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Roflamycoin — a new channel-forming antibiotic

Pavel Grigorjev [a], Rolf Schlegel [b], Hans Thrum [b] and Lev Ermishkin [a]

[a] *Institute of Biological Physics, Academy of Sciences of the U.S.S.R., Pushchino, Moscow Region (U.S.S.R.) and* [b] *Central Institute of Microbiology and Experimental Therapy, Academy of Sciences of the G.D.R., Jena (G.D.R.)*

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Ion permeability of lipid bilayers was studied in the presence of a new antifungal pentaene antibiotic, roflamycoin, the structure of which differs considerably from that of the well-known polyene channel-former amphotericin B. Both of them, however, show the property of increasing the membrane permeability only in the case of sterol-containing membrane when added on both its sides. The conductance is strongly dependent on the concentration of the antibiotic in the solutions and of sterol in the membrane. Unlike the amphotericin B channels, roflamycoin channels are potential-dependent and have short lifetime (approx. 1 s) and high conductance (approx. 100 ps in 1 M KCl), which increases linearly with the salt concentration and is not blocked by the familiar blockers of amphotericin B channels. The two antibiotics seem to have a common mechanism of channel formation, viz. the formation starts from two semi-pores assembled in the opposite monolayers from several molecules of the antibiotic and sterol. However, the inner diameter of the roflamycoin channel is larger because of the different antibiotic-to-sterol ratio in the channel aggregate. It is believed that the difference in the ratio is due to the presence of the methyl group in the polyene chain of roflamycoin, and the considerable difference in lifetimes of the two types of channels depends on the terminal groups of the antibiotics.

Introduction

Experiments with ion channels formed in both artificial and cell membranes by antibiotics of the well-defined structure such as gramicidin A, amphotericin B and alamethicin are at present the only way of understanding how the structures of channel-forming substances and of channels themselves determine the permeability properties of ion pathways across membranes. Among these substances, the polyene antibiotics seem to be of the greatest interest because, firstly, there are several natural antibiotics of known chemical constitution and, secondly, they can be modified at different parts of the molecule. Some of the polyene antibiotics with large lactone ring have been shown to form ion channels. Three of them, studied at the level of single channels, amphotericin B, nystatin and mycoheptin form long-living potential-independent channels in sterol-containing phospholipid bilayers. The experiments [1] with these antibiotics of closely related structure revealed that alterations in various parts of the polyene molecule influence different properties of the channels: (1) neutralization of the charged groups results in shorter lifetimes in the open state; (2) hydrogenation of a double bond in the polyene chain makes the channel formation more difficult; (3) variations in the hydrophylic chain of the lactone ring affects the channel conductance and selectivity.

The present paper deals with another channel-forming polyene antibiotic, roflamycoin, which differs considerably in its chemical constitution from the previously studied ones. As roflamycoin

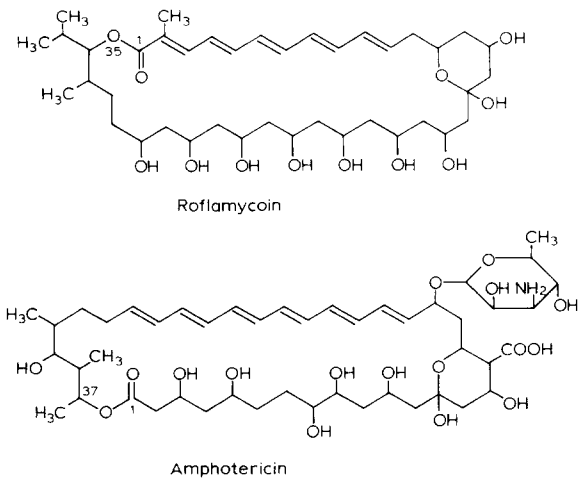

Fig. 1. Structural formulae of roflamycoin and amphotericin B.

was shown to form ionic channels in lipid bilayers [2], the aim of the further studies was to characterize the properties of the channels.

