The significant threat posed by biological agents (e.g. anthrax, tetanus, botulinum, and diphtheria toxins) (Inglesby, T. V., O'Toole, T., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Friedlander, A. M., Gerberding, J., Hauer, J., Hughes, J., Mcdade, J., Osterholm, M. T., Parker, G., Perl, T. M., Russell, P. K., and Tonat, K. (2002) J. Am. Med. Assoc. 287, 2236–2252) requires innovative technologies and approaches to understand the mechanisms of toxin action and to develop better therapies. Anthrax toxins are formed from three proteins secreted by fully virulent Bacillus anthracis that competitively bind to the heptameric PA63, forming the PA20 and PA63 (4, 5).

**EXPERIMENTAL PROCEDURES**

**Proteins**—Purified PA63, LF, and EF proteins (List Biological Laboratories Inc.) from Bacillus anthracis were used for these studies.

**Antibody**—Hybridoma PA63 1G3–1–1 was prepared by fusing spleen cells from female BALB/c mice injected with PA63 with SP2/O-Ag14 myeloma cells and subcloning positive hybridomas twice by limiting dilution (9, 10). Ascites was produced in BALB/c female mice and was clarified by centrifugation. This ascites monoclonal antibody, 1G3–1–1, prevents binding of LF to PA63 through steric hindrance (9).

**Electrophysiology**—Channel recordings were carried out with planar lipid bilayer membranes as previously described (11). Briefly, solvent-free diphtanylo phosphorylcholine membranes were formed on a 50–100-μm diameter hole in a thin Teflon partition. The partition separated two identical Teflon chambers that each contained ~2 ml of aqueous solution (0.1 M KCl, 5 mM MES, pH 6.6). Voltage was applied across the membrane via Ag-AgCl electrodes. The current was amplified using a patch-clamp instrument (Axon Instruments 200B), recorded with an analog to digital converter (Axon Instruments Digidata 1322), and analyzed off-line. A negative transmembrane potential drove anions from the cis to the trans chamber. Details of this particular method were summarized elsewhere (12).

Channels were formed by adding small aliquots (~100 ng) of the purified PA63 to the aqueous electrolyte solution bathing one side of the membrane (herein called cis). The formation of individual channels was indicated by the stepwise increases in ionic current monitored at +50 mV applied potential. During recording of the ionic current in the steady-state experiments, the membrane potential was held constant at either +50 or −100 mV. To determine the instantaneous current-voltage (I-V) relationships, we generated current by applying brief (0.5 s) voltage pulses (typically +200 to −200 mV in 10-mV steps) across the membrane and averaged the first 100 ms of the signal to obtain the instantaneous (I-V) relationship.
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FIGURE 1. A lethal factor decreases the conductance of PA$_{63}$ channels reconstituted in a planar bilayer membrane. The method for membrane formation and ionic current recording was described elsewhere (12). In the absence of LF, there was a persistent ionic current through $~14$ PA$_{63}$ channels. The addition of $3$ nm LF to the same side of the chamber (cis) that PA$_{63}$ was added caused the current to decrease. Each downward step corresponds to the virtually complete blockade of individual channels. The dashed line indicates zero current. The solutions on both sides of the membrane contained $0.1$ M KCl, $5$ mM MES, pH $6.6$, and the applied potential was $+50$ mV. $B$ and $C$, the LF-induced blockade of voltage-gated PA$_{63}$ channels is reversed by negative applied potential. In the absence of LF ($B$), the PA$_{63}$ ionic current spontaneously decreases over several min. The decrease in current is more rapid for greater magnitudes of the applied potential and for negative voltages. $C$, the addition of $3$ nm LF blocks virtually all the ionic current for positive potentials. In contrast, some current flows for negative applied potentials. In panels $B$ and $C$, the membrane contained $~36$ PA$_{63}$ channels.

Fluorescent-based Kinetic Assay—Known concentrations of serially diluted free LF or unknown concentrations of complexed LF were incubated with $20$ µM optimized peptide substrate (13). Kinetic measurements were made every minute for $30$ min using a fluorescence plate reader (Tecan Safire). The peptide has an excitation peak at $324$ nm and an emission peak at $395$ nm. A calibration curve was established using free LF as a standard. The complexed LF concentration was determined using the standards. The concentration of complexed LF was also confirmed by enzyme-linked immunosorbent assay as described previously (14).

