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Non stochastic distribution of single channels in planar lipid bilayers

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Abstract

The selectivity of the planar lipid bilayers modified by two channel-forming proteins (α-toxin *S. aureus* and colicin Ia) was examined. It was established that in all cases the value of zero current potential depended on the amount of open ion channels and increased with the number of channels (from one to about 5–7). These facts point out both the interactions among ion channels and their non stochastic distribution on the membrane surface.

Keywords: Ion channel; Lipid bilayer; Non stochastic distribution

1. Introduction

At present there is no doubt that ion channels together with transporting ATPases and carriers are the main elements for basic cell functions such as generation of membrane potential, nerve impulse, reception etc. The structure and functions of ion channels are under intensive study. For both types of membrane (natural or artificial) investigators have assumed that there were N independent ion channels in the membrane plane [1–3], although about ten years ago the first experimental data about complicated or perhaps cluster organization of some ion channels were found [4,5]. Moreover, the theoretical analysis of the interaction among macromolecules which were situated in organized areas pointed out that there was a long distance interaction between them [6,7]. Despite this almost all theoretical descriptions of ion channel properties and behavior were based on the assumption of independent channels. That is why we decided to examine this assumption.

2. Material and methods

The α-toxin *S. aureus* was a gift of Dr. K.D. Hungerer of the Behringwerke Laboratory (Marburg, Germany). The colicin Ia was a gift of Prof. J. Konisky (University of Illinois, Champagne Urbana, USA). Chromatographic pure phosphatidylcholine was obtained from eggs by the method described earlier [8]. Azolectin was purified by acetone extraction [9]. Cholesterol was purchased from Sigma and used as received. Other chemicals were analytical grade.

Planar bilayer lipid membranes (BLM) were formed at room temperature (25 ± 2°C) by the technique of Montal and Mueller [10] by apposition of two monolayers. Monolayers from a 10 mg/ml solution of the lipids in n-hexane were spread on the water surface of chambers of a Teflon experimental cell and after evaporation of the solvent a membrane was formed on a hole in a 25 μm thick Teflon partition separating two buffered salt solutions (2, 4 or 10 ml, dependent upon the experimental cell used). The hole (0.1–0.4 mm in diameter) was pretreated with either a 4% solution of hexadecane or 2% solution of vaseline in n-hexane. Experiments were done under voltage-clamp conditions. During the bilayer formation, the peak current in response to constant voltage pulses (amplitude, ±5–10

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mV; duration, 2–4 ms; frequency, ~ 500 Hz) or capacity current in response to triangle voltage signal (peak to peak amplitude ~ 10 mV; frequency, ~ 500 Hz) was monitored continuously. Water double distilled in glass was used in the preparation of all buffer solutions.

The current going through the bilayers was measured with Ag/AgCl electrodes connected via salt bridges (3% agar with 3 M KCl) in series with a voltage source and a current amplifier. Disbalance of the potential between electrodes was controlled and was not larger than 1 mV during experiments.

The use of KCl-filled bridges and KCl solutions (in the experiments presented in this paper) allowed to avoid many problems such as: (i) a large diffusion potential (which usually appeared at the boundary of an electrode-solution); (ii) a difference between potentials of two used Ag/AgCl electrodes in the presence of different concentrations of KCl in the *cis* and *trans* compartments of the experimental cell; (iii) a large diffusion trans-membrane potential that appeared in the zone of contact (through nonspecific leakage in particular) of two solutions with different salt concentrations caused by different motions of a cation and an anion. In our case all mentioned potentials were not larger than 1 mV and constant during the experiment.

