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Properties of *Pseudomonas aeruginosa* exotoxin A ionic channel incorporated in planar lipid bilayers

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Acidic conditions induce the incorporation of *Pseudomonas aeruginosa* exotoxin A into phospholipid planar bilayers and the formation of pores permeable to electrolytes. Channel openings occur as single events, although they may occasionally cluster in bursts. In 100 mM KCl, the elementary single channel current amplitude is 3.1 pA (at a transmembrane voltage of 100 mV), the mean open time is 1.3 ms, while bursts may last for several seconds. Noise analysis gave results identical to single channel analysis. Voltage pulse protocols and continuous cycling voltage ramps showed that the toxin channel is voltage dependent, having a higher probability of being open at positive voltages.

Lipid bilayer; Ion channel; pH dependence; Surface potential; Toxin translocation; *Pseudomonas aeruginosa*

1. INTRODUCTION

It has been shown that the insertion into model membranes of bacterial toxins (pathogenic for man, animals or plants) increases membrane permeability by the formation of transmembrane ionic pores. In some cases (e.g. cytolysins), the increase of membrane permeability represents the main step of the toxic activity itself [1]. In other cases, the formation of a transmembrane pore seems to be a necessary prerequisite for the expression of the toxicity which eventually develops as intracellular enzymatic activity [2,3]. This seems to be the case for botulinum, tetanus and diphtheria toxin [4,5] and *Pseudomonas aeruginosa* exotoxin A [6]. These toxins present common permeabilization properties and similar response to variations of some physico-chemical parameters, thus suggesting that their interaction with membranes is regulated by a related molecular mechanism.

The main prerequisite for fast incorporation into the membrane phase and subsequent channel formation is acidification of the ionic solution. The reaction in fact proceeds much slower and requires a higher toxin concentration at less acidic pH. Also required is the presence of negatively charged lipids in the target membrane.

2. MATERIALS AND METHODS

Pseudomonas aeruginosa exotoxin A (PextoxA) was purchased from the Swiss Vaccine Institute (Bern, Switzerland) as a lyophilysed powder, dissolved in water and used without further purification. According to the supplier the reconstituted toxin is more than 97% pure by HPLC, and it has a mean lethal dose of 0.3 μg/ml on mice. Diphytanoylphosphatidylcholine (PC) and phosphatidylserine (PS) were obtained from Avanti Polar Lipids, Birmingham, AL. Asolectin (Al, soy bean lecithin) was provided by Sigma Chemical Co., St. Louis, MO, and purified according to Kagawa and Racker [7].

Formation of planar bilayers by apposition of two monolayers is described in [8]. The records were obtained using a List EPC7 amplifier (List Electronic, Darmstadt, Germany) in the voltage-clamp mode. The voltage of the PextoxA-free compartment was defined as the reference voltage. Membrane current was recorded with a commercial VHS video cassette recorder equipped with a digital audioprocessor (Sony PCM 601 ESD) modified according to Bezanilla [9]. The recordings were filtered through a KEMO VBF8 filter (Kemo, Beckenham, UK) and digitized off-line using an Instrutech A/D/A board (Instrutech, Elmont, NY) interfaced to a 4Mega ST Atari personal computer.

The power spectrum of the signal was calculated off-line and (under the hypothesis that the signal was due to the contribution of only one channel) it was fit with a single Lorentian distribution, i.e.

$$S(f) = A/(1+(2\pi\tau f)^2) \quad (1)$$

where

$$A = 2\tau\sigma^2, \quad (2)$$

where σ is the standard deviation of the current and $1/2\pi\tau$ is the cut-off (half power) frequency. For a simple 2-state model in which α and β denote the rate constants from the closed to the open state and from the open state to the closed state, respectively, in general $\tau = 1/(\alpha+\beta)$ whereas the mean open time is given by $t_o = 1/\beta$. Moreover:

$$\sigma^2 = i^2 p(1-p) \quad (3)$$

where i is the single channel current and p is the probability of finding the channel open.

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3. RESULTS AND DISCUSSION

At neutral pH the addition of up to 100 μg/ml PextoxA to either asolectin or PC:PS membranes did not increase the ionic permeability of these membranes for several hours. However, in accordance with experiments performed with phospholipid vesicles [6], at acidic pH (5.5) 50 μg/ml PextoxA induced the permeabilization of the bilayer a few minutes after its addition to the aqueous solution. Current signals showed fluctuations typical of ion channel formation (Fig. 1).

