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Channels produced by spider venoms in bilayer lipid membrane: mechanisms of ion transport and toxic action

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The selectivity of ion channels produced by latrotoxin obtained from a black widow spider venom and by venom from the spider *Steatoda paykulliana* in bilayer phospholipid membrane was studied. Experimental current-voltage curves of these channels were used for the estimation of parameters of a two barrier model of their energy profiles. Selectivities of both types of channels are similar. Alkaline earth cations are permeable, the permeability increasing in the order $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$. In contrast transition metal cations block the channel, their efficiency decreases in the order: $Cd^{2+} \gtrsim Ni^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+}$ (*Steatoda paykulliana* spider venom) and $Cd^{2+} > Co_2^{+} > Ni^{2+} > Zn^{2+} > Mn^{2+}$ (latrotoxin). Amplitudes of current carried by corresponding ions are mainly determined by the depth of the potential well for this ion, i.e., by its affinity to the cation binding site in the channel. The channels are also permeable to monovalent cations but they do not bind them. Selectivity for monovalent cations depends on Ca^{2+} concentration at the cis-side of membrane in the micromolar range. However, the addition of Ca^{2+} to the trans-side up to 10 mM does not affect currents carried by monovalent ions. It is suggested that venom-induced calcium channels have two conformational states with different selectivities which interconvert upon binding one calcium ion. Possible general schemes for the organisation of calcium channels in excitable membranes are also discussed. Finally, using a mathematical model of synaptic transmission, possible mechanisms of toxic action of spider venoms are considered.

Introduction

Elucidation of the molecular mechanisms of ion transport in biological membranes is one of the most important problems of modern membranology. Great advances in this direction were achieved using model systems simulating selective pathways for ion transfer through membranes [1]. However, a vast majority of these systems is permeable only to monovalent cations. Therefore, isolation, identification and extensive investigation of channel-forming substances selective to Ca^{2+} and Mg^{2+} is of great importance. These studies may provide valuable information concerning molecular mechanisms which control the functioning of putative calcium channels in different excitable cells [2].

Comparatively recently, Finkelstein and coworkers [3] isolated a neurotoxin (latrotoxin) from a black widow spider venom which forms ionic channels permeable to divalent cations in phospholipid bilayers. It was shown [4] that, in some respects, ion-transporting properties of these

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channels resemble those found for calcium channels of excitable membranes. Sokolov et al. [5] observed the formation of ionic channels in bilayer lipid membranes by *Steatoda paykulliana* spider venom. Their properties are similar to those of latrotoxin. Moreover, it was shown [6] that this venom, like latrotoxin, induces a sharp increase of the frequency of miniature endplate potentials followed by its decrease and a complete block of synaptic transmission. These facts indicate a close similarity between the mechanisms of toxic action of latrotoxin and *S. paykulliana* spider venom.

This paper is devoted to a detailed study of the selectivity of channels formed by both toxins. We discuss also possible general schemes of organisation of calcium channels and probable mechanisms of toxic action of spider venoms.

Materials and Methods

In our work we used crude venom from the *Steatoda paykulliana* spider and latrotoxin isolated from the venom of black widow spider (*Latrodectus mactans tredecimguttatus*). Crude venoms were eluated by 0.02 M Tris-HCl buffer (pH 8.05) from the venom glands of adult female spiders. These venom solutions were frozen and stored at $-70°C$. The solution was thawed before using. Latrotoxin was isolated by means of FPLC liquid chromatography (Pharmacia) with a 'Mono-Q' column. The eluate contained 0.02 M Tris-HCl buffer (pH 8.05) with an NaCl concentration gradient. The latrotoxin peak was eluated with 0.33 M NaCl. By observation of polyacrylamide gel electrophoresis and immunoelectrophoresis, homogeneous protein of 130 kDa molecular weight was obtained. Its LD_{50} was 20 mg/g of mouse body weight. This value approaches that from Grasso [7].

Bilayer lipid membranes were formed by the Mueller et al. [8] technique across a 0.6 mm diameter hole in an Teflon cup placed in a glass cell. The membranes were made from a mixture of phosphatidylcholine and cholesterol, containing the lipids at a weight ratio of 2:1. Heptane was used as solvent with a lipid concentration of 20 mg/ml. Phosphatidylcholine was isolated from egg yolk according to Ref. 9. Cholesterol was from 'Serva'. Electrolyte solutions bathing the membrane contained 10 mM Tris-HCl (pH 7.4) and the required quantities of chloride salts of different metal cations.

Two Ag/AgCl electrodes connected with both compartments were used to transmit current through the system under voltage-clamp conditions. Current versus time plots and current-voltage curves were made on an Endim 620.02 X-Y recorder.

To remove venom not bound to the membrane and to change ionic composition of the solution at the cis-side of the membrane we applied a perfusion procedure [10]. In all experiments toxins were added at the cis-side of the bilayer lipid membranes in the concentration 0.5 μg/ml and 10^{-9} M for *S. paykulliana* spider venom and latrotoxin, respectively. The potential at the trans-side of the membrane was taken as zero. All experiments were carried out at room temperature, 20–22°C.

Theory

For the description of the energy profile of channels formed by spider venoms in lipid bilayers we used a two-barrier model. This is the simplest model approximating the channel energy profile (Fig. 1) which assumes the single ion occupancy of the channel. A single channel current is given by the difference of unidirectional fluxes each equal to the product of the occupation number of the empty or ion-containing states of the channel times the rate constant for exit from this state [11]. In the case of two permeant ions A and B, a lengthy but straightforward formula leads to Eqn. 1 (below) e and z are the electron and the ion charges, respectively, C_i is its concentration from the cis- or trans-side of the membrane, k_j and k_{-j} are the rate constants of ion transfer across j-th potential barrier in opposite directions.

Contrary to earlier investigations [12], the effects of surface potential on ion-transport processes in the channel were not taken into account, because membranes containing only neutral lipids were used. The potential dependence of

$$i = e\frac{z_A(k_1^A k_2^A C_{out}^A - k_{-1}^A k_{-2}^A C_{in}^A)(k_{-1}^B + k_2^B) + z_B(k_1^B k_2^B C_{out}^B - k_{-1}^B k_{-2}^B C_{in}^B)(k_{-1}^A + k_2^A)}{(k_{-1}^A + k_2^A)(k_{-1}^B + k_2^B) + (k_1^A C_{out}^A + k_2^A C_{in}^A)(k_{-1}^B + k_2^B) + (k_1^B C_{out}^B + k_{-2}^B C_{in}^B)(k_{-1}^A + k_2^A)} \quad (1)$$

