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Transient Permeability Induced by Cationic Derivatives of Amphotericin B in Lipid Membranes*

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Individual ionic channels were shown to be formed through brain phospholipid membranes containing cholesterol, by two-sided addition of cationic derivatives of amphotericin B. At concentrations between 10^{-8} and 10^{-7} M, the resulting conductance appeared to be transient. Equilibrium between different antibiotic assemblies inside the membrane was demonstrated by the kinetics of conductance decrease following washing out the antibiotic. To explain the transient characteristics of the induced conductance, it is proposed that the antibiotic, present in solution under self-associated form, binds the membrane and forms pores, then dissociates in the bilayer in a non active monomeric form. This observation may be of importance to explain the delivery of oligonucleotides into mammalian cells, by cationic derivatives of amphotericin B.

Key words: amphotericin B, cationic, membrane conductance, permeability, circular dichroism, oligonucleotide delivery

It is well known that heptaene macrolide antibiotics such as amphotericin B (AmB) form transmembrane pores and channels through sterol-containing membranes [1,2]. These channels, the structure of which has recently been the object of molecular modeling [3,4], are permeable to univalent ions and organic compounds [5–7]. In an effort to understand the mechanism by which cationic derivatives of amphotericin B allow the delivery of nucleic acids into mammalian cells without killing them [8,9], we developed a study of the membrane conductance inducement in the presence of four of these cationic derivatives. Interestingly, it appeared that at low antibiotic concentration the kinetics of membrane conductance (as a result of channel formation) is non-monotonic. We propose a mechanism for this observation, based on the intramembranous dissociation of the antibiotic oligomers after their binding to the lipid bilayer.

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* Dedicated to Prof. E. Borowski on the occasion of his 75th birthday.

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EXPERIMENTAL

AmB was purified and chemically modified with respect to the polar amino and carboxyl groups [10] by Dr. V.A. Vainshtein from the Technological Institute of Antibiotics and Enzymes (Saint Petersbourg, Russia). Alkyl derivatives were prepared (Figure 1), where R = CH_3: metamphocin (ε_{382} = 137500); R = C_2H_5: etamphocin (ε_{382} = 144000); R = C_3H_7: propamphocin (ε_{382} = 140000); R = C_4H_9: butamphocin (ε_{382} = 137500).

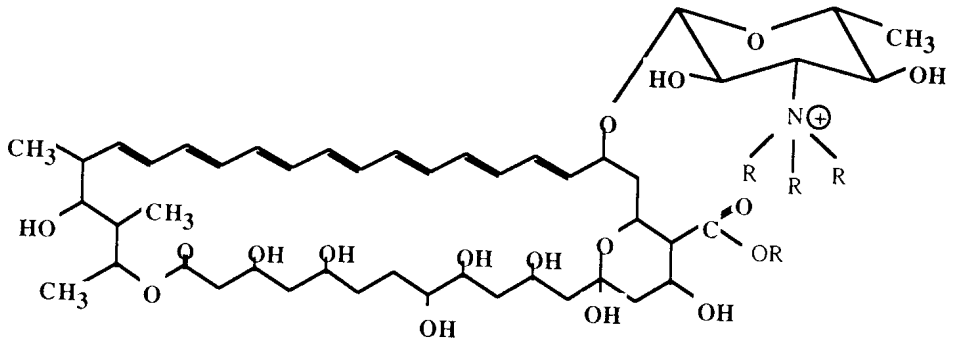

Figure 1. Alkyl derivatives of amphotericin B.

