Whole-cell Voltage Clamp Measurements of Anthrax Toxin Pore Current*

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Protective antigen (PA) of anthrax toxin binds cellular receptors and forms pores in target cell membranes, through which catalytic lethal factor (LF) and edema factor (EF) are believed to translocate to the cytoplasm. Using patch clamp electrophysiological techniques, we assayed pore formation by PA in real time on the surface of cultured cells. The membranes of CHO-K1 cells treated with activated PA had little to no electrical conductivity at neutral pH (7.3) but exhibited robust mixed ion currents in response to voltage stimuli at pH 5.3. Pore formation depended on specific cellular receptors and exhibited voltage-dependent inactivation at large potentials (>60mV). The pH requirement for pore formation was receptor-specific as membrane insertion occurs at significantly different pH values when measured in cells specifically expressing tumor endothelial marker 8 (TEM8) or capillary morphogenesis protein 2 (CMG2), the two known cellular receptors for anthrax toxin. Pores were inhibited by an N-terminal fragment of LF and by micromolar concentrations of tetrabutylammonium ions. These studies demonstrated basic biophysical properties of PA pores in cell membranes and served as a foundation for the study of LF and EF translocation in vivo.

Anthrax toxin, secreted by Bacillus anthracis, belongs to the A-B family of bacterial toxins and consists of three protein components. The “B” component, protective antigen (PA),2 binds target cell receptors and is responsible for translocation of the two “A” components, lethal factor (LF) and edema factor (EF), to the cytoplasm of the affected cell (1). The action of LF and EF, a metalloprotease (2) and a soluble adenyl cyclase (3), respectively, have various effects on cells, eventually leading to death of the infected host. PA is an 83-kDa protein that binds either of two known cellular receptors, capillary morphogenesis protein 2 (CMG2) (4) and tumor endothelial marker 8 (TEM8) (5). Cleavage of PA at a furin-sensitive site follows receptor binding and releases an N-terminal 20-kDa fragment, leaving a mature 63-kDa protein bound to the receptor (6). This allows interaction with other cleaved PA molecules and leads to the formation of ring-shaped, heptameric “prepore” structures on the cell surface (7). LF and EF bind competitively to the prepore, and the resulting complexes are internalized by endocytosis (8, 9). Enzyme digestion by a vesicular membrane proton pump induces a conformational change in the prepore that allows it to form a membrane-spanning pore (10). LF and EF are believed to translocate through this pore to the cytosol. Because LF and EF are nontoxic in the absence of PA, a better understanding of the translocation process may reveal useful drug targets for disrupting the damaging effects of the toxin after anthrax infection.

In vivo systems for the study of translocation in real time remain elusive, and instead, most relevant data on the process come from indirect biochemical studies (11) and from experiments in planar lipid bilayers (12, 13). The biophysical properties of PA pores have been partially characterized in planar lipid bilayers (14, 15). Like some other A-B toxins (16, 17), purified PA forms ion-permeable pores in diphytanoyl phosphatidylcholine membranes. Pore formation is dependent on pretreatment of PA with trypsin to yield the active 63-kDa species and also on acidic pH on the side of the membrane (cis) to which the protein is added. The observed membrane currents show relatively nonspecific, large-conductance pores that are blocked especially well by tetraethylammonium ions. Planar lipid bilayers have also been used to study translocation of LF and EF (12, 13).

A real-time cellular assay for translocation would complement planar lipid bilayer studies. Importantly, PA monomer addition to artificial membranes results in pore formation in the absence of cellular receptors, but receptor-deficient cells are unaffected by anthrax toxin (5). Because the cellular receptors for PA may form some structural component of the pore in vivo (18, 19) and because of differences in lipid composition, it is reasonable to ask whether the overall structure of the pores formed in artificial membranes accurately reflects that found in plasma or endosomal membranes. A second consideration is the possibility that in vivo translocation involves additional cellular factors. Translocation of the related Pseudomonas exotoxin A (20), diphtheria toxin catalytic domain (21), and botulinum toxin C21 (22) has been reported to require additional ATP-dependent steps for complete translocation to the cytoplasm. Naturally, if additional and unknown cellular factors are necessary, reconstitution of the translocation process in vitro becomes more difficult.

Our ultimate goal was to develop a patch clamp-based assay to measure LF or EF translocation in real time. Here we used whole-cell patch clamp electrophysiology to characterize PA pores in cell membranes. We showed that pore formation in plasma membranes requires cellular receptors, and we measured basic biophysical properties of these pores, comparing our recordings with those made in artificial bilayers. We found that the pores are effectively blocked by tetrabutylammonium ions and the N-terminal, PA-binding domain of LF. Lastly, using engineered cell lines, we found that pore formation is pH-dependent but that the dependence ultimately resides in the receptor for PA.