The current pattern observed is diverse, however, and usually a fluctuation of about 30 pS was present, either as current bursts due to the occurrence of several short openings separated by short closures (Fig. 1A) or as isolated current transitions separated by long closures (Fig. 1B). Sometimes, transitions of larger conductance were also observed, suggesting the existence of either multiple conductance levels or a high cooperativity among several pores. As an example, analysis of 672 current transitions in a record particularly rich in large events obtained at +100 mV showed that, together with the usual amplitudes of 3.1 pA, channels of 19.1 pA and 36.9 pA were also present; the probability of occurrence in this particular record was 43, 11 and 46%, respectively.

The existence of several conductance states has also been observed in native channels controlling ionic transport through cell membranes [10,11].

In the rest of this paper we will focus on the typical (smallest) elementary transitions occurring either as isolated events or in current bursts. Accurate analysis of their amplitude and duration is necessary to certify that these signal fluctuations can be ascribed to the gating mechanism of ionic pores, especially in the case of very rapid kinetics, such as those in Fig. 1.

Since a preliminary analysis did not show significant differences in the conductance or the open lifetime of single-channels occurring either as isolated events or in current bursts, statistics were obtained from openings appearing in both types of signals. The experimental distributions of the open lifetime and amplitude of 1,600 events were fit with exponential and Gaussian distributions (Fig. 2A and B, respectively). A mean open time t_o=1.33 ms, and a mean current level i=3.1 pA at a transmembrane voltage of +100 mV were obtained. We verified that the other two amplitudes also have mean lifetimes in the order of 1 ms.

