Rimantadine Effects on the Elasticity of Bilayer Lipid Membranes and on Ion Transport through Gramicidin D Channels

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Abstract. The effect of the antiviral preparation rimantadine on lipid bilayer membranes (BLM) was studied by measuring the modulus of elasticity in the direction normal to the surface ($E_\perp$) and by estimating the conductance Λ, the lifetime τ of single gramicidin D channels (GRD), and the coefficient of nonlinearity β of current voltage characteristics (IVC) of GRD-modified BLM. Rimantadine induced a nonmonotonic change in $E_\perp$ of BLM prepared from a mixture of egg lecithin with cholesterol: at relatively low rimantadine concentrations (0 — 40 μg/ml) $E_\perp$ first increased, reached a maximum and started to decrease. The effectivity of rimantadine was dependent on the cholesterol concentration in the BLM. Changes in $E_\perp$ suggest an increased ordering of the lipid bilayer at low rimantadine concentrations and formation of clusters of the preparation at concentrations exceeding those necessary to obtain maximal values of $E_\perp$ for the given BLM lipid composition. Rimantadine concentrations between 40 — 100 μg/ml decreased both the conductance and the GRD channel lifetime by approximately 20 percent, affected the degree of IVC nonlinearity and superlinearity of GRD-modified membranes, which suggests some effect on the height of the barrier at the ionic channel mouth and in its centre.

Key words: Rimantadine — Bilayer lipid membranes — Mechanical properties — Ion transport — Gramicidin D

Introduction

Rimantadine (methyl-1-aminoadamantane hydrochlorid, m.w. 179.3) is widely used for the treatment and prophylaxis of viral diseases. Its effect on biological

membranes, in particular influenza virion membranes (Kharitonenkov et al. 1979, 1981), erythrocyte shape (Tverdislov et al. 1985) and deformability (Cherny et al. 1989), as liposomes (Kharitonenkov et al. 1979, 1981), bilayer lipid membranes (BLM) (Tverdislov et al. 1985; Simonova et al. 1986; Cherny et al. 1989) have been thoroughly studied. Rimantadine has been shown to raise BLM conductance and to change modulus of the elasticity in the direction normal to membrane surface, $E_{\perp}$. Rimantadine effects were largely dependent on the lipid composition of the membrane studied. The drug was able to "rigidize" azolectin membranes as well as to "fluidize" dioleyllecithin and/or bovine brain lipid BLM (Cherny et al. 1989). Rimantadine induced substantial changes in the shape and mechanical properties of erythrocytes. Concentrations of 10 μg/ml were sufficient to smoothen echinocyte spicules, thus inducing erythrocyte transition to normo- and stomatocytes (Tverdislov et al. 1985). Moreover, due to the aggregation of membrane proteins, rimantadine induced nonhomogenous deformability of erythrocyte membranes (Cherny et al. 1989).

Rimantadine interaction with nonhomogenous BLM can be expected to result in nonmonotonic changes of mechanical properties of the membranes. The aim of the present work was to study processes of rimantadine interaction with lipid bilayers, to estimate the nature of changes in mechanical properties of BLM with strongly heterogenous structure of the lipid bilayer. This will, at least to a define degree, model rimantadine interactions with structurally inhomogenous membranes, such as the erythrocyte membrane, the inhomogeneity being the result of aggretation of the cytoskeleton membrane proteins. Cholesterol was used to produce inhomogenous BLM structure; at concentrations exceeding 0.2 mole fraction cholesterol forms clusters in BLM (see Hianik et al. 1984).

To shed more light on the mechanisms of rimantadine interactions with lipid bilayers, also rimantadine effects on the conductance of single gramicidin D (GRD) channels and on the dynamics of cation transport through GRD ionic channels were studied. This is important for the understanding of rimantadine interactions with membranes of excitable cells which contain ionic channels. From the practical point of view (medicine and pharmacology), these issues are of interest as they help to estimate physical parameters of membranes upon their simultaneous interaction with two different drugs, the antiviral preparation rimantadine and the antibiotic gramicidin.

Materials and Methods

BLM were formed according to the method of Mueller et al. (1962) on a circular hole (approx. 0.5 mm in diameter) in the wall of a teflon cup. The lipid solution contained egg lecithin (Kharkov Plant of Chemical Preparations, USSR) and cholesterol (Fluka) in *n*-heptane (Fluka) (20 mg/ml).

