Haematoporphyrin Changes the Mechanical Properties of Lipid Bilayer Membranes

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Abstract. The interactions between haematoporphyrin (HP) and bilayer lipid membranes (BLM) were studied. A weak effect of HP on BLM conductivity was observed at HP concentrations ranging between 10^{-6} and 3×10^{-5} mol/l. Modulus of elasticity in the direction normal to the membrane plane ($E_\perp$) and dynamic viscosity coefficient (η) were measured, both exhibiting HP-induced decrease by 22–31% in the dark. In this case, membrane potential V_m became negative and reached a value close to −50 mV. Under illumination by low-intensity (1 mW) He-Ne laser ($\lambda = 632$ nm) the values of parameters $E_\perp$ and η of the HP-modified membranes increased by 41–66% , and V_m decreased to −20 mV. Upon removing HP from the solution by perfusion, irreversible changes in mechanical properties of the HP-modified membranes induced by the laser light were observed. The reason could be the formation of stable complexes of HP with the lipid molecules. HP binds to membrane noncooperatively, with a binding constant $K \sim 10^5$ l/mol.

Key words: Haematoporphyrin — Bilayer lipid membranes — Laser illumination — Mechanical properties

Introduction

Photodynamic therapy is known as a promising method for diagnosis and treatment of cancer (Henderson and Belnier 1989). The key factors determining the efficiency of this method are the selectivity of accumulation of a photosensitizing dye in tumor tissue and its ability to produce cytotoxic agents under illumination. Haematoporphyrin (HP) is one of the photosensitizers commonly used for the purposes of photodynamic therapy. The main features of HP accumulation and localization in the cell membranes have already been reported (Chernyaeva et

al. 1988), yet the mechanisms of HP interaction with membranes have not been well understood. Since considerable dye accumulation in the cell plasma membrane has been reported (Chernyaeva et al. 1988) the mechanisms of the interaction are of high interest.

The purpose of our investigation has been to study the interactions of HP with artificial bilayer lipid membranes (BLM). BLM consist of lipids and hydrocarbon solvent, and are commonly used as models of cell membrane (Tien 1974). The effects of HP on mechanical characteristics of BLM were studied in the dark and under illumination by a He-Ne laser. The electrostriction technique was used to measure modulus of elasticity in the direction normal to the membrane plane ($E_\perp$), the dynamic viscosity coefficient (η), and electric capacity (C) and membrane potential (V_m).

Materials and Methods

BLM were prepared according to method of Mueller et al. (1962) from a solution of palmitoylphosphatidylcholine (POPC) and/or a mixture of POPC and cholineplasmalogen (CHPG) (weight ratio 5:1) in *n*-heptane (Fluka) at a concentration of 20 mg/ml. Phospholipids were prepared according to the methods described by Hermetter and Paltauf (1982), and were kindly supplied by Dr. A. Hermetter (Technical University Graz, Austria). Choline plasmalogen is an important component of cell membranes (Hermetter 1988); the presence of this lipid in BLM makes the properties of BLM close to those of biomembrane lipid bilayers.

Membranes were formed at the tip of a teflon tube, 0.8 mm in diameter. An AgCl electrode was placed inside the tube. KCl (0.1 mol/l) was used as the electrolyte (pH 7.2). HP (Serva), dissolved in redistilled water, was added at different concentrations ranging from 10^{-6} to 3×10^{-5} mol/l to one membrane side. At the used concentrations HP induces photodynamic destruction of cell (Chernyaeva et al. 1988). To study the photodynamic action of HP, the membrane was illuminated with the light of a He-Ne laser ($\lambda = 632$ nm) with a power of ~ 1 mW.

The conductivity was estimated by the widely used technique of Hladky and Haydon (1972) based on the measurement of currents through membranes under direct voltage of 20 mV. The membrane currents were measured using a low-bias current operating amplifier (WSH 223, Tesla, see Dostál 1981) with high input impedance.

The values of $E_\perp$ and η provide information about the structural state of the inner (hydrophobic) part of BLM. These parameters were measured using the special electrostriction method as described by Passechnik and Hianik (1977). The basic principle of the method is as follows. Alternating voltage $V = V_0 \sin 2\pi ft$ (where V_0 is the voltage amplitude, f is its frequency, and t is time) applied to a membrane produces pressure $p = C_s V^2/2h$ (where C_s is the specific membrane capacity and h is the membrane thicknes), resulting in the membrane attenuation due to electrostriction.

