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Biochimica et Biophysica Acta 1758 (2006) 29–37

BIOCHIMICA ET BIOPHYSICA ACTA

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Transient permeability induced by alkyl derivatives of amphotericin B in lipid membranes

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Received 15 June 2005; received in revised form 5 December 2005; accepted 29 December 2005

Available online 25 January 2006

Abstract

Individual ionic channels were shown to be formed in the brain cholesterol containing phospholipid membranes by two-sided addition of the amphotericin B alkyl derivatives. At concentrations between 10^{-8} and 10^{-7} M, the resulting conductance appeared to be transient. Existence of different antibiotic assemblies was justified by the kinetic analysis of the membrane conductance decline following the antibiotic washing out. In order to account for the transient characteristics of the induced conductance, it was proposed that the antibiotic oligomers incorporate into the membrane from the aqueous phase, form channels aggregating with cholesterol, and then dissociate in the bilayer into non-active degraded oligomeric or monomeric forms.

Keywords: Amphotericin B; Alkyl derivatives of amphotericin B; Transient permeability in membrane; The kinetic of the membrane conductance; Washing out the antibiotic; Dissociate; Non active monomeric form

1. Introduction

Amphotericin B is an effective drug at the treatment of systemic fungal infections [1–3]. Although this drug possesses valuable chemotherapeutic properties, its mechanism of action remains largely unclear, especially on the molecular level. It was discovered that amphotericin B induced a sterol-dependent growth of the lipid membrane conductivity [4–6]. The effectiveness of the antibiotic sharply rises at the increased cholesterol concentration [5,6]. Amphotericin B induces a preferential (but not ideal) anionic conductance in the cholesterol-containing lipid membranes [6–8]. A characteristic feature of the amphotericin B (and other polyenes) effect on the lipid membranes is the strong dependence of the membrane conductance upon the antibiotic concentration [5,6]. The membrane permeability is enhanced for ions, water molecules, and non-electrolytes [9,10] at the addition of amphotericin B to both sides of the lipid bilayers [5,6]. Penetration coefficients for hydrophilic non-electrolytes rise in the inverse proportion to their size [9]. Glucose and larger molecules do not penetrate through the membrane in the presence of amphotericin B [9,10]. All aforementioned data allowed to conclude that amphotericin B forms pores in lipid membranes with the effective radius of 4 Å [9,10]. A molecular model of this pore had been proposed [11–13]. According to this model, the pore consists of two cylindrical half-pores each of which is formed by eight antibiotics and eight intercalated between them sterol molecules. The polyene molecules are oriented at the right angle to the membrane surface, and two half-pores are held together in the middle of the bilayer by the hydrogen bonds between hydroxyl groups located at the end of each polyene molecule [12]. The other end of each molecule has the charged amino sugar and carboxyl groups, which are anchored in the aqueous phase [11–13]. The charged groups are located near

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doi:10.1016/j.bbamem.2005.12.011

the pore entrance. The lining of the half-pore is formed by the hydrophilic hydroxyl groups of the lacton ring.

Single ionic channels formed in lipid membranes by amphotericin B were observed and examined [14–17]. These channels are permeable to univalent ions and organic compounds [16,18–21]. To date, it is well known that amphotericin B channels are characterized by conducting and non-conducting states [14–16]. Individual ionic channels perform a large number of transitions between these two states during their lifetime [15,16]. The channel conductivity is independent of the cholesterol membrane concentration and pH variation from 3.0 to 8.0 [16]. The conductivity and selectivity of the amphotericin B channel does not depend on changes in the charged carboxyl and amino groups [16]. These properties strongly depend on the structure of the antibiotic lactone ring polar chain [16,22]. Computer molecular simulations of the amphotericin B–cholesterol complex revealed that the orientation of the lactone ring hydroxyl groups determines its conductance and selectivity [23,24]. The transformation from conducting to non-conducting states is possibly attributed to the conformational transition between complexes with a wide and narrow pore diameter [23]. The presence of the opposite charges on the carboxyl (−) and amino (+) groups provides for the long time stability (about 240 s) of the channel conducting state [16]. The loss or neutralization of the single charged group caused by a chemical modification or pH shift leads to the substantial decrease (40- to 100-fold) of the channel conducting state lifetime [16]. This was accounted for by the molecular modeling according to which the interactions between the charged groups of the neighboring antibiotic molecules in the amphotericin B-cholesterol complex strongly contribute to its stability [23]. It is obvious that modifications of the amphotericin B primary structure may lead to a new data concerning its physical, chemical, and pharmacological properties. Whereas many of these properties have been elucidated by experimental [18] and modeling [16,23,24] techniques, the important features of the channel formation dynamics upon the incorporation of the antibiotic and its oligomers into the lipid bilayers have been studied more poorly [17]. In this communication, we present kinetic and relaxation experiments developed in order to study the membrane conductance inducement in the presence of alkyl derivatives of amphotericin B. Based on the experimental data, the important mechanisms of the channel formation from the antibiotic oligomers with the following channel dissociation have been revealed. The dependence between concentration of several antibiotic alkyl derivatives and multi-channel conductance inactivation of the membrane with the two-sided antibiotic action has been demonstrated. The membrane modification by the alkyl derivatives led to a transient (biphasic) kinetics of the membrane conductance resulting from the channel formation at low antibiotic concentration. It was observed that polyene channels have an additional non-conducting states. These states were assigned to a disassembled membrane bound antibiotic–cholesterol complexes. Experiments showed that the single channels immediately transform into the non-conducting states. The following formation and activity of the channels have been controlled by the variation of the membrane antibiotic concentration. The molecular mechanism of the intra-membranes dissociation of the antibiotic oligomers after their binding to the lipid bilayer was described. The new non-conducting states of the antibiotic assemblies bound to the membrane were justified.

