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Cholesterol-dependent gramicidin A channel inactivation in red blood cell membranes and lipid bilayer membranes

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The exchange diffusions of tracer cations ($^{22}Na^+$, $^{86}Rb^+$) are studied on gramicidin-A-treated red blood cell (RBC) membranes. A time-dependent decrease in cation permeability has been observed and has been considered to be the result of a channel inactivation process. The channel inactivation appears at 20 and 30 °C but not at a temperature as low as 6 °C. The gramicidin A channel inactivation can be monitored by a conductivity decay of molecular lipid membranes (BLM) prepared either from cholesterol or from a mixture of cholesterol and phospholipids but not of pure phosphatidylethanolamine. The role of cholesterol in the channel inactivation is discussed.

Introduction

Recent intensive studies on the amino-acid sequences of proteins forming ion channels in cell membranes [1–3] have to some extent permitted their secondary and tertiary structures to be deduced, and thus certain suggestions on the general shape of ion channels and their arrangements in cell membranes can be made. Taking into account the great complexity of ion-channel protein structures, it is easy to understand the attempts to use more simple model systems to gain an insight into the molecular structure and function of ion channels.

It is well known that ion permeability of artificial lipid bilayer membranes (BLMs) may be obtained by incorporation of channel-forming compounds into the membranes. A great number of substances of quite different chemical structure: detergents [4], polyene antibiotics [5], small peptides [6] and high-molecular-weight proteins [7,8] appear to behave as channel formers, inducing conductivity of lipid bilayers with characteristic, discrete, current fluctuations. One of the best-studied channel formers is gramicidin A, a linear pentadecapeptide with alternating L- and D-amino acids. For a long time, only a dimer head-to-head helical structure with a narrow water pore inside the helix was considered as a proven model for channel organization [9–12]. Meanwhile, new data showed that rather than a dimer, at least a tetramer or larger molecular aggregates should be considered as the structures forming the channel [13]. Some properties such as single-channel population, or conductance of gramicidin A channels were found to be dependent on the lipid composition of bilayer membranes and the temperature [14–16].

In our previous work [17,18], gramicidin A has been induced into red blood cell (RBC) membranes to study the properties of the channels when they are organized in a living membrane. It has been shown that the penetration of the antibiotic molecules into the RBC membrane is a highly cooperative process and more than 600 gramicidin molecules per cell should be present to induce cation permeability of the membranes of all RBCs. Below this antibiotic concentration two populations of RBCs could be observed from the kinetic data, one with membranes modified by the channel former and the other free of the modifier action. However, a time-limited action of the modifier may also correspond to the kinetic data obtained.

There are few data on the kinetics of gramicidin A action on membranes. Only the increase in lipid bilayer conductance after addition of gramicidin A to the solutions in contact with the membranes has been discussed [19]. It was stated that either quadratic or exponential

Abbreviations: BLM, bilayer lipid membrane; PE, phosphatidylethanolamine; RBC, red blood cell.

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dependence of the bilayer conductivity on time was characteristic for the initial phase of the antibiotic action. It is obvious that after that initial phase the conductance should reach a plateau if an equilibrium or a steady state is established. But in the course of our previous study carried out on spherical lipid bilayer membranes [20], it was noted that to maintain high and constant membrane conductivity during periods longer than 20–40 min it had been necessary to add gramicidin A into the bathing solution at regular intervals. This showed that the modifier added to the system was somehow inactivated with time in respect to its channel-forming ability. Until now, time-dependent channel inactivation has been reported only for polyene antibiotics incorporated into BLM [21]. If the same kind of inactivation process takes place in the case of gramicidin A action on RBC membranes and the rate of the inactivation is high enough, the isotope equilibrium should not be reached until the membrane permeability has significantly decreased.

In this work, the kinetics of ion flux through gramicidin-A-doped RBC membranes are studied and compared with those investigated in of Mueller's- and Montal-Mueller's type lipid bilayers.