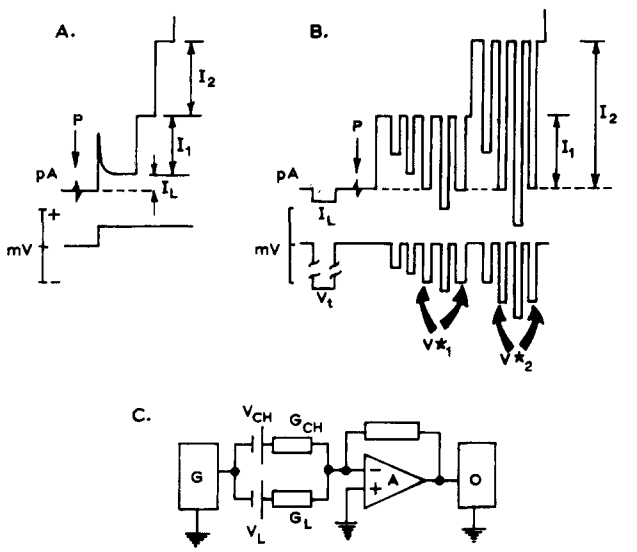

Fig. 1. Protocols of measurement of both the ion channel conductance (at symmetrical concentration of KCl (A); the value of zero current potential (at 3-fold KCl gradient (B); and a principal electrical scheme of measurement of the membrane properties (C). (A) Current trace: arrow pointed out a moment of addition of channel-forming protein. I_L, I_1 and I_2 are the currents going through leakage, through first and second channels, respectively. Dotted line marks zero current. P with arrow indicates moment of addition of a channel-forming protein. Voltage trace points to an algorithm of change of value of transmembrane potential during an experiment. (B) Current trace: arrow pointed out moment of addition of channel-forming protein. I_L, I_1 and I_2 are the currents going through leakage, through one and two channels, respectively. Dotted line marks the virtual zero current. P with arrow indicates a moment of addition of a channel-forming protein. Voltage trace points an algorithm of change of value of transmembrane potential during an experiment. V_1^* and V_2^* are the appeared zero current potentials for one and for two open ion channels in bilayer. V_t is a test potential. It was usually equal to 50 mV. It was applied at nonmodified membrane in order to determined a basal conductance (G_L) of a bilayer. G_L was calculated as ratio I_L / V_t. (C) G, voltage source; A, amplifier (current-voltage converter); O, oscilloscope; V_L and V_{CH} are leakage's and channel's 'batteries'; G_L and G_{CH} are leakage and channel 'conductances'.

Membrane current was amplified by a U5-11 current-voltage converter or any of two other amplifiers based on microchips K284UD1A and OPA111, respectively. The amplifier signal was monitored with a storage oscilloscope (C8-13 or Nicolett 201) and recorded on a strip chart or tape recorder. The trans compartment was connected to the virtual ground. After the membrane was completely formed and stabilized, a portion (a few microliters) of the stock solution of a channel-forming protein was added only to one compartment of the experimental cell to concentrations of approx. 30 ng/ml and 200 ng/ml for colicin Ia and α-toxin *S. aureus*, respectively. The stock solution of α-toxin *S. aureus* (400 μg/ml) consisted of 100 mM KCl, 10 mM Tris-citrate buffer, pH 7.0. The stock solution of colicin Ia (60 μg/ml) consisted of 1000 mM KCl, 2 mM EDTA, 10 mM $CaCl_2$, 5 mM Hepes, pH 8.0. In some experiments the channel-forming proteins were already present prior to the formation of the membrane. Such a change of the protocol did not influence the results obtained. Conductance and selectivity of the channels obtained by both protocols were indistinguishable. The solutions in both compartments were magnetically stirred during the experiment. Sometimes stirring was stopped a few minutes after addition of the protein. It decreased the current noise and did not change the measured values of conductance and zero current potential.

The conductance of ion channels G in symmetrical solutions is defined as $G = I/V$ where I is the transmembrane current flowing through the channels and V is the fixed potential (Fig. 1A). Basal conductance of nonmodified BLM was less then 4 pS for PC bilayers and approx. 5 pS for azolectin bilayers.