2. Material and methods

Amphotericin B was purified and chemically modified with respect to the amino and carboxyl groups by Dr. V.A. Vainshtein from the Technological Institute of Antibiotics and Enzymes , Saint Petersburg, Russia. The following alkyl derivatives were prepared (Fig. 1): metamphocin ($R=CH_3$, $\varepsilon=1375$), ethamphocin ($R=C_2H_5$, $\varepsilon=1440$), prophamphocin ($R=C_3H_7$, $\varepsilon=1400$), and butamphocin ($R=C_4H_9$, $\varepsilon=1375$); here ε is the extinction coefficient of antibiotics. Amphotericin B and its alkyl derivatives were dissolved in dimethyl sulfoxide (DMSO) solution in the concentration of 1 mg/ml. Stock-solutions of amphotericin B and its alkyl derivatives in DMSO were prepared just before the experiments. The antibiotics were added to one or two sides of the membrane at different concentration ranging from 10^{-8} M to 5×10^{-7} M in the independent experimental sets. All experiments were performed at pH 6.5 and room temperature. Each amphotericin B derivative was studied separately.

3. Experimental

Bilayer lipid membranes were obtained by the standard technique [25] on the 0.2-mm diameter hole in a teflon cell. The membranes were formed with total ox brain phospholipids extracted by the method of Folch et al. [26] and freed of the neutral phospholipids by the acetone extraction according to Kates [27]. The brain phospholipids were obtained by dissolving in chloroform–methanol solution. Phospholipids were stored at the concentration of 10 mg/ml in the chloroform/methanol (2/1, v/v) mixture at 0 °C. Before experiments, the total ox brain phospholipids were dried in the nitrogen atmosphere in order to remove the solvent traces. Finally, the phospholipids were transferred to n-heptane and dissolved in it at the concentration of 20 mg/ml. In some preparations, the re-crystallized cholesterol (Sigma, Saint Louis, USA) was added to the brain phospholipids so as to achieve the phospholipid:cholesterol molar ratio of 20:1 or 2:1. Histidine or phosphate at 5×10^{-3} M concentration was added to the aqueous solutions in order to stabilize pH. Optically black lipid membranes were formed on the teflon cell hole from the phospholipid–cholesterol solution in n-heptane. The value of the membrane conductance in an absence of the antibiotic was 2–3 pS in 2 M KCl. The electrical measurements of the single ionic channels, kinetics of the multi-channel conductivity variation, and the relaxation time constant of the membrane conductivity after washing out the antibiotic were performed under voltage-clamp conditions. The current across the membrane was

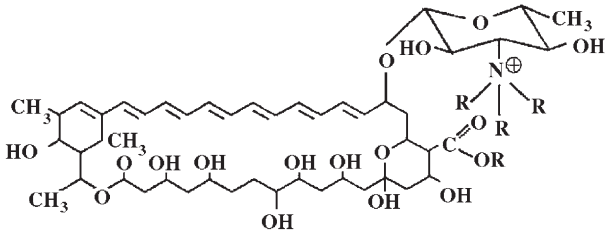

Fig. 1. Structures of the amphotericin B derivatives.

measured with a Keithley 301 electrometric amplifier — USA. An Endim-XY recorder was used to record the current. After the membrane formation, adequate aliquots of each of antibiotics were added to one or two sides of the membrane. The relaxation experiments were performed at 3×10^{-7} M concentration of the antibiotic, which was added to one side of the membrane during 30 min. Then, the antibiotic was washed out from aqueous solution by the perfusion method in the sucrose density gradient [28]. The antibiotic was removed from the solution containing 10% of sucrose by a two-channel peristaltic pump. The washing solution was fed with the pump to the pan bottom, and the initial solution was synchronously drawn off from above. At the low speed of feeding, solutions of different density do not mix and the border between them is seen clearly. The moment of the membrane passing through the border is monitored perfectly. This method is particular useful for studding of the non-equilibrium membrane conduction at the output of the experiment. The membrane's inactivation was evaluated by the relaxation time constant τ, which equals the e-times decrease in the membrane conductance from the starting point of the measurement. The membrane conductivity was determined at different time intervals after the alkyl derivatives of amphotericin B were washed out of the membrane. The existence of the membrane bound non-conducting forms of the channel complexes was testified by the amphotericin B addition to the membrane opposite sides. Note, the addition of the antibiotics to both membrane sides at the same concentration (3×10^{-7} M) leads to a much more intensive channel formation over the aforementioned time (30 min).

4. Results and discussion

4.1. Induction of permeability

Fig. 2 demonstrates the conductance rise occurred as a result of the trans-membrane channel formation at the two-sided addition of the amphotericin B alkyl derivatives to the membrane with the phospholipids: cholesterol molar ratio of 20:1. The increase is in accord with that observed for nystatin [14] and amphotericin B [15]. Individual channels formed by these antibiotics differ only slightly in conductance. It was shown that the conductance of the single channels was declining from 7.5 pS to 4 pS versus the rising length of the alkyl chains bound to the polar groups. Selectivity of these channels is roughly the same: the zero current potentials at the KCl gradient of 2M–0.2M are rather close and equal 42 ± 3 mV. The induced conductance and sign of the membrane potential correspond to the anion selectivity, i.e., a preferential permeability for Cl^- in comparison with K^+.

As can be seen from the recordings in Fig. 2, the channels become temporary non-conducting then render conduction again; but the channels formed by the alkyl derivatives of amphotericin B differ those formed by amphotericin B itself since they remain conducting for a shorter period of time. The channels are less stable since the alkyl chains of the antibiotics may shield the positively charged ammonium groups and neutralize the negatively charged carboxyl group (Fig. 1). The interactions between these groups substantially contribute to the stability of the antibiotic–cholesterol complex [23]. The mean conducting state time is 4.5 ± 0.3 s. At pH 6.5, the alkyl derivatives are generally more effective when added to two sides of the membrane relative to its one side.