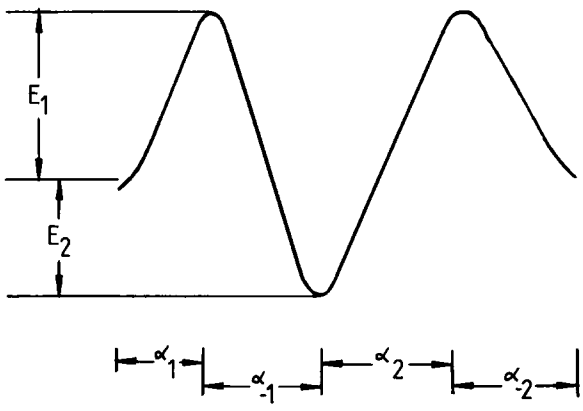

Fig. 1. Two-barrier model of the channel energy profile.

rate constants was written as follows:

$$k_j = k_{j0} \exp(-z\alpha_j FU/RT);$$

$$k_{-j} = k_{-j0} \exp(z\alpha_{-j} FU/RT) \quad (2)$$

where U is applied voltage, α is the relative distance from the bottom of the potential well to the peak of the potential barrier (so called electrical distance, Fig. 1), and k_{j0} and k_{-j0} are rate constants for $U = 0$. To reduce the number of model parameters, we assumed that all barriers have the same height and are symmetrically shaped, i.e., $\alpha_j = \alpha_{-j}$. Thus, the rate constants were determined in the following way:

$$k_{10} = k_{-20} = \mathscr{K}\frac{kT}{h}\exp\left(-\frac{E_1}{RT}\right);$$

$$k_{-10} = k_{20} = \mathscr{K}\frac{kT}{h}\exp\left(-\frac{E_1 - E_2}{RT}\right) \quad (3)$$

where E_2 is the depth of the potential well separating two barriers, k and h are the Boltzmann and Plank constants, respectively, T is absolute temperature and $\mathscr{K}$ is the transmission coefficient taken as unity. In this formulation the values of E_i correspond to free energy of an ion in the channel. The value of E_2 was used for calculation of the dissociation constant of the corresponding ion with the channel. According to Kostyuk et al. [12], it can be written as the ratio between 'off' and 'on' rate constants:

$$\frac{k_{-10}}{k_{10}} = \exp(-E_2/RT) \quad (4)$$

Thus, ionic permeability of the channel to a certain cation in the framework of this two-barrier model can be described using only three parameters: E_1, E_2 and $\alpha = 2\alpha_1 = 1 - 2\alpha_2$.

The total ionic current through the membrane modified by spider venoms is proportional to the single channel current and the channel density. Although the concentration of toxins in the solutions was constant in all experiments, the channel density could be slightly different. Therefore we calculated the proportionality coefficient between the total current and single channel current by minimizing the mean square error between the experimental and the corresponding theoretical current-voltage curve. From Eqns. 1–3, we see that using this approach one cannot determine the absolute height of the potential barriers. We obtained this value from measurements of the single channel conductance. The height of the potential barrier for other divalent cations was calculated using the measured value of reversal potential U_0 in symmetric conditions. From Eqns. 1–3 it can be shown that this value does not depend on other model parameters and is given by the equation:

$$E_1^X - E_1^{Ca} = RT \ln \frac{C_{out}^X \exp(-2U_0F/RT) - C_{in}^{Ca} \exp(2U_0F/RT)}{C_{in}^X \exp(-2U_0F/RT) - C_{out}^{Ca} \exp(2U_0F/RT)} \quad (5)$$

Results

After addition of both types of spider venom to the solution at the cis-side of the bilayer lipid membrane we observed a large increase of transmembrane current. Usually discrete changes of conductance were observed (Fig. 2a, b). The amplitude histograms for these unit events had a single sharp maximum (Fig. 2c, d) corresponding to unitary conductance of 100 pS (latrotoxin) and 20 pS (*S. paykulliana* spider venom). These data were used for estimation of the barrier height of the channels for Ca^{2+} (parameter E_1^{Ca}). Calculating single channel currents according to Eqn. 1 we obtained $E_1^{Ca} = 9.5$ and 9.1 RT for latrotoxin and *S. paykulliana* spider venom, respectively.

Current-voltage curves recorded for these chan-

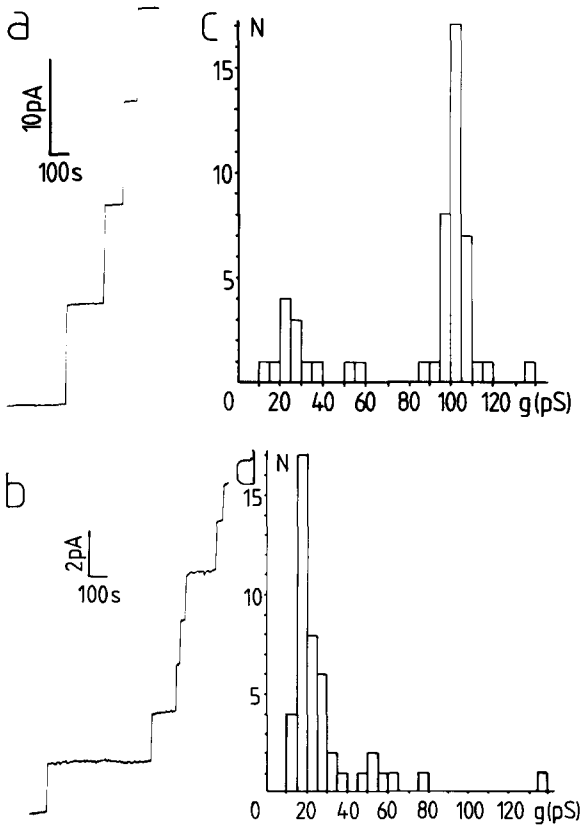

Fig. 2. Conductances of single channels formed by latrotoxin and *S. paykulliana* spider venom in bilayer lipid membrane. Current fluctuations of the membrane at +100 mV were applied. (a) latrotoxin at a concentration of 10^{-9} M and (b) *S. paykulliana* spider venom at a concentration of $5 \cdot 10^{-7}$ g/l were added to the cis-side of the membrane which separated solutions containing 10 mM Ca^{2+}. An amplitude histogram of conductance fluctuations of the membranes under the same conditions for latrotoxin (c) and *S. paykulliana* spider venom (d) was made.

