acids 259–487 of the mature protein) (33). We choose the amino acids Glu272, Glu280, Glu341, Glu346, Glu399, and Asp426. A recent publication demonstrated the important role of Phe427 (the so-called Φ-clamp) in PA for LfN transport (35). Phe427 in PA corresponds to Phe428 in C2II. Mutants of Phe428 were also constructed: F428A, F428Y, F428W, and F428D. Fig. 1, B and C, shows the localization of all mutated amino acids in the soluble PA-heptamer (33). Besides single mutations, some double and triple mutants of the different residues were also generated. All mutants were expressed in E. coli BL21 cells and purified as described previously (36).

**Heptamerization of the C2II Mutant Proteins**—Wild type C2II is able to form SDS-stable oligomers (heptamers) (22). To check if heptamerization is influenced by the mutations, the majority of the mutant proteins were activated by trypsin and subjected to 7% SDS-PAGE without heating (Fig. 2). Wild type C2II was also run on SDS-PAGE after heating (lane 1). It showed a similar aggregation tendency as shown previously (22). C2II wild type and most of the mutants formed SDS-stable oligomers. However, the single mutant E399A, the double mutants E399A/D426A and E399A/F428A (data not shown), and the triple mutant E399A/D426A/F428A (not shown) showed weaker oligomer bands, indicating a smaller stability of the oligomers. This result suggests that residue Glu399 could be involved in oligomerization of the binding component or influences the SDS stability of the heptamer.

**Channel Formation of the C2II Mutants in Artificial Lipid Bilayer Membranes**—A cellular receptor, a hybrid, and/or complex carbohydrate structure is involved in the binding of C2II on the surface of cells (23). A receptor is not required for formation of ion-permeable channels by activated C2II in black lipid bilayer membranes (28). To check if the mutants also showed membrane activity, experiments were performed in which activated C2II mutants were added at a defined concentration (about 25 ng/ml) to one side of black lipid bilayer membranes (28). To check if the mutants also showed membrane activity, experiments were performed in which activated C2II mutants were added at a defined concentration (about 25 ng/ml) to one side of black lipid bilayer membranes. The subsequent increase of the membrane current at 20 mV was steep for about 20 min. Only a small further increase (as compared with the initial one) occurred after that time. The membrane conductance measured 60 min after the addition of C2II mutants was taken as a measure of the membrane activity of the proteins. Mutants E399A and D426A had a lower membrane activity than wild type C2II by a factor of about 3–5. The double mutant E399A/D426A and the triple mutant E399A/D426A/F428A showed almost no pore forming activity in the lipid bilayer assay. Proteins with the mutations E272A, E280A, E341A, E346A, and F428A (Y, W, and D) had the same high membrane activity as wild type C2II. This result suggested that pore formation is not affected by the mutations with the exception of E399A and D426A, which both considerably reduced membrane activity of the mutants.

**Binding of Chloroquine to C2II Mutants**—To study the effect of the mutation on the C2II channel, the biophysical properties of the channels were investigated in single channel experiments. Fig. 3 shows current recordings of wild type C2II and different C2II mutants following reconstitution in lipid bilayer membranes. The recordings of both wild type and mutant proteins showed the typical step-like characteristics, which are caused by the superposition of the long-lived C2II channels (28, 43). Each single step represents the reconstitution of a heptamer formed by C2II or by a C2II mutant in the diphytanoyl phosphatidylcholine membranes. Some of the mutants had the same single channel conductance as wild type C2II (see Table 2). For others, it differed considerably from wild type C2II (40 pS) and varied from 5 to 140 pS in 150 mM KCl, 10 mM MES, pH 6 (20 mV applied voltage). The changes appeared to be dependent on size, property, and position of the amino acid introduced in exchange for the genuine one. This finding suggested that some of the selected amino acids (in particular Glu399, Asp426, and Phe428) are exposed to the water-filled vestibule of the channel and therefore are able to influence the biophysical properties of the C2II heptamer. The single channel distributions of most mutants were fairly homogeneous, suggesting well defined channels. This was not the case for the D426A mutants, where a wide conductance range was observed. Fig. 4 shows histograms of conductance fluctuations observed with mutant D426A and wild type C2II. The conductance of mutant D426A showed considerable variations, indicating that amino acid Asp426 is important for channel stability and size.