Here, we discovered that in the presence of the alkyl derivatives of amphotericin B the membrane conductance has to be described by a biphasic kinetics since the initial conductance growth was followed by its decay (Fig. 3). After 30 min, the conductance of the bilayer doped with the alkyl derivatives taken at the same concentration was almost of that observed in the absence of the antibiotics. The relation between the membrane conductance kinetics and antibiotic concentration was revealed (Fig. 4). At the high 5×10^{-8} M antibiotic concentration, the membrane conductance converted from the

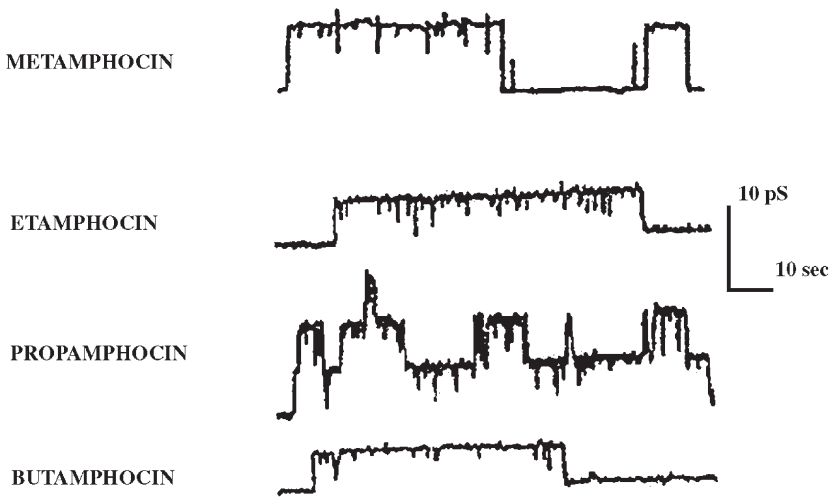

Fig. 2. Time dependence of the discrete membrane conductance induced by the alkyl derivatives of amphotericin B. Concentration of antibiotics: metamphocin: 2×10^{-8} M, etamphocin: 2×10^{-8} M; propylamphocin: 3×10^{-8} M; butamphocin: 4×10^{-8} M. The applied potential was 200 mV. Aqueous solution: pH 6.5, 2M KCl, temperature 24 °C. Membrane composition: 20:1 molar ratio of phospholipid:cholesterol.

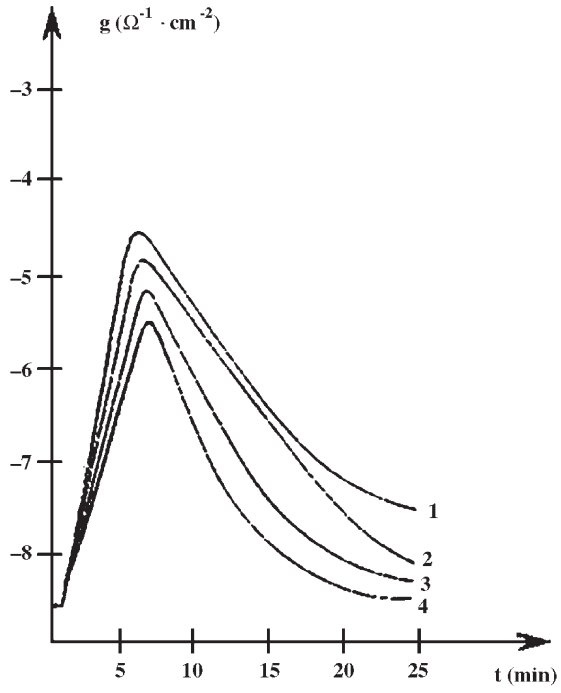

Fig. 3. Variation as a function of time of the membrane conductance induced by the alkyl derivatives of amphotericin B added at the same concentrations of 5×10^{-8} M to both sides of the membrane. 1: metamphocin; 2: etamphocin; 3: propylamphocin; 4: butamphocin. Each experiment is performed separately. Aqueous solution: pH 6.5, 2 M KCl, temperature 24 °C. The applied potential was 200 mV. Membrane composition: 2:1 molar ratio of phospholipid: cholesterol.

biphasic kinetics to the monotonic one (Fig. 4). The phenomenon of the membrane conductance decay (Fig. 3) may originate from the decline in the: (1) channel conductance, (2) mean lifetime of the channels in the conducting state, and (3) total number of the conducting channels. Based on a spectral composition of the conductance fluctuations induced by the alkyl derivatives of amphotericin B [29,30], it was concluded that the two first factors did not influence on the kinetic type of the membrane conductance but the third one seems to be more probable The level of the 1/f noise in the presence of metamphocin increased in 10 times as compared with that of amphotericin B [29,30]. The coincidence of spectra measured in different zones of the membrane conductance alteration (growth, maximum, and steady state) indicates that the only one form of the conducting channel occurs [29,30].

The found kinetics may clarify the transient permeability observed upon the amphotericin B addition to hepatocyte [31] or Baby Hamster Chinese Ovary cells [32], and the two-stage action of amphotericin B on mammalian cells, such as an inducement of permeability to K^+ and cell killing at the low and high antibiotic concentration respectively [33]. In these cells, the return of the trans-membrane permeability to a lower level after its initial burst enables the membrane pumps – such as Na^+/K^+ ATPase – to restore the initial intracellular homeostasis.

Based on the aforementioned experimental data, a plausible molecular mechanism of the biphasic permeability inducement has been proposed. Like the parent compound, the alkyl derivatives of amphotericin B exist in water as a concentration dependent balance between monomeric and self-associated forms. The similar balance may take place in lipid membranes but only the self-associated form is capable to induce permeability across the cholesterol-containing membranes [34]. Upon the antibiotics binding, re-equilibration between monomers and self-associated forms takes place in the membrane. The share of the oligomeric forms exponentially grow with the rise of the monomeric ones since the order of the association reaction exceed the first order dissociation one. At low antibiotic concentration the re-equilibration takes place in the favor of monomers, followed by the reduction of the initial level of the channel-forming oligomers. However, at the higher antibiotic concentrations ($>3\times10^{-7}$ M) in the aqueous solution, permeability does not decrease with the time since the re-equilibration process is shifted towards the bound oligomeric complexes, yet the membrane may become saturated with the monomers.

