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Interaction of α-latroinsectotoxin from *Latrodectus mactans* venom with bilayer lipid membranes

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Abstract

α-Latroinsectotoxin (LIT) from *Latrodectus mactans* venom increased the conductance of bilayer lipid membranes (BLM) by inducing channel like activity. The channels formed had a maximal single channel conductance of 5 pS in 10 mM $CaCl_2$ solution. This process occurred more rapidly in symmetrical 10 mM $CaCl_2$ solution than in equimolar KCl or NaCl. The LIT induced conductance showed pronounced rectification, that was dependent upon the face of the BLM to which the LIT was applied. This suggests that the LIT molecules incorporate into the bilayer lipid membrane in an oriented manner. The ion channels formed in bilayer phospholipid membrane by LIT are cation selective. The permeability of divalent cations decreased in the order $Ba^{2+} > Ca^{2+} > Mg^{2+} > Cd^{2+} > Zn^{2+}$ (Zn^{2+} and Cd^{2+} blocked effectively LIT channels with the ratio of Ca^{2+}_{trans} and Cd^{2+}_{cis} or Zn^{2+}_{cis} of 1:1). Selectivity of LIT to monovalent cations was not high and was Ca^{2+} sensitive. Our data suggest that LIT has at least two Ca^{2+}-binding sites, a high affinity site and low one (pK of binding is 2.4). As a result, the binding kinetics of Ca^{2+} with the toxin shows a high positive cooperativity (Hill coefficient, (h) = 5.95) and that dimerization might be a prerequisite to channel formation. Temperature dependence of conductance of LIT treated lipid bilayers in 100 mM KCl and 10 mM $CaCl_2$ solutions was also determined: 18.9 ± 2.11 kJ/mol and 28.537 ± 1.678 kJ/mol, respectively.

Keywords: Lipid bilayer; Ion channel; Calcium ion channel selectivity; Latroinsectotoxin; Latrotoxin

1. Introduction

LIT, a high molecular weight protein from black widow spider venom acts as a specific insectotoxin causing rapid transmitter release from insect nerve endings [1,2]. A high affinity LIT receptor has been identified in insect nerve tissue [2] and the DNA nucleotide sequence coding LIT precursor has been defined [3]. It has been suggested that the structure and physiological actions of LIT were the same in many respects to that of latrotoxin (LT) [1–6]. The toxic action of LT appears to be mediated by the formation of cation-selective channels [7–10]. In the present study we investigate the interaction of LIT with BLM and characterize the properties of the ion channel it forms.

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2. Materials and methods

2.1. Latroinsectotoxin isolation

LIT was isolated from *Latrodectus mactans tradecimguttatus* venom or from venom glands by the method described in [1,2].

2.2. Bilayer membranes formation

Plate membranes were formed by the method described in [11] across a 0.6 mm diameter hole in a Teflon cup from the solution of phosphatidylcholine (Kharkov factory of biopreparations, Ukraine) and cholesterol (Serva, Germany) at a weight ratio of 2:1) in n-heptane (total lipid concentration of 20 mg/ml). The solution bathing the membrane contained 10 mM Tris-HCl (pH 7.4) (Sigma, USA) and the required quantity of metal chlorides. The internal volume of the Teflon cup was 1 ml, whilst the

external compartment contained 9 ml of solution. Membrane formation was monitored using reflected light with an MBC-9 binocular microscope (St. Petersburg Optico-Mechanical Plant LOMO, Russian Federation). The membrane was illuminated via a light guide from a low-voltage lamp situated behind a screening case.

2.3. Conductance measurements

Voltage-clamp recordings of membrane current were made using silver chloride electrodes immersed in a 2 M KCl solution with 0.2 M KCl agar bridges attached to a high resolution voltage clamp amplifier. The polarization potential between the electrodes did not exceed 1–1.5 mV. The transmembrane voltage was controlled using a Shch-4300 digital voltmeter (Zhitomir Measuring Instruments Plant, Ukraine). This device enabled us to apply either a constant potential difference across the BLM of up to ±100 mV or a voltage ramp of 100 mV/min. The potential difference was referenced to the *trans*-side of the membrane which was defined as zero. Membrane currents were recorded on an ENDIM 620.02 type XY-recorder (Schlenhein, Germany). The recording method is shown in more detail in [12]. The majority of experiments were carried out at room temperature (20–24°C). To investigate the temperature dependence, a system of semi-conductor thermobatteries TEMO-3 (Lvov Factory Electronpribormach, Ukraine) allowed us to vary the temperature using a temperature ramp or to induce steady-state changes in temperature within ±0.5°C. Continuous mixing of the bathing solutions minimized the errors due to uneven warming or cooling.

