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PROPERTIES OF AMPHOTERICIN B CHANNELS IN A LIPID BILAYER

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Summary

Properties of individual ionic channels formed by polyene antibiotic Amphotericin B were studied on brain phospholipid membranes containing cholesterol. The ionic channels have a closed state and an open one (with conductance of about 6.5 pS in 2 M KCl). The conductance value of an open channel is independent of cholesterol concentration in the membrane and of pH in the range from 3.5 to 8.0. The voltage-current characteristics of a single channel are superlinear. Zero current potential value in the case of different KCl concentrations in the two solutions indicates preferential but not ideal anionic selectivity of a single channel. Channel conductivity grows as the electrolyte concentration is increased and tends to a limiting value at high concentrations. A simple model having only one site for an ion was shown to represent satisfactorily an open channel behaviour under different conditions. An individual ionic channel performs a large number of transitions between the open and closed states during its life-time of several minutes. Rate constants of these transitions depend on the kind and concentration of salt in aqueous solutions. The switching system functioning is not influenced by an ion situated inside the pore.

Introduction

In 1968 Andreoli and Monahan [1] and Cass and Finkelstein [2] discovered that Amphotericin B causes a sterol-dependent increase in the conductance of lipid membranes. The drug is ineffective for sterol-free membranes at any concentration. As the concentration of cholesterol (or certain other sterols) is increased effectiveness of the antibiotic sharply rises [3]. Amphotericin B induces preferential (but not ideal) anionic conductance in the cholesterol-containing membranes [4,5]. A characteristic feature of the effect of Amphotericin B (and other polyenes) on the lipid membrane is the strong dependence of membrane conductance upon concentration of the antibiotic. The drug is much

less effective when it makes contact with only one side of the membrane. It increases permeability of the membrane not only for ions but also for water and nonelectrolytes and this permeability increases proportionally to electric conductivity [6,7]. Permeation coefficients of the membrane for hydrophylic nonelectrolytes increase in inverse proportion to the size of the latter. Glucose and larger molecules do not penetrate the membrane in the presence of Amphotericin B [6].

All the above data have led to the conclusion that Amphotericin B forms in the lipid membrane pores with effective radius about 4 Å [6,7]. A molecular model of such a pore has been proposed [8,9,10]. According to this model, the pore is formed of two half-pores and is built into across the membrane. Each half-pore consists of 8 antibiotic and 8 sterol molecules [8,10]. Only recently were the conductance changes resulting from formation of one such pore, a single ionic channel, detected and experimental conditions described [11]. The present paper describes properties of the ionic channel formed in a lipid bilayer in the presence of Amphotericin B.

Methods

Black lipid membranes were obtained by the standard technique [12] on a hole 0.2 mm in diameter in a teflon cell. The membranes were formed of total brain phospholipids extracted by the method of Folch et al. [13] and freed of neutral phospholipids by acetone extraction according to Kates [14]. Phospholipids were stored at 10 mg/ml in chloroform/methanol (2 : 1 v/v) mixture at 4°C. Before the experiment, phospholipids were transfered to *n*-heptane at 20 mg/ml and 1 mg/ml of re-cristallized cholesterol was added. Histidine or phosphate at 5 mM concentration were added to aqueous solutions to stabilize pH. Conductance values of unmodified membranes in 2 M KCl were 2—3 pS.

The current across the membrane was measured at a constant membrane potential with a Keithley-301 electrometer amplifier. The current was fed by Ag-AgCl electrodes with agar bridges filled with 3 M KCl. An Endim XY-recorder was used to record the current.

Amphotericin B and its water-soluble sodium salt were kindly provided by Dr. E.D. Etingov, Institute of Antibiotics, Leningrad. Stock solutions of the antibiotic were renewed weekly to ensure effectiveness of the drug and reproducibility of the results.