The equilibrium between membrane bound superstructures of amphotericin B and its derivatives was testified by the following data obtained by the removal (washing out) of the antibiotics from the membrane.

4.2. Antibiotic washing out of membranes

The process of removal of the alkyl derivatives from the membrane is characterized by different kinetics, depending on the one- or two-sided addition. The differences were monitored

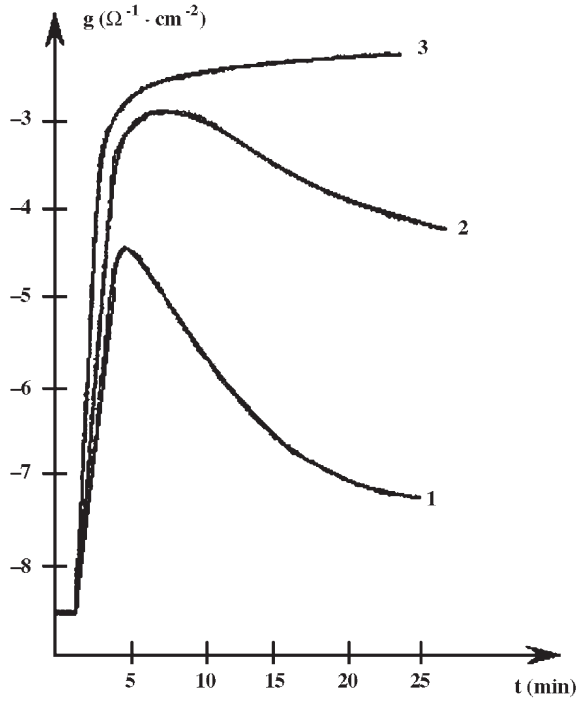

Fig. 4. Variation as a function of time of the membrane conductance induced by metamphocin added at various concentrations to both sides of the membrane with the phospholipid:cholesterol molar ratio of 2:1. 1: 5×10^{-8} M; 2: 10^{-7} M; 3: 3×10^{-7} M. Aqueous solution: pH 6.5, 2 M KCl, temperature 24 °C. The applied potential was 200 mV.

in two sets of experiments. In the first one, amphotericin B or metamphocin were added at a concentration of 3×10^{-7} M to one membrane side, kept in the aqueous solution for 30 min, and then washed out. After increased time lag, amphotericin B was added to the opposite side at the same concentration that induced a rapid jump of conductance. A plateau was reached in less than 3 min (data is not illustrated); the amphotericin B-induced conductance increase was measured at that moment (Fig. 5). The level of the plateau decreased monotonically with the time elapsed after the antibiotic removal. It should be noted that with an initial addition of amphotericin B instead of the alkyl derivatives, the same experiments resulted in a more rapid decrease of conductance. To comprehend these results, let us recall first, that at the concentration studied, no conductance is observed upon the one-sided addition of the antibiotic alkyl derivatives; the same has been observed for amphotericin B [14,16,35]. Let note that single channels have been detected upon the one-sided addition of amphotericin B at very low concentration [36] that might be below the self-association threshold. However, it may be assumed that its very low lifetime would not be sufficient to induce a significant conductance in the multi-channel system. The conductance jump observed after the addition of the 3×10^{-7} M amphotericin B derivatives to membranes containing metamphocin on the opposite side is similar to one that has formerly been observed with amphotericin B and nystatin [6] or amphotericin B methyl ester and N-acetyl amphotericin B [16]. It was assumed that the composite channels were formed by the half-pores of different composition. Similarly, the one-sided addition of the anion-specific amphotericin B with the cation-specific levorin on the other side resulted in the conducting channels with selectivity and conductivity determined by amphotericin B.

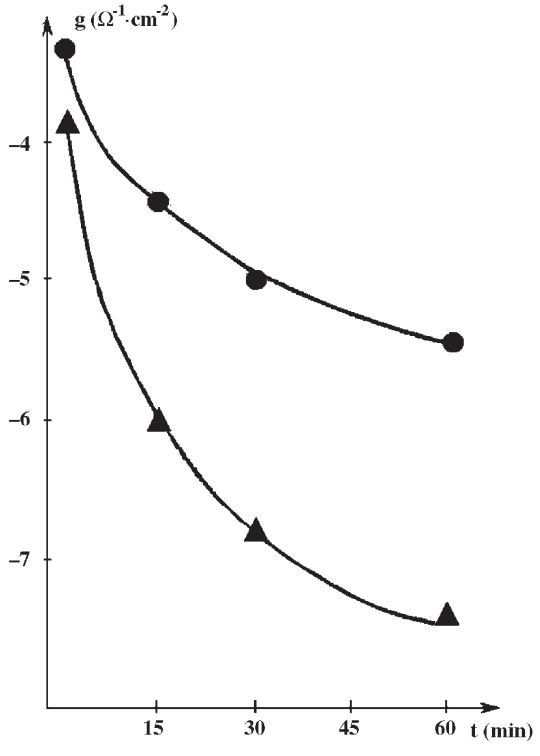

Fig. 5. Kinetics of the conductance decrease after removal of metamphocin (triangles) or amphotericin B (circles) added to one side of the membrane at the initial concentration of 3×10^{-7} M (after removal of the antibiotic, 3×10^{-7} M amphotericin B was added at a different time intervals on the opposite membrane side in order to induce conductance). The applied potential was 100 mV. Aqueous solution: pH 6.5, 2M KCl; temperature 24 °C. Membrane composition: 2:1 molar ratio of phospholipid:cholesterol.

