THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 43, pp. 32335–32343, October 27, 2006

Anthrax Edema Factor, Voltage-dependent Binding to the Protective Antigen Ion Channel and Comparison to LF Binding*

Received for publication, July 10, 2006, and in revised form, August 23, 2006 Published, JBC Papers in Press, September 5, 2006, DOI 10.1074/jbc.M606552200

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Anthrax toxin complex consists of three different molecules, the binding component protective antigen (PA, 83 kDa), and the enzymatic components lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa). The 63-kDa N-terminal part of PA, PA_{63}, forms a heptameric channel that inserts at low pH in endosomal membranes and that is necessary to translocate EF and LF in the cytosol of the target cells. EF is an intracellular active enzyme, which is a calmodulin-dependent adenylate cyclase (89 kDa) that causes a dramatic increase of intracellular cAMP level. Here, the binding of full-length EF on heptameric PA_{63} channels was studied in experiments with artificial lipid bilayer membranes. Full-length EF blocks the PA_{63} channels in a dose, temperature, voltage, and ionic strength-dependent way with half-saturation constants in the nanomolar concentration range. EF only blocked the PA_{63} channels when PA_{63} and EF were added to the same side of the membrane, the *cis* side. Decreasing ionic strength and increasing transmembrane voltage at the *cis* side of the membranes resulted in a strong decrease of the half-saturation constant for EF binding. This result suggests that ion-ion interactions are involved in EF binding to the PA heptamer. Increasing temperature resulted in increasing half-saturation constants for EF binding to the PA_{63} channels. The binding characteristics of EF to the PA_{63} channels are compared with those of LF binding. The comparison exhibits similarities but also remarkable differences between the bindings of both toxins to the PA_{63} channel.

The plasmid-encoded tripartite anthrax toxin comprises a receptor binding moiety termed protective antigen (PA)[2] and two enzymatically active components, edema factor (EF) and lethal factor (LF) (1–3). The monomeric anthrax PA is the binding component of the AB toxin Anthrax. It is a cysteine-free 83-kDa protein that binds to two possible receptors: a ubiquitously expressed integral membrane receptor (ATR) and also to the LDL receptor-related protein LRP6, which can both be involved in anthrax toxin internalization (4, 5). PA_{83} present in the serum or bound to receptors is processed by a furin-like protease to a 63-kDa protein PA_{63} (6, 7). PA_{63} spontaneously oligomerizes in the serum and/or on the cell surface into a heptamer. The heptamer may bind up to three molecules of EF and/or LF with high affinity (K_d ~1 nM) (8–10). However, the number of LF molecules that are able to bind to the PA_{63} channel is still a matter of debate. Our own data suggest a single hit process which could mean that the first LF molecule that is bound to the channel blocks also the channel (11). A recent cryo-electron microscopic study suggested that LF interacts with four successive PA_{63} molecules within a PA_{63} heptamer, which makes it questionable that three LF molecules could simultaneously bind to the PA_{63} heptamer with the same or a similar affinity (12).

The membrane-bound complex of PA_{63} is clathrin-mediated endocytosed together with LF and/or EF (13). EF is a calmodulin-dependent adenylate cyclase (89 kDa) that causes a dramatic increase of intracellular cAMP level, altering water homeostasis, and intracellular signaling. In addition, EF is believed to be responsible for the edema found in cutaneous anthrax and it is a very effective inhibitor of T cell activation and proliferation (2, 14–16). The structure of the mushroom-shaped membrane-bound PA_{63} heptamer is not exactly known. However, based on the crystal structure of the water-soluble homoheptameric complex (7) and the structure of the structurally related, also homoheptameric channel formed by *Staphylococcus aureus* α-hemolysin (17), a hypothetical model has been proposed. In this model, formation of a β-barrel channel requires: (*a*) the unfolding of a Greek key motif (strands $2\beta_1$-$2\beta_4$) to form a β-hairpin and (*b*) the association of seven β-hairpins in the PA_{63} heptamer to form a 14-stranded, membrane-spanning β-barrel with a diameter of about 1.4 nm (18–19). This conformational change is presumably induced by endosomal acidification, which may protonate histidines in the loop and key region of the heptameric PA_{63} protein. The negatively charged PA_{63} channel interacts with positively charged quaternary ammonium ions, which have a structure similar to that of the N-terminal ends of LF and EF (20–22). Studies with a truncated form of LF, LF_N with a length of 263 amino acids suggest that LF

* This work was supported by the Deutsche Forschungsgemeinschaft (SFB 487, project A5), by the Fonds der Chemischen Industrie, by the Anthrax-Euronet Coordination Action (FP6, contract no. 503616) and by the Consorzio Interuniversitario Biotecnologie (CIB). 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.

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[2] The abbreviations used are: PA, protective antigen; EF, edema factor; LDL, low density lipoprotein; MES, 4-morpholineethanesulfonic acid; SPR, surface plasmon resonance; NTA, nitrilotriacetic acid; LF, lethal factor.

interacts with its N-terminal end with the PA_{63} channel (23). LF_N is also able to block PA_{63} channels in a voltage-dependent and reversible way, which suggested that the PA_{63} channel could be the pathway of delivery of LF into the cytosol of the target cells (24). Similar results were also obtained with full-length LF, which blocks the PA_{63} channel in a voltage- and ionic strength-dependent way (11, 25). In addition, evidence has been presented that the PA_{63} channel represents the pathway of LF transport into the cytosol of the target cells driven by voltage and pH gradient (24, 26). Similarly, it has been demonstrated that also EF binds to the PA_{63} channel *in vivo* and *in vitro* but the binding process has not been studied in such detail as the interaction between full-length LF and the PA_{63} channel (10, 11, 25, 27).

