Characterization of *Actinobacillus actinomycetemcomitans* leukotoxin pore formation in HL60 cells

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Abstract

The mechanism of cell death induced by *Actinobacillus actinomycetemcomitans* leukotoxin (LTX) has been investigated with flow cytometry and patch electrode recording using cultured HL60 cells. The kinetics of propidium iodide (PI) positive staining of HL60 cells was measured as a function of LTX concentration at 37°C. Results showed a concentration-dependent decrease in the t½ times. Cell kill was slow at < 1 µg/ml LTX concentrations with fewer than 50% of the cells killed after 1 h; at 1 µg/ml, the t½ times ranged from ~ 15 to 30 min. At higher concentrations, the t½ times decreased rapidly. The rate of cell kill was appreciably slowed at 20°C. HL60 whole cell currents were recorded with patch electrodes. Immediately following exposure to high concentrations of LTX, large currents were recorded suggesting that the membrane potential of these cells had collapsed due to the large conductance increases. At low toxin concentrations, rapid conductance fluctuations were seen suggestive of a limited number of toxin-mediated events. Cells exposed to low concentrations of LTX exhibited these conductance fluctuations for up to 1 h, whereas toxin-insensitive cells were unaffected by long exposures to high concentrations of toxin. Our results are consistent with LTX-induced pores in susceptible cells which overwhelm the ability of the cell to maintain osmotic homeostasis causing cell death. © 1998 Elsevier Science B.V.

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1. Introduction

*Actinobacillus actinomycetemcomitans* leukotoxin (LTX) is a member of the RTX (Repeats in ToXin) family of pore-forming cytotoxins/hemolysins which are produced by a diverse group of gram-negative bacteria [1–10]. The bacteria are important in both human and veterinary pathology, and they produce various infections in humans including infective endocarditis and several forms of periodontal disease [11–14]. The leukotoxin is a 116 kDa water-soluble protein expressed on the surface of the bacteria [15]. Based on the amino acid sequence, LTX was divided into four regions designated N-terminal, central, repeat and C-terminal. The N-terminal region shares sequence homology with the C-terminal pore-forming domain of Colicin Ia [1,16,17]. Since Colicin Ia forms membrane pores [18], the sequence conserva-
tion raised the possibility that LTX may also form pores in susceptible cell membranes. The central region of LTX shares homology with several related toxins and contains two acylation sites, similar to *Escherichia coli* hemolysin [16,19], which are required to produce functionally-active toxin. The repeat region consists of multiple copies of a 9-amino acid cassette which is involved in recognition of the target cell. The C-terminal region appears to regulate interactions with secretory proteins and is required for translocation of the toxin protein to the bacteria surface. In contrast to many bacterial toxins, LTX exhibits a marked cytolytic specificity, destroying human polymorphonuclear leukocytes and macrophages without destroying erythrocytes, platelets and most other cell types [20]. The toxin also exhibits a marked species specificity such that leukocytes of Old-World monkeys and great apes are susceptible to the toxin but not leukocytes of New-World monkeys [21]. Two long-standing questions about LTX are what is the molecular basis of the cytolytic specificity of the toxin and how does LTX kill susceptible cells.

The first question was recently answered by a series of experiments showing that LTX binds to a β2 integrin, lymphocyte function-associated antigen 1 (LFA-1) [22]. LFA-1 is expressed on immune cell surfaces matching the profile of cytolytic targets. LTX binding to LFA-1 receptors appears to be a mandatory first step in a multistep process that enables the toxin to undergo the requisite transition from the water-soluble form to the membrane-inserted form. In the absence of LFA-1 receptors, the membrane-inserted form of the toxin is unable to form and the toxin has no cytotoxic activity. Here we investigate the question of how the toxin kills target cells. A number of bacterial toxins insert into the target cell membrane and form ion permeable channels that dissipate essential cell gradients leading to rapid cell death [2,7]. The homology with the pore-forming domain of Colicin Ia as well as a number of previous studies [23,24] suggest that LTX forms an ion channel, but no electrophysiological evidence for channel activity in susceptible cells has been presented to date. Indirect evidence that LTX forms membrane pores has been obtained from experiments with artificial bilayers [2,23]. In our experiments, when LTX was added to the bathing solution of an artificial bilayer, no channel activity was seen [23]. If, however, LTX was added to the lipid monolayer before forming the bilayer, large conductance fluctuations were seen in the bilayer. We interpreted this result to imply that the aqueous form of the toxin will not spontaneously incorporate into a bilayer, but if the toxin is partially unfolded, as likely happens at the lipid monolayer–water interface, insertion into the membrane occurs and channels are formed. These are also consistent with LTX being required to interact with a cell surface receptor in order to facilitate toxin activation.