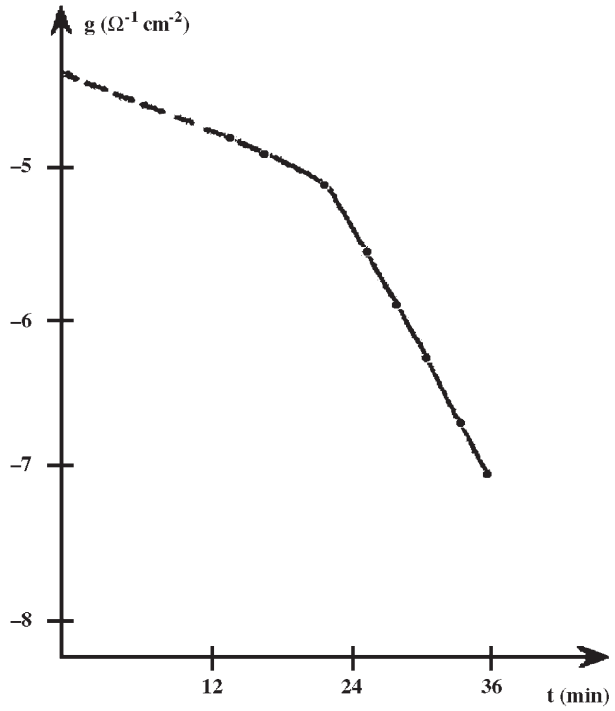

Fig. 6. Kinetics of the conductance decrease after removal from the one membrane side of metamphocin added to its two sides at the initial concentration of 2×10^{-7} M. The applied potential was 100 mV. Aqueous solution: pH 6.5, 2 M KCl, temperature 24 °C. Membrane composition: 2:1 molar ratio of phospholipid:cholesterol.

In the second set of experiments (the similar experiments were described in [29,30]), metamphocin was added at the concentration of 2×10^{-7} M to both sides of the membrane, was kept in the aqueous solution for 30 min, and then washed out. Conductance was recorded with an increased time lag after washing out of the antibiotic (Fig. 6). All data were obtained with the same membrane. A similar experiment was done by Cass et al. [8], but because of the temperature variation along the relaxation step these data is not comparable with ours. In our experiment, the conductance behaviour can be presented by two phases: a period of slow conductance alteration («induction period») and a period of exponential decrease. It turned out that the relaxation time constant τ depends on the initial antibiotic concentration (Fig. 7). For low antibiotic concentration, τ is close to zero.

The «induction period» is not observed in the experimental set performed with the one-sided addition of metamphocin. Therefore, it might be assigned to a step, which is not observed in the first set: dissociation of an assemblage controlling the trans-membrane permeability in the thick lipid bilayers studied here. In such bilayers – according to the currently accepted model – the inactivation of the functional channel consisting of two coupled half-pore barrels could be related to its dissociation

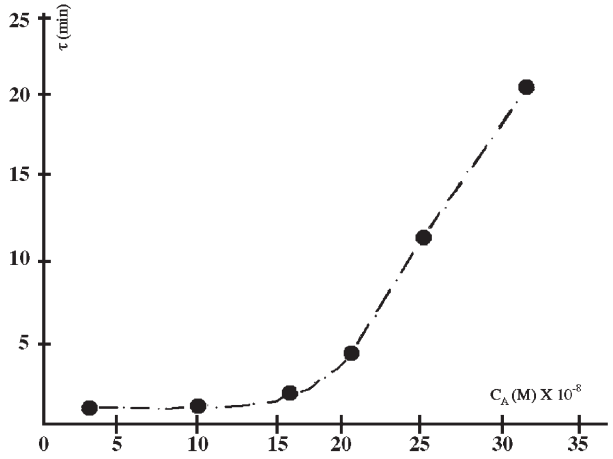

Fig. 7. Influence of the initial concentration of metamphocin (C_A) on the exponential phase conductance decrease after removal of the two side added antibiotic from the one membrane side. The applied potential was 100 mV. Aqueous solution: pH 6.5, 2 M KCl, temperature 24 °C. Membrane composition: 2:1 molar ratio of phospholipid:cholesterol.

into two separated barrels, each of whish is not able to span the membrane and therefore to provide the conducting pathway for permeant ions. The exponential conductance decay with the similar τ for both set of experiments could be assigned to the disassembly of the double half-pores channel into a degraded associates or monomers. The dependence of τ versus the antibiotic concentration implies a non-linear kinetics of the channel-complex formation-dissociation reaction. Two aforementioned molecular mechanisms might be suggested for account of such kinetics: the second order reaction for the two half-pores model or a higher order reaction for the channel-oligomers/monomers degradation model. Possibly, amphotericin B and its alkyl derivatives have the same structure of the contact zone between two half-pore ends in the functional channel since the chemical modifications of the derivatives are implemented on the opposite ends of the pore. Taking into account that the channel inactivation could be associated with the lateral dislocation of one half-pore relative to another, the values of τ for all antibiotics have to be similar. However, the τ differences obtained in our experiments indicate that interactions in the contact area between two half-pores do not contribute to the τ variations. The model of the conformational transition between open and closed state of the channel does not fit the experimental data as well since it is described by the first order kinetics with the concentration independent τ. Thus, the τ-concentration dependence is probably related to the dissociation of the functional channel into degraded oligomers and monomers followed by their migration through the membrane–water interface.

5. Conclusion

The question: what physical processes in membrane systems do affect the conductance kinetics, has to be often addressed for interpretation of experimental data. The shape of kinetic curves of membrane related phenomena could depend on electrical, mechanical, diffusive, and chemical processes. It was shown that electrical processes in lipid membranes do not impact substantially the channel forming kinetics [28,29]. For example, assembling of ion channels is not voltage-dependent, i.e., is not the function of the electrical field applied to the membrane. On the other hand, switching of electric field through different intervals after the membrane formation and the beginning of the channel forming process as well as switching of the voltage polarity during the process do not affect the shape of kinetic curves [30]. Apparently, this proves a virtual lack of coupling between determining kinetics electrical and chemical processes. The only area where the electrical field can affect the kinetic curve is located in its primary slightly distort part, which appears as a consequence of the capacitive current [30]. But it is easy to show that such influence is negligible because of the rapidity of this process in comparison with typical timings of the channel forming kinetics. Actually, capacity of the bilayer membrane is ~0.5 μF/cm^2, and specific resistance of the membrane in our experiments – within 20 s after the beginning of the channel forming process – is 10^7 Om·cm^2. The product of these values (time constant) does not exceed 5 s and is much smaller than kinetic timings. It is reasonable to neglect the mechanical processes, i.e., the membrane geometry alterations, because during the kinetic unfolding the antibiotics were added to the system after the membrane formation (10 s). As far as diffusive processes are concerned, for amphotericin B and other channels formed by polyene antibiotics diffusive limitations for entrance of ions into the pores are incidental [15].

