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Comparative analysis of latrotoxin channels of different conductance in planar lipid bilayers. Evidence for cluster organization

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It has been established that channels induced by *Latrodectus tredicimguttatus* α-toxin (LT) in lipid bilayers have a cluster organisation. So far as: (i) The LT-channels had practically identical sizes of its water pores ($r = 9.4 \pm 0.6$ Å) independently on the lipid composition of planar bilayer lipid membrane (BLM) although their conductances might differ from each other more than 10 times (100 mM KCl (pH 7.5)). (ii) affinity of permeable ions to channels had a small variation with distinct group of BLM, although LT-channels conductances varied from 112 ± 8 pS till 1110 ± 40 pS for phosphatidylcholine-BLM and from 75 ± 6 pS till 170 ± 14 pS for phosphatidylserine-BLM. (iii) Ca/K selectivity was greater in negatively charged membranes but did not also depend on the channel amplitude for the same BLM. Cation-anionic selectivity was identical for all studied channels.

Introduction

Practically every type of ion channel in biological and model membranes has some conducting and closed states. The several kinds of ion-channel structures have been discussed for a long time in order to explain this phenomenon [11]: the channel may have constant or variable size and charge constitution as unitary water pores or it may be organized as a cluster. However, sufficient proofs of the suggested structures do not exist yet. The reliable way to choose among the structures is not yet available. In our opinion, it should include the determination of both the charge constitution of comparable ion channels and the size of their water pores. In this way we examined ion channels forms by *Latrodectus tredicimguttatus* α-toxin (LT) in planar bilayer lipid membranes (BLM).

The conductance of LT-channels could differ a lot from each other [2–4]. Detailed comparative analysis of the properties of six types of LT-channel incorporated in two kinds of BLM allow us to conclude that these channels have a cluster organization.

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Abbreviations: LT, *Latrodectus tredicimguttatus* alpha-toxin; BLM, planar bilayer lipid membranes; PC, phosphatidylcholine; PS, phosphatidylserine; Ch, cholesterol; PEG, poly(ethylene glycol); Tris, 2-amino-2-hydroxymethyl propane-1,3-diol.

Materials and Methods

Channel-formers. Black widow spider venom has been obtained from the glands (fresh or lyophilized as noted in the text) of the spider (*Latrodectus tredicimguttatus*) by extraction of the glands.

α-Latrotoxin has been purified on Mono-Q-column by FPLC-system (Pharmacia) as described earlier [4].

Lipids. TLC-pure phosphatidylcholine (PC) and phosphatidylserine (PS) were obtained from fresh hen eggs and ox brain, respectively, according to the method described in Ref. 5. Cholesterol (Ch) was obtained from Sigma (Munich, Germany).

Chemicals. Polyethylene glycols with average molecular weights and hydrodynamic radii (r, Å), MW/r: $300/r = 6.0 \pm 0.2$ (Koch-Light), $400/r = 7.0 \pm 0.3$ (Schuchardt); $1000/r = 9.4 \pm 0.3$ (Austranal Preparate); $1500/r = 10.5 \pm 0.1$; $2000/r = 12.2 \pm 0.1$; $3000/r = 14.4 \pm 0.4$; $4000/r = 19.2 \pm 0.3$ and $6000/r = 25.0 \pm 0.3$ (Loba) were used.

Ethyleneglycol ($r = 2.62 \pm 0.03$), glycerine ($r = 3.08 \pm 0.02$), glucose ($r = 3.7 \pm 0.1$), sucrose ($r = 4.67 \pm 0.05$) and other chemicals were made in the USSR.

Hydrodynamic radii of nonelectrolytes are taken from Ref. 6.

Planar phospholipid bilayer membranes (BLM). These have been prepared by the method of Mueller et al. (1967) [7] using a 2–4% (w/v) lipid solution in n-octane. In general, two kinds of BLM were used: neutral membranes formed from a PC/Ch (4:1, w/w; PC-BLM) and negatively charged ones formed from PS (PS-BLM).

The aqueous phase was buffered with 5 mM Tris-HCl (pH 7.5) and usually contained 100 mM KCl (except in the cases of salt-dependence experiments and measurement of biionic potential values). In experiments on pore-size determination, the water phase contained 10–30% (w/v) nonelectrolyte.