Results and Discussion

Membrane conductance in the presence of small amounts of Amphotericin B

Fig. 1 shows several records of random membrane current variations with time in the presence of Amphotericin B. The current jumps observed are apparently due to the formation of channels with two states: the open (about 6.5 pS in 2 M KCl) and the closed one. The probability of channel formation rises slightly as the membrane potential is increased. The conductance value of an open channel is independent of cholesterol concentration in the membrane-forming solution.

Current jumps were observed only upon addition of Amphotericin B to both

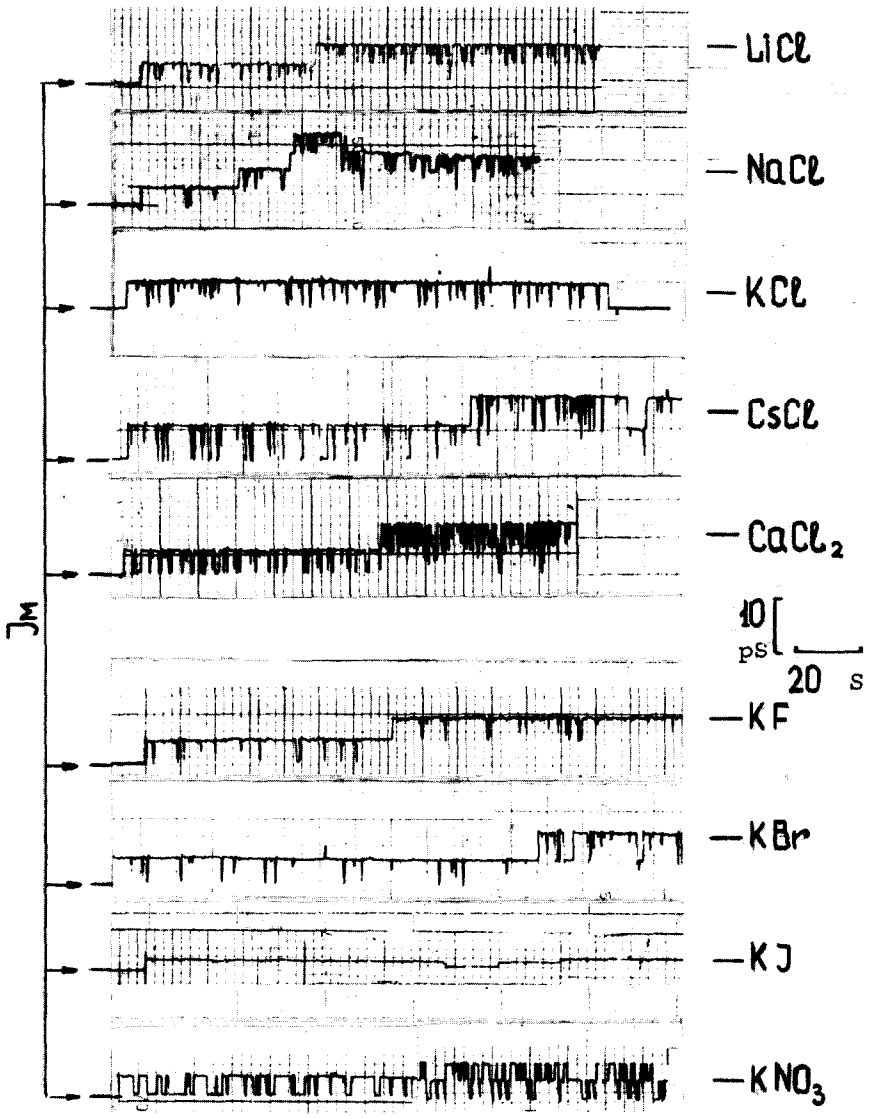

Fig. 1. Membrane conductivity change with time at a constant membrane potential of 200 mV in 2 M solutions of different salts. Concentrations of Amphotericin B: LiCl, $2 \cdot 10^{-7}$ M; NaCl, $1 \cdot 10^{-7}$ M; KCl, $5 \cdot 10^{-8}$ M; CsCl, $5 \cdot 10^{-8}$ M; $CaCl_2$, $1 \cdot 10^{-7}$ M; KF, $1 \cdot 10^{-7}$ M; KBr, $1 \cdot 10^{-7}$ M; KJ, $5 \cdot 10^{-7}$ M; KNO_3, $2 \cdot 10^{-7}$ M.

aqueous solutions. Marty and Finkelstein [15] reported an increase of membrane conductance with Amphotericin B added at high concentration to one solution. We also found that addition of 10^{-4} M Amphotericin B to one side of a cholesterol-containing film (2 : 1 weight ratio phospholipid : cholesterol) increased the membrane conductance about 10-fold, but no discrete current jumps were seen.