The effect of the KCl concentration on single channel conductance was studied in additional experiments (see Table 3). Previously, we found that the single channel conductance, G, of wild type C2II was dependent on the square root of the KCl concentration, which indicated point charge effects on the channel properties (28). This is in contrast to measurements with the C2II mutants E399A and D426A (see Table 3). In these cases, the single channel conductance was an almost linear function of the aqueous KCl concentration (see “Discussion”). The concentration dependence of the single channel conductance of the C2II mutants F428A and F428Y was similar to that of wild type C2II, but their conductance was larger than that of wild type C2II, suggesting a larger effective channel diameter.
Binding of Chloroquine to C2II Mutants

Zero Current Membrane Potentials—To obtain information about the selectivity of the C2II-mutant channels zero-current membrane potential measurements were performed in the presence of salt gradients. After reconstitution of 100–1000 mutant channels into membranes, the salt concentration of the aqueous phase on one side of the membranes was increased 3-fold from 150 to 450 mM. Thereafter, the zero current potential was measured. In all cases, the more diluted side of the membrane became positive, which indicated preferential movement of cations through the C2II mutant channel (i.e., the mutant channels were cation-selective, similar to those formed by wild type C2II). With the exception of mutant F428Y, all mutants showed somewhat weaker cation selectivity than wild type. The magnitude of change with respect to wild type depended on the position and the nature of the introduced amino acid and the number of mutations. The zero current membrane potentials for KCl are given in Table 4 together with the ratios of the permeability $P_{\text{cation}}$, divided by $P_{\text{anion}}$, as calculated from the Goldman-Hodgkin-Katz equation (38).

Stability Constant of the 4-Aminoquinolone Binding to C2II Mutants—Chloroquine and related compounds (see Fig. 5 for the structure of chloroquine, primaquine, or quinacrine) bind to the C2II channel in vitro and in vivo and thereby block it (28, 29). To investigate the influence of the mutations on the binding of 4-aminoquinolones, we performed titration experiments in the following way. Activated C2II mutant proteins were added to the cis side (the side of the applied potential) of a black lipid bilayer membrane in a concentration of about 50 ng/ml, leading to an increase of conductance by many orders of magnitude caused by reconstitution of C2II channels into the membrane. About 60 min after the addition of protein, the conductance was virtually stationary. At this time, concentrated solutions of chloroquine, primaquine, or quinacrine were added to the aqueous phase on one or both sides of the membrane while stirring to allow equilibration. Subsequently, the C2II-induced membrane conductance decreased as a function of the concentration of the added compound on one or both sides of the membrane (see Fig. 6, A and B). The data of Fig. 6, A and B, and of similar experiments were analyzed using Lineweaver-Burk plots according to Equation 2. The good fit of the experimental data by the straight line in Fig. 6, B and C ($r = 1.000$) suggests that the interaction between chloroquine and the C2II channel represents a single hit binding process. From the data of Fig. 6A, a stability constant, $K_s$ of $(8.85 \pm 0.04) \times 10^4$ M$^{-1}$ (half-saturation constant $K_{1/2} = 11.3 \pm 0.1$ µM) was calculated from a least-squares fit for the binding of chloroquine to the C2II channel.