Activation energy of ion transport through LIT treated BLM was determined as in [13].

3. Results

3.1. Incorporation of LIT into BLM

The effects of ion substitution

After introduction of LIT into the cell *cis*-compartment an increase of BLM conductance was observed. The increase in BLM conductance by LIT was achieved much more readily in the solution of 10 mM $CaCl_2$ than in equimolar KCl. LIT (0.1 μg/ml) induces a BLM current when using 10 mM $CaCl_2$, however in equimolar KCl, 0.7 mg/ml LIT (applied to the near membrane area) was required to produce an equivalent change in membrane current (Fig. 1B).

Amplification of the current recordings showed that the apparent linear changes in membrane current consists of small step-wise increases in membrane current suggesting that the LIT-induced current results from discrete channel activity. Estimates of the conductance of these single channels (at +100 mV in 10 mM $CaCl_2$) suggested a maximal conductance of 5 pS. The conductance of single channels increased in 100 mM KCl solution (Fig. 1C) which provided a means of measuring the rate of incorporation of LIT into the BLM (Table 1).

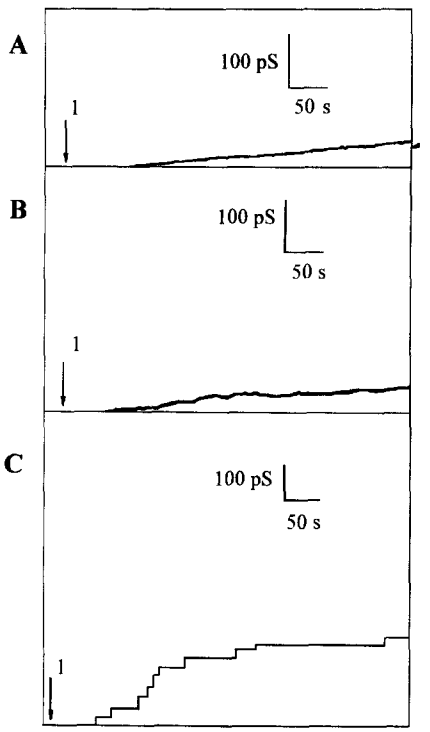

Fig. 1. Channel forming activity of LIT under a constant voltage (+100 mV) after addition of: (A) 0.1 μg/ml LIT in symmetric 10 mM $CaCl_2$; (B) 0.7 mg/ml LIT just to the nearest membrane area in symmetric 10 mM KCl. (C) Stepwise increase in current, driven by a constant voltage (+50 mV), after introduction of 0.1 μg/ml LIT to the around membrane area in symmetric 100 mM KCl. 1, protein was added to the *cis*-compartment.

The effect of trans-BLM potential

The rate of change of LIT-induced BLM current was investigated at different *trans*-BLM potentials. Toxin was applied to the membrane and after reaching a stable rate of conductance change, the rate was then monitored at different potentials (Table 1). By utilizing our estimates for single channel conductance (Fig. 2) we were able to calculate the incorporation frequency (ν) at several *trans*-BLM potentials. Our results (Table 1) suggest that incorporation of LIT into BLM was independent of membrane voltage.

Table 1
Lack of an effect of membrane potential on the frequency of LIT channel formation in BLM

V (mV)	−100	−50	50	100
ν	2.77±0.1 (4)	2.66±0.2 (3)	1.83±0.5 (4)	3.07±0.1 (4)

V, membrane potential; ν, frequency of ion channel formation in BLM as quantity of channels incorporating into BLM per 1 min. ± standard error; the number of measurements is shown in brackets.

