Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2209–2213, April 1989
Biophysics

Anthrax toxin: Channel-forming activity of protective antigen in planar phospholipid bilayers

(*Bacillus anthracis*/voltage-dependent channels/pH-dependent channels)

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Communicated by A. M. Pappenheimer, Jr., December 27, 1988

ABSTRACT **The three separate proteins that make up anthrax toxin—protective antigen (PA), edema factor (EF), and lethal factor (LF)—act in binary combinations to produce two distinct reactions in experimental animals: edema (PA + EF) and death (PA + LF). PA is believed to interact with a membrane receptor, and after proteolytic processing, to mediate endocytosis and subsequent translocation of EF or LF into the cytosol. PA can be separated, after mild trypsinolysis, into two fragments, PA_{65} (65 kDa) and PA_{20} (20 kDa). We demonstrate that trypsin-cleaved PA is capable of forming cation-selective channels in planar phospholipid bilayer membranes and that this activity is confined to the PA_{65} fragment; PA_{20}, LF, and EF are devoid of channel-forming activity. These PA_{65} channels exhibit pH-dependent and voltage-dependent activity—a property reminiscent of the channels formed by the two-chain proteins diphtheria, tetanus, and botulinum toxins.**

The pathogenesis of *Bacillus anthracis*, the causative agent of anthrax, depends on two important virulence factors: an antiphagocytic poly(D-glutamic acid) capsule and "anthrax toxin." The latter term refers collectively to three proteins encoded by plasmid pXO1 (1): edema factor (EF; 89 kDa), lethal factor (LF; 83 kDa), and protective antigen (PA; 85 kDa), the last so named because of its use in vaccines to generate antibodies which protect against anthrax infection. These three toxin components have no known biological effect when administered individually to experimental animals, but they act in binary combinations to produce two distinct reactions. Intradermal coinjection of PA and EF (a combination termed "edema toxin") produces edema, while coinjection of PA and LF (a combination termed "lethal toxin") causes death in susceptible animals. EF has been shown to be a calmodulin-dependent adenylate cyclase (2), whereas the molecular action of LF remains unknown. (For a general review of anthrax toxin see ref 3.)

EF must penetrate to the cytosolic compartment to contact calmodulin and substrate ATP, and it is generally assumed that LF also penetrates to the cytosol, where it is believed to inactivate an essential cellular component. Although the mechanism(s) by which EF and LF are translocated to the cytoplasm is poorly understood, PA clearly plays a central role. Leppla and coworkers (4, 5) have proposed a mechanism of toxicity in which (*i*) PA binds to a receptor on the cell surface; (*ii*) a small N-terminal piece of PA (PA_{20}; 20 kDa) is removed proteolytically, leaving the larger C-terminal piece (PA_{65}; 65 kDa) bound to the receptor; (*iii*) EF or LF binds to PA_{65}; (*iv*) the receptor-PA-EF or receptor-PA-LF complex undergoes receptor-mediated endocytosis; and finally, (*v*) EF or LF (within an endocytic or derivative vesicle) undergoes membrane translocation and is released into the cytosol.

Recent studies on LF (6) suggest that toxicity requires passage through an acidic vesicle, a step reminiscent of the pathway leading to intoxication by diphtheria, tetanus, and botulinum toxins. Indeed, the entire sequence of events is quite analogous. The latter toxins, which are synthesized as single-chain polypeptides, are functionally two-chain molecules whose N-terminal portion is (or is presumed to be for tetanus and botulinum toxins) an enzyme that can be readily dissociated from the rest of the molecule by proteolytic "nicking" and reduction, and which requires the C-terminal portion of the molecule for translocation into the cytosol from an acidic vesicle (7, 8). With anthrax toxin, the two effector moieties, EF and LF, are *ab initio* separate from the fragment (PA_{65}) involved in their translocation (Fig. 1).

