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Interaction of the Antivirus Agents Remantadine and Amantadine with Lipid Membranes and the Influence on the Curvature of Human Red Cells

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Abstract. Surface potential difference, conductance, and elasticity changes of bilayer lipid membranes induced by the antivirus drugs amantadine and remantadine were measured. An influence on the human erythrocyte shape was shown. Both drugs are stomatocytogenic. The adsorption at the cytoplasmatic membrane was electrophoretically proved. The heat-induced vesiculation is partly inhibited. No microvesicles were observed. Instead, large tails which did not detach from the cell body were seen.
The general conclusion is that these amphiphilic adamantane derivatives are membrane agents which modify membrane interaction processes, possibly by influencing the bending properties.

Key words: Antivirus drugs — Membrane structure — Membrane properties — Erythrocyte — Vesiculation

Introduction

In recent years derivatives of adamantane, amantadine (1-aminoadamantane) and remantadine (α-methyl-1-adamantanemethylamine), have been widely used as antivirus chemotherapeutics (Tilley and Kramer 1981). These compounds efficiently inhibit the reproduction of a variety of enveloped lipid-containing viruses in

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infected cells, such as influenza type A, German measles, herpes, Rous sarcoma, and also some paramyxo-, arbo-, and adenoviruses (Davies et al. 1964; Cochran et al. 1965; Schulman 1968; Smorodintsev et al. 1970). In spite of the widespread clinical use of adamantane derivatives the molecular basis of their antivirus action is not yet clear.

It has been demonstrated that adamantane derivatives either inhibit the adsorption of enveloped viruses at cell membranes (Koff and Knight 1979) or other early stages of the virus cell interaction (Davies et al. 1964; Cochran et al. 1965; Schulman 1968; Smorodintsev et al. 1970; Koff and Knight 1979; Skehel et al. 1977). It has been suggested that amantadine and remantadine probably effect ribonucleoprotein deproteination, i.e. the separation of the M-protein from the viral ribonucleoprotein (Narmanbetova et al. 1982). Interestingly, with bacteriophage PM 2 it was found that adamantane derivatives inhibit virus assembly but not the early reproduction steps (Cupp et al. 1975). Similarly, it has been reported that amantadine is able to prevent blood platelet aggregation, possibly by blocking the release reaction (Colman et al. 1977).

The physico-chemical properties of the adamantane derivatives clearly imply that these compounds are typical membrane perturbers (Morse et al. 1982) as they consist of a hydrophilic positively charged aminogroup and a hydrophobic moiety. Indeed, it has been proved that they are incorporated into virus, artificial and cell membranes and significantly change the viscoelastic (Kharitonenkov et al. 1979) and electrostatic (Kharitonenkov 1982; Tverdislov et al. 1982) membrane properties.

In the present work we continued the study on the interaction of amantadine and remantadine with bilayer lipid membranes consisting of phosphatidylcholine and phosphatidylcholine-cholesterol. Taking into account that the above drugs can change the difference in the interfacial free energy between the two membrane interfaces we argued that they can influence the spontaneous curvature of biological membranes, which is an important parameter in membrane deformation, adhesion and fusion (Evans and Parsegian 1983). In our experiments effects of these drugs on red blood cell shape and vesiculation was studied. As a matter of fact, these mechanical membrane processes reflect to some extent related membrane properties which are essential for the process of virus penetration, self-assembly, and the virus exocytosis from the cell membrane.

We used human erythrocytes as a model system because its shape transformation represent a sensitive system for studying membrane curvature changes. By means of cell electrophoresis and ESR studies with fatty acid spin labels we investigated the adsorption of the two adamantane derivatives at the erythrocyte membrane. We studied the shape effect of these substances on erythrocytes at different electrochemical conditions. In addition, heat-induced vesiculation in the presence of amantadine and remantadine was also studied.

Materials and Methods

Interaction of amantadine and remantadine with bilayer lipid membranes (BLM): BLM were made either of egg yolk phosphatidylcholine (SIGMA) or a mixture of phosphatidylcholine with cholesterol (3: 1, w/w). Amantadine was purchased from SERVA and remantadine was synthesized in the Institute of Organic Chemistry, AN LSSR, Riga. The lipids were dissolved in heptane (SIGMA) in a concentration of 30 mg/ml. The membranes were formed on a teflon diaphragma with a hole (∅ 0.8 mm), separating two identical solutions (200 mmol/l KCl, 5 mmol/l Tris, pH 7.4, 20 °C). Tris was purchased from SERVA. KCl was recrystallized twice.