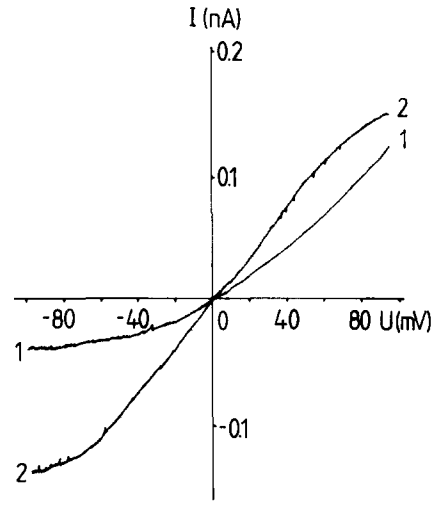

Fig. 3. Current-voltage curves of bilayer lipid membranes treated by latrotoxin. (1) latrotoxin was added to the cis-side of bilayer lipid membrane at a concentration of 10^{-9} M; (2) latrotoxin was added to the both sides of the bilayer lipid membrane at the same concentration. The membrane separated solutions containing 10 mM Ca^{2+}.

nels were usually concave (Fig. 3). The decrease of their steepness for negative potentials was connected, probably, with the closure of channels. Making membrane potential more negative we observed some increase of channel closing frequency. Unfortunately, we could not study these phenomena qualitatively because of the extremely long duration of the mean open time for these channels (see Fig. 2a, b).

Our experimental data confirm the observation made by Grasso and co-workers [13] about asymmetric incorporation of latrotoxin in planar bilayer membrane. This can be illustrated by the fact that after introduction of the venom at the cis-side of bilayer lipid membrane its introduction at the trans-side of the membrane transformed the concave current-voltage curve into an essentially linear one (Fig. 3).

Fig. 4 shows that elevation of the Ca^{2+} concentration at the cis-side of the membrane increased the current at positive potentials. This effect was accompanied by a shift of the reversal potential whose values coincide within $\pm$ 1 mV of the Nernst equilibrium potential for Ca^{2+}. From this we may conclude that the channels formed by spider venoms in bilayer lipid membranes are permeable only to Ca^{2+}, whereas $Tris^+$ and Cl^-, also present in the bathing solution, are not permeable.

Parameters of a two-barrier model of the energy profile of channels formed by venoms in bilayer membrane were found by minimizing the mean square difference between corresponding experimental and theoretical current-voltage curves calculated using Eqns. 1–3. The best fit of all experimental current-voltage curves was achieved for $\alpha = 0.3$. Thus, for every ion X considered in the model (other than Ca^{2+}) we had to determine

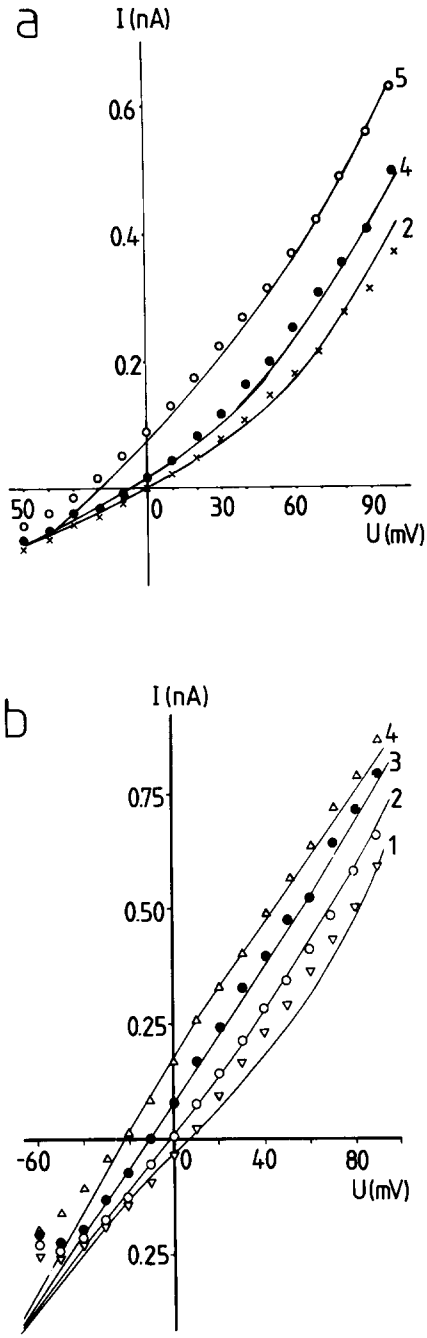

Fig. 4. Experimental (points) and theoretical (curves) current-voltage characteristics of the channels formed by spider venoms in bilayer lipid membrane. (a) latrotoxin, (b) *S. paykulliana* spider venom. Solution at the bilayer lipid membrane trans-side contained 10 mM Ca^{2+}. Cis-side solution contained: (1) 5 mM, (2) 10 mM, (3) 20 mM, (4) 50 mM and (5) 100 mM Ca^{2+}.

only the depth of the potential well (parameter E_2^X), because the height of the potential barrier E_1^X was estimated independently of the measured reversal potential in biionic symmetrical conditions ($C_{trans}^{Ca} = C_{cis}^X = 10$ mM, Figs. 5 and 6). Current-voltage curves and the quality of their approximation for both toxins were quite similar. Figs. 4–6 show representative examples of such fitting of experimental data by our model. Its parameters for different divalent cations are listed in Table I. Overall agreement between the theoretical and experimental data indicates that our model, despite it simplicity, adequately describes the selectivity properties of the channels formed by the spider venoms in bilayer lipid membrane. The improvement of the approximation of experimental data can be achieved using more sophisticated models, e.g., a one-ion three barrier model or taking into account the effects of ion-ion interaction. However, this effect seems to arise only due to the addition of extra parameters to the model and does not correspond with better representation of the energy profile in the channel (see also Discussion).

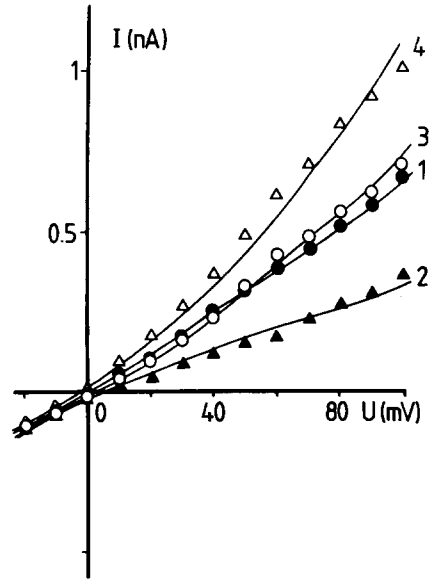

Fig. 5. Experimental (points) and theoretical (lines) current-voltage characteristics of the channels formed by latrotoxin in bilayer lipid membrane. The solution at the trans-side of the bilayer lipid membrane contained 10 mM Ca^{2+}. The cis-side solution contained 10 mM Ca^{2+} (1), Mg^{2+} (2), Sr^{2+} (3) and Ba^{2+} (4).