The selectivity of the ion channels was measured in the presence of a 3-fold KCl concentration gradient. Zero current potential is defined as a potential (V^*) that is necessary to apply to the experimental cell in order to reach a virtual zero transmembrane current equal to that before a channel appeared (Fig. 1B). The relationship between V^* and the potential (V_{CH}) generated by a channel in the asymmetrical system becomes clear from the analysis of a simple electrical scheme that approximates a modified membrane and is pointed out in Fig. 1C. One can model a leakage of a bilayer (surrounded by a different salt concentration) with a resistor connected with a small battery. Zero mV is fixed by voltage-clamp. The out-put signal of an amplifier at this state of the system was taken as the virtual zero. The appearance of a channel in the membrane is simulated by a second resistor and a battery. The potential of the latter battery (V_{CH}) reflects the selec-

tivity of a channel, which is a subject we are interested in. The applied external potential (V^*) that leads to reach the virtual zero value of the transmembrane current can be estimated carefully. At this state the value of V_{CH} is related with V^* by following equation:

$$V_{CH} = V^*(G_L + G_{CH})/G_{CH} \quad (1)$$

where G_L and G_{CH} are the conductances of a pure bilayer (leakage) and a channel, respectively. The value of G_L (basal conductance of nonmodified bilayer = leakage) was measured before channel-former was added in bathing solution. The sum $(G_L + G_{CH})$ was calculated as ratio I_{CH}/V^*, where I_{CH} is the current going through the channel at zero mV fixed potential; V^* is the appeared (measured) zero current potential (see Fig. 1B). In our cases the maximal value of the ratio $(G_L + G_{CH})/G_{CH}$ was always less than 1.1.

The diffusion between compartments through a hole in the partition did not change visibly the transmembrane gradient during the experiments. The examination of it was carried out in a special study in a 100 mM KCl/100 mM NaCl *cis/trans*-system. The concentration of KCl in the *trans* compartment (4 ml) was monitored by a K-sensitive valinomycin-electrode. It was found that even after the successful formation of 40 bilayers (during 2 h) less than 4 μl of the KCl solution could come from the *cis* compartment, which is less then 0.1% from the total volume of the compartment. This means that the possible change of the gradient during our zero current potential experiments is negligible ($\leq 0.33\%$).

3. Results and discussion

It was established that the value of zero current potential of BLM modified by both α-toxin *S. aureus* and colicin Ia depended on the amount of open ion channels and increased with the number of channels (from one to about 5–7) till the steady-state value that was typical of a bilayer containing a large amount of channels.

In order to determine if the ion channels were independent from each other in lipid bilayer we used the most simple system – planar lipid bilayer membranes modified by some channel-forming proteins (α-toxin *S. aureus* and colicin Ia). These proteins were chosen because the properties of the ion channels formed by them have been intensively investigated during the last 10 years. Moreover, some experimental data pointed out that the ion channels obviously have a single water pore (for review see [11–14]). To describe some of the properties of the ion channels the independence hypothesis was also taken [15].

From the many features of the ionic channels we chose to investigate the selectivity – the cation/anion in particular. This parameter was taken because it is very sensitive to the distribution of charges around ion channel mouths. We expected to solve the problem by the comparative analysis of zero current potential values for membranes containing a few open ion channels. Since, if the ion channels are distributed stochastically on membrane plane, the zero current potential would not depend on the amount of open channels. In the opposite case we would expect to find a change of the parameter.

In our experiments we used such ion concentrations and pH values of the medium which allowed us to register reliably the ion channel amplitudes and to get an almost maximal selectivity of the bilayers containing a large amount of the channels.