Membrane compression by alternating electric field results in modulation of alternating current flowing through the membrane. A current component with frequency $3f$ and amplitude A_3 occurs in the current with frequency f and amplitude A_1 ($A_1 = 2\pi fCV_0$), whereby $A_3 \ll A_1$. The capacity of the membrane to change its thickness in response to the action of an external force is characterized by modulus of elasticity in direction

perpendicular to the membrane plane, $E_\perp$. Parameter $E_\perp$ can be described by

$$E_\perp = 3C_s V^2 A_1/4hA_3 \quad (1)$$

As shown previously (Passechnik and Hianik 1991), for many simple systems, parameter η can by determined from

$$\eta = E_\perp \sin\varphi/2\pi f \quad (2),$$

where φ is the phase shift. Phase shift expresses the delay in membrane deformation following pressure application, due to the existence of internal friction; the parameter φ can be determined using a phasometer. Amplitude A_1 can also be used to determine electric capacity of a membrane:

$$C = A_1/2\pi f V_0 \quad (3).$$

Membrane potential V_m is produced as the result of adsorption of charged particles to the membrane. In this case, the second current harmonic with amplitude A_2 and frequency $2f$ will be generated in addition to the third current harmonic (Carius 1976). The value V_m is then calculated by

$$V_m = V_0 A_2/4h \quad (4)$$

This means that it is sufficient to measure amplitudes A_1, A_2, A_3 and phase shift φ to determine $E_\perp, \eta, C$ and V_m. This can be done with the use of a standard electronic apparatus including resonance amplifiers (Passechnik and Hianik 1977). Voltage $V_0 = 75$ mV with frequency $f = 1$ kHz was used. The kinetics of changes of parameters A_1, A_2, A_3 and φ were recorded and on-line computer processed to yield the kinetics of $E_\perp, \eta, C$ and V_m. $E_\perp$ was computed using parameters $C_s \sim 4 \times 10^{-3}$ F/m^2, and $h \sim 5$ nm, as typical of lecithins (Hianik et al. 1984a). To compute the data, a PMD-85 (Tesla) microcomputer equipped with a LJSP-2 (Centre of Physiological Sciences, Slovak Academy of Sciences) A/D converter was used. The software used to calculate the data was developed in our laboratory. The values of C_s and h were similar for BLM from POPC and those from POPC + choline plasmalogen. This was suggested by measurements of BLM capacity on an aperture with a large diameter ($d \sim 1.5$ mm) to eliminate effects of the torus.

Results and Discussion

Addition of HP into the electrolyte can lead to its adsorption on the membrane surface, incorporation into the lipid bilayer and penetration across the membrane. In all these cases structural transitions of the membrane can be induced and, as a result, the bilayer becomes permeable to ions. Therefore, BLM conductivity was measured first under the influence of HP in the dark and under illumination.

1. *BLM conductance in the presence of HP*

Addition of HP into the electrolyte at $10^{-6} - 3 \times 10^{-5}$ mol/l slightly increases the BLM conductivity. As compared with nonmodified membranes with an average conductivity of 23.4 ± 2.9 pS, the conductance of modified BLM did not increase more than twice, reaching 25–50 pS. Illumination of modified membranes with laser light for 30 min was followed by an increase of BLM conductance up to 75–100 pS. As soon as the light was switched off the conductance decreased to 25–75 pS. Hence, HP, in particular under illumination, changes BLM conductance. These changes are, however, substantially less than those induced by other biologically active substances (Hianik et al. 1984b), and are comparable with the conductance of a gramicidin A unit channel (Hladky and Haydon 1972).

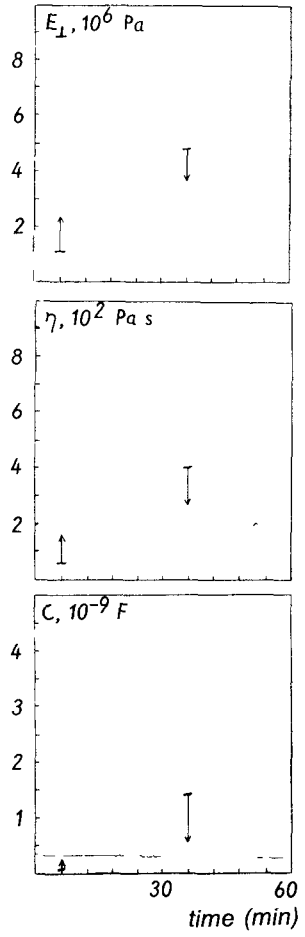

Figure 1. Kinetics of changes of parameters $E_\perp$ (a), η (b) and C (c) of nonmodified BLM from POPC under illumination with the light of a He-Ne laser. ↑ laser on, ↓ laser off.