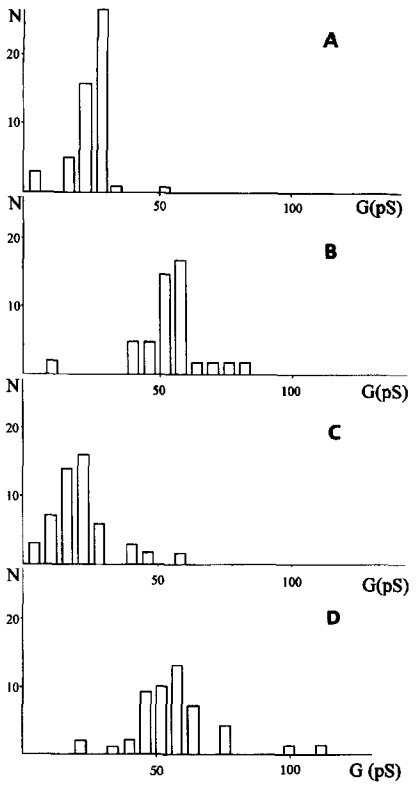

Fig. 2. Amplitude histograms of single channel conductance in the bilayer lipid membrane treated with LIT. Current increases on the membrane at: (A) 50 mV; (B) 100 mV; (C) −50 mV; (D) −100 mV. LIT at a concentration of 0.6 μg/ml was added to the *cis*-side of the membrane which separated solution containing 100 mM KCl.

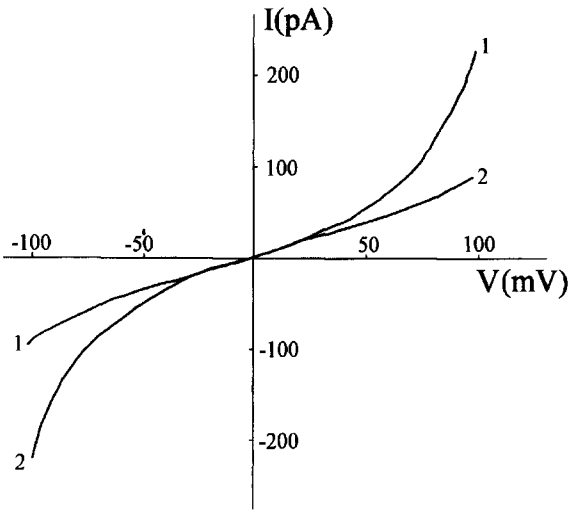

Fig. 3. Current-voltage curves of bilayer lipid membrane treated by LIT. 0.1 μM LIT was added: 1, from the *cis*-side; 2, from the *trans*-side. The solution contained 10 mM $CaCl_2$ on both sides.

3.2. Properties of channels formed by LIT in BLM

Potential-dependence of the LIT-induced membrane current

The current-voltage relationship (I/V) was determined using a ramp protocol (−100 mV to +100 mV, 2 min). LIT applied to the *cis*-side of the membrane induced a membrane current that showed pronounced voltage-dependent rectification in all of the salt solutions tested (Ca^{2+}, Na^+, 10 mM; K^+, 10 mM or 100 mM). The rectification was quite pronounced, passing 2.8 ± 0.4 times more current at +100 mV than at −100 mV. A typical curve is shown in Fig. 3, curve 1. Incorporation of LIT from the *trans*-side (Fig. 3, curve 2) inverted the voltage sensitivity of the I/V curve, more current now passing at −100 mV than at +100 mV. This suggests that the orientation of the toxin in the membrane determines its voltage sensitivity.

Ionic selectivity

The voltage-ramp protocol also allowed us to determine the reversal potential of the LIT-induced current whilst changing the composition of the bathing solutions either side of the membrane. Placing 5 mM $CaCl_2$ on the *trans*-side of the membrane and 50 mM $CaCl_2$ on the *cis*-side of the membrane resulted in a −30 ± 3 mV shift in the reversal potential. This coincides with that predicted by the Nernst equation for a calcium selective conductance. Utilizing other divalent ions we were able to estimate a permeability sequence for the ion channels formed by LIT: $Ba^{2+} > Ca^{2+} > Mg^{2+} > Cd^{2+} > Zn^{2+}$ (Zn^{2+} and Cd^{2+} blocked the LIT channel) (Fig. 4). For the monovalent cations the permeability sequence was: $Li^+ < Rb^+ < Cs^+ < Na^+ < K^+$ (Table 2). Application of Cd^{2+} (1 mM) or Zn^{2+} (1 mM) on to a LIT-induced current in 10 mM Ca^{2+} solution decreased the inward current by 39.5 ± 3% and 52 ± 2%, respectively (Fig. 5). This ability of both Cd^{2+} and Zn^{2+} to inhibit the LIT-induced current in 10 mM