Various concentrations of cholesterol were employed: 0.11; 0.2; and 0.33 mole fraction cholesterol. The experiments were performed at $T = 20\,°C$.

Rimantadine interaction with BLM was studied by three different methods: 1. By measuring the modulus of elasticity in the direction normal to membrane surface, $E_\perp$; 2. By determining the conductance A, and the lifetime τ of single GRD ion channels; and 3. by determining the dynamics of K^+ ion transport through GRD-modified BLM from current-voltage characteristics (IVC). Rimantadine (Olajnchim), Gramicidin D (P-l Biochemical) as well as other preparations used were of analytical purity. Let us present a short description of the techniques and methods employed for the present experiments.

Measurement of modulus of elasticity in the direction normal to membrane surface $E_\perp$

BLM elasticity in the direction normal to membrane surface is expressed by the modulus of elasticity $E_\perp$, which characterizes the membrane ability to decrease its thickness in response to pressure p acting on it. Pressure p acting on a membrane can be produced by applying to the membrane electrical voltage U. According to the method developed by Passechnik and Hianik (1977), alternating electrical voltage $U = U_0 \sin 2\pi ft$, where f is the frequency and U_0 is the amplitude, compresses the membrane with pressure $p = C_s U^2 / 2h$, where C_s is the specific membrane capacitance, and h is the thickness of the hydrophobic part of membrane. Membrane capacitance depends on the electrical voltage: $C = C_0(1 + \alpha U^2)$, where C_0 is BLM capacitance at $U = 0$ mV) and α is the coefficient of electrostriction. Due to the nonlinear dependence of capacitance on voltage, membrane current $i = \mathrm{d}(CU)/\mathrm{d}t$ contains with frequency $3f$ and amplitude A_3 in addition to that with frequency and amplitude f and A_1 respectively. Parameter $E_\perp$ can be determined by the mechanical membrane model (Passechnik and Hianik 1990) as

$$E_\perp = 3C_s U_0 A_1 / 4hA_3$$

Hence, only amplitudes A_1 and A_3 of membrane current harmonics have to be measured to determine parameter $E_\perp$; this can be done using e.g. a common electronic device and resonance amplifiers (see Passechnik and Hianik 1977). Alternating voltage with amplitude $U_0 = 140$ mV and frequency 1000 Hz was applied to the membrane during the measurements. The following values of parameters C_s and h were used for individual BLM with respect to cholesterol content (mole fraction cholesterol) to calculate $E_\perp$: 0.11 mole fraction — $C_s = 3.4 \times 10^{-3}$ F m^{-2}, $h = 5.5$ nm; 0.20 mole fraction — $C_s = 3.6 \times 10^{-3}$ F m^{-2}, $h = 5.2$ nm; 0.33 mole fraction — $C_s = 3.9 \times 10^{-3}$ F m^{-2}, $h = 4.8$ nm (see Hianik et al. 1984). The experiments were performed as follows. Parameters A_1 and A_3 were allowed to stabilize (approx. 10 min following the membrane formation), and rimantadine dissolved in the same electrolyte as that used to form the membrane (0.2 mol l KCl + 5 mmol l tris-HCl, pH 7.4) was added to one side of the membrane. Maximal rimantadine concentration in the electrolyte was 100 μg ml. No substantial increase of BLM conductance has been observed with similar concentrations (Tverdislov et al. 1985). Hence, it were the effects associated with the adsorption of the preparation that could be studied.

Conductance measurements of single ionic channels

The conductance of single GRD ionic channels was measured using the method described by Haydon and Hladky (1972). One calomel electrode was immersed into the electrolyte in one compartment and connected to a direct voltage source. The other electrode was immersed into the other compartment of the teflon cup and was connected to the high-resistance input of an operational amplifier type WSH 223 (Tesla) (see Dostál 1981). The apparatus allowed recording of currents $i < 10^{-13}$ A using a TZ 4100 recorder (Laboratorni přistroje, Praha) with a time discrimination of approx. 10 ms. GRD (approx. 10^{-13} mol l) was added to the electrolyte (0.2 mol l KCl, pH 7.4) at

both membrane sides. As soon as the discrete change in membrane conductance, typical of GRD, appeared rimantadine (40; 60; or 100 μg ml) was added into the electrolyte at one membrane side. Then, approx. 5 min were allowed to elapse before starting the recording of single rimantadine-modified GRD channels.