If alternating current is applied to the membrane, the nonlinearity of the system (caused by electrostriction in this case) results in appearence of multiple frequency current components. The ratio of the amplitudes of the second and third harmonics characterizes the surface potential difference ΔU, and the ratio of the first and third harmonics determines the transversal elasticity modul $E_{\perp}$ of the membrane. A detailed description can be found elsewhere (Passechnik 1981; Shimane et al. 1984).

The BLM area was calculated from the magnitude of membrane capacitance. In Fig. 1 each point represents the average of about seven different membranes measured.

Erythrocyte shape: Blood cans were obtained from the blood bank (Berlin—Lichtenberg). The blood was washed by three centrifugations ($500 \times g$, $2 \times 2000 \times g$, 5 and 10 min, respectively) in physiological saline (150 mmol/l NaCl, 5.8 mmol/l phosphate buffer, pH 6.8) to remove the buffy coat and the plasma. The erythrocytes obtained this way were used directly for the experiments, or two subsequent washings in 30 mmol/l NaCl-sucrose (300 mOsm) followed. The additionally washed cells were then treated with nystatin (50 μg/ml, 10% erythrocyte suspension) for 1 hour at 4 °C. Nystatin was then removed by washing in physiological saline at 37 °C (10 min, $2000 \times g$). In this way cells with a changed transmembrane potential difference were obtained (Glaser 1982).

The shape of the erythrocytes was determined by direct microscopic observation. A drop of the suspension was put onto a coverslip and the occurrence of different shapes was observed in an inverted microscope. The frequencies of three basic forms, echinocytes, normocytes, and stomatocytes were multiplied by the shape factor ($+4$ — stomatocytes, 0 — normocytes, and -4 — echinocytes) and the resulting number was divided by the total cell count. Differences between two independent experiments were usually in the order of 10—25%, especially at intermediate shapes. The subjective error from point to point in one experiment was much smaller. Throughout the experiment the cell suspension (cell concentration 0.5%) was continuously incubated with amantadine, or remantadine at 20 °C and 37 °C. Nontreated cells were in parallel to ensure that there was no time-dependent variation in the shape of untreated cells.

Erythrocyte vesiculation: The cells were preincubated with amantadine (26.6 mmol/l, cell concentration 5%) for several minutes at 40 °C. Then, the cells were heated up to 50 °C at a rate of 0.5 grd/s in a thin glass vessel. After 20 s the cell suspension was rapidly coolled down to 18 °C. Photographs were taken to study the vesiculation process (see Coakley and Deeley 1980).

ESR-measurements: The labeling was performed as described elsewhere (Herrmann et al. 1982). The remaining dissolved label I (10,3) [2-(3-carboxypropyl)-2-decyl-4, 4-dimethyl-3-oxazolidinyloxyl; Reanal, Budapest] and I (1,14) [2-(14-carboxytetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazolidinyloxyl; Syva, Palo Alto] were removed by repeated centrifugations. The cell suspension was then treated with amantadine (26.6 mmol/l, cell concentration 5%) for 1 hour at room temperature. After spinning down the cell suspension the sediment was used for ESR-measurements (Varian E-3 or ESR 231, ZWG). From the resulting spectra of the label I (10, 3) the order parameter was determined according to (Griffith and Jost 1976):