Similar experiments were performed with most of the mutants, with the exception of the double mutant E399A/D426A, which had a very low reconstitution rate (see above).
binding to the mutants D426A ($K_S = 2,500 \mu M$) and F428A ($K_S = 3,700 \mu M$), suggesting that these two residues play a major role in binding of these compounds. Substitution of Phe428 by aspartate causes a similar major impact on binding of the 4-aminooquinolones, and the half-saturation constant increased to $K_S = 3,400 \mu M$ for F428D. Interestingly, the replacements of Phe428 by the aromatic amino acids tyrosine and tryptophan also resulted in a substantial increase of the half-saturation constant of chloroquine binding to 170 and 240 $\mu M$, respectively. Glu399 is also involved in chloroquine binding but less than Asp426. E399A had a half-saturation constant, $K_S$, of 250 $\mu M$ that is by a factor of 25 higher than wild type. Similar effects as described above for chloroquine binding could also be found in titration experiments with quinacrine and primaquine (see Table 5).

Titration experiments of the C2II mutants containing the mutation D426A showed an interesting feature. As pointed out above, these mutants had a very low membrane activity, which means that only a few channels inserted into the membranes. When no further increase in conductance was observed, increasing amounts of concentrated 4-aminooquinolone solutions were added to both sides of the membrane to determine the stability constants of their binding to the C2II mutant channels (Fig. 7). First, at low concentrations, conductivity decreased somewhat because of partial blocking of the channels. However, the membrane conductance strongly increased again at higher concentrations, indicating a further insertion of

Table 5 demonstrates that the mutations strongly affected the affinity of chloroquine to the mutated C2II channels and decrease the stability constant for the binding of the 4-aminooquinolones. The strongest effect was observed for chloroquine binding to the mutants D426A ($K_S = 2,500 \mu M$) and F428A ($K_S = 3,700 \mu M$), suggesting that these two residues play a major role in binding of these compounds. Substitution of Phe428 by aspartate causes a similar major impact on binding of the 4-aminooquinolones, and the half-saturation constant increased to $K_S = 3,400 \mu M$ for F428D. Interestingly, the replacements of Phe428 by the aromatic amino acids tyrosine and tryptophan also resulted in a substantial increase of the half-saturation constant of chloroquine binding to 170 and 240 $\mu M$, respectively. Glu399 is also involved in chloroquine binding but less than Asp426. E399A had a half-saturation constant, $K_S$, of 250 $\mu M$ that is by a factor of 25 higher than wild type. Similar effects as described above for chloroquine binding could also be found in titration experiments with quinacrine and primaquine (see Table 5).

Titration experiments of the C2II mutants containing the mutation D426A showed an interesting feature. As pointed out above, these mutants had a very low membrane activity, which means that only a few channels inserted into the membranes. When no further increase in conductance was observed, increasing amounts of concentrated 4-aminooquinolone solutions were added to both sides of the membrane to determine the stability constants of their binding to the C2II mutant channels (Fig. 7). First, at low concentrations, conductivity decreased somewhat because of partial blocking of the channels. However, the membrane conductance strongly increased again at higher concentrations, indicating a further insertion of
additional channels or stabilizing effects on already inserted ones. Possibly, chloroquine and related compounds bind to some kind of oligomeric state of the mutants that are deficient in pore formation and somehow stabilize the structure and/or trigger membrane insertion followed by pore formation.

Ref. 29 demonstrated that chloroquine binding to C2II channels is asymmetrically dependent on the side of addition. To test this for the C2II mutants, titration experiments were carried out where chloroquine was added only to one side of the membrane (the cis side, the side of the addition of C2II mutants). These data are also included in Table 5. Depending on the mutation, all mutants displayed different binding affinities if chloroquine was added to different sides of the membrane, denoting also a considerable asymmetry of binding. However, the asymmetry of chloroquine binding to the C2II mutants was considerably smaller than in the case of wild type C2II. This is presumably caused by the reduced binding affinity and the higher permeability of chloroquine through the C2II mutant channels.