Material and Methods

Tracer exchange diffusion measurements on RBCs

Human blood from healthy volunteers stabilized by citrate buffer and stored at 4°C not longer than 3 days was used. The blood was centrifuged and the red blood cells were washed three times with a solution of the following composition (in mmol/l): 130 KCl/20 NaCl/2.5 $CaCl_2$/1 $MgCl_2$/27 sucrose (pH 7) and then resuspended in the same solution to obtain a haematocrit value (H) of 0.4. The suspension was taken into a shaking bath and the antibiotic gramicidin A dissolved in ethanol was added into the suspension. The final concentration of ethanol in the RBC suspension did not exceed 1% (v/v). Such ethanol concentration was shown to produce no effect on the ionic permeability of the RBC membranes. The suspension was incubated for 30 min at 35°C then cooled to a given temperature (6, 20 and 30°C) and incubated further for either 10 or 110 min. Radioactive isotopes of cations ($^{86}Rb^+$, $^{22}Na^+$) were then added to the suspension. Samples of the suspension were taken at given intervals. The samples were centrifuged and the radioactivity of aliquots of the supernatants was measured by a γ scintillation counter (Gamma, Hungary).

The transport of tracers is characterized by the amount of radioactive ions left in the external medium after a time period t and expressed as the percentage of the total activity of suspension ($N(t)$). The curves characterizing the cation influx are presented in a semilogarithmic plot; $-\ln(N(t)/N_\infty - 1)$ over time, where N_∞ is the percentage of radioactivity in the external medium at the tracer equilibrium state. Estimated curves are fitted to the experimental points. The slope of curves is the rate coefficient of influx of tracer cation i (k_i).

To prove that the Na^+/K^+ pump does not provide an additional pathway for cations, except those through gramicidin A channels, the exchange-diffusion measurements were repeated under the same conditions but in presence of 10^{-5} mol/l ouabain, whereupon no differences in kinetic data were found.

Electric conductance measurements on BLM

Spherical bimolecular lipid membranes of a large area (up to 2 cm^2) were prepared according to the method described in detail elswhere [20,22]. The membrane was formed from 1.5% (w/w) solution of bulk ox brain lipids plus cholesterol (1:4 phospholipids-to-cholesterol molar ratio) in 4:3:2 (v/v) chloroform/tetradecane/methanol mixture. The BLM was bathed in 0.16 mol/l RbCl (pH 6).

Planar bimolecular lipid membranes were formed by Montal-Mueller's method [23]. The area of the membranes did not exceed $1.2 \cdot 10^{-5}$ cm^2. For membrane formation a solution of 0.04% of a specific lipid dissolved in hexane was applied at the interface of the 0.1 M RbCl solution and the air.

The electrical conductance of the lipid bilayers was determined by a standard reference method [20] with the aid of high-resistance electrometers (TR-1501, Hungary), or the voltage clamp set-up constructed on the basis of a 41K Analog Device (U.S.A.). Nonpolarizing Ag/AgCl electrodes were used to establish contact between the salt solutions separated by lipid bilayer membranes.

Ox brain lipids were extracted according to the method of Folch [24].

PE was isolated from bulk ox brain lipids by coloumn chromatography using the chloroform/methanol (9:1, v/v) solution. For column chromatography silica gel (Woelm, F.R.G.) was used. The purity of PE was checked by thin-layer chromatography.

Gramicidin A obtained from Sigma was dissolved in ethanol. The ethanol concentration in electrolyte solutions never exceeded 0.03% (v/v). It was proved that such a concentration of ethanol produces no effect on the conductance of BLM. All other chemicals used were of analytical grade. Bidistilled water was used in all experiments.

Results and Discussion

The exchange diffusion of Rb^+ and Na^+ cations has been investigated on gramicidin-A-treated RBC membranes. Fig. 1 shows the time-courses of $^{22}Na^+$ and $^{86}Rb^+$ influx determined at 20°C. The gramicidin A was added to the RBC suspension to a final concentration of $1 \cdot 10^{-8}$ mol/l in extracellular solution and then

the dispersion of RBC was divided into two portions. Tracers ($^{86}Rb^+$, $^{22}Na^+$) were added to the first one in the 40 min after the addition of gramicidin A, while in the second they were added 100 min later.