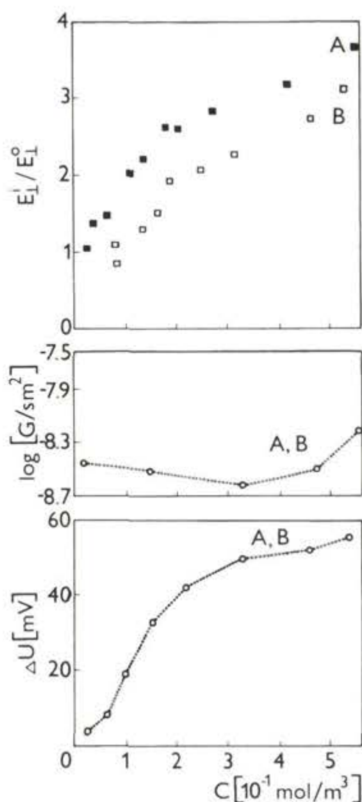

Fig. 1. Conductivity (G), surface potential difference (ΔU) and elasticity increase ($E^{i}_{\perp}/E^{0}_{\perp}$) of phosphatidylcholine (A) and phosphatidylcholine/cholesterol (B) membranes as functions of remantadine concentration. The curves for conductivity and surface potential are identical for both A and B. The maximum standard deviation in conductivity measurements was about 30%, in ΔU about 5 mV and in $E^{i}_{\perp}/E^{0}_{\perp}$ about 10%. 200 mmol/l KCl, 5 mmol/l Tris (pH 7.4, 20 °C).

$$S=\frac{(T_{\parallel}-T_{\perp})(T_{xx}+T_{yy}+T_{zz})}{[T_{zz}-(T_{xx}+T_{yy})/2](T_{\parallel}+2\bar{T}_{\perp})} \tag{1}$$

$$\bar{T}_{\perp}=T_{\perp}+1.32+1.86\log_{10}(1-S_0) \tag{2}$$

where $T_{\parallel}$ and $T_{\perp}$ are the outer and inner hyperfine splittings, respectively. The $\boldsymbol{T}$-tensors were taken from a paper by Seelig (1976). S_0 is calculated by Eq. 1 using $T_{\perp}$ instead of $\bar{T}_{\perp}$. According to Suda et al. (1980), an apparent correlation time was evaluated for I (1, 14) by adopting the equation of Butler and Smith (1978):

$$\tau=6.65\times10^{-10}\Delta H_0[(I_0/I_{+1})^{\frac{1}{2}}+(I_0/I_{-1})^{\frac{1}{2}}-2] \tag{3}$$

Table 1. Electrophoretic mobility (EPM) of human erythrocytes treated with amantadine and remantadine as a function of time. pH 7.4, 20 °C. EPM-values given in 10^{-8} m^2/Vs.

NaCl (mmol/l)	Time (min)	10	30	60
10	Control	1.80 ± 0.15	1.94 ± 0.10	1.81 ± 0.15
	5.32 mmol/l Amantadine	1.57 ± 0.15	1.71 ± 0.05	1.58 ± 0.15
	0.464 mmol/l Remantadine	1.66 ± 0.10	–	1.59 ± 0.15
150	Amantadine control	0.93 ± 0.05	0.98 ± 0.05	1.01 ± 0.05
	5.32 mmol/l Amantadine	0.945 ± 0.05	0.95 ± 0.05	0.99 ± 0.05
	0.464 mmol/l Remantadine	0.90 ± 0.05	–	0.91 ± 0.05
	Remantadine control	0.90 ± 0.05	–	0.91 ± 0.05

where I_{+1}, I_0 and I_{-1} are the peak-to-peak heights of the low, intermediate and high field line, respectively, and ΔH_0 is the width of the central resonance line in gauss.

Results

Interaction of amantadine and remantadine with BLM

Fig. 1 shows the effect of remantadine on the surface potential of BLM. Curves A refer to pure phosphatidylcholine and curves B to phosphatidylcholine-cholesterol membranes. The presence of cholesterol changes the original elasticity modul from 8×10^5 Pa to 7.3×10^7 Pa. In addition, the influence of different solvents on the elasticity change was investigated. Data obtained with decane and hexadecane have been very similar to those reported herein for heptane (El-Karadagi 1982). This similarity of the relative values with different solvents shows that elasticity changes with the increasing remantadine concentration were probably not caused by an artificial solvent flow to the menisc. An increase in the surface potential difference suggests an asymmetric adsorption of remantadine. At high concentrations a sudden increase in the conductivity was observed (not shown). This occurred at concentrations above 1.16 mmol/l and was accompanied by a sharp decrease in elasticity, suggesting that, at this saturating remantadine concentrations, the membrane itself lost its stability.