In light of the similarity of anthrax toxin to these other toxins and the fact that they (9, 10), and in particular their heavy chains (11, 12), form ionic channels in planar phospholipid bilayer membranes, we undertook to determine whether any of the components of anthrax toxin had channel-forming activity. Our studies reveal that nicked PA (that is, PA which has been treated by mild trypsinization), but not unnicked PA, forms channels which exhibit both voltage-dependent and pH-dependent activity; LF and EF are devoid of channel-forming activity. In addition, we demonstrate that channel-forming activity is confined to the PA_{65} fragment. We describe in this communication results of studies with these anthrax PA_{65} channels, compare them with the channels formed by diphtheria, tetanus, and botulinum toxins, and speculate about their possible relevance to the process of toxin action on cells.

MATERIALS AND METHODS

Purification of Toxin Components. Anthrax toxin was prepared from the noncapsulogenic Sterne strain of *Bacillus anthracis* by using a modification of the method outlined by Leppla (4). Cells were grown in tightly capped 4-liter flasks containing 2 liters of R medium (13) buffered with 100 mM Tris·HCl, pH 7.5. Cultures were incubated at 37°C for 20 hr with rotary shaking (100 rpm). Large-volume (8-liter) culture supernatants were collected by using a Pellicon tangential flow system with 0.45-μm pore-size membranes (Millipore). The cell-free filtrate was concentrated 10-fold by ultrafiltration through PTGC membranes (molecular mass cut-off, 10,000) in a Minitan system (Millipore). Protein was precipitated overnight at 4°C by the addition of $(NH_4)_2SO_4$ to 70% saturation. The precipitate was collected by centrifugation at 18,000 × *g* for 45 min and resuspended in 8 ml of 20 mM ethanolamine·HCl, pH 9.0. The crude toxin preparation was dialyzed until the conductivity of the dialysate was equivalent to that of the buffer.

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Abbreviations: EF, edema factor; LF, lethal factor; PA, protective antigen; DPhPC, diphytanoyl phosphatidylcholine; Pr_4NBr, tetrapropylammonium bromide.

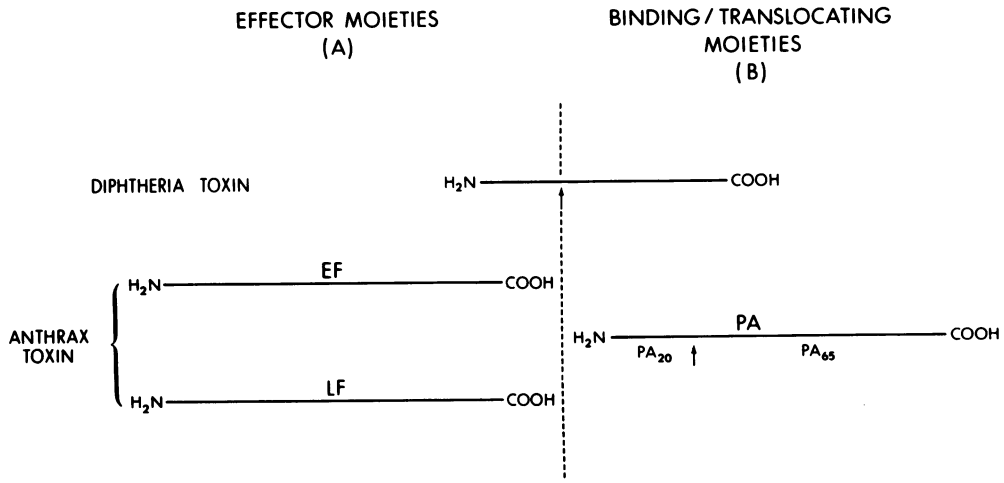

FIG. 1. Schematic diagram of diphtheria toxin and anthrax toxin, comparing the functional domains of the anthrax tripartite toxin with those of diphtheria toxin, a dichain toxin. The lengths of the proteins are drawn approximately to scale. Diphtheria toxin has a molecular mass of ≈58 kDa; LF, EF, and PA each have a molecular mass of ≈85 kDa. Arrows indicate sites of proteolytic cleavage.

