Interaction of Clostridium perfringens Iota-Toxin with Lipid Bilayer Membranes

DEMONSTRATION OF CHANNEL FORMATION BY THE ACTIVATED BINDING COMPONENT Ib AND CHANNEL BLOCK BY THE ENZYME COMPONENT Ia*

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The interaction between model lipid membranes and the binding component (Ib) of the ADP-ribosylating iota-toxin of Clostridium perfringens was studied in detail. Ib had to be activated by trypsin to result in channel formation in artificial lipid bilayers. The channels formed readily by Ib had a small single-channel conductance of about 85 picosiemens in 1 M KCl. Channel function was blocked in single-channel and multichannel experiments by the enzymatic component Ia in a pH-dependent manner. The strong Ia-mediated channel block of Ib occurred only when the pH was at least lowered to pH 5.6. The single-channel conductance showed a linear dependence on the bulk aqueous KCl concentration, which indicated that the channel properties were more general than specific. Zero current membrane potential measurements suggested the Ib channel has an ~6-fold higher permeability for potassium ions than for chloride. The selectivity ratio changed for salts composed of cations and anions of different mobility in the aqueous phase, again suggesting that Ib formed a water-filled general diffusion pore. Asymmetric addition of activated Ib to lipid bilayer membranes resulted in an asymmetric voltage dependence, indicating its full orientation within the membrane. Titration experiments with chloroquine and different tetraalkylammonium ions suggested that the Ib channel was blocked by these compounds but had only a weak affinity to them. In vivo measurements using Vero cells demonstrate that chloroquine and related molecules also did not efficiently block intoxication of the cells by iota-toxin. The possible role of Ib in the translocation of iota-toxin across the target cell membrane is discussed.

Clostridium perfringens iota-toxin is a member of a family of toxins that ADP-ribosylate actin (see Refs. 1–4 for reviews). Other members of this toxin group are the closely related Clostridium difficile toxin; PA, anthrax protective antigen; pS, picosiemens.

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1 The abbreviations used are: Sb, binding component of C. spiroforme toxin; Ib, binding component of iota-toxin; Ia, ADP-ribosylating iota-toxin; PA, anthrax protective antigen; pS, picosiemens.
essential for intoxication of target cells in the case of C2 toxin of C. botulinum (23, 24). On the other hand, it has also been demonstrated that the binding components of C2 toxin, iota-toxin, and the anthrax protective antigen share common structural properties, which suggests a common mode of action (11, 22, 25, 26).

Intracellularly acting toxins have to internalize their enzymatic domains into the cytosol to generate the toxic activity. In a number of recent studies, it has been shown that these intracellularly acting toxins are able to produce channels in the target cell membrane and in lipid bilayer membranes. Examples for this are diphtheria toxin (27–29), neurotoxins such as tetanus toxin (30) and botulinum neurotoxin (27, 31, 32), and the binding components of anthrax toxin and the C2 toxin (33–35). At least for diphtheria toxin, some evidence has been presented (36, 37) that toxin translocation may occur through the channel formed by its own translocation domain, although the role of the channel in the translocation of diphtheria toxin is still a matter of debate (38). Here we report that the activated Ib is able to induce the formation of small ion-permeable channels in artificial lipid bilayer membranes. The channels are cation-selective on the basis of an excess of negatively charged groups in or near the channel. Channels were observed in many different salts, suggesting that its diameter is at least 1 nm. The inhibition of channel formation by Ib and inhibition of intoxication by iota-toxin by a variety of different compounds was studied in vitro and in vivo.

MATERIALS AND METHODS

Materials—Chloroquine and related compounds 4-aminoquinadin, 4-amodiaquine, primaque, quinine, and quindine were obtained from Sigma. All salts were obtained from Merck (Darmstadt, Germany, unless otherwise stated). Dulbecco’s modified Eagle’s medium containing 30 g of glucose, 20 g of yeast extract, and 0.5 g of cysteine-HCl/liter (pH 7.2) under anaerobic conditions. Unprocessed Ib was produced from C. perfringens strain TS133 harboring the recombinant plasmid pMRP384, and Ib was produced from strain 667 harboring pMRP147. Ib and Ib components were purified as described previously (19). Ib (200 μg/ml) was activated by incubation in 20 μg/ml trypsin for 30 min at room temperature followed by the addition of 100 μg/ml trypsin inhibitor (19). Rabbit anti-Ib antibodies were obtained as has been described previously by immunizing the animals with activated Ib (21). They were used as whole serum.

