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Permeation of Divalent Cations Through α-Latrotoxin Channels in Lipid Bilayers: Steady-State Current-Voltage Relationships

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Summary. α-Latrotoxin, a polypeptide neurotoxin known to cause massive release of transmitter from vertebrate nerve terminals, is thought to act by forming cation-selective channels in plasma membranes. This paper describes the steady-state current carried by Ca^{2+}, Sr^{2+} and Ba^{2+} through pores of α-LaTx molecules incorporated in artificial bilayer membranes made of neutral lipids. Even when the solutions separated by the membrane are identical, the *I-V* relations rectify strongly, the current being higher when the side to which the toxin is added is positive. The polarity of the rectification is consistent with the hypothesis that the mechanism of action of the toxin is, at least in part, that of promoting inwardly directed flow of cations, and thus, accumulation of Ca^{2+} and other ions in the intracellular spaces. The dependence of the *I-V* characteristics on voltage and Ca^{2+} concentration is well described by a one-site, one-ion model for a channel. Three parameters of the model are deduced: the binding constant of the site for Ca^{2+}, $K = 1.5$ M^{-1} (or $K = 7$ M^{-1} when activities are used instead of concentrations); the "electrical" distance of the site from the toxin-containing solution, $\alpha = 0.3$; the free energy difference between the two barrier peaks, $\Delta F = 0.26$ kT. The values of the parameters deduced by studying the channel in the presence of Ca^{2+} give theoretical curves that also fit the data with Sr^{2+} and Ba^{2+}, indicating a low level of discrimination among these three cations.

Key Words α-Latrotoxin channels · Ca^{2+} permeability · two-barrier model

Introduction

Natural neurotoxins provide a useful set of reagents for the study of the nerve cell function, since their potent toxic action is frequently the consequence of specific interactions with key elements of the cell. In this respect, α-Latrotoxin, the polypeptide neurotoxin isolated from the venom of the Italian black widow spider (*Latrodectus tredecimguttatus*), certainly constitutes one of the most proper examples, since it is a specific probe for the study of presynaptic function and neurotransmitter secretion (Frontali et al., 1976; Kelly et al., 1979; Howard & Gundersen, 1980). On the basis of the pre-existing experimental evidence, the mechanism of action of α-Latrotoxin on its target structures (neuromuscular junction, rodent brain synaptosomes, cloned neurosecretory cell lines), can be assumed to involve, after the primary interaction with the acceptors of the membrane, insertion of the molecule into the cellular bilayer, followed by the expression of its channel functions (Grasso et al., 1980: Meldolesi et al., 1984; Wanke et al., 1986). The plausibility of this hypothesis is supported by the fact that α-Latrotoxin increases the conductance of bimolecular lipid membranes via formation of ionic pores (Finkelstein, Rubin & Tzeng, 1976), and also by the finding that insertion of the toxin in bilayers occurs with a specific polarity, as is strongly suggested by the relation between the direction of the current rectification and the side of the membrane to which the toxin is added (Robello et al., 1984).

In view of the central role of calcium in transmitter secretion, we deemed it important, and thus undertook, to characterize the electrical properties of α-Latrotoxin channels in the presence of divalent cations. In this paper, we present a study of the steady-state current-voltage relations for these channels, when inserted in lipid bilayers and interposed between solutions of Ca^{2+}, Sr^{2+} and Ba^{2+} at various concentrations. The data, which are characterized by rectification of the conductance and by a clear dependence of the *I-V* curves on ion concentration, are satisfactorily described by a simple Eyring model, requiring only three fitting parameters.

Materials and Methods

Solvent-free membranes were obtained using a technique similar to that described by Montal and Mueller (1972). Monolayers of a mixture (1 : 1 molar ratio) of L-α-lecithin (egg lecithin) and cholesterol (Chol) dissolved in hexane (10 mg/ml), were spread on the aqueous solutions, and bilayers were formed across a 200-μm diameter hole in a 12.5-μm thick Teflon® partition, separating two Teflon chambers. Both egg lecithin and Chol were from Calbiochem.