In this study, we demonstrate that also full-length EF is able to block the PA_{63} channel in a dose and ionic strength dependent way with half-saturation constants in the nanomolar range. The block of the PA_{63}-induced membrane conductance is almost symmetrical when EF is added to the *cis* side, the same side of addition of PA_{63}. Furthermore, the binding affinity of EF to the PA_{63} heptamers is highly voltage dependent and the stability constant for EF binding to the channel strongly increases with increasing voltage when it has a positive sign at the *cis* side. The results suggest that the PA_{63} heptamers contain a high affinity binding site for EF inside the channel vestibule that is localized to the *cis* side. Binding of EF is highly ionic strength-dependent and moderately temperature-dependent.

MATERIALS AND METHODS

Anthrax Protective Antigen PA_{63}—Nicked anthrax protein PA_{63} from *Bacillus anthracis* was obtained from List Biological Laboratories Inc., Campbell, CA. 1 mg of lyophilized protein was dissolved in 1 ml of 5 mM HEPES, 50 mM NaCl, pH 7.5 complemented with 1.25% trehalose. Aliquots were stored at −20 °C. Channel formation by PA was stable for months under these conditions.

Cloning, Expression, and Purification of Anthrax Edema Factor—The EF gene from the plasmid pMMA187 (28) was PCR-amplified using the following primers: 5′-AAAGGATCCATGAATGAACATTACACTGAG-3′ (forward) and 5′-AAAGAGCTCTTATTTTTCATCAATAATTTTTTGG-3′ (reverse). The obtained PCR fragment was digested with BamHI and SacI and inserted in pRSET A (Invitrogen). The cloned sequence was confirmed by DNA sequencing. EF was expressed in BL21 DE3 (Novagen Inc.) in a native form, fused to a N-terminal His_6 tag and purified by FPLC with a Cu-charged Hitrap chelating column (Amersham Biosciences) following the protocol described in the Recombinant Protein Handbook (Amersham Biosciences). The His_6 tag was removed from His_6-EF by incubation with enterokinase leaving five exterior residues, DRWGS, at the N terminus.

Lipid Bilayer Experiments—Lipid bilayer experiments were performed as described previously (29) using diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster AL) dissolved in *n*-decane as membrane-forming lipid. Membrane formation occurred across a small circular hole (0.4 mm^2) in the thin wall separating two compartments in a Teflon chamber. PA_{63} was added from concentrated protein solutions either immediately before membrane formation or after the membranes had turned black to the *cis* side of the membrane. The temperature was maintained at 20 °C during most of the experiments. In some others the temperature in the membrane cell was kept at 5 °C or at 35 °C to measure the effect of temperature on EF binding to the PA_{63} channels. The aqueous salt solutions were buffered with 10 mM MES-KOH, pH 6. Membrane conductance was measured after application of a fixed membrane potential with a pair of silver/silver chloride electrodes with salt bridges inserted into the aqueous solutions on both sides of the membrane. The potentials applied to the membranes throughout the study refer always to those applied to the *cis* side, the side of addition of PA. Similarly, positive currents were caused by positive potentials at the *cis* side and negative ones by negative potentials at the same side. Membrane currents were measured using a homemade current-to-voltage converter made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope and recorded on a strip chart recorder.

Binding Experiments with EF—The binding of EF and His-EF to the PA_{63} channel was investigated with titration experiments similar to those used previously to study the binding of 4-aminoquinolones to the C2II and PA_{63} channels and LF to the PA_{63} channel in single- or multi-channel experiments (11, 22, 30). The PA_{63} channels were reconstituted into lipid bilayers. About 30 min after the addition of PA_{63} to the *cis* side of the membrane the rate of channel insertion in the membranes was very small. Then concentrated solutions of EF or His_6-EF were added to one or both sides of the membranes while stirring to allow equilibration. The results of the titration experiments, *i.e.* the block of the PA_{63} channels, were analyzed using Langmuir adsorption isotherms (11, 31). This means that the conductance as a function of the EF concentration was analyzed using Lineweaver-Burk plots. K is the stability constant for EF or His_6-EF binding to the PA_{63} channel. The half-saturation constant, K_s (also known as K_d) of its binding is given by the inverse stability constant $1/K$.

RESULTS

Binding of Anthrax Edema Factor to the PA_{63} Channel—The stability constant for the binding of EF to the PA_{63} channel was measured in multi-channel experiments, performed as follows. Recombinant PA_{63} was added to black lipid bilayer membranes in a concentration of about 1 ng/ml (corresponding to about 16 pM for PA_{63} or at maximum about 2 pM for $(PA_{63})_7$) to the *cis* side of a black lipid bilayer membrane while stirring from the concentrated stock solution (10 μg/ml). The aqueous phase on both sides of the membrane contained 150 mM KCl, 10 mM MES-KOH, pH 6. 30 min after the addition of the protein, the rate of conductance increase had slowed down considerably at an applied membrane potential of 10 mV (see Fig. 1*A*). At that time small amounts of a concentrated EF solution were first added to the aqueous phase on the *trans* side of the membrane (the side opposite to the addition of PA_{63}). This addition had no effect on membrane conductance. However, when EF was added to the *cis* side of the membrane the PA_{63}-induced membrane conductance decreased in a dose-dependent manner. Fig. 1*B* shows an experiment of this type in which increasing