The channel-forming substances from aqueous stock solutions were added only to the *trans*-compartment of experimental cell to final concentrations that were enough for the formation of single channels in BLM. The addition was followed by vigorous mixing of the solution with magnetic bars.

Electric measurement. The conductance of single-ion channels was measured at voltage-clamp conditions (50 mV). The current through the BLM was measured with Ag/AgCl electrodes connected in series with a voltage source and a current amplifier. The amplifier signal was monitored with a storage oscilloscope and recorded on a strip chart or tape recorder. The *trans*-compartment was connected to the virtual ground and the voltage sign was referred to it. The cation transference number was calculated from zero-current potentials measured in the presence of three-fold KCl concentration gradient: 40 mM/120 mM (*cis*/*trans*). Conductivity of the solutions was measured by Radelkis OK 102/1 conductivity meter.

All experiments were performed at 25 ± 1°C.

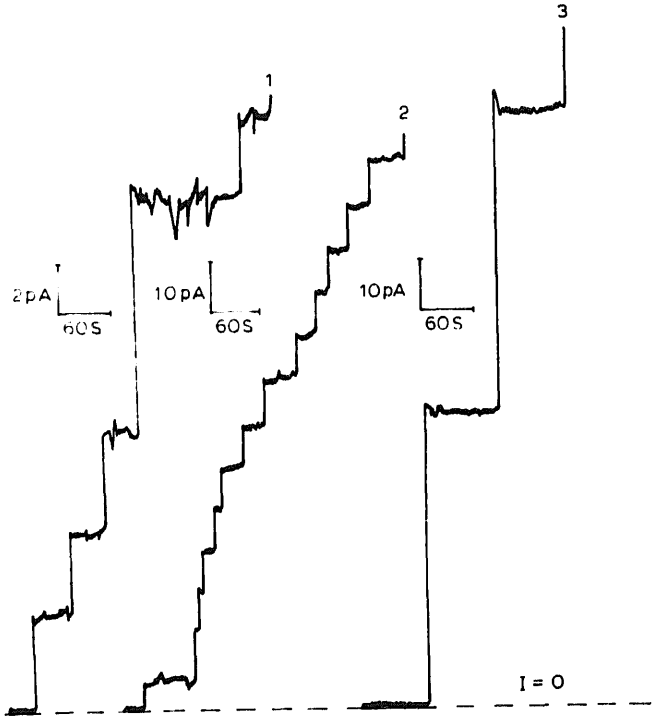

Fig. 1. Stepwise increase of membrane currents induced by some preparations of latrotoxin. Channels have been generated by whole venom which was extracted from lyophilized venom glands by KCl-containing buffer. 1, PC-BLM; 2, PS-BLM; 3, influence of purified α-latrotoxin subjected to a freeze-thaw procedure twice, on PS-BLM current. Dashed line correspond to zero current level.

Results and Discussion

In accordance with recent data [2–4], we established that the conductance of the LT-channel strongly depended on the nature of receiving venom, on the procedure of LT purification and on the lipid composition of BLM. In Fig. 1, the examples of such recordings are shown. Corresponding histograms are represented in Fig. 2. It can be seen that the conductance of LT-channels could differ a lot from each other. For further analyses, we chose the following six conducting-types of LT-channels: 74 ± 6 pS (A); 169 ± 14 pS (B); 112 ± 8 pS (C); 415 ± 20 pS (D); 565 ± 25 pS (E) and 1110 ± 40 pS (F) in 100 mM KCl.

The LT-channels tested may be separated to three groups. The first group consists of A- and B-type channels incorporated in phosphatidylcholine(PC)-BLM. The second group included C- and D-type and the third E- and F-type in phosphatidylserine(PS)-BLM. Both the treatment of the channel-forming component and the BLM composition were different between these groups.

In general, the differences of conductances among LT-channels may be determined by at least three causes: (i), by differences of charges distributed near the ion-channels' mouths; (ii), by differences in the water pores size; (iii), by the number of unitary water pores in a cluster.

