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Actinobacillus actinomycetemcomitans leukotoxin forms large conductance, voltage-gated ion channels when incorporated into planar lipid bilayers

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Abstract

Actinobacillus actinomycetemcomitans leukotoxin is a member of the bacterial RTX (repeats in toxin) toxin family, produced by a diverse group of Gram-negative pathogens. Members of this group of toxins, although similar in sequence, differ in target cell specificity with *Actinobacillus actinomycetemcomitans* leukotoxin demonstrating a unique species-and cell-type specificity. Purified *A. actinomycetemcomitans* leukotoxin added to pre-formed POPE/POPS lipid bilayers showed no spontaneous incorporation (to concentrations of 250 ng/ml). Reproducible channel activity was seen when the bilayer was reformed from lipid monolayers in the presence of toxin (50 ng/ml) in one of the aqueous chambers. Control experiments with heat-inactivated toxin did not display channel activity under the same experimental conditions. The channel behavior showed a complex pattern of multiple conductance levels of 118, 262 and 406 pS in solutions containing 0.140 M NaCl. The first two states showed voltage-dependent channel gating with approximately equal but opposite apparent gating charges of 1.4 electrons. A model accounting for the multiple conducting states and gating properties is presented.

Keywords: Leukotoxin; Planar bilayer; Ion channel; Bacterial toxin; Single channel conductance; Multiple conductance state; Membrane pore; RTX toxin; (*A. actinomycetemcomitans*)

1. Introduction

Actinobacillus is a member of the family Pasteurellaecae, a group of nonenteric, fermenting, Gram-negative rods, which are of considerable importance in both human and veterinary medicine. While there are many *Actinobacillus* species found in animals, only *A. actinomycetemcomitans* is routinely cultured from humans. This organism has been associated with a variety of infectious disease processes in man including endocarditis, brain abscesses, osteomyelitis, subcutaneous abscesses and periodontal disease [1–3]. The pathogenesis of *Actinobacillus* infections is not entirely understood, but *Actinobacillus actinomycetemcomitans* produces several factors, which alone or in combination, may account for its virulence. Of these, the most extensively studied is a relatively large (116 kDa), heat labile protein leukotoxin [4]. In addition to representing a potential virulence factor in infections caused by this organism, *A. actinomycetemcomitans* leukotoxin is also of interest because it possesses a unique biological specificity, killing only cells of the monomyelocytic lineage of man and some higher non-human primates [5,6]. The primary sequence of the leukotoxin [7–9] suggested that it is a member of the bacterial RTX toxin (repeats in toxin) [10] family produced by a diverse group of Gram-negative pathogens [11–16]. The family was so named

Abbreviations: RTX, repeats in toxin; POPE, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine; PC, phosphatidylcholine; PS, phosphatidylserine; *I/V*, current vs. voltage relation.

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because it contains a repeat domain, consisting of multiple copies of a nine amino acid cassette with a consensus sequence of GGXGXDX(L|I|V|W|Y|F)X. The repeats appear to be involved in Ca^{2+} binding and are essential for cytolytic activity since deletion of this region renders the toxin inactive [7,17]. In addition, a region of hydrophobic amino acid residues which may function to span the target cell membrane is also conserved within the members of the RTX family.

It is likely that all members of the RTX toxin family exert their lethality through a common mechanism. Evidence from numerous studies (reviewed in [18]) suggests that the toxins can create pores in target cell membranes which cause the cells to swell and burst. This process termed 'osmotic lysis', is a consequence of water influx associated with the transmembrane equilibration of ions (primarily sodium, potassium, and chloride) normally maintained by metabolism in composition gradients. Effectively, the pores overwhelm the cell's ability to maintain its osmotic equilibrium. Note that this can also happen as a result of general metabolic inhibition or of specific interferences with essential 'pumping' mechanisms in the cell membrane, so lysis by itself does not constitute evidence for pore formation [19]. Pore formation has, however, been directly demonstrated in artificial planar bilayer experiments with toxins from *Escherichia coli* [20,21], *Actinobacillus pleuropneumoniae* [22]and from both *Proteus vulgaris* and *Moganella moganii* [23]. Recently, channel activity from *A. actinomycetemcomitans* was also reported (Fig. 2, [24]), however, the channel properties were not characterized. Although the experimental conditions vary with regard to both lipid composition of the bilayers and salt concentration, all toxin channels conducted ions in the range of 200–400 pS [25]. These experiments provide compelling evidence that toxin mediated pores are responsible for cell death but they do not explain the unique target cell specificities.