The stability constant of chloroquine binding to C2II wild type is ionic strength-dependent (29).
check whether this is also the case for chloroquine binding to C2II mutants, stability constants were also determined for its binding to E399A, F428A, and F428Y in 1 M KCl instead of 150 mM (Table 5). With the exception of E399A, the half-saturation constants increased with increasing ionic strength of the aqueous phase, suggesting that also point charges influence chloroquine binding to some of the C2II mutants. The rate of the increase of the half-saturation constant from 150 mM to 1 M was approximately the same as for wild type C2II.

**DISCUSSION**

In previous studies, we could demonstrate that chloroquine and other 4-aminoquinolones are able to block C2-mediated intoxication of target cells in vivo and reconstituted C2II channels in vitro (29, 32, 43). Binding of chloroquine and related compounds to C2II is highly ionic strength-dependent. This suggests that ion-ion interactions between the positively charged molecules and the channel-forming heptamers are at least partially responsible for their high affinity to the C2II channel (32). Channel block occurs also when C2I is added to the cis side of C2II reconstituted into lipid bilayers at very low C2I concentration (26). Binding of EF/LF to PA is also highly ionic strength-dependent, which is presumably caused by a number of negatively charged amino acids localized within the vestibule of the PA channel (35, 44–46). The close homology between C2II and PA was used in this study to identify negatively charged residues in C2II that could be responsible for this ion-ion interaction between the C2II heptamer and the 4-aminoquinolones. These negatively charged amino acids could also be involved in C2I binding (33, 34). The corresponding C2II mutants and additional mutants where the Φ-clamp was also mutated were studied here with respect to heptamerization, channel properties, and binding of 4-aminoquinolones.

**Heptamerization and Pore Formation Are Influenced by Mutations E399A and D426A**—After proteolytic activation, C2II, PA of anthrax toxin, iota b of iota toxin, and the VIPs form heptamers (12, 33, 47). These heptamers are essential for binding and component-induced channel formation in biological and artificial membranes (12, 28, 29, 41, 48). Here the influence of the mutation of several different amino acids on heptamerization as a prerequisite for channel formation was investigated. Most C2II mutants, such as E272A, E280A, E341A, and E346A, formed readily SDS-resistant heptamers. Similarly, SDS-resistant heptamers were also observed for mutants of F428 irrespective of whether the phenylalanine of the Φ-clamp was replaced by different aromatic residues or was changed into aspartic acid or alanine. However, SDS-resistant heptamer formation was only little influenced. On the other hand, D426A, which does not form defined channels and has an extremely small membrane activity, shows SDS-resistant oligomers.

Most mutants form stable and defined channels in lipid bilayer membranes. These are the mutants E272A, E280A, E341A, and E346A. The channel-forming activity of these mutants was approximately the same as for wild type C2II, indicating again that these mutations had no influence on the structure and function of the C2II heptamer. Similarly, mutation of the phenylalanine in position 428 (the Φ-clamp) had only a small influence on channel formation and stability. However, the mutation had a substantial influence on single channel conductance and on 4-aminoquinolone binding (see below). Glu399 seems to be important for oligomerization or for SDS stability of the heptamer but not for formation of stable pores. D426A, on the other hand, seems to form SDS-stable oligomers, but it has an extremely low membrane activity and forms channels that are not well defined. This mutation in a double or triple mutant has the same characteristics as the single mutation, which means that it was difficult to analyze them because of the low reconstitution rates. Interestingly, titration experiments of the mutant D426A and the double and triple mutants containing the same mutation with chloroquine and related compounds showed an interesting feature. When chloroquine was added to a few channels, the membrane activity of the mutants strongly increased, indicating an increasing reconstitution rate in the presence of chloroquine. Possibly, chloroquine binds to the binding site inside preformed heptamers and triggers their conformational change into the membrane-active form.

