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A cytolytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers

Barbara H. Knowles, Michael R. Blatt*, Mark Tester*, Jane M. Horsnell, Joe Carroll, Gianfranco Menestrina+ and David J. Ellar

*Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, *Department of Botany, University of Cambridge, Downing Street, Cambridge CB2 3EA, England* and *+Dipartimento di Fisica, Universita' di Trento, I-38050 Povo, Italy*

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In order to determine the mechanism of action of the 27 kDa mosquitocidal δ-endotoxin of *Bacillus thuringiensis* var. *israelensis* we have studied its effects on the conductance of planar lipid bilayers. The toxin formed cation-selective channels in the bilayers, permeable to K^+ and Na^+ but not to *N*-methylglucamine or Cl^-, showing very fast, cooperative opening and closing. Channel opening was greatly reduced in the presence of divalent cations (Ca^{2+}, Mg^{2+}) and the effect was reversed when these ions were removed. These results are consistent with our proposal that *B. thuringiensis* toxins act by a mechanism of colloid-osmotic lysis.

Endotoxin, δ-; Ion channel; Planar lipid bilayer; (*Bacillus thuringiensis* var. *israelensis*)

1. INTRODUCTION

Numerous theories have been proposed to account for the mechanism of action of the insecticidal protein δ-endotoxins synthesised by *Bacillus thuringiensis* (reviews [1,2]). We proposed a model in which the activated toxin first binds to a specific receptor on the plasma membrane of susceptible cells (accounting for the specificity of the toxins) followed by the generation of small pores in the plasma membrane. This leads to equilibration of ions and small molecules across the membrane followed by osmotic influx of water, cell swelling and lysis [2]. Biochemical evidence indicated that several *B. thuringiensis* toxins with different insect specificity acted by the same mechanism, generating pores of 0.5–1.0 nm in radius [2–4]. However, it was not possible, using a whole-cell system, to determine whether the toxin molecules themselves inserted into the membrane to create a pore, or whether they caused the opening of an existing ion channel or pump. The 27 kDa mosquito toxin of *B.t.* var. *israelensis* is known to interact with certain phospholipids [5] and to make phospholipid vesicles permeable to $^{86}Rb^+$ [6] without the requirement for a specific receptor. It is therefore a suitable candidate for studies of mechanism of action in the absence of cell membrane proteins. It is possible to record currents through single ion channels in a planar lipid bilayer, and this method has been used to study other pore-forming protein toxins [7,8]. We have employed this technique to clarify the mode of action of the 27 kDa *B.t.* var. *israelensis* toxin in a simple system in which any channels observed can only have been formed by the toxin molecules themselves.

Correspondence address: D.J. Ellar, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

2. MATERIALS AND METHODS

B.t. var. *israelensis* IPS-78 was obtained from Professor H. de Barjac (Institut Pasteur, Paris). The 27 kDa toxin was

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solubilised at 1 mg/ml from the δ-endotoxin crystal as described in [9], substituting K_2CO_3 for Na_2CO_3. Alternatively, the 27 kDa toxin synthesised by *B. subtilis* containing the cloned toxin gene was purified as in [10] and solubilised as above. In both cases the 27 kDa protoxin was cleaved by endogenous proteases to an active toxin of 25 kDa.

Planar bilayers were painted across a 0.3 mm hole in a styrene copolymer cup using a decane solution of 40 mM 1-palmitoyl-2-oleoylphosphatidylethanolamine (PE; synthetic, Avanti Polar Lipids, Pelham, AL) to form a neutral bilayer separating solutions in the cup ('cis' side) and an outer chamber ('trans' side). Electrical connections were achieved via 500 mM KCl/2% agar bridges to two Hg:HgCl:KCl half cells. Connections to the cup ('cis') were taken to ground, while the trans side was taken to a List EP/C-7 amplifier (List Electronics, Darmstadt). Potentials are recorded as the difference trans with respect to cis.