We report here the initial characterization of *A. actinomycetemcomitans* toxin channels in artificial bilayers formed from a 1:1 POPE/POPS monolayers. Of particular interest is our finding that channels did not spontaneously insert into pre-formed bilayers even at relatively high toxin concentrations of ~ 2.16 nM (250 ng/ml) which is about 30-times higher than required for cell kill [26]. Despite the lack of spontaneous insertion, channel activity was reproducibly observed when the bilayer was broken and reformed in the presence of toxin in the chamber suggesting to us that destabilizing interactions at the water-soluble toxin-membrane interface might be necessary to induce assembly of the pore structure in the membrane environment. The observed channel conductances are complex with multiple open states and voltage-dependent gating. The techniques applied here should prove useful in exploring the unique biological specificity of this toxin in addition to providing a method for studying the channel properties of the toxin.

2. Materials and methods

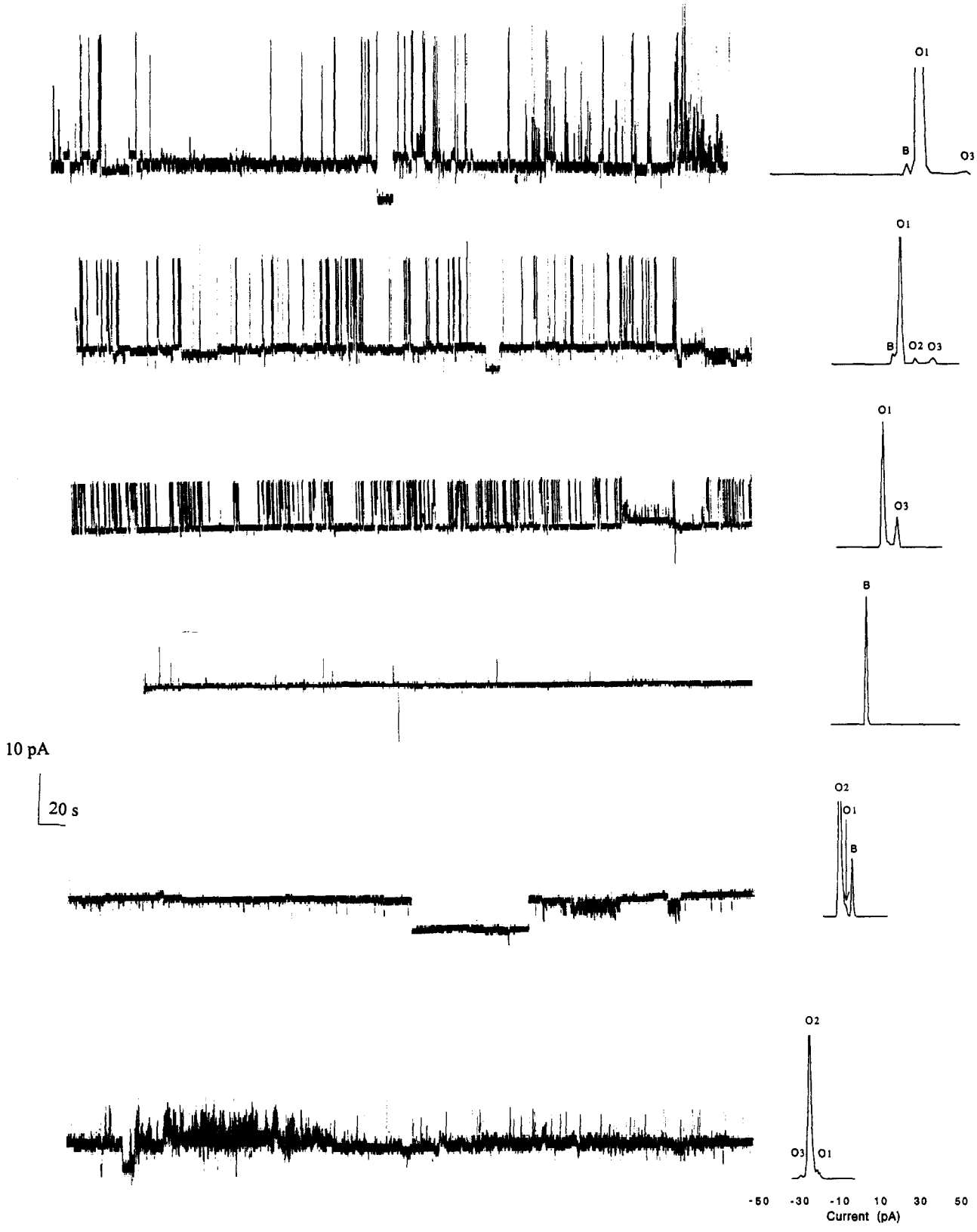
2.1. Extraction and purification of A. actinomycetemcomitans leukotoxin