Electrophoretic mobility and shape effect of amantadine and remantadine. Table 1

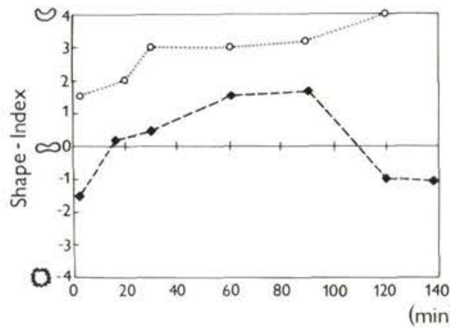

Fig. 2. Human erythrocyte shape change in the presence of remantadine as a function of time. Drug concentrations: Open symbols — 1.16 mmol/l, filled symbols — 0.464 mmol/l, physiological saline, 37 °C, pH 6.8. Typical experiments.

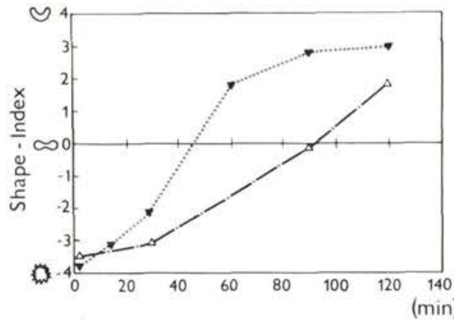

Fig. 3. Human erythrocyte shape change in the presence of amantadine as a function of time. Drug concentrations: Open symbols — 2.66 mmol/l, filled symbols — 5.32 mmol/l, physiological saline, 37 °C, pH 6.8. Typical experiments.

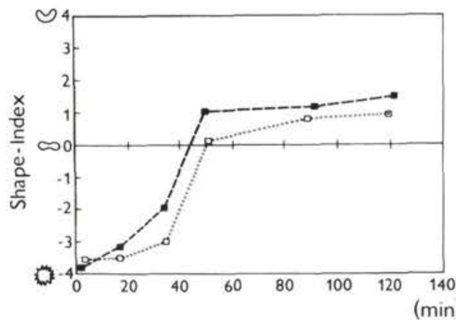

Fig. 4. Human erythrocyte shape change in the presence of remantadine as a function of time. Drug concentrations: Open symbols — 0.464 mmol/l, filled symbols — 1.16 mmol/l, 30 mmol/l NaCl, sucrose, pH 6.8, 37 °C, nystatin pretreated. Typical experiments.

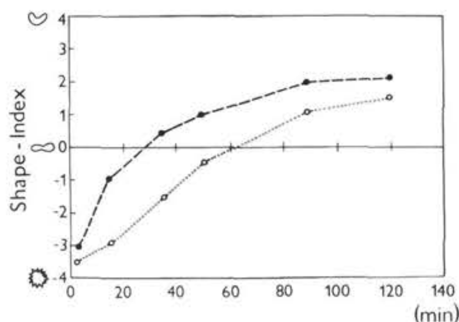

Fig. 5. Human erythrocyte shape change in the presence of amantadine as a function of time. Drug concentrations: Open symbols — 2.66 mmol/l, filled symbols — 5.32 mmol/l, 30 mmol/l NaCl, sucrose, pH 6.8, 37 °C, nystatin pretreated. Typical experiments.

shows the electrophoretic mobility of amantadine-treated red cells compared with the mobility of control cells at two different electrolyte concentrations as a function of time. Only at low ionic strengths, amantadine reduced the electrophoretic mobility significantly, whereas at physiological ionic strengths the mobility of treated and untreated cells was similar. Similar results were obtained with remantadine.

Figs. 2 and 3 show the influence of remantadine and amantadine on the shape of human erythrocytes at 37 °C in physiological saline.

Figs. 4 and 5 compare the shape effect of the drugs on nystatin-pretreated cells. The general conclusion is that in all cases both drugs had a stomatocytogenic effect. Remantadine was efficient at lower concentrations in comparison with amantadine. The shape transformation did not depend on temprature. At 20 °C the same transition was observed, proceeding however more slowly. The efficient drug concentration found coincided with the respective saturation range observed in the BLM studies.

Erythrocyte vesiculation. In contrast to control cells amantadine-treated cells were characterized by the formation of long tails (Fig. 6) which rarely detached from the membrane. Only few large vesicles were observed. The number of microvesicles was drastically reduced. Sometimes it seemed as if the tails were consisting of preformed vesicles where the last stage of pinching off had been inhibited (see the vesicle in dark field, Fig. 6*a*).