The electrolytic solutions were prepared using Carlo Erba RPE salts and were buffered to pH 7.5 with 10 mM Tris-chloride (Trizma; Sigma Chemical Co.). All other reagents were of the purest grade commercially available.

α-LaTx was purified from the venom of Italian Latrodectus spiders according to a published procedure, the degree of purity having been tested by established criteria for homogeneity (Grasso, 1976). α-LaTx was added to only one of the two compartments separated by the membrane. Such compartment will be referred to as the *cis* side and the other as *trans*. All the experiments were performed at room temperature.

Electrical Recordings

Electrical signals were recorded with Ag-AgCl electrodes. The variable potential was applied to the *trans* side, while the *cis* one was kept at virtual ground. The methods for current recording used standard techniques described in detail previously (Robello et al., 1984). To test the membrane thickness prior to dissolving the toxin, capacitance measurements were performed by simultaneously applying square waves of 40 mV both to the bilayer and to a confront circuit with a variable RC, and by checking the signal difference on an oscilloscope. The two signals were equalized with a properly calibrated potentiometer, capable of varying the time constant of the confront circuit and thus providing a direct reading of the membrane capacitance.

Data Processing

Minuit routines, a package from the C.E.R.N. program library, running on a VAX 11/780 computer (Digital Equipment Corp.), were used for curve fitting. These programs allow one to estimate unknown parameters in theoretical expressions by minimizing the sum of the squares:

$$\Sigma|N_{obs} - N(\nu)|^2$$

where N_{obs} are the observed values of the measured physical quantities, and $N(\nu)$ those expected from the model for assigned values of the fitting parameters.

Results

I-V Characteristics in the Presence of the Alkali-Earth Cations, Ca^{2+}, Sr^{2+} and Ba^{2+}

The steady-state conductive properties of α-LaTx channels incorporated in lipid bilayers have been analyzed in the presence of the cations, Ca^{2+}, Sr^{2+} and Ba^{2+}. When small amounts of the purified toxin are introduced (final concentration of 2 to 3 μg/ml), and a constant voltage is applied across the membrane, the current varies stepwise, strongly suggesting the opening of ionic pores. Although channel closure is seen occasionally (*see* Fig. 1), the prevailing tendency for the channels, once they are open, is to remain so, showing a behavior similar to that reported previously for experiments with univalent cations (Finkelstein et al., 1976; Robello et al., 1984).

Fig. 1. Current steps, observed several minutes after addition of α-LaTx to the *cis* side (final concentration of 2 to 3 μg ml $^{-1}$) of a membrane, made of a 1 : 1 molar ratio of egg-lecithin and cholesterol, and interposed between symmetrical solutions of 100 mM $CaCl_2$, buffered at pH 7.5 with 10 mM Tris-HCl. A 40-mV negative potential was applied to the *trans* side, while the *cis* side was at virtual ground. The conductance of the unmodified membrane was 10 pS. All the experiments were carried out at room temperature

The effects of Ca^{2+} were investigated in symmetrical solutions of $CaCl_2$ at several concentrations (5, 10, 30, 100, 300, 500 mM), all buffered at pH 7.5. The mechanism of channel formation by the toxin seems to depend on the type of cation present in the solution. Unlike the case of univalent cations, the formation of channels in the presence of $CaCl_2$ occurred with very low frequency, the delays between addition of the toxin and channel appearance ranging from several minutes to more than one hour. Thus, while a statistical analysis of the amplitude of the unit current steps as a function of ion concentration could be made for potassium ions (Lassa, 1985), a similar study would have been impractical in the present case. However, one advantage of the slow rate of toxin incorporation was that, in many cases, the current-voltage relations could be obtained with few channels in the bilayer, and without variation of the number of channels during the voltage sweep. The absolute magnitudes of the single-channel conductance varied considerably. Values of about 100 pS, as can be deduced from the record in Fig. 1, are typical in 100 mM $CaCl_2$, although a ten times smaller value has also been seen for the same salt concentration. At 500 mM