Experiments with Black Lipid Membranes—Black lipid bilayer membranes were formed from a 1% solution of diphantoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane as described previously (39). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole with a surface area of 0.3 mm² across which the membranes were formed. Activated iota (Ib) was added from a concentrated stock solution to the aequous phase bathing a membrane in the black state. The temperature was kept at 20 °C throughout. The membrane current was measured with a pair of Ag/AgCl electrodes with salt bridges switched in series with a voltage source and a current amplifier (Keithley 427). Zero-current membrane potential measurements were performed by establishing a salt gradient across membranes containing 100–1,000 Ib channels as has been described earlier (40).

In Vivo Experiments Using Vero Cells—Vero cells were grown in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum in 96-well plates at 37 °C in an atmosphere with 5% CO₂. For the in vivo experiments with iota-toxin, the growth medium of the Vero cells was replaced with Dulbecco’s modified Eagle’s medium-5% fetal calf serum containing chloroquine and related compounds (100 μg/well) for 1 h at 37 °C prior to the addition of the iota-toxin. The concentration of chloroquine and related compounds was selected such that the highest concentration did not induce any cytotoxic effects on the Vero cells. Then the binding and the toxin components of iota, C. perfringens, and C2 toxin (3 × 10⁻⁸ M) and serial 2-fold dilutions were added to the cells. The monolayers were incubated for 5 h at 37 °C, and the toxic-specific cytotoxic effects were monitored by phase contrast microscopic observation. The toxin titer corresponded to the lower concentration of toxin, which induced a morphological alteration in 50% of cells. The results are expressed as percentages of the inhibition of the cytotoxic effects as compared with those obtained with the toxin without chloroquine and related compounds.

RESULTS

Channel Formation by Activated Ib—In the first set of experimental conditions, we studied the interaction of native Ib with lipid bilayer membranes made of diphantoyl phosphatidylcholine/n-decane. Only a small number of channels were observed under these conditions even when very high concentrations of Ib (up to 10 μg/ml) were added to the aequous phase bathing black lipid membranes, which means that native Ib did not show a high membrane activity. Channel formation was frequent, however, when Ib was activated with trypsin. Fig. 1A shows a single-channel recording of current fluctuations of a lecithin membrane observed with 100 ng/ml activated Ib in 1 m KCl solution. The single-channel conductance was on average 85 pS at a membrane potential of 50 mV, and the channels had a long lifetime of at least 5 min under the conditions of Fig. 1A.

Fig. 2A demonstrates that the fluctuations were fairly homogeneous. Only occasionally, we observed conductance steps that were twice that of the 85-pS channel, which may indicate that two channels were formed at once. The formation of channels by Ib in lipid bilayer membranes was not a rare event. It is noteworthy that with 100 ng/ml activated Ib, more than 1,000 channels were formed within about 30 min in a diphantoyl phosphatidylcholine/n-decane membrane with a surface area of about 0.3 mm².
substrates of the open channel. 

To study the interaction between Ia and Ib in more detail, and in particular also the pH effect on Ia-Ib interaction, we performed multichannel experiments with membranes that contained many Ib channels. Fig. 3 shows an experiment of this type. Ib was added in a concentration of 1 μg/ml to one side of a black membrane bathed in 150 mM KCl, pH 6. After about 20 min, the conductance increase caused by the reconstitution of Ib channels slowed down considerably. At this time, 500 ng/ml Ia was added to the same side of the membrane (the cis-side, left side arrow). Only a rather small decrease occurred, indicating that the effect of Ia on the Ib channels was small under these conditions. After about 8 min, the pH was lowered at the cis-side to pH 5.6 (right side arrow). This led to a substantial decrease of the membrane conductance, indicating a block of the Ib channels. To rule out the possibility that the closure of the Ib channels is caused by the pH, we also performed experiments in which the pH was lowered to pH 5.6 before the addition of Ia. Fig. 4 shows such an experiment: Ib was added in a concentration of 2 μg/ml to one side of a black membrane bathed in 150 mM KCl, pH 6 (arrow at the left-hand side of Fig. 4). Subsequently the membrane conductance increased by more than 3 orders of magnitude within about 20 min. At 25 min after the addition when the conductance was stationary, the pH was lowered at the cis-side to pH 5.6 by adding a defined amount of HCl (second arrow from the right). This had no effect on the membrane conductance. 7 min later, 2 μg/ml Ia was added while stirring to the cis-side of the membrane (arrow on the right side in Fig. 4). The addition of Ia resulted in a rapid decrease of the membrane conductance, indicating the block of the Ib channels (see also the inset in Fig. 4, which represents the original strip chart record). It is noteworthy that the decrease of pH and addition of Ia only influenced Ib-mediated membrane conductance when it occurred at the cis-side of the membrane. The addition of Ia to the trans-side or lowering the pH at the same side when Ia was already present did not influence the conductance of the Ib channels.