Stock-solutions of the antibiotics were prepared in dimethyl sulfoxide, just before the experiments. Bilayer lipid membranes were obtained by the standard technique [11] on a hole 0.2 mm in diameter in a teflon cell. The membranes were formed with total ox brain phospholipid extracted by the method of Folch *et al*. [12] and freed of neutral phospholipids by acetone extraction according to Kates [13]. Phospholipids were stored at 10 mg/mL in choroform/methanol (2/1, v/v) mixture at 0°C. Before experiments, phospholipids were transferred to n-heptane at 10–20 mg/mL, and 2–10 mg/mL of recrystallized cholesterol (Sigma, Saint Louis, USA) was added. Histidine or phosphate at a concentration of 5×10^{-3} M were added to the aqueous solutions to stabilize pH. The value of membrane conductance in the absence of antibiotic was 2–3 pS in 2 M KCl. The single ionic channels, the kinetics of multichannels conductance variation, the relaxation kinetics of conductance after washing out the antibiotic were performed under voltage-clamp conditions. The current across the membrane was measured with a Y 5-9 (as a Keithley) electrometric amplifier and recorded with an Endim - XY recorder. A solution containing 10% sucrose was used for removal of the antibiotic from one side of the membrane by a two-channel peristaltic pump.

Circular dichroism spectra were recorded at room temperature with a Jobin-Yvon Mark V dichrograph. Spectral wavelengths are given +/- 1 nm. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient (10^3 cm^{-1} M^{-1}).

RESULTS AND DISCUSSION

Circular dichroism measurements and self-association of the cationic derivatives of AmB. Circular dichroism spectra of the alkyl derivatives of AmB in 2 M KCl aqueous solution were recorded between 300 and 450 nm region, where heptaene antibiotics present electronic absorption and dichroic bands. Heptaene antibiotics in aqueous solutions undergo self-association in a concentration-dependent manner and it is well known [14] that the dichroic spectrum changes from four weak positive

bands (monomers) to a strong dichroic doublet centered around 340 nm (oligomers) as concentration is increased. In our experimental conditions, corresponding to those were transient permeability occurs (see below), that is between 10^{-8} and 10^{-7} M, we observed this dichroic doublet (Figure 2). The intensity of the doublet, between these two limits of concentration, increased from $\Delta\varepsilon = 180$ to $\Delta\varepsilon = 200$. Therefore, we can affirm that, at these concentrations, AmB alkyl derivatives are to some extent under self-associated form and susceptible to form pores across cholesterol-containing membranes [15].

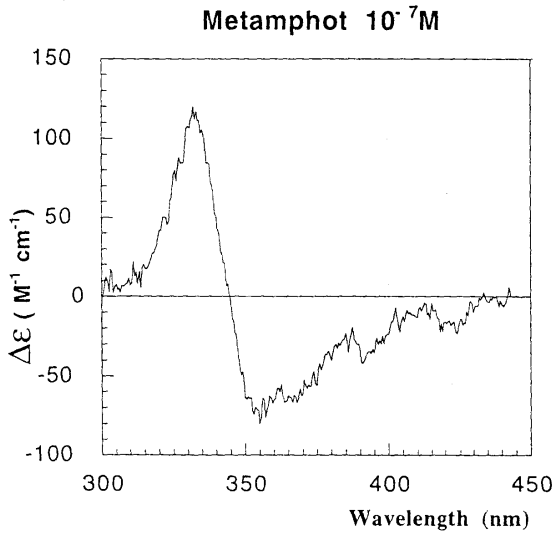

Figure 2. Circular dichroism spectra of 10^{-7} M metamphocin in 2 M KCl aqueous.

Induction of permeability. The results presented in Figure 3 demonstrate that the increased conductance observed upon two-sided addition of alkyl derivatives of AmB (Figure 4) to membranes constituted of phospholipid and cholesterol (20/10 molar ratio), results from transmembrane channel formation. This is in accordance with what has been observed with nystatin [16] or AmB [17]. The conductance of these single channels decreased from 7.5 pS to 4 pS with increasing length of the alkyl chains borne by the polar group. Anion-selective conductance was induced and the membrane potential was approximately 42 mV for a 10-fold KCl gradient. As a conclusion, modification of AmB at the polar head did not modify the AmB channel characteristics. The new observation is that below 3×10^{-7} M, the initial increase of conductance was followed by its decay. At 5×10^{-8} M, after 30 min, the bilayer conductance was almost that observed in the absence of antibiotic. The same non-monotonic kinetics of conductance change was also observed with AmB at 37°C (data not shown).