The average life time of a channel in the membrane is more than 4 min (mean for 30 channels). During this time a channel is converted many times into

the closed state and then into the open one. As the antibiotic concentration in aqueous solutions is increased one can observe formation of several channels. In one of such records made using 5 M CsCl solutions from 2 to 7 conductance levels, differing from one another by the same value were observed for a long period of time. The frequencies of short switch-offs from the second, third etc. levels to the preceding ones were 0.76, 1.14, 1.65, 1.9, 2.3 and 3.3 s^{-1}, respectively. It is easily seen that the frequencies are approximately proportional to the level numbers. This must be the case when the membrane contains identical and independently functioning channels with two states. The frequency of short transitions of each channel into the closed state is 0.39 s^{-1}.

As was shown earlier [11], channel formation depends on the salt used and its concentration. No discrete conductance level shifts were observed in 2 M solutions of acetyl choline chloride, choline chloride, potassium rodanide and potassium propionate, lithim and potassium sulfate, and potassium phosphate. The distribution function of single channel conductance in 2 M KCl for 200 mV is presented in Fig. 2. The figure shows that 6.5 pS channels as well as channels of lower conductance value are formed.

The relationship between channel conductance and salt activity presented below were obtained by averaging conductance for about 10 channels. The vol-

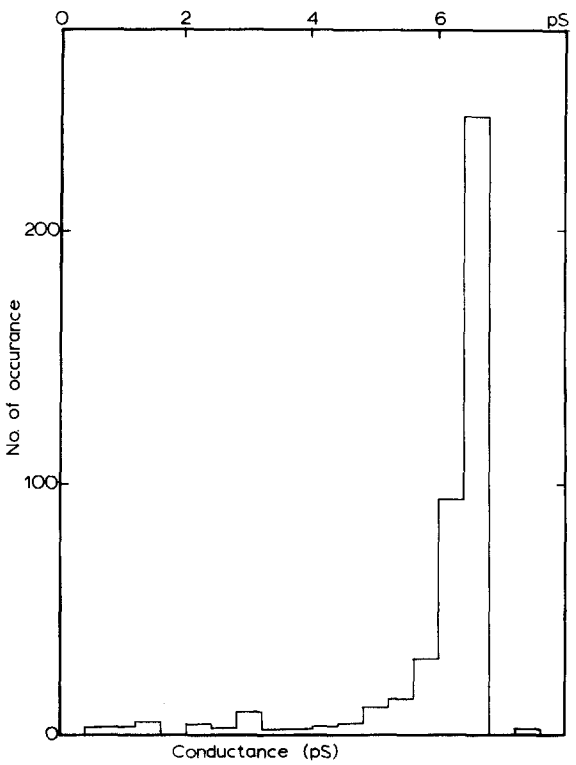

Fig. 2. Distribution function of Amphotericin B channels conductance. The abscissa is channel conductance, the ordinate is the number of observed channels with conductance within the range indicated on the abscissa. For each membrane the jumps of formation of 2 to 3 channels were recorded. 200 mV, 2 M KCl, pH 6.5, 24°C.

tage-current characteristics were obtained on the channels with the most probable conductance value.