**Inhibition of Channel Formation by anti-Ib Antibodies**—To test whether the channels formed by Ib in lipid bilayer membranes were specific for the presence of Ib or caused by an unspecific artifact, we performed experiments with anti-Ib polyclonal antibodies. The addition of 1 μL/ml serum containing the antibodies had no effect on the conductance of the channels that were already reconstituted into the membranes when the aqueous phase contained 100 ng/ml activated Ib. However, reconstitution of additional channels was not observed when the antibodies were added to the cis-side, the side of the addition of Ib. The addition of the antibodies to the trans-side had no influence on channel formation. Reconstitution of Ib channels could not be observed when 400 ng of Ib was preincubated with 5 μl of antiserum before its addition to the aqueous phase.

**Single-channel Analysis**—The single-channel conductance was a linear function of the KCl concentration in the bulk aqueous phase (Table I). This indicates that the Ib channel does not contain a binding site for potassium ions or chloride inside the channel or a cluster of negatively charged groups, as has been observed for the C2-II channel (34) or the channel formed by the anthrax protective antigen (PA) (33). Single-channel experiments were also performed in other salt solutions. These experiments were done to get some insight on the biophysical properties of the Ib channel. The results are also included in Table I and show that cations had a strong influence on the single-channel conductance. This result is consist-

**Fig. 2.** Histograms of the probability of the occurrence of certain conductivity units observed with membranes formed of diphytanoyl phosphatidylcholine/decane in the presence of activated Ib from *C. perfringens*. The aqueous phase contained 1 mM KCl and 100 ng/ml activated Ib (A). The applied membrane potential was 50 mV; T = 20°C. The average single-channel conductance was 85 pS for 230 single-channel events. The data were collected from five different membranes. The aqueous phase contained 1 mM KCl, 320 ng/ml activated Ib, and 80 ng/ml Ia (B). The applied membrane potential was 50 mV; T = 20°C. The average single-channel conductance was 60 pS for 185 single-channel events. The data were collected from three different membranes. Note also that the 85 pS was observed besides substrates of the open channel.

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**pH-promoted Block of Ib Channels by Ia**—Evidence has been presented that certain toxins enter the target cell via permeation through the channels formed by the binding component (36). To test whether the enzymatic component Ia interacts with the channels formed by activated Ib, we performed single-channel experiments in the presence of both binding component and enzyme. In the first set of experimental conditions, Ib was preincubated with an equal concentration of Ia, and both were added to the aqueous phase bathing lipid bilayer membranes. In single-channel experiments, we found a drastic effect of the enzyme component on the open probability of the Ib channels when it was preincubated with Ia. The channels frequently switched to substrates in the presence of Ia when the pH of the aqueous phase was slightly decreased to pH 5.6 (Fig. 1B). A histogram of the current fluctuations observed under these conditions is given in Fig. 2B. The average single-channel conductance of all current fluctuations (i.e. the open channels (85 pS) and the Ia-mediated substates of the Ib channel (about 50 pS)) was about 60 pS.
ent with the assumption that the Ib channel is cation-selective. The ionic selectivity of cations was Rb$^+$ > Cs$^+$ > K$^+$ > Na$^+$ > Li$^+$ ≈ Tris$^+$, which means that the permeability of the cations through the channels followed approximately their mobility sequence in the aqueous phase. This suggests that the Ib channel is water-filled and has inside only a small field strength and no small selectivity filter (i.e. no binding site). On the other hand, the single-channel conductance did not vary very much for the alkali ions Na$^+$, Li$^+$, and the large organic Tris$^+$, which suggests that the cation selectivity of the Ib channel is limited. In agreement with this, we observed that the single-channel conductance decreased in potassium acetate, indicating also that anions can enter the channel and influence the conductance of the cations.

**The Ib Channel Conducts Quaternary Ammonium Ions**—As mentioned above, *C. perfringens* iota-toxin shares some simi-

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**FIG. 3. Block of Ib-induced membrane conductance by Ia.** The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, pH 6, and 1 µg/ml activated Ib added to the cis-side of the membrane. 20 min after the addition of Ib, 500 ng/ml Ia (arrow on the left-hand side) was also added to the cis-side of the membrane. About 8 min later, the pH at the cis-side was lowered to pH 5.6 (arrow on the right-hand side). The temperature was 20 °C, and the applied voltage was 50 mV at the cis-side. For further explanations, see “Results.”