In the experiments described in this report, we have examined the kinetics of toxin-dependent cell kill as a function of toxin concentration and temperature using flow cytometry with HL60 cells. These experiments were undertaken to better understand the mechanisms involved in toxin-mediated kill and to establish the appropriate conditions for toxin application in patch electrode recording. Conductance changes in HL60 cells were then monitored following LTX exposure using whole cell patch clamp recording. At high toxin exposures, toxin-induced currents resulted in rapid cell death. At low toxin exposures, rapid conductance fluctuations were observed suggestive of a single or few toxin-mediated events. The activity produced in response to low levels of LTX was stable for more than an hour suggesting that cells can tolerate a limited toxin exposure without the collapse of the cell membrane gradients.

2. Materials and methods

2.1. Cell culture

HL60 cells were grown in suspension in RPMI 1640 media with either 5% heat-inactivated fetal bovine serum (Media I) or with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids, 1% gentamycin, 1% MEM vitamin solution (Media II). Cells were grown in humidified air with 5% CO₂ at 37°C and subcultured 3–4 times a week.

2.2. Purification of LTX

*A. actinomycetemcomitans*, strain JP2, used in this study was extracted and purified as previously re-
The strain was grown in PYG medium (5 g Bactopeptone, 5 g Tryptone, 10 g yeast extract, 10 g dextrose, 8 g CaCl₂, 8 g MgSO₄, 40 mg KH₂PO₄, 400 mg NaHCO₃, 80 mg NaCl in 1 l of dH₂O) for 24 h at 37°C in an atmosphere of 5% CO₂. After washing the bacteria twice in phosphate-buffered saline (pH 6.5), leukotoxin was extracted and purified by a modification of the procedure previously described. Briefly, the bacteria were incubated at 37°C for 1 h in 2% polymyxin B sulfate in 0.01 M phosphate buffer, pH 6.5 with protease inhibitors (5 mM EDTA, 10 mM ε-amino caproic acid, 0.1 mM PMSF, 5 mM sodium tetrathionate). After incubation, bacterial cells and debris were removed by centrifugation at 7000 rpm for 30 min at 4°C and the supernatant was passed over a sulfopropyl Zeta Prep disk (60 mm, CUNO, Meriden, CT) and after washing, the leukotoxin-containing fraction was eluted with 1.0 M NaCl. The eluent was concentrated, re-equilibrated in 0.01 M phosphate buffer, pH 6.5 and applied to a 5/5 Mono S (Pharmacia, Piscataway, NJ) column and a NaCl gradient (0–0.2 M NaCl over 10 min; isocratic at 0.2 M NaCl for 30 min; and 0.2–0.7 M NaCl over 10 min) was passed over the column. Leukotoxin-containing fractions identified with anti-leukotoxin monoclonal antibody were recovered from the 0.2–0.7 M NaCl fractions, concentrated and subjected to gel filtration on a Superose 6 column (Pharmacia).

2.3. Flow cytometry

HL60 cells were washed by centrifugation at 1000 rpm for 6–10 min and suspended at ~2 x 10⁶/ml in Solution B containing 140 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 2 mM CaCl₂ (unless otherwise noted), 1.2 mM MgSO₄, 6 mM glucose, 25 mM HEPES, pH 7.3. Cell samples were analyzed by flow cytometry using propidium iodide (PI) staining, an indicator of cell death, on a Coulter EPICS Elite (Coulter, Hialeah, FL). PI (final concentration 10 μg/ml) and LTX were added to cells at t = 0 and PI fluorescence was monitored at a constant temperature. Controls in each experiment monitored the amount of cell death due to PI alone over time. Cells were considered to be PI positive, if fluorescence intensities were greater than the intensity of 95% of the control cells.