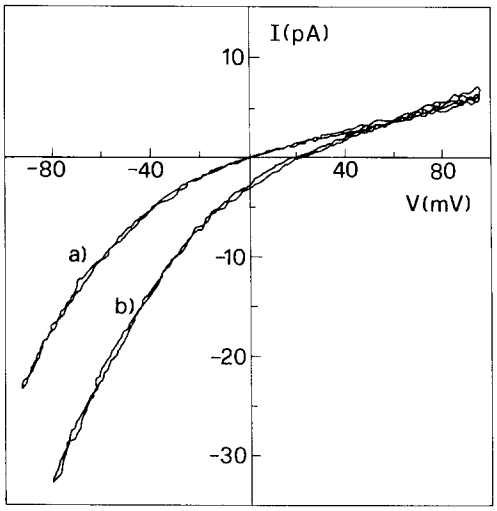

Fig. 2. Steady-state current-voltage relationships in toxin-treated membranes. The toxin was added to the *cis* side. The *I-V* curves were obtained by applying a slow voltage ramp (4 mV/sec) to the *trans* side, and recording the current response. $V = V(trans) - V(cis)$. *a*) Symmetrical solutions of 20 mM $CaCl_2$, buffered at pH 7.5 with 10 mM Tris-HCl. *b*) Fourfold gradient of Ca^{2+}, obtained by adding $Ca(NO_3)$ to the *cis* side. The intersection of the *I-V* curve with the abscissa gives the "zero current potential," showing an ideal Nernstian behavior for Ca^{2+}

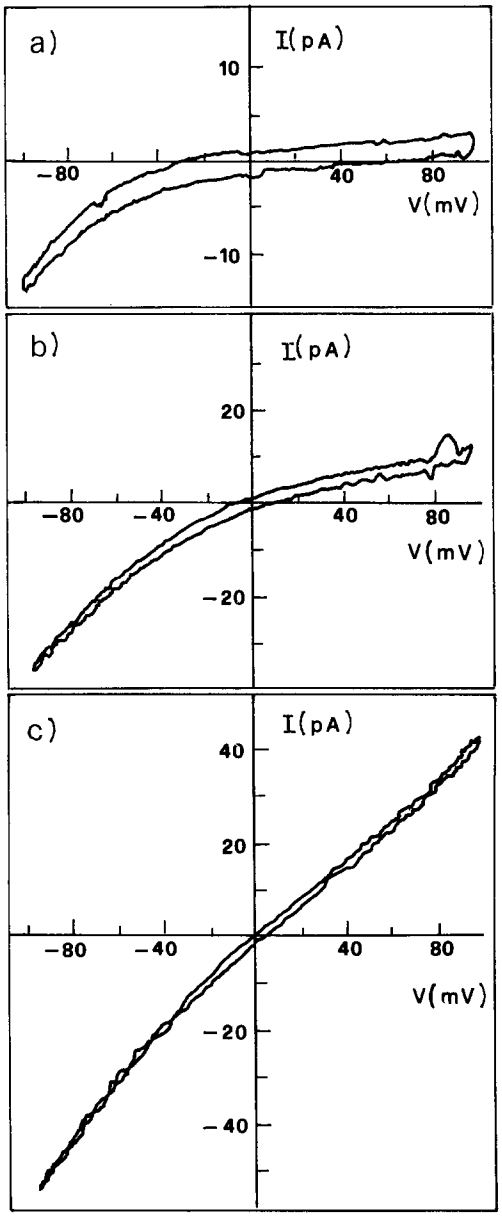

Fig. 3. Current-voltage characteristics after addition of the toxin to the *cis* side of a membrane interposed between symmetrical solutions of $CaCl_2$. *a*) 5 mM $CaCl_2$; *b*) 100 mM $CaCl_2$; *c*) 500 mM $CaCl_2$. All the solutions were buffered at pH 7.5

$CaCl_2$, which is the highest concentration used, the maximum and the minimum conductance values were 225 and 30 pS, respectively. Despite the considerable variability of the current steps, they were consistently lower than those measured for comparable concentrations of monovalent cations (Finkelstein et al., 1976; Robello et al., 1984).