**FIG. 4. Effect of pH on the block of Ib-induced membrane conductance by Ia.** The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM KCl, pH 6, and 2 µg/ml activated Ib added to the cis-side of the membrane (arrow on the left-hand side). 25 min after the addition of Ib, the pH of the aqueous phase was lowered to pH 5.6 at the cis-side, which did not influence the membrane conductance (second arrow from the right side). 7 min later, 2 µg/ml Ia was added to the cis-side (arrow on the right-hand side). The temperature was 20 °C, and the applied voltage was 50 mV at the cis-side. The inset shows the original strip chart recording of membrane current after the addition of Ia (arrow).
larities with *C. botulinum* C2 toxin and the anthrax PA. For these channels, it has been demonstrated that the addition of ammonium chloride and methylammonium chloride to the external media inhibit the translocation of the enzyme component of C2 toxin (C2-I) through the cytoplasmic membrane of target cells (23). To test whether quaternary ammonium ions have any influence on the channel function of Ib and channel formation in lipid bilayer membranes (41), we performed single channel experiments with a variety of them. Interestingly, we observed current fluctuations for all salts (Table II), which means that Ib similar to C2-II conducts these cations. The single-channel conductance of some of these salts was considerably larger than in the corresponding 1 M KCl solution. This suggests a special architecture of the channel, because NH$_4^+$ and potassium ions have approximately the same ion radii and hydrated ion radii but have a different three-dimensional structure.

**Selectivity of the Channel Formed by Ib**—The selectivity of the Ib channel was measured in zero-current membrane potential measurements in the presence of salt gradients. After incorporation of a large number of channels in membranes bathed in 100 mM KCl, 5-fold salt gradients were established across the membranes by the addition of small amounts of concentrated KCl solution to one side of the membrane. In all cases, the more diluted side of the membrane became positive, which indicated preferential movement of cations through the Ib channel, i.e. it is cation-selective as suggested from the single-channel data (compare Tables I and III). Analysis of the zero-current membrane potentials using the Goldman-Hodgkin-Katz equation (equation 40) showed that the permeability ratio $P_{cation}/P_{anion}$ followed the aqueous mobility of the ions (Table III), which is consistent with the assumption that Ib forms a general diffusion pore.

### Table I

Average single-channel conductance, $G$, of the channel formed by the activated binding component Ib of iota-toxin in different salt solutions

| Salt        | Concentration $c$ | Single-channel conductance $G$ |
|-------------|-------------------|--------------------------------|
|             | $\mu$             | Ib $\mu S$ | C2-II $\mu S$ |
| LiCl        | 1.0               | 35         | 60          |
| NaCl        | 1.0               | 40         | n.m.$^a$    |
| KCl         | 0.01              | n.m.$^a$  | 12          |
|             | 0.03              | 3          | 25          |
|             | 0.1               | 9          | 55          |
|             | 0.3               | 30         | 80          |
|             | 1.0               | 85         | 150         |
|             | 3.0               | 180        | 380         |
| RbCl        | 1.0               | 125        | n.m.$^a$    |
| CaCl        | 1.0               | 110        | n.m.$^a$    |
| K$^+$-Acetate (pH 7.0) | 1.0 | 55 | 120 |
| Tris-Cl     | 1.0               | 28         | n.m.$^a$    |

$^a$ n.m., not measured.

### Table II

Average single-channel conductance, $G$, of the Ib channel in different 1 M ammonium salt solutions

| Salt                        | Single-channel conductance $G$ |
|-----------------------------|--------------------------------|
|                            | Ib $\mu S$ | C2-II $\mu S$ |
| KCl                         | 85         | 150         |
| NH$_4$Cl                    | 170        | 250         |
| (CH$_3$)$_2$NH$_2$Cl         | 160        | 270         |
| (C$_2$H$_5$)$_2$NH$_2$Cl     | 110        | 230         |
| (C$_2$H$_5$)$_4$NCl          | 42         | 175         |
| (C$_2$H$_5$)$_4$NCl          | 10         | 50          |
| (CH$_3$)$_4$NCl              | 70         | n.m.$^a$    |
| (C$_2$H$_5$)$_4$NCl          | 8.0        | n.m.$^a$    |

$^a$ n.m., not measured.

### Table III

Zero-current membrane potentials, $V_m$, of diphytanoyl phosphatidylcholine/n-decane membranes in the presence of Ib of *C. perfringens* measured for a 5-fold gradient of different salts $V_m$ is defined as the difference between the potential at the dilute side (100 mV) and the potential at the concentrated side (500 mV). The pH of the aqueous salt solutions was 6 unless otherwise indicated; T = 20 °C. The permeability ratio $P_{cation}/P_{anion}$ was calculated with the Goldman-Hodgkin-Katz equation (40) from at least 3 individual experiments.