RESULTS AND DISCUSSION

Fig. $1A$ shows an ionic current recording for $14$ PA$_{63}$ channels ($~3.8$ pA/channel for an applied potential $V = +50$ mV) in a planar phospholipid bilayer membrane. The introduction of LF ($~3$ nm) causes a rapid and virtually complete reduction in PA$_{63}$ channel current as revealed by the stepwise decrements (Fig. $1A$). Previous reports at pH $6.6$ and $5.5$ suggested that various factors, such as tetraalkylammonium ions and the N-terminal binding fragments of both EF and LF (i.e. EF$_{N}$ and LF$_{N}$), can cause a decrease in the PA$_{63}$ channel conductance (15–19). In these earlier studies, the ionic current blockades were removed by greater...
transmembrane voltages (16–19). For instance, voltages greater than +40 mV increased \( \text{PA}_{63} \) channel conductance at pH 5.5 following the blockade by \( \text{LF}_N \) (19). However, although no data were shown, the authors state that current blockade relief is not seen at pH 6.6 for \( \text{LF}_N \). Interestingly, this is exactly the case for the full-length LF-induced blockade that we report here at pH 6.6 (see Figs. 1 and 2 and below).

In the absence of LF (Fig. 1B), the current through many \( \text{PA}_{63} \) channels decreases relatively slowly, and this gating effect is voltage dependent (16, 17). The greater the magnitude of the applied potential, the more rapidly the channels gate to lesser conductance states. In addition, the current decays more rapidly for negative potentials than for positive voltages of the same magnitude (Fig. 1B). In the presence of LF (Fig. 1C), the blockade of \( \text{PA}_{63} \) channel conductance is highly asymmetric with respect to the sign of the transmembrane potential. That is, the ionic current is virtually nonexistent for positive potentials, whereas negative voltages (i.e. −50 and −100 mV) produce significant current through the \( \text{PA}_{63} \) channels even in the presence of LF. In contrast, LF added to the \( \text{trans} \) chamber had no effect on the \( \text{PA}_{63} \) conductance for either sign of the potential (data not shown). In addition, LF had no effect on channels formed by the pore-forming toxin \( \text{Staphylococcus aureus} \) \( \alpha \)-hemolysin (data not shown). These experiments demonstrate that the creation of a rectifying ion channel by LF binding to the cap domain of \( \text{PA}_{63} \) is not only site specific but also pore-forming toxin specific. Similar results were observed for EF binding to the channel formed by \( \text{PA}_{63} \).

The intrinsic gating of the \( \text{PA}_{63} \) channel (Fig. 1B) makes accurate determination of LF-(\( \text{PA}_{63}\)) or EF-(\( \text{PA}_{63}\)) binding parameters difficult. However, measuring the \( \text{PA}_{63} \) instantaneous \( I-V \) relationship, without and with either LF or EF, avoids that issue. In particular, several hundred ms-long voltage pulses (e.g. +120 to −120 mV, in 10-mV steps) are applied across the membrane in the absence or presence of ~2.5 nM LF (Fig. 2, A and B, respectively). EF binding to the \( \text{PA}_{63} \) channel changes the \( I-V \) relationship from slightly nonlinear to strongly rectifying, as was observed for full-length LF (13). Note that EF causes virtually complete extinction of the ionic current only for positive voltages. The \( I-V \) relationship for a similar experiment is shown in Fig. 2C. These functional measurements and the analysis below demonstrate that the interaction between (\( \text{PA}_{63}\)) and EF or LF cannot be described as merely a passive interaction of anthrax toxin complex biosensor.
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FIGURE 3. The (PA$_{63}$)$_7$, ion channel as a rapid diagnostic anthrax biosensor. A, application as a therapeutic screen demonstrated by inhibition of LF binding to the PA$_{63}$ channels. The instantaneous I-V relationship of PA$_{63}$ channels (filled squares), PA$_{63}$ channels in the presence of a monoclonal antibody (1G3–1-1) (open circles), and PA$_{63}$ channels with the monoclonal antibody bound followed by the addition of ~3 nM LF (half-filled pentagons). B and C, use of the method as a diagnostic tool to detect anthrax lethal toxin. B, lethal toxin (the complex of LF and (PA$_{63}$)$_7$) purified from the serum of a guinea pig infected with fully virulent B. anthracis spontaneously forms rectifying channels. The concentration of LF in the complex with (PA$_{63}$)$_7$, in the chamber is ~ 30 pM. C, typical I-V relationship for the lethal toxin complex demonstrates that lethal toxin (~60 nM LF in the complex with (PA$_{63}$)$_7$) formed in vivo is detectable with our method. D, the concentration and activity of LF in the complex determined with a fluorescence-based kinetic assay. The enzymatic activity of free LF at different concentrations was measured directly (open symbols). A calibration curve derived from these LF standards is used to determine the concentration of LF bound to the PA$_{63}$ channel in serum (filled squares).

Our electrophysiological results (Figs. 1 and 2) demonstrate that both full-length LF and EF markedly occlude PA$_{63}$ ion conductance only at positive applied potentials. The block may persist for zero transmembrane potential.