Actinobacillus actinomycetemcomitans (*A. actinomycetemcomitans*), strain JP2, used in this study was obtained from Dr. N. Taichman (Department of Pathology, University of Pennsylvania, PA). The strain was grown in PYG medium (5 g Bacto-peptone, 5 g Trypitcase-peptone, 10 g yeast extract, 10 g dextrose, 8 mg $CaCl_2$, 8 mg $MgSO_4$, 40 mg K_2HPO_4, 400 mg $NaHCO_3$, 80 mg NaCl in 1 l of dH_20) for 24 h at 37°C in an atmosphere of 5% CO_2. After washing the bacteria twice in phosphate-buffered saline (pH 6.5), leukotoxin was extracted and purified by a modification of the procedure described by Tsai et al. [27]. 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 proteinase 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 eluant was concentrated, reequilibrated 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 [28] were recovered from the 0.2–0.7 M NaCl fractions, concentrated and subjected to gel filtration on a 10/30 Superose 6 column (Pharmacia, Piscataway, NJ). Protein was determined by the Bradford procedure [29]; the leukotoxin routinely gives IC_{50} values on HL60 cells of 5 to 30 ng/ml [26] as assessed by trypan blue exclusion.

2.2. Planar bilayer experiments

Lipids (POPE: 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine and POPS: 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine) were purchased as 10 mg/ml solutions in chloroform from Avanti Polar Lipids, Alabaster, AL, USA. Aliquots were taken of each in equal volume (typically 40 μl), mixed, and the solvent evaporated and replaced by HPLC-grade n-pentane (Sigma, St. Louis, MO, USA) to give a total lipid concentration of 2 mg/ml. Planar bilayer membranes were formed in a 200 μm hole (created by electrical discharge) in a Teflon film, pretreated with 10 nl of a 1% solution of squalane in pentane. The film separated a pair of 2 ml chambers in a Teflon block. The chambers were cleaned with chromic/sulfuric acid; they were thoroughly rinsed with

water and ethanol before use. The pH 7 bath solutions (electrolyte buffer) contained 0.14 M NaCl, and 10 mM Tris-HCl, 1 mM $CaCl_2$. 10 to 20 μl of lipid in n-pentane was added to the surface of the bath solutions in each chamber, and bilayer membranes were formed by monolayer apposition [30] by raising the bath levels using



plastic syringes. Membrane capacitance was monitored on an oscilloscope using a 100 Hz, 100 mV amplitude triangle-wave voltage input. Typical capacitance values were between 200 and 400 pF, consistent with bilayer formation over most of the available hole area. Formation of bilayers stable for sufficient times to perform further experiments usually required repeated formation, sometimes with the addition of more lipid solution to promote stability. Membrane voltage was controlled and currents measured using an EPC-7 patch-clamp amplifier (List Medical Systems, Greenvale, NY), an 8-pole lowpass Bessel filter (Frequency Devices, Haverhill, MA) set to 500 Hz, a LabMaster D/A converter, and a TL-1 DMA interface (Axon Instruments, Foster City, CA, USA). Currents were recorded on digital tape (Recorder Model #200, A.R. Vetter, Rebersburg, PA), and later analysed using the programs 'Fetchex' and 'Fetchan' in the pClamp 6.0 software package (Axon Instruments)