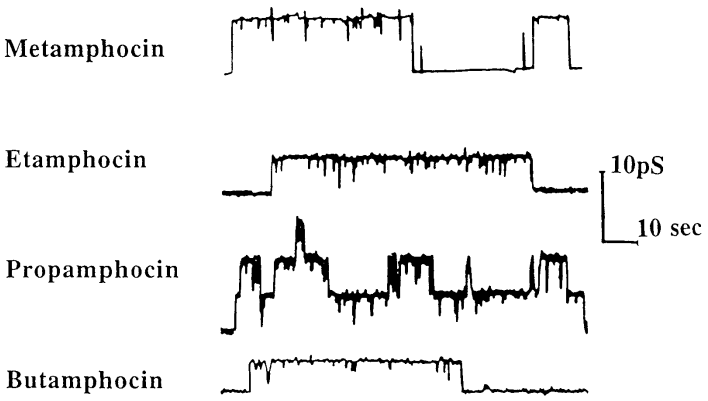

Figure 3. Time dependence of the discrete membrane conductance induced by alkyl derivatives of AmB. Concentration of antibiotics: metamphocin: 2×10^{-8} M, etamphocin: 2×10^{-8} M; propamphocin: 3×10^{-8} M; butamphocin: 4×10^{-8} M. Membrane composition: phospholipid/cholesterol: 2/1 molar ratio.

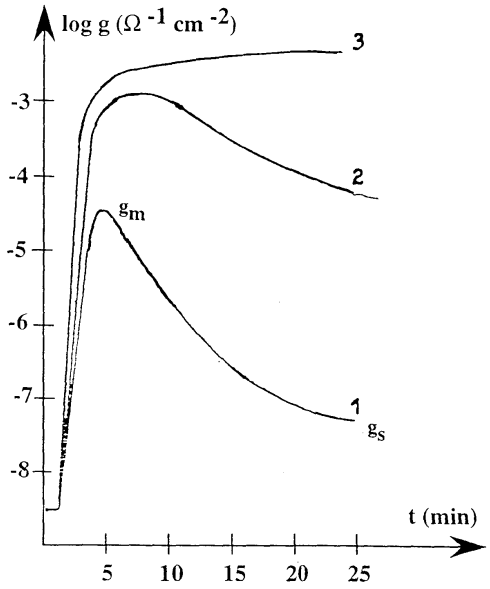

Figure 4. Variation as a function of time, of the membrane conductance induced by metamphocin added at various concentrations to both sides of a membrane (phospholipid/cholesterol: 2/1 molar ratio). 1: 5×10^{-8} M; 2: 10^{-7} M; 3: 3×10^{-7} M.

This decrease of membrane conductance can take its origin in the decrease of 1) channel conductance, 2) mean lifetime of the channels in conducting state, 3) the total number of conducting channels. The last reason seems the more probable. We have studied the spectral composition of conductance fluctuations induced by metamphocin. The level of 1/f noise in the presence of metamphocin increased

10-fold as compared with that of AmB. The coincidence of spectra measured in different zones of the membrane conductance alteration (growth, maximum and steady state) indicates that only one form of conducting channel occurs.

Altogether these results may explain the transient permeability observed upon addition of AmB to hepatocytes [18] or Baby Hamster Chinese Ovary cells [19], and the two-stage action of AmB on mammalian cells (at low concentration inducement of permability to K^+, at high concentration cell killing) [20]. In these cells, after the initial burst of transmembrane permeability, its return to a lower level enables the membrane pumps such as Na^+/K^+ ATPase to restore the initial intracellular concentration. In light of our results, a similar behaviour may be expected to occur with alkyl derivatives.