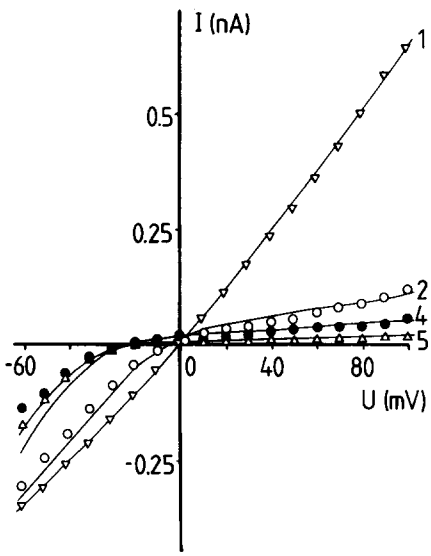

Fig. 6. Experimental (points) and theoretical (lines) current-voltage characteristics of the channels formed by *S. paykulliana* spider venom in bilayer lipid membrane. The solution at the trans-side of the bilayer lipid membrane contained 10 mM Ca^{2+}. The cis-side solution contained 10 mM Ca^{2+} (1), Mn^{2+} (2), Co^{2+} (4) and Cd^{2+} (5).

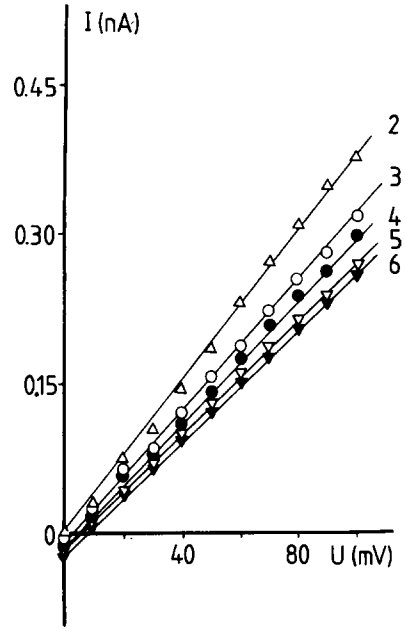

Fig. 7. Permeability of the channels formed by *S. paykulliana* spider venom for monovalent cations. The solution at the bilayer lipid membrane trans-side contained 10 mM K^+. The cis-side solution contained 10 mM K^+ (2), Rb^+ (3), Cs^+ (4), Na^+ (5) and Li^+ (6).

The potential well in our model of the channel energy profile corresponds with the binding site in the channel. According to Eqn. 2, the affinity of this site can be expressed as the dissociation constant K_d. Table II lists the values of $pK_d = -\log K_d$ obtained using this model.

Channels formed in bilayer membranes by spider venoms were permeable also to monovalent cations. Currents carried by different alkaline metal cations in biionic symmetrical conditions were comparable in magnitude (Fig. 7). The best fits for the corresponding current-voltage curves were obtained when the depth of the potential well was set at zero. In other words, contrary to alkaline earth cations, these ions are not bound in the channel. Therefore, for a description of their current-voltage curves, we used the Goldman equation which treats ion transfer of the channel as one-dimensional diffusion. Using this equation and the experimental data presented in Fig. 7 we calculated permeabilities for monovalent cations

TABLE I

PARAMETERS OF TWO-BARRIER MODEL (IN *RT* UNITS) OF THE ENERGY PROFILES OF CHANNELS FORMED BY SPIDER VENOMS IN PLANAR BILAYER MEMBRANE

Parameter	Mg^{2+}	Ca^{2+}	Sr^{2+}	Ba^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}	Zn^{2+}	Cd^{2+}
E_1 [a]	9.8	9.5	9.7	9.4	9.8	8.7	9.5	9.5	9.5
E_2	−4.2	−3.8	−3.4	−3.4	−5.8	−7.4	−6.0	−5.8	−7.8
E_1 [b]	9.5	9.1	9.4	9.1	8.8	8.7	8.3	7.7	8.3
E_2	−4.8	−4.6	−4.1	−4.0	−7.0	−7.6	−9.4	−8.9	−9.4

[a] Latrotoxin.
[b] *S. paykulliana* spider venom.

X relative to potassium permeability. The values of $P_X/P_K = \exp(-U_0F/RT)$ were obtained from the measurements of reversal potentials U_0 in symmetrical biionic conditions ($C^K_{trans} = C^X_{cis} = 10$ mM). For *S. paykulliana* spider venom the following sequence was obtained $P_K : P_{Rb} : P_{Cs} : P_{Na} : P_{Li} = 1 : 0.85 : 0.8 : 0.7 : 0.65$. A similar series was obtained for latrotoxin $P_K : P_{Rb} : P_{Cs} : P_{Na} : P_{Li} = 1 : 0.8 : 0.85 : 0.9 : 0.5$. In the latter case, however, we used other experimental conditions ($C^{Ca}_{trans} = C^X_{cis} = 10$ mM).

The permeability of channels to monovalent cations is dependent on Ca^{2+} concentrations at the cis- but not at the trans-side of membrane. In these experiments, Ca^{2+} concentration was varied from 0.01 μM to 50 mM. Concentration of free Ca^{2+} in a micromolar range was fixed by the addition of certain amounts of Ca^{2+} to the solution containing 1 mM EDTA. Concentration of free Ca^{2+} was calculated using the values of initial Ca^{2+} and EDTA concentrations and the apparent dissociation constant for the Ca-EDTA complex equal to $2 \cdot 10^{-8}$ M at pH 7.2 [14].

Fig. 8a shows that Ca^{2+} present at the cis-side of the membrane caused suppression of potassium current through the latrotoxin-induced channels and a shift of reversal potential. This 'blocking' effect of Ca^{2+} was potential independent within the whole range of membrane potentials applied. In contrast with increasing Ca^{2+} content at the trans-side of the membrane up to several millimoles we did not observe any changes of either reversal potential or the current amplitude. Nevertheless, when Ca^{2+} ions were present at the trans-side of the membrane the reversal potential shifted after addition of small amounts of Ca^{2+} to the cis-side of bilayer lipid membrane. In this case the amplitude of 'outward' current increased at positive potentials (Fig. 8b). For *S. paykulliana* spider venom we observed the same effects.