For experiments to characterize single channel activity, bilayers were first made with no toxin in the chambers in order to establish quiet baseline activity at potentials of ± 100 mV. 2 μg samples of lyophilized toxin, purified from *A. actinomycetemcomitans* bacteria, were dissolved in 100 μl of the electrolyte buffer solution and 3 to 5 μl aliquots were added to the '*cis*' chamber, opposite to that of the amplifier ground. We calculate each 5 μl aliquot to add 0.57 nM (67 ng/ml) of leukotoxin. For the experiments testing for spontaneous incorporation, the solution was stirred gently by repeated raising and lowering of levels, staying above the bilayer aperture to avoid breaking the bilayer. The potential was maintained constant at various values and currents were recorded at an amplifier gain (20 mV/pA) sufficient to detect possible channel incorporation events. For the experiments where toxin channel activity was detected, 3 to 5 μl of toxin solution was added to the *cis* side after a stable bilayer had been formed and then, after the bilayer had broken (either adventitiously or, if necessary, by a sharp rap to the chamber), the bilayer was reformed as described above and the potential was again held at different values between ± 100 mV. This same procedure was used for control experiments using heat-inactivated toxin and polymyxin B. When channel activity was observed, currents were recorded on tape at a series of voltages for long time periods in order to obtain data for off-line analysis.

3. Results

With pre-formed 1:1 PE/PS bilayers, no spontaneous channel incorporation was seen upon *cis*-side addition of *A. actinomycetemcomitans* toxin, at concentrations as high as 250 ng/ml, between 9-and 50-fold the typical IC_{50} concentration for HL-60 cells. Breaking and reforming the bilayer in the presence of these high concentrations of toxin produced large currents suggesting the incorporation of many channels. These bilayers were not analyzed further. We found, however, that single channels could routinely be incorporated into the bilayer by breaking and reforming the bilayer in the presence of ~ 50 ng/ml of toxin on one side. Bilayers showing single channel activity were stable for times as long as several hours and showed no significant evidence of additional toxin incorporation. During the time of the experiments which varied from one to several hours, the channels appeared to have stable gating and conductance properties. Channel activity from continuous 9 min records of a bilayer showing single channel activity are shown in Fig. 1A. Current histograms of these data are shown in Fig. 1B. The histograms provide a quantitative, time averaged representation of the baseline and channel current levels. States assigned on the assumption of linear current voltage relationships (B, O1, O2, O3) are indicated near corresponding maxima identified in the histograms. Where a particular assignment is missing the corresponding state contributed too little to the total to show up as a peak in the histogram, even though it could be detected in the records. Only the histogram for +50 mV shows maxima corresponding to all four assigned states.

Fig. 2A shows the linear current–voltage relations which were the basis for the state assignments. The least squares lines yield slope conductance values of 230 ± 6, 348 ± 4, 489 ± 34, and 636 ± 38 pS. Subtraction of the baseline value gives conductances of 118 ± 10, 259 ± 40, and 406 ± 44 pS for the three observed open levels. To describe the apparent voltage-dependent gating behavior of these apparent conductance states, the Fig. 1 histograms were analyzed into Gaussian peaks and the fractional contribution of each assigned histogram peak to the total area was determined for histograms at each voltage. Fig. 2B shows the results. Level O1 fraction increases with increasing positive voltage and, in an apparently mirror-symmetric