It is noteworthy that Sellman et al. (44) described a similar behavior for pore formation and heptamerization for related PA mutants whose structure is presumably highly homologous to that of C2II. They demonstrated that the PA mutant D425A was not impaired in receptor binding and oligomerization, but translocation of the enzymatic components, SDS stability, and pore-forming activity were decreased. Mutant PA E398C also exhibited some altered behavior in the above mentioned properties but caused only a minor functional defect (44). It was suggested that PA mutant D425A is no longer able to undergo conformational changes required for formation of an SDS-resistant oligomer and that this property could be a prerequisite...
for pore formation. The precise structure of the membrane form of any of the binding proteins is still not known, but our data confirm the results of Ref. 44 for the related mutations D426A (corresponding to PA D425A) and E399A (corresponding to PA E398C) and also demonstrate that the structure of the C2II heptamer could be influenced or stabilized by chloroquine and related compounds. Other residues are possibly also important for the formation of the C2II heptamer. In a recent publication (49), it has been demonstrated that residue Asp426 of PA is essential for the interaction during pore formation of protective antigen. Asp426 seems to interact with Lys397, presumably through a salt bridge between the oppositely charged side chains. Similar salt bridges are probably not present in C2II. However, it has been suggested that the two glutamines Gln398 and Gln427 play a similar role in C2II (50).

Selected Mutations Alter Single Channel Conductance—The single channel conductance of the mutants E272A, E280A, E341A, and E346A was not affected as compared with that of the wild type C2II, which agrees with their high membrane activity. However, mutations of the residues Glu399, Asp426, and Phe428 resulted in substantial change of the single channel conductance, indicating that the three residues are exposed to the water-filled vestibule of the channel and thus influence ion flux. Substitution of the negatively charged amino acids Glu399 and Asp426 by alanine caused a decrease of single channel conductance from 40 pS in 150 mM KCl in wild type C2II to 13 and 20 pS, respectively, probably because seven negatively charged residues within the vestibule of the channel were replaced by neutral ones. It has to be mentioned that the mutant D426A was special because its single channel conductance displayed a wide range of values in contrast to all of the other mutations. Presumably, the broad histogram has to do with an undefined structure of the mutant C2II heptamer. Interestingly, the double mutant E399A D426A did not lead to any further decrease of single channel conductance, indicating that additional charges influence its conductance. One possibility is Glu307, which is directly localized in the channel interior (43). Glu307 is presumably also responsible for the fact that all mutants are still cation-selective, although the selectivity decreased somewhat for the mutants E399A, D426A, and F428A (see Table 4).

Mutation of residue Phe428 also resulted in a substantial change of single channel conductance. Substitution of phenylalanine by alanine, aspartic acid, or tyrosine led to an increase, whereas replacement by tryptophan led to a decrease of single channel conductance. Alanine had the biggest effect (140 pS as compared with 40 pS for wild type C2II), because its side chain has a smaller size than the phenyl group. This is a little astonishing, because F428D (G = 110 pS) adds seven additional putative charges to the constriction of the channel. However, the vicinity of the central constriction is presumably the result of Glu399 and Asp426 already being highly acidic; therefore, the additional seven aspartic acids in position 428 are presumably not all negatively charged. Tyrosine is a little more hydrophilic, which results in a small increase of the single channel conductance of the F428Y mutant (G = 60 pS). The dramatic decrease of the single channel conductance of the mutant F428W (G = 5 pS) is not very astonishing. The bulky tryptophan side chains obviously restrict the entrance of the C2II channel even further than the Φ-clamp formed by the seven phenylalanines. Nonlinear relationships between single channel conductance and KCl concentration were also observed in this study for most of the mutants (see Tables 2 and 3). The ionic strength dependence of the single channel conductance of some of the mutants was analyzed in detail using the previously proposed equations based on the Debeye-Hückel theory (40). The experimental points in Fig. 8 (conductance of wild type C2II and the mutants F428A and F428Y as a function of the KCl concentration) were fitted according to a combination of Equations 1–4 of the Appendix of Ref. 28. The data for wild type C2II could be sufficiently well explained (see the broken line in Fig. 8) using 1.4 negatively charged groups (q = 2.24 × 10⁻¹⁹ coulombs) and a diameter of 0.7 nm for the channel, similar to that shown previously (28). The data points for F428A could also be fitted using the same equations and assuming the same number of negatively charged groups. However, the channel diameter had to be increased to 1.0 nm to obtain a reasonable fit (see the solid line in Fig. 8). This result is consistent with an important role for the Φ-clamp in C2II channel function, because the seven Phe428 residues restrict the channel size. The experimental data of the F428Y mutant could also be reasonably well explained using the parameters of wild type C2II and a somewhat higher conductance of the channel in the absence of point net charges (q = 2.24 × 10⁻¹⁹ coulombs; r = 0.7 nm; dashed line in Fig. 8). Fig. 8 also demonstrates that the single channel conductance of the mutant E399A was a linear function of the KCl concentration, because a least-squares fit of the experimental points had a slope of 1 (see the dotted line in Fig. 8) in clear difference from the curve for wild type C2II (broken line in Fig. 8). A similar conclusion could also be drawn for the D426A mutant. However, the data were, by far, not as precise as those for E399A.
because of the broad single channel distributions observed for this mutant.