ESR-measurements. The apparent correlation time of I (1, 14) and the order parameter of the I (10, 3) spin label have been evaluated at different temperatures. The absolute values and the temperature dependence of both parameters as well as

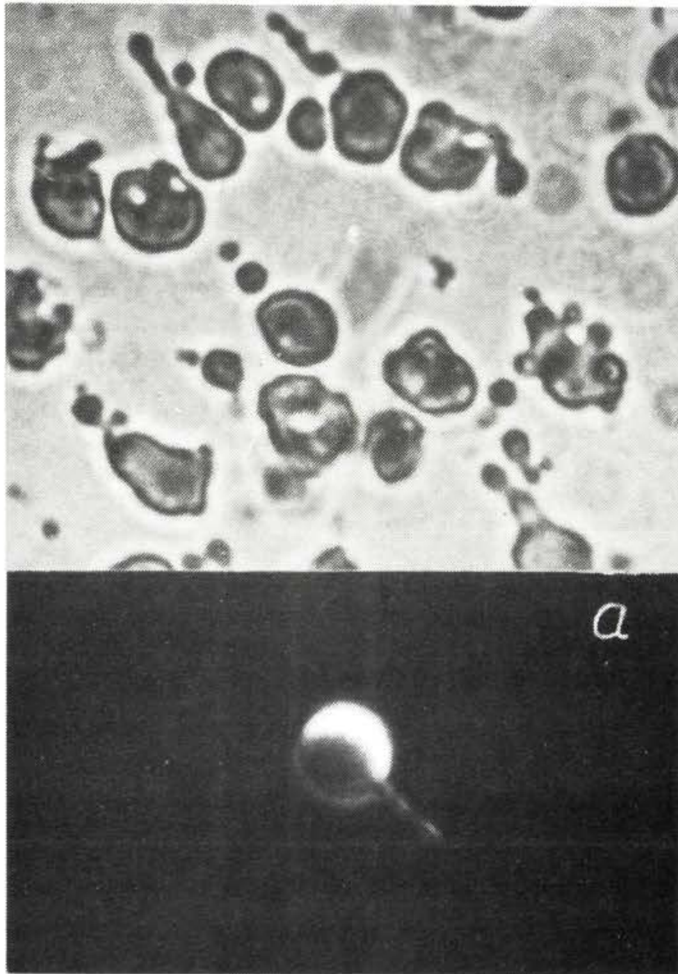

Fig. 6. Light microscopic observations of tail formation of amantadine-treated human erythrocytes heated for 20 s at 50 °C. *A*: Observation in dark field shows the spatial nature of the vesicle chains.

the the shape of the membrane spectra of untreated human erythrocytes were in agreement with those reported previously (Suda et al. 1980; Herrmann et al. 1982). The correlation time for amantadine-treated erythrocytes was shorter by about 10% as compared with control values. The order parameter was only slightly smaller (Fig. 7). However, the differences observed were significant (see legend to Fig. 7) at all temperatures used. A marked difference in the *I* (1, 14) spectra is shown in Fig. 8. Here the altered high field line (marked by arrows) indicates a structural rearrangement due to the presence of amantadine.

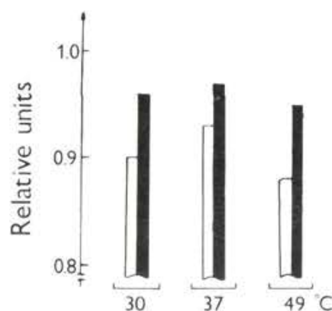

Fig. 7. Relative changes of the order parameter (S_A/S_E, open columns) and the apparent correlation time (τ_A/τ_E, filled columns) of the membrane spectra of (10,3) and I (1,14), respectively, incorporated into the human erythrocyte membrane. S_A, τ_A — amantadine-treated human erythrocytes (see Materials and Methods). S_E, τ_E — untreated human erythrocytes. The relative error of measurements was about 2%. Each value represents the mean of at least 3 independent measurements. Using t-test statistics for paired values, differences between the values of untreated and amantadine-treated cells were significant at $\alpha = 0.01$. The obtained values of the order parameter and the apparent correlation time were 0.7 and 2×10^{-9} s, respectively, which is in agreement with previously published results (Suda et al. 1980; Herrmann et al. 1982).