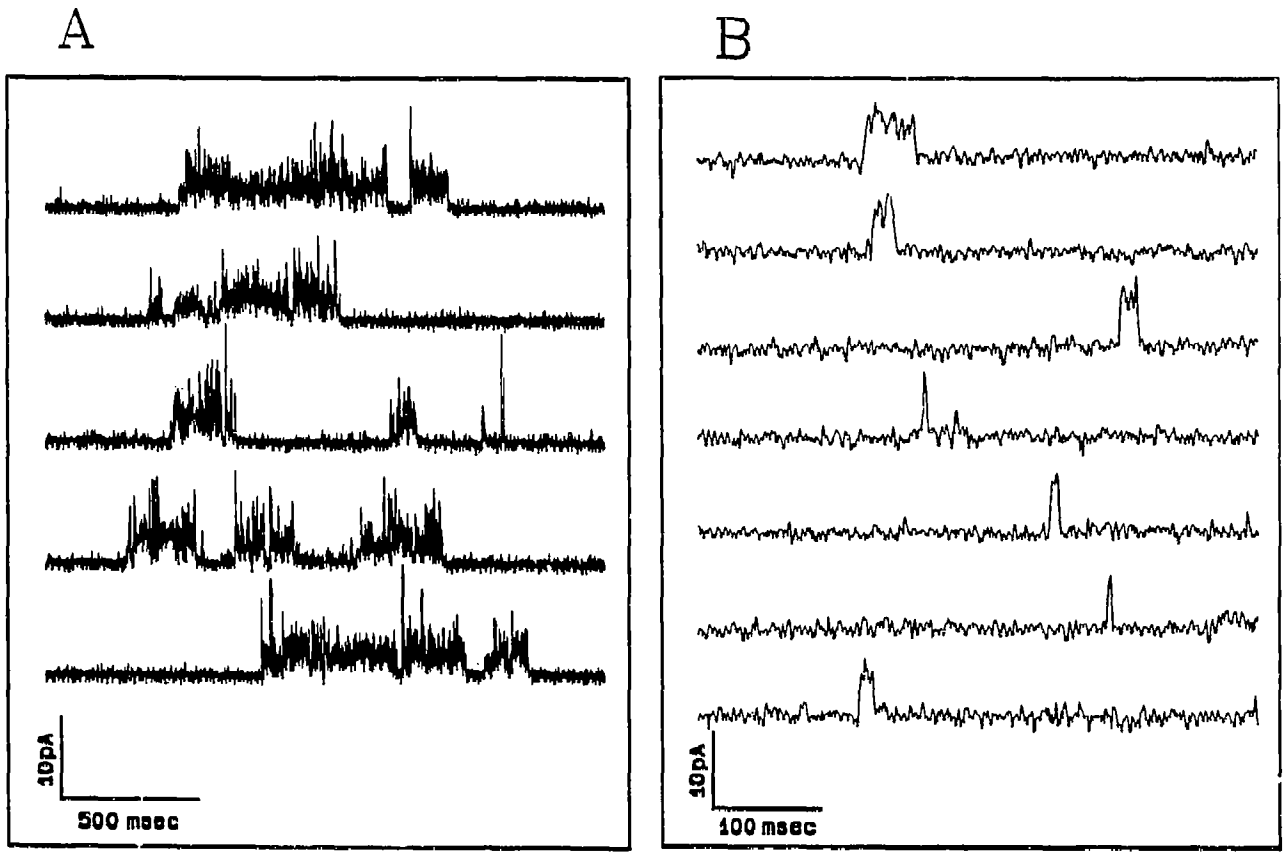

Fig. 1. (A) Typical single-channel current bursts recorded in Al membranes. (B) Isolated transitions observed in PC:PS (1:1) membranes; for clarity longer events were selected. In both cases 50 μg/ml PextoxA was added to the ionic solution bathing the membrane. The applied voltage was +100 mV. Signals were low-pass filtered at 1 kHz, digitized at a sampling rate of 0.5 ms per point and played back on an HP laser printer using specially designed high-resolution plot programs. Ionic solutions were symmetric: KCl 100 mM at pH 5.5.

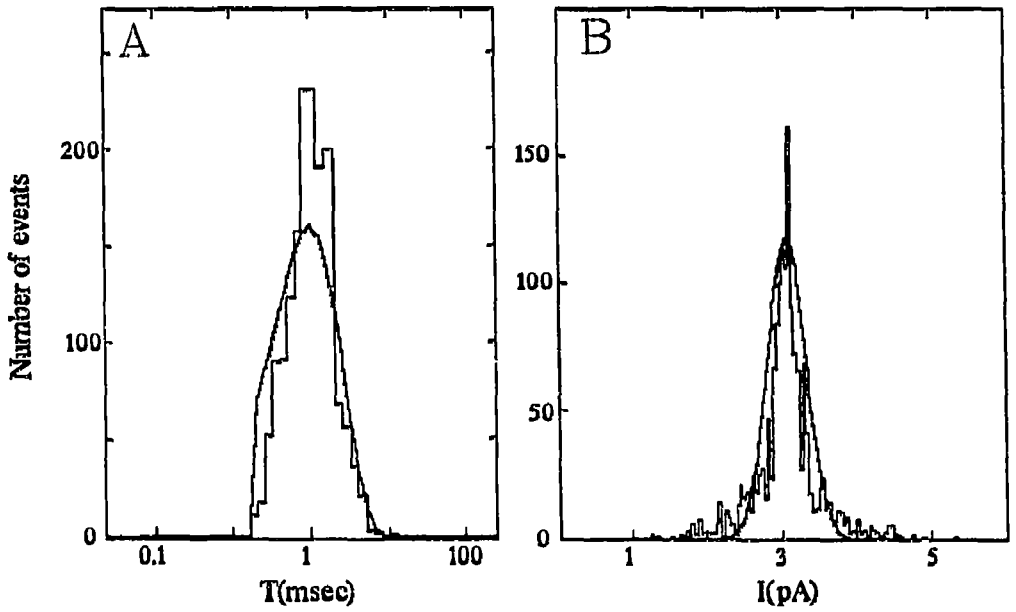

Fig. 2. Single-channel open time distribution (A) and current amplitude (B), obtained from 1,600 single openings in Al membranes. The best fit of experimental data gave a mean open time, t_o=1.33 ms, and a mean current, i=3.1±0.4 pA . In panel A, an open-time hystogram is displayed (according to Sigworth and Sine [19]) with a logarithmic time axis. This display overwhelms limitations of traditional (linear) dwell-time histograms where two or more components can hardly be resolved on one time scale. The main advantages of this logarithmically binned histogram are that exponential components appear as peaked functions and the position of the peak corresponds to its time constant. Experimental conditions are as in Fig. 1A.

Noise analysis was performed on current bursts similar to those reported in Fig. 1A. Applying Eqns. 1 and 2 we obtained σ^2= 0.25 pA^2 and τ=1 ms (Fig. 3). Both these parameters are in full agreement with those obtained from the analysis reported in Fig. 2. In fact, from the single channel event analysis we obtained i=3.1 pA and p=0.02, which inserted into Eqn. 3 to give σ^2= 0.18 pA^2, in good agreement with the noise analysis value. Furthemore, for PextoxA we note that β is much larger than α and hence $\tau \approx 1/\beta = t_o$ (which is the mean open time, 1.3 ms from Fig. 2A). This provided further evidence that signals in bursts were due to the opening of ionic channels.