The $^{86}Rb^+$ influx registered 40 min after the gramicidin addition (Fig. 1, curve 3) evidenced a one-exponent diffusion process, while the influx of $^{22}Na^+$ (Fig. 1, curve 2) could be represented only by a combination of at least two exponents. The rate coefficients of the exchange diffusion of $^{86}Rb^+$ and $^{22}Na^+$ were estimated (in the case of $^{22}Na^+$, k_{Na} was obtained from the first exponent of the kinetic curve) and a k_{Rb}/k_{Na} ratio of 4.2 was found, which appeared to be close to the Rb^+/Na^+ selectivity determined for gramicidin-A-treated RBC membranes [18] and not much different from that of bimolecular lipid membranes [25,26]. The slope of the second exponent of the $^{22}Na^+$ influx characterizing the influx rate of $^{22}Na^+$ in the interval 100–160 min after the application of the channel former (Fig. 1, curve 2) was found to be only 2–3-times higher than that in the control experiment where no gramicidin A was added to the RBC suspension (Fig. 1, curve 1). When the measurement of the exchange diffusion of these tracer cations was started 140 min after addition of gramicidin A, the kinetic curve of $^{86}Rb^+$ influx should have been also deconvoluted into two exponents (Fig. 1, curve 5). The rate coefficients, k_{Rb} and k_{Na}, obtained from the initial portions of the corresponding kinetic curves (Fig. 1, curves 5 and 4) appeared to be about 4-times less than when they were estimated from the kinetic curves measured 40 min after the addition of gramicidin A. The k_{Rb}/k_{Na} ratio obtained from the first exponent of the kinetic curves 5 and 4 in Fig. 1 was found to be 3.9. The slopes of the second exponent were about the same for both tracer cations and were again only 2–3-times steeper than the slope of the exponent in the control experiment (Fig. 1, curve 1).

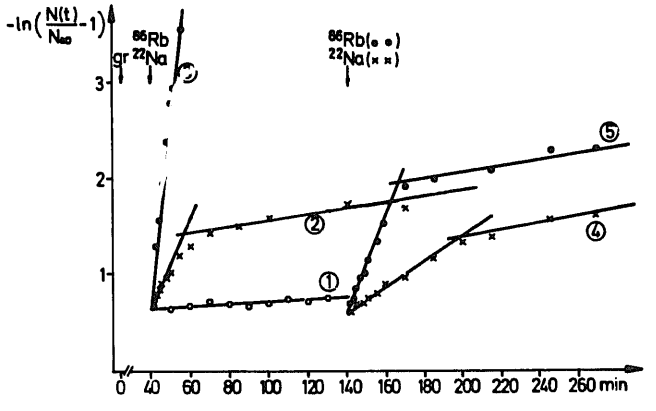

Fig. 1. Effect of gramicidin A on the influx of $^{22}Na^+$ (curves 2, 4) and $^{86}Rb^+$ (curves 1, 3, 5). Extracellular solution (in mmol/l): 130 KCl/20 NaCl/2.5 $CaCl_2$/1 $MgCl_2$/27 sucrose (pH 7); *H*, 0.4; *t*, 20 °C; gramicidin A concentration: curve 1: control, curves 2–5: $1 \cdot 10^{-8}$ mol/l of outside solution. (Addition of gramicidin A, as well as that of the tracers into the media, are shown by arrows).

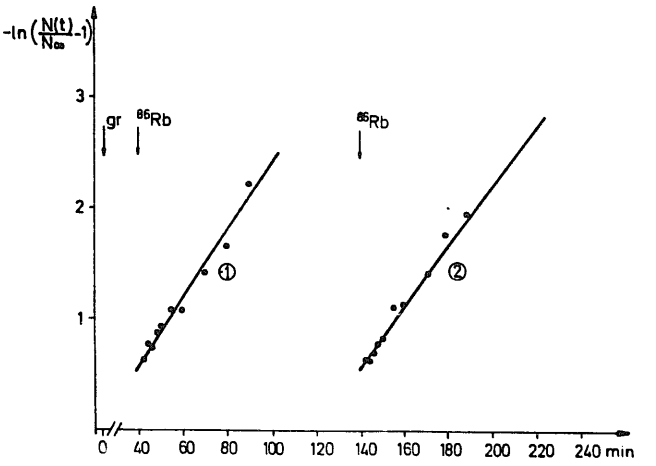

Fig. 2. Time-courses of $^{86}Rb^+$ influx through gramicidin-A-treated RBC membranes at 6 °C. For experimental conditions, see Fig. 1.

The results presented showed obvious changes with time in cation permeability of the gramicidin-A-treated RBC membranes. The decrease of k_i with time i.e., the decrease of gramicidin-induced RBC cation permeability may be a result of a reduction in either the number of ion channels or the integral time of their open state, or both. Such a process may be called time-dependent inactivation of the channels. It should be noted that during the time when the channel-inactivation process was observed, the cation-selectivity properties of channels did not change *, the k_{Rb}/k_{Na} ratio was found to be about 4, for the two portions of gramicidin-A-treated RBCs. At the very first approximation the rate of the gramicidin A channel inactivation in RBC membranes may be characterized by a half-time ($t_{1/2}$) of about 70 min.