Roflamycoin is an antifungal carbonyl-conjugated pentaene antibiotic produced by *Streptomyces roseoflavus* JA 5068 [3,4]. Recently, its constitution was described by Schlegel et al. [5]. This antibiotic possesses a cyclic macrolide ring with 35 carbon atoms, a methyl-substituted pentaene chromophore conjugated with lactone carbonyl, and a hemiketal structure formed by a keto group in position C-17. The molecular weight of roflamycoin determined by mass spectrometry is 738, the molecular formula is $C_{40}H_{66}O_{12}$. The structural formulas of roflamycoin and amphotericin B are shown in Fig. 1. Besides the difference in the polyene chain, roflamycoin differs from the heptaene antibiotic amphotericin B in all other parts of the molecule. The hydrophylic part (C-19–C-31) of roflamycoin consists of a uniform line of OH-groups in the β-position. In contrast to amphotericin B, which contains an amino sugar and a carboxylic group, there are no charged groups in roflamycoin.

Within the group of pentaene antibiotics, roflamycoin differs from flavofungin and filipin in both chemical structure [5] and membrane activity [2].

Methods

The standard technique [6] was used for formation of a planar lipid bilayer on a hole of 0.3 mm diameter in a teflon cell. The membranes were formed from a *n*-heptane solution of total brain phospholipids free of neutral phospholipids. The required sterol concentrations were obtained by addition of sterol to the lipid solution. The lipids contained 9% of cholesterol, unless otherwise specified. Unbuffered aqueous solutions of electrolytes at 20°C and pH 5.6 ± 0.2 were used.

Stock solutions of reflamycoin were always freshly prepared by dissolving pure crystalline samples ($E^{1\%}_{1\,cm} = 860$) in dimethylsulfoxide.

As roflamycoin is unstable under light, the glass cuvette was protected from external light. Illumination by the lamp of a binocular microscope took place only during the short period of membrane formation and addition of the antibiotic.

Results and Discussion

Conductance of multi-channel membrane

Roflamycoin was ineffective even at high concentration (10^{-5} M) when added to the solution only on one side of the membrane, no matter what was the sterol content of the membrane. However, as little as 10^{-8} M of the antibiotic added to the solutions on both sides of the membrane caused an increase in the conductance of cholesterol-containing membrane *. Fig. 2 shows the relationship between the steady-state conductance and the increasing cholesterol concentration in the membrane-forming lipid solution. The dependence is very steep (slope 6–8 in the double-log plot) similarly to that observed for amphotericin B [7]. Even a steeper relationship was observed between the bilayer conductance and roflamycoin concentration in the two solutions. Fig. 3 shows such relationships for cholesterol (curve 1) and ergosterol-containing membranes (curve 2). Two major differences from the case of amphotericin B should

* Amphotericin B makes thin membranes permeable when added on only one side. Thus, amphotericin works when applied to one side only of red blood cells, some types of lipid vesicles and lipid bilayers [10]. 10^{-5} M of roflamycoin added on one side also increases the conductance of asolectin-heptane bilayers by 3–4 orders (in 1 M KCl). However, both amphotericin B and roflamycoin added to one side of heptane-containing bilayers of brain lipids do not affect ion permeability. Presumably, this membrane is relatively thick.

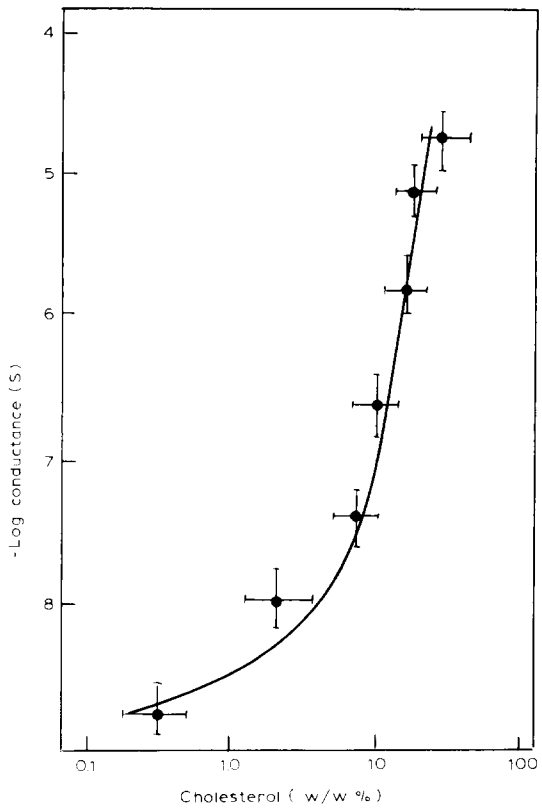

Fig. 2. Steady-state conductance vs. the ratio of cholesterol/phospholipids + cholesterol (w/w %) in the membrane-forming solution. 0.5 M KCl and 80 nM roflamycoin.

be noted. Firstly, both curves are at least twice as steep as those with amphotericin B, indicating that more antibiotic molecules are required to form the ionic pathways in the case of roflamycoin. Secondly, roflamycoin poorly discriminates between ergosterol and cholesterol, whereas amphotericin B is much more effective in the case of ergosterol-containing membranes [8]. Poor sterol selectivity of roflamycoin channels should result in the higher toxicity of the antibiotic.