_Mutations E399A, D426A, and F428A Strongly Affect the Binding Affinity of 4-Aminoquinolones to the C2II Channel—_4-Aminoquinolones, such as chloroquine, primaquine, or quinacrine, are able to block the C2II-induced membrane conductance in vitro and intoxication in vivo_ (29). Binding of these drugs is ionic strength-dependent and asymmetric with respect to the side where they were added. The results of titration experiments of C2II mutant channels with the same compounds strongly suggest that some of the mutated amino acids, in particular Glu399, Asp426, and Phe428, but not all of the others, play a major role in the binding of these molecules. Residue Glu399 is most likely located at the cis side of the vestibule near the channel entrance. Its mutation to alanine resulted in a 25-fold weaker binding affinity for titration with chloroquine added to both sides. Similarly, the mutation D426A, which is presumably localized next to the phenylalanines of the F-clamp, results in an even lower binding activity on the order of 250 times lower, indicating that Asp426 is also highly involved in the binding process of the 4-aminoquinolones.

Not only charged residues contribute to the binding of the 4-aminoquinolones to the C2II channel. The mutation F428A decreases the stability constant for chloroquine binding to the C2II channel by a factor of almost 400, from 10^5 m^-1 to 270 m^-1. This is definitely the strongest effect of all of the mutations on chloroquine binding. Alanine alone is not able to create such a big effect. Replacement of Phe428 by a charged amino acid has approximately the same effect, because the F428D mutant channel has also a very low affinity for chloroquine binding. A strong increase of the half-saturation constant for chloroquine binding was also observed for the C2II mutants F428W and F428Y. However, the aromatic residues tryptophan and tyrosine can at least partially replace phenylalanine in these cases, which means that the effect of these mutations on chloroquine binding was less pronounced than for nonaromatic amino acids. Double and triple C2II mutants do not add much to this picture, which means that the affinity for chloroquine did not decrease much further for these mutants as compared with single mutations. Possibly, other neutral or charged groups also contribute to chloroquine binding, which is definitely very weak for all double and triple mutants. One possible candidate is, for instance, Glu307, which is localized inside the C2II channel (43).

In previous studies, we could show that binding of 4-aminoquinolones exhibits a considerable asymmetry for addition from the cis side of the membrane (the side of the membrane where the C2II mutants were added) as compared with the trans side (32). This has been explained by assuming that the binding site is localized within the vestibule of the C2II channel, where Glu399, Asp426, and Phe428 are localized. Chloroquine molecules added to the trans side have to cross the channel first before they can be bound. Since the permeability of the channel is limited for the bulky compounds, the half-saturation constant is higher for chloroquine addition to the trans side (see Table 5) (32). Mutations E399A, D426A, F428A, and F428D resulted not only in a much smaller affinity for chloroquine binding but also in a highly reduced or almost complete loss of binding asymmetry from the cis as compared with the trans side. The mutation of Glu399, Asp426, and Phe428 has a qualitatively similar effect on binding affinity of the other 4-aminoquinolones, quinacrine and primaquine, to the C2II mutant channels as on chloroquine binding, suggesting that the molecular basis of their binding is more or less the same as with chloroquine. Nevertheless, they show some difference in binding activity, which presumably has to do with the individual structure of the single 4-aminoquinolone, as has been discussed in detail elsewhere (29, 40).