Transition metal cations block both calcium and potassium currents through the channels formed by latrotoxin with about the same efficiency. Fig. 9 shows corresponding current-voltage curves recorded in solution is containing $C^{Ni}_{cis} = 10$ mM and $C^K_{trans} = 10$ mM or $C^{Ca}_{trans} = 10$ mM. It can be seen that the suppression of 'outward' calcium or potassium current at positive potentials was about 90%. In the case of potassium current a

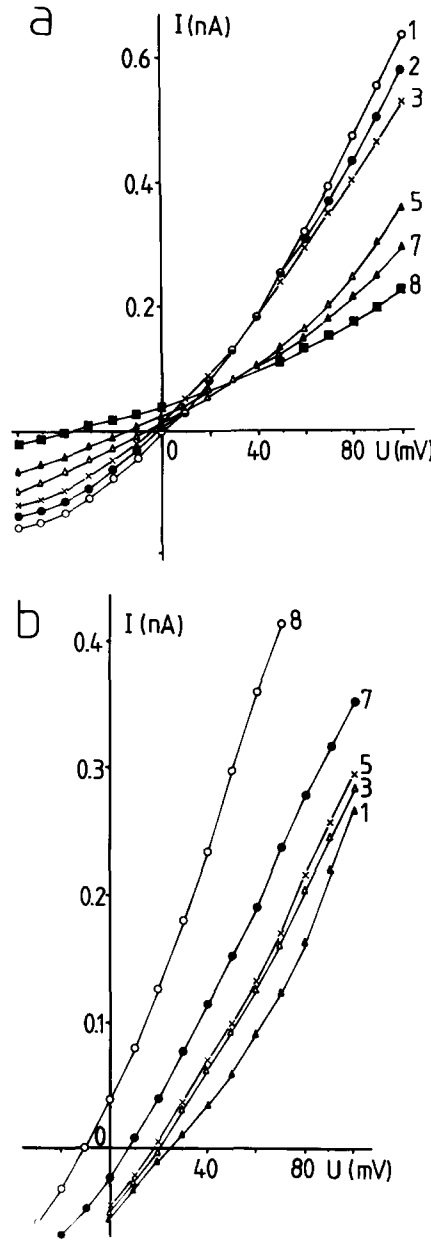

Fig. 8. Influence of Ca^{2+} on K^+ permeability of the channels formed by latrotoxin in bilayer lipid membrane. Solution at the bilayer lipid membrane trans-side contained 10 mM K^+ (a) and 10 mM Ca^{2+} (b). The cis-side solution contained 10 mM K^+. The cis-side concentration of Ca^{2+} was: 0 (1), 0.03 μM (2), 0.2 μM (3), 0.1 mM (5), 1.5 mM (7) and 9 mM (8).

shift of the reversal potential was also observed. It had the same absolute value as in the inverted system ($C^{Ca}_{trans} = C^K_{cis} = 10$ mM, Fig. 8b) but was of

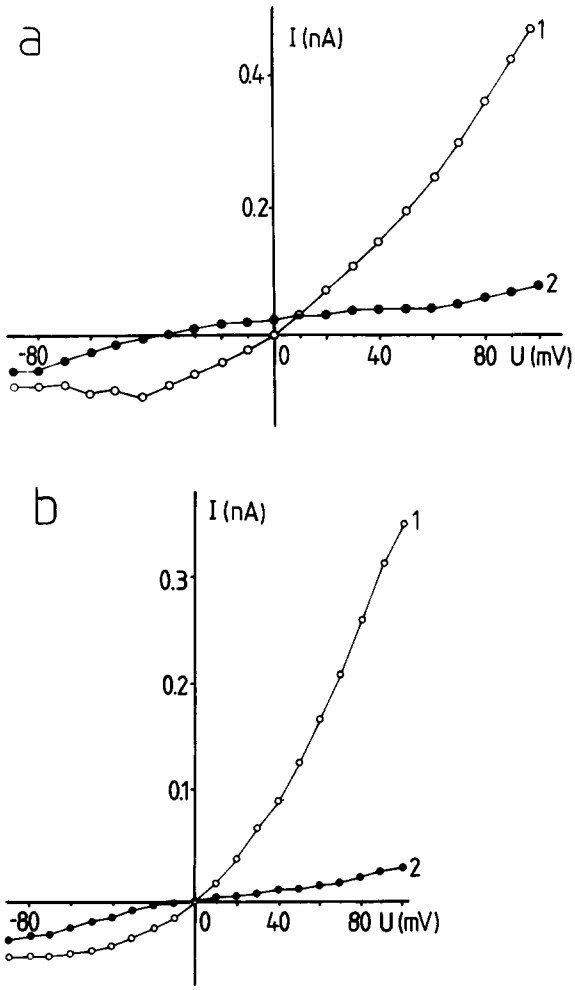

Fig. 9. Blocking of latrotoxin channels by Ni^{2+}. (a) potassium current, (b) calcium current. (1) current-voltage curve of the channels at symmetrical conditions ($C^{K}_{cis} = C^{K}_{trans} = 10$ mM (a) and $C^{Ca}_{cis} = C^{Ca}_{trans} = 10$ mM (b)), (2) the substitution of the penetrating cation at the cis-side of the bilayer lipid membrane for 10 mM Ni^{2+}.

the opposite sign because K^+ replaced Ni^{2+} at the cis-side of the membrane.

Neither nifedipine nor verapamil, which are considered specific blockers of Ca^{2+} conductance, affected the currents through venom-induced channels in bilayer membranes.

Discussion

Isolation and identification of spider venoms as inducers of passive Ca^{2+} transport through bilayer membranes is an important step in our understanding of molecular mechanisms of Ca^{2+} transport processes in excitable membranes. These toxins are proteins with molecular weight of about 130 kDa. It should be mentioned that estimation of the molecular weight of the calcium channels from skeletal muscle membranes gave a value of 136 kDa [16].

Contrary to other artificial ion-transporting systems and those isolated from different native sources, which increase the permeability of bilayer membranes mainly for monovalent cations [1], latrotoxin and *S. paykulliana* spider venom form transmembrane channels permeable to alkaline earth metal cations. These channels possess some properties similar to the calcium channels of excitable membranes. First of all, their incorporation in bilayer membrane is a strongly asymmetric one. Secondly, the probability of their opening depends on the membrane potential. Such potential dependence of channel 'activation' is, however, less great than for putative calcium channels. The mean open time of these channels is usually about several minutes, being considerably larger than that of calcium channels of excitable cells [2].

TABLE II

VALUES OF pK_d FOR COMPLEXES OF DIVALENT CATIONS WITH SPIDER VENOMS, CALCIUM CHANNELS OF NERVE CELLS AND AMINO ACIDS

System	Mg^{2+}	Ca^{2+}	Sr^{2+}	Ba^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}	Zn^{2+}	Cd^{2+}
Latrotoxin	1.8	1.7	1.5	1.5	2.1	3.2	2.6	2.5	3.4
S. paykulliana spider venom	2.0	1.9	1.7	1.6	2.9	3.2	4.0	3.7	4.0
Mollusc neurones [a]	–	2.0	1.7	1.0	3.5	3.2	–	–	4.3
Mammalian neurones [a]	–	1.3	1.3	0.7	2.6	2.5	–	–	4.3
Amino acids [b]	1.9	1.5	1.1	1.0	2.8	4.3	5.2	4.6	4.0

[a] Data taken from Ref. 12.
[b] Data taken from Ref. 21.