As was established earlier [16] the properties of ion channels induced by α-toxin *S. aureus* depend on salt concentration and pH of medium. The conductance of the ion channel changes practically in proportion with the concentration of the electrolyte (from $\sim$ 100 pS at 100 mM KCl (pH 7.5) to $\sim$ 2000 pS at 2000 mM KCl) and increases (at 100 mM KCl) from $\sim$ 100 pS to $\sim$ 180 pS while pH decreases from 9.0–7.0 to 4.0. As the increase of the concentration of the electrolyte decreases the selectivity of the channel, the optimal conditions (in our opinion) to test the existence of dependence of selectivity of the ion channel on their amount in the membrane are: (i) the concentration of KCl equals approx. 100 mM and (ii) pH is ranging from 7.0–7.5, where the conductance of the channel is approx. 100 pS and zero current potential for membrane contained a large amount of the channels at 3-fold gradient KCl through the membrane (40:120 mM, *cis/trans*, pH 7.0) is about -13.5 mV.

Colicin Ia is a member of the ion channel-forming family of colicins (for reviews see [17–19]). In the presence of negatively charged lipids in lipid bilayers the K ion is usually more permeable than Cl through the colicin-induced channels [20–23]. The ion channel conductance (which consists of a few pS at 100 mM solution of 1-1 electrolyte) and the P_K/P_{Cl} ratio for membrane were modified by colicin's increase when the pH of the bathing solution changes from acid to alkaline. The increase of the electrolyte concentration also increases the conductance of the single ion channels. For reliable registration of the ion channel (which is necessary to experiment) its conductance should be equal to some dozen pS. Which is why in order to investigate the properties of the ion channel induced by colicin Ia we have to use a large electrolyte concentration (1.0–3.0 M KCl) with pH 7.0–8.0 because only under these conditions did the ion channel conductance reach the appropriate value. The zero current potential for membrane containing a large amount of channels at 3-fold gradient KCl through membrane (3.0:1.0 M, *cis/trans*, pH 8.0) was about -16.0 mV.

When a channel-forming protein from a stock solution was added in a small quantity to the aqueous solution bathing the bilayer membrane, the membrane conductance started to increase in a stepwise fashion. The occurrences of these conductance steps are specific to channel-forming protein. The conductance increments are not ideal uniform

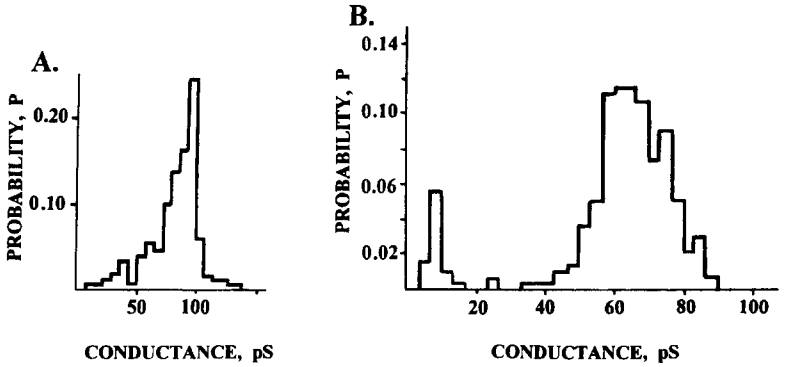

Fig. 2. The amplitude histogram of conductance fluctuations in voltage-clamped planar lipid bilayer membranes treated by *S. aureus* α-toxin (A) and colicin Ia (B). (A) The solution in both compartments of the experimental cell contained 100 mM KCl, 10 mM Tris-citrate buffer, pH 7.0. The toxin was added to the *trans* compartment. Potential was 50 mV. BLM was formed from PC/Chol mixture (3:1; w/w). More than 250 ion channels were registered (5–7 channels per membrane), bin width 7 pS. All other conditions of experiment are such as in Materials and methods. (B) The solution in both compartments of the experimental cell contained 1.5 M KCl, 0.002 M EDTA, 0.01 M $CaCl_2$, 0.005 M Hepes. pH was adjusted by 1.0 M KOH till pH 7.0. Planar bilayer lipid membranes were formed from azolectin purified from neutral lipids by acetone extraction. The colicin Ia was added to *cis*-compartment. Clamped potential was equal to 50 mV. More than 200 ion channels were registered (5–7 channels per membrane), bin width 3.3 pS. All other conditions of experiment are such as in Materials and methods" and Fig. 1A.