| Salt                        | $V_m$/mV | $P_{cation}/P_{anion}$ |
|-----------------------------|----------|------------------------|
| Ib                          |          |                        |
| KCl                         | 25       | 5.5                    |
| LiCl                        | 9.8      | 1.8                    |
| KCH$_3$COO (pH 7)           | 29       | 8.1                    |
| C2-II                       |          |                        |
| KCl                         | 31       | 9.8                    |
| LiCl                        | 30       | 9.3                    |
| KCH$_3$COO (pH 7)           | 31       | 10.2                   |

membrane (the cis-side), and the conductance increase was followed for about 20 min. At this point, we applied different positive and negative potentials (with respect to the cis-side) to the membrane starting from 20 mV. Then we repeated the experiment with 30, 40, 50, and 60 mV. Fig. 5 shows the results with 70 mV. We applied first 70 mV (left side) and then −70 mV (right side) to the cis-side of the membrane. Only for negative potential at the cis-side did the membrane current decrease in an exponential fashion. For positive potentials at the cis-side, the current did not decrease even when the membrane potential was as high as 130 mV (data not shown). This result indicated asymmetric insertion of Ib into the membranes. The addition of the protein to both sides of the membrane resulted in a symmetric response to the applied voltage (data not shown). The data of the experiment of Fig. 5 and similar experiments were analyzed in the following way: the membrane conductance ($G$) as a function of voltage, $V_m$, was measured when the opening and closing of channels reached an equilibrium, i.e. after the exponential decay of the membrane current following the voltage step $V_m$. $G$ was divided by the initial value of the conductance ($G_0$, which was a linear function of the voltage) obtained immediately after the onset of the voltage. The data of Fig. 6 correspond to the asymmetric voltage-dependence of Ib (mean of three membranes) when the protein was added to the cis-side. The results indicated full orientation of the Ib channel when the protein was added only to one side of the membrane.
decane. The membranes were formed from diphytanoyl phosphatidylcholine. The aqueous phase contained 1M KCl and 100 ng/ml activated Ib. The membrane potential ($V_m$) of a membrane refers to that on the cis-side of the membrane. The voltage refers always to that on the cis-side of the membrane. Addition to the trans-side only had no effect on membrane conductance. Fig. 7 demonstrates that the membrane conductance decreased as a function of the chloroquine concentration. The data of Fig. 7 and of similar experiments with tetramethylammonium were analyzed using the following equation (Eq. 1), derived previously from carbohydrate-mediated block of the carbohydrate-specific LamB-channel of the *Escherichia coli* outer membrane (43).

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G_{\text{max}} - G(c)/G_{\text{max}} = K\cdot c/(K\cdot c + 1) \quad \text{(Eq. 1)}
$$

$G_{\text{max}}$ is the maximum membrane conductance before the first addition of chloroquine. $G(c)$ is the membrane conductance at a given chloroquine concentration. $c$. Eq. 1 means that the titration curve given in Fig. 7 can be analyzed using a Lineweaver-Burk plot as shown in Fig. 8. The straight line in Fig. 8 corresponded to a stability constant, $K$, of 1,240 1/m (half saturation constant $K_S = 0.81$ mM). The mean value of the stability constant for chloroquine binding to the Ib channel was (1,500 ± 400) 1/m ($K_S = 0.67$ mM). It has to be noted that the binding of chloroquine occurred exclusively from the cis-side of the membrane (the side of the addition of Ib). No decrease of conductance was observed when chloroquine was added to the trans-side of the membrane.

Similar titration experiments were performed with tetramethylammonium and tetraethylammonium chloride because symmetric tetraalkylammonium ions block the PA channel (41, 44). The affinity of these compounds to the Ib channel was even smaller, as observed above for chloroquine. The stability constants for tetramethylammonium and tetraethylammonium binding were about 43 1/m ($K_S = 23$ mM) and 88 1/m ($K_S = 11$ mM), respectively, when the ions were added to the cis-side of the membrane. Addition to the trans-side only had no effect on the Ib-mediated ion conductance. It seems to be likely that the low binding affinity of these ammonium compounds to the Ib channel is responsible for their small inhibitory effect on cell intoxication *in vivo* (6). It is noteworthy that the titration of the Ib-induced membrane conductance could also be measured on the single-channel level. The addition of tetramethylammonium at the half-saturation constant ($K_S = 23$ mM) to the aqueous 1 M KCl concentration resulted in a decrease of the single-channel conductance from 85 to about 40 pS (data not shown).

Interestingly, the ionic strength of the aqueous phase had a substantial effect on the binding of chloroquine and the tetraalkylammonium ions. Decreasing the potassium chloride concentration from 1 to 0.1 M resulted in a decrease of the half-saturation constant for chloroquine binding from 0.67 to 0.22
constant $K$, for chloroquine binding to iota $b$ of $1.240 \text{ M}$. Similarly, the half-saturation constants for tetraethylammonium binding decreased from $11 \text{ mM}$ at $1 \text{ M KCl}$ to $2.7 \text{ mM}$ at $0.1 \text{ M KCl}$. This result indicates that charges are probably involved in the chloroquine and tetraalkylammonium ion-mediated block of the Ib channel from the cis-side.