Channels induced by these venoms bind divalent cations. This binding is responsible for the selectivity properties of these channels. Alkaline earth metal cations, whose affinity to the corresponding channel binding site is relatively weak ($pK_d \approx 2$), are permeable cations, whereas transition metal cations possessing a larger affinity are less permeable and effectively block Ca^{2+} conductance. The same principles also hold for calcium channels in excitable membranes [2].

However, channels formed by spider venoms do not interact with the typical organic calcium channel inhibitors, nifedipine and verapamil. They also do not inactivate during prolonged depolarisation. But it must be noted that these properties are also not universal for calcium channels in excitable membrane.

Recently, Finkelstein and co-workers [17] incorporated the voltage-dependent calcium channels in the bilayer membrane by fusion of vesicles preapred from the membrane fraction of *Paramecium cilia*. They found two types of channel with different conductance (1.5 and 30 pS, respectively). The channels with greater conductance had selectivity properties similar to spider venom-induced channels.

In the present work we made a detailed study of cation permeability of channels formed by spider venoms in the planar bilayer membrane. The parameters of the channel energy profile for different divalent cations were approximately the same for both toxins (Table I). Calculated dissociation constants were also close to those of calcium channels of excitable membranes (Table II).

For transition metal cations K_d values for Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} form the sequence known from inorganic chemistry as the Irwing-Williams series established from the studies on the binding of these cations with nitrogen- and oxygen-containing ligands. By averaging the data compiled by Martell and Smith [18] for K_d values of complexes of alkaline earth and transition metal cations with amino acids and short peptides in aqueous solutions, we obtained the mean K_d values listed in Table II. It is known [19] that these cations interact with amino acids forming coordination bonds with nitrogen belong to an amino group and oxygen of a carboxylic group. Therefore, while calculating these mean K_d values we did not take into account sulfur-containing amino acids and histidine which provide additional bonds for coordination of metal cations and consequently have much larger pK_d values.

Close similarity between pK_d values for complexes of metal cations with spider venoms and putative calcium channels possibly indicate that a common mechanism is responsible for permeability properties of model and native calcium channels. That is, ion passage through the channel includes its binding at some site located inside the channel. The stronger an ion binds to this site, the lower its permeability and the higher its blocking potency. As revealed by comparison with amino acids, this channel binding site may be formed by one amino group and one carboxylic group. Some deviations of pK_d for different systems may be attributed to differences in spatial arrangement of the groups forming this binding site.

Channels produced by spider venoms in the bilayer lipid membrane were permeable to monovalent cations. Their permeability depends on the presence of Ca^{2+} at the cis-side of the membrane. In contrast, changes of Ca^{2+} concentration at the trans-side of the membrane did not affect currents carried by monovalent cations.

Similar effects were also observed for the calcium channels in excitable membranes. In mollusc neurones the calcium channels are able to pass Na^+ and other monovalent cations after removing all Ca^{2+} from the extracellular solution by EDTA or other Ca-chelating agents [14]. On the other hand, no such effect was observed during intracellular application of EDTA. Kostyuk and colleagues [14] explained this effect as a release of Ca^{2+} from some high affinity Ca-binding site of the channel located at the external side of the membrane. This site was suggested as controlling the permeability of the channel to divalent and monovalent ions by inducing some conformational transition of the channel leading to alteration of its selectivity.

Recently these results were confirmed by Almers and McCleskey [20] for the calcium channels in frog skeletal muscle, and by Fukushima and Hagiwara [21] for mouse myeloma cells. These authors, as well as Hess and Tsien [22] for heart cells, found evidence of calcium current reversal when internal solution contained monovalent cat-

ions. For an explanation of the results obtained, these authors used the model suggested by Almers and McCleskey [20] and Hess and Tsien [22]. The essential feature of this model is the assumption of two high affinity Ca^{2+}-binding sites with pK_d = 6–7 located on the ionic pathway inside the channel and strong repulsion between ions when they fill these sites. If the channel contains one calcium ion, all potential barriers and wells rise and for the second ion the effective dissociation constant will be equal to

$$K'_d = \exp((E_0 + E_c)/RT)$$

where E_0 is the initial depth of the potential well and E_c is the energy of ion-ion repulsion. For $E_0 = -15\ RT$ corresponding to $pK'_d = 6.5$ and $E_c = 10\ RT$ $pK'_d = 2.3$. Thus, this model can explain the ability of calcium channels to pass monovalent ions at a Ca^{2+} concentration below 0.1 μM, the block of these currents by Ca^{2+} and other divalent cations for larger Ca^{2+} concentrations as well as the appearance of pure calcium current at $C_{Ca} > 1$ mM and its saturation upon increasing Ca^{2+} concentration.

Despite the simplicity and capability of the model to describe qualitatively some experimental results, it has serious shortcomings. First of all, the presence of high-affinity sites in any channel with a given conductance put upper limits on the height of barriers. If these binding sites have $K_d \cong$ 0.1 μM for the channels with conductance of 10–100 pS, the height of potential barriers must be lower than 3 RT. This is several RT lower than the diffusion limit for Ca^{2+} in water corresponding to $E_{diff} = 8.7\ RT$ [20]. Until now, in the studies of the energy profiles of different ionic channels the inequality

$$E_{barrier} \gtrsim E_{diff} \qquad (6)$$

was always fulfilled. It was also used earlier [14,23] as an argument against the possibility of the existance of such high-affinity Ca^{2+}-binding sites on the ionic pathway in the calcium channel of mollusc neurones. Although this conclusion was made on the basis of the one-ion model, inclusion of ion-ion repulsion will only strengthen it. Indeed, the one-ion model is the limiting case for any multi-ion model when ion-ion repulsion becomes so great that the channel at any one time can contain at most one ion. In general, it can be shown [24,25] that lowering the strength of ion-ion repulsion, i.e., transition from the one-ion to the multi-ion model with the same energy profile, results in the decrease of the channel conductance.

The suggestion about the possible existence of low barriers ($E_{barrier} \leqslant 3\ RT$) in the calcium channels and similarity of high-affinity binding sites to those present in such Ca-binding proteins as calmodulin, troponin C etc. also contradict direct measurements of rate constant k_{on} for interaction of Ca^{2+} with these proteins. According to experimental data [26], the values of $_{on} < 10^8\ M^{-1} \cdot s^{-1}$. These values correspond to $E_{barrier} > 9.2\ RT$, i.e., they satisfy inequality (Eqn. 6). Moreover, a sequential filling of several Ca^{2+}-binding proteins [26] occurs without significant changes in their pK_d values. This evidence also conflicts with the model with strong mutual repulsion between divalent ions in the calcium channel.