Finally, we will examine the influence of chemical factors on the membrane conductance kinetics. Kinetic curve characteristics are reproduced either while the antibiotics are injected into the system after the preliminary incubation of membranes in the non-modified species or during formation of membranes in the antibiotics containing solutions. Kinetic curves are reproduced as well at the repeated introduction of the antibiotics after their preliminary washing out. We are prone to state that the membrane conductance kinetics is determined by the presence of monomeric and different oligomeric forms of the antibiotics in the membrane–water system as well as transitions between them.

We assumed that the observed shape of the conductance curves could be attributed to the decrease of: 1) conduction of the single channels; 2) the lifetime of the conducting channels, and 3) the total quantity of the conducting channels. The last factor is most probable, since our studies showed that neither the conduction of the single channels, nor the lifetime of the channels at the state of conduction changed substantially. Yet, the analysis of the conductance spectral structure fluctuation induced in the lipid bilayer doped with the methylated analogue of amphotericin B support our conclusion [30].

For accounting of the observed biphasic conductance kinetics we assumed that an assembling of the functional channel complexes takes place at the beginning of the membrane conductance growth, and the conductance decline is connected with the following decay of the conductive structures to monomeric, dimeric or other degraded oligomeric non-conductive structures. It was shown earlier that polyenes and their analogs can exist in monomeric or associated forms in

the water solutions with the balance depending on the solutes concentration [37,38]. Both forms are present in a sufficiently large quantity in order to be built into the membrane. Presence of the associated forms of the antibiotics in the water solution was detected by a circular dichroism technique [37]. Heptaene antibiotics aggregate in water with the level of aggregation is directly related to their concentration [37,38]. In salt solutions at low concentrations (1×10^{-8} M), the monomeric forms are dominant, but at higher concentrations the rate of the non-linear association reaction prevails over the rate of the first order dissociation one and the equilibrium between the forms is shifted towards the associates that is clearly seen on the circular dichroism spectrum with the maximum of absorption at 340 nm [38]. Due to the conjugate double bond system, amphotericin B and its alkyl derivatives have their own absorption spectra in the wave length interval of 300–450 nm.

Let assume that the antibiotics incorporate into the membrane from the water phase as a stable oligomeric units with the number of polyene molecules corresponding to that of the factional half-pore. Aggregating with cholesterol these units could form functional channels, which later would disassemble to a non-conducting degraded oligomers and monomers that correspond to the observed growth of conduction to a certain level and its following decay respectively (Fig. 4, curves 1 and 2). After inactivation of the membrane conductance (Fig. 4, curve 1), injection of amphotericin B from any side of the membrane leads to a sharp rise of conductivity, in spite of the fact that amphotericin B at neutral pH is not effective from one side of the membrane [6,8]. Our experiment shows clearly that at the certain low antibiotic concentrations, the disassembling of the conducting channels takes place in the membrane and can be reversed again by injecting of the additional portion of amphotericin B from any side of the membrane. The presence of the non-conducting antibiotic forms can be validated by washing out of the alkyl derivatives after their fixation on one side of the membrane with following membrane conductivity induction by the injection of amphotericin B from the opposite membrane side. It is known that the washing out time of amphotericin B at the one-sided membrane injection (the antibiotic supposedly forms the half-pore complexes) at neutral pH is 1–3 min [8]. Our experiments show that the amphotericin B alkyl derivatives retain in the membrane sufficiently longer after washing out of the antibiotics from the water solution. Injection of amphotericin B or metamphocin in concentration of 3×10^{-7} M from one membrane side during 30 min with the following antibiotics washing out demonstrated sufficient increase of conductivity even after 60 min of the antibiotics removal. The conductivity was detected by injecting of the initial amphotericin B from the opposite side of the membrane in order to induce the formation of the conducting channel consisting of two bound half-pores. It is possible to detect the kinetics of washing out of the non-conductive structures from the membrane one side by testing the conductivity induced by amphotericin B added to the opposite side with a certain time step.

Studies of the non-aqueous solutions of polyene antibiotics may serve as an additional argument in the favor of the assumption that after the decay of the functional channels the distribution of different antibiotic forms is shifted towards degraded non-conducting subunits. It is known that the mother solutions of the antibiotics contain dimethylsulfoxide (DMSO). The antibiotic conduction in the membrane–water–DMSO systems is very small [28]. In salted water solutions, the membrane conductivity depends on DMSO concentration in water. At the volumetric water:DMSO ratio of 10:1, conductivity and sensibility of the membrane doped with antibiotics become extremely high. The membrane sensitivity is a minimal at the antibiotic concentration corresponding to the single channel formation. As the DMSO concentration rises, the membrane antibiotic sensitivity decreases, though tangent of the concentration–conductivity curve does not change. But at 50% and more concentration of DMSO in water, the antibiotics do not induce an increase in the membrane conductivity. It was shown [28] that the monotonic growth of the DMSO concentration gradually transfers the active antibiotic associates into the inactive degraded or monomolecular forms.

The described above experimental results, regarding the antibiotic removal after achieving of the steady state conductivity level (Fig. 4, curve 3), can be interpreted on the basis of the two stage relaxation kinetics. We assume that before washing out the equilibrium occurs in the membrane between conducting and non-conducting antibiotic complexes and monomers. After antibiotics removal from the aqueous phase non-conducting less associated forms starts diffusing from the membrane to the aqueous phase along the created concentration gradient. The reaction rate of the channel disassembling prevails over the reversed one that is detected as the conductivity decline. At the first stage of the decline (induction period), the reaction rate of the channels disassembling could be offset by the rate of the coupling of the functional half-pores retaining in the membrane immediately after the removal of the antibiotic from the water phase. When the number of the retaining half-pores becomes small the rate of the channel disassembling completely “overwhelms” the reversed one that is detected as the second stage of the exponential conductivity decrease.