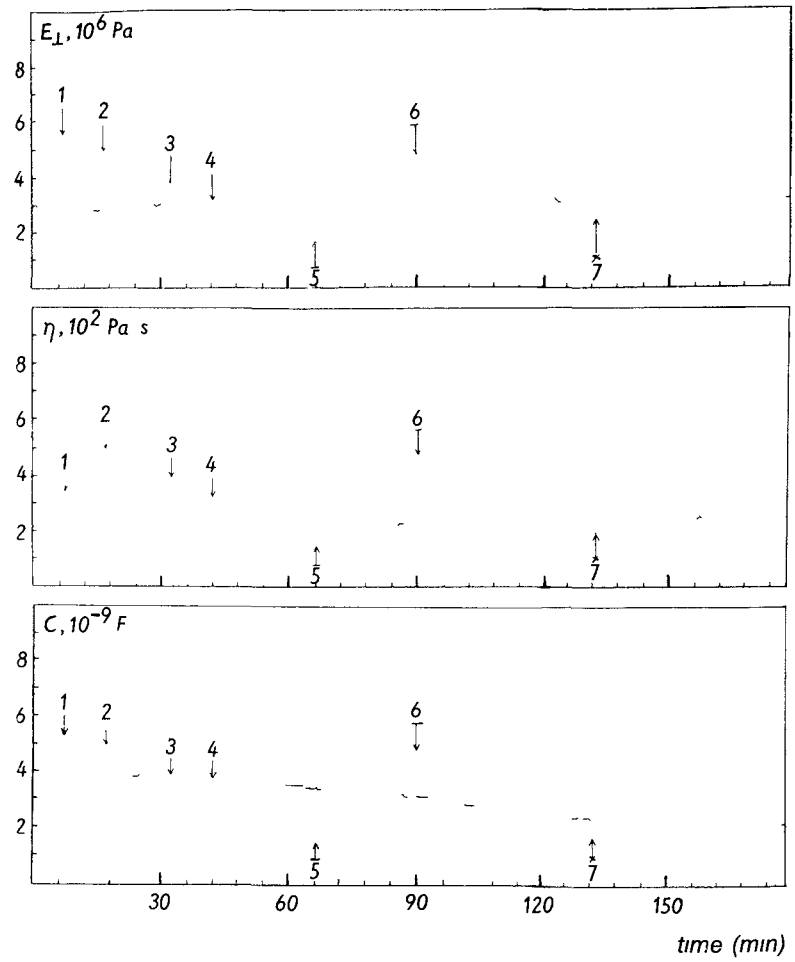

Figure 2. Kinetics of changes of parameters $E_\perp$ (a), η (b) and C (c) after addition of HP into the electrolyte· 1, 10^{-6}; 2, 3×10^{-6}; 3, 10^{-5}; 4, 3×10^{-5} mol/l. 5, laser on; 6, laser off; 7, electrolyte perfusion with 0.1 mol/l KCl without HP.

2. *HP – induced changes of parameters* $E_\perp, \eta, C$ and V_m

Figure 1 shows the kinetics of parameters $E_\perp, \eta, C$ of a nonmodified membrane from POPC in the dark and under illumination. The arrows indicate turning the laser on and off. It can be seen that all the parameters exhibit no time evolution and that they do not respond to the illumination.

The addition of HP into the electrolyte (30 min after the membrane forma-

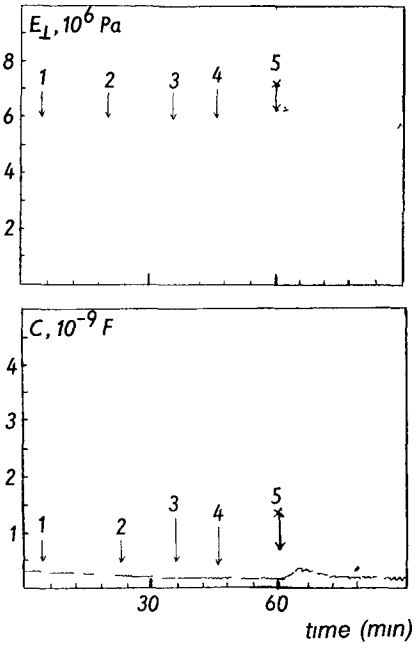

Figure 3. Kinetics of parameters $E_\perp$ (a) and C (b) after addition of HP into the electrolyte 1, 10^{-6}, 2, 3×10^{-6}, 3, 10^{-5}, 4, 3×10^{-6} mol/l in the dark 5, electrolyte perfusion with 0 1 mol/l KCl withouth HP