Fig. 1. *Actinobacillus actinomycetemcomitans* leukotoxin channel activity and normalized current histograms of the data. (A) Protein was incorporated into POPE/POPS bilayers as described in Materials and methods. Continuous current records were collected and stored on tape; data were digitized using Fetchex and analyzed with Fetchan. The records were low-pass filtered at 50 Hz for display. Calibration: 20 s and 10 pA. (B) Total current histograms from 9 min, constant voltage segments were made using the Fetchan program with a 250 Hz sampling rate. Voltages were, from top to bottom, +75, +50, +25, 0, −25, and −50 mV. The current scale was corrected for current offset (introduced by the Bessel filter) by subtraction of the current value for the zero mV record. Assignment of the four states denoted B (baseline) and O1 to O3 noted for the histograms were arbitrarily assigned based on Fig. 2A (see below).

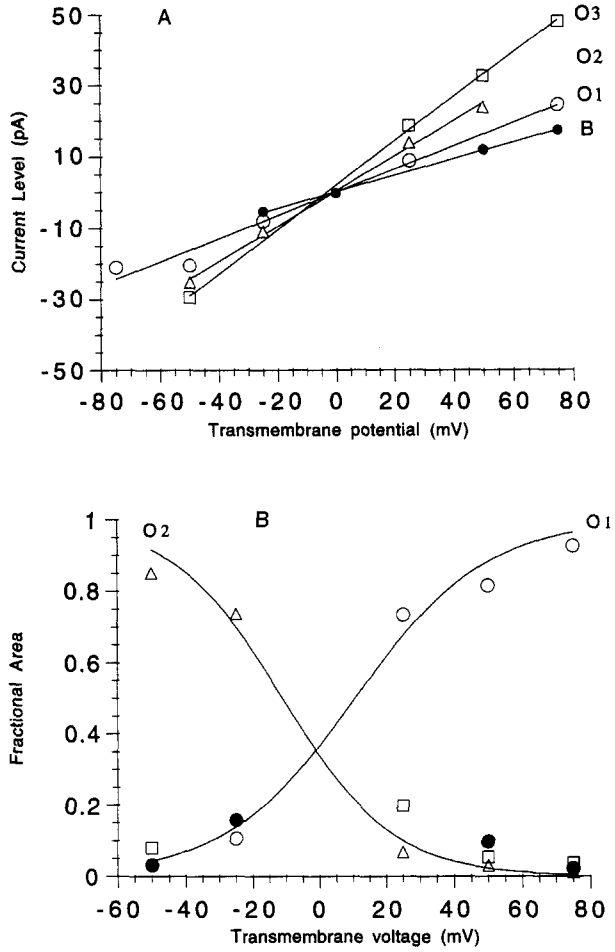


Fig. 2. (A) Single channel current levels determined from current-time traces are plotted against holding voltage. The values determined for the different current levels were plotted against voltage and specific state assignments made on the assumption of linearity in the channel state's current voltage curve. The resulting curves are drawn with least-squares-fit lines (Kaleidagraph) forced through the origin in order to determine the reported slope conductances. Levels are B (solid circles), O1 (open circles), O2 (open triangles), and O3 (open squares). Slopes were used to compute the assigned state conductances. (B) Channel state open probabilities plotted against holding potential. The open state probabilities were estimated by determining area fractions assigned to each state in the current histograms using the program 'Peakfit' (Jandel Scientific) assuming Gaussian distributions for each state's current level. Area contributions of states B (solid circles), O1 (open circles), O2 (open triangles), and O3 (open squares) to the histograms of Fig. 2. Lines are least-squares fits to the equation: $y = (1 + \exp(25.7z(V - V_0)))^{-1}$ where y = fractional area, V = mV holding potential, V_0 is the voltage (mV) where $y = 0.5$, and z is defined as the 'gating charge'. The 25.7 factor is equal to thermal energy (RT) in milli-electron volt units at 25°C. Fit values for the two curves O1 and O2 were, respectively: $V_0 = 13 \pm 6$, -17 ± 7 and $z = 1.3 \pm 0.2$, -1.5 ± 0.2.

manner, the level O2 open probability decreases. Baseline and O3 levels appear to exhibit a maximum near zero voltage. The fractional open time for O1 and O2 levels were fit to a thermodynamic model often used to describe voltage-gated ion channels [31]. The fit parameters gave very nearly the same value but opposite signs of the gating charge (± 1.4 electrons) and 'intrinsic' asymmetry (± 15 mV mid-points).