in size but distributed over a certain range. It is possible to point out nonidentical conformation states among ion channels because of the flexibility in some parts of their protein chain. The histograms of the conductance steps observed with α-toxin *S. aureus* and colicin Ia are shown in Fig. 2. Most of the channels induced by α-toxin *S. aureus* (at 100 mM KCl, pH 7.0) had conductance within range between 85 pS and 110 pS. Most of the events induced by colicin Ia (at 1.5 M KCl, pH 7.0) were situated in a conductance range between 50 and 85 pS. Those results are in a good agreement with the expected values of parameters of ion channel induced by colicin Ia [19] and with the established recently data [16,24] for channel induced by *S. aureus* α-toxin.

To determine if the selectivity of an ion channel depends on amount of channels incorporated in bilayer mem-

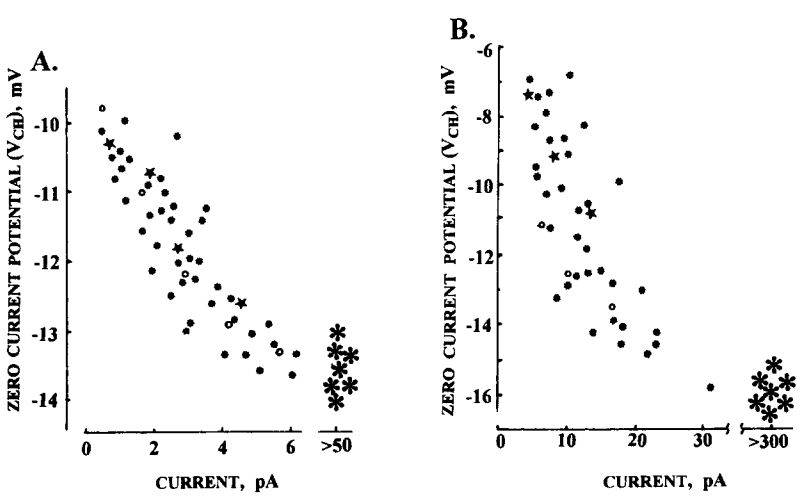

Fig. 3. Dependencies of the real zero current potentials (V_{CH}) on value of current going through ion channels induced by α-toxin *S. aureus* (A) and by colicin Ia (B). (A) 3-fold KCl concentration gradient 40 mM/120 mM (*cis/trans*) was used. Solutions were buffered by 10 mM Tris-citrate (pH 7.0). BLM was formed from PC/Chol mixture (3:1; w/w). The protocol of the experiment was as noted in Fig. 1B. The α-toxin *S. aureus* was added in the *trans* compartment of the experimental cell. All other conditions of experiment are such as described in Materials and methods and the text. The wide distribution of data reflects no homogeneity of the ion channel conductance (Fig. 2A) and interaction among ion channels in the membrane plane. Symbols: ●, the bilayers contained a few open channels; ○ and ⋆, the data obtained on two separate bilayers during consequent opening ion channels; ∗, the bilayers with (integral) macroscopic conductance (from 50 to about 500 channels). Any dependence of the zero current potential on the bilayer conductance (among the membranes with macroscopic conductance) was not observed. (B) 3-fold KCl gradient 3 M/1 M (*cis/trans*) was used. The basic buffer solution contained: 2 mM EDTA, 10 mM $CaCl_2$, 5 mM Hepes, pH 8.0. Bilayers were formed from azolectin. The protocol of experiment was as in Fig. 1B. The colicin Ia was added in the *cis* compartment of the experimental cell. All other conditions of experiment are such as described in Materials and methods and the text. Symbols: ●, the bilayers contained a few open channels; ○ and ⋆, the data obtained on two separate bilayers during consequent opening ion channels; ∗, the bilayers with (integral) macroscopic conductance (from 50 to about 500 channels). Any dependence of the zero current potential on the bilayer conductance (among the membranes with macroscopic conductance) was not observed.