Properties of the open channel

Fig. 3 shows the relationships between conductance of a single channel and activity of several salts. All the curves tend to saturation at high electrolyte activity. The value of maximum conductance depends on both the anion and cation type. The conductance in solutions of alkaline metal chlorides increases with the cation cristal radius. Among potassium halide solutions, maximum conductance is observed for the chloride. In fitting the calculated curve for LiCl, g_{200} values of 5 and 6 pS for 4 and 5 M solutions, respectively, were taken into account, which are not shown in Fig. 3 due to the high activity coefficient. The voltage-current characteristics of channels in symmetric electrolyte solutions are superlinear for all salt kinds and concentrations. Fig. 4 shows voltage-current characterstics of the channel when KCl concentrations in the two aqueous solutions are the same or different. For KCl concentration of 1 and 3 M in the two solutions, respectively, the zero current potential $V_0 = 17.0 \pm 2.0$ mV. The solution with the smaller KCl concentration is negative with respect to the other solution. The transport numbers $T_{Cl} = 0.85$ and $T_K = 0.15$ indicate preferential, but not ideal, anionic selectivity of a single channel. Zero current potential for a membrane with a large number of channels separating 1 and 3 M KCl solutions is 18.5 ± 0.5 mV, which agrees with the value for a single channel within the error range. An interesting feature of voltage-current characteristics in Fig. 4 is their crossing in the region of positive potentials. The anion in this case goes from the 1 M KCl solution through both channels studied. Even though the force exerted on the anion is higher in the 1 : 1 M system at high positive potentials, the current is higher in the 1 : 3 system. This effect is apparently due to cation contribution to the current. Evidently, the cation flux at

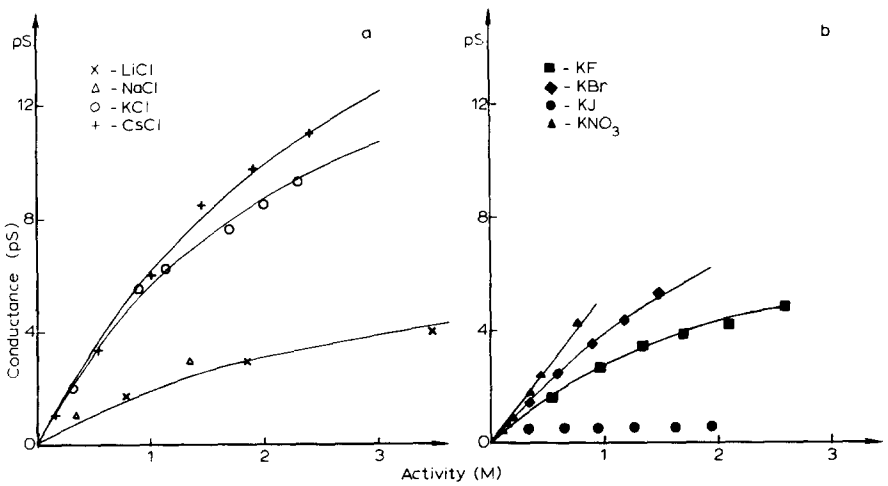

Fig. 3. Relationship between conductance of individual channels and salt activity: a, different cations; b, different anions. Sodium thiosulfate at 1 mM was added to KI solutions to avoid oxidation of iodide to iodine. The solid lines were fitted to the experimental point by the least squares method according to Eqn. 1.