An alternate mechanism describing the membrane conductivity decline after the antibiotics removal might be proposed. In this case, the conducting structures dissociate into separated half-pores which diffuse through the membrane–water interface in the direction of the bulk water. Since the concentration of non-conducting structures in the membrane are high, the secondary assembling of conducting channels might take place resulting in the observed non-monotonic kinetics. The mechanism of the half pores migration may not fit the τ-concentration dependence and τ differences observed for amphotericin B and its alkyl derivatives Indeed, since the diffusion of the separated half-pores from the membrane to water could be described by the first order kinetics the relaxation time should not depend on the antibiotic concentration. All antibiotics may have the same structure of the contact area between two half-pores in the functional channels that does not account for the observed τ-differences related – in the frame of this model – to the pores translocations in the lateral directions during the channel inactivation.

We can assume that the τ value depends on the power of the electrostatic interaction between the forming half-pore polyene molecules. It is known that the initial amphotericin B forms stable channels with a long lifetime at the state of conducting due to the electrostatic coupling of amine and carboxylic groups of the neighboring polyene molecules [16,23]. The loss or neutralization of the single charged group caused by a chemical modification or pH shift lead to the weakening of the electrostatic coupling between antibiotic molecules that in its turn leads to the substantial decrease of the conducting channel lifetime since it could disassemble to monomers and other non-conducting oligomeric structures.

It is known that amphotericin B and its alkyl derivatives induce permeability to K^+ in the cholesterol containing membranes only if they form self-associated aggregates in the incubation medium [34]. There are two water-soluble biologically active antibiotic forms: monomers and oligomeres [34]. The incorporation of the water-soluble self-associated antibiotic forms into the membrane is leading to the formation of the individual ionic channels. Here, we found that the multi-channel membrane conductance induced by the polyene antibiotics has to be described by the non-monotonic biphasic kinetics. We suggested that this is the result of the consecutive transformations of the membrane bound polyene aggregates between conducting and degraded non-conducting oligomeric and monomeric forms.

The non-monotonic kinetics of the membrane conductance, induced by the alkyl derivatives of amphotericin B, fits the three states kinetic model consisting of one conducting state which is surrounded by two non-conducting ones (1↔2↔3) [39]. The non-conducting state 1 is associated with two separate non-conducting half pores, which "meet" in the membrane and form the conducting channel (state 2). Later, the conducting channel disassembles to monomers and other non-conducting structures (state 3). This three states model of the channel formation and disassembling is able to generate the non-monotonic kinetics depending on the antibiotic concentration and the reaction rates between two last states (2↔3) [39].

When the concentration of the antibiotic in the membrane is low, the conducting channel transforms from the state 2 to 3 ($2 \rightarrow 3$), since the rate of the $2 \rightarrow 3$ transformation is higher than that of $2 \leftarrow 3$. The transformation $2 \rightarrow 3$ is the first order reaction, whereas the reverse one ($2 \leftarrow 3$) is a higher order reaction. At much higher antibiotic concentrations (3×10^{-7} M) the membrane is saturated with monomeric forms that makes the $2 \leftarrow 3$ reaction rate (secondary assembling of the channel) grated than the $2 \rightarrow 3$ one, and the only monotone growth of conduction is observed on the kinetic curve (Fig. 4, curve 3). The final balance between conducting (2) and non-conducting (3) states would be shifted towards the conducting one. Thus, the kinetic type of the membrane conduction is defined by the concentration dependent distribution of the antibiotics between different membrane bound oligomeric and monomeric forms along with the molecular exchange rates between them.

We realize that the proposed molecular model might be not the only possible one describing our experimental data. However, in any other model, the main principals put in the basis of the ours have to be conserved, particularly the existence of the self-catalyzed reactions shunting the process of the conducting structures formation.

Our studies contribute at the molecular level in the attempts to solve the important problem: how do polyene antibiotics induce the transient permeability in cell membranes? We have to acknowledge that some attempts to prove the existence of monomers and non-conducting self-associated forms of amphotericin B and its alkyl derivatives bound to the lipid membrane have been performed before [29,30]. Like ours, they were based on the analysis of the membrane conductance kinetics induced by the antibiotics removal (washing out) from the aqueous medium.

Acknowledgements

Authors are grateful to Prof. J. Bolard, Head of the Laboratory of the Physical and Chemical Properties of Biomolecules at the Pierre and Marie Curie University, Paris for cooperation at the performing of this work.