Similar to the case of monovalent cations (Finkelstein et al., 1976), the membrane potential at zero current indicated a clear selectivity for cations also in the presence of $CaCl_2$. As is shown in Fig. 2, a fourfold gradient of Ca^{2+} concentration across the membrane, obtained by addition of the appropriate amount of $Ca(NO_3)_2$ to one side, caused a 20-mV shift of the zero-current membrane potential. The fact that this value is actually 1 or 2 mV higher than that expected from the Nernst equation is probably due to junction-potential effects at the electrodes. The finding of a cation-versus-anion selectivity with divalent cations has also been reported previously (Krasilnikov, Ternovsky & Tashmukhamedov, 1982).

The current-voltage relationships are clearly nonohmic and show rectification even when the channel separates identical solutions. A similar rectification had also been seen in the presence of Na^+ (Robello et al., 1984), although it is clearly more pronounced with divalent cations (Fig. 3). When going from *trans* to *cis* (upper-right quadrant in Figs. 2, 3 and 4), the current is always smaller than when it flows in the opposite direction. At low calcium concentrations, the positive current tends either to saturate or to asymptotically approach a low-slope straight line. Despite the variability of the unit-current steps, the characteristics of the rectification changed with ion concentration in a consistent way. The Table, which gives the ratios of the positive to negative currents at +90 and −90 mM, respectively, clearly indicates that the rectification

Table. Current rectification ratios as a function of calcium concentrations (in moles per liter)

Ca =	0.005	0.01	0.03	0.1	0.3	0.5
R^a =	0.15	0.17	0.26	0.32	0.4	0.76

[a] R = Ratios between channel currents recorded at +90 and −90 mV of applied voltage in symmetrical salt solutions. The current values were derived from the *I-V* curves at six different calcium concentrations.

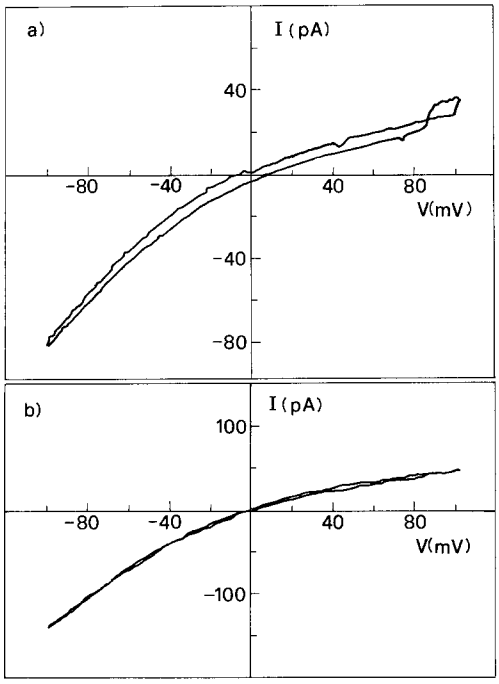

Fig. 4. *I-V* relationships for treated membrane (the toxin being added to the *cis* side) in the presence of *a*) 100 mM $BaCl_2$; *b*) 100 mM $SrCl_2$. The temperature and the pH were as in the previous Figures

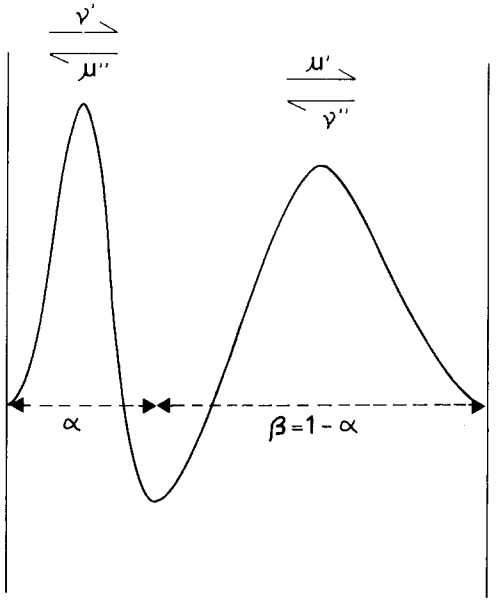

Fig. 5. Barrier model for ion movement through a one-site channel. The free energy profile refers to the case of zero applied field. The energy peaks lie halfway between adjacent sites (symmetrical barriers). The rate constants depend on the applied potential according to the Eyring rate-reaction theory

becomes attenuated when the ion concentration is increased. This trend is also illustrated in Fig. 3, where three *I-V* curves are shown at different Ca^{2+} levels. At high calcium, the shape of the curve is almost symmetrical and slightly supralinear, whereas, at 5 mM, the conductance in the positive region is much lower than in the negative one. As will be shown in the next section, this behavior can be reconciled with a simple "one-ion" model for the channel.