Voltage dependence of the membrane conductance

The measurement of steady-state current-voltage characteristics of the membranes in the presence of roflamycoin revealed a strong voltage dependence of the conductance. Comparisons between normalized conductance-voltage dependences for the two antibiotics are shown in Fig. 4. Curve 1 and curve 2 characterize membranes activated with roflamycoin and with amphotericin B, respectively. It is seen that the conductance is more potential-dependent in the case of roflamycoin.

Anion-cation selectivity

Bilayers with reflamycoin channels are well permeable for univalent anions and cations, and less permeable for divalent ions.

Fig. 5 shows zero-current potentials as functions of the ratio A_i/A_o, a 1 : 1-valent electrolyte in the inner (i) and outer (o) compartments, where A_o is the constant concentration (100 mM) and A_i the variable concentration. Dashed lines stand for ideal anion and cation selectivities of the membrane (upper and lower parts of Fig. 5, respec-

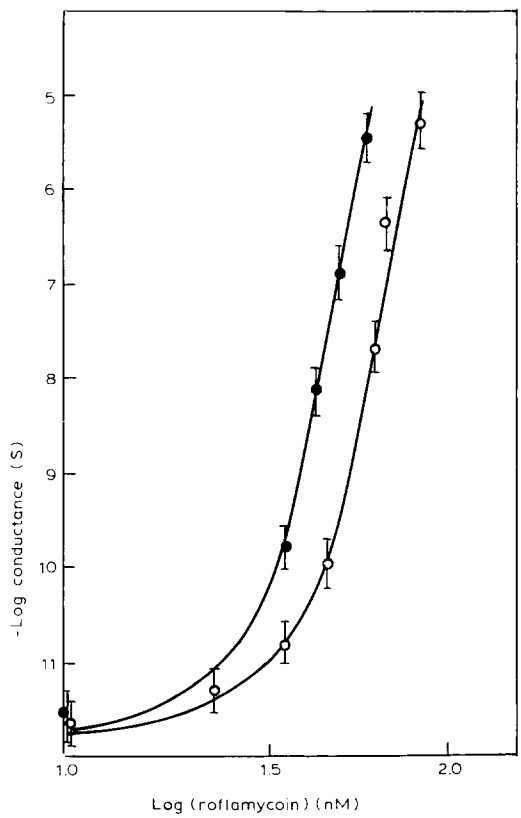

Fig. 3. The relation between the steady-state membrane conductance and the concentration of roflamycoin in the two aqueous phases at constant ion concentration (0.5 M KCl) and constant lipid composition. Curve 1, phospholipids/cholesterol/*n*-heptane = 20 mg : 2 mg : 1 ml. Curve 2, phospholipids/ergosterol/*n*-heptane = 20 mg : 2 mg : 1 ml. Membrane potential, 120 mV.