REFERENCES

1. Considine, R. V., and Simpson, L. L. (1991) *Toxicon* 29, 913–936
2. Aktories, K., Wille, M., and Just, I. (1992) *Curr. Top. Microbiol. Immunol.* 175, 97–113
3. Barth, H., Blöcker, D., and Aktories, K. (2002) *Nawyn Schmiedeberg's Arch. Pharmacol.*** 366, 501–512
4. Simpson, L. L., Stiles, B. G., Zepeda, H. H., and Wilkins, T. D. (1987) *Infect. Immun.* 55, 118–122
5. Schering, B., Bärmann, M., Chhatwal, G. S., Geipel, U., and Aktories, K. (1988) *Eur. J. Biochem.* 171, 225–229
6. Popoff, M. R., Rubin, E. J., Gill, D. M., and Boquet (1988) *Infect. Immun.* 56, 2299–2306
7. Perelle, S., Gibert, M., Bourlioux, P., Corthier, G., and Popoff, M. R. (1997) *Infect. Immun.* 65, 1402–1407
8. Gülke, I., Pfeifer, G., Liese, J., Fritz, M., Hofmann, F., Aktories, K., and Barth, H. (2001) *Infect. Immun.* 69, 6004–6011
9. Popoff, M. R., and Boquet, P. (1988) *Biochem. Biophys. Res. Commun.* 152, 1361–1368
10. Simpson, L. L., Stiles, G. B., Zepeda, H., and Wilkins, T. D. (1989) *Infect. Immun.* 57, 255–261
11. Han, S., Craig, J. A., Putnam, C. D., Carozzi, N. B., and Tainer, J. A. (1999) *Nat. Struct. Biol.* 6, 932–936
12. Leuber, M., Orlak, F., Schiller, B., Sickmann, A., and Benz, R. (2006) *Biochemistry* 45, 283–288
13. Mock, M., and Fouet, A. (2001) *Annu. Rev. Microbiol.* 55, 647–671
14. Collier, R. J., and Young, J. A. (2003) *Annu. Rev. Cell Dev. Biol.* 19, 45–70
15. Ohishi, I., Iwasaki, M., and Sakaguchi, G. (1980) *Infect. Immun.* 30, 668–673
16. Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K. H., and Habermann, E. (1986) *Nature* 322, 390–392
17. Vandekerckhove, J., Schering, B., Bärmann, M., and Aktories, K. (1988) *J. Biol. Chem.* 263, 696–700
18. Geipel, U., Just, I., Schering, B., Haas, D., and Aktories, K. (1989) *Eur. J. Biochem.* 179, 229–232
19. Reuner, K. H., Presek, P., Boschek, C. B., and Aktories, K. (1997) *Eur. J. Cell Biol.* 43, 134–140
20. Wiegers, W., Just, I., Müller, H., Hellwig, A., Traub, P., and Aktories, K. (1991) *Eur. J. Cell Biol.* 54, 237–245
21. Ohishi, I. (1987) *Infect. Immun.* 55, 1461–1465
22. Barth, H., Blöcker, D., Behlke, I., Bergsmaj-Schutter, W., Brisson, A., Benz, R., and Aktories, K. (2000) *J. Biol. Chem.* 275, 18704–18711
23. Eckhardt, M., Barth, H., Blöcker, D., and Aktories, K. (2000) *J. Biol. Chem.* 275, 2328–2334
24. Ohishi, I., and Yanagimoto, A. (1992) *Infect. Immun.* 60, 4648–4655
25. Barth, H., Hofmann, F., Olenik, C., Just, I., and Aktories, K. (1998) *Infect. Immun.* 66, 1364–1369
26. Blöcker, D., Pohlmann, K., Haug, G., Bachmeyer, C., Benz, R., Aktories, K., and Barth, H. (2003) *J. Biol. Chem.* 278, 37360–37367
27. Simpson, L. L. (1989) *J. Pharmacol. Exp. Ther.* 251, 1223–1228
28. Schmidt, A., Benz, R., Just, I., and Aktories, A. (1994) *J. Biol. Chem.* 269, 16706–16711
29. Bachmeyer, C., Benz, R., Barth, H., Aktories, K., Gilbert, M., and Popoff, M. R. (2001) *FASEB J.* 15, 1658–1660
30. Zhang, S., Finkelstein, A., and Collier, R. J. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 16756–16761
Binding of Chloroquine to C2II Mutants