With Sr^{2+} and Ba^{2+} in the solutions, the insertion of the protein in the bilayer occurred more readily than with Ca^{2+}, even though the electrical properties examined, such as the order of magnitude of the current steps and the characteristics of the *I-V* curves were very similar. Experiments have been carried out with 100 mM $BaCl_2$, as well as with different concentrations of $SrCl_2$ (10, 30 and 100 mM). In the latter case, the rectification varied with ion concentration in the same way as it did with Ca^{2+}. Current-voltage curves are shown in Fig. 4 for both Sr^{2+} and Ba^{2+}. The ratio of the conductance at +90 and −90 mV is about 0.36 for both ions at 100 mM, a value that is also close to that for Ca^{2+} (0.32) at the same concentration.

A Barrier-Model for the Channel

The current-voltage curves shown in the previous section have been fitted by a simple one-ion model for a channel, which is capable of accounting both for the nonlinearity of the curves and for the concentration dependence of the rectification. It is assumed that the channel can be schematized by only two energy barriers with one site in between, the barrier peaks being located halfway between the internal site and the interfacial openings. A schematic diagram is given in Fig. 5. Since the channel is selective to cations, and since we deal with cases in which there is only one permeant cation species in the solutions, we can also assume that there is only one type of occupied site.

According to Eyring rate theory, the rate con-

stants for entering the channel are proportional to the aqueous ion concentrations and depend exponentially on the free-energy difference per unit mole (in units of RT) between the interfacial sites and the peaks of the adjacent barriers. Denoting with α the "electrical distance" between the mouth of the channel at the *cis* side, and the internal site, we shall have

$$\nu' = \bar{\nu}' c' e^{\alpha z u/2}; \; \nu'' = \bar{\nu}'' c'' e^{-(1-\alpha) z u/2} \tag{1}$$

where z is the valency, u $(= u' - u'')$ is the transmembrane potential in units of RT/F, c' and c'' are the external concentrations, and $\bar{\nu}'$ and $\bar{\nu}''$ are quantities independent of the applied potential. Analogous expressions describe the rate constants for ion transfer from the internal site into the external solutions

$$\mu' = \bar{\mu}' e^{z(1-\alpha)u/2}; \; \mu'' = \bar{\mu}'' e^{-z\alpha u/2}. \tag{2}$$

If the total number of channels per unit area, N^T, is viewed as the sum of those that are empty N_e, and those that are occupied N_o, so that

$$N_e + N_o = N^T \tag{3}$$

an expression for the mean steady-state current through the single channel can be easily calculated using standard procedures (e.g. *see* Läuger 1973). Introducing the following definitions: $\nu = \bar{\nu}''/\bar{\nu}'$, $K = \bar{\nu}''/\bar{\mu}' = \bar{\nu}'/\bar{\mu}''$ (K = binding constant to the internal site), and considering the case of a membrane interposed between similar solutions containing only one permeant ionic species ($c' = c'' = c$), the ratio of the conductance at the voltage u, $g(u)$, to that near zero voltage, $g(0)$, is found to be, for divalent cations

$$\frac{g(u)}{g(0)} = \frac{\sinh u}{u} \cdot \frac{(1+\nu)(1+Kc)}{e^{-\alpha u} + \nu e^{(1-\alpha)u} + Kc(e^{\alpha u} + \nu e^{-(1-\alpha)u})}. \tag{4}$$

Three independent parameters appear in the equations: ν, K and α. All our experimental curves, which were obtained at different concentrations of $CaCl_2$ (5, 10, 100, 300 and 500 mM), have been fitted to Eq. (4), allowing the three parameters to vary independently. Although different in shape, such curves are quite well fitted by the following set of values:[1]