To examine the contribution of the first of these possibilities we determined both the dependencies of LT-channel conductances on the KCl concentration and its selectivity. It was found that the shapes of all dependencies of LT-channel conductances on salt concentrations were similar to each other and looked usually appeared as saturation curves (Fig. 3). To describe these dependences quantitatively we expressed the data in a Michaelis-Menten relationship:

$$G = G_{max} \cdot \{[KCl]/(K_d + [KCl])\} \quad (1)$$

where K_d is an apparent constant of dissociation, G_{max} is the upper limit of ion-channel conductance and [KCl] is KCl concentration.

The K_d and G_{max}, which were in good agreement with the experimental data, were fitted by computer simulations and are represented in Table I. It can be

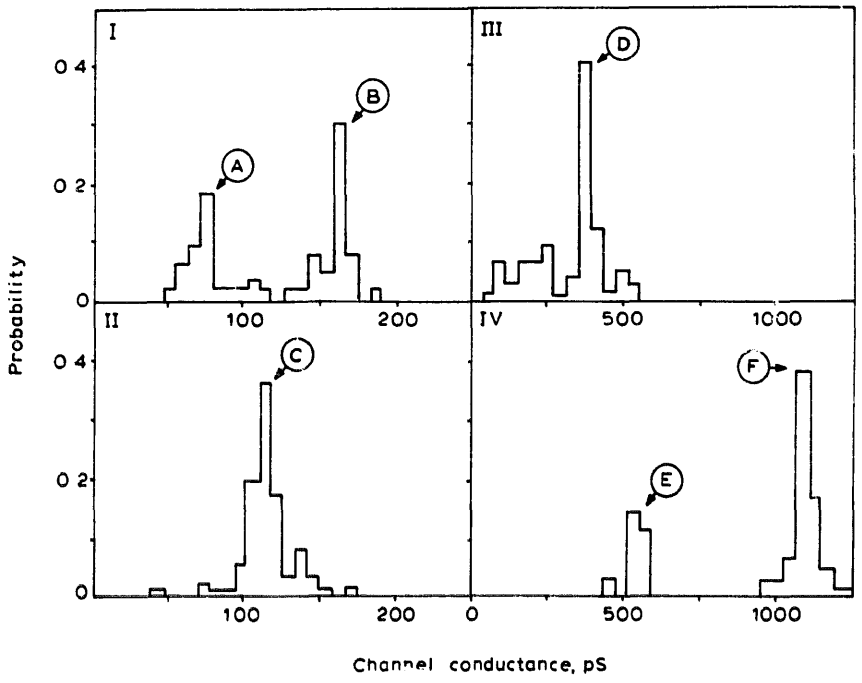

Fig. 2. Amplitude histogram of conductance fluctuations in voltage-clamped BLM treated by different preparations of latrotoxin. I, II, Channels like those in the 1 and 2 traces of Fig. 1. III, channels induced by venom which had been extracted from glands by 10 mM Tris-HCl buffer, pH 8.0; PS-BLM. IV, channels like that in the 3 trace of fig. 1. Letters A, B, C, D, E, F in circles note the type of channels used for further analysis. Ordinate: probability P to observe a channel of a given conductance during an experiment. Number of analyzed events: I, 70; II, 99; III, 76; IV, 98.

seen that the K_d values for LT-channels incorporated in PC-BLM were about 10-times less than the analogous parameters for LT-channels inserted in PS-BLM. Perhaps this difference is a result of the influence of negatively-charged BLM surfaces on both concentration and distribution of the ions near the ion-channels' mouths. The variation of K_d for different LT-channels within distinct membrane groups depended on pretreatment of the toxin however these were small or negligible.

The Ca/K selectivity of the LT-channels depended on BLM composition but was independent of the conductance of LT-channels. Biionic potentials (V_m) for C-, D-, E- and F-types of LT-channel (in PS-BLM) were about 40 mV in a system containing 10 mM $CaCl_2$/20 mM KCl. The '+' sign was in the KCl compartment, indicating that Ca permeability was higher than the K permeability. The relative Ca-permeability of both LT-A and LT-B channels (in PC-BLM) were considerably less, where the V_m reached only 28 mV. The differences between C-, D-, E- and F-type of LT-channel and the A- and B-type were independent on ion-channel conductance and may also be explained by the influences of lipid charges.