The permeabilization induced by PextoxA is strongly

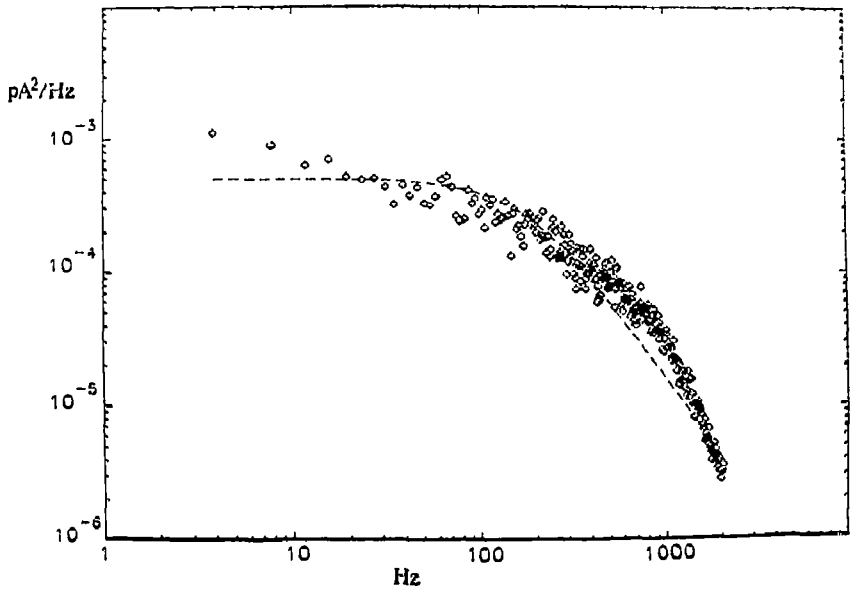

Fig. 3. Power spectrum (shown as double logarithmic plot) obtained from the current response at +100 mV in a PC:PS (1:1) membrane. Signals were stored on videorecorder and analyzed off-line after they were low-pass filtered with a cut-off frequency of 2 kHz and digitized at a sampling interval of 250 μs. Experimental data were best fit with a single Lorentian distribution with A=5·10^{-4} pA^2 and $1/2\pi\tau$=160 Hz (dashed line). Other conditions are as in Fig. 1.

voltage dependent, since the probability of the channel being in the open state is much higher at positive transmembrane potentials (i.e. when the voltage is positive in the compartment containing the toxin) and is almost zero at negative voltages. Fig. 4 shows the voltage dependence of the macroscopic current observed in an asolectin membrane after the incorporation of PextoxA channels. Each data point is the mean current obtained in response to a voltage which was continuously cycled between −100 and +100 mV, and after sorting the current values in 24 bins. The very low bare phospholipid membrane conductance remains almost unchanged at negative transmembrane voltages, while a very steep current increase can be observed at positive values.

The single channel conductance does not change with applied voltage (at least between 0 and 60 mV, data not shown), however, applying the Boltzmann distribution to a 2-state system [12] and since the macroscopic current, I, is far from saturation, experimental data can be fit (as observed also with other pore forming peptides [13]) by an exponential function of the type:

$$I=A\cdot V\cdot\exp(ze(V-V_0)/KT) \quad (4)$$

where z is the number of elementary charges reoriented by the transmembrane potential, V, during pore opening, V_0 is the voltage at which 50% of the channels are open and e, K and T have the usual meaning. A is a parameter representing the single channel conductance times the number of active pores present in the membrane phase. The best fit of data in Fig. 3 gave the following values for the three parameters:

A=457 nS, V_0=262 mV, z=0.6

Results in Fig. 4 refer to Al membrane, but we observed similar voltage dependence in PC:PS membranes. We also tested whether the current rectification observed at positive voltages was a genuine property of the PextoxA channel or was dependent on the voltage ramp protocol. Therefore, square-voltage pulses were applied to the membrane and current responses consistent with those reported in Fig. 4 were observed, i.e. channels were suddenly activated at +100 mV, and deactivated in fractions of a second when the transmembrane potential was switched to −100 mV.