Another argument against the presence of such high-affinity Ca^{2+}-binding sites on the ionic pathway comes from the studies on the transition metal block of currents carried by monovalent and divalent ions. Almers and McCleskey [20] observed that transition metal cations block calcium and sodium currents through calcium channels with the same efficiency. They suggested an additional binding site for these inorganic calcium channel blockers because these cations can be expected to bind to the postulated high-affinity binding site much more strongly than Ca^{2+}, and their pK_d must be lower than $pK_{Ca} \approx 6$. This suggestion, however, contradicts the results of extensive studies on calcium channels [27] which convincingly demonstrated a competition between Ca^{2+} and these cations, indicating a common binding site. Our results are also consistent with these findings: 10 mM Ni^{2+} almost equally suppressed both Ca^{2+} and K^+ currents formed by spider venoms. Moreover, their block was clearly potential dependent, being stronger at positive potentials. This demonstrates that the Ni^{2+} binding site is located inside the channel and, probably, situated on the ionic pathway. If the hypothetical high-affinity binding sites are also located on the same pathway we can hardly imagine why they are not revealed in such experiments.

The last and the strongest argument against the model of calcium channel permeability suggested by Almers and McCleskey [20] concerns the asymmetry of monovalent ions current block by Ca^{2+} and other divalent cations. Their model implicitly suggests that Ca^{2+} must block currents carried by monovalent ions being applied at any side of the membrane with almost equal potency. Experiments made on mollusc neurones [14] have shown that sodium current through the calcium channels is really suppressed during the internal perfusion with solution is containing 0.5 mM Ca^{2+}. This effect developed slowly and was maximal in about 20–30 min which was considerably longer than the characteristic time needed for complete change of the perfusing solution (3–5 min). Moreover, the kinetics of sodium current through calcium channels suppression by F^- was essentially the same. Thus, these findings probably reflect indirect action of Ca^{2+} on the channel which is mediated by the system of cyclic nucleotides. A close interrelationship between intracellular Ca^{2+}, cyclic nucleotides and performance of calcium channels was convincingly shown in the studies made by Kostyuk and colleagues [28].

In our experiments, Ca^{2+}-mediated changes of potassium current through the channels induced by spider venoms in the bilayer membranes were also observed only after addition of Ca^{2+} at the cis-side of the membrane (Fig. 8).

All the facts discussed above must be kept in mind when building some reasonable models of calcium channel permeability. Unfortunately, the present version of the model suggested by Almers and McCleskey [20] and Hess and Tsien [22] cannot cope with these difficulties.

In our opinion the model suggested by Kostyuk et al. [14] is more adequate. It suggests the presence of high-affinity Ca^{2+}-binding sites which control the permeability of the channel for divalent and monovalent cations inducing some (not detected yet) conformational transitions. This high-affinity site has probably the same features as Ca^{2+}-binding sites of some specific proteins [29] and is located on the outer side of the membrane (in the case of spider venoms this is the cis-side of the membrane).

These permeability changes were observed in our experiments with spider venoms. We observed a small but statistically significant shift of the reversal potential for potassium current in the presence of micromolar Ca^{2+} at the cis-side of the membrane. This effect reflects some changes of Ca^{2+} and/or K^+ permeability of the channel. At present we cannot quantitatively describe them. If we suppose that the channel has two conformational states and each energy profile can be described by a two-barrier model for the case of one penetrating ion, we readily obtain the model considered by Läuger et al. [11]. This model contains more parameters than a simple two-barrier model. Except for two extra constants describing the conformational transition of the empty channel for the description of transfer of any ion through the channel, one must know six additional constants. Two of them correspond to conformational transition of ion-containing channel and four represent ion jumps across two potential barriers in the second conformation state. Surely there is little hope for guessing these rate constants and finding their potential dependence using only current-voltage curves measured in different experimental conditions. Kinetic measurements of open- and closed-time distributions of channels are necessary to provide additional information. In the results section we mentioned that the experiments of this kind, in the case of spider venoms, are extremely difficult due to the long duration of the open state of these channels.

However, some evidence supporting the hypothesis concerning the role of conformational transitions in the process of ion transfer through the calcium channels can be extracted from the studies on excitable membranes using the patch-clamp method. In different neuronal preparations the distribution of open times was shown to be bi-exponential [30]. A modern description of kinetic behaviour of calcium channels is based on a scheme $C_1 \leftrightharpoons C_2 \leftrightharpoons 0$ containing two closed and one open states. The second closed state may actually be open but may contain a high-affinity Ca^{2+}-binding site. Then, as it was shown above, it will be non-conducting open state.

Recently Nelson [31], for the calcium channels in rat brain, found that both the conductance and the mean open time of channels are dependent on the type of penetrating cation. In accordance with the results of studies on microscopic currents,

channel conductance decreases in the order $Ba^{2+} > Sr^{2+}$, $Ca^{2+} > Mn^{2+}$ but the mean open time corresponds to the inverted series $Mn^{2+} > Sr^{2+}$, $Ca^{2+} > Ba^{2+}$, whereas the mean closed time does not depend on the type of penetrating cation. This correlation suggests that ion affinity to the calcium channel may not only affect its conductance but also its kinetic behaviour. We hope that future studies will resolve this important problem and that additional data will provide the information needed to construct the unified picture of functional organization of calcium channels.

Appendix

For the description of synaptic transmission a simplified version of the model suggested by Llinas et al. [32,33] was used. This model is based on the following assumptions: (a) the action potential in a nerve fiber leads to an increase of Ca^{2+} permeability in the presynaptic ending. (b) Ca^{2+} enters the presynaptic ending and activates some proteins which (c) induce the fusion of acetylcholine-containing vesicles with the membrane. (d) released acetylcholine molecules migrate to the postsynaptic membrane and (e) activate acetylcholine receptors which generate a postsynaptic current. Below we list equations which describe all these stages.

(a) action potential was approximated by the following analytic expression:

$$U(t) = \frac{160}{(1+(t/2-1)^2)^2} - \frac{80}{1+(t/2-1)^2} - 70 \tag{7}$$

where time t is in ms and membrane potential $U(t)$ is in mV. (b) calcium current in the presynaptic membrane was described by the expression:

$$I_{Ca} = g_{Ca} m^5 \frac{K C_{out}^{Ca} \exp(-U/12.5) - C_{in}^{Ca}}{1 + K C_{out}^{Ca} \exp(-U/12.5)} \tag{8}$$

where $g_{Ca} = 40$ nA/cm^2 is the limiting Ca^{2+} conductance, $C_{out}^{Ca} = 10$ mM, $C_{in}^{Ca} = 0$, Ca^{2+}-binding constant $K = 100$ M^{-1} is the parameter of the model, m^5 is the relative stationary number of calcium channels in the open state. The values of $m(t)$ during action potential were found from the differential equation:

$$\frac{dm}{dt} = -(\gamma_1 + \gamma_2)m + \gamma_1 \tag{9}$$

Rate constants in this equation were determined as

$$\gamma_1 = a_1 \exp(0.06U); \ \gamma_2 = a_2 \exp(-0.01U)$$

where U is in mV, $a_1 = 2.76$ ms^{-1} and $a_2 = 0.14$ ms^{-1}. The changes of Ca^{2+} concentration in the presynaptic ending were described by equation:

$$\frac{dC_{in}^{Ca}}{dt} = \delta I_{Ca} - \beta C_{in}^{Ca} \tag{10}$$

where δ is the scale coefficient determined by dimensions of the presynaptic ending and β is the rate constant for Ca^{2+} extrusion from the ending. Expressing I_{Ca} in μA/cm^2 and C_{in}^{Ca} in μM we used the dimensionless form of this equation, putting $\delta = 1$.