Determination of the coefficient of nonlinearity of the current-voltage characteristics of the membrane

The current-voltage characteristics were determined using the methods described in detail by Flerov et al. (1981) and Passechnik et al. (1985). The employed method of direct determination of current-voltage characteristics (IVC) of modified membranes is based on the recording of the third current harmonic generated in BLM with nonlinear current-voltage relationship. Here, we shall show but the principal relationships. In first approximation, membrane IVC can be expresed as $i = g \cdot U(1 + \beta U^2)$, where g is the conductance, β is the coefficient of nonlinearity. If alternating voltage $U = U_0 \sin 2\pi ft$ with a sufficiently low frequency is applied to a membrane to eliminate the capacitive current component, current with frequency $3f$ and amplitude A_3 will flow through the membrane in addition to the current component with frequency f and amplitude A_1. The coefficient of nonlinearity is then determined by

$$\beta = -4A_3(1 + rg)^3 E_0^2 \cdot A_1,$$

where r is the resistance of both the electrodes and the electrolyte, $g = A_1/(E_0 - rA_1)$, and E_0 is the amplitude of the alternating voltage applied to the system electrodes — electrolyte — membrane. For the total voltage amplitude E_0 and that of voltage decrement at the membrane holds: $U_0 = E_0 - A_1 r$. Consequently, amplitudes A_1 and A_3, and resistance r can be used to determine membrane IVC pattern. In our experiments alternating voltage with amplitude $E_0 = 100$ mV and frequency $f = 40$ Hz was employed.

We have shown in a previous paper (Hianik et al. 1987) that the IVC pattern of gramicidin D-modified BLM, determined by the sign of the coefficient of nonlinearity β, depends on the electrolyte concentration. GRD-modified BLM from a mixture of egg lecithin and cholesterol (0.11 mole fraction cholesterol) have sublinear IVC ($\beta < 0$) at electrolyte (KCl) concentrations $c < 0.15$ mol/l, and the ion transport is limited by the entrance area of the ion channel. At $c = 0.15$ mol/l KCl, IVC is linear ($\beta = 0$), whereas it is superlinear ($\beta < 0$) at $c < 0.15$ mol/l KCl, and the ion transport across the membrane is determined by the internal energy barrier of the ion channel. Therefore, it is of interest to study the effects of rimantadine on the ion transport dynamics across GRD channels under conditions of markedly nonliner IVC, i.e. at very low and very high concentrations. In our experiments, BLM from egg lecithin-cholesterol (0.11 mole fraction cholesterol) mixture were formed in 0.03 or 3.0 mol/l KCl (pH 7.4). The experiments were performed as follows. Immediately after a membrane formation, 40 μg ml rimantadine was added to one side of the membrane. At this concentration, rimantadine was observed to induce the largest changes in modulus of elasticity E_1 for BLM of above lipid composition. As soon as parameters A_1 and A_3 stabilized (after 15 – 20 min), GRD in a final concentration of 10^{-9} mol/l was added into the electrolyte at both membrane sides. Then, the dependence $A_1(A_3)$ was recorded using an X – Y plotter, and the value of parameter β (by the relationship given above) was determined.