2.4. Estimation of time required to kill ~50% of the cells

The percentage of PI positive cells vs. time was fit to a form of the Hill equation to estimate the time required to kill 50% of the cells in TableCurve (Jandel Scientific). The percentage of PI positive cells vs. time could be described by the equation:

\[ \text{% PI Positive cells} = \text{Initial} + B_0 \left(1 + \frac{t}{t_k}\right)^{N_h} \]

The fit estimates of the number of cells dead at the outset of the measurements (Initial), the cooperativity, \( N_h \), and the maximal percentage of cells killed, \( B_0 \). The parameters in this equation cannot be interpreted uniquely without knowing the mechanism of cell kill, but \( t_k \) roughly measures the time required to kill a significant fraction of the cells (50% if Initial = 0, \( B_0 = 100\% \), \( N_h = 1 \)). This equation was chosen to represent the small but significant initial lag and subsequent rapid increase in cell kill observed in the experiments.

2.5. Whole cell current recording

To allow whole cell recording with patch pipettes, HL60 cells in Media I were washed twice in Solution A with 5 mM glucose (Solution A: 137 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.8 mM MgCl₂, 10 mM HEPES, pH 7.3). Aliquots of cells were added to round glass coverslips in tissue culture dishes with Solution A and allowed to attach to the glass. Glass coverslips were placed in the recording chamber after the cells attached. Cells were used from 10 min to several hours after plating, depending on the condition of the cells. In some experiments, HL60 cells grown in Media II were placed directly on glass coverslips without washing. Both procedures were used in the experiments reported here. The major difference is that better whole cell seals were obtained with cells grown in Media II. The 4 x 12 mm Plexiglass recording chamber with a glass coverslip bottom was placed in a Dagan TC-10 Temperature Controller (Dagan, Minneapolis, MN) containing Solution A on a Nikon inverted microscope. When currents were not being recorded, Solution A continuously flowed into the chamber; outflow was via a fine suction capillary. All patching was done at 26°C.
Patch pipettes of 5–8 MΩ were pulled from Dagan LG16 borosilicate glass as previously described [26,27]. Pipette tips were coated with Sylgard and heat polished with a Narishige MF-83 fire polisher (Narishige Scientific Instrument Lab., Tokyo, Japan). The pipette solution contained 140 mM KCl, 1.1 mM EGTA, 0.1 mM CaCl₂, 2.0 mM MgCl₂, 10 mM HEPES, pH 7.3. Recording electrodes were connected to the headstage of a Dagan 8900 patch clamp amplifier; Ag/AgCl electrodes in 200 mM KCl agar bridges were inserted into a standard polystyrene electrode holder (E.W. Wright, Guliford, CT). Similar agar bridges were used for both the ground electrode and a bath reference amplifier. The output was filtered at 1 kHz (eight-pole Bessel, model 902LPT, Frequency Devices, Haverhill, MA) and digitized with a Digidata 1200 A/D converter Axon Instruments, Foster City, CA). Capacitative current was adjusted with capacitance compensation controls on the amplifier. Macroscopic currents were recorded, stored and analyzed using the pCLAMP 6 program on a 80486 PC. To follow the time course of the entire experiment, the holding potential and currents were continuously recorded on VHS tape using a Vetter PCM recorder (A.R. Vetter, Rebersburg, PA) and video recorder. These tapes were later used for single channel analysis with pCLAMP 6.

Macroscopic current–voltage (I–V) relations were obtained from either steady-state current traces or instantaneous voltage ramps. For steady-state currents, the membrane potential was held at −50 mV for 50 ms and then a series of increasing 50 ms voltage steps was applied from −80 mV to 80 mV in 20 mV increments. I–V relations were determined by averaging the currents during the voltage pulse duration. Changes in the I–V relations immediately after establishing the whole cell configuration are discussed in the results. Ramp I–Vs were generated from a 74-ms linear voltage ramp from −80 to +80 mV followed by a decreasing, symmetrical ramp. Correction for linear capacitative offset currents produced by stray capacitance between the preamplifier input and ground was partially compensated by digitally combining the up and down ramps as described elsewhere [26]. In general, ramp I–Vs were used following the application of high concentrations of LTX in order to follow the rapid changes in macroscopic currents before the loss of the seal.