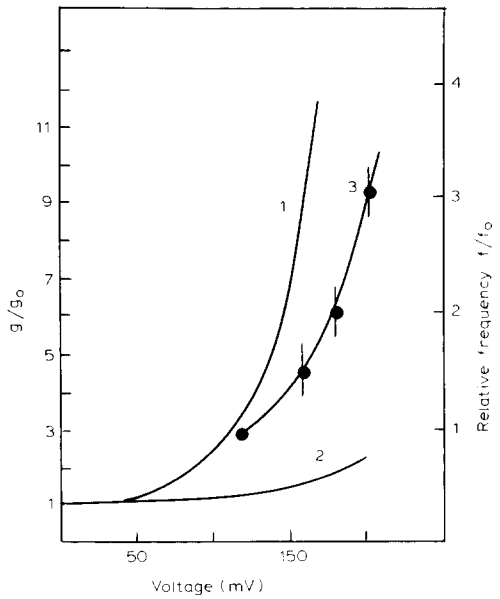

Fig. 4. The relation between steady-state conductance and membrane voltage: 1, for roflamycoin; 2, for amphotericin B. The g/g_0 ratio is plotted along the left vertical scale. g_0 is the conductance measured at 25 mV, i.e., in the linear region of $I-V$ curve. Points and curve 3 represent the relation between the membrane voltage and the relative frequency of opening of a single channel (right scale). Curves 1 and 2 were obtained for 1 M KCl and $3 \cdot 10^{-7}$ M antibiotic; curve 3 for 1 M KCl and 10^{-8} M roflamycoin.

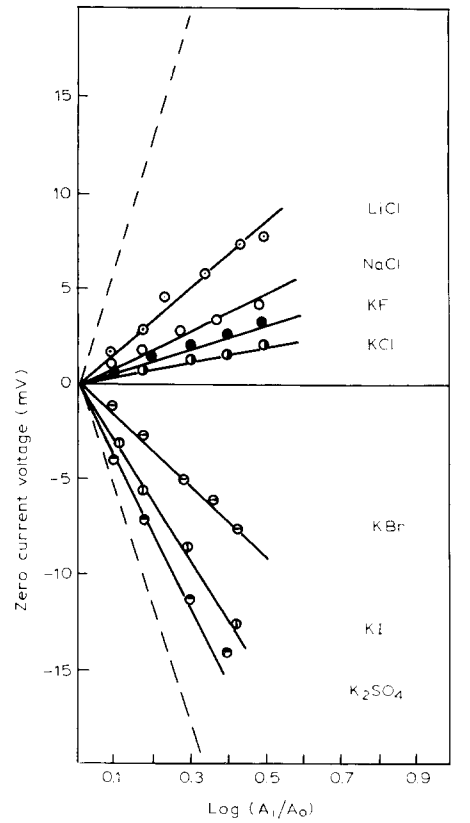

Fig. 5. Zero-curret voltage as a function of the ratio of salt concentrations in the inner (A_i) and outer (A_o) solutions. The voltage is potential in the inner minus this in the outer solution. A_o was kept constant (100 mM) and A_i was increased. The membrane conductances were in the range of 10^{-7}–10^{-6} S.

tively). Anion selectivity was preferable in solutions of LiCl, NaCl and KCl, and cation selectivity in KBr, KI and K_2SO_4 solutions.

Moreover, the membranes with roflamycoin channels were permeable to SO_4^{2-} anions, as opposed to data in Ref. 6. This is probably due to a larger diameter of the roflamycoin channels.

Block of roflamycoin channels

Many organic substances with molecular size of 6–8 Å have been shown to block amphotericin B channels [9]. Neutral blockers such as urea, ribose and glucose cause potential-independent block. Cations like tetramethyl-, tetraethyl- and phenyltrimethylammonium (in millimolar concentration) and propionate anion lead to potential-dependent block. Substances of larger molecular size like tetrabutylammonium and sucrose are less effective. It has been concluded that the blocker molecule entering the channel occludes it, and the most effective blockers are those the molecular sizes of which fit well the interior diameter of the channel. None of these substances blocks roflamycoin channels even at rather high (50 mM) concentration.

Roflamycoin channels are blocked only by such relatively large anions as ADP^{3-} and ATP^{2-}. Addition of 2 mM ADP into the electrolyte solution caused a decrease of conductance by a factor of 1.85 if potential of the solution was negative (-130 mV). The value of the factor was only 1.45 if the potential was positive. This potential dependence of the effect, though weak, shows that ADP molecule enters into the channel. These experiments, together with the observation that roflamycoin channels are permeable for sulfate anions, suggest that the diameter of a roflamycoin channel is larger than that of an amphotericin B channel.

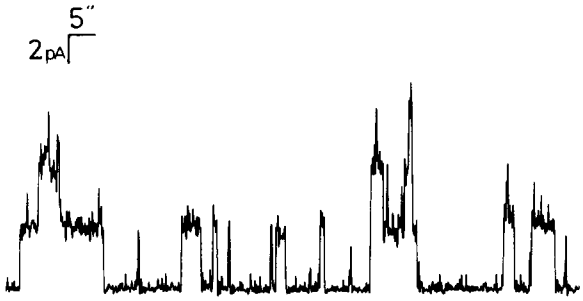

Fig. 6. Single-channel current fluctuations at 10^{-8} M roflamycoin in 0.5 M KBr; membrane voltage = 110 mV. Similar fluctuations were recorded with other 1:1-valent salts (see Table I). The upward deflection corresponds to channel opening.