On the contrary, the cation-anion selectivities of all tested LT-channels were independent of surface charge

TABLE I

Selectivity, binding properties and size of latrotoxin channels with different conductances

G is the mean conductance of channels pool corresponding to types A, B, C, D, E, F (see Figs. 1 and 2 for notes). V is the zero current potential in the biionic system 10 mM $CaCl_2$/20 mM KCl. K_d and G_{max} are the parameters of the Michaelis-Menten equation. R is the effective radius of the pore (see text for details).

N	Channel type	BLM composition	G (pS)	V (mV)	K_d (mM)	G_{max} (pS)	R (Å)
1	A	PC+Ch (4:1)	74± 6	28.4±1.2	582±9	675± 2	8.8±0.8
2	B	PC+Ch (4:1)	169±14		818±7	2013±11	9.8±0.9
3	C	PS	112± 8	40.7±2.3	67±1	185± 1	9.7±1.3
4	D	PS	415±20	39.6±7.0	64±1	702± 4	9.3±0.3
5	E	PS	565±25	38.3±1.3	28±1	770± 2	9.0±0.3
6	F	PS	1110±40		28±1	1500± 3	9.5±0.3

of BLM (cation-transference number was 0.98 ± 0.02 at pH 7.5; calculated from zero-current potentials measured in the presence of three-fold KCl concentration gradient: 40 mM/120 mM (*cis*/*trans*)). The apparent paradox is a result of the limit upper level of cation transference number. Thus, it is impossible to record an influence of BLM surface charge on K/Cl selectivity.

Taken together, the data from these experiments indicate that differences in LT-channels charge constitution exist but they can not themselves determine the large differences of LT-channel conductances.

To determine the effective diameter of the LT-channel water pores we carried out the method based on registration of the influence of nonelectrolyte on single-ion-channel conductances. The substantiation [6,8,9] and some results of the method applications [10–12] were published recently.

In accordance with this method, we found that small nonelectrolytes decreased the ion-channel conductance whereas large nonelectrolytes only slightly increased it, although all tested nonelectrolytes decreased conductivity of the used buffer. To estimate the permeability of channels to nonelectrolytes we used parameter ν determined as the relative change of channel conductances normalized by relative change of bath solution conductivities:

$$\nu = (G \cdot G_n) \cdot \varkappa / (\varkappa - \varkappa_n) \cdot G \qquad (2)$$

where G and G_n are the conductances of the channel in the absence and presence of nonelectrolyte; $\varkappa$ and $\varkappa_n$ are corresponding values of buffer conductivity. It was found that ν varied from $\geqslant 1$ for permeable nonelectrolytes to $\leqslant 0$ for impermeable ones [6,8,9].

The results of the application of this method to the LT-channels are shown in Fig. 4. It is clear that LT-channels, which had different conductances, under the same condition had practically identical effective radii of their water pores. This value was close to 9.4 ± 0.6 Å. Precise values for all of the channel types are presented in Table I.

Therefore, the observed large differences of LT-channel conductances can not be explained by differences of their radii.

Thus, only one possibility remains that can account for the large differences in single-channel conduc-