2.6. LTX application

It was important to use stable patches in order to distinguish the effects of LTX from constitutive channel activity and/or seal breakdown. The ability to achieve tight whole cell seals varied greatly depending on the condition of the cells and type of growth media. Cells maintained at low densities produced the best results. Generally, patches with low resistance seals < 1 GΩ were unstable and either ruptured or the resistance decreased substantially within the first 5 min. These cells were discarded. Usually, seals pre-screened in this manner lasted up to an hour without decreasing in resistance and rarely ruptured unless provoked. Long-lasting cells were used to determine the effects of high concentrations of LTX as well as to monitor the effects of lower concentrations over long times. In all experiments, steady whole cell seals > 1 GΩ were obtained about 5–10 min prior to toxin application. In order to conserve the amount of toxin required for these experiments, toxin was manually applied with pressure using a separate patch pipette (tip diameter ~100 μm) back-filled with toxin in the tip and positioned near the recording electrode. The concentrations of the toxin in the pipette are reported for each experiment.

3. Results

3.1. Kinetics of LTX-induced cell kill

The kinetics of cell kill at various toxin concentrations were determined by flow cytometry with HL60 cells using propidium iodide (PI) as an indicator of cytotoxicity. PI is normally excluded from cells but once a cell membrane is permeabilized, PI interacts with cellular DNA and RNA to form a highly fluorescent conjugate [27]. Our results show that LTX acts rapidly, in a dose-dependent manner, to kill cells and that all cells are susceptible to the toxin at concentrations greater than 5–10 μg/ml.

Fig. 1A,B shows the kinetics of LTX-induced cell kill at 37°C in two separate experiments. In Fig. 1A, the tₖ times, defined in Section 2, were estimated to be 29 min at 1 μg/ml, 12.5 min at 2 μg/ml and 7.7 min at 4 μg/ml. In the experiment shown in Fig. 1B,
cell kill was faster at all concentrations illustrating the variability between experiments. The $t_k$ times were 16.4 min, 3.4 min and 2.9 min for 1 $\mu$g/ml, 4 $\mu$g/ml and 8 $\mu$g/ml, respectively. Based on these two experiments as well as other observations, it would appear that the cells vary somewhat in their sensitivity to kill perhaps relative to the culture conditions or receptor expression at the time of the experiment since these two experiments were performed with the same batch of purified toxin. Control experiments were performed with a resistant cell, murine SP2 myloma cells [24]. At times as long as 30 min, less than 11% of the cells were PI positive, similar to the PI control in this experiment, at 4 $\mu$g/ml of LTX.

The variability between experiments makes it difficult to analyze the kinetics of cell kill using flow cytometry. Nevertheless, several features were seen

![fig1](image_url)

**Fig. 1.** (A) Concentration dependence of LTX-induced cell kill. Using flow cytometric analysis, the percentage of PI positive cells was followed over time at three toxin concentrations and a zero toxin concentration control (squares). Data were fit (solid lines) to a nonlinear form of the Hill equation. The data at 4 $\mu$g/ml (triangles) were fitted with a half-time of 7.7 min and an $N_h$ of 1.6; the maximal kill was 92%. At 2 $\mu$g/ml (circles), the half-time was 12.5 min and the $N_h$ was 1.7 with maximal kill at 52%. At 1 $\mu$g/ml (diamonds), toxin data were fit with a half-time of 29 min, an $N_h$ value of 1.0 and the maximal kill was 72%. The maximal kill in this determination was obviously affected by the scatter in the data at longer times. This concentration was the final sample tested and the time course was limited by the available number of cells. The experiment was done at 37°C. (B) A separate experiment showing the concentration-dependence of cell kill. The experiment followed the same protocol as A. Data were fitted to the Hill equation and the half-times were 2.9 min at 8 $\mu$g/ml, 3.4 min at 4 $\mu$g/ml and 16.4 min at 1 $\mu$g/ml. The $N_h$ values for these concentrations were 2.0, 1.9 and 1.0 and the maximal kill values were 93%, 77% and 83%, respectively. Data in the diamonds were cells exposed to 0.1 $\mu$g/ml of LTX. The PI control showed about the same % kill over time as the low concentration exposure. (C) Temperature dependence of LTX-induced cell kill. The kinetics of LTX-induced cell kill was monitored at three temperatures; the control, with PI but no LTX added, was measured at 37°C. These data were also fitted to estimate the half-times. At 28°C, the half-time was 6.4 min with a maximal kill of 96% and an $N_h$ value of 2.35. At 37°C, the half-time was 7.2 min with maximal kill at 93% and an $N_h$ of 2.7. At 20°C, the half-time was 18.0 min with a maximal kill of 94% and an $N_h$ of 2.1. The toxin concentration was 2 $\mu$g/ml.
in all experiments and may provide insights into the underlying processes involved in kill. First, at concentrations of toxin below $\sim 1 \mu g/ml$, there was no appreciable cell kill. For example, in Fig. 1B, the total fraction of PI positive cells at 0.1 $\mu g/ml$ is similar to PI control (not shown) of 17% after 30 min. At higher concentrations, the number of PI positive cells increased with time, but often approached a limiting value less than 100% which was related to the concentration. This concentration-dependent limit of cell kill is consistent with an irreversible toxin–cell interaction which requires cooperation of multiple toxin molecules, either alone or in association with a receptor. The idea of cooperativity was supported by the Hill coefficients at least for concentrations above 1 $\mu g/ml$. These higher concentrations had cooperativity indexes between 1.6 and 2.0 (details given in Figure legend), whereas the cooperativity at 1 $\mu g/ml$ in both experiments was 1.0.