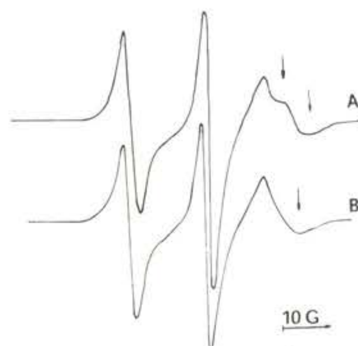

Fig. 8. Membrane spectra of I (1, 14) incorporated into human erythrocytes. A — control cells, B — amantadine-treated cells. 37 °C, arrows indicate differences in the high field line.

It should be pointed out that the apparent correlation time (Eq. 3) is a semiempirical way to characterize membrane fluidity. The formalism used is only valid in the range from 10^{-9} s to 10^{-11} s and on the condition that the sample studied is homogenous and isotropic. This is certainly not the case in the human erythrocyte membrane, as indicated by the composite shape of the I (1, 14) spectra. There are at least two or three different spin label populations with different motion properties. We have tried to simulate the spectra, using the formalism of Israelachvili et al. (1975). We distinguished different phospholipid

environments by various wobble angles holding the rotation frequency constant. However, the results were not satisfying. Therefore we assume that besides spatial effects also dynamic factors have to be taken into account.

Discussion

The results presented herein have shown that both derivatives, amantadine and remantadine, are efficient membrane agents. They affect both artificial and biological membranes. Adsorption of both drugs to BLM causes the appearance of potential differences between both sides of the membrane and results in an increase of the transverse elasticity modul. The change in conductivity was observed only after adsorption saturation had been achieved. Previously it was shown that addition of remantadine in concentrations more than 0.46 mmol/l to asolectin BLM and 0.15 mmol/l to BLM from oxidized cholesterol causes a significant increase in conductivity. These data suggest that the lipid composition of the BLM is important in the process of interaction of the drugs with membranes.

Let us now estimate the number of molecules adsorbed to the membrane. At physiological pH they carry a positive unit charge ($pK_a \sim 9.5$). If the dissociation constant does not change with the adsorption and the Guy-Chapman approach is valid (Aityan and Belaya 1980) the charge density of the BLM surface may be estimated. At a remantadine concentration of about 0.3 mmol/l using the experimental value of $\Delta U = 50$ mV we obtain a value of 4×10^{-2} C/m^2 for the charge density. This corresponds to an average distance between two amantadine or remantadine molecules of about 2 nm at saturation conditions. Remantadine, in contrast to amantadine, has two additional methyl groups. This explains the higher affinity of remantadine to BLM membranes (cf. El-Karadagi 1982).

Electrophoretic experiments with human erythrocytes have conclusively proved that both drugs also adsorb to the plasma membrane itself. If there also were an adsorption within the glycocalyx at physiological salt concentrations, the electrophoretic mobility should decrease. However, only a relatively small decrease of the electrophoretic mobility was observed in our experiments at 10 mmol/l NaCl. This difference can be understood, taking into account the 5—10 nm thick polyelectrolyte layer. This layer completely screens any change in the surface charge at the adjacent membrane surface, if the Debye-Hückel length is considerably smaller than the layer thickness. In contrast, in a 10 mmol/l NaCl solution the screening distance is about 3 nm, and thus comparable to the layer thickness. These considerations can also be made quantitatively to estimate the change in the charge density at the membrane surface (Donath and Pastushenko 1980). If we neglect the interaction of the negatively charged glycocalyx molecules with the positive amantadine molecules incorporated into the membrane we get a charge density of about 3.5—4×10^{-3} C/m^2. This is by an order of magnitude smaller than the value

obtained with artificial membranes. However, this difference should not lead to confusion, because these two ways of determining surface charge densities usually result in different charge densities. The reason is that the method of determining the electric properties by using AC-currents senses the potential exactly in the plane that separates the hydrophobic part of the membrane from the head group region, whereas the electrokinetic methods sense electric potentials at the solution side of the membrane head groups.