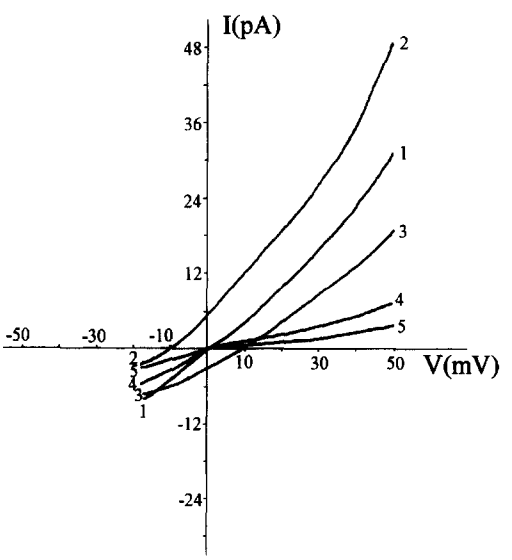

Fig. 4. Current-voltage curves of bilayer lipid membrane modified with LIT under bi-ionic conditions. From the *cis*-side: (1) 10 mM $CaCl_2$; (2) 10 mM $BaCl_2$; (3) 10 mM $MgCl_2$; (4) 10 mM $CdCl_2$; (5) 10 mM $ZnCl_2$. From the *trans*-side: 10 mM $CaCl_2$.0.1 μM LIT was added to the *cis*-side of the cell.

Table 2
Relative permeability or conductance of bilayer lipid membrane modified by LIT to Ca^{2+} with respect to other cations

Ion	Bi-ionic potential U (mV)	$P_{Ca^{2+}}/P_I$	$G_{Ca^{2+}}/G_I$
Ba^{2+}	-10 ± 1 (3)	0.71	0.66 ± 0.01
Ca^{2+}	0 ± 0.05 (5)	1.0	1.0 ± 0.03
Mg^{2+}	10 ± 2.5 (3)	2.21	1.78 ± 0.4
Cd^{2+}	– (3)	–	4.82 ± 0.3
Zn^{2+}	– (3)	–	9.11 ± 0.28
K^+	20.5 ± 2.5 (3)	1.81	
Na^+	22.3 ± 2.7 (4)	2.04	
Cs^+	25.3 ± 1.7 (3)	2.51	
Rb^+	27.2 ± 2 (4)	2.82	
Li^+	29.3 ± 3 (3)	3.23	

The first column represents a shift of reversal potential data calculated from the intercept of the current-voltage curve under bi-ionic conditions: 10 mM $CaCl_2$ from *trans*-side and 10 mM salt ($BaCl_2$, $MgCl_2$, etc.) from *cis*-side of the membrane. LIT was added to the *cis*-side at a final concentration 0.1 μg/ml. The second column represents the relative permeability ratio data calculated from the shift of reversal potentials. P_I or G_I represents the permeability or conductance, respectively, of the other cations. The third column represents the relative conductance under the same bi-ionic condition of column 2, except conductances which were measured under constant voltage (50 mV). The final level of conductance in the initial $CaCl_2$ solution was the same in all experiments. The $\pm$ are the standard errors, and numbers in parentheses represent the number of experiments.

Ca^{2+} solution probably results from their low permeability and may also reflect stronger binding of zinc ions a site within the toxin molecule. In support of this, perfusion of 50 ml $CaCl_2$ at the *cis*-side restored completely the initial conductance measured before the introduction of Cd^{2+} while it was possible, by careful washing (100 ml), to reverse the Zn^{2+}-induced inhibition by $78\pm2\%$ only. Similar results were obtained in the experiments conducted to estimate the divalent cation permeability sequence (Table 2) where re-perfusion of the calcium chloride solution (50 ml) at the *cis*-side restored the LIT-induced conductance by $78\pm2\%$ and $62\pm1\%$ after measurements had been made in Cd^{2+} and Zn^{2+} solutions, respectively.

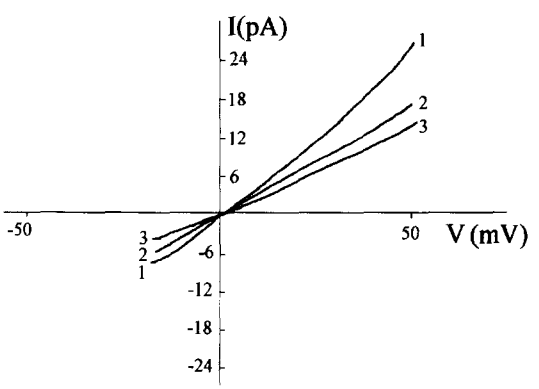

Fig. 5. Blocking of LIT channels by zinc or cadmium ions: (1) membrane washing solution contained 10 mM $CaCl_2$ from both sides; (2) 1 mM Cd^{2+}, (3) 1 mM Zn^{2+} was added from *cis*-side. 0.1 μM LIT was introduced at the *cis*-side.