Another feature of the kinetics of cell kill was the initial time lag. Although not appreciated in the time course of the data shown in Fig. 1A,B, this lag phase is readily apparent in the next experiment, Fig. 1C, which examines the effect of temperature on cell kill. Previous work showed that incubating LTX at 60°C inactivates the toxin [24]. On the other hand, reducing the assay temperature to 20°C greatly retarded the ability of the toxin to kill target cells. It was important to establish a temperature for patch clamp recording where we could expect reasonable cell kill within 15 to 30 min, the average lifetime of a patch since patch recording is typically done at room temperature. At 2 $\mu g/ml$ LTX at assay temperatures of 20, 28 and 37°C the $t_k$ times were 18.0 min, 6.4 min and 7.2 min, respectively. In this experiment, cells were preincubated at the assay temperature for ~10 min before the toxin was added. The results show that the rate of kill is similar at 28 and 37°C but is appreciably slowed by reducing the temperature to 20°C. At long times, the total fraction of cells killed is roughly the same at all three temperatures. The next set of experiments examined the effects of LTX on single cells.

3.2. Electrophysiological measurements of LTX-induced effects on HL60 cells

Whole cell currents were recorded from HL60 cells in a temperature-controlled bath held at 26°C. Patching at higher temperatures was problematic because it was difficult to obtain stable seals and the
cells died much faster than cells held at either 26°C or room temperature. Two voltage protocols were used to determine the current–voltage (I–V) relations as described in Section 2. The I–V records generally showed small currents with weak outward rectification in the absence of toxin exposure. In response to the voltage steps, the currents activated rapidly and displayed no inactivation. During the first 5 min after obtaining a seal, the I–V often became more linear as the cell interior was dialyzed with the pipette solution. Also, during this initial settling period, the currents sometimes decreased due to an increase in the seal resistance.

Previous electrophysiological studies with HL60 cells reported potassium and chloride currents that produce outward rectification [28,29]. Contributions from these currents are directly related to the concentration of permeant or blocking ions in the bath and in the pipette. Since our experimental protocol was not designed to block or eliminate these constitutive currents, the weak outwardly rectifying I–Vs most likely reflected contributions from these currents. In our experiments, cells were selected for toxin application if and only if the whole cell currents were stable for at least 5 min. This time window seemed adequate to eliminate fragile cells. All cells selected with these criteria, unless perturbed in some unintended way, were stable for long periods of time.

LTX was applied to selected cells following the criteria mentioned above. Various concentrations of toxin were used in the application pipette as described in Section 2. Fig. 2A shows whole cell currents recorded from a cell prior to and 90 s following a pulse of toxin from the pipette which contained 200 µg/ml. The concentration of toxin at the cell surface, although not known exactly, was less than that in the pipette due to dilution into the bath. The dispensing pipette was positioned as close as feasible to the clamped cell, and, at this high concentration, the effects of the toxin were very fast as shown in Fig. 2. Immediately after this I–V was

![Fig. 3. Morphological changes in HL60 cells following toxin exposure. (A) Morphological changes in the cells were seen following 200 µg/ml LTX exposure. The first photograph was taken 35 min following LTX exposure. Several healthy HL60 cells, about 10 µm in diameter, are shown at the lower edge of the field (note the arrows) and appear to be unaffected by the toxin. The toxin reached most of the cells in this field. Fig. 4B shows another coverslip 12 min after exposure to the same concentration of toxin. The patch electrode with the recording cell attached is visible in this field. This photograph shows the beginning stages of visible LTX-induced kill. Fig. 4C shows another coverslip 55 min following exposure at 200 µg/ml LTX. All of the cells in this frame were affected by the toxin.](image_url)
recorded, the amplifier current saturated, indicating a large decrease in the cell membrane resistance. In four other patches in which concentrations of toxin from 67 to 200 μg/ml were applied, similar increases in the current were seen indicating loss of the membrane resistance within 3 min after applying the toxin.