Connections were also made from the cup, via a series of stopcocks, to perfusion bottles so that the solutions in the cup could be exchanged during an experiment. Perfusate in the cup was removed by aspiration. All solutions were filtered through 0.22 μm cellulose acetate fibre filters. Solutions were buffered at pH 9.5 with 10 mM 3-cyclohexylamino-1-propanesulphonic acid (CAPS).

Measurements were carried out at room temperature (20–22°C) and current and voltage data were recorded on a Sony Betamax video tape recorder after digitization at 22 kHz (PCM701-ES, Sony, Japan). Recorded currents were first filtered at 10 kHz with a 6-pole Butterworth filter. Experiments were started by adding an aliquot of toxin preparation to the cup while stirring, and then monitoring the (voltage-clamped) current signal on an oscilloscope. Once activity was observed the solution (and excess toxin) in the cup was removed by perfusion.

3. RESULTS AND DISCUSSION

Experiments carried out using the toxin synthesised by *B. thuringiensis* or *B. subtilis* generated identical results, ruling out the possibility that the results were due to contamination with other *B.t.* var. *israelensis* toxins or by *B. subtilis* proteins. When toxin was added at 30 μg/ml on the cis side of a bilayer in symmetrical 300 mM KCl at pH 7.5 no change in the conductance of the bilayer was seen even after 30 min, however when the pH was raised by adding free base (*N*-methylglucamine) channels were seen in 1 min. Subsequent experiments were therefore carried out at pH 9.5.

The toxin generated channels in the planar lipid bilayer which showed very fast flickerings and could not be resolved entirely, even with a 10 kHz bandwidth. Opening and closing seemed to be highly cooperative, i.e. channels tended to open or close together (fig.1). In order to minimise the number of channels present in each bilayer, we washed the 'cis' chamber with fresh buffer as soon as channel activity was observed, to remove unbound toxin molecules. There is no evidence that *B.t.* var. *israelensis* toxin channels can detach from the bilayer once formed: washing out the chambers did not decrease channel activity. Because of the cooperativity of the channels and their rapid opening and closing rates, it was not possible to study the kinetics of individual channels, or their voltage dependence, in detail. Fig.1 shows the presence of at least 4 toxin channels in one bilayer. The bilayers we used usually contained several channels which could open simultaneously. The conductance of a single channel was approx. 40 pS in symmetrical 300 mM KCl. This compares with 60–98 pS for *Staphylococcus aureus* α-toxin in 100 mM KCl [7].

The channels were highly cation-selective. Fig.2

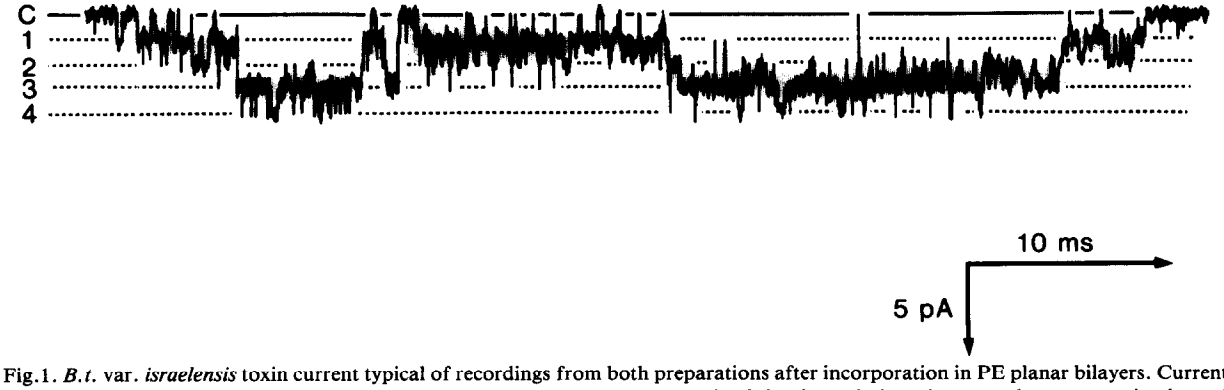

Fig.1. *B.t.* var. *israelensis* toxin current typical of recordings from both preparations after incorporation in PE planar bilayers. Current quantizations are indicated on the left (C, closed; 1,2, etc., open). Over much of the time window shown, at least one toxin channel was open. Recording parameters: [KCl], 1000 mM (cis) to 300 mM KCl (trans); clamp voltage, 9 mV; low-pass cut-off, 10 kHz (−3 dB).