EXPERIMENTAL PROCEDURES

Materials—Proteins were produced and purified as described previously (23). Highly concentrated, trypsin-activated PA was diluted to 1...
In 20 Tris/150 NaCl solution, pH 8.5, and added to cultured cells to a final concentration of 10 μM, at 4 °C for 1 h prior to experiments.

Cell Culture—CHO-R1.1 cells stably expressing either human CMG2 or human TEM8 (24) were maintained under G418 selection (600 μg/ml; Invitrogen) and cultured in carbonate-buffered Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) containing 10% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin, under 7% CO2. Cells were seeded onto round coverslips 12–36 h before recording.

Solutions—Solutions were designed to mimic physiological conditions and allow pH manipulation. Extracellular (bath) solutions contained (in mM): 140 NaCl, 3 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 10 MES, and 10 dextrose. In HEPES-free externals, Bis-Tris was substituted for HEPES. Solutions were adjusted to pH values between 4.9 and 7.3 with NaOH. Internal (pipette) solution contained (in mM): 110 KCl, 10 EGTA, 5 CaCl2, 1 MgCl2, 10 HEPES. Internals were adjusted to pH 7.2 with HCl. Calculated free calcium was 195 nM in the HEPES-containing solution using Maxchelator’s WEB-MAXC standard. A stock solution of 1 M 2-deoxy-D-glucose, 200 mM sodium azide, and 4 μM bafilomycin A1 (all from Sigma) was diluted into cell cultures (1:20) 45 min prior to incubation with PA.

Electrophysiology—Whole-cell recordings were made using an EPC-9 (HEKA Electronics) amplifier, and data were acquired using Pulse software (HEKA Electronics). Current traces were acquired between 0.5 and 20 kHz, depending on the protocol, and filtered at 2.9 kHz. The holding potential was 0 mV. The intersweep interval was 5 s. Pipettes were pulled from filamented glass capillary tubes (1B150F-4; World Precision Instruments) with resistances that ranged from 2.2 to 4.0 MΩ. Toxin-treated cells were transferred to a recording chamber. All experiments were performed at room temperature (22–24 °C). Bath solution changes were accomplished by gravity perfusion, and LF_Na was added directly by pipette.

Planar lipid bilayers were painted onto a 200-μm aperture of a 1-ml Delrin cup, using 3% 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine in n-decane. The cis (side to which PA and tetrabutylammonium (TBA) were added) chamber contained extracellular solution, pH 5.3. The trans chamber contained pipette solution, pH 7.2. Trypsin-activated PA was added to a concentration of 90 nM. Macroscopic currents were recorded using a Warner bilayer clamp BC-525C (Warner Instruments) and an ITC-16 interface (Instrutech) at 50 Hz in response to voltage commands using an Axograph 4.0 (Axon Instruments). Indicated voltages refer to the trans chamber.

Data Analysis—Analysis was performed using Pulse software and Microsoft Excel. Current amplitudes at −100 mV were averaged over a 100-ms period preceding the voltage-ramp. For I-V plots showing inactivation, amplitudes were averaged over a 60-ms period at the beginning and end of the voltage step. The periods were chosen to avoid capacitative transients. To calculate the percentage of inactivation, current at 0 mV was subtracted from both data sets. Data for TBA inhibition and the pH dependence of pore insertion were fit using the Sigmoidal Dose Response fitting tool in Origin (OriginLab Corp.) with upper and lower asymptotes fixed at 1 and 0, respectively.

RESULTS

We chose CHO-K1 cells for these studies because of their demonstrated susceptibility to anthrax toxin and because they exhibit few endogenous membrane conductances. Nonetheless, because we expected pore formation by PA to be pH-dependent, we characterized the properties of CHO-K1 cell membranes at acidic pH. Fig. 1A shows that at neutral (7.3) pH, these membranes are electrically silent when subjected to a 2-s voltage-ramp from −100 to +100 mV. When the cell chamber was perfused with the same solution at pH 5.0, however, a large, outwardly rectifying current appeared. This current, which varied in amplitude from cell to cell, is similar to a proton-activated chloride current previously observed in CHO-K1 cells (25).