Conductance and kinetic parameters of single channels

Membrane current fluctuations characteristic of opening and closing of single ion channels, were registered under low roflamycoin concentration (10^{-8} M) in aqueous volumes on both sides of the membrane.

Single-channel current fluctuations in 0.5 M KBr at 110 mV are shown in Fig. 6. The same form of currents was recorded for other 1 : 1-valent salts. The channel conductances were 10–20-times higher than those of amphotericin B channels under the same conditions. For example, the single roflamycoin channel conductance in 1 M KCl at 20°C was 77 pS and that of amphotericin B was only 4 pS. On the other hand, the mean values of dwell-times of the open states of roflamycoin channels were 10–100-times less. They were 0.7, 0.5 and 0.2 s in 1 M KI, KCl and KF, respectively. The conductance of roflamycoin channels was proportionally increasing with electrolyte concentration and was unsaturating even at as high concentration as 1 M (Fig. 7) in contrast to the amphotericin B channels [6]. Again this is probably due to the larger diameter of roflamycoin channels. Single-channel conductances in 1 M electrolytes at 180 mV voltage and 20°C are given in Table I. It can be seen that the conductances are increasing from left to right and also down the column, which in both cases correlates with the direction of increasing the crystal radii of the ions. Such a behavior might be expected for a rather wide water-filled pore in which ions move according to their mobility in water. However, quantitative inspection of the data shows that ion permeation through these channels cannot be described by a simple electro-diffusion model taking no account of the interaction between permeating ions. Indeed, in the absence of any interaction, the permeability coefficient for a given ion should be independent of the counterion type, which is not the case. Two types of relationships between per-

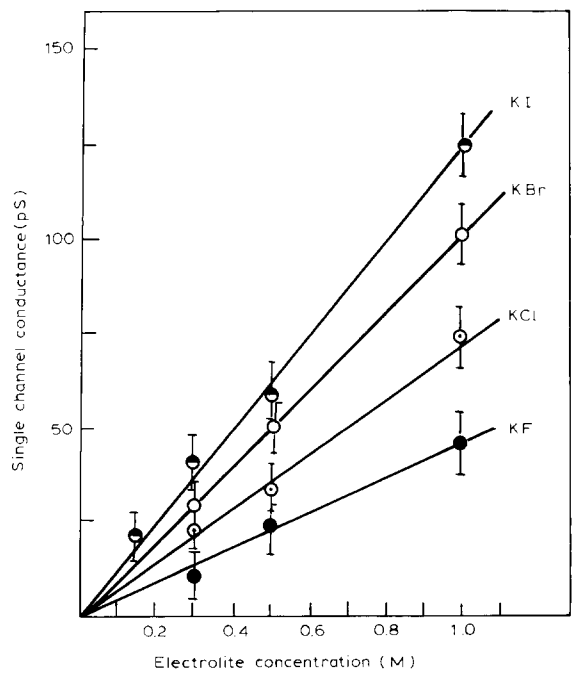

Fig. 7. The relation between the single roflamycoin channel conductance and electrolyte concentration. Membrane voltage = 180 mV.

TABLE I

SINGLE-CHANNEL CONDUCTANCES (pS) IN 1 M SOLUTIONS OF DIFFERENT SALTS AT 180 mV AND 20°C

For example, the channel conductance in 1 M NaCl (54 pS) is at the crossing of the Na row and the Cl column.