Results

Rimantadine-induced alterations of mechanical BLM characteristics

Rimantadine addition to the electrolyte in contact with BLM induced a growth

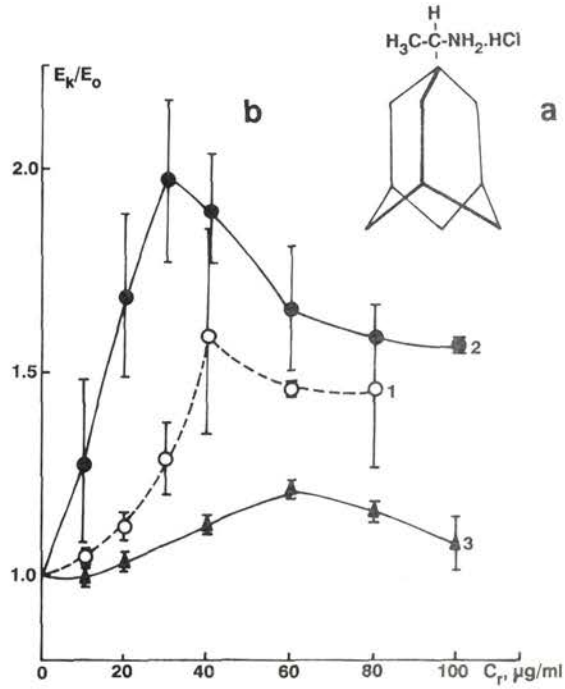

Fig. 1. *a:* Structural formula of rimantadine. *b:* The dependence of relative change in modulus of elasticity E_k/E_o of BLM prepared from egg lecithin with various cholesterol contents on rimantadine concentration in the electrolyte. 1—0.11; 2—0.2; 3—0.33 mole fraction cholesterol.

of the relative modulus of elasticity of membranes from egg lecithin containing various cholesterol concentrations (Fig. 1). The relative change of modulus of elasticity E_k/E_o (where E_k is the modulus of elasticity measured in the presence of the respective rimantadine concentrations c_r and calculated from steady-state values of A_1 and A_3; E_o is the modulus of elasticity of the same membrane prior to rimantadine administration) was dependent on the rimantadine concentration in the electrolyte. The largest changes of E_k/E_o were observed for membranes with the highest rigidity ($c = 0.2$ mole fraction cholesterol) (Fig. 1*b*, curve 2). The rimantadine effect was minimal at 0.33 mole fraction cholesterol (strongly inhomogenous membranes, curve 3), whereas the dependence $E_k/E_o(c_r)$ was intermediate (i.e. between curves 2 and 3) for the relatively low cholesterol concentration ($c = 0.11$ mole fraction, curve 1). A typical peculiarity of all the three curves is their nonmonotonicity. With the increasing rimantadine concentration, parameter E_k/E_o first increased reaching a maximum, and decreased thereafter. The maximum on the E_k/E_o curve depended on the cholesterol

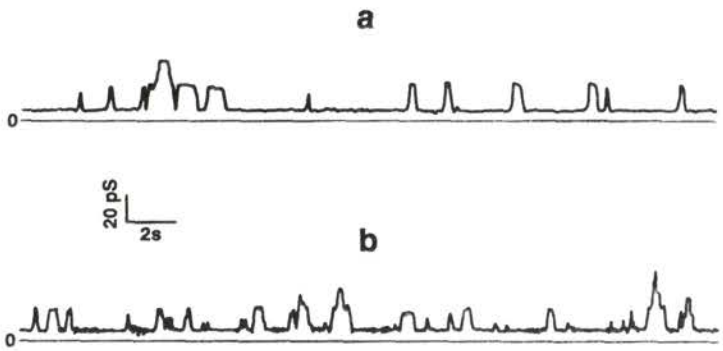

Fig. 2. The dynamics of changes in BLM conductance. BLM from egg lecithin plus 0.11 mole fraction cholesterol, modified by small amounts of gramicidin D. *a:* In the absene of rimantadine, *b:* 100 μg/ml rimantadine.

concentration in the membrane, and appeared at different rimantadine concentrations $(c_r)_{max}$ in the electrolyte: $(c_r)_{max} = 30\ \mu$g/ml for 0.2 mole fraction cholesterol; $(c_r)_{max} = 60\ \mu$g/ml for 0.33 mole fraction; and $(c_r)_{max} = 40\ \mu$g/ml for 0.11 mole fraction cholesterol.

Rimantadine effects on parameters of single GRD channels

Fig. 2 illustrates a typical pattern of the conductance change kinetics of a single GRD channel in a BLM from egg lecithin-cholesterol (0.11 mole fraction) mixture in the absence (*a*) and in the presence of 100 μg/ml rimantadine in the electrolyte (*b*). Fig. 3 shows the corresponding distribution histogram of channel conductances. Obviously, rimantadine alters the distribution of channel conductances, shifting it towards low values. Table 1 summarizes the results and suggests that the lower concentration (40 μg/ml) of rimantadine tested did not affect ion channel lifetime. Rimantadine concentrations in the electrolyte higher than that induced substantial changes in parameter τ, whereas the relative change of conductivity $\Delta\Lambda/\Lambda_0$ grew linearly with the increasing rimantadine concentration. To make the results obvious, parameters Λ and τ ($\Delta\Lambda/\Lambda_0$ and $\Delta\tau/\tau_0$) for $c_r = 60$ and 100 μg/ml were related to the respective values for control membrane ($c_r = 0$), since measurements for various rimantadine concentrations were made with different membranes of identical lipid composition. Nevertheless, the membranes may have differed in their physical characteristics, such as modulus of elasticity, which most probably was due to variations of hydrocarbon solvent concentration in the lipid bilayer during BLM formation according to Mueller (see Passechnik et al. 1981).