31. Zhang, S., Udho, E., Wu, Z., Collier, R. J., and Finkelstein, A. (2004) Biophys. J. 87, 3842–3849
32. Bachmeyer, C., Orlik, F., Barth, H., Aktories, K., and Benz, R. (2003) J. Mol. Biol. 333, 527–540
33. Petosa, C., Collier, R. J., Klöppel, K. R., Leppa, S. H., and Liddington, R. C. (1997) Nature 385, 833–838
34. Schleberger, C., Hochmann, H., Barth, H., Aktories, K., and Schulz, G. E. (2006) J. Mol. Biol. 364, 705–715
35. Krantz, B. A., Melnyk, R. A., Zhang, S., Juris, S. J., Lacy, D. B., Wu, Z., Finkelstein, A., and Collier, R. J. (2005) Science 309, 777–781
36. Blöcker, D., Barth, H., Maier, E., Benz, R., Barbieri, J. T., and Aktories, K. (2000) Infect. Immun. 68, 4566–4573
37. Benz, R., Janko, K., Boos, W., and Läuger, P. (1978) Biochim. Biophys. Acta 511, 305–319
38. Benz, R., Janko, K., and Läuger, P. (1979) Biochim. Biophys. Acta 551, 238–247
39. Benz, R., Schmid, A., and Vos-Scheperkeuter, G. H. (1987) J. Membr. Biol. 100, 12–29
40. Orlik, F., Schiffer, B., and Benz, R. (2005) Biophys. J. 88, 1715–1724
41. Knapp, O., Benz, R., Gibert, M., Marvaud, J. C., and Popoff, M. R. (2002) J. Biol. Chem. 277, 6143–6152
42. Nüssi, S., Collier, R. J., and Finkelstein, A. (2002) Biochemistry 41, 1445–1450
43. Blöcker, D., Bachmeyer, C., Benz, R., Aktories, K., and Barth, H. (2003) Biochemistry 42, 5368–5377
44. Sellman, B. R., Nüssi, S., and Collier, R. J. (2001) J. Biol. Chem. 276, 8371–8376
45. Neumeyer, T., Tonello, F., Dal Molin, F., Schiffer, B., Orlik, F., and Benz, R. (2006) Biochemistry 45, 3060–3068
46. Neumeyer, T., Tonello, F., Dal Molin, F., Schiffer, B., and Benz, R. (2006) J. Biol. Chem. 281, 32335–32343
47. Blöcker, D., Behlke, J., Aktories, K., and Barth, H. (2001) Infect. Immun. 69, 2980–2987
48. Blaustein, R. O., Koehler, T. M., Collier, R. J., and Finkelstein, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2209–2213
49. Melnyk, R. A., and Collier, R. J. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 9802–9807
50. Christensen, K. A., Krantz, B. A., and Collier, R. J. (2006) Biochemistry 45, 2380–2386