To determine whether PA forms ion-conducting pores in CHO-K1 cell membranes under similar conditions, we incubated the cells with trypsin-activated PA monomers (10 nM) prior to recording and repeated the experiment. Fig. 1B shows that when the bath pH was lowered to 5.0 in the presence of PA, a new conductance emerged. In the presence of extracellular chloride ions, both inward and outward current developed as pH was lowered. When sulfate was substituted for...
chloride in the extracellular solution, the outward current decreased at the most positive potentials, and the resulting steady-state current was S-shaped. Offline subtraction of the current recorded in the absence of chloride from the initial current, recorded in the presence of chloride, yielded a current almost identical to that shown in Fig. 1A.

The current that persisted in the absence of chloride at acidic pH (PA current) was only observed after prior incubation with PA, and its reversal potential was around ~12 mV, consistent with conductance of mixed cations. The peculiar shape of the I-V relation shown in Fig. 1B resembles that initially observed for PA in planar lipid bilayers (14). Inward conductance is not linear, probably due to voltage-dependent blockade by HEPES present in our recording solutions (15). Outward conductance is similarly nonlinear, due to a noticeable voltage-dependent inactivation that occurs at membrane potentials >60 mV and higher. From this, we concluded that PA forms ion-conducting pores in CHO-K1 cell membranes at acidic pH and that a contaminating chloride current is also present at pH 5.0.

Anthrax toxin action depends on the presence of cellular receptors for PA. Because the formation of heptameric receptor-PA complexes on the cell surface triggers their endocytosis, we treated cells with 2-deoxy-D-glucose, sodium azide, and bafilomycin A prior to incubation with PA. This mixture reduces endocytosis and receptor degradation and boosts the number of receptor-PA complexes at the plasma membrane available for recording in the whole-cell configuration (24, 26). This treatment, and the use of recording solutions of pH 5.3, rather than 5.0, greatly reduced the chloride current in subsequent recordings (see Figs. 1, C and D, and 3A for example), perhaps because the chloride current depends on cellular ATP production.

To determine whether pore formation depends on receptors in cell membranes, we tested CHO-R1.1 cells, in which both of the known receptors for anthrax toxin are absent (5). Fig. 1C shows currents recorded from a CHO-R1.1 cell in the absence of PA. At both pH 7.3 and pH 5.3, the CHO-R1.1 membranes are largely electrically silent at potentials between ~100 and 100 mV, and the proton-activated chloride current is largely absent. PA-treated cells (10 nM) gave identical results (Fig. 1D). From this, we concluded that pore formation by PA depends on the presence of cellular receptors for PA.

To characterize the basic biophysical properties of PA pores in the plasma membrane, we used HEPES-free recording solutions and a stable CHO-R1.1 cell line that overexpresses CMG2, the higher affinity cellular receptor for anthrax toxin. After incubation with PA and establishment of the whole-cell configuration, cells were subjected to a voltage-step protocol at pH 5.3. Holding the cell membranes at potentials from ~100 to 100 mV at 20-mV intervals elicited steady-state currents (Fig. 2A). We plotted mean current amplitudes from three cells as a function of membrane potential (Fig. 2B) and found linear (ohmic) pore conductance over this potential range. We used tetrabutylammonium ions (>1 mM), a known inhibitor of PA pore conductance, to block this mostly linear current, thus distinguishing it from a simple leak in the whole-cell voltage-clamp configuration (not shown, see Fig. 3B for examples). When the duration of each step in the protocol was lengthened to 3 s, we observed current inactivation at potentials greater than +40 and below ~60 mV (Fig. 2C). We then plotted current-voltage relationships for initial and steady-state current to show the effects of inactivation (Fig. 2D). In three cells, current amplitude declined by an average of 34% ± 8.6% after 3 s at 100 mV. Inactivation at potentials greater than 75 mV was still apparent, even when the length of the voltage-ramp protocol used in Fig. 1 decreased to 500 ms (not shown).

Large positive potentials in the trans chamber of planar lipid bilayer experiments were reported to eliminate conductance through PA pores almost completely (14). This discrepancy with our measurements could result from differences in the recording solutions, and we therefore tested for inactivation in planar lipid bilayers using the same solutions. Fig. 2, E and F, show currents recorded through PA pores in an artificial bilayer, in response to the same voltage commands used in Fig. 2C and the I-V plots for initial and steady-state currents. Under these conditions, inactivation was much more pronounced at positive (trans) potentials and largely absent at negative potentials. In five membranes, current amplitude declined by an average of 94% ± 2.9% after 3 s at 100 mV.