A proposed model for the voltage-dependent mechanism of pore formation by PextoxA, based on the toxin primary sequence [14] and its X-ray 3D structure [15], is shown in Fig. 5. Helices A and B of domain II, the domain of the toxin which mediates its translocation across cell membranes [16], are thought to be involved in channel formation. These two α-helices are rather amphiphilic and are connected by a positively charged loop containing three arginines and one histidine. They are also linked by a disulphide bridge. We propose that upon acidification of the medium the two helices be-

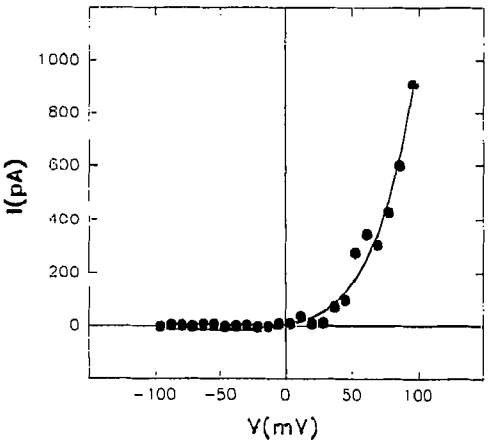

Fig. 4 Macroscopic current–voltage properties of Al membrane following the incorporation of PextoxA ionic channels. The voltage-dependent permeability increase was obtained on the addition of 50 μg/ml of PextoxA to the ionic solution bathing the membrane. Data points in the figure were obtained from the average of three voltage ramps ranging from −100 to +100 mV. The current was digitized at a frequency of 2.4 points per mV and then sorted in the 24 bins which divided the entire voltage range. The solid line is a best fit of Eqn. 4 providing A=457 nS, V_0=262 mV and z=0.6. Other conditions are as in Fig. 1.

come less tightly packed to the rest of the molecule (through the formation of a 'molten globule' intermediate [17]) and undergo a surface interaction with negatively charged head-groups of phospholipid bilayers (configuration a in the figure). The two helices become partially embedded into the hydrophobic phase (lying flat on the plane of the membrane) whereas the posi-

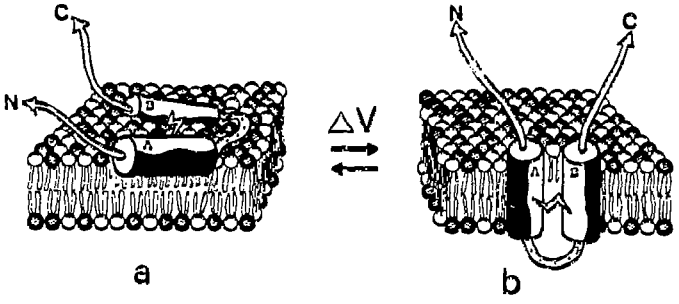

Fig. 5. Hypothetical model for the voltage-dependent mechanism of pore formation by PextoxA. The membrane contains 50% of PS. Negatively charged head-groups are shaded. The region of PextoxA shown to interact with the lipid membrane is the one encompassing residues 253–300. It includes the two α-helices of domain II called A and B [16], which are linked by a disulphide bond between Cys-265 and Cys-287 (indicated) and are connected through a hinge containing a cluster of four positive charges (Arg-274, Arg-276, Arg-279 and His-275). Helix-A is largely apolar whereas helix-B is more amphiphilic. The hydrophobic region is shown as solid. In configuration a, the hinge interacts electrostatically with the negative lipid headgroups. A positive voltage on the side of the toxin pushes this loop to the opposite side of the membrane where it is stabilized by the electrolyte (configuration b). Activation of the toxin in vivo requires reduction of the disulphide bridge and nicking of the loop on the C-side of Arg-279, indicated by a break.

tively charged arg-loop electrostatically interacts with the negatively charged lipid headgroups. Application of a positive voltage on the side of the toxin flips the arg-loop through the lipid phase to reach the opposite side of the membrane where it is stabilized again by electrostatic interaction. This triggers the insertion of the two helices into the membrane in a perpendicular position (configuration **b**). Pores are then formed from this state by lateral aggregation of two or more monomers which dispose the amphiphilic helices such that their hydrophobic part faces the lipid tails whereas the hydrophilic part lines the interior of the pore.

The formation of the inserted state may be relevant for intoxication, being involved in the step of translocation of the toxin from the endosome to the cytosol. In fact it has been shown [18] that upon reduction of the disulphide bridge between helices A and B, and after nicking the arg-loop at Arg-279 (i.e. next to helix B), helix B (with the enzymically active C-terminal domain) is internalized into the cytosol, whereas helix-A (with the N-terminal receptor-binding domain) remains bound to the endosomal membrane. This can be readily understood starting from the inserted state that we propose. Helix-A is the most hydrophobic and it is further stabilized in the inserted position by the presence of the arg-loop, whereas helix-B, which is much more hydrophilic, once disjoined from helix-A, can move to the cytosol thus starting the translocation of the enzymically active part of the molecule.

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