As for the mechanism of this two-phase inducement of permeability, a possible explanation is the following: we have shown that similarly to the parent compound, the cationic derivatives of AmB are present in water under monomeric and self-associated form, in a concentration dependent manner. Both forms bind to membranes but the self-associated form, only, induces permeability through cholesterol-containing membranes [21]. Inside the membrane, reequilibration between monomers and self-associated forms take place, in favour of monomers, thus reducing the initial level of channel-forming oligomers. However, for high concentrations (that is above 3×10^{-7} M), there is enough monomers in the aqueous solution to saturate the membrane in monomers. The bound oligomeric antibiotic does not dissociate and the permeability does not decrease with time. That equilibrium between different AmB or AmB derivatives superstructures exists inside the membrane is demonstrated by the following data on the antibiotic removal from the membrane, resulting from washing.

Washing out of antibiotics from membranes. Removal of cationic derivatives from the membrane, presents different kinetic characteristics, depending on their one-sided or two-sided addition. They were put in evidence in two types of experiments.

1) In the first case, amphotericin B or metamphocin were added at a concentration of 3×10^{-7} M to one side of the membrane, kept in aqueous solution for 30 min, then washed out. After increasing time lags, amphotericin B was added to the opposite side at a concentration of 3×10^{-7} M, which induced a rapid jump of conductance. A plateau was reached in less than 3 min (data not shown); the amphotericin B-induced increase of conductance was measured at that moment (Figure 5). The level of the plateau decreased monotonically with the increase in the time elapsed after antibiotic removal. It should be noted that with an initial addition of AmB instead of cationic derivatives, the same experiments resulted in a more rapid decrease of conductance.

To interprete these results, let us recall first, that at the concentration studied, no conductance is observed upon one-sided addition of the cationic derivatives of AmB. This is in agreement with what has been described with AmB [7,22,23]. It should be noted that single channels have been observed upon one-sided addition of AmB at a very low AmB concentration [24], that is below the threshold of self-association. However, it may be assumed that their very low duration time may not be sufficient to

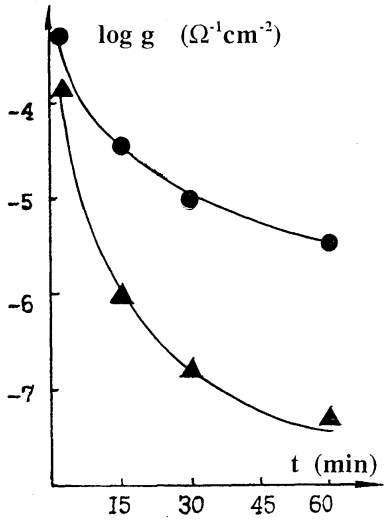

Figure 5. Kinetics of conductance decrease after removal of metamphocin (triangles) or amphotericin B (circles) added to *one* side of the membrane at an initial concentration of 3×10^{-7} M (3×10^{-7} M AmB was added on the opposite side to induce conductance). The applied potential was 100 mV. Aqueous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

induce significant conductance. The conductance jump observed after addition of 3×10^{-7} M AmB derivative to membranes containing metamphocin on the opposite side is similar to what has formerly been observed with AmB and nystatin [25] or amphotericin B methyl ester and N-acetyl AmB [23]. It was assumed that composite channels were formed with half-pores of different composition. Similarly, the one-sided addition of the anion-specific AmB and that of the cation-specific levorin to the other side, resulted in conducting channels, their selectivity and conductivity being determined by the AmB molecules.

2) In the second case, the same kind of experiment as already described [26,27] was performed: metamphocin was added at a concentration of 2×10^{-7} M to both sides of the membrane, was kept in aqueous solution for 30 min, then washed out. Conductance was recorded for increasing time lags after washing (Figure 6). All data were obtained with the same membrane. It should be noted that a similar experiment was done by Cass *et al.* [28], but the data cannot be compared to ours because temperature was varied along the relaxation step.

In our experiment, the conductance relaxation presented two-phases: a period of slow conductance alteration (« induction period ») and a period of exponential decrease. The exponential phase could be characterized by the relaxation time constant τ, the time necessary for an e-fold conductance decrease. τ depends on the initial antibiotic concentration as shown in Figure 7. For low antibiotic concentration, τ is close to zero.