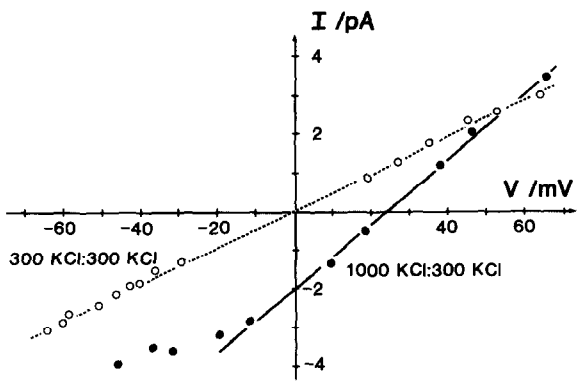

Fig.2. Single-channel, current/voltage curves for *B.t.* var. *israelensis* toxin incorporated into a PE planar bilayer. Regression lines to the two data sets give current reversal potentials of −1.6 (300:300 KCl) and 23.4 mV (1000:300 KCl), with single-channel conductances of 48 and 84 pS, respectively. Points negative to −20 mV in 1000:300 KCl were not included in the fitting.

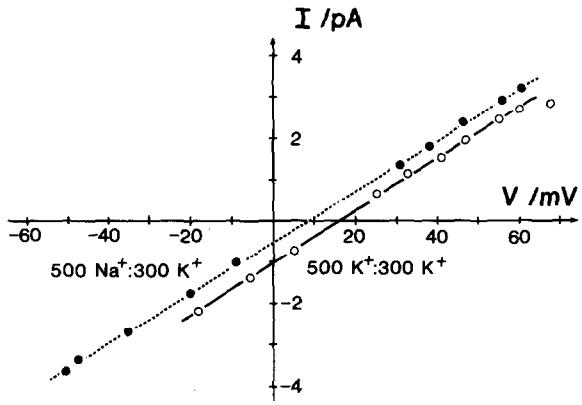

Fig.3. *B.t.* var. *israelensis* toxin channel selectivity for K^+ over Na^+. Single-channel, current-voltage curves for one PE planar bilayer. Regression lines to the two data sets give current reversal potentials of 15.9 (500:300 KCl) and 8.7 mV (500 NaCl:300 KCl), with single-channel conductances of 63 and 61 pS, respectively.

shows that the current/voltage (*I/V*) curve in symmetrical 300 mM KCl was linear and had an equilibrium potential close to zero (−1.6 mV), while in 300 mM KCl (trans):1000 mM KCl (cis) the equilibrium potential shifted to 23.4 mV, close to the value predicted by the Nernst equation. The equilibrium selectivity was close to 23:1 for $K^+:Cl^-$, correcting for K^+ activities in free solution [11]. Indeed, when KCl was replaced by equimolar *N*-methylglucamine chloride no current was recorded at any voltage tested, showing that neither ion could pass through the channel (not shown). Replacing KCl with NaCl on the cis side (fig.3) gave a reversal potential of 8.7 mV, indicating a slight selectivity for K^+ over Na^+ (permeability ratio $P_{Na}/P_K = 0.75$).