PA, EF, and LF were purified by using an anion-exchange chromatography apparatus (FPLC; Pharmacia LKB), as described by Quinn *et al.* (14), with the following changes. Toxin was chromatographed on a Mono Q HR 5/5 column in 20 mM ethanolamine·HCl buffer, pH 9.0 (5), with a linear 40-ml 0–0.4 M NaCl gradient. PA purity was greater than 90% as assessed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (15) and Western blotting methods.

Trypsin Cleavage of Protective Antigen. Purified PA (20 μM) in 20 mM ethanolamine·HCl, pH 9.0, was incubated with trypsin (1 μg/ml) at 37°C for 30 min. The reaction was stopped by addition of phenylmethylsulfonyl fluoride (Sigma) to 1 mg/ml or soybean trypsin inhibitor (Sigma) to 5 μg/ml. PA_{65} and PA_{20} were separated by anion-exchange chromatography on Mono Q as described above. Column fractions were assayed by SDS/PAGE. Protein purity was assessed with Coomassie blue-stained SDS/PAGE gels of pooled fractions.

Membrane Formation and Measurements. Most experiments were performed on planar phospholipid bilayer membranes formed at room temperature by the brush technique of Mueller *et al.* (16) across a 1-mm diameter hole in a Teflon partition separating two Lucite compartments, each containing 3 ml of identical salt solutions. Generally, the salt solutions were 100 mM KCl/1 mM EDTA/10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, and the membranes were formed either from a 3% solution of diphytanoyl phosphatidylcholine (DPhPC) or from a 5% solution of asolectin in *n*-decane. After the membranes were completely black, the desired component or fragment of anthrax toxin was added to one compartment (defined as the cis compartment) to concentrations ranging from 0.1 ng/ml to 1.2 μg/ml, and records were then taken. The pH values of the solutions were changed during the course of an experiment by additions of small volumes of concentrated buffer solutions—citrate or Mes [2-(*N*-morpholino)ethanesulfonic acid]—to one or both compartments; changes in salt concentrations or additions of other ions were also made with small volumes of concentrated solutions. Both compartments were continuously stirred with small magnetic stir bars throughout the course of an experiment. A few experiments, confirmatory in nature, were performed on so-called "hydrocarbon-free" bilayers (17).

Experiments were done under voltage-clamp conditions with a single pair of Ag/AgCl electrodes that made electrical contact with the solutions in the compartments through 3 M KCl agar bridges. The membrane conductance (g) in symmetric salt solutions is defined as the current flowing through the membrane (I) divided by the transmembrane voltage (V), $g = I/V$, where V is the potential of the cis compartment; the potential of the trans compartment is taken as zero. The conductance of a membrane prior to addition of anthrax toxin components was less than 50 pS. The applied voltages and the current responses were displayed on a Narco physiograph chart recorder. DPhPC was obtained from Avanti Polar Lipids, asolectin was phosphatidylcholine type IIS from Sigma and was purified by the method of Kagawa and Racker (18), and *n*-decane (99+%) was from Aldrich. All anthrax toxin components were stored as aliquots frozen at −20°C; once thawed, they were kept at 4°C. At a concentration of ≈600 μg/ml, pH 9.0, and 4°C, PA_{65} showed little loss of channel-forming capability after storage for several months. Tetrapropylammonium bromide (Pr_4NBr; purum grade) was purchased from Fluka.

RESULTS

Proteolytic Nicking of PA. Mild treatment of PA with trypsin yielded two fragments of estimated molecular masses 65 kDa (PA_{65}) and 20 kDa (PA_{20}) (5), and the trypsin cleavage products were separated by ion-exchange chromatography (see *Materials and Methods*). PA_{65} eluted as a single peak ([NaCl] = 0.35 M), whereas PA_{20} eluted as three peaks ([NaCl] = 0.12–0.15 M). The microheterogeneity may reflect cleavage by trypsin at different closely spaced sites. Fig. 2 shows an SDS/polyacrylamide gel of intact PA, PA treated with trypsin, and purified PA_{65} and PA_{20}.