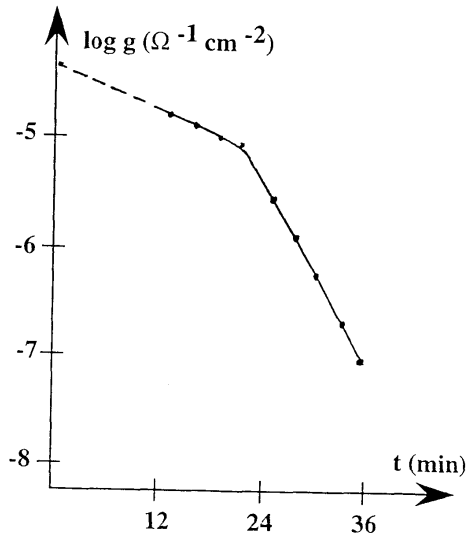

Figure 6. Kinetics of conductance decrease after removal from one side, of metamphocin added to *two* sides of the membrane at an initial concentration of 2×10^{-7} M. Experimental conditions as in Figure 4.

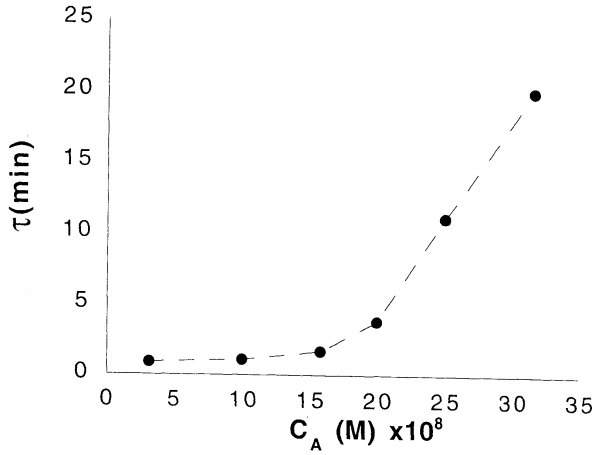

Figure 7. Influence of the initial concentration in metamphocin (C_A) on the kinetics of conductance decrease in the exponential phase, after removal from one side of the antibiotic added to *two* sides of the membrane. Experimental conditions as in Figure 4.

The « induction » period is not observed in the experiments performed with the one-sided addition of metamphocin. Therefore, it may be assigned to a step not observed in the first study: dissociation of the assemblage enabling trans membrane permeability to develop, that is dissociation of two coupled half-pores (according to the current model adopted for the kind of lipid bilayers studied here). The period of exponential decrease, with a relaxation time constant similar to that observed in the first

case, that of one-sided addition, can either be assigned to a displacement inside one lipid layer of the equilibrium between half-pores and monomers, leading to the disassembly of the half-pores and/or to the release of monomers in the aqueous medium.

CONCLUSIONS

Our study explains at the molecular level, how polyene antibiotics can induce transient permeability in cell membranes. Such a property has recently been used to deliver nucleic acids into mammalian cells. As a matter of fact amphotericin B 3-dimethylaminopropyl amide (AMA), a cationic derivative of AmB [29], was shown to bind oligonucleotides and to facilitate their internalization into mammalian cells. As a result, an anti-MDR antisense oligonucleotide could be delivered by AMA and inhibit the expression of P-glycoprotein [8,9]. Circular dichroism and electronic microscopy have clearly demonstrated the interaction of the cationic derivatives with the negatively charged nucleic acids [8] and given some characteristics of the complex. Concerning the mechanism of internalization data are missing but it was assumed that cationic derivatives of AmB induce, like their parent compound, transient transmembrane permeability, which enables cells to recover from the initial « injury » and allows entry of the nucleic acid. The present study shows that transient pore formation is a possibility to be considered. To what extent the mechanism proposed in the present study can be extended to oligonucleotides and the presence of nucleic acids may interfere with this process is under current study in our laboratory.

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