	F	Cl	Br	I
Li		38		
Na	27	54	68	84
K	43	77	100	128
Rb		125		

meability coefficients for cation, P_c, and anion, P_a, were derived. The first one was from the data of Table I:

$$G = \frac{F^2}{RT} \cdot (P_c + P_a) A$$

and the second one from the initial slope of the curves in Fig. 5:

$$\left(\frac{dV}{d(\ln A_i/A_o)} = \frac{RT}{F} \cdot \frac{P_c - P_a}{P_c + P_a} \right)$$

where R is the gas constant; T, temperature and F, Faraday. The values of P_{K^+} were found to be $4.0 \cdot 10^{-18}$, $9.0 \cdot 10^{-18}$, $16.0 \cdot 10^{-18}$ and $23.6 \cdot 10^{-18}$ l/s with F^-, Cl^-, Br^- and I^- as counterions, respectively. The P_{Cl^-} coefficients were $6.2 \cdot 10^{-18}$, $7.8 \cdot 10^{-18}$ and $10.2 \cdot 10^{-18}$ l/s for Li^+, Na^+ and K^+ salts, respectively. Therefore, some kind of interaction between permeating ions of opposite signs does take place inthe roflamycoin channels. Another characteristic property of the anion-cation selectivity of the roflamycoin channels is its strong dependence on temperature, as it has been calculated from the data presented in Fig. 8; the ratio of potassium-to-chloride permeability coefficients is 0.9 at 21 °C and 4.4 at 6 °C.

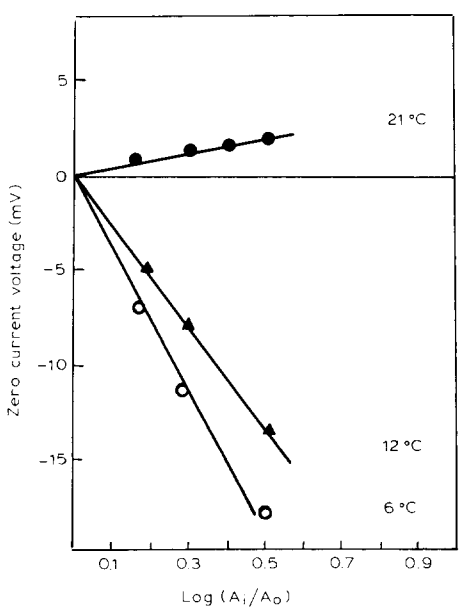

Fig. 8. The relations between the zero-current voltages and the A_i/A_o ratio at different temperatures of KCl solution. Other experimental conditions as in Fig. 5.

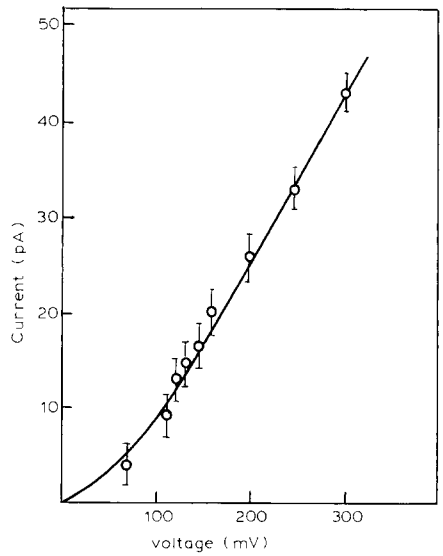

Fig. 9. The current-voltage curve of a single roflamycoin channel in 1 M KI.

A current-voltage curve of a single roflamycoin

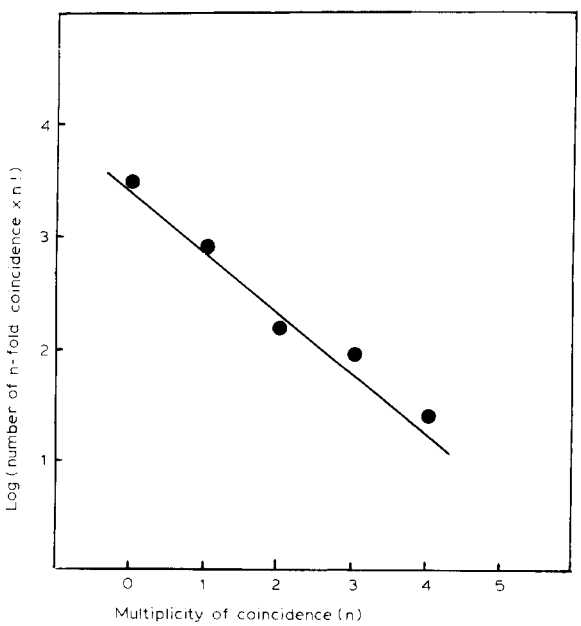

Fig. 10. Log_{10} $(E_n \cdot n!)$ is plotted along vertical scale as function of n. E_n is frequency of occurrence of n simultaneously open channels. This quantity is obtained by sampling the current at 200-ms intervals. The straight line corresponds to the Poisson distribution $E_n = Nx^n \cdot e^{-x}/n!$ with $x = 0.3$, and $N = 3900$ is the number of 200-ms intervals in the analyzed current recording.

channel is shown in Fig. 9. The channel conductance does not depend on voltage in the range from 60 to 300 mV, in contrast to the strong voltage dependence of steady-state conductance of the membrane with many roflamycoinc channels (see Fig. 4).