The effect shown in Fig. 2B provides a method to probe the interaction between EF or LF and the PA$_{63}$ channel. Fig. 2D illustrates the I-V relationships for PA$_{63}$ channels in the absence of LF and for five different concentrations of LF. For increasing [LF] and $V > 0$, the instantaneous current decreases markedly with respect to that for [LF] = 0. In contrast, for $V < 0$, LF has only a slight effect on the instantaneous current. The ratio of the current in the presence of LF to that in the absence of LF for $0 \leq$ [LF] $\leq$ 2.5 nM for three different positive applied potentials is shown in Fig. 2E. In each case, an increase in LF concentration monotonically decreases the PA$_{63}$ current for $V > 0$.

To estimate the apparent binding constant of LF to the PA$_{63}$ pore, we assume that one LF molecule binds reversibly to the channel (21) and can thereby block it. A least squares best fit of this simple model to the data in Fig. 2E suggests that the apparent dissociation constant is $K_D \approx$ ~50 pM. Despite the good fit of the model to the data, LF may bind even more strongly to PA$_{63}$. For example, the actual concentration of LF might be lower if LF binds to trace amounts of PA$_{63}$ (~10 pm) in solution. The apparent binding constant deduced from the effect of LF on the PA$_{63}$ channel I-V relationship (Fig. 2E) is consistent with estimates from cell-based and Biacore surface plasmon resonance (SPR) assays (~10 pm to 2.5 nM) (22-24). However, unlike SPR, the electrophysiology method described here provides a functional test of PA$_{63}$ and LF and is obtained in minutes rather than days as is required by cell-based methods.

Although we do not yet understand the physical mechanism for EF- and LF-induced rectification of the PA$_{63}$ channel, the schematic in Fig. 2F illustrates several hypothetical models for the effect. There are at least two possible mechanisms that can account for the LF-induced current decrease for $V > 0$ (Figs. 1 and 2). LF could be forced into the pore entrance, perhaps by the small electric field gradient near the pore mouth, and thereby occlude the channel (Fig. 2F, top panel). Alternatively, LF might be driven completely through the channel (not illustrated). EF or LF channel blockade reversal by negative potentials may be the result of their removal from the PA$_{63}$ ion conduction pathway like a swinging gate or the complete separation from the PA$_{63}$ channel (Fig. 2F, bottom panel).

The (PA$_{63}$)$_7$-LF interaction (Fig. 2) might prove useful for understanding the mechanism by which PA, LF, and EF traverse the endosomal membrane through further characterization of the protein-protein interactions. Another promising prospect includes identifying and screening compounds and/or antibodies that disrupt toxin function or interaction. To test this hypothesis, we use a previously characterized monoclonal antibody, 1G3–1-1, that prevents binding of LF to PA$_{63}$ through steric hindrance (9). Briefly, previous studies showed that 1G3–1-1 neutralized LF toxin both in vivo and in vitro. In vivo, this monoclonal antibody protected 100% of animals injected with lethal toxin (i.e. LF bound to the PA$_{63}$ peptamer). In vitro, the monoclonal antibody prevented LF from binding to the protective antigen and neutralized the cytolytic activity even if lethal toxin was formed prior to monoclonal antibody addition (9). Although it is known that this antibody inhibits the binding of LF to PA, to date there have been no reported studies showing the effect of antibodies on the channel conductance. In our
electrophysiological studies, the antibody had virtually no effect on the $I-V$ relationship of PA63 channels but completely inhibited LF-induced blockade (Fig. 3A). As a control, incubating the channels with bovine serum albumin (150 nM) had no effect on LF-induced PA63 channel blockade (data not shown). Thus, the effect of the antibody is specific.

We conclude that the results in Fig. 3A provide the basis for a quantitative biosensor to rapidly screen for one type of anthrax therapeutics. Agents that bind to the PA63 channel (and do not cause current blockades) or to LF would inhibit LF-induced rectification of the instantaneous $I-V$ relationship for PA63 channels. Other potentially useful anthrax therapeutics could conceivably bind to and occlude the PA63 pore.

In a recent study, we showed the presence of a functionally active complex in the serum of infected animals (14). To demonstrate the applicability of this method for diagnostic purposes, we first characterized the channel properties of anthrax lethal toxin complex purified from the serum of an infected guinea pig. Serum samples contain a large number of contaminating cellular and bacterial proteins that may possibly interfere with the channel recordings. Hence, size exclusion chromatography was used to remove most of the small contaminating proteins, although the fraction corresponding to the high molecular weight complex may also contain some contaminating proteins. The purified in vivo (PA$_{63}$)-LF complex, at an initial LF concentration of $\approx 30$ pM in the bilayer chamber, formed strongly rectifying channels (Fig. 3, B and C), as do PA$_{63}$ channels after the addition of LF (e.g. Fig. 2D) and the complex formed in vitro (14). These results clearly demonstrate the physiological relevance of the rectifying channel in vivo. In addition, they support earlier results that suggested that PA$_{63}$ was complexed with lethal factor in the blood of infected animals (25). The concentration and activity of

![Molecular models of the PA$_{63}$ heptamer and the PA$_{63}$ heptamer-LF complex.](image)
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The in vivo formed complex was determined using a fluorescent-based kinetic assay (Fig. 3D) as described under “Experimental Procedures.” Having identified and characterized the conducting properties of the purified infected complex, we may eventually be able to isolate the signal of the complex from the noise generated by contaminating serum proteins. Studies are ongoing to detect the complex in serum samples at various stages of infection.