**Effect of pH on Ib Conductance**—The acidification of the endosomes seems to be an essential step for the Ib-promoted translocation of Ia in the cytosol (22). Furthermore slightly acid pH promotes the Ia-mediated block of Ib. To check whether the pH has an effect on the properties of the Ib channels, we lowered the pH on that side of the membrane where Ib was added (the cis-side) by the addition of increasing amounts of HCl while stirring. The decrease of the pH had no effect on the open probability of the Ib channels down to pH 4.8 in agreement with the above described effect on membrane conductance in the presence of Ib (Figs. 3, 4, and 9). When the pH was lowered further, the channel started to close. At pH 3.7, about 50% of the channels were closed. The solid line of Fig. 9 demonstrates that one single protonated group could be responsible for the effect of pH on the open probability of the Ib channel. Single-channel measurements within the pH range from 5 to 9 suggested that the conductance was virtually independent of pH. At pH 4, the single-channel conductance was about 70 pS, which means that the pH influenced mainly the open probability of the Ib channels and to a smaller extent their single-channel conductance. The decrease of the pH on the trans-side had again no effect on the Ib-mediated membrane conductance.

**Chloroquine Does Not Block Iota-Toxin and C. spiroforme Toxin Intoxication of Vero Cells in Vivo**—In vivo experiments, we studied the effect of chloroquine and its analogues on iota, C. spiroforme, and C2 toxin intoxication of Vero cells. The Vero cells were first incubated with different concentrations of chloroquine up to $1 \text{ mM}$ for 1 h. Then iota-toxin was added in a concentration of $1.5 \times 10^{-8} \text{ M}$ to the cells, a concentration that is sufficient to cause redistribution of the actin cytoskeleton and rounding up of Vero cells. The effect of chloroquine and related compounds on the cells was tested in control experiments, and these compounds were added to the cells at maximum concentrations, which did not induce cytotoxic effect on the Vero cells. From all compounds tested in these experiments, only primaquine was toxic and could not be used in in vivo experiments. They demonstrate that chloroquine and related compounds had no inhibitory effect on the intoxication of Vero cells with iota- and C. spiroforme toxin, but chloroquine and some of the related compounds efficiently blocked C2 toxin-mediated intoxication of Vero cells.

**DISCUSSION**

The Activated Ib Binding Component of the ADP-ribosylating Iota-Toxin Forms Channels in Lipid Bilayer Membranes—In this study, we demonstrated that the binding component of iota-toxin (Ib) forms channels in artificial lipid bilayer membranes. The formation of channels by Ib was only observed with the activated binding component but not with non-activated Ib and not with Ia, which is the intracellular active component. This result suggests that the activation of the binding component with trypsin is a prerequisite of channel formation by Ib. Trypsin and other proteases release a 20-kDa fragment from the N-terminal end of the native ~100-kDa binding component and lead to the active form of Ib. Most importantly, the proteolytic activation of Ib is also necessary for the toxin action in intact cells, i.e. the translocation of Ia across the cytoplasmic membrane (19). The observation that non-activated Ib did not form...
many channels in lipid bilayer membranes of different composition makes it unlikely that the conductance fluctuations are caused by unspecific artifacts. Also, these channels could not be initiated by trypsin because the trypsin used in our investigation did not contain any channel-forming impurity. The formation of channels by impurities is also completely unlikely when the results of the experiments using Ib antibodies are considered. Channel formation by activated Ib was completely inhibited when Ib was preincubated with polyclonal anti-Ib antibodies. These experimental results strongly suggest that the interaction of activated Ib with lipid bilayer membranes is a very specific process and not an artifact.

The transport of several different bacterial toxins is accompanied with the formation of channels in artificial and biological membranes. Examples are channel formation by the heavy chains of tetanus toxin (27, 30, 45, 46), *botulinum* neurotoxins (27), diphtheria toxin (28, 29, 47, 48), C2 toxin (34), and anthrax toxin (33, 35). Here we demonstrate that the binding component of the binary *C. perfringens* iota-toxin is also capable of channel formation. It is noteworthy that the formation of channels by activated Ib was not a rare event. The addition of 100 ng/ml activated Ib to the aqueous phase bathing an artificial membrane was able to increase the conductance of lipid bilayer membranes considerably, and more than 1,000 channels could be formed in a 0.3 mm² membrane surface. Higher Ib concentrations led to the formation of an even higher number of channels.