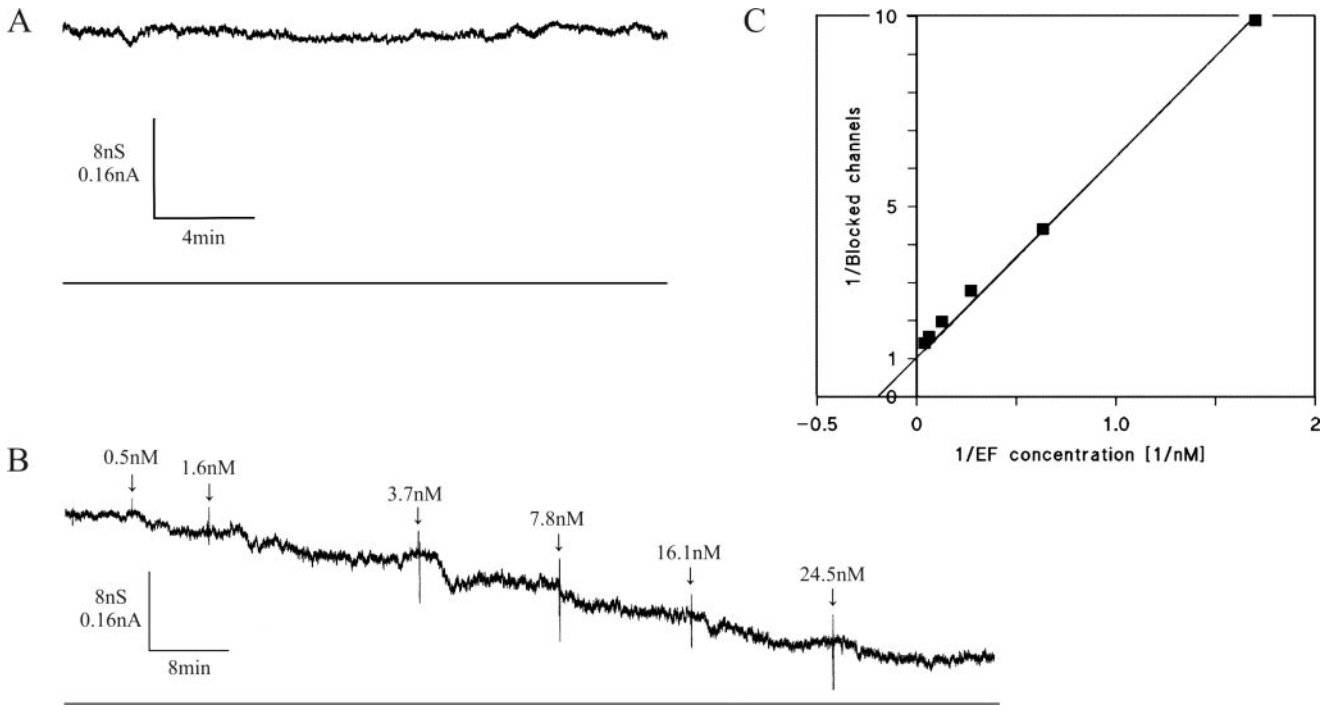

FIGURE 1. **Titration of PA_{63}-induced membrane conductance with EF.** *A*, recording shows the time course of the current for about 25 min before addition of EF. The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane. It contained about 200 channels. The aqueous phase contained 1 ng/ml PA_{63} protein (added only to the *cis* side of the membrane), 150 mM KCl, 10 mM MES, pH 6. The temperature was 20 °C, and the applied voltage was 10 mV. *B*, titration of PA-induced membrane conductance with EF added to the *cis* side of the membrane of *A*. EF was added at the concentrations shown at the *top* of the panel. Note that EF blocks only the PA_{63} channels when it is added to the *cis* side of the membrane. *C*, Lineweaver-Burk plot of the inhibition of the PA_{63}-induced membrane conductance by EF. The *straight line* was obtained from linear regression of the data points taken from *B* ($r = 0.9997$) and corresponds to a stability constant *K*, for EF binding to PA_{63} of $(1.89 \pm 0.06)\cdot10^8$ l/M (half-saturation constant $K_S = (5.3 \pm 0.14)$ nM).

concentrations of EF (arrows) were added to the *cis* side of a membrane containing about 200 PA_{63} channels. The membrane conductance decreased after addition as a function of the EF concentration. The time between EF addition and start of decrease varied somewhat because of slow diffusion of EF in the aqueous phase. The data of Fig. 1*B* and of similar experiments were analyzed using Equation 1 used previously for the fit of the data of carbohydrate-mediated block of the maltose and maltooligosaccharide-specific LamB channel and also for the block of the PA_{63} channel with LF and His_6-LF (11, 32).

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

$G(c)$ is the conductance of the PA_{63} channels in the presence of an EF concentration c. *K* is the stability constant (corresponding to a half-saturation constant $K_S = 1/K$) for EF binding to the channels and G_{max} is the maximum membrane conductance (when no EF was added to the aqueous phase).

Equation 1 shows that the titration curves could be analyzed using Lineweaver-Burk plots as it is shown in Fig. 1*C* for the data of Fig. 1*B*. The good fit of the experimental points by a least squares fit (the *straight line* in Fig. 2 ($r = 0.9997$)) suggests that the interaction between EF and the PA_{63} channels (*i.e.* the channel block) represents a single hit process. The fit corresponded to a stability constant, *K*, of $(1.89 \pm 0.06)\cdot10^8$ l/M (half-saturation constant $K_S = (5.3 \pm 0.14)$ nM). In another set of experimental conditions, the experiment was repeated with a variety of different KCl concentrations instead of 150 mM KCl. The results showed that the stability constant for EF binding to

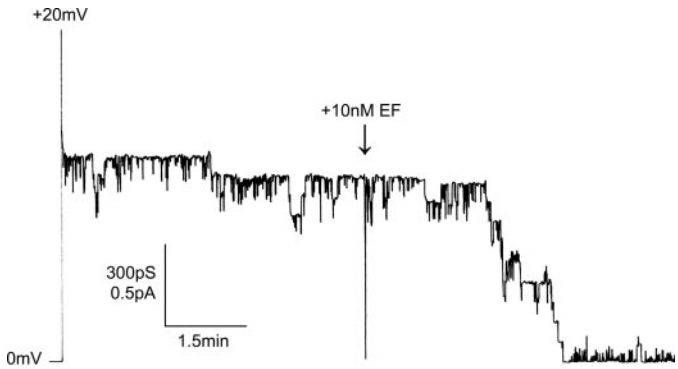

FIGURE 2. **Block of PA_{63} channels by EF observed on the single channel level.** 20 mV were applied to the *cis* side of a diphytanoyl phosphatidylcholine/*n*-decane membrane containing 10 PA_{63} channels. About 4 min after the application of the voltage EF was added in a concentration of 10 nM to the *cis* side of the membrane (*arrow*). Note that all PA_{63} channels subsequently closed caused by binding of EF to the channels.

the PA_{63} channel was highly ionic strength-dependent and increased with decreasing ionic strength from 300 mM KCl to 50 mM by a factor of more than fifty (from $0.29\cdot10^8$ l/mol to $1.6\cdot10^9$ l/mol; see Table 1). These results suggest that at least part of the binding process between the PA_{63} channel and EF is controlled by ion-ion interaction similar as in the case of full-length LF (11).