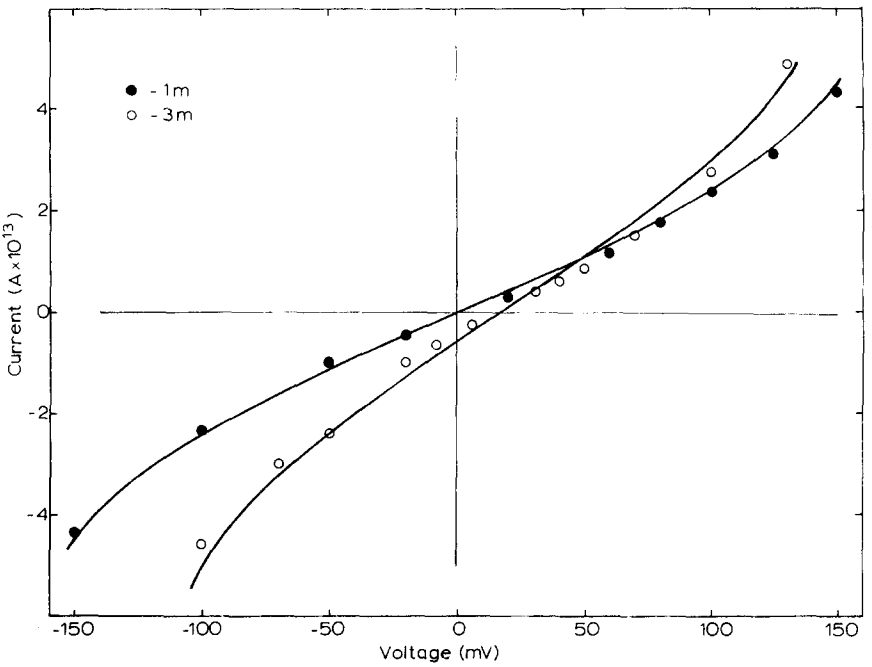

Fig. 4. Voltage-current characteristics of individual channels. KCl concentration in the outer solution is 1 M in both cases. The inner KCl concentrations are 3 M (○) and 1 M (●). Potential of the inner solution with respect to the outer one is indicated. The solid curves are calculated for the two-barrier model with the parameters given in Table I.

positive potentials must be higher in the 1 : 3 M system than in the 1 : 1 M system due to both the higher electromotive force exerted upon the cation and to higher salt concentration.

Relationships between zero-current potential and activity ratio in the two salt solutions obtained on a many-channel membrane are presented in Fig. 5. All the relationships are linear from the activity ratio of 1 to about 0.1 and reach their limiting values at greater differences between the activities. The channel conductance is independent of pH within the pH range of 3.5 to 8. The temperature coefficient of conductance of an open channel, Q_{10}, equals 1.3 (g_{200} is 2.0 pS and 2.7 pS at 11 and 23°C, respectively, in 1 M KNO_3).

An open channel model

It is convenient to discuss the obtained data by comparing them with predictions of a simple model. In describing voltage-current characteristics, which are close in shape to $\sinh (VF)/(4RT)$ function for all ion concentrations and salt types, consideration may be restricted to symmetrical two-barrier models with one potential well for ions in the channel. We thus suppose that there is never more than one ion in the channel. Well depths and barrier heights for cations and anions may be different. Let α_a and α_c be rate constants of entry of anion and cation, respectively, into the well from solution at zero membrane potential; the rate constants of release into solution are β_a and β_c. Let θ_a and θ_c be probabilities of the well being occupied by anion and cation, respectively, at a given moment. Stationary fluxes of monovalent anion and cation over the two

barriers are

$$j_a = \alpha_a A_1(1-\theta)\exp(-\varphi/4) - \beta_a\theta_a \exp\varphi/4$$
$$= \beta_a\theta_a \exp(-\varphi/4) - \alpha_a A_2(1-\theta)\exp\varphi/4$$
$$j_c = \alpha_c A_1(1-\theta)\exp(\varphi/4) - \beta_c\theta_c \exp(-\varphi/4)$$
$$= \beta_c\theta_c \exp\varphi/4 - \alpha_c A_2(1-\theta)\exp(-\varphi/4)$$

where φ is dimensionless membrane potential $\varphi = \frac{FV}{RT}$, A_1 and A_2 are salt activities in the two solutions and $\theta = \theta_a + \theta_c$ is an occupation coefficient of the well.