Based on these measurements, the PA formed simple ion-conducting pores in plasma membranes. The pores formed in these membranes only in the presence of cellular receptors and as a result of a conformational change that occurs at acidic pH, converting from membrane-bound prepro to membrane-spanning pore that includes a 14-strand β-barrel (27, 28). Since they are not “gated” like conventional voltage- or ligand-gated channels (once inserted in the membrane, they apparently do not “close”), we could not employ any online leak subtraction strategy. For this reason, in all subsequent recordings, we used solutions containing 10 mM HEPES (internal and external) and 2-s voltage-ramps from ~100 to +100 mV. The characteristic S-shaped I-V relation that results from these conditions allowed us to distinguish current flowing through PA pores more easily from the leak current that occasionally contaminates whole-cell recordings.

Previous studies indicate that PA pore conductance is inhibited both by LF (29, 30) and by the tetraklylaminonium ions (15). Accordingly, we tested the blocking ability of the N-terminal domain of LF (LF180, residues 1–263) and TBA on plasma membrane PA pores. Fig. 3A shows the block (gray) of initial current at pH 5.3 (black) by 30 nM LF180 in CMG2-expressing cells. Inhibition was rapid and asymmetric, with pores becoming partially unblocked at positive potentials. Similarly, we characterized the inhibition of PA pores at pH 5.3 by TBA. Fig. 3B shows an example of TBA block at 30 μM. Bath pH was lowered to 5.3, and TBA-containing solution was perfused into the recording chamber until the current amplitude reached steady state (gray). At micromolar concentrations, TBA block was also asymmetric. For this reason, percentage inhibition was always calculated using current amplitudes at ~100 mV. Much higher concentrations of TBA (1–10 mM, added directly by pipette) blocked conductance of PA at all potentials. Fig. 3C shows an inhibition curve for TBA under these conditions (squares). Inhibition was almost complete at 300 μM and minimal at 10 μM. The calculated IC50 for TBA from these data is 67 ± 9.5 μM (n = 4 cells). In artificial membranes, the K50 for TBA binding to PA pores was reported as 8 μM. Because this discrepancy could arise from differences in recording solutions (chiefly pH and the presence of HEPES), we used our patch clamp solutions to determine the K50 of PA for TBA pores in a planar lipid bilayer. Fig. 3D shows the inhibition curve for TBA under these conditions (triangles). The calculated IC50 for TBA from these data is 150 ± 8.9 μM (three membranes).

Planar bilayer studies indicate that TBA inhibition of PA pores results from binding to a ring of phenylalanine side chains that protrude into the barrel of the three-dimensional pore structure (13). These phenylalanines prove critical to LF translocation through the pore. Mutation of the phenylalanine residue in PA (Phe-427) that serves as the binding site for TBA should abolish the blocking ability of this cation. Fig. 3E shows that this is in fact the case. Substitution of an alanine at this site (F427A) yielded a protein that was similar to wild-type PA in its pH dependence for pore formation and voltage-dependent inactivation at positive potentials, but its conductance at negative potentials was markedly different, remaining linear from 0 to ~100 mV (black). As expected, 300 μM TBA had no effect on pores formed by F427A (gray). The
absence of voltage-dependent inhibition of the mutant pores by HEPES present in the normal recording solutions (10 mM), combined with the lack of inhibition by TBA, suggested that the voltage-dependent block by these two compounds occurs by a similar mechanism.

Biochemical characterization of the PA prepore in complex with either CMG2 or TEM8 receptors suggests that the pH dependence of prepore-to-pore conversion in solution and in cells depends on the specific receptor (24). We used current as a read-out to measure pore formation directly in cells engineered to express CMG2 or TEM8 receptors in a receptor-null background. We measured inward current amplitude at −100 mV to avoid contaminating chloride currents. CMG2-expressing cells were treated with PA and exposed to a series of increasingly acidic bath solutions following establishment of whole-cell configuration. Pore formation began at pH 5.5, reaching a maximum at pH 5.1. Fig. 4, A and B, show inward currents from one cell and the time course of current development. Pore current was not observed in solutions of pH greater than 5.5, and decreasing the pH below 5.1 did not result in additional current. Fig. 4C shows fractional current amplitude as a function of bath pH. The half-maximal pH for pore insertion into CMG2-expressing cell membranes is 5.3 ± 0.01 (n = 5 cells). Fig. 4, D and E, show inward currents and the time course of pore formation in TEM8-expressing cells. Pore formation occurred at higher pH values and over a narrower pH range, when PA was bound to TEM8. Current was not observed at pH greater than 6.3, and pH values below 6.1 did not result in additional current. The half-maximal pH for pore insertion in TEM8-expressing cells is 6.2 ± 0.02 (Fig. 4F; n = 6 cells).
DISCUSSION