Effect of EF on the PA Channel at Single Channel Level—In additional measurements we studied EF-induced block of the PA channel on the single channel level. PA was added in a very small concentration (about 0.1 pM) to the *cis* side of a black membrane formed of diphytanoyl phosphatidyl-choline/*n*-

TABLE 1

Stability constants *K* for the inhibition of the PA_{63} channel by EF and His_6 EF in lipid bilayer membranes

The data represent means of at least three individual titration experiments. The standard deviation was typically less than 20% of the mean values. K_S is the half-saturation constant. Constants for LF and His_6-LF binding are given for comparison (Neumeyer *et al.*, 11). The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained the indicated KCl concentration and about 1 ng/ml PA_{63}, T = 20 °C.

Ionic strength	Temperature	EF		LF	
		K	K_S	K	K_S
mM	°C	$10^8\ M^{-1}$	nM	$10^8\ M^{-1}$	nM
50	20	16.1	0.62	22.5	0.44
150	5	4.2	2.4	n.m.[a]	n.m.
150	20	1.45	6.9	3.62	2.76
150	35	1.10	8.8	n.m.	n.m.
300	20	0.29	34.5	0.45	22.2
1000	20	0.037	280	0.046	220
Ionic Strength	**Temperature**	**His_6-EF**		**His_6-LF**	
		K	K_S	K	K_S
50	20	7.9	1.27	31.6	0.316
150	20	62.5	0.16	55.2	0.181
300	20	13.7	0.73	41.6	0.240
1000	20	3.4	2.93	5.4	1.86

[a] n.m., not measured.

decane. After the reconstitution of about twelve PA channels in the membrane, the voltage was set to 20 mV at the *cis* side. EF was added first to the *trans* side of the membrane. No effect of EF on the PA channels could be observed (data not shown). Then the recording of Fig. 2 started. About 6 min after the onset of the voltage EF was added to the *cis* side at a concentration of 10 nM. The interaction between PA heptamers and EF resulted in a complete and reversible blockage of the ionic current through the channels. Only some occasional flickers of the channels were observed indicating that the lifetime of the open state of the PA heptamer was very small.

Effect of Temperature on EF Binding to the PA_{63} Channels—Most of the experiments were performed at 20 °C. However, it has been demonstrated in a previous study using surface plasmon resonance (SPR) studies that EF binding to PA_{63} is temperature-dependent (10). To check if a similar temperature effect on EF binding was also observed in lipid bilayer membranes we performed some experiments at temperatures other than 20 °C. The results of EF binding to PA_{63} channels at 5 °C and 35 °C are also included in Table 1. The half-saturation constant of EF binding decreased for decreasing temperature from 6.9 nM at 20 °C to 2.4 nM at 5 °C. Similarly, the half-saturation constant increased to 8.8 nM when the temperature was increased to 35 °C.

His_6-EF Has a Higher Affinity than EF for the PA_{63} Channel—For easy purification EF was expressed in *Escherichia coli* with a His_6 tag at the N-terminal end. For most experiments, the His_6 tag was removed by enterokinase treatment. His_6-EF exhibited an even higher affinity for binding to the PA_{63} channel similar as has previously been found for a truncated form of LF, LF_N, or LF_{263} (11, 23, 33). The half-saturation constant for His_6-EF binding to the PA_{63} channel was on average 0.16 nM (K = $6.25{\cdot}10^9$ 1/M) at an ionic strength of 150 mM KCl; which means that the stability constant for binding for His_6-EF was roughly a factor of ten higher than that without the His_6 tag (see Table 1). Binding of His_6-EF to the PA_{63} channels was much less ionic strength-dependent than EF. The influence of the partially positively charged His_6 tag on EF binding indicates again that the interaction between the N-terminal end of EF and the PA_{63} channels is based on ion-ion interactions similarly as discussed in the case of LF_N (23).

The Binding of EF to the PA_{63} Channel Results in Symmetric Current Voltage Curves—Binding of full-length LF to the PA_{63} channels resulted in asymmetric current voltage curves showing a diode-like behavior (11, 25). For negative voltages applied to the *cis* side no or only little binding of LF could be detected whereas dose-dependent channel block was observed for positive potentials. Current voltage relationships were measured with PA_{63} channels with and without EF to check if EF binding caused also diode-like current-voltage curves. Initial currents (after the onset of the voltage) through the open PA_{63} channels were almost symmetrical with the exception of some channel inactivation at low ionic strength and high negative potentials at the *cis* side (see Fig. 3*A*) (21, 22). Starting with −20 mV applied to the *cis* side the PA_{63} channels started to close whereas positive voltages up to 150 mV did not induce channel gating (data not shown). Addition of EF to the *cis* side resulted in a decrease of the membrane current similar to that of the titration experiments. However, the membrane current after addition of EF was almost symmetrical as Fig. 3*B* shows.

Fig. 3*B* demonstrates that the PA_{63} channels closed for positive potentials higher than 20 mV when EF was present at the *cis* side. This result suggested that EF binding was voltage-dependent (see below). The closing of PA_{63} channels at negative potentials is not caused by EF binding as Fig. 3*A* indicated. It is caused by slow PA_{63} channel closure when negative potentials higher than −20 mV were applied to the *cis* side of the membrane (20, 22).