tion) induced decreases of $E_\perp$ and η by 22–31% compared to their initial values, and a slight decrease of parameter C (Fig 2) These changes might be due to a modification of the membrane structure by the HP entering the layer of the outer polar heads of the lipid molecules thus allowing the hydrophobic tails more space to move

Simultaneously, the absolute value of the membrane potential V_m decreased to 50 mV In this case, the membrane charges negatively at the side to which HP had been added, as in these experimental conditions HP molecule is in anionic or dianionic form It should be noted that the changes of parameters $E_\perp, \eta$ and V_m were mainly unidirectional, in a majority of cases the membrane capacitance did not change Occasionally, decreased or increased BLM capacity was observed, probably connected with membrane bulging due to osmotic pressure upon the addition of high HP concentrations However, membrane bulging has no effect on parameters $E_\perp$ and η (Passechnik and Hianik 1991)

Illumination of HP-modified membranes resulted in increases of $E_\perp$ and η by 41–66%, and in a further slight decrease of membrane capacity C (Fig 2) In absolute values, membrane potential V_m decreased by 30 mV No further changes of $E_\perp$ and η were observed after the laser was turned off In some instances (6 out of 11 membranes) disruption of membrane occurred 15–30 min after the laser had been turned off Shortly before the membrane disrupted, $E_\perp$ increased,

whereas capacitance C remained unchanged. The changes of $E_\perp$ and η after laser illumination were irreversible, as shown by electrolyte exchange. This suggests that, as a result of illumination, HP incorporated into the membrane, forming stable complexes with the lipid bilayer. Perfusion of HP-modified membranes in darkness resulted in returning of $E_\perp$ and η to the respective initial values for nonmodified membrane (without HP) (Fig. 3). To reach this, electrolyte perfusion had to be maintained during $\sim$ 10 min.

Similar results were obtained also with BLM from a mixture of POPC + choline plasmalogen.

To study the specificities of HP binding to membranes, the dependence of relative changes of membrane potential ($\Delta V_m = (V_m - V_{m0})/V_{m0}$, where V_{m0} is the potential of the nonmodified membrane) was plotted for different HP concentrations in the electrolyte using Scatchard coordinates ($\Delta V_m/c$ vs. ΔV_m). Examples of these dependences are shown in Fig. 4 for BLM from POPC (curve 1) and POPC + choline plasmalogen (curve 2). It can be seen that the dependences have different slopes. For the above relationship holds

$$\Delta V_m/c = c^{m-1}K(N - \Delta V_m) \tag{5},$$

where K is the binding constant, N is the number of binding sites on the membrane, $\Delta V_m/c$ represents the distribution coefficient of the concentrations of the free and bound agent, and m is Hill coefficient. In our case, as it follows from the above dependences (Fig. 4), $m = 1$. It seems that the process of HP binding with the membrane is non-cooperative, i.e. binding of one molecule does not depend on the binding of other molecules:

$$\Delta V_m/c = KN - K\Delta V_m \tag{6}$$

As it is seen from Eq. (6), the slopes of curves 1, 2 (Fig. 4) may be used to determine the binding constant K for both types of BLM: $K_{\mathrm{POPC}} = (9.97 \pm 2.83) \times 10^4$ l/mol (5 membranes) and $K_{\mathrm{POPC+CHPG}} = (4.79 \pm 1.16) \times 10^4$ l/mol (6 membranes). These values do not differ statistically significantly from each other (Student's test). Thus, the binding constant of HP does not differ in dependence on the lipid composition, and is comparable with those of hydrophobic fluorescent probes (Vladimirov and Dobretsov 1980). Once again, this result points to a high affinity of HP to lipids. Hence, the interactions of HP with BLM in the dark and under illumination result in changes of parameters $E_\perp, \eta, C$ and V_m. The decreases of $E_\perp$ and η following HP addition into the electrolyte suggest a decreased membrane ordering. Very probably, HP distorted the packing of the polar region of membrane lipids, allowing more space for the hydrocarbon chains of lipids to move. In contrast, $E_\perp$ and η increase under illumination. This could be due to the formation of clusters of HP and lipids. As a matter of fact, laser excitation