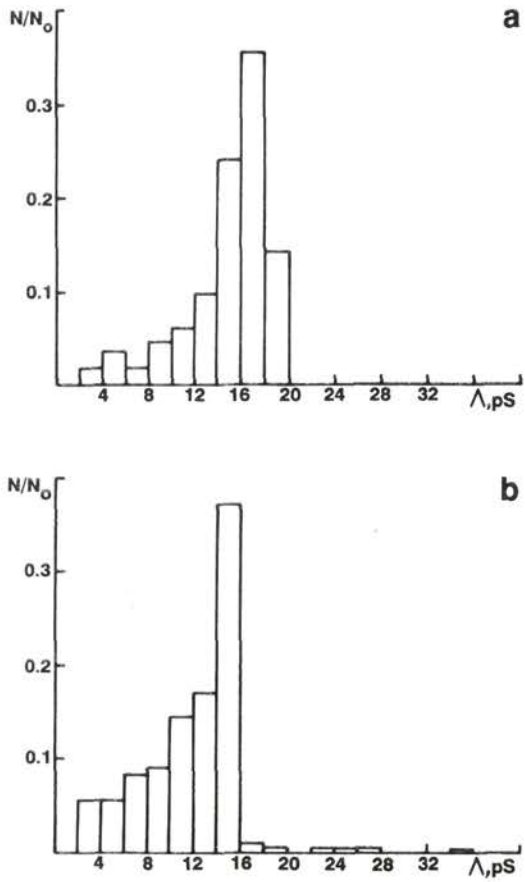

Fig. 3. Distribution of relative channel numbers N/N_o (N_o is the total number of ion channels, N is the number of ion channels corresponding to the given range of conductance) according to the conductance Λ. *a:* In the absence of rimantadine; *b:* In the presence of 100 μg/ml rimantadine.

Table 1. The dependence of single gramicidin D channel conductance Λ and lifetime τ and their relative changes Δ/Λ_o and $\Delta\tau/\tau_o$; on rimantadine concentration in the electrolyte ($\Delta\Lambda = \Lambda_o - \Lambda_r$; $\Delta\tau = \tau_o - \tau_r$; indexes: o — in the absence of rimantadine, r — in the presence of rimantadine).

Rimantadine concentration (μg/ml)	$\Lambda \pm$ SE (pS)	$\tau \pm$ SE (s)	$\Delta\Lambda/\Lambda_o$	$\Delta\tau/\tau_o$	Number of channels N
0	11.32 ± 0.27	0.25 ± 0.07			1012
40	9.28 ± 0.56	0.24 ± 0.06	0.18	0.04	514
60	9.16 ± 0.26	0.19 ± 0.06	0.19	0.24	230
100	8.83 ± 0.21	0.21 ± 0.07	0.22	0.16	231