The Enzymatic Component Ia Blocks the Ib Channel—As we pointed out above, the binding component Ib is essential for the uptake of the enzymatic Ia component into the target cell. In single- and multichannel experiments, we studied the effect of Ia on Ib-mediated conductance. At pH 6 and above, we observed only a minor decrease when Ia was added to the cis-side of the membrane (the side of the addition of Ib). However, when the pH was lowered to pH 5.6 at the cis-side, the effect on the Ib channels was more substantial, and their conductance decreased to something like 30–40% of the open configuration. Single-channel conductance experiments revealed indeed that the Ib channel was partially blocked by the addition of Ib at pH 5.6. It is noteworthy that both the Ia-promoted block of Ib and the effect of pH only occurred when Ia was added to the cis-side or when the pH was lowered at the cis-side in the presence of Ib.

Properties of the Ib Channel—The Ib channel had a single-channel conductance of about 85 pS in 1 M KCl, which is considerably smaller than the single-channel conductances of the PA and the C2-II channels (see Refs. 33 and 34 and Table I). The single-channel fluctuations were fairly homogeneous as judged from the histograms. We observed only occasionally channels that had about twice the size of 85 pS, indicating the simultaneous reconstitution of two Ib channels. The Ib was found to be moderately cation-selective without any indication for the presence of point negatively charged groups, as has been found for the C2-II channel (34). This is consistent with the assumption that the Ib is water-filled and with the observation that its conductance is a linear function of the bulk aqueous concentration of KCl in contrast to the C2-II channel, which shows a dependence of the single-channel conductance of the square root of the aqueous KCl concentration (Table I). Ammonium and some other tetraalkylammonium ions had a higher single-channel conductance than potassium despite similar ion and hydrated ion radii. This is an interesting feature, which has also been found for the PA and the C2-II channels (34, 41). It means presumably that in or near the channel, a binding site for tetraalkylammonium ions exists, which probably has to do with the function of the binding component in binding and transport of the enzymatic component across the endosomal

**Fig. 9.** *Titration of the Ib-induced membrane conductance with protons.* Increasing concentrations of HCl were added to the cis-side of the membrane (the side of the addition of 100 ng/ml Ib) and resulted in a decrease of the number of open channels. The solid line corresponds to the best fit of the experimental data using the acid-base titration curve with a pK of 3.7. The aqueous phase contained 1 M KCl, pH 6; T = 20 °C.

**Table IV**

| Compound       | Concentration | Iota-toxin C. spiroforme toxin | C2 toxin | % inhibition | % inhibition |
|----------------|---------------|-------------------------------|----------|--------------|--------------|
| Chloroquine    | 1.0           | 0                             | 96       | 0            |              |
| Aminoquinidine | 0.5           | 0                             | 87.5–93.7| 0            |              |
| Quinine        | 0.06          | 0                             | 50–75    | 0            |              |
| Amodiaquin     | 0.1           | 0                             | 75       | 0            |              |
| Primaquine     | 0.06          | 0                             | 0        | 0            |              |
| Quinidine      | 0.25          | 0                             | 0        | 0            |              |

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membrane (see below). Negatively charged amino acids are presumably involved in this binding site; otherwise, the ionic strength dependent binding of tetraalkylammonium ions and chloroquine cannot be understood.

The Ib channel showed voltage-dependent gating. Starting with about –60 mV applied to the cis-side of the membrane (the side of the addition of the activated Ib), the current through the channels decreased in an exponential fashion (Fig. 5). For opposite potential at the trans-side of the membrane, the current was not influenced. This result indicated asymmetric insertion of Ib into the membrane. Possibly, a large hydrophilic part of the binding component is exposed to the aqueous phase on the cis-side of the membrane (i.e. the side of the addition of the protein), whereas the more hydrophobic channel-forming domain (the hairpin) crosses the membrane and leads to a transmembrane channel similar to what has been suggested for PA (49). This asymmetric distribution of the protein results in an asymmetric response toward the sign of the membrane potential. The interesting point is that the iota-toxin (binding component and toxin) is present on the external surface of the cell, which means that the trans-side is negative, caused by the membrane potential (from about –60 to –70 mV). This means that the Ib channel is open under in vivo conditions.

**Inhibition of the Channel Function by Chloroquine and Tetraalkylammonium Ions**—The PA and the C2-II channel can be blocked by tetraalkylammonium ions and chloroquine, respectively (34, 41, 42). Similarly, the Ib channel could also be blocked by these compounds, but the half-saturation constants of their binding to the channel was considerably smaller, as has been observed in the case of PA or C2-II. The half-saturation constant for chloroquine binding to C2-II is 3.5 μM at 0.1 M KCl (42), which has to be compared with a value of about 220 μM in the case of Ib. Similar large variations can be noted when tetraalkylammonium binding to the PA and the Ib channels is considered (41), which means that their affinity to the Ib channel is considerably smaller. It has been suggested that chloroquine and tetraalkylammonium compounds inhibit acidification of the endosome, which is a prerequisite for the translocation of toxin into intact cells (24). Therefore, it is possible to speculate that the inhibition of channel conductance by chloroquine and drug-induced impairments of endocytotic processes are related events, and again, suggest a functional link between channel formation and endocytosis. To study the effect of the pH on channel formation, we performed multichannel and single-channel measurements at different pH values. The results suggest that the single-channel conductance is only slightly influenced by pH. However, the open probability decreased drastically with decreasing pH, indicating that one group with a pK of about 3.7 is involved in channel gating.