References

[1] J. Brajtburg, J. Bolard, Carrier effects on biological activity of amphotericin B, Clin. Microbiol. Rev. 9 (1996) 512–531.
[2] S. Hartsel, J. Bolar, Amphotericin B: new life for an old drug, TIPS 17 (1996) 445–449.
[3] S.B. Zotchev, Polyene macrolide antibiotics and their applications in human therapy, Curr. Med. Chem. 10 (2003) 211–223.
[4] T. Andreoli, M. Monahan, The interaction of polyene antibiotics with thin lipid membranes, J. Gen. Physiol. 52 (1968) 300–325.
[5] A. Finkelstein, A. Cass, Permeability and electrical properties of thin lipid membranes, J. Gen. Physiol. 52 (1968) 145–172.
[6] Kh. Kasumov, E. Liberman, Ionic permeability of bimolecular lipid membranes in the presence îf polyenå antibiotics. I: Nystatin and amphotericin B, Biofizika 17 (1972) 1024–1031 (in Russian).
[7] V.W. Dennis, N.W. Stead, T.S. Andreoli, Molecular aspects of polyene- and sterol-dependent pore formation in thin lipid membranes, J. Gen. Physiol. 55 (1970) 375–400.
[8] A. Cass, A. Finkelstein, V. Krespi, The ion permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B, J. Gen. Physiol. 56 (1970) 100–124.
[9] R.W. Holz, A. Finkelstein, The water and nonelectrolite permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B, G. Gen. Physiol. 56 (1970) 125–145.
[10] T.E. Andreoli, V.W. Dennis, A.M. Weigl, The effect of amphotericin B on the water and nonelectrolyte permeability of thin lipid membranes, J. Gen. Physiol. 53 (1969) 133–156.
[11] T.E. Andreoli, On the anatomy of amphotericin B-cholesterol pores in lipid bilayer membranes, Kidney Int. 4 (1973) 337–345.
[12] A. Finkelstein, R. Holz, Aqueous pores created in thin lipid membranes by polyene antibiotics nystatin and amphotericin B, in: G. Eisenman (Ed.), Membranes, 2, Marcell Dekker, Inc, New York, 1973, pp. 377–407.
[13] B. De Kruyff, R. Demel, Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes: III. Molecular structure of the polyene–antibiotic–cholesterol complexes, Biochim. Biophys. Acta 339 (1974) 57–70.
[14] L. Ermishkin, Kh. Kasumov, V. Potseluyev, Single ionic channels induced in lipid bilayers by polyene antibiotics, Nature 262 (1976) 698–699.
[15] L. Ermishkin, Kh. Kasumov, V. Potseluyev, Properties of amphotericin Â channels in lipid bilayer, Biochim. Biophys. Acta 470 (1977) 357–367.
[16] Kh. Kasumov, M. Borisova, L. Ermishkin, V. Potseluyev, A. Silberstein, V.

Vainshtein, How do ionic channel properties depend on the structure of polyene antibiotic molecules? Biochim. Biophys. Acta 551 (1979) 229–237.
[17] W. Huang, Z. Zhang, X. Han, J. Tang, J. Wang, S. Dong, E. Wang, Ion channel behavior of amphotericin B in sterol-free and cholesterol- or ergosterol-containing supported phosphatidylcholine bilayer model membranes investigated by electrochemistry and spectroscopy, Biophys. J. 83 (2002) 3245–3255.
[18] J. Bolard, How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim. Biophys. Acta 864 (1986) 257–304.
[19] S. Hartsel, C. Hatch, W. Ayenew, How does amphotericin B work? Studies on model membrane systems, J. Liposome Res. 3 (1993) 377–408.
[20] B. Cohen, Amphotericin B toxicity and lethality: a tale of two channels, Int. J. Pharm. 162 (1998) 95–106.
[21] C. Garcia-Chaumont, O. Seksek, B. Jolles, J. Bolard, A cationic derivative of amphotericin B as a novel delivery system for antisense oligonucleotides, Antisense Nucleic Acid Drag Dev. 10 (2000) 177–184.
[22] Kh. Kasumov, N.Kh. Mekhtiev, S.D. Karakozov, Potential-dependent formation of single conducting ionic channels in lipid bilayers induced by the polyene antibiotic levorin A2, Biochim. Biophys. Acta 644 (1981) 369–372.
[23] V.E. Khutorsky, Structures of amphotericin B–cholesterol complex, Biochim. Biophys. Acta 1108 (1992) 123–127.
[24] V.E. Khutorsky, Ion coordination in the amphotericin B channel, Biophys. J. 71 (1996) 2984–2995.
[25] P. Mueller, D.O. Rudin, H.T. Tien, W.C. Wescott, Methods for the formation of single bimolecular lipid membrane in aqueous solution, J. Phys. Chem. 67 (1963) 534–535.
[26] J. Folch, M. Less, G.H. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[27] M. Kates, Techniques of lipidology. Isolation, analysis and identification of lipids. North-Holland, Publishing Company-Amsterdam. London American Elsevier Publishing Co. Inc.-N.Y. (1972) 117–118.
[28] V. Ibragimova, I. Alieva, D. Aliev, Amphotericin B channel conductance inactivation, Cytology (in Russian) 45 (2003) 804–811.
[29] Kh. Kasumov, O. Malafriev, Research of membrane conductivity relaxation kinetics in the presence of amphotericin B alkyl derivatives, Stud. Biophys. (Berlin) 99 (1984) 137–142.
[30] O. Malafriev, Kh. Kasumov, S. Bezrukov, On the polyene channels in inactivation, Biol. Membr. (in Russian) 8 (1991) 633–637.
[31] A. Binet, J. Bolard, Recovery of hepatocytes from attack by the pore former amphotericin B, Biochem. J. 253 (1988) 435–440.
[32] R. Demaimay, K. Adjou, C. Lasmezas, F. Lazarini, K. Cherifi, M. Seman, J.P. Deslys, D. Dormont, Pharmacological studies of a new derivative of amphotericin B, MS-8209, in mouse and hamster scrapie, J. Gen. Virol. 75 (1994) 2499–2503.
[33] V. Joly, J. Bolard, P. Yeni, In vitro models for studying toxicity of antifungal agents, Antimicrob. Agents Chemother. 36 (1992) 1799–1804.
[34] P.H. Legrand, E. Romero, B. Cohen, J. Bolard, Effects of aggregation and solvent on the toxicity of amphotericin B to human erythrocytes, Antimicrob. Agents Chemother. 36 (1992) 2518–2522.
[35] A. Marty, A. Finkelstein, Pores formed in lipid bilayer membranes by nystatin, J. Gen. Physiol. 65 (1975) 515–526.
[36] R. Brutyan, P. McPhee, On the one-sided action of amphotericin B on lipid bilayer membranes, J. Gen. Physiol. 65 (1996) 515–526.
[37] Ph. Legrand, A. Vertut-Doi, J. Bolard, Comparative internalization and recycling of different amphotericin B formulations by macrophage-like cell line J774, J. Antimicrob. Chemother. 37 (1996) 519–533.
[38] J. Mazerski, J. Bolard, E. Borowski, Self-association of some polyene macrolide antibiotics in aqueous media, Biochim. Biophys. Acta 719 (1982) 11–17.
[39] L. Ermishkin, Count state of sodium channel, Biofizika 21 (1976) 94–100 (in Russian).