The results of the shape experiments performed show that both compounds cause stomatocyte transformation upon prolonged incubation independent of the transmembrane potential difference. Again, remantadine is more efficient than amantadine. A stomatocytogenic effect is usually observed after the addition of cationic substances (Deuticke 1968). Another important point is that increased temperature enhances the rate of the shape transition. These findings suggest that the structure of the membrane is changed due to the incorporation of amantadine or remantadine molecules. The shape of red cells is controlled by a large variety of parameters. Up to now there has been no overall theory explaining the red cell shape. One of the most popular concepts is the bilayer couple model (Sheetz and Singer 1974). In its original version it explains the overall convex or concave shape as a result of the difference in the surface area between the two membrane leaflets. However, how this possible area difference originates remains unclear. There are several possibilities, such as contraction of the cytoskeleton, intercalation of substances, electrostatic effects due to repulsion of charges in the membrane plane, conformational changes of molecules. Another line of red cell shape theories stresses the bending properties of the membrane (Deuling and Helfrich 1976). Within this concept lateral diffusion is also taken into account. It may create areas of different bending resistance (Markin and Glaser 1980; Markin 1981). At the moment we cannot explain the shape effect of these drugs in a simple manner. For example, considering the bilayer couple model we should expect an incorporation in the inner membrane leaflet, thus leading to an overall expansion of the inner membrane surface and consequently to the formation of stomatocytes. An alternative explanation is that the outside positive charge decreases the stress due to the reduction of the glycocalyx repulsion. The last point is that the conic shape of the molecules itself can create a spontaneous bending of the membrane. In some experiments it was observed that after long incubation the shape change was reversible. This could be explained by a transbilayer redistribution process.

The long (about 1 hour) characteristic time of the shape change may be related to protein diffusion in the cell membrane. It is quite unreasonable that the adsorption itself is so slow. Indeed, electrophoretic experiments show that the surface charge does not change within this period. Also, vesiculation is affected by amantadine after much shorter time. So we would favour a mechanism of the adamantane derivative action on the red cell shape transition which includes lateral

diffusion of membrane components (or transbilayer redistribution) as these characteristic times are just of the same order as the shape transition observed (Sheetz et al. 1980; Haest et al. 1978). This idea concerning the influence of adamantane derivatives on lateral membrane properties has also been confirmed by BLM-experiments. Here, adsorption isotherms of non-Langmuir nature were observed (El-Karadagi 1982).

Using a saturation function of the type

$$Y = \frac{K\, x^n}{1 + K\, x^n} \tag{4}$$

where K is the association constant and n the cooperativity coefficient, the value of n was calculated from the saturation behaviour to be approximately 1.33 (El-Karadagi 1982). This indicates an interaction of the substances incorporated. Another point is the conic shape of adamantane derivatives. It should be expected that these molecules prefer the inner leaflet of a convex or the outer leaflet of a concave membrane area. It seems reasonable to explain the inhibited vesiculation and the tail formation in this way. Indeed, if concave membrane areas appear it should be expected that amantadine and remantadine would accumulate there, thus minimizing the bending energy. On the other hand, the accumulation of these molecules then dictates the curvature of this particular area and it may well be that a further increase in the negative curvature is not favoured.

The ESR-results suggest a disturbance in the membrane structure following to the addition of amantadine. Membranes of amantadine-treated human erythrocytes seem to be altered towards a higher disturbed structure resulting in an enhanced membrane fluidity. This is consistent with findings obtained with phospholipid bilayers (Colman et al. 1977) and other biological membranes (Cupp et al. 1975). These results could be explained by a spacing effect of amantadine, the latter increasing the distance between lipid molecules. This enhances the angular amplitude of motion of the flexible CH_2 chains of phospholipids as well as of spin labeled fatty acids. Due to the opposite charge of amantadine and spin labeled fatty acids a preferential interaction between both could be expected.

Summarizing these data we suggest that the antivirus effect of adamantane derivatives can be explained by altered membrane properties induced by amantadine and remantadine incorporation. It is possible that curvature changes can play a significant role as an alternative mechanism to previously suggested pH-effects (Ohkuma and Poole 1978). But also, the lipid-protein interaction may be affected because the neighbourhood of the membrane proteins is probably characterized by high local curvatures due to surface tension effects (Israelachvili 1977).

At the present we are not able to answer in detail the question concerning the antivirus effect of amantadine and remantadine, as the particular steps of the virus

infection are not very well characterized with respect to the biophysical phenomena involved. It is certain that several membrane processes are of importance. Maybe these substances do not affect one particular step but modify some essential steps of the virus infection cycle (membrane fusion, uncoating and self-assembly). That these substances are not simply specific virus protein affecting agents is easily proved when considering the large body of data