**Biological Implications of the Channels Formed by Ib**—The primary sequence of Ib shares significant homology with the primary sequences of PA and C2-II (4). It has been found that the binding components of anthrax and C2 toxins form oligomers (probably heptamers) in the target cell membrane and in artificial lipid bilayers (24, 50). PA contains a flexible loop forming an amphipathic β-hairpin with alternating hydrophobic and hydrophilic residues (50). Benson et al. (49) showed strong evidence that the loops from the seven protomers combine to form a transmembrane 14-stranded β-barrel where the hydrophobic residues face the lipid and the hydrophilic residues face the lumen of the channel. Similar antiparallel, amphipathic β-strands with a length of about 24 amino acids are conserved in Ib and C2-II (51) (Fig. 10). The interesting point is that the putative channel-forming domain of Ib does not contain negatively charged amino acids in contrast to C2-II (one glutamic acid residue) or PA (two glutamic acid residues and one aspartic acid residue). The negatively charged residues of PA and C2-II are presumably responsible for the high affinity of chloroquine (C2-II) and tetraalkylammonium ions (PA) to the channel in vitro and the block of C2-I-mediated intoxication of Vero cells in vivo even when endocytosis is blocked by bafilomycin A1 (42) and when the external side of the target cells is acidified (24).

Cytotoxicity induced by C2 toxin is blocked by alkalinizing agents such as weak bases (tetraalkylammonium ions, chloroquine, and analogues) and ionophore (monensin). Iota-toxin and C. spiroforme toxin, which are structurally related to C2 toxin, probably share a common mechanism of entry into cells. These toxins are probably internalized by receptor-mediated endocytosis (3). However, iota-toxin and C. spiroforme toxin are not inhibited by tetraalkylammonium ions, chloroquine, and analogues (Table IV), and monensin (22). One possibility is that the routing of C2 toxin and iota-toxin and the translocation of their enzymatic component is performed in two different endosomes, one being submitted to acidification, which could be inhibited by weak bases, the other which does not require an acidification or which is not inhibited by weak bases. This is supported by the fact that C2 toxin and iota-toxin recognize different cell surface receptors, a carbohydrate for C2 toxin (24), and a membrane protein for iota-toxin (21), which could be important to drive the intracellular trafficking. Another possibility could consist of a different structure of the channels formed by C2-II and Ib. The presence of negatively charged groups (only one Gln) in C2-II channels, which are able to bind cations such as tetraalkylammonium ions, could explain their inhibitory effects on the internalization of C2-I. In contrast, this negative motif is not conserved in Ib and Sb, and the channels formed by these binding components have a low affinity for tetraalkylammonium ions in vitro and intoxication in vivo. This possibly accounts for the fact that tetraalkylammonium ions, chloroquine, and analogues do not inhibit the cytotoxicity induced by iota-toxin and C. spiroforme toxin (22).

Bafilomycin A1, which blocks the vesicular ATPase, was reported to prevent cytotoxicity mediated by C2 toxin (22, 24). We found that bafilomycin A1 (100 nm) inhibits C2 toxin and also iota-toxin activity in Vero cells (data not shown). This suggests that both C2 toxin and iota-toxin require a pH gradient for the translocation of the enzymatic component. Our data suggest that binding of Ia to Ib occurs only at slightly acid pH. Thus it seems that we could mimic the in vivo situation in lipid bilayer experiments. Taken together, it could mean that at the endosome membrane, iota-toxin and C2 toxin could have a common mechanism of entry into the cytosol, a different structure in the channels formed by the binding components could

![FIG. 10. Comparison of the putative channel-forming domains of PA, C2-II, and Ib. The multiple sequence alignment results were obtained using the BCM Search Launcher.](http://www.jbc.org/doi/fig/10.1074/jbc.M109.110023)
explain why tetraalky lammonium ions and chloroquine inhibit C2 toxin and the anthrax toxins but not iota-toxin.

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Interaction of Clostridium perfringens Iota-Toxin with Lipid Bilayer Membranes: DEMONSTRATION OF CHANNEL FORMATION BY THE ACTIVATED BINDING COMPONENT Ib AND CHANNEL BLOCK BY THE ENZYME COMPONENT Ia

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