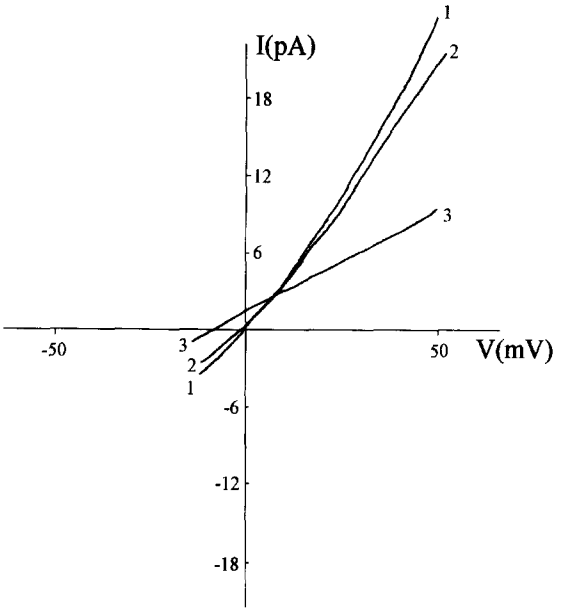

Fig. 6. Influence of Ca^{2+} on K^+ current through bilayer lipid membrane treated by LIT. A 10 mM KCl solution was present at both sides of membrane. The *cis*-side concentration of Ca^{2+} was: (1) 0; (2) 0.03 μM; (3) 0.5 μM.

The effect of calcium ions on selectivity of LIT channels

LIT-induced currents in K^+ solution were reduced in the presence of submicromolar concentrations of calcium ions when added to the *cis*-side of the membrane. The data shown in Fig. 6 represents that 'free' calcium concentrations used inhibit the LIT-induced current and shift the reversal potential negatively. Moreover the inhibition was independent of voltage and therefore the direction of current flow. 'Free' calcium concentrations were achieved by the addition of $CaCl_2$ to 10 mM KCl containing EGTA. Concentrations of 'free' calcium were calculated by the standard method [14].

In calcium solution the amplitude of the LIT-induced conductance was dependent on calcium concentration (Fig. 7). The conductance increases steeply in a sigmoid manner with calcium concentration with a threshold at 1.5 mM calcium and saturating at 3.5 mM. The binding constant and cooperativity coefficient were determined in Hill coordinates ($K_h = 10^{2.4}$ M^{-1}, $h = 5.95$).

The effect of temperature

Varying the temperature in the range +3°C to 22–24°C did not affect the resting conductance of the BLM in the absence of LIT. However the conductance of LIT channels was increased by elevating temperature. Conversely, decreasing temperature reduced the LIT conductance. The conductance changes induced by temperature calculated at steady state temperatures were consistent with those found using the temperature ramp method. The calculated activation energy (E_a) for K^+ ion transport through LIT-containing membranes (18.9 ± 2.11 kJ/mol) differed from that for a 100 mM KCl solution (3.903 ± 0.84 kJ/mol) (Fig.