Fig. 4 shows current-voltage curves of the open and the EF-mediated partially closed PA_{63} channels by 3 nM EF, which decreased the current of the open channels by about 50%. Higher concentration of EF resulted in a higher block of the channels in agreement with the titration experiments. The current always corresponded to that of the initial currents immediately after the onset of the voltage (*i.e.* before the exponential decay of the current, see Fig. 3*B*). The curves demonstrate that the asymmetry of the channels was limited, which means that EF blocks also the PA_{63} channels when negative potentials were

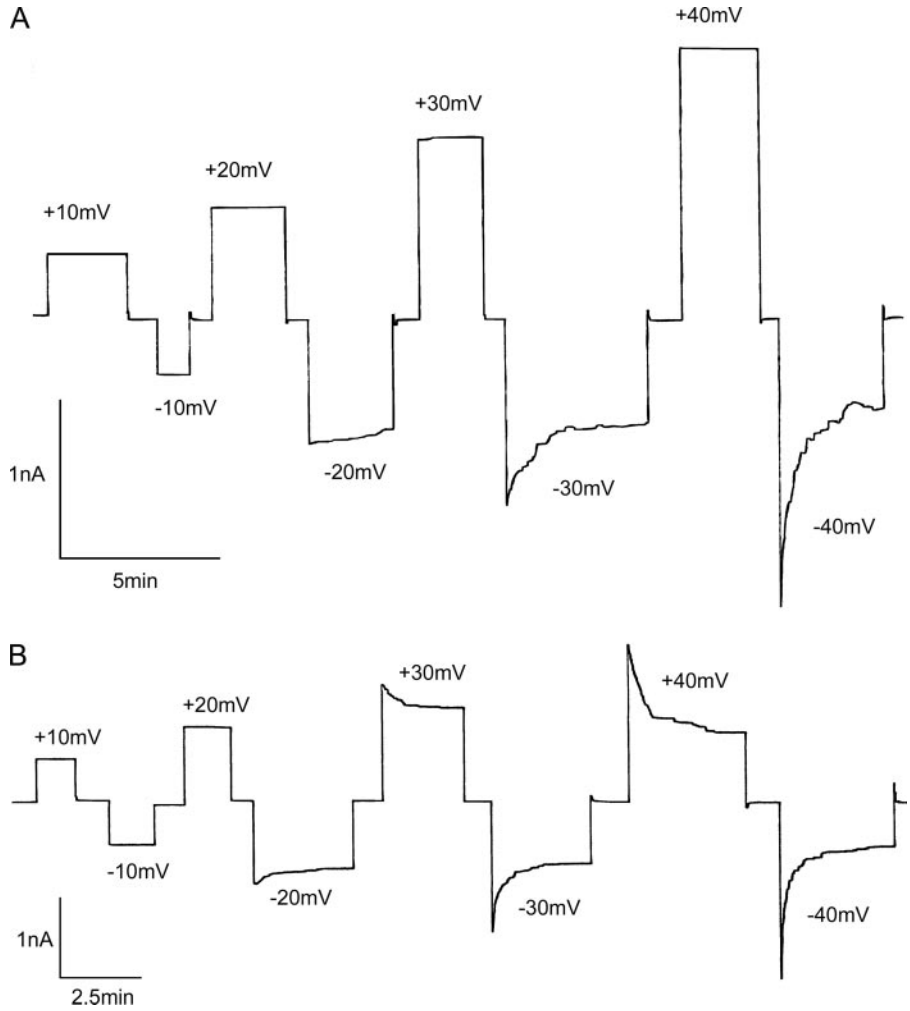

FIGURE 3. *A*, current response of PA channels. Voltage pulses between ± 10 and ± 40 mV were applied to a diphytanoyl phosphatidylcholine/*n*-decane membrane in the presence of 1 ng/ml PA_{63} protein (added only to the *cis* side of the membrane). The aqueous phase contained 150 mM KCl, 10 mM MES, pH 6. The temperature was 20 °C. Note that the current through the PA_{63} channels inactivated only when negative potentials were applied to the *cis* side of the membrane. *B*, current response of PA channels in the presence of EF. Voltage pulses between ± 10 and ± mV to a diphytanoyl phosphatidylcholine/*n*-decane membrane in the presence of 1 ng/ml PA_{63} protein (added only to the *cis* side of the membrane). The aqueous phase contained 150 mM KCl, 10 mM MES, pH 6, and 1.8 nM EF added to the *cis* side. The temperature was 20 °C.

applied to the *cis* side of the membranes. This result is in some contrast to measurements of the current-voltage curves of LF-blocked PA_{63} channels because block of the PA_{63} channels was reversed at negative potential applied to the *cis* side (see Fig. 4; and Refs. 11 and 25).

The Stability Constant of EF Binding to the PA_{63} Channels Is Voltage-dependent—The current immediately after the onset of the voltage was a linear function of the applied potential irrespective if EF was present or not (see Fig. 4). However, the exponential decay of current following a positive voltage step suggested that binding of EF to the PA_{63} channel occurs in a voltage-dependent way (see Fig. 3*B*), which means that the stability constants increased with increasing positive voltage at the *cis* side that was not observed in controls (see Fig. 3*A*). Fig. 5 shows an experiment in which we studied the effect of positive potentials on EF binding in detail. PA_{63} was reconstituted in a lipid bilayer membrane. When the membrane conductance became stationary EF was added to the *cis* side of the membrane at a concentration of 2 nM while stirring to allow equilibration. The membrane conductance decreased as a result of the addition of EF at a membrane potential of 10 mV. Then the voltage at the *cis* side was increased in steps of 10 mV. Whereas 10 and 20 mV did not cause any effect on EF binding, which means that the current was stationary after application of voltage, higher voltages starting with 30 mV resulted in a considerable further decrease of the PA_{63}-induced membrane conductance. The decrease followed an exponential function, with a voltage-dependent relaxation time (see Fig. 5). This result indicated that channels, which were not blocked before by EF at zero voltage closed as a result of the higher voltage. This result suggested an increase of the stability constant of binding up to very high voltages an effect that has not been observed with full-length LF and the truncated form LF_N (11, 23).