Addition of 5 mM $CaCl_2$ in 300 mM KCl to the cis side greatly reduced the frequency and number of channel openings (fig.4). Channel activity was recovered on flushing the cis chamber with 300 mM KCl without $CaCl_2$ (fig.4). Addition of 5 mM $MgCl_2$ to the cis side had a similar effect to $CaCl_2$ and was also reversible (not shown). This effect is probably more than simple charge masking, since channel activity was not recovered over a wide voltage range (−65 to 72 mV), and a neutral PE bilayer is not likely to have appreciable surface charge (although the hydrophilic surface of the toxin channel may be charged). A number of diverse pore-forming agents are inhibited by divalent cations [12] although the mechanism is unknown. The cytolytic activity of a mosquitocidal toxin from *B.t.* var. *darmstadiensis* 73-E-10-2 is significantly reduced in the presence of 5 mM Ca^{2+} or Mg^{2+} [13].

The formation by *B.t.* var. *israelensis* 27 kDa toxin of channels permeable to K^+ and Na^+ is compatible with the proposal that this toxin acts by colloid-osmotic lysis [2]. Equilibration of these ions across the insect cell membrane would lead to an influx of water and subsequent cytolysis. Sacchi et al. [14] presented indirect evidence to suggest that the lepidopteran toxins of *B.t.* var. *kurstaki* and var. *thuringiensis* increased the permeability of brush border membrane vesicles of lepidopteran midgut columnar cells to K^+ but not Na^+ or H^+. To our knowledge no similar experiments have been carried out on the effect of *B.t.* var. *israelensis* toxins on the ion permeability of their natural target cells: the midgut epithelium of mosquito and blackfly larvae [15,16]. However, since both insects live in fresh water and must maintain blood ion levels three orders of magnitude higher than the water in which they live [17] generation of pores in their midgut cells can explain the observed pathogenic effects of *B.t.* var. *israelensis* toxins. The midgut pH of insects susceptible to *B.t.* var. *israelensis* toxins is very high [17]. It is therefore

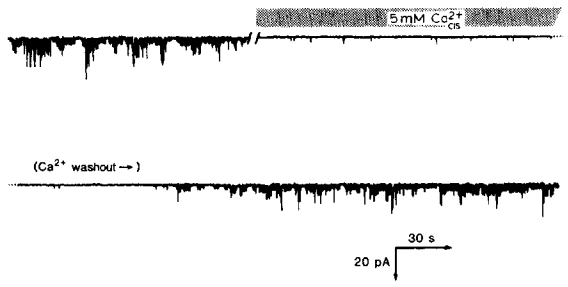

Fig.4. *B.t.* var. *israelensis* toxin channel response to Ca^{2+}. Low-speed trace of channel activity in symmetric 300 mM KCl and 10 mM K^+-CAPS buffer, pH 9.5. After a control recording (top left), 5 mM $CaCl_2$ was introduced on the cis side (exposure period indicated by shading above trace) and the recording was restarted. Ca^{2+} washout was carried out while recording (lower trace). The lag time for channel activity to recover is roughly equivalent to the time for Ca^{2+}-free buffer to clear the perfusion line. Upper and lower traces in Ca^{2+} are contiguous.

interesting to note that a high pH was required before any channels were observed in planar lipid bilayers. The present results do not allow us to predict whether the channels formed by *B.t.* var. *israelensis* 27 kDa toxin are generated by a single toxin molecule or by toxin oligomers, although kinetic evidence supports the proposal that oligomers are required to form a cytolytic lesion [18]. Our results do not address the question of toxin specificity: activated 27 kDa toxin is lytic to a wide range of eukaryotic cells in vitro [9] and can make liposomes [6] and planar lipid bilayers permeable to ions, but in vivo its toxicity is confined to dipteran larvae. Specific receptors have been identified for *B. thuringiensis* lepidopteran toxins [3,19]. We have evidence to suggest that in addition to its ability to bind certain phospholipids, *B.t.* var. *israelensis* 27 kDa toxin has a specific receptor present on dipteran but not lepidopteran cells (Armstrong et al., unpublished).

In conclusion, we have demonstrated that the 27 kDa mosquito toxin of *B.t.* var. *israelensis* generates cation-selective channels in a planar phospholipid bilayer. These channels show rapid opening and closing, which is affected by the presence of divalent cations. Our results provide further support for the proposal that this toxin acts by a mechanism of colloid-osmotic lysis.

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