Solving these four equations for j_a, j_c, θ_a and θ_c, one obtains membrane current $I = F(j_c - j_a)$. For symmetrical solutions ($A_1 = A_2 = A$) the current is

$$I = 2F(\alpha_c + \alpha_a)\, A \cdot \sinh(\varphi/4)\left[1 + A\left(\frac{\alpha_c}{\beta_c} + \frac{\alpha_a}{\beta_a}\right)\right]^{-1} \tag{1}$$

$(\alpha_c + \alpha_a)$ and $(\alpha_c/\beta_c + \alpha_a/\beta_a)$ were found by the least square fit of Eqn. 1 to experimental points of Fig. 3 ($g_{200} = \frac{I}{V}$ for $V = 200$ mV). Limiting value of g_{200} (for $A \to \infty$) and $A_{0.5} = (\alpha_c/\beta_c + \alpha_a/\beta_a)^{-1}$ are given in Table I. One more relation between α_c and α_a was found from zero current potential value (Fig. 5). According to the model dependence of this potential on salt activities ratio is

$$\varphi_0 = \ln\left(\frac{\alpha_c}{\alpha_c} + \frac{A_1}{A_2}\right) \Big/ \left(\frac{\alpha_c}{\alpha_a}\cdot\frac{A_1}{A_2} + 1\right) \tag{2}$$

It is more convenient to determine α_a/α_c value from the initial region of $(\varphi_0 - A_1/A_2)$ curve.

For $A_1 \simeq A_2$

$$\frac{d\varphi_0}{d\ln A_1/A_2} = \left(1 - \frac{\alpha_c}{\alpha_a}\right) \Big/ \left(1 + \frac{\alpha_c}{\alpha_a}\right) \tag{3}$$

Experimental values of $d\varphi_0/d\ln A_1/A_2$ and calculated α_c and α_a for several kinds of salt are given in Table I.

TABLE I

g_{200}, limiting value of channel conductivity (at $A \to \infty$); $A_{0.5}$, salt activity at which channel conductivity equals half of the limiting conductivity. g_{200} and $A_{0.5}$ were found by the least squares method according to Eqn. 1. $d\varphi_0/d\ln(A_1/A_2)$ values were found by the slope of $V_0(\lg A_2/A_1)$ curves (Fig. 5) at initial points. α_a, α_c, β_a and β_c, rate constants of two barrier models.

Salt	KCl	CsCl	KF	KBr	KNO_3	LiCl
g_{200} (pS)	20.2	27.0	9.6	17.9	20	7.75
$A_{0.5}$ (M)	2.66	3.5	2.53	3.71	3	2.4
$d\varphi_0/d\ln(A_1/A_2)$	0.70	0.64	0.96	0.76	0.41	0.64
$\frac{\alpha_a}{\beta_a} + \frac{\alpha_c}{\beta_c}$ (cm^3/mol)	376	285	395	270	340	417
$10^{15} \times \alpha_a$ (cm^3/s)	1.85	1.8	1.05	1.21	1.07	0.8
$10^{15} \times \alpha_c$ (cm^3/s)	0.33	0.39	0.02	0.17	0.44	0.17

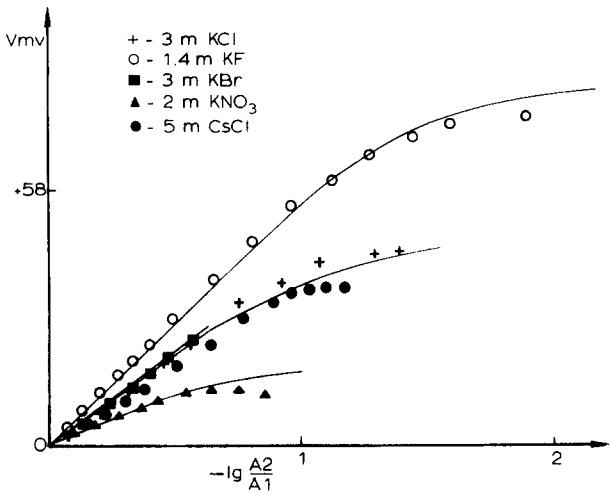

Fig. 5. Zero current potential as a function of a logarithm of the salt activity ratio in the two solutions. The outer solution activity A_2 was varied. The constant activity A_1 is 5, 3, 1.4, 3 and 2 M for CsCl, KCl, KF, KBr and KNO_3, respectively. At the beginning of each measurement the antibiotic concentration was adjusted to obtain membrane conductivity corresponding to 200—500 channels. Potential of the inner solution with respect to the outer one is indicated.