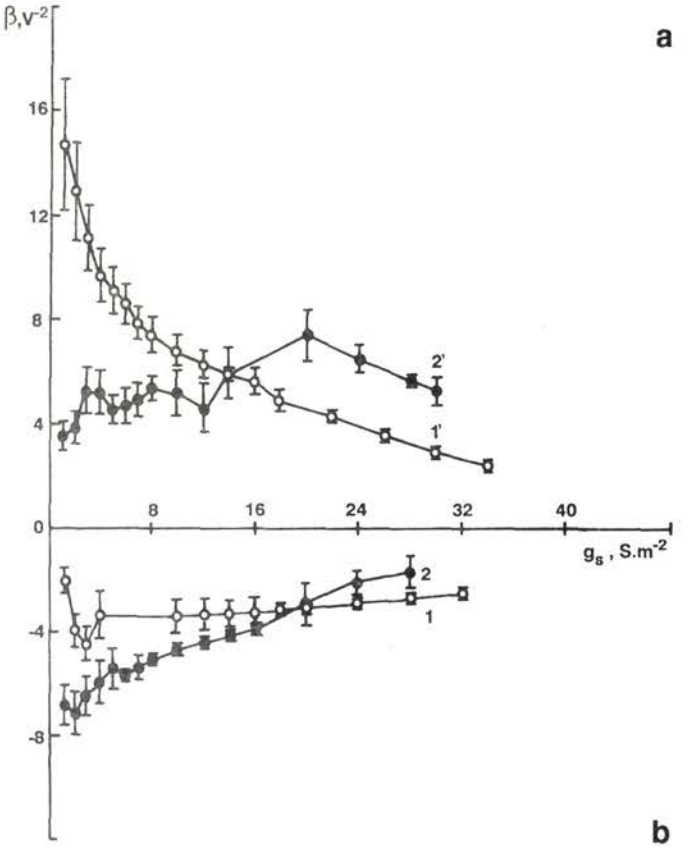

Fig. 4. The dependence of coefficient of nonlinearity β of IVC for GRD-modified BLM from egg lecithin plus cholesterol (0.11 mole fraction) on the specific membrane conductance for two different electrolyte concentrations: 3 mol l KCl (*a*) and 0.03 mol l KCl (*b*) in the absence of rimantadine (1,1') and in the presence of 40 μg ml rimantadine (2,2').

Rimantadine-induced changes in ion transport dynamics

To explain the possible rimantadine effect on the ion transport dynamics, the effects of rimantadine on coefficient of nonlinearity β of current-voltage characteristics of GRD-modified membranes were studied. Membranes of lipid composition identical with that employed for previous experiments (egg lecithin-cholesterol (0.11 mole fraction) mixture) were used. Due to gradual formation of ion channels, the presence of GRD in the electrolyte induced a growth of specific BLM conductance, reaching values of approx. 30 S/m^2. It should be

noted that rimantadine significantly affected the kinetics of the GRD-modified membrane conductance growth. In the presence of rimantadine, identical values of specific BLM conductance were obtained with a delay of 30—100 min as compared with experiments in the absence of the drug. Similar effects could be observed earlier with BLM with high cholesterol contents (see Hianik et al. 1987). Obviously, the rather compact membrane structure in the presence of the drug makes ion channel formation more difficult. A certain role in the slow process of gramicidin incorporation is played also by the positive surface charge produced as a result of rimantadine adsorption to the BLM (Simonova et al. 1986). This is due to the fact that neutral pH rimantadine molecule carries a single elementary positive charge (Fig. 1*a*).

Fig. 4 shows the dependence of coefficient of nonlinearity β on specific BLM conductance g_s in the absence of rimantadine (curves 1 and 1'), and in the presenced of 40 μg/ml rimantadine curves 2 and 2') for two different electrolyte concentrations: 3 mol/l KCl (curves 1' and 2') and 0.03 mol l KCl (curves 1 and 2). It is obvious that both in the presence and in the absence of rimantadine, parameter β changes with the increasing specific membrane conductane. In agreement with the report of Passechnik et al. (1985), this change can be associated but with a change in coefficients of nonlinearity of ionic channels, as parameter β is of non-additive nature. Most likely, this phenomenon is analogical to, although less marked than, that typical of changes of coefficient of nonlinearity β in dependence on amphotericin B-modified specific BLM conductance (Feigin et al. 1982). Fig. 4 shows that at the low electrolyte concentration (0.03 mol/l) rimantadine shifts the value of coefficient β with the range of low membrane conductances towards negativity; this might suggest some growth of the energy barrier in the channel mouth area. At higher conductances however, the value of β is shifted towards positivity. At the electroylte concentration of 3 mol/l, when the IVC of GRD-modified BLM is superlinear ($\beta < 0$), the dependence $\beta(g_s)$ is changed as well. The parameter β of rimantadine-modified BLM increases at high specific membrane conductances.