The effects of LTX on HL60 cells were visually striking. Fig. 3 shows HL60 cells in the field of the patching microscope after 200 μg/ml of LTX had been applied. Fig. 3A shows healthy cells at the bottom edge which are small, round and display distinct dark outlines, whereas cells affected by the toxin in the remaining field of view are large, flat, irregular cells with a granular-appearing nucleus not seen in the healthy cells. Fig. 3B shows another field of cells around the patch electrode as the effects of the toxin become evident. By the time these effects are visible in the microscope, usually several minutes, the seal is gone. Fig. 3C shows a field of cells after 55 min of toxin exposure. These cells no longer have distinct membranes, and no healthy cells are seen in the field. In experiments where large currents were not seen after the application of toxin, the most likely reason was that toxin did not reach the intended cell. This interpretation was supported by visual inspection of the patching field which showed no evidence of toxin effects on the general neighborhood of the cell.

To prolong the period during which electrical measurements could be made and to examine the single channel behavior, experiments were done using lower concentrations of toxin in the dispensing pipette. In Fig. 2B, I–V’s are shown following the application of 30 μg/ml LTX in the dispensing pipette. In this and similar experiments, the application of low concentrations of toxin resulted in the induction of a small inward current and a reduction in the in-situ outward current. The control I–V shows the characteristic outwardly rectifying shape seen in HL60 cells (Fig. 1 [28]). The I–V recorded 30 min after toxin application shows the increase in inward current and the decreased outward current producing the inward rectification. Consistent with this small current induction, low concentrations of toxin produced no obvious changes in cell morphology over times as long as an hour.

As a control, high concentrations of LTX were applied to human K562 erythroleukemia cells under patch electrode voltage clamp as described for HL60 cells. These cells have been shown to be resistant to LTX in previous experiments [24]. Unlike the rapid responses to toxin application seen with HL60 cells, these cells showed no evidence of toxin-mediated pore formation even for times up to 30 min.

3.3. LTX-induced conductance fluctuations

Previous work showed that purified LTX induced relatively small, discrete conductance fluctuations in planar bilayer membranes [23]. The application of 30 μg/ml of LTX to HL60 cells resulted in the appearance of conductance fluctuations, Fig. 4, somewhat suggestive of the type of voltage-dependent channel fluctuations.
activity seen in bilayers but with very rapid fluctuations between conductance levels. In the top trace recorded at +50 mV, the membrane is relatively nonconductive with the only channel activity being that of a small conductance, long lifetime constitutive channel observed in the absence of toxin. This channel appears to be active at positive potentials in the presence of toxin as well. Toxin effects are seen at 0 mV and at -30 and -50 mV. As the membrane potential decreases, the LTX-induced conductance fluctuations increase. At -50 mV, the fluctuations are large and nearly continuous. The control shows a small, long-lived channel at -30 mV but almost no activity at -50 mV consistent with the $I-V$s recorded before toxin application.

The small constitutive channels seen at +50 mV were more active in the control recording and this feature was seen in other experiments as well sug-

![Amplitude histograms](image)

Fig. 5. Amplitude histograms of 90 s current recordings for control and toxin-exposed cell. Data shown in the previous figures were used to generate 90-s histograms before and after the addition of toxin. As suggested by the channel diary in Fig. 4, there is no appreciable increase in channel activity at positive potentials. Toxin activity is clearly seen by the increase in the events at negative holding potentials.
gesting that the toxin can inhibit constitutive channel activity in some way. The conductance of this channel was ∼60 pS. At −50 mV, the control record shows little channel activity in the absence of toxin in contrast to the very active membrane following the addition of toxin. The largest current fluctuations observed in this trace correspond to a conductance of 400 pS, but these events are brief and might represent the simultaneous opening of multiple channels. The conductance transitions between the closed and open states are very rapid, and no long-lived channel activity is seen making it difficult to characterize unitary events.