The increase of the stability constant for binding could be calculated from the data of Fig. 5 and similar experiments by dividing the initial current (which was a linear function of voltage) by the stationary current after the exponential relaxation and multiplying the ratio with the stability constant derived at 10 mV. Fig. 6 summarizes the effect of the positive membrane potential on the stability constant *K* for EF binding as a function of the voltage. Starting with 30 mV *K* started to increase and reached with about 60–70 mV a maximum. At that voltage *K* was roughly 10 times greater than at 10 mV. For higher voltages the stability constant decreased probably because of secondary effects of the high voltage on the PA_{63} channel or on EF binding. This may explain the discrepancy between our results and those of Ref 25, who found half-saturation constants for EF binding in the picomolar range. Part of this discrepancy is presumably caused by the 50 mV that was applied by Ref. 25 to the membranes. The rest is presumably caused by the lower ionic strength used by Ref. 25 (100 mM) compared with 150 mM used here (see below).

DISCUSSION

Full Size EF Binds in a Single Hit Process to the PA_{63} Channel with Half-saturation Constants in the Nanomolar Range—In this study we investigated the interaction between full size EF and the PA_{63} channel in detail. Experiments with a few channel in a membrane demonstrated complete EF-mediated channel block from the *cis* side. Titration experiments with membranes containing a large number of PA_{63} channels suggested that full

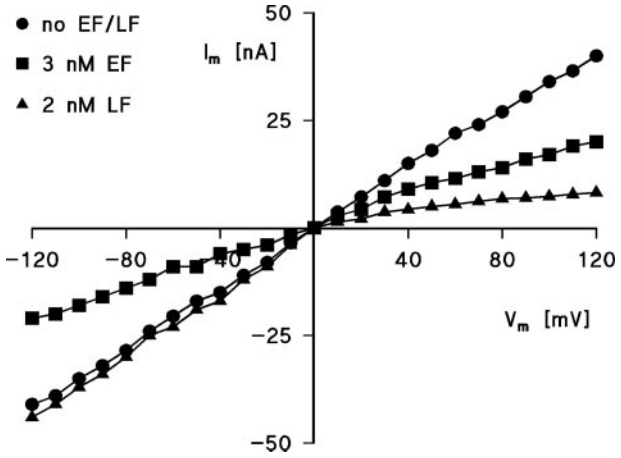

FIGURE 4. **Current-voltage relationships of open and EF-mediated closed PA_{63} channels.** The *full circles* show PA_{63} channels in the absence of EF and LF. The *full squares* show an experiment where PA_{63} channels were blocked with 3 nM EF added to the *cis* side. The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 ng/ml PA_{63} protein (added only to the *cis* side of the membrane) and 150 mM KCl, 10 mM MES, pH 6. The temperature was 20 °C. The sign of the voltage corresponds to that on the *cis* side of the membrane. The *triangles* show an experiment where 2 nM LF (*full*) was added to the *cis* side of another membrane (taken from Ref. 11). Note the completely different current-voltage curves in the presence of EF and LF.

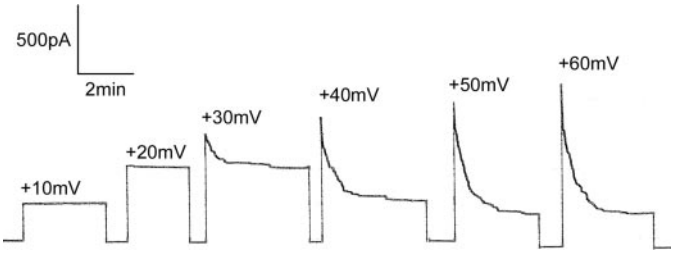

FIGURE 5. **Voltage dependence of PA_{63} channel blocked with 2 nM EF.** EF was added to the *cis* side of the membrane. The PA_{63}-induced membrane conductance was titrated with 2 nM EF that caused decrease of the initial current by about 40%. Then voltage steps with increasing voltage and positive sign were applied to the *cis* side. The aqueous phase contained 1 ng/ml PA_{63} protein (added only to the *cis* side of the membrane) and 150 mM KCl, 10 mM MES, pH 6. The temperature was 20 °C. Note that the voltage steps at 30, 40, 50, and 60 mV were all followed by an exponential decrease of the current indicating that the stability constant *K* for LF binding to the PA_{63} channels increased as a result of the applied membrane potential.

size EF blocks the PA_{63} channel from the *cis* side at very low half-saturation constants. Addition to the *trans* side had no effect on the channels indicating a highly asymmetric structure of the PA_{63} channels (19, 34). The three-dimensional structures of the monomeric and heptameric prepore forms of PA_{63} are known (7), which suggest that the membrane spanning domain of the PA_{63} channel is a 14-stranded β-barrel (18, 19), similar to the structure of α-toxin of *Staphylococcus aureus*, which forms also a heptamer with some sort of vestibule on the *cis* side of the membrane (17). Only a small part of the PA_{63} heptamer is localized inside the membrane. The vestibule and the mushroom-like protrusion of the PA_{63} channel are formed by the majority of the amino acids of the PA_{63} monomer. It is the binding place for the toxins, which has been demonstrated by many mutations of the toxins and also of PA (8, 26, 27, 34, 35).

The half-saturation constant of about 7 nM has to be compared with an equilibrium constant, K_d, calculated from kinetic data of EF binding to amine-coupled PA_{63}, in surface plasmon

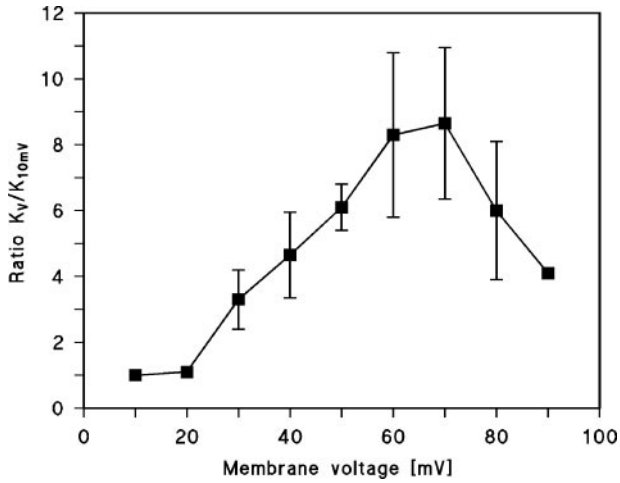

FIGURE 6. **Voltage dependence of EF binding to the PA channel.** The stability constants of EF binding to the PA_{63} channel are given as a function of the applied membrane potential taken from experiments similar to that shown in Fig. 5. Means of four experiments are shown.