The question now arises whether some lipid components of the membrane in the vicinity of the gramicidin A channel play a role in the inactivation process. The temperature-dependence of this phenomenon may give us an answer. For this purpose, ^{86}Rb exchange diffusion through gramicidin-A-treated RBC membranes was determined at 6 °C, which is considered to be below the structural transition of the major lipids of the RBC membranes [28,29], and at a higher temperature (30 °C) where the lipids are in liquid crystalline state. As can be seen in Fig. 2, at 6 °C there is no sign of any decrease in rate coefficient of the $^{86}Rb^+$ exchange diffusion for as long as 200 min. The transport curves are straight lines in semilogarithmic representation. The rate coefficient found was 0.03 min^{-1} and appeared to be 6.5-times less than that determined for the same conditions at 20 °C. Consequently for k_{Rb} a Q_{10} value of 4.6 could be

* Cationic specificity of gramicidin-A-induced self-diffusion in RbC membranes allows us to exclude the possibility of the existence of a recently described ion-nonspecific leak in the membranes found at 1000-times higher concentration of the antibiotic in the media [27].

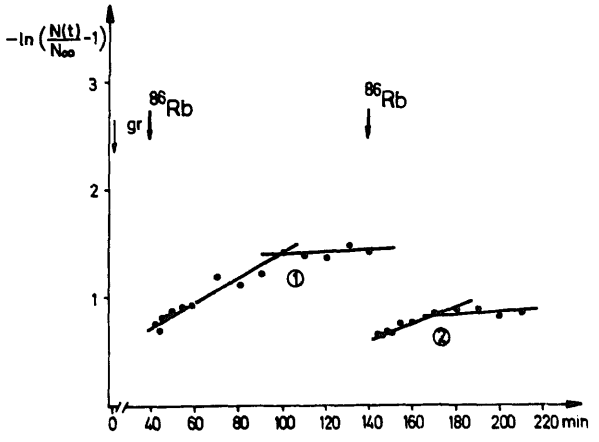

Fig. 3. Time-course of $^{86}Rb^+$ influx through gramicidin-A-treated RBC membranes at 30 °C. For experimental conditions, see Fig. 1.

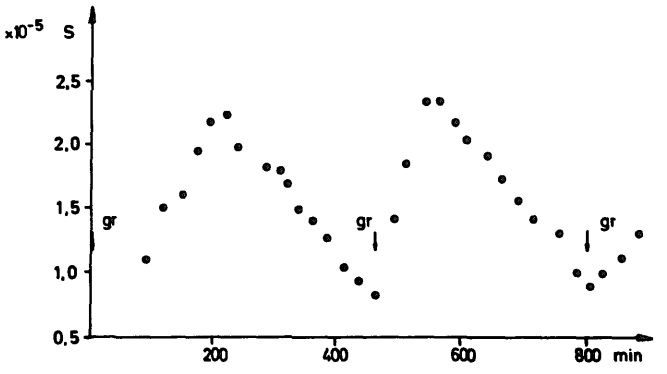

Fig. 4. Effect of gramicidin A on the conductance of spherical BLM prepared from a mixture of bulk ox brain lipids and cholesterol in a phospholipid/cholesterol molar ratio of 1:4. Area of BLM, 1.7 cm^2; bathing solution, 0.16 mol/l RbCl (pH 6); t, 27 °C. 10 μl of a 10^{-4} mol/l gramicidin stock solution was added to the outside bathing solution of 10 ml at the time indicated by arrows.

estimated, which seems to be too high to be explained as a decrease in the rate of cation movement through a water-filled channel. Such high Q_{10} value may be the result of the different structure of lipids in the vicinity of the gramicidin channels above and below the phase-transition temperature [15,16,30].