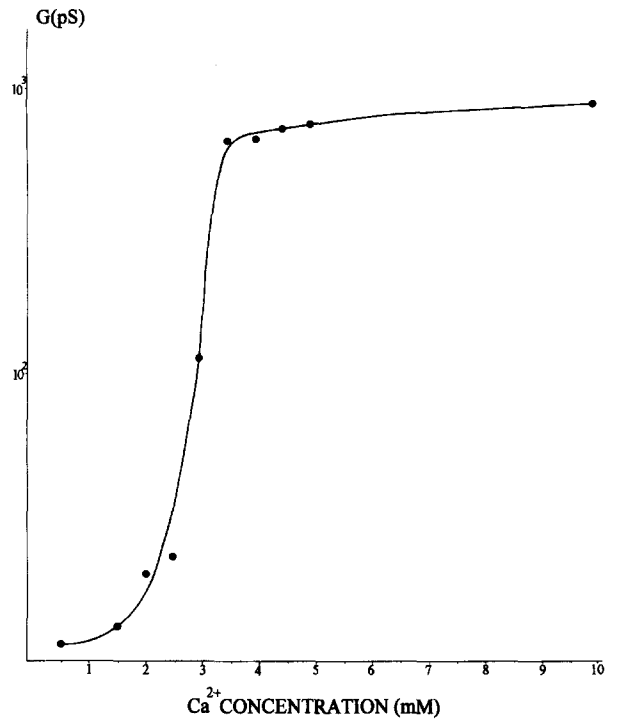

Fig. 7. Bilayer lipid membrane conductance against Ca^{2+} concentrations in the presence of 16 μg/ml of LIT. The membrane bathing fluid consisted of the indicated Ca^{2+} concentrations from both sides. The conductance was continuously monitored under 50 mV voltage clamp conditions. The conductances indicated are those obtained after steady state. Each point represents a new membrane.

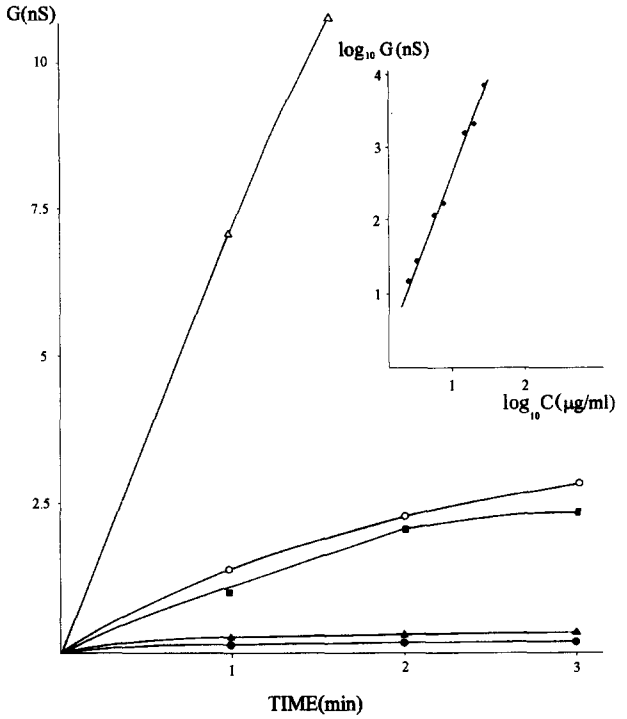

Fig. 9. Time-response of bilayer lipid membrane conductance in the presence of various concentrations of LIT. The membrane washing solution contained from both sides 10 mM $CaCl_2$. The protein concentration were: 2.4 μg/ml, 4 μg/ml, (●) 5.6 μg/ml, (▲) 8 μg/ml, (■) 16 μg/ml, (○) 24 μg/ml, (△) 32 μg/ml. The conductance was continuously monitored under 50 mV voltage. Each point represents a new membrane. The inset is a log-log plot of the data on conductance at 1 min in Fig. The inclination angle of this relationship to abscissa axis was indicated as (β) (see Results).