resonance (SPR) measurements, which yielded 1.3 nM for EF at 150 mM NaCl, whereas for NTA-coupled PA_{63}, an equilibrium constant, K_d, for EF binding of 1.8 nM was observed (10). The results of the titration experiments at low voltage described here are in good agreement with data from SPR measurements with full size EF derived earlier (10). Similarly, binding of full size EF to PA_{63} was also measured in a study using L6 cells using different concentrations of ^{125}I-labeled EF at 4 °C (10). Half-saturation for EF binding was obtained at a concentration of 0.7 nM. This means that the data are in good agreement with our data and results of the SPR measurements (10). Our lipid bilayer system allows good control of temperature during the experiments. Lower temperature resulted in a decrease of the half-saturation constant, whereas higher temperature led to its increase (see Table 1). It is noteworthy that the temperature effects described here show again good agreement with SPR measurements with NTA-coupled PA_{63} (10). We calculated an activation energy of 32 ± 10 kJ/mol from the temperature dependence of the stability constant of EF binding given in Table 1, which agrees sufficiently well with an activation energy of about 55 kJ/mol derived by Ref. 10 for the off-rate constant of EF binding to PA_{63} in SPR experiments. The activation energy of the half-saturation constant K_d for EF binding to binding to PA_{63} in SPR experiments is about 45 kJ/mol (10), again in good agreement with our data.

The fit of the titration experiments using the Langmuir adsorption isotherm suggests that the EF-mediated block of the PA_{63} channel is a single hit process similar to the block of full size LF to the PA_{63} channel (11, 25). This is in some contrast to the literature where it has been reported that up to 3 molecules of LF and/or EF can bind to the PA_{63} channel (8, 36). Our results suggest that the first EF molecule that binds to the binding-site in the vestibule of the channel leads already to channel block irrespective of the maximum number of possible binding places for EF on the PA_{63} channel (36). On the other hand, data presented in a recent cryo-electron microscopy study suggest that there might not be enough place within the vestibule of the PA_{63} channel for binding three molecules of LF and/or EF (12).

The reason for this is the observation that a single LF molecule interacts with four successive PA_{63} molecules in a PA_{63} heptamer. The molecular masses of LF and EF are very similar, which means that it is impossible that three EF molecules bind to the PA_{63} channel with the same affinity to the binding site. The transport efficiency of the toxins into the target cells is independent from the number of bound toxin molecules to the PA heptamer indicating the independent translocation of them (23).

Full-length His_6-EF showed a much higher affinity for the PA_{63} channel than LF (see Table 1). It is noteworthy that this was also observed for a truncated form of LF (LF_N or LF_{263}) (24, 33). This result suggested that the six, partially charged imidazole groups of the His_6 tag are somehow involved in binding of His-EF to the PA_{63} channel. This result agrees with previous reports that His_6-LF and LF bind with their N termini to the PA_{63} heptamers (24, 33). These considerations agree with data of other studies (20–22) where it has been shown that quaternary ammonium groups of tetraalkylammonium ions and 4-aminoquinolones, such as chloroquine, quinacrine, and fluphenazine, may be involved in binding of LF and EF to the PA_{63} channel.

Voltage Increases Binding Affinity of EF to the PA_{63} Channels—LF binding to the PA_{63} channel is highly asymmetric with respect to the sign of the applied membrane potential, which leads to an asymmetric diode-like current-voltage curve (11, 25). EF binding to the PA_{63} channel was in contrast to this more or less symmetrical for positive and negative potentials at the *cis* side. Channel block was also observed when the side of addition of PA_{63} and EF, the *cis* side, was negative, which means that channel block was not reversible under these conditions. This result indicates that EF binding to the PA_{63} channel is different to that of LF binding. This is in some contrast to the work of Halverson *et al.* (25) who found also a diode-like behavior for EF binding. The reason for this difference is not clear. Voltage dependence of EF binding has nothing to do with channel inactivation that was observed at low ionic strength and negative voltages (compare Figs. 3*A* and 5) (20, 22, 37).

Positive potentials resulted in an increased affinity of the PA_{63} channel for EF binding. Starting with 30 mV the stability constant for EF binding to the channel increased up to about 10-fold for voltages of 60–70 mV. For higher voltages the stability constant decreased slightly, indicating a maximum for the stability constant at about 60–70 mV. This result points to some difference for the influence on EF binding as compared with binding of LF or its truncated form LF_N (LF_{263}) (24). The strong voltage dependence of EF binding could explain the different half-saturation constants published in different studies. Ref. 25 measured half-saturation constants for EF binding to the PA_{63} channel in the picomolar range. However, they measured binding at 50 mV, which means that their half-saturation constant should be lower than ours by a factor of about six (see Fig. 6). In addition, in Ref. 25 an ionic strength of 100 mM was used, which also considerably lowers half-saturation constant of LF binding to the PA_{63} channel (see below).

The voltage dependence of EF binding has probably also some implication for the binding of EF to the PA_{63} channel bound to target cells (*i.e.* macrophages) because of their membrane potential of about −60 to −80 mV (38). This means that EF binding to target cells could be enhanced when the target cell is polarized. Similarly, an effect of membrane voltage on EF binding can also be expected in endosomes because of the action of the H^+-ATPase and several ion channels that result in positive potentials inside the endosomes (39). However, the magnitude of endosomal potentials is not known, which means that it is not clear if they are able to decrease the half-saturation constant of EF binding to the PA_{63} channels within the endosomes. It seems to be more important for efficient intoxication of the target cells that the PA_{63} channels on their surfaces are all decorated with EF before clathrin-dependent endocytosis occurs.