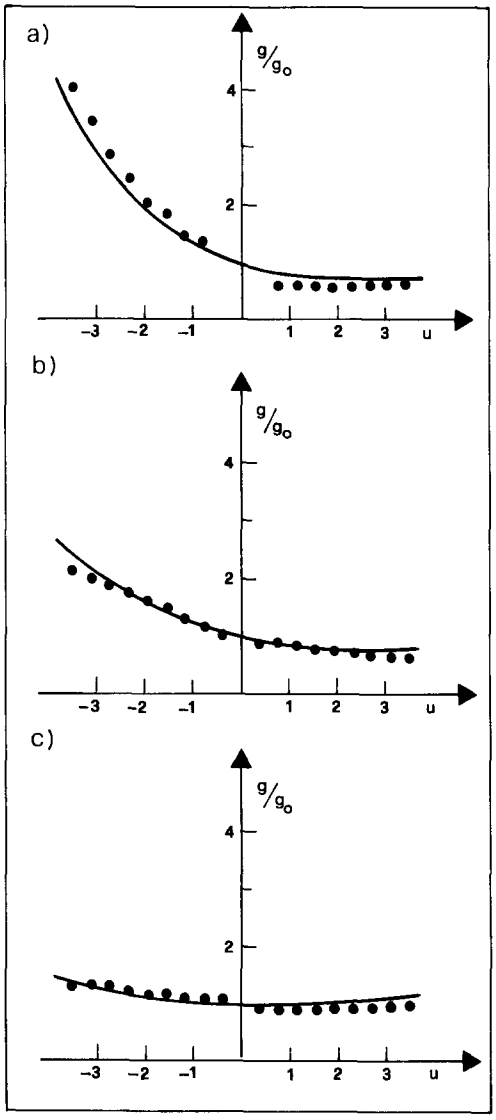

Fig. 6. Theoretical curves for the normalized conductance of the toxin channels in the presence of three solutions of $CaCl_2$. *a*) 5 mM. *b*) 100 mM. *c*) 500 mM. The solid lines were obtained from Eq. (4), using the values of the parameters ($K = 1.5$ M^{-1}, $\nu = 1.3$, $\alpha = 0.3$), deduced by simultaneously fitting the results of all the experiments with $CaCl_2$. The points were derived from the *I-V* relations of Fig. 3. u is the potential in units of RT/F

$$\nu = 1.3, \; K = 1.5 \text{ M}^{-1}, \; \alpha = 0.3. \tag{5}$$

According to the formalism of Eyring theory, the value of 1.3 for ν would correspond to a free energy difference between the peaks of 0.26 kT. The comparison between theoretical curves and the data for the conductances, normalized to their zero-current value, is presented in Fig. 6 for symmetrical solutions of $CaCl_2$ at the three concentrations: 5, 100, 500 mM. In order to estimate the experimental values for $g(u)/g(0)$ (namely, the filled circles in Fig. 6,

[1] If the data are fitted using ionic activities instead of concentrations (as deduced from the activity data for $CaCl_2$, and using the Debye-Hueckel theory to calculate the activities for the calcium ions), the best-fitting value for the binding constant K becomes 7 M^{-1}, while the values for ν and α remain unchanged.

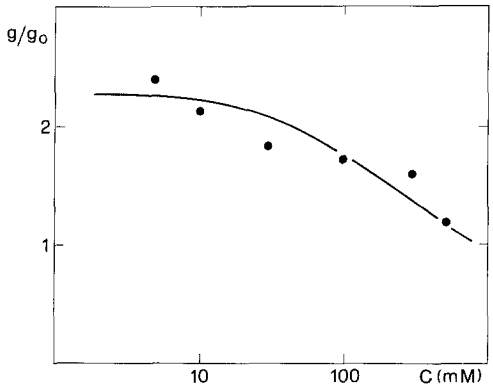

Fig. 7. Normalized conductance at a fixed voltage of α-LaTx channels (conductance at 60 mV divided by the conductance near zero voltage) as a function of $CaCl_2$ concentration. The curve is drawn according to our model for the same values of the parameters as in Fig. 6. The experimental points are derived from the *I-V* curves at six different salt concentrations

as well as in Figs. 7 and 8), the data points for $g(u)$ were obtained directly from the *I-V* relationships by calculating I/V, while the limiting values for the $g(0)$ were deduced by drawing the tangents to the *I-V* curves at the origin and by estimating their slope. The solid curves were deduced from Eq. (4), using the parameters given in Eq. (5).