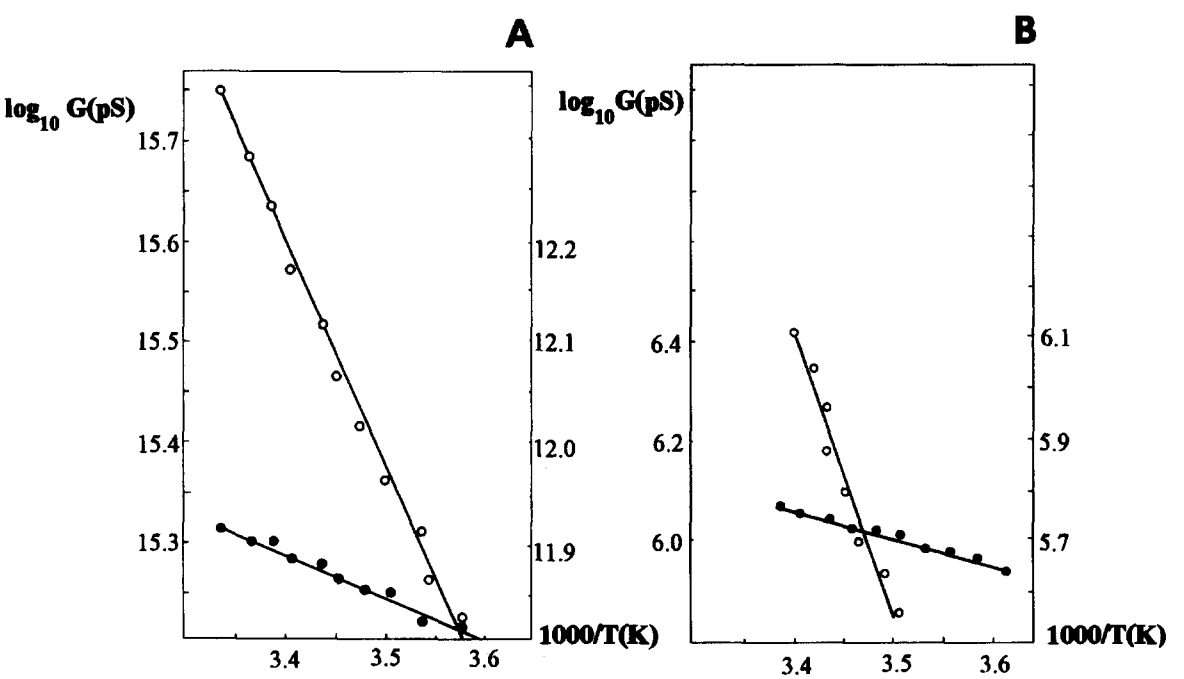

Fig. 8. Temperature relationship of LIT-induced conductance of bilayer lipid membrane. The membrane bathing fluid contained: (A) 100 mM KCl. The conductance was continuously monitored under 50 mV voltage clamp conditions. (●) protein was not added (left); (○) protein was added from *cis*-side of membrane (right); (B) 10 mM $CaCl_2$. The conductance was continuously monitored under: 50 mV voltage clamp conditions. (●) protein was not added (left), (○) protein was added from *cis*-side of membrane (right). Each point represents an average data of three experiments.

8A). The energy of activation measured in calcium chloride solution was higher (28.537 + 1.678 kJ/mol) than that obtained in K^+ solution and also than that found in the absence of BLM (4.978 ± 0.079 kJ/mol) (Fig. 8B). It should be noted that E_a of calcium ion transport measured for LT under similar circumstances (8.534 ± 0.839 kJ/mol) did not differ significantly from that obtained without BLM.

The effect of different concentrations of LIT on BLM conductance

Binding of LIT to the BLM was concentration dependent and could be described by a Langmuir saturation curve. The protein concentration was varied between 0.16 μg/ml and 40 μg/ml. Threshold for activation was at a concentration of 2.4 μg/ml and maximum values were reached at 32 μg/ml. Subsequent increases in toxin concentration decreased membrane stability and led to its eventual rupture. The rate of incorporation of toxin into BLM was determined as the change of conductance per minute (Fig. 9), which when plotted on double-log coordinates was linear (Fig. 9 inset, tg $\beta = 2.5$).

4. Discussion

LIT is a high-molecular weight protein (120 kDa) that has a pronounced ability, at low concentrations (4 nM), to induce an increase in the frequency of miniature excitatory postsynaptic potentials (MESPs) at the insect neuromuscular junction, whilst the nerve endings at the frog neuromuscular junction were unaffected [2].

Highly specific binding of ^{125}I-labelled LIT with membranes from insect nerve tissue and the absence of ^{125}I-LIT binding to rat brain synaptosomes suggest the existence of LIT receptors on insect nerve endings [2]. The presynaptic effects of LIT (but not the binding of ^{125}I-LIT or LIT) depend on the presence of divalent cations (Ca^{2+}, Mg^{2+}) [2]. It has been proposed that the physiological action of LIT is mediated by an increase in the presynaptic nerve membrane permeability to divalent cations.

In the present work it was shown that LIT forms ion channels in BLM, the conductance of which is much lower than that measured for LT channels under the same recording conditions [15]. The voltage-dependent asymmetry of the I/V curve was dependent upon to which side of the membrane the LIT was applied. This suggested that LIT incorporates into BLM in a specific orientation that this orientation determines the current passing capability of the channels (Fig. 3), as has been proposed for LT channels [9,15].

Taking into account the shape of I/V plot of BLM modified by many channels, it may be supposed the potential-dependence of single LIT channel.

The frequency of ion channel formation in BLM obtained with LT was dependent upon membrane potential and it was proposed [16] that its incorporation depended on positive charged groups on the membrane penetrating part of the molecule. However, we noted no significant effect of transmembrane potential on the frequency of LIT channel formation in BLM (Table 1).