Nicked PA and PA_{65} Form Channels. When intact (i.e., unnicked) PA is added at concentrations of up to 1.2 μg/ml to a planar phospholipid bilayer separating identical 1 M KCl solutions buffered at pH 5.5, there is little effect on membrane conductance; occasionally one can see a few discrete single channels with a conductance of ≈165 pS. In contrast, if nicked PA (i.e., PA which has been treated by mild trypsinization) is added at only 1/20th of this concentration to the same or an identical untreated membrane, the membrane conductance increases enormously within seconds (Fig. 3); at much lower concentrations of nicked PA, single channels are seen, similar to those observed with unnicked PA (Fig. 3 *Inset*). Thus proteolytic nicking of PA appears necessary for channel-forming activity, and the minimal activity sometimes seen with unnicked PA may be a consequence of mild proteolysis. Crude preparations of LF and EF

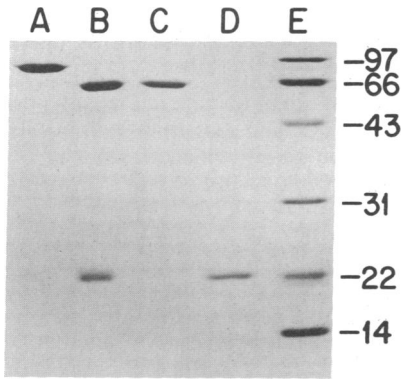

FIG. 2. Limited proteolytic cleavage of PA by trypsin. Samples were electrophoresed on an SDS/11.25% polyacrylamide gel and stained with Coomassie blue. Lanes: A, unnicked PA; B, nicked PA; C, purified PA_{65}; D, purified PA_{20}; E, molecular mass standards, as indicated, in kDa.

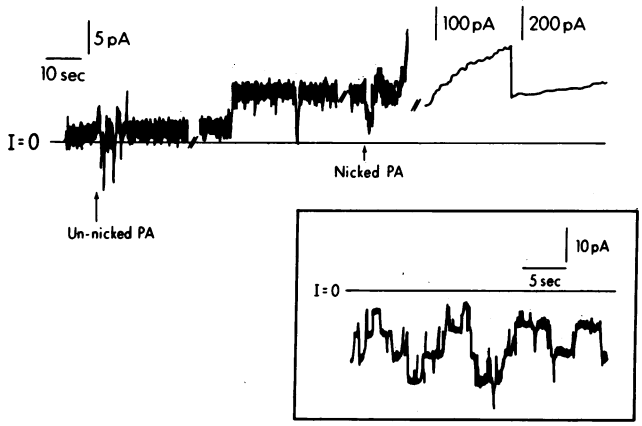

FIG. 3. Comparison of channel-forming abilities of unnicked and nicked PA. At the first arrow, unnicked PA was added to a concentration of 1.2 μg/ml to one side of an asolectin membrane separating identical salt solutions (1 M KCl/5 mM $CaCl_2$/0.1 mM EDTA/10 mM Mes, pH 5.5). The membrane was held at a potential (V_{cis}) of +30 mV. For 15 min there was essentially no effect of unnicked PA on membrane conductance. [During the first break in the record (12 min), a few samples of very dilute nicked PA were added. The single channel which appears after the break may have resulted from either the unnicked PA or the dilute nicked PA.] At the second arrow, 3 min after the appearance of the channel, nicked PA was added to a concentration of 51 ng/ml. Seconds later, the current can be seen to increase rapidly, indicating the opening of many channels. Note the changes in the current scale after the third break (10 sec) in the record. (*Inset*) Current fluctuations resulting from the opening and closing of single channels with a unitary conductance of ≈165 pS. Nicked PA was added to a concentration of 0.56 ng/ml to an asolectin membrane. The membrane was held at a potential of −50 mV; therefore, downward jumps in current indicate channel openings. Solutions were identical to those above; the record was filtered at 10 Hz.

do not display any channel-forming activity when added at concentrations of 0.4 μg/ml under similar conditions. Nicked PA was separated into its component fragments, PA_{65} and PA_{20}, and these fragments were examined for channel-forming activity. We find that PA_{65} possesses channel-forming activity indistinguishable from that of nicked PA, whereas PA_{20}, even at concentrations of 0.4 μg/ml, exhibits no such activity. Therefore, the channel-forming activity of nicked PA resides exclusively in the PA_{65} fragment.