We examined the voltage dependence of the conductance properties of the toxin by constructing amplitude histograms of 90-s recordings at several membrane potentials. These histograms are compared before and after toxin exposure in Fig. 5. At +50 and +30 mV, there is little difference between the control and toxin histograms. Toxin activity is dramatically increased at −30 and −50 mV, suggesting a voltage-dependent activity. Single channels could not be resolved into separate histogram peaks, consistent with multiple conductance states of short lifetimes.

The membrane conductance activity induced by LTX was seen in eight other cells three of which maintained stable activity for >30 min. In five additional cells, higher concentrations of toxin were applied. These cells displayed a higher level of activity, but the cells did not last as long and stable recordings could not be obtained at all potentials. Current fluctuations appeared at various times after the application of toxin. This variability may be due, in part, to the local diffusion patterns in the chamber at the cell surface. In some cases, the toxin-induced channels appeared within minutes of toxin application but in others, the delay was as long as 15 min. Considering the variability, we observed in the kinetics of cell kill using flow cytometry, the cell response times may reflect unexamined parameters related to cell properties as well.

4. Discussion

We have studied the effects of a bacterial protein toxin, LTX, in sensitive and insensitive cells. Following exposure to concentrations of toxin in the range of 5 µg/ml, rapid cell death ensues. When recording the conductance of a single cell following exposure to high concentrations of LTX, large increases are seen within seconds of toxin exposure in toxin-sensitive cells but no changes are seen in insensitive cells. Cells exposed to high concentrations undergo morphological changes consistent with rapid cell death. At lower concentrations of toxin, rapid conductance fluctuations were observed but were not well-resolved. These conductance changes did not exhibit the long-lived, stable conductances exhibited by many voltage-gated ion channels [30], however the fluctuations could be observed for periods as long as an hour without a loss of the membrane seal suggesting they were LTX-mediated.

Under conditions where toxin-induced membrane disruption is less than catastrophic, the conductance showed a striking voltage-dependence. At positive potentials, almost no conductance increases were seen after toxin exposure, whereas at negative potentials, the activity was persistent. Interestingly, a similar voltage dependence was observed for the toxin-induced individual state transitions in bilayer experiments, demonstrating that the pore activity of LTX is, to a large extent, associated with the toxin alone. The LTX-mediated activity in bilayers and cells differed in conductance lifetimes, however. In patches, conductance transitions were always rapid with several apparent levels of conductance between 150 and 400 pS, whereas in bilayers, channel openings had much longer lifetimes and conductance levels 118, 262 and 406 pS were observed. These differences may reflect the different lipid environment of the toxin in bilayers as opposed to cells. It is also possible that a number of LTX molecules associate to form a pore. The observation of multiple LTX conductance states suggests that oligomerization of LTX molecules is a possibility.

4.1. The kinetics of cell kill are complex

The kinetics of cell kill at various toxin concentrations were determined by flow cytometry using PI as an indicator of cytotoxicity. At concentrations of toxin less than 1 µg/ml, the total fraction of PI positive cells was small, even over periods up to an hour. At intermediate (e.g., 1 and 2 µg/ml) concentrations, PI positive cells appeared to approach a
limiting value considerably less than 100%. At higher concentrations, there was a steep increase in the rate of cell kill suggesting that cell kill might require cooperation of multiple toxin molecules, either alone or in association. The observation of a plateau value much less than 100% kill at low toxin concentrations also suggests that the toxin–cell interaction responsible for killing is either irreversible or very slow on the time scale of these experiments. Finally, as seen most prominently in Fig. 1C, there appears to be a time lag between exposure to toxin and the initiation of cell killing. Altogether, the kinetics indicates that cell killing is an irreversible, multi-step process involving the interaction of multiple toxin molecules with a single cell. Based on these observations, we propose a multistep mechanism for cell kill that involves (a) the toxin binding to a cell surface receptor, (b) the irreversible membrane insertion of the toxin or toxin complex, (c) the formation of an oligomeric pore, and (d) subsequent overwhelming of cell repair mechanisms when a critical number of pores have been formed. Future experiments may be able to test each of these specific steps in the toxin-mediated cell kill.