The activation effect of a positive potential on cation transport through channels in LIT (Fig. 3) or LT [9,15] treated BLM suggests that positive charge outside nerve endings at the rest state probably stimulates calcium influx after toxin binding with excitable membranes.

The larger increase in BLM conductance induced by LIT in 10 mM calcium solution when compared to equimolar KCl suggests that calcium ions (by acting directly on the toxin molecule, as with LT [15]) might be a factor facilitating toxin incorporation and that this may be of physiological significance at the insect neuromuscular junction.

The divalent cation selectivity established for LIT ($Ba^{2+} > Ca^{2+} > Mg^{2+} > Cd^{2+} > Zn^{2+}$) differs from that of LT by the transposition of Cd^{2+} and Zn^{2+} [15]. The permeability of LIT channels for the monovalent cations ($K^+ > Na^+ > Cs^+ > Rb^+ > Li^+$) is not exactly the same with that determined for LT channels. At the same time by the disposition in the Eizenman's ranges the given sequence is close to the determinated one in [15] (between 6–7).

The investigation of the effect of submicromolar calcium ions (0.03–0.5 μM) on the conductance of LIT channels in 10 mM KCl solution showed that Ca^{2+} could block the influx of K^+ and cause a negative shifts in the reversal potential (Fig. 6). Higher concentrations of Ca^{2+} could probably produce the values described by the equation of Goldman-Hodgkin-Katz [17]. Similar data obtained for LT were interpreted according to the model proposed by Kostjuk and co-workers [18]. In this model calcium ions are proposed to interact with the structure of channel thereby regulating its permeability to di- and monovalent cations and to induce conformational rearrangements of the channel resulting in altered permeability. This structure contains the high affinity component of calcium binding probably similar to Ca^{2+}-binding centres of some proteins. This binding site is probably located at the *cis*-side of the membrane and away from the membrane binding region as transmembrane potential had no effect on the Ca^{2+}-dependent blocking of both LIT and LT channels [15]. The existence of such a region is confirmed by the high cooperativity of calcium binding (Fig. 7). The dose–response curve for calcium ion concentration showed that a maximal conductance increase was achieved at a Ca^{2+} concentration of 3.5 mM, suggesting the existence of a second, low-affinity calcium binding site ($K_h = 10^{2.4}$ M^{-1}), which is analogous to that found in LT channels (Ca^{2+} binding constant was $10^{1.7}$ M^{-1}) [15]. Low-affinity calcium binding sites have been characterized for Ca^{2+}-channels in neurones from mammals ($pK = 2$) [19], moluscs ($pK = 1.3$) [20], and those formed in BLM by

Steatoda paykulliana venom ($pK = 1.9$) [15] and it has been suggested that this site is located within the channel [19]. The Hill coefficient determined for LIT ($h = 5.95$) allows us to suppose that the low-affinity site in the LIT molecule is formed by 12 ionogenic groups.

Conductance measurements at different temperatures showed that the E_a for K^+ transport through LIT channels differs from that found for K^+ in free solution, this indicates that K^+ must overcome a greater activation barrier to pass through the channel. These data suggest an interaction of the ionogenic groups of the protein with K^+ (Fig. 8A). The increased E_a for calcium may be explained by a more expressed effect of LIT channel pore diameter on the passing of larger size ions through the channel. E_a of Ca^{2+}-transport via LIT channel was slightly higher than that calculated for LT in a two barrier model [15]: 11.6 and 9.7 in RT, respectively.

It should be noted that E_a has been measured for calcium ion transport across LT treated membrane (8.534 $\pm$ 0.839 kJ/mol) is lower than that of LIT (Fig. 8B) and while quite close to E_a of calcium ions transport through free solution (without BLM) of 10 mM Ca^{2+} that compares favorably with data about lower conductance of LIT channels and it probably results from smaller diameter of their pores.

The dose–response curve for LIT can be described by a first order relationship (Fig. 9). The slope of nearly 2 (Fig. 9, inset) suggests that LIT needs to combine in to a dimer before forming a conducting channel as has been suggested for LT [21].

From the data obtained we conclude that LIT, like LT forms channel-like structures in BLM which are similar in order of cation selectivity and in potential-dependence of ion transport with potential-dependent calcium channels of excitable membranes [18–20]. These properties of LIT molecules may be important to physiological action of LIT. However, the observed differences between LIT and LT channels especially their permeability sequences for cations and the measured unitary conductance of their single channels, suggest that LIT channels are quite distinct from those induced by LT.

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