Effects of pH on Channel Activity. In 0.1 M KCl and in the presence of 1 mM EDTA, the rate at which PA_{65} inserts into membranes and forms channels, as manifested by the rate of conductance increase (dg/dt), is a very steep function of the cis (the compartment to which protein is added) pH. For example, at symmetric pH 7.4, a few channels might appear over the course of several minutes following addition of PA_{65} to ng/ml concentrations. However, when the cis pH is lowered to below 6.8 (with HCl, Mes buffer, or citrate buffer), there is a dramatic (>10^4-fold) increase in dg/dt (Fig. 4); lowering the pH below 6.5 does not result in further stimulation. To determine if this increase in rate reflects an increase in the binding of PA_{65} to the bilayer or an increase in the rate of insertion and opening of bound PA_{65} channels, we performed the same experiment but first perfused the cis compartment with toxin-free solution at pH 7.4 before lowering the cis pH. Under these conditions there was only a small increase in dg/dt, implying that low cis pH triggers a binding step, as opposed to significantly gating the channel.

This impressive effect of cis pH on channel activity is dependent on both salt concentration and the presence of EDTA. In EDTA-free solutions of 0.1 M KCl, PA_{65} forms channels at symmetric pH 7.4 at rates comparable to those seen in the presence of EDTA after the cis pH is lowered. In addition, in the absence of EDTA there is no further stimulation of activity with the lowering of the cis pH. It is possible that EDTA binds a trace metal contaminant (e.g., Cu, Al, Zn,

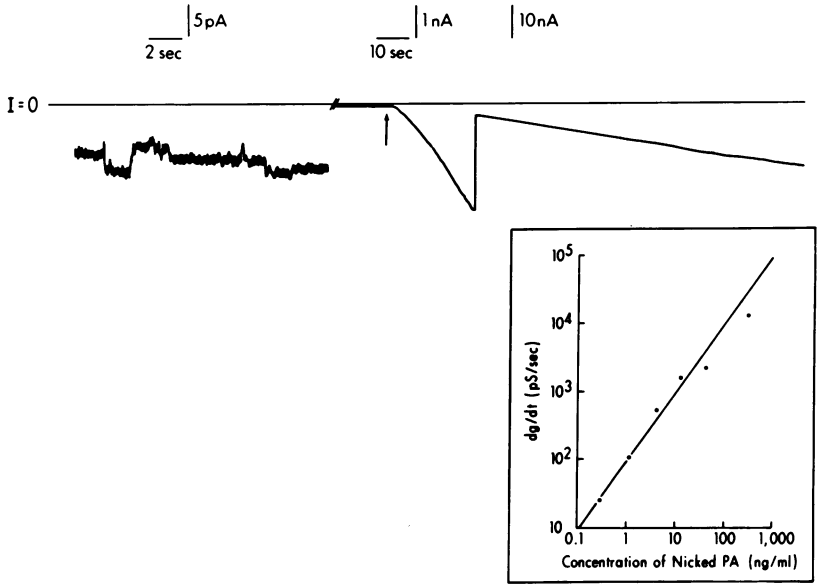

FIG. 4. Effect of cis pH on channel activity. Prior to the start of the record, PA_{65} was added to a concentration of 4.3 ng/ml to a DPhPC membrane separating symmetric solutions at pH 7.4 (100 mM KCl/1 mM EDTA/10 mM Hepes). The membrane was held at a potential of −30 mV. At this cis pH only a few channels can be seen. Four minutes later, at the arrow, the cis pH was lowered to 6.5 by addition of Mes buffer to a concentration of 8 mM. Within seconds there is a rapid, linear increase of current with time. Note the changes in the current scale. (*Inset*) Logarithmic plot of dg/dt vs. concentration of nicked PA. The line is drawn with a slope of 1. PA was added, in increasing concentrations, to an asolectin membrane held at a potential of +50 mV. Five seconds after each addition, the slope of the chart recorder current trace was measured, and this value was converted to dg/dt. The interval between additions was 4–9 min. Salt solutions were 1 M KCl/5 mM $CaCl_2$/0.5 mM EDTA/10 mM Mes, pH 5.5