We have used patch clamp electrophysiology to record ionic currents through pores formed by anthrax toxin protective antigen in CHO-K1 and CHO-R1.1 cell plasma membranes. Pore currents showed a linear, mixed cation conductance, with some voltage-dependent inactivation, and inhibition by TBA and LF

FIGURE 4. pH dependence of pore formation resides in the receptor for PA. A and D, inward currents from cells expressing CMG2 (A) or TEM8 (D) at the indicated extracellular pH. B and E, time course of pore insertion as indicated by current amplitude measured at −100 mV in CMG2- (B) and TEM8- (E) expressing cells. C and F, pH response curves for pore insertion in CMG2- (C) and TEM8- (F) expressing cells. Half-maximal pH for CMG2 is 5.34 ± 0.01 (n = 5 cells); for TEM8, the value is 6.23 ± 0.02 (panel F, n = 6 cells).

FIGURE 3. Protective antigen pores are blocked by LF and TBA. A, CMG2 cell current at pH 5.3 before (black) and after (gray) the addition of 30 nM LF. B, example currents (pH 5.3) from CHO-R1.1 cells overexpressing CMG2 in the absence (black) and presence (gray) of 100 μM TBA. Much higher concentrations of TBA (~5 mM) block the pore completely. C, inhibition of PA pores by TBA in cells (squares) and in planar lipid bilayers (triangles). IC50 is 67 ± 9.5 μM in cells (n = 4) and 150 ± 8.9 μM in bilayers (n = 3). D, PA mutant F427A forms pores at pH 5.3 (black) that are not blocked by 300 μM TBA (gray).
membranes depended on cellular receptors, whereas pore formation in artificial membranes does not. This was despite the 100-fold higher concentrations of activated PA used in cell experiments. The high affinity binding of PA to its receptors suggested that the receptor might remain a structural component of the heptameric pore through the membrane penetration and possibly the protein translocation processes. The crystal structure of CMG2 bound to the prepore indicates at least some difference in pore structure and/or behavior in plasma membranes when compared with artificial membranes, suggested at least some difference in pore structure and/or behavior in these two microenvironments.

Pore formation depended on cellular receptors and on acidic pH, but the pH range depended on the receptor involved. The biological importance of this receptor effect on prepore-pore conversion may lie in the location along the endosomal-sorting pathway at which LF or EF is delivered to the cytoplasm. Our work confirms the findings of Rainey et al. (24), who showed by other means that the pH dependence for pore insertion resides in the receptor. Although the mechanism for voltage-dependent inactivation is not known, it is unlikely to play an important role in anthrax toxin physiology as potentials of high magnitude are unlikely to occur across endosomal membranes. At the same time, the difference in degree of inactivation, as well as the 2-fold difference in IC50 for TBA in plasma membranes when compared with artificial membranes, suggested at least some difference in pore structure and/or behavior in these two microenvironments.

In these experiments, the number of receptors present in a given cell seemed to determine the amount of pore current recorded. We recorded less current from "native" CHO-K1 cells than from those overexpressing CMG2 or TEM8. (Over 10–15 cell passages, the amount of current recorded from the stable cell lines gradually declined, to nearly zero, most likely due to gradual loss of stable receptor expression.) Because the interaction between PA and CMG2 is extremely tight, with a reported Kd of 170 pm (31), it is reasonable to assume near total receptor occupancy at the working concentration of 10 nM PA. Interestingly, this concentration seemed high enough to saturate the receptors in both the CMG2- and the TEM8-expressing cell lines because the currents measured in the two cell lines were similar in amplitude. This suggests that the effective receptor affinities for PA are similar despite their apparent differences in affinity for monomeric PA (31, 32). If PA binding and pore formation were simply a function of receptor affinity for monomeric PA, measured currents would presumably have been much smaller in TEM8-expressing cells treated with 10 nM PA. The similarity of effective receptor affinities may reflect predicted high avidity values of the heptameric PA63 prepore when bound to either CMG2 or TEM8.

The experiments described here provided a biophysical characterization of receptor-bound PA pores as ion-permeable complexes at the cell surface. The basic protocol for measuring current in these experiments represents a sensitive assay for pore formation in plasma membranes by PA and could probably be adapted for other pore-forming bacterial toxins (33, 34). This whole-cell technique may ultimately prove suitable for the study of catalytic factor translocation. However, because the membrane integrity of the recorded cell often deteriorates several minutes after pore insertion, further refinement of these methods will likely be required for translocation measurements, and single-pore measurements may be more appropriate.

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