This means that the number of channels opened at any given moment depends on the membrane potential. The points in Fig. 4 represent relative frequencies of channel openings. The frequency increases exponentially (with a factor of e) per 60 mV (approx.), as compared with exponential increase per 45 mV for the integral conductance. This difference is probably due to the longer lifetimes in the open state at higher membrane potentials.

Statistics of single-channel activity

The statistical analysis of long-term recordings of current fluctuations of single roflamycoin channels (e.g., see Fig. 6) shows that the probability of opening and closing events is given by the Poisson distribution. This means that an elementary conducting unit, a single-channel, has only two states, closed and open, and the state of each channel does not depend on the presence of another. To prove this statement, the logarithm of $(E_n \cdot n!)$ values was plotted as a function of n. E_n means the number of n-fold current pulses recorded during a given period; n stands for the number of simultaneously open channels. As seen in Fig. 10, the experimental points are well approximated by the straight line. It means that opening of the roflamycoin channels is a Poisson process.

Conclusion

It is worthwhile to underline once more the similarities and differences in the properties of roflamycoin- and amphotericin B-induced conductances in planar lipid bilayers, and consider them with relation to the constitutions of the polyenes. Very steep conductance-sterol concentration and conductance-antibiotic concentration relations have been observed for both polyenes. These facts together with the observation that roflamycoin, similarly to amphotericin B, is more effective when added on both sides of the membrane, suggest that roflamycoin and amphotericin B channels are formed by a similar general mechanism, i.e., both channels are composed of two barrel-like half-pores assembled in the opposite monolayers of the membrane. In accordance with the well-known model of an amphotericin B channel [10,11], the two half-pores face aqueous solution with the charged groups and are attached to each other by H-bonds between the terminal OH-groups in the C-35 position. In the roflamycoin channel, half-pores may be stabilized by Van der Waals' interaction between terminal methyl groups (at C-34 and C-35), polar OH-groups at C-15 and C-17 face aqeous solutions.

More detailed inspection of the curves in Fig. 3, as well as observation of single-channels reveals significant differences in the parameters of channels created by the two antibiotics. First, the dependence of the number of channels on roflamycoin concentration is unusually steep. Second, though no direct measurements of channel diameter by nonelectrolytes of different size, like those performed by Holz and Finkelstein [12] on amphotericin B channels, have been done so far with roflamycoin, the indirect data show that roflamycoin channels are wider (12 Å in diameter). This evidence includes higher channel conductance, permeability to divalent sulfate ions, blocking of channels by relatively large organic anions and inability of small organic ions and nonelectrolytes to block them. We propose that a half-pore containing 12–16 roflamycoin molecules and 6–8 sterol molecules might explain both the observed steepness of the relationship between conductance and the antibiotic and sterol concentrations, and the low selectivity of open channels. The different stoichiometry of these channels is probably due to the additional methyl group in the position C-2 in the region of the polyene chain.

Strong potential dependence of the integral steady-state conductance and dependence of the channel opening frequency on membrane voltage could probably be explained by the length of the roflamycoin molecule. Indeed, the macrolide ring of amphotericin B consists of 37 C-atoms and that of roflamycoin consists of 35. Besides, roflamycoin contains no amino sugar and carboxyl groups. The Corey-Pauling-Koltun (CPK) space-filling models of these antibiotics show that the length of the roflamycoin molecule is of about 24.5 Å [2] and

that of amphotericin B 27 Å. Thus, it can be assumed that reduction of the bilayer thickness under the action of external electric field may enhance the probability of the roflamycoin channel formation to a higher degree than the formation of amphotericin B channels.

The stability of the amphotericin B pore has been shown to depend on the electrostatic interaction of amino and carboxyl groups of neighboring molecules in the half-pores [1]. The absence of such groups in the roflamycoin molecule could be a reason for the shorter lifetime of roflamycoin channels in the open state.

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