When the tracer exchange diffusion was determined at 30 °C (Fig. 3) the shape of the kinetic curves obviously changed, indicating a pronounced inactivation of gramicidin channels with time. At least two exponents were again necessary to fit the curves. The slope of the first one was found to be an order of magnitude less than that measured at 20 °C (compare Fig. 1, curve 3 to Fig. 3, curve 1). This 10-fold decrease in the cation permeability of the gramicidin-A-treated RBC membranes indicates the start of the channel-inactivation process within 40 min of the addition of gramicidin A into RBCs. The inactivation decreased with time, as the slope of the initial part of the transport curve representing the transport rate between 140 and 170 min after application of gramicidin A (Fig. 3, curve 2) appeared to be 1.5-times less than that obtained from the kinetic curve for the first portion of RBCs (Fig. 3, curve 1).

The kinetic data obtained at different temperatures demonstrate that the gramicidin channel inactivation results from interactions of one or several lipid components with the channel former and may be related to temperature-dependent lateral diffusion of lipid molecules in the membrane [31]. Above all, cholesterol seems to be responsible for the inactivation as, according to our preliminary results when cholesterol is removed from the membrane by liposome treatment, the sign of channel inactivation can not be observed for as long as 150 min.

To elucidate the role of different lipids in the channel-inactivation process, bimolecular lipid membranes were used as models, as these membranes could be prepared from different lipids.

It was already mentioned that on spherical BLMs made from a mixture of bulk ox brain lipids and cholesterol some indication of channel inactivation had been observed [20]. In this work special studies have been performed on this type of BLM. As presented in Fig. 4, after addition of gramicidin A, the conductivity of the membrane increases gradually, reaching a maximum value, then starts to decrease. The half-time of the conductance decrease is about 130 min. The conductivity of this BLM can be increased again by a new addition of channel former.

The Mueller-type spherical BLMs have a small amount of lipid solution located in the torus of the membrane. The decrease in the membrane conductance with time may be explained as a result of redistribution of the gramicidin between the lipid bilayer and the excess of lipid solution in the torus. To be sure that the observed phenomenon is an inactivation process and not the result of channel-former redistribution, another set of experiments has been performed on Montal-Mueller's-type BLM, where the amount of

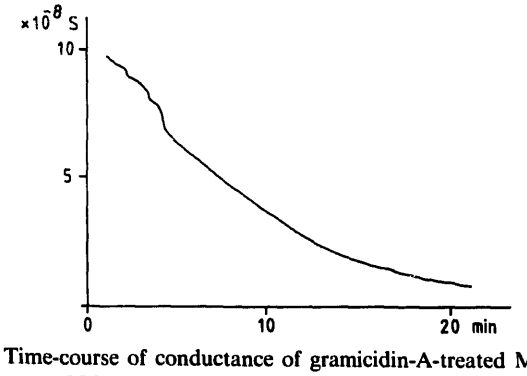

Fig. 5. Time-course of conductance of gramicidin-A-treated Montal-Mueller-type BLM prepared from bulk ox brain lipids of a phospholipid/cholesterol molar ratio of 1:2. Area of BLM, $1.2 \cdot 10^{-5}$ cm^2; bathing solution, 0.1 mol/l RbCl (pH 6); t, 22 °C; gramicidin concentration, 10^{-10} mol/l, the antibiotic was added to the bathing solution just before BLM formation.

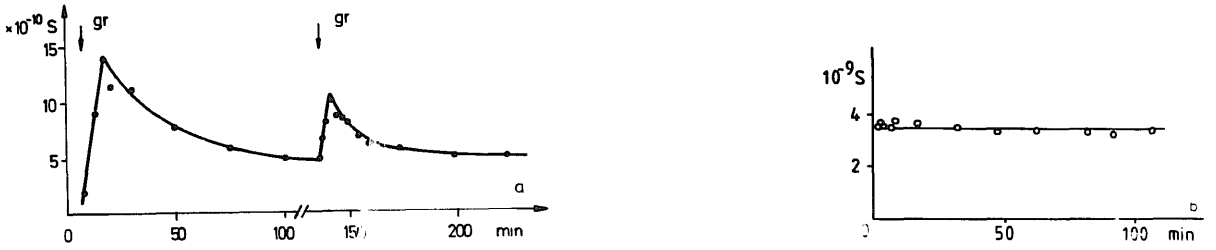

Fig. 6. (a) Effect of gramicidin A on the conductance of Montal-Mueller-type BLM prepared from PE and cholesterol in a molar ratio of 1:4. Area of BLM, $1.2 \cdot 10^{-5}$ cm^2; bathing solution, 0.1 mol/l RbCl (pH 6); t: 27°C. 2 µl of 10^{-5} mol/l gramicidin stock solution was added to the bathing solution of 2 ml at the time indicated by arrows. (b) Time-course of conductance of gramicidin-A-treated Montal-Mueller-type BLM prepared from PE. Area of BLM, $1.2 \cdot 10^{-5}$ cm^2; bathing solution, 0.1 mol/l RbCl (pH 6); t, 22°C; gramicidin concentration, $2 \cdot 10^{-10}$ mol/l. The antibiotic was added to the bathing solution before BLM formation.

organic solvent at the edge of the bilayer is negligible or in any case should be much smaller than that in Mueller-type membranes.