A control experiment was performed to confirm that the channel activity was due to the leukotoxin and not a contaminant. It was established in previous studies that this particular toxin loses cytotoxic activity after an hour exposure in solution to 60°C [29]. A sample of toxin was therefore dissolved in our standard buffer, split into two vials and one vial heated in a 60°C water bath for 1 h. After forming a PE/PS bilayer in the absence of toxin, 70 ng/ml additions of heat-inactivated *A. actinomycetemcomitans* leukotoxin were used as in previous experiments with intact toxin. No channel activity was seen in more than 50 min of observation following re-formation of the bilayer after toxin addition. As the protein concentration was increased to a total of 400 ng/ml, the noise of the bilayer was increased but no conductance steps similar to those shown in the records in Fig. 1 were seen.

A prominent feature of these experiments is the relatively large baseline currents as evidenced by the 230 pS slope of the baseline (B) current–voltage curve (Fig. 2A). This feature was evident in all our experiments. Since the *A. actinomycetemcomitans* leukotoxin used in these experiments was extracted with polymyxin B, a possible ionophore, we investigated the effect of adding polymyxin B alone to the bilayer chamber. Controls were performed with the same lipids as used in the leukotoxin experiment and the experiment was performed in the same manner. Initially, bilayers formed from monolayers were exposed to small aliquots of polymyxin B solution in electrolyte buffer added to the *cis* chamber. No channel activity was seen. The bilayer was then reformed and monitored again. In total, 20 ng/ml of polymyxin B was added to the *cis* chamber which would be equivalent to a 50% by weight contamination of the toxin. Although no channel activity (as evidenced by 'on-off' gating) was seen, the increasing amounts of polymyxin B did produce increases of bilayer 'leakage' conductance (from 62 pS without polymyxin to 420 pS with 20 ng/ml). It is therefore possible that some of the increased leak conductance seen in the experimental records is due to a non-specific polymyxin contribution. The ability of several antibiotics, including polymyxin B, to form ion channels in planar bilayers was investigated by Lopez-Escalera et al. [32] who saw no channel activity in the presence of polymyxin B alone (R. French, personal communication).

4. Discussion

4.1. Relationship to biological activity

The results of these experiments show that *A. actinomycetemcomitans* leukotoxin forms ion-conducting pores in lipid membranes, a mechanism for its biological activity that was previously suggested on the basis of osmotic

protection experiments [29]. The properties of the *A. actinomycetemcomitans* leukotoxin channels observed in these experiments are qualitatively consistent with previous work on the cytotoxicity of the toxin. In cell-kill experiments, it was shown that heat inactivates the toxin. In our experiments heat-inactivated toxin was unable to form the characteristic channels. Also, in cell-killing experiments, osmolytes smaller than maltose afforded no protection of cells from lysis [29], a finding consistent with a toxin-induced pore of defined size. We find the toxin to be capable of forming transmembrane ion channels with defined conductance states. The primary differences we observe between our experiments and those involving cells are:

1. Concentrations of the toxin that result in 50% kill on sensitive cells such as polymorphonuclear leukocytes, monocytes, and human leukemic cell lines were 5 to 30 ng/ml, from 2-to 10-fold lower than concentrations used in our experiments.
2. Pore incorporation into PE/PS lipid bilayers did not occur spontaneously but rather required re-forming the bilayer from monolayers after adding the toxin to the *cis* chamber.