Charge Effects of EF Binding to the PA_{63} Channel—The data shown in Table 1 demonstrate a considerable dependence of EF binding on the bulk aqueous KCl concentration. The stability constant, K, for EF binding to the PA_{63} channel decreases about 400-fold for an increase of the ionic strength by a factor of 20 (from 50 mM to 1 M KCl). This means that the square root of K is proportional to the ionic strength. This result suggests that ion-ion interactions are involved in EF binding to the PA_{63} channel besides other interactions such as those between F427 and LF/EF, which may be involved in pore formation of PA_{63} and subsequent toxin translocation in cells (26, 35). These ion-ion interactions are caused by negatively charged groups localized in the vestibule of the channel and their interaction with positive charges at the N-terminal end of EF and LF (8, 40, 41). A quantitative description of the effect of charges on the binding of EF to the PA_{63} channel may be given by the Debye-Hückel theory used previously to describe the effect of charges on LF binding (11). It has previously also been used to explain ionic strength effects on conductance of channels containing point net charges such as RTX toxins, mycobacterial porins, and the C2II and PA_{63} channels (22, 42, 43, 44). Effects of point charges on membrane channels have also been described in other studies (45–47). In case of a negative charge, q, in an aqueous environment a negative potential Φ is created that is dependent on the distance, r, from the charge in Equation 2.

$$\Phi = \frac{q \cdot e^{\frac{r}{l_D}}}{4\pi \cdot \epsilon_0 \cdot \epsilon \cdot r} \qquad \text{(Eq. 2)}$$

ϵ_0 ($= 8.85 \times 10^{-12}$ F/m) and ϵ ($= 80$) are the absolute dielectric constants of vacuum and the relative constant of water, respectively, and l_D is the so-called Debye length that controls the decay of the potential in the aqueous phase in Equation 3.

$$l_D^2 = \frac{\epsilon \cdot \epsilon_0 \cdot R \cdot T}{2F^2 \cdot c} \qquad \text{(Eq. 3)}$$

c is the bulk aqueous salt concentration. R is the gas constant ($r = 8.31$ J/(mol °C), T the absolute temperature ($T = 293$ K) and F is Faraday's constant ($F = 96{,}500$ As/mol). The concentration of the monovalent cations near the channel increases because of the negative potential Φ. Their concentration, c_0^+, at the channel is given by Equation 4.

$$c_0^+ = e^{\frac{-\Phi \cdot F}{R \cdot T}} \qquad \text{(Eq. 4)}$$

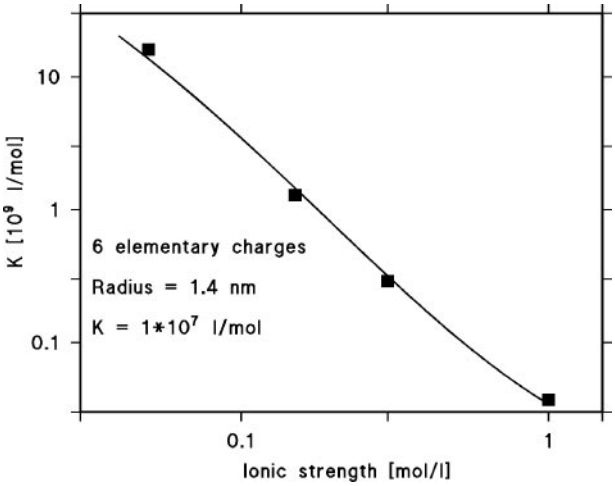

FIGURE 7. **Stability constants of EF binding to the PA_{63} channel given as a function of the ionic strength.** The experimental data were taken from Table 1. The *solid line* shows the fit of the stability constants as a function of the ionic strength of the aqueous phase (equal to the KCl concentration) using Equations 2, 3, 4, and 5 and by assuming that six negatively charged groups ($q = -9.6 \cdot 10^{-19}$ As) are located within the vestibule of the channel and that its radius is ~0.7 nm. The stability constant of EF binding in the absence of charges was 10^6 l/mol corresponding to a half-saturation constant of 1 μM.

The negative charges are localized on the *cis* side of the PA_{63} channel, which is the binding site for EF. This means that the accumulated positively charged N terminus of EF virtually increases the stability constant for EF binding as a function of the ionic strength c in the aqueous phase in Equation 5,

$$K(c) = K^* \cdot c_0^+ / c \qquad \text{(Eq. 5)}$$

where K^* is the stability constant for the binding reaction when no charges influence the binding process. A best fit of the data of Table 1 was obtained using Equations 2, 3, 4, and 5 by assuming that 6 negatively charged groups ($q = -9.6 \cdot 10^{-19}$ As) are located within the channel vestibule and that its radius is ~0.7 nm. The results of this fit are shown in Fig. 7 for Equation 5. The solid line represents the fit of the stability constant of binding *versus* ionic strength (*i.e.* the KCl concentration) by using the Debye-Hückel theory, and the parameters mentioned above together with a stability constant for binding $K^* = 10^6$ l/(mol) when no charge is involved. The figure demonstrates that the influence of the surface charges is rather small at high ionic strength, *c, i.e.* small l_D (see Equation 3). The numbers of negative charges involved in the accumulation of cations within the channel have to be considered as tentative because the relative dielectric constant in the vicinity of the charge is not known. This is discussed in detail elsewhere (42, 43). On the other hand, the diameter of the channel opening in the vicinity of the charges appears to be more precise. A comparison of the ionic strength dependence of EF binding with that of LF demonstrates that the parameters of ionic strength dependence were very similar (11). Only the stability constant K^* (*i.e.* the stability constant in the absence of charges, which corresponds to that at very high ionic strength) varies somewhat between toxins. This result indicates that EF and LF binding to the PA_{63} channel are highly related processes. From this point of view both toxins have the same probability to penetrate inside the cells as it was also found *in vivo* (48).

Acknowledgment—We thank Cesare Montecucco for helpful discussions.

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Anthrax Edema Factor, Voltage-dependent Binding to the Protective Antigen Ion Channel and Comparison to LF Binding

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J. Biol. Chem. 2006, 281:32335-32343.
doi: 10.1074/jbc.M606552200 originally published online September 5, 2006