or Fe) which may bind to the toxin and affect its interaction with the bilayer. In 1 M KCl, even in the presence of EDTA, there is full channel-forming activity at pH 7.4 (see Fig. 5).

Kinetics, Dose Response, and Ion Permeability. Addition of PA_{65} to a compartment whose pH is 7.0 or lower (or lowering the pH of the toxin-containing compartment to below 7.0) produces an essentially linear increase of membrane conductance with time (dg/dt), at clamping voltages ranging from −40 mV to +40 mV (Fig. 4); this rate remains constant over the usual time course of an experiment (10–30 min). The failure to reach a steady-state conductance implies that insertion is an essentially irreversible step; indeed, after perfusion of the cis compartment with toxin-free solution, there is no discernable decrease in the conductance. The lack of any significant concavity in the time response of the membrane's conductance after toxin addition (or lowering of pH) suggests that a single channel-forming unit inserts into the bilayer from solution. Consistent with this is our finding that the slope of the logarithmic plot of dg/dt vs. toxin concentration is approximately unity (Fig. 4 *Inset*).

When, after several minutes of clamping the membrane potential at a modest voltage (−30 mV to +30 mV) and observing channel insertion, the voltage is subsequently pulsed to larger negative values (<−40 mV), the conductance displays a much different voltage dependence, suggesting that insertion and subsequent gating exhibit differences in the effects of voltage. Specifically, once channels are inserted they can be closed by large negative voltages (half of the channels close within 5 sec) and rapidly reopened by positive voltages (in less than 1 sec) (Fig. 5). In addition, at voltages greater than +50 mV and less than −80 mV, the "instantaneous" membrane current saturates (Fig. 5 and its *Inset*); this phenomenon may be explored by studies at the single-channel level. (The slight decline in current seen at voltages greater than +50 mV and less than −80 mV is probably a result of very rapid gating which was not resolvable at our time resolution; this too will be clearer from single-channel analysis.)

PA_{65} forms channels which exhibit almost ideal selectivity for univalent cations. With 0.1 M KCl on one side of a DPhPC membrane and 0.7 M KCl on the other (an activity ratio of 5.8), we measured a reversal potential of 39 mV, cation selective (ideal would be 45 mV). Similar results were obtained with NaCl. Reversal potential measurements with Pr_4NBr yielded virtually Nernstian potentials, indicating essentially ideal selectivity for Pr_4N^+. These data also imply, since Pr_4N^+ is clearly permeant, that a lower limit on the channel's diameter is 9 Å.

DISCUSSION

It has previously been shown that the heavy chains of diphtheria, tetanus, and botulinum toxins form pH-dependent and voltage-dependent channels in planar phospholipid bilayer membranes (11, 12). A striking feature of the channel-forming activity of these toxins is the similarity between the conditions for which channel formation is maximal in lipid bilayers and the conditions which are believed to arise in an endocytic vesicle. In particular, these toxins show a marked stimulation in channel-forming activity