Discussion

The considerable change in the modulus of elasticity $E_\perp$ of BLM, induced by rimantadine, suggests that the drug molecules are incorporated into the membrane, and this results in a compact ordering of lipid hydrocarbon chains. This conclusion agrees with the results of Simonova et al. (1986) who measured treshold potentials and could show that rimantadine adsorbs to the BLM

surface and incorporates into a certain depth of the lipid bilayer. In analogy with the cholesterol effect on the viscoelastic characteristics of membranes, as discussed previously (Hianik et al. 1984) it can be assumed that the initial growth of E_k/E_o within the range of low rimantadine concentrations is associated with the drug-induced tight ordering of the lipid bilayer. The decrease of E_k/E_o after $E_k/E_o(c_i)$ maximum is likely the result of the formation of clusters in the membrane: probably, rimantadine aggregates or form complexes with lipids. The stronger effect of rimantadine mechanical characteristics of BLM with lipid composition corresponding to maximally ordered bilayer ($c = 0.2$ mole fraction cholesterol) as compared to less ordered membranes suggests that rimantadine molecules interact more strongly with molecules of egg lecithin and cholesterol than do cholesterol molecules with egg lecithin.

This means that the rimantadine effect requires a certain degree of "membrane inhomogeneity" to occur; the modifier aggregates in dependence on this factor and changes the mechanical characteristics of the bilayer. The examples of rimantadine and cholesterol actions on mechanical BLM characteristics show that aggregation processes accompanied by cluster formation result in significant changes of mechanical membrane characteristics. It may well be that it is those effects that underlie the different elasticities of different membrane regions in erythrocytes (due to the formation of aggregates of peripheral proteins of the cytoskeleton, which is necessary for the cell to assume and stabilize a certain shape).

The different effectivity of rimantadine action on mechanical properties of lipid bilayer in dependence on the drug concentration has significant practical consequences (selection of optimal drug doses and/or raising the effectivity of the drug action).

The results obtained in measuring parameters Λ, τ, and β of the channel-former gramicidin D-modified BLM have confirmed and extended our understanding of rimantadine-induced changes of mechanical membrane characteristics. The decreased conductance of ion channels in the presence of rimantadine may be the result of decreased near-membrane K^+ concentrations due to repulsion by the positive rimantadine charge. This idea is supported by the observation of a shortened lifetime τ of GRD ion channels at high rimantadine concentrations; this shortening may be associated with a decreased GRD dimer stability due to the decreased concentration of K^+ at the membrane surface. This can be assumed based on the report of Kolb and Bamberg (1977) who showed that a decrease of KCl concentration from 1.0 to 0.1 mol/l results in a threefold reduction of parameter τ for gramicidin channels. These conclusions were but partialy confirmed by the measurements of coefficient of nonlinearity β. The shift of parameter β towards negative values at relatively small specific BLM conductances (Fig. 4) might suggest some effect of rimantadine on the

growth of the energy barrier to ion passing at the channel mouth. It should be noted that similar results have been obtained also by Bajči (1987) under identical experimental conditions, but with an electrolyte concentration $c = 0.2$ mol/l KCl; at this concentration, parameter β for BLM without rimantadine approaches zero. On the other hand, if rimantadine exclusively acted on gramicidin channel mouth area, coefficient of nonlinearity β was of membrane IVC would be expected to show minimal changes at high electrolyte concentrations (3 mol/l KCl) when IVC is superlinear ($\beta < 0$) and cation transport is limited by the energy barrier at the central part of the channel. However, the value of β changes also in this case (Fig. 4). The decrease within the range of low g_s values could be explained by a changed mouth area/central channel area energy barrier ratio. However, the growth of β at high g_s values suggests that rimantadine affects the central energy barrier, most likely by acting on the hydrophobic membrane moiety.

It can be concluded from what has been shown above that rimantadine acts on both the surface and the central, hydrophobic membrane area. The mechanisms whereby rimantadine can alter membrane structure remain unknown. The results obtained however suggest that 1. the membranotropic effect of rimantadine is stronger than the effect of cholesterol on lipid bilayer ordering; 2. the effect of rimantadine depends on the structural state of the lipid bilayer, in particular on the degree of its inhomogeneity; 3. rimantadine affects both the entrance and the central energy barriers to ion transport through gramicidin D channels. The significance of the above conclusions for pharmacology and therapy is strengthened by the fact that observed changes of lipid bilayer characteristics and ion transport occured within the range of rimantadine concentrations which also show antiviral activity (25—100 μg/ml in the extracellular environment). The rimantadine-induced changes in the polar and hydrophobic membrane regions most likely play a decisive role in the intraction of viruses with the cell plasma membrane: changes in the structure of the latter hamper the entering of viruses into the cytoplasm.

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