4.2. Role of LFA-1 in pore formation

Identification of LFA-1 as the cell surface receptor for LTX [92] strengthens the idea that a toxin-receptor complex is a required initial step in the process of cell kill by LTX. Since bacterial protein toxins are water-soluble and yet must form an integral membrane pore in the target cell, they must find a way to overcome the natural energy barrier to unfolding. Evidence from other bacterial protein toxins [31–34] suggests that a partial unfolding of the water-soluble form of the toxin is required for membrane insertion. Presumably, the toxin undergoes partial refolding to the lipid-soluble form once insertion is achieved. In order to achieve this dichotomous existence, some assistance is required to initiate the unfolding of the toxin. In the bilayer experiments, the partial unfolding is likely achieved by the partitioning of the toxin into the lipid monolayer–water interface. In leukocytes and related cells, LFA-1 binding could provide an efficient mechanism to lower this barrier and catalyze a partial unfolding of the toxin leading to exposure of sequestered hydrophobic residues that interact with the cell membrane lipid environment.

4.3. The role of LTX-mediated conductances in cell kill

The electrophysiological features of cell death seen with high concentrations of LTX exposure can be compared to changes seen in patch clamp recordings for magainin antimicrobial peptides, perforin-mediated cytotoxicity and haemolysin A toxin (HlyA). The relatively nonselective, large conductance pores created by all of these cytotoxic molecules are associated with large ion fluxes, diminishing electrochemical gradients, intracellular Ca\(^{2+}\) overload and characteristic changes in cell morphology. A number of differences in electrophysiological properties distinguish each of these pore types. Magainin peptides gave conductances ranging from 680 to 1900 pS in planar bilayer [35,36]. In patch recordings, exposure to magainins resulted in the rapid loss of membrane integrity at high concentrations and low, sustained levels of activity at low concentrations [27]. Perforin results in large conductance channels in susceptible cells. The conductances range from 440–1400 pS and the channels have long open times [37–39]. The pore is thought to involve an aggregation of 4 to 20 perforin molecules. HlyA is a member of the RTX family and shares some features with LTX but does not exhibit the species and cell-type specificity of LTX. HlyA formed large conductance pores in human macrophages which showed a large single channel conductance with long lifetimes, cation selectivity with little discrimination among different cations and a nearly linear current–voltage relationship [40]. In bilayer experiments, the channel properties were similar to those seen in macrophages [41]. LTX-induced conductances range from 150–400 pS in both bilayer and patch recordings with a characteristic voltage-dependent activity. In HL60 cells, persistent activity was recorded ~50 and ~30 mV while very little activity was observed at positive holding potentials. The major difference between the bilayer and whole cell activity was seen in the kinetics. In lipid bilayer, the channel openings were well-resolved even with 50 Hz filtering, but in the whole cell recordings, long-lived channel events were not resolved at 1 kHz filtering. The whole cell kinetics, while difficult to
analyze, are not inconsistent with channel formation, however. For example, cGMP-activated channels in retina photoreceptors show rapidly fluctuating conductances similar to the LTX-mediated conductances we observe in the HL60 cells [42,43]. It is certainly possible that the lipid composition as well as the LFA-1 receptor alter the LTX conductance properties.

The well-known signalling role of LFA-1 receptors [44,45], as well as previous work showing that LTX elevates intracellular Ca\(^{2+}\) levels [24], suggest that LTX may also be involved in an additional cytotoxic pathway at low toxin concentrations. It is possible that a limiting number of LTX-mediated pores could lead to an elevation of intracellular Ca\(^{2+}\) without causing collapse of the electrochemical gradients of the cell. In this case, the overload of intracellular Ca\(^{2+}\) could lead to the well-recognized forms of Ca\(^{2+}\)-induced cell damage [46]. In addition to inducing DNA fragmentation and mitochondrial damage, elevated Ca\(^{2+}\) can directly affect cytoskeletal organization and induce surface blebs [47]. Evidence from flow cytometry, morphologic and gel electrophoresis studies have shown that cells treated with low doses of LTX show signs that are consistent with apoptosis or programmed cell-death [48]. These data suggest that downstream signalling events may be initiated by LFA-1 binding in addition to the rapid, direct, toxin-mediated cell kill seen in patch clamped cells. There is, in fact, precedence for two distinct pathways in cytotoxic T lymphocyte destruction of target cells [37]. The non-perforin pathway triggers apoptosis through the Fas receptors on the target cell surface. In the case of LTX-mediated cell death, the physiological role of LTX-mediated apoptosis remains to be understood.

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References