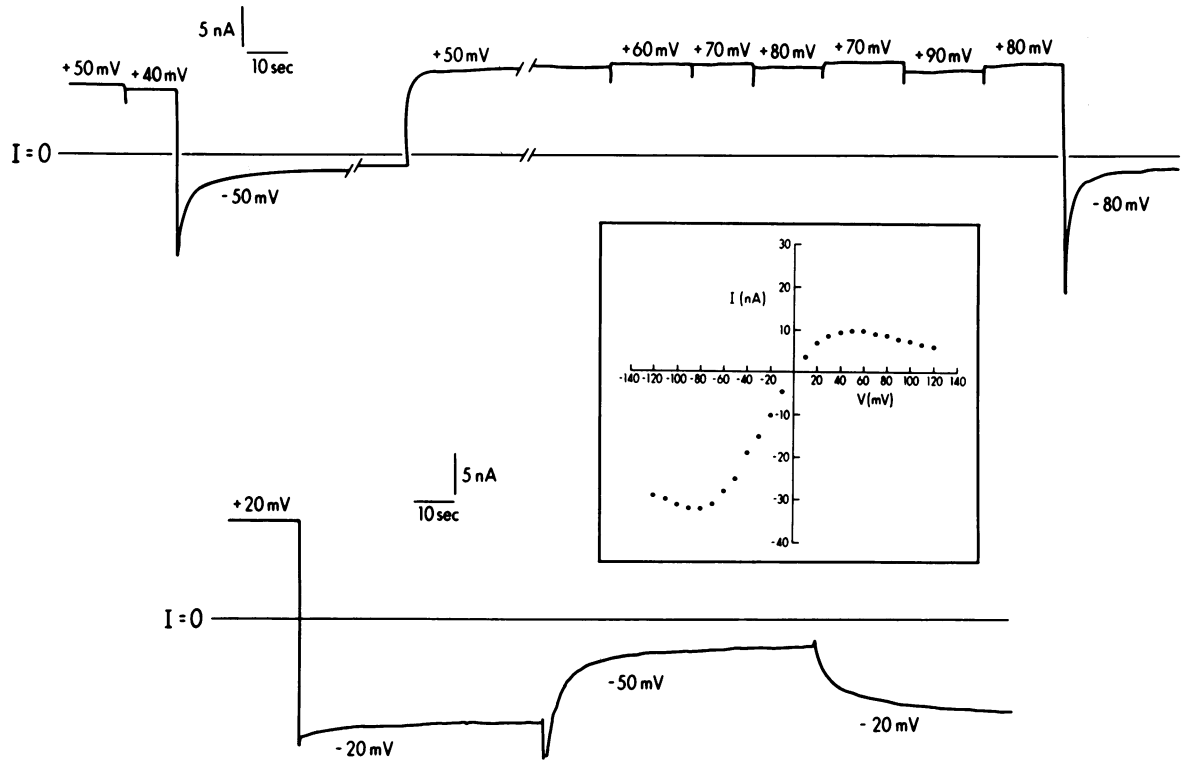

FIG. 5. Voltage dependence of anthrax PA_{65} channels. Nicked PA was added to a concentration of 0.3 μg/ml to an asolectin membrane separating symmetric salt solutions at pH 7.4. The record begins 8 min after toxin addition. The first and second breaks in the record in the top trace are of 3-min and 1-min duration, respectively; the lower trace begins 35 min later. Downward vertical deflections in the top trace represent changes in applied voltage. Note the saturation in the current at positive voltages and the voltage-dependent gating at negative voltages. (In the lower trace, the initial current response to the −50-mV stimulus is truncated because of limitations in the pen excursion.) Saturation of current at large negative voltages is not shown in this figure but is seen in the *I–V* curve in the *Inset*. Salt solutions were 1 M KCl/5 mM $CaCl_2$/0.1 mM EDTA/10 mM Hepes, pH 7.4. (*Inset*) "Instantaneous" *I–V* plot. PA_{65} was added to a concentration of 3.8 ng/ml to a DPhPC membrane separating symmetric solutions (100 mM KCl/1 mM EDTA/10 mM Hepes, pH 7.4), and the cis pH was then lowered to 6.5 by addition of Mes buffer. After 20 min, *I–V* data for this curve were generated by pulsing to the voltages shown and recording the instantaneous (within 0.5 sec) currents.

upon lowering of the cis pH. In the present study we demonstrate that one of the components of the tripartite anthrax toxin, PA, forms channels in planar phospholipid bilayer membranes and that this activity is greatly stimulated, as it is for the toxins mentioned above, by a lowering of the cis pH.