Solid curves in Figs. 3—5 are calculated according to the two-barrier model, using parameters from Table I. For calculation of curves in Fig. 4 (asymmetrical conditions) α_c/β_c is taken to negligible compared to α_a/β_a. The curves' shape and the limiting values of potential in Fig. 5 are satisfactorily represented by the model.

Thus, the two-barrier model with a single ion site in the pore is a good representation of channel behaviour under different experimental conditions. According to this model, the voltage-current characteristics are superlinear and their shape is independent of salt concentration. Fig. 4 shows that voltage-current characteristics calculated according to this model cross over under symmetrical and asymmetrical conditions similarly to the experimental ones.

Serious limitations of this model are revealed in comparing rate constants for different ions. The values α_c found for salts with the same cation must be independent of the anion, and values for a given anion must be independent of the cation type. As is seen from Table I this is observed for neither α_c or α_a. It is possible that interaction of ions with a pore is not confined to simple competition for one potential well and some alternative models have to be considered.

Salt-dependence of channel switching system

This part of the paper describes properties of a system, which controls the opening and closing of a channel. Its functioning is shown to be independent of membrane potential but to be specifically influenced by the type and concentration of electrolyte solutions.

It can be seen from Fig. 1 that the channel dwell times in the two states are salt-dependent: they depend on both the cation and the anion kind. Cation charge also affects the switching off frequency, which is apparent from comparison of channel records in $CaCl_2$ with the rest. By contrast to the other salts,

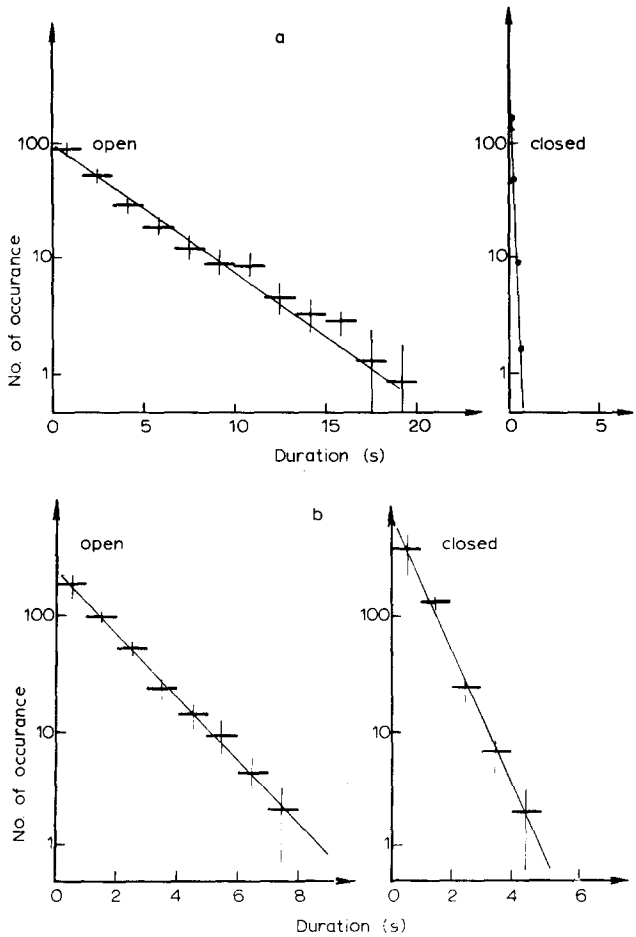

Fig. 6. Distribution of dwell time in the open (left) and the closed (right) states. Semilogarithmic scale. A, 3 M KCl, 200 mV; B, 2 M KNO_3, 200 mV. pH, 6.5; t = 24°C.

the channel dwell time in the closed state in KNO_3 is longer. Fig. 6A and B show distribution functions of dwell times in the open and closed states for channels in KCl and KNO_3. All the distribution functions are single-exponentials. Average dwell times obtained from the distribution functions differ significantly for potassium nitrate and potassium chloride. The value of τ_c is independent of KCl activity in a wide range of the latter. The value of τ_0 rises approximately linearly with increase in KCl concentration: τ_0 is 1.9 s in 0.5 M KCl and 5.4 s in 3.5 M KCl.