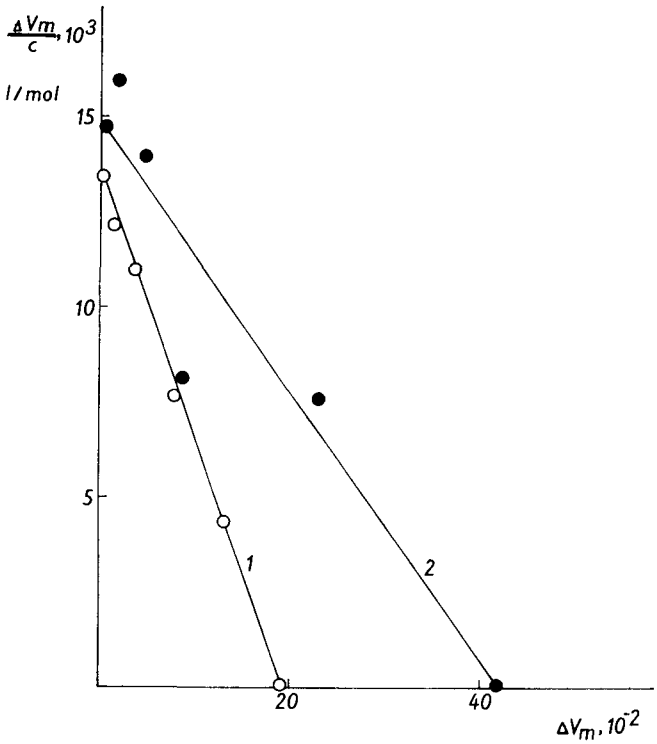

Figure 4. A typical example of the HP concentration dependence of relative changes of membrane potential, ΔV_m Scatchard plots for BLM from POPC (1) and POPC + choline plasmalogen (2)

and subsequent conversion to a metastable triplet state may be followed by the HP molecule losing an electron and forming an ionic bond with the negatively charged carboxyl group of the lipid polar head The result is condensation of the polar membrane area (that induces an increase of the ordering of the BLM hydrophobic area) In this case, there is a certain analogy with the condensation action of Ca^{2+} ions Interaction of Ca^{2+} with BLM also increase membrane $E_\perp$ (Hianik et al 1984c) HP fluorescence also suggests a stable bond to be formed between excited molecules of HP and lipids (the fluorescence spectra of HP contain two intensive bands at 615 and 675 nm) Chernyaeva et al (1988) showed HP fluorescence spectra to shift in the lipid environment from 615 nm and 675 nm to 630 and 690 nm respectively This suggests that HP forms complexes with lipids Thus, the action of light results in irreversible changes of parameters $E_\perp$ and η of BLM, and in membrane disruption Probably, membrane disruption is the result

of BLM inhomogeneities occurring at the sites of HP aggregates with lipids As a result of the condensation effect, the hydrocarbon solvent can be pushed out of the aggregates into the other parts of the lipid bilayer The thickness of the solvent-free parts of BLM will be smaller and these parts will be more exposed to the action of the electrostriction pressure $p = C_s V^2/2h$ Membrane instability results with subsequent disruption

The results obtained reflect but a single aspect of the HP – cell interaction, namely the action of HP on biomembrane lipid bilayer As discussed above, this does not result in any substantial changes of BLM conductance However, HP is well known to accumulate in cells (Chernyeva et al 1988, Henderson and Belnier 1989) Probably, HP penetrates into the cell through the plasma membrane due to inhomogeneities occurring at the protein/lipid interface However, as suggested by fluorescence studies, even in this case, HP binds to lipids (Chernyaeva et al 1988) Hence, the interaction of HP with lipids could be one mechanism underlying its cytotoxic action This might be due to the formation of clusters of HP and lipids laser excitation and subsequent conversion to a metastable triplet state can be fol lowed by the HP molecule losing an electron and forming an ionic bond with the negatively charged carboxyl group of the lipid polar head The result is conden sation of the polar membrane area, resulting in an increase of the ordering of the BLM hydrophobic area

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