Figs. 1–3 demonstrate that LF and EF bind to and block the PA63 channel at positive applied potentials. This observation raises the question: why is the PA63 channel blocked only at positive potentials? Recent experiments (26) and molecular modeling (27) indicate that the (PA63)7-channel bears a striking similarity to the crystal structure of the S. aureus α-hemolysin channel (28). According to the model for the PA63 channel, it has a mushroom-shaped cap domain and a long stem (Fig. 4A and B) that consists of 14 strands that comprise an anti-parallel β-barrel. The model illustrates how three LF molecules might bind to the PA63 channel. Because the LF- and EF-induced blockades are sensitive to electric field polarity, it is reasonable to speculate that an electrostatic or electokinetic mechanism is involved. The LF binding site on the PA63 channel is mostly basic (Fig. 4C); it is possible that when the transmembrane potential polarity is reversed, the net positive or negative surface charges of the LF molecule (Fig. 4D) cause small mechanical shifts in its position, resulting in PA63 channel blockade.

Based on this hypothetical model, the blockade of the channel by LF for positive applied potentials could be caused by either a physical occlusion (by LF) or a change in the PA63 channel structure induced by the binding of LF. The view shown in Fig. 4B suggests that even a small shift in the location of a single LF molecule could block the pore entrance. It is conceivable that a positive (or negative) potential would drive LF into (or out of) the ionic conduction pathway as discussed above.

Because the LF-induced block of the PA63 channel can be triggered by very small positive potentials or removed by very small negative potentials, the portion of LF that occludes the pore may lie close to or be attached to the pore mouth at all times. For example, we found that when a steady membrane potential is reversed from negative to positive polarity (50 mV) in the presence of LF at ~2 × 10^12 molecules/cm^3 (Fig. 1), the occlusion occurs in less than 50 ms with no subsequent increase in current (data not shown). Simple but conservative model calculations indicate that this rapid occlusion cannot be explained by the LF flux arriving at the channel entrances via diffusion from the bulk (<2 LF molecules/s) or by LF molecules being driven there by the small electric field (~5 × 10^-9 V/cm) in the bulk electrolyte solution. The slightly reduced ionic current for negative membrane potentials observed in the presence of LF (Fig. 1) would also be consistent with LF strongly bound near the channel openings but only partially blocking the ionic current. Additional experimental results and analysis are needed to determine the mechanism by which the applied potential modulates the LF-induced PA63 current blockages.

Electrophysiology measurements of PA63 channel blockade by EF_N (18) and LF_N (19) and cell-based studies that demonstrate the LF- and EF_N-induced blockade of PA63-doped cells (20) have led to the hypothesis that at pH 5.5 LF and EF are translocated through PA63 channels that have a diameter of ~1.1 nm (16, 17). Our results using the physiologically relevant full-length LF and EF show that the LF-induced PA63 current blockades are persistent (Fig. 1). The blockades remain even when a steady membrane potential is reversed from negative to positive polarity (50 mV) in the presence of LF at ~10^12 molecules/cm^3 (Fig. 2). The marked voltage-dependent, diode-like effect suggests that PA may control the direction of ion flow across cell membranes. The toxins may also influence the transmembrane potential, as is true for other rectifying channels (for review see Ref. 32). By controlling the endosomal pH and H^+ influx, PA could create an ideal environment for toxin translocation and/or lysis of the endosome. Exploitation of this effect as an innovative means of characterizing anthrax toxin interactions is only one demonstration of the value of this technique. In comparison to other methods, such as monoclonal antibody assays measuring competition between radiolabeled LF and unlabeled LF or EF to PA (22), cell-based cytotoxicity assays (23), and surface plasmon resonance measurements (24), the approach reported here is a rapid quantitative tool to determine the effective concentrations/binding constants or disruption between LF or EF with PA63. This report demonstrates three exciting applications of this method as a biosensor for anthrax diagnostics. First, full-length LF or EF can be rapidly quantitated at low concentrations. Second, therapeutic agents against anthrax can be rapidly screened. Third, purified lethal toxin from infected animal blood can be detected at low concentrations (~30 pM). The application of the methods described herein will enhance efforts to render anthrax toxin ineffective.

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