Both of these differences could be related to differences between our totally artificial bilayer system and the much more complex membrane composition of living cells. For example, the negative charges of the PS-containing membranes would repel entry of negatively charged protein segments. Also, the conversion of the water soluble toxin to its membrane associated 'pore' form could require or be aided by the presence of specific, cell membrane-associated receptors for the toxin. That such receptors might be present in toxin-sensitive cells is suggested by the significant species specificity exhibited by the *A. actinomycetemcomitans* toxin. Receptor-toxin interaction would not only increase the effective toxin concentration near the membrane surface but also could contribute to destabilizing the water-soluble form of the toxin thereby promoting membrane incorporation. Such a mechanism for toxin insertion into membranes has been suggested for the large, pore-forming toxin from *Bacillus thuringiensis* [13]. The primary sequence of *A. actinomycetemcomitans* toxin [7] does contain features (> 20 residue hydrophobic and amphipathic helical sequences) which could accommodate such a mechanism. Also, it is probable that lipid composition will play a role in the toxin insertion mechanism. For example, it has been shown [20] that soybean lipid (asolectin) membranes favor the spontaneous insertion of the non-specific hemolytic RTX toxin from *E. coli* (HylA). This general issue of toxin specificity will be interesting to investigate in future studies.

4.2. Channel properties

The current-time records and current level histograms obtained for membrane-incorporated toxin (Fig. 1) show a challenging complexity. Multiple levels of conductance, changes in open time with voltage, and a relatively large apparent 'baseline' conductance prompted our analysis of the records in terms of a baseline and three different

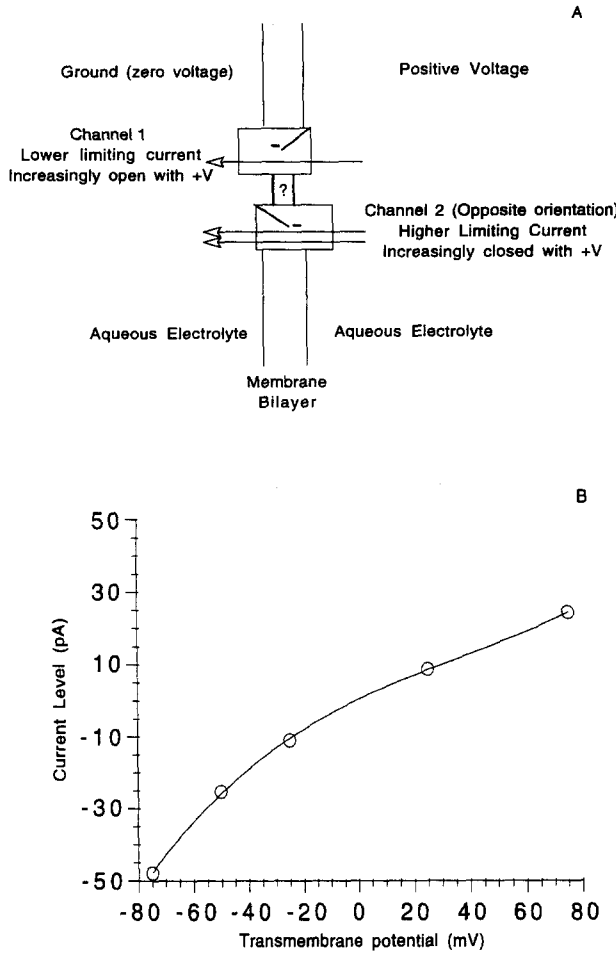


Fig. 3. (A) Diagram indicating proposed origins of the different conductance states observed in these experiments. The directions of the arrow lines traversing each channel correspond to the direction of positive ion current. The intrinsic asymmetry of the channels is represented by their asymmetric displacement from the membrane center and also by having two lines to represent the larger intrinsic current flow for channel 2. The '?' in the area connecting the two structures denotes our uncertainty concerning their independent incorporation into the membrane. The negative signs (−) shown near the 'flappers' at the each channels' end represent the gating charges which, upon increasing attraction to increased positive potential, move to increase either the open (Channel 1) or closed (Channel 2) probability of the channels. Note that the decision to represent the charges as negative is an arbitrary one based solely on the convention of representing gating charges as electrons; positively charged 'flappers' could be disposed to accomplish the same effect. (B) Theoretical I/V relation based on reassignment of conductance levels. Re-analysis of data assigning 'O1' currents to channels conducting current in the same direction as (negative) gating charge movement and 'O2' (except for +75 mV; see Discussion) currents to similar or identical channels conducting current in the opposite direction from their gating charge movement. The line is a cubic polynomial fit to the data: $y = 0.361 + 0.367x - 0.002x^2 + 2.02x^3$ with y = current (pA) and x = voltage (mV). Subtraction of the previously determined linear baseline slope yields a zero-voltage slope conductance of 137 pS.