The kinetic data on the conductance changes for Montal-Mueller-type membrane prepared from the same bulk ox brain lipids with addition of cholesterol are shown in Fig. 5. In this experiment the gramicidin was added to the bathing solution of BLM before the membrane formation, so that the registration of membrane conductance started from a high level, 10^{-7} S, which was characteristic of the gramicidin-A-modified BLM and decreased by a nearly exponential decay with a $t_{1/2}$ of 5 min. These results show that diminishing the amount of free-lipid solution does not eliminate the channel inactivation, since an even faster inactivation is observed than in the case of spherical BLM. Pope et al. [32] were the first two show that when cholesterol was added to phospholipid BLM modified by gramicidin A, the conductivity of the channels as well as the mean time of the open state of channels decreased. This, together with our finding that channel inactivation was observed in membranes of high cholesterol content (cholesterol/phospholipid ratio in RBC membrane is about 1:1 and in the BLM used is 4:1), indicates that this inactivation phenomenon might be dependent on the presence of cholesterol in the membrane. This suggestion might be proved by a comparison of the kinetics of gramicidin-induced conductivity of BLM prepared from a specific lipid with or without addition of cholesterol. An example of such experiments is presented in Fig. 6. Montal-Mueller's membranes formed from the mixture of cholesterol and PE taken at a molar ratio of 4:1 gave a typical picture of the pronounced decrease of gramicidin-A-induced BLM conductivity (Fig. 6a). When the membranes were prepared from the same lipid but without cholesterol no sign of the inactivation was observed for a period of up to 110 min (Fig. 6b).

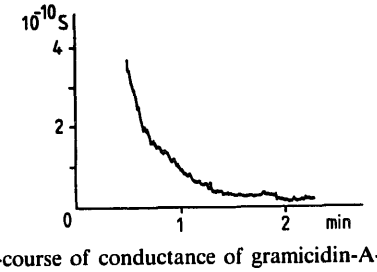

Fig. 7. Time-course of conductance of gramicidin-A-treated Montal-Mueller-type BLM prepared from cholesterol. Area of BLM, $1.2 \cdot 10^{-5}$ cm^2; bathing solution, 0.1 mol/l RbCl (pH 6); t, 22°C; gramicidin concentration, 10^{-8} mol/l. The antibiotic was added to the bathing solution before BLM formation.

It was of interest to test the time-course of electric conductivity of cholesterol * BLM. The decrease of gramicidin-A-induced conductivity with time for such a membrane is presented in Fig. 7. The inactivation process in this case was extremely fast ($t_{1/2} = 13$ s). This may be evidence for the interaction of gramicidin A with cholesterol being indeed responsible for inactivation of the modifier. The result of special experiments has shown the strong dependence of the inactivation rate on the molar ratio of phospholipid-to-cholesterol in the membrane (manuscript under preparation).

It should be noted that, during the final phase of conductance decay in the course of inactivation, the integral membrane conductance was reduced to the single-channel conductivity typical for those induced by gramicidin A. It is obvious that for the detailed analysis of the inactivation process, special studies on time-dependent changes of the distribution of conductances of single channels and on their open-to-closed state time ratios should be undertaken. This work now is in progress.

The data presented above explain the peculiarities of the time-course of the exchange diffusion of alkali ions in gramicidin-A-modified RBC membranes. The established fact of the pronounced influence of cholesterol on that inactivation process is especially interesting as an indicator for an active role of cholesterol in the transformation of ion-channel structures with time. The

* The experiments were carried out under normal room atmosphere and the oxygen present in the solutions in contact with the bilayer might have oxidized some part of the cholesterol in the bilayer. Thus, some amount of oxidized cholesterol might have been present as a bilayer component.

latter transformations may be in some way related to a protective role of cholesterol in the living cell membrane permeability [33].

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