Fixing the potential u, and considering c as the independent variable, Eq. (4) expresses the normalized conductance as a function of the permeant ion concentration. In Fig. 7, experimental values for the conductance at six different calcium concentrations are shown and compared with the theoretical curves for the set of parameters given in Eq. (5). Finally, Fig. 8 shows the extent to which the theoretical curves that describe the *I-V* relations for Ca^{2+} are also able to fit those for Sr^{2+} and Ba^{2+}. As can be seen, the agreement is adequate, although, with the latter two ions, the conductance increases less steeply than in the case of Ca^{2+} when the current flows from the *cis* to the *trans* side.

Discussion

Relevance of the Bilayer Studies to the Biological Effects of the Toxin

There is substantial evidence that α-LaTx, a protein component of the black widow spider venom, acts at the presynaptic nerve terminals by stimulating massive release of neurotransmitter (Frontali et al., 1976). The results of several studies on vertebrate synapses, as well as on cells of the neurosecretory PC12 cell line (Grasso et al., 1980), are consistent

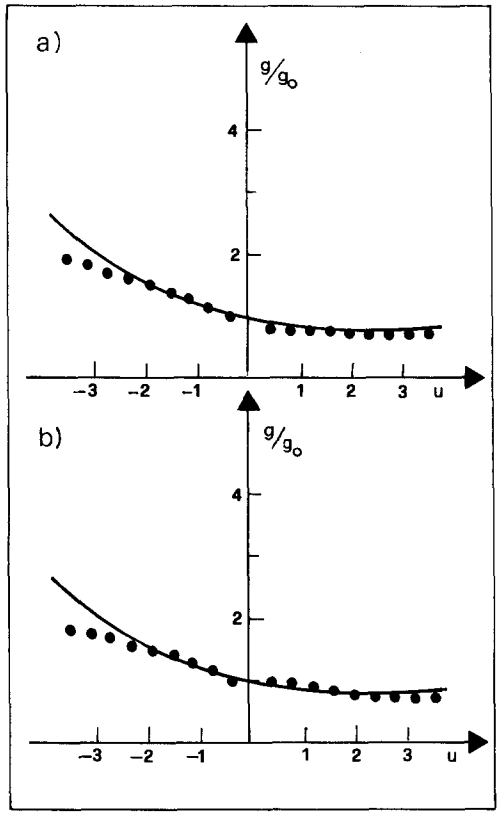

Fig. 8. Normalized conductance in the case of barium and strontium salts. *a*) 100 mM $BaCl_2$. *b*) 100 mM $SrCl_2$. The solid lines were calculated from the model using the same parameters as for calcium. The experimental points were deduced from the *I-V* curves of Fig. 4

with the hypothesis that the mode of action of the toxin is, at least in part, via the formation of ionic channels through which Ca^{2+} and other ions flow into the intracellular compartment of the presynaptic terminals. In view of these findings, it seemed interesting to incorporate the toxin in lipid bilayers and to study its electrical properties in the presence of divalent cations. Similar studies with monovalent cations have been reported previously (Finkelstein et al., 1976; Robello et al., 1984), although the current-voltage relationships were not analyzed in detail. The most apparent feature of the steady-state *I-V* curves with divalent cations is their rectification, even in symmetrical solutions; the conductance being always higher when the toxin-containing solution is held at positive potentials. Since the channels are selectively permeable to cations, the direction in which the current is higher corresponds to that of greater permeability to cations. It is important to note that, if the orientation of channel insertion in artificial bilayers is the same as in the cell membranes, the direction of greater permeabil-

ity to cations through the toxin channels inserted in the latter would correspond to that of inward flow. In this respect, the channel behavior in bilayers is consistent with the hypothesis that the toxin-mediated release of transmitter may be due to the formation of transmembrane channels facilitating the inward movement of cations.