The increase in channel activity seen after lowering the cis pH is predominantly a reflection of an increased rate of binding of PA_{65} to the bilayer from the cis solution; we do observe, however, a small (2- to 3-fold as compared to >1000-fold) increase in dg/dt arising from channels already in the bilayer. The differing effects of cis pH on channel activity in the absence and presence of EDTA imply an interaction of PA_{65} with some transition metal. This interaction, combined with the observation that maximal stimulation of channel-forming activity occurs (in the presence of EDTA) in the pH range 6.5–6.8, suggests to us that the titration of 1 or more of the 10 histidine residues present in PA_{65} (19) may play an important role in channel formation. In the absence of EDTA, transition metals, which are present in trace quantities, may bind to histidine, thereby essentially titrating the group and resulting in high activity even at pH 7.4. One would then expect, and we indeed observe, that subsequent lowering of pH yields little or no stimulation in activity.

Channels formed by PA_{65} are strongly cation selective, having about a 20:1 preference for K^+ over Cl^-, and are almost ideally selective for the quaternary nitrogen ion Pr_4N^+. Their permeability to Pr_4N^+ indicates a diameter of at least 9 Å. The channels display voltage-dependent gating, turning off at large negative voltages and on at positive voltages. At voltages above +50 mV and below −80 mV the "instantaneous" current through the channels saturates.

Although the channel-forming activity of PA_{65} is qualitatively similar to that of the heavy chains of diphtheria, tetanus, and botulinum toxins, there are several quantitative differences: (*i*) maximal channel-forming activity of these latter toxins is achieved at pH values below 5.0 (12), whereas maximal activity of PA_{65} is reached at about pH 6.8; (*ii*) channel-forming activity of diphtheria, tetanus, and botulinum toxins is very sensitive to trans pH (12), whereas the activity of PA_{65} is not; (*iii*) these toxins form channels much less efficiently in decane-containing bilayers than in so-called hydrocarbon-free bilayers, whereas PA_{65} is equally active in both; (*iv*) the channels formed by these toxins are weakly anion or cation selective, depending on the pH (12), whereas the PA_{65} channel is strongly cation selective and does not exhibit a substantial change in selectivity with pH. It is interesting that despite the difference in ion selectivity, all of these channels have a large diameter as judged by the ions that are permeant.

Of particular interest to us is the connection between the ability of a hydrophobic portion of these toxins to form channels in lipid bilayers and the fact that a separate protein or polypeptide fragment of the toxin must somehow be translocated across an endocytic vesicular membrane. In the case of anthrax toxin, channel-forming activity has been confined to the C-terminal 65-kDa portion of the PA molecule, PA_{65}, which is believed to arise biologically from the proteolytic nicking of PA into two fragments (the other fragment being PA_{20}) (see Fig. 1). It is this channel-forming PA_{65} fragment which, when attached to a membrane receptor, is believed to bind EF and LF and then mediate their entry into the cytosol from an acidified endocytic vesicle. It has been suggested that, in light of the similarity of conditions required for formation of channels in bilayers and intoxication in cells, the aqueous pores formed by the heavy chains of the two-chain (diphtheria, tetanus, and botulinum) toxins may accommodate the passage of their light chains in an unfolded conformation (11, 12). Similarly, the channel formed by PA_{65} may serve an analogous function, providing an aqueous pore in the endosomal membrane through which EF and LF might traverse.

We are grateful to Dr. S. H. Leppla of the U.S. Army Medical Research Institute (Frederick, MD) for generously providing anti-PA and anti-LF antisera and samples of anthrax toxin components, which served as standards for our purification. We also thank Dr. S. L. Welkos (U.S. Army Medical Research Institute) for providing ref. 19 prior to publication. This work was supported by National Institutes of Health Grants GM 29210-11 (A.F.), AI-22021 and AI-22848 (R.J.C.), and Medical Scientist Training Grant T32GM7288 from the National Institute of General Medical Sciences (R.O.B.). Partial support was also received from the Shipley Institute of Medicine (R.J.C.).

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