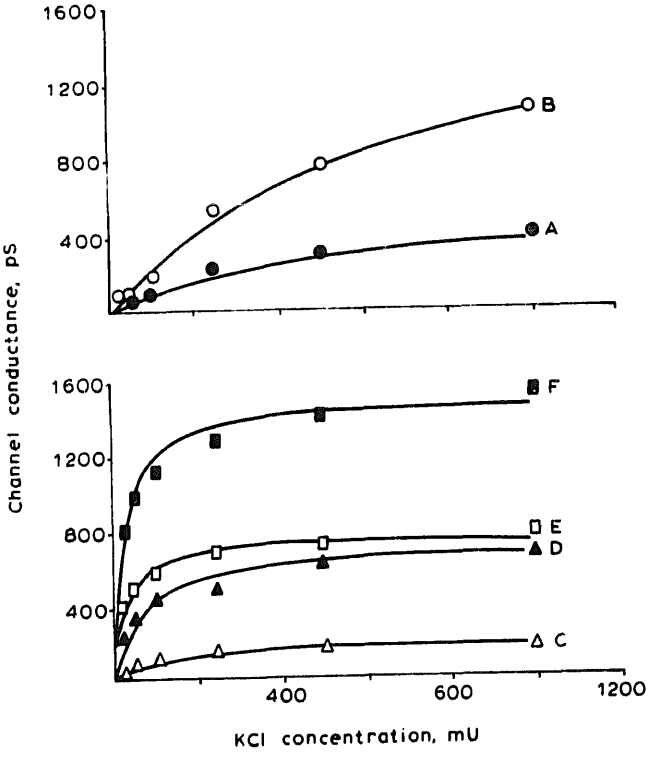

Fig. 3. Conductance of latrotoxin channels inserted in PC-BLM (top) and in PS-BLM (bottom) as a function of KCl concentration. Symbols ○, ●, △, ▲, □, ■ correspond to A, B, C, D, E and F-types of LT-channels (for details see the Fig. 2 and the text). Solid lines were calculated according to Michaelis-Menten relationship [1] with parameters presented in Table I.

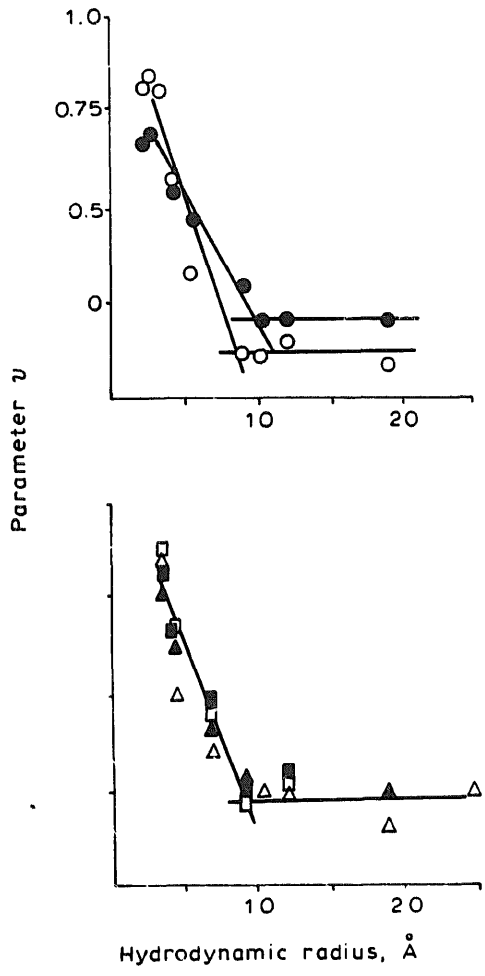

Fig. 4. Parameter ν as a function of hydrodynamic radii of nonelectrolytes. Symbols are same as used in Fig. 3. Left, PC-BLM; right, PS-BLM. In these experiments the water phase contained 100 mM KCl, 10–30% nonelectrolyte and 5 mM Tris-HCl buffer (pH 7.5).

tances of LT-channels within each group: LT-channels are clusters of unitary channels.

For example, B-channels probably consist of three subunits like the A-channels, because G_{max} of B-type channels were about 3-times more than A-type (see Table I). Analogically, D-channels may consist of 3–4 C-type channels and F-channels consist of 2 E-type channels.

What type is the LT-channel of those examined which is unitary? It may be the C-type, which has the smallest G_{max}. Our present evidence suggests that C-type channels, incorporated to PC-BLM, should have a conductance about 15-20 pS at 100 mM KCl. It should be noted that this value for LT-channel conductance is similar to that estamated by the patch-clamp technique on pheochromocytoma PC-12 cells [13].

LT is a very labile protein that can easily undergo structural modifications. Such modifications can change the charge constitution of the induced ionic channels. LT is also able to aggregable in solution. The aggregation process can also occur in membranes, resulting in the formation of oligomeric channels or clusters.

It should be noted that the apparent conductance of a registered single LT-channel can not be an exact sum of the conductances of smaller channels, because there are both the interaction between unitary channels in cluster and structural changes of channel protein. However, it does not alter our main conclusions: (i) investigation of both the charge constitution and the water-pore size of several conducting states of one kind of ion channel permits an estimate of the structural organization. (ii) LT-channels in lipid bilayer are clusters.

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