Channel Rectification

Since asymmetric current-voltage curves are seen in bilayers made from monolayers of the same lipid and separating identical solutions, it seems plausible to assume that the rectification originates from properties inherent to the pore structure. Rectifying *I-V* relationships in symmetrical solutions can be modeled in various ways; for example, by extending the continuous Nernst-Planck formalism to include a nonsymmetrical barrier shape. However, this approach is not suitable for explaining the effects of ion concentration on the rectification, unless a dependence on ion concentration and voltage for the partition coefficient is somehow introduced in the model. These effects, instead, are accounted for quite naturally by the two-barrier model described in the previous section, where the fitting of the *I-V* curves only requires the barriers to have different heights, and the internal site to be located off center. One of the parameters, namely the binding constant K, should also be independently deducible by studying the value of the single-channel conductance (at a fixed voltage) as a function of ion concentration. Although this study was difficult to carry out with divalent cations (due to the variability of the step sizes and the low frequency of the events), a similar analysis has been done with potassium ions (Lassa, 1985), demonstrating that the average value of the conductance saturates with ion concentration, and that the binding constant determined with this method has the same value as that deduced by fitting the conductance ratios.

Alternative mechanisms that would account for the rectification are possible. For example, although the lipid used was neutral, a charged group at one end of the channel might have altered the ion concentration near to one of the openings, thus producing asymmetric boundary conditions and rectifying current-voltage relations. Screening of this group would then explain the decrease of the rectification at high ion concentration. However, while in this case the screening effect of divalent cations would be expected to be greater than that of monovalent cations, the opposite is shown by the experiments, since the rectification is more pronounced in 100 mM $CaCl_2$ than in the same concentration of NaCl (Robello et al., 1984).

Still another possibility to consider is that in which the conductance of the single pore would be small and ohmic, and the "apparently" continuous variations of the *I-V* slope would be due to a voltage-dependence of the fraction of open channels. However, this hypothesis would not be easily reconcilable with the finding that the conductance ratio, $g(u)/g(0)$, varies with the ion concentration in the solutions; unless, of course, one were to make the more elaborate assumption that the probability of channel opening is a function of both voltage and ion concentration.

In sum, although alternative possibilities cannot be excluded, the model we use seems quite attractive on account of its simplicity and its ability to fit several data with a small number of parameters.

Variability of the Current Steps

As we have already mentioned, the sizes of the current steps were quite variable and not clearly recognizable as multiples of a common unit value. However, the characteristics of the rectification were very well determined at a given ion concentration, and did not depend either on the size of the steps or on the number of steps prior to the voltage sweep. A possible explanation for this variability may be that the ions in solution, particularly the divalent ones, promote the formation of molecular aggregates, so that clusters of channels would get incorporated in the membrane at once. This assumption would account for the similarity of the conductance ratios at a given ion concentration, since, in this case, the variability of the steps would simply reflect the variability of the number of channels in the cluster, without implying changes in the single-channel properties. Pursuing this hypothesis, it is easy to realize, from the data obtained, that the conductance of the "true" single channel would be expected to be quite small. In fact, since 30 pS is the smallest conductance step measured for 0.5 M $CaCl_2$ (and therefore represents an upper limit for the "true" value of the single-channel conductance at that concentration), the corresponding upper limits for the single-channel conductance values at lower salt concentrations can be deduced from the binding constant, $K = 1.5\ M^{-1}$, and from the equation, easily deducible from the model

$$\frac{g(0)_1}{g(0)_2} = \frac{C_1}{C_2} \cdot \frac{1 + Kc_2}{1 + Kc_1} \tag{6}$$

which gives the ratio of the zero-current conductances at two different ion concentrations. For 0.1

and 0.005 M of $CaCl_2$ one finds 9 and 0.5 pS, respectively.

The hypothesis that the actual single-channel conductance may be small is also consistent with recent patch-clamp studies of α-LaTx channels incorporated in PC12 cell membranes (Wanke et al., 1986). In solutions of 150 mM NaCl, steps of currents were found that corresponded to a conductance of 15 pS, a value that is one order of magnitude smaller than that observed with artificial bilayers in similar ionic conditions.

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