(c) the formation of the active complex of Ca^{2+} with cytoplasmic protein P whose activated form is $P^* \cdot Ca$ is thought to induce the fusion of acetylcholine-containing vesicles with the presynaptic membrane was described by equation:

$$\frac{d(P^* \cdot Ca^{2+})}{dt} = \mathcal{K}_1 [Ca^{2+}]_{in} - \mathcal{K}_2 [P^* \cdot Ca^{2+}] \tag{11}$$

where $\mathcal{K}_1 = 10$ ms^{-1} and $\mathcal{K}_2 = 16$ ms^{-1} are the rate constants for complex formation and its inactivation, respectively.

(d) the diffusion of acetylcholine (ACh) in the synaptic gap was considered instantaneous, (e) the activation of acetylcholine receptors (AChR) after their interaction with agonist was approximated by the following equation:

$$d[AChR^* \cdot ACh]/dt = k_g [AChR \cdot ACh] - k_{-g} [AChR^* \cdot ACh] \tag{12}$$

where $k_g = 20$ ms^{-1} and $k_{-g} = 4$ ms^{-1} are the rate constants for activation and inactivation of acetylcholine receptor.

The amplitude of post-synaptic current J_{post} was taken proportional to the number of activated acetylcholine receptors [AChR* · ACh].

This model adequately describes the main fea-

tures of synaptic transmission – the existence of delay in development of post-synaptic current and its characteristic time dependence. Using this model, we were also able to reproduce the effects of synaptic facilitation and depression.

We have no idea about the relation of interaction of spider venoms with bilayer lipid membrane and presynaptic membrane. Our speculation is that a specific latrotoxin receptor in presynaptic membrane can accelerate the latrotoxin molecules incorporation into presynaptic membrane, while the insertion of toxin into bilayer lipid membrane is a common mechanism of its interaction with any membrane. We think that it is reasonable to consider the similarity of latrotoxin-induced channels in artificial and presynaptic membrane.

Using model parameters for Na^+, K^+, Mg^{2+} and Ca^{2+} we calculated current-voltage curves for the channels formed by venoms in the membrane bathed in solutions whose ionic composition is close to intercellular fluid and the cytoplasm (Fig. 10). We see that at the resting potential of the presynaptic membrane equal to -70 mV calcium current is half of the total current.

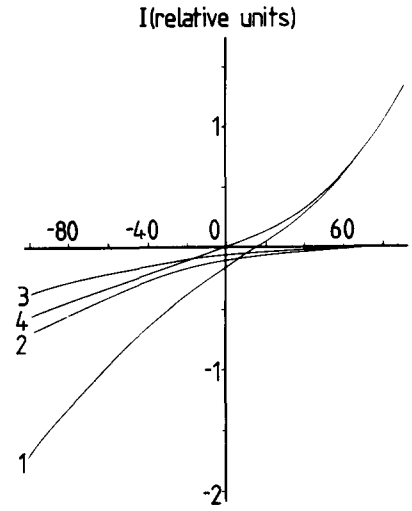

Fig. 10. Current-voltage curves of latrotoxin channels calculated in the framework of the two-barrier model of its energy profile. (1) total current, (2) calcium current, (3) magnesium current and (4) the sum of the sodium and potassium currents. The ion concentration for calculations were taken: $C_{out}^{Ca} = C_{out}^{Mg} = 10$ mM, $C_{in}^{Ca} = C_{in}^{Mg} = 0$, $C_{in}^{K} = C_{out}^{Na} = 130$ mM and $C_{out}^{K} = C_{in}^{Na} = 10$ mM.

According to Llinas et al. [32,33], acetylcholine release into the synaptic cleft is triggered by the increase of intracellular Ca^{2+} content in the presynaptic ending. Spider venoms may induce this effect by two mechanisms: (a) causing membrane depolarization by increasing inward current permeability at resting potential that may lead to the opening of putative calcium channels; or (b) raising Ca^{2+} permeability by formation of specific Ca^{2+}-selective channels.

We rejected the first mechanism because, according to our estimates, the magnitude of current induced by spider venoms in presynaptic membranes at their concentrations of 0.5 μg/ml (*S. paykulliana* spider venom) and 10^{-9} M (latrotoxin) must be about 5 nA/cm^2. This value is two orders lower than the amplitude of outward potassium current in squid giant synapse [32,33] and is insufficient to depolarize the membrane.

Spider venom seem to provide independent and direct pathways for entry of Ca^{2+}. We investigated the effect of increase of intracellular Ca^{2+} content on the amplitude of postsynaptic current (Fig. 11).

Postsynaptic current increases when the concentration of Ca^{2+} inside the presynaptic ending is raised. This dependence is non-linear and a sharp increase of J_{post} was observed for Ca^{2+} concentration above 30 nM. Addition of spider venoms to normal Ringer solutions induced the increase of frequency of spontaneous miniature potentials [6,15]. Both the amplitude of postsynaptic current and frequency of miniature potentials are determined by the quantity of acetylcholine released into the synaptic cleft. Therefore, com-

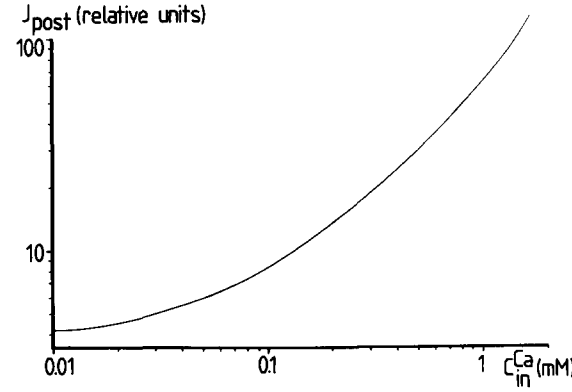

Fig. 11. Postsynaptic current amplitude versus intracellular Ca^{2+}.

paring the results of the present work with the above-mentioned experimental data we may conclude that toxic action of spider venoms may be mediated by the increase of membrane permeability for Ca^{2+}.

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