Variation of pH in the range of 3.5 to 8.0 does not affect the properties of the switching system. Temperature coefficients for τ_0 and τ_c in 1 M KNO_3 are close to 3 which corresponds to activation energy of transitions between the two states about 15 kCal/mol.

It is possible that the switching system is influenced by an ion situated inside the pore. The next experiment (Fig. 7) shows this is not the case. A membrane with one Amphotericin B channel freed solution I of 2 M KCl and solution II of 2 M KNO_3. Channel current records were obtained for membrane voltage

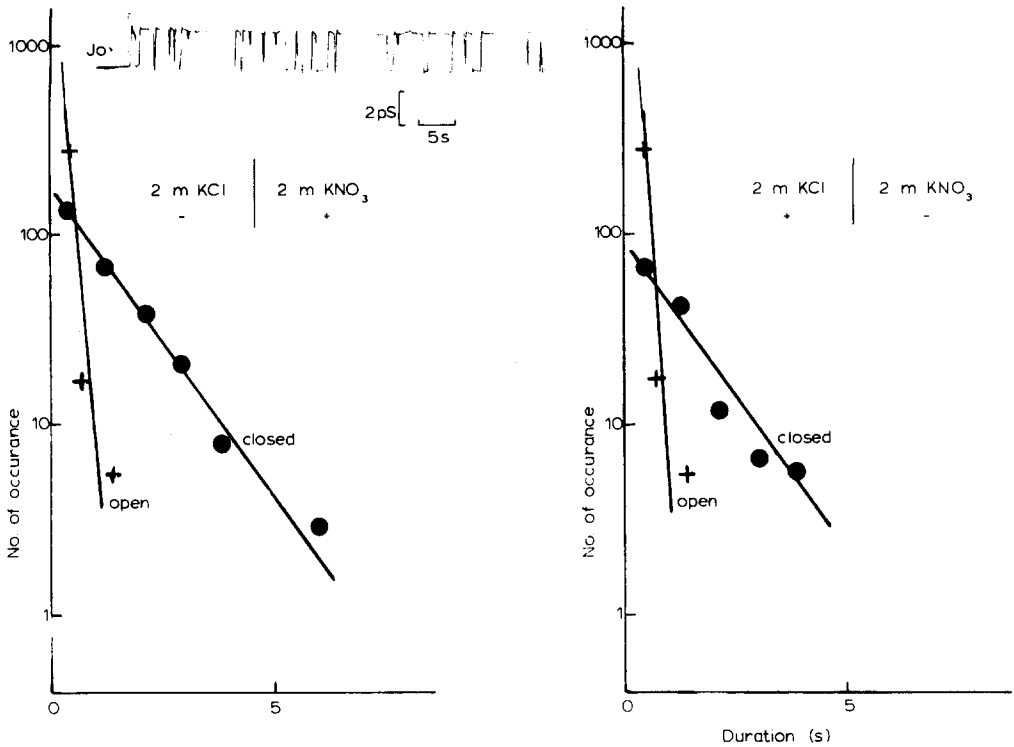

Fig. 7. Distribution function of dwell-times of a single channel in a membrane formed between solutions of 2 M KCl and 2 M KNO_3. Left-current flows from solution of KNO_3, right current from KCl. pH , 6.5; $t = 24°C$.

of +200 mV and −200 mV. When current passes from solution II to solution I the channel is mainly occupied by Cl^-. For another current direction NO_3^- passes through the channel. However, distribution functions of dwell times are the same for two current directions. Thus, the functioning of the switching system does not depend on which ions pass through the channel. Probably, salt dependence of Amphotericin B channel switching is a result of influence of solution content on the physical state of lipid bilayer or on charge groups of antibiotic molecules which form a channel. Further experiments are necessary to decide between these two possibilities.

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