brane the zero current potential was assayed in the presence of a 3-fold KCl concentration gradient. When the membrane was formed and it properties were stabilized a zero mV was fixed and a few microliters of channel-forming protein were added in one compartment of the experimental cell. In lag time (this value depended upon the amount of added channel-forming protein and usually was some dozen seconds because the chosen pH value is far from that necessary for maximal channel-forming activity of the proteins) stepwise increase of transmembrane current was observed. We have tried to measure the zero current potential for all current steps. By a temporary change of fixed potential (pulse) it instantly changed to a new value for a few seconds (see protocol of experiment in Fig. 1B). If the used value was less than zero current potential it could not bring the transmembrane current to the virtual zero level. If the temporary fixed potential exceeded zero current potential the resulted transmembrane current became the opposite direction (opposite sign). It was situated on the other side from the virtual zero level. The accuracy of the determination of value of zero current potential was less than ± 0.2 mV. The existence of measuring ion channel was verified continuously during inter pulses interval (while zero mV potential was fixed). The verification was especially important for colicin Ia channels because their function strongly depends on a value of transmembrane potential [25].

It was found that the real zero current potentials (V_{CH}) of modified membranes that contained one open channel were always less than that of the bilayers that contained a lot of the channels. The increase in the amount of open ion channels (from 1 to about 5–7) led to increase V_{CH} till the value typical for bilayers with macroscopic conductance (Fig. 3 A and B). The presented data were obtained from seven different experiments (for each channel-forming protein) on some dozen membranes. It was very difficult to present the dependence of V_{CH} value against the amount of the channels directly because of the wide distribution of the ion channel conductance (Fig. 2) and the interaction among ion channels in the membrane plan (our hypothesis). Which is why we offer the dependence of the V_{CH} value against the induced transmembrane current (at zero mV fixed potential) only. It needs to be noted that at one membrane the dependence of the V_{CH} against the amount of ion channel was always stronger and observed at the experiments with both used types of protein channel-former. At one membrane the insertion each followed channel in bilayer was followed by the simultaneous increase of the total selectivity of channels (Fig. 3).

The analogous dependence of the selectivity on the amount of open ion channels was observed recently for a channel induced by toxic factor produced by *Sh. flexnera* 2a [26] but the authors did not explain it. We think that the data actually point out the inter-influence among a few open ion channels. Of course many types of forces could take part in the channel-channel interaction observed. Some of them act over short distance and others over large distance. The electrostatic inter-influence among single ion channels appeared indeed because we registered the change of selectivity of the ion channels during their appearance (open) in a membrane. The electrostatic interaction is realized by the most long distance force that allows us to explain the data. In our experimental condition the distance (Debye length) was not more than a few nm (in the case of α-toxin channels) and a few Å (in the case of colicin Ia channels). These values are comparable with ion channel sizes but it is negligibly small as compared with the bilayer area. In the latter case we should assume in addition that during channel-channel interaction some structural reorganization of ion channel structure occurred because the Debye Length was very small.

The obtained results allow us to assume that the first channel: (i) creates some condition around itself that facilitates the construction or opening of the following ones; (ii) is like a center of 'crystallization' of ion channels in membrane plane. The organized structure looks like a cluster where the ion channels have not a hard synchronization.

The analyses of cation-anion selectivity of cluster-organized ion channels formed by latrotoxin in lipid bilayers [27] additionally supports the assumptions made. In that case the selectivity increased when the conductance of the single channel (and hence the amount of unitary channels in cluster) was increased.

In general, the obtained data allow us to conclude that open ion channels are not distributed stochastically on the membrane plane. It is possible that a long distance non-electrostatic interaction (that was noted above [6,7]) improves initial aggregation of monomers of channel-forming molecules or/and preformed nonconducted channels in membrane plane.

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