channel conductance levels (Fig. 2). Which of these levels dominated the current records depended on the sign and magnitude of the holding voltage (Fig. 3). We calculate conductance states of 118, 262 and 406 pS after baseline subtraction and assuming linear current–voltage curves. These values are in the range of conductances reported for other RTX toxins in similar electrolyte conditions. A recent report showed a channel conductance state of ~ 400 pS produced by exposing decane-containing PE/PS bilayers to unspecified concentrations of recombinant *A. actinomycetemcomitans* leukotoxin produced by *E. coli* [24]. Smaller channels or partial closings of the large channel were evident in this record (Fig. 2B). Only one channel opening at 30 mV was reported and the electrolyte solutions were not specified. Channels were also shown from the hemolysin from *E. coli* (HylA) and the leukotoxin from *Pasturella hemolytica* (LktA), toxins closely related in sequence to the *A. actinomycetemcomitans* leukotoxin. In previous studies, the *E. coli* hemolysin HylA was reported to form ion channels from concentrations in the range of 100 to 1000 ng/ml by spontaneous insertion into asolectin (soybean lipid mixture) bilayers [20]. These channels had a conductance of ~ 500 pS at 20 mV in (apparently) unbuffered pH 6, 0.15 M KCl. Interestingly, a smaller conductance sub-state of 170 pS at 100 mV was reported, suggestive of at least some voltage-dependent gating. No channels were reported to form when bilayers were made using pure (single component) lipids. An independent study [21] of an *E. coli* hemolysin showed spontaneous insertion into egg PC and 1:1 PC/PS bilayers at 0.3 nM (ca. 30 ng/ml) toxin concentration in Hepes-buffered, pH 7 0.05 M KCl. Channels had a conductance linear with KCl concentration, giving a value of ~ 250 pS for 0.1 M KCl, and showed significant voltage gating of their opening between ±20 mV. Another related toxin, *A. pleuropneumoniae* hemolysin, was shown to spontaneously produce channels in 1:1 PS/PE bilayers formed from patch pipettes [22]. The conductance estimated from the open channel IV was 400 pS in a solution containing 140 mM NaCl and 1.5 mM Ca with physiological ions. The open channel IV was reported to be linear and no voltage gating was reported between ±60 mV. Considering the many differences in experimental conditions, it is not surprising that the channels we report differ somewhat in their detailed characteristics.

4.3. Speculations on the observed voltage gating

The observed differences in the voltage dependencies of the O1 and O2 open probabilities shown in Fig. 2B suggest that O1 and O2 currents are associated with oppositely oriented channels in the membrane. If the channels are identical, the differences in the conductance values themselves could be accounted for by single channel rectification due to an intrinsic electrical asymmetry [17]. This particular situation is represented in Fig. 3A. The extent of possible single channel rectification can be explored by reversing the signs of the currents and voltages for the O2 state and combining the data with the current voltage data for the O1 state. Consistent with the expectation that O2 should always be present to observe O3, we reassign the 'O3' state in the Fig. 1 histogram for +75 mV to state 'O2'. The resulting, hypothetical current–voltage curve shown in Fig. 3B displays a significant degree of asymmetry and non-linearity. The slope conductance calculated for zero voltage (in 150 mM NaCl with 1 mM Ca^{2+} at pH 7.2) is 137 pS, somewhat smaller than values calculated for the individual open states represented in Fig. 2A, but still a moderately large conductance channel. We emphasize that this interpretation of our channel data is highly speculative and that considerably more experimental work will be needed to fully characterize the voltage-dependent gating and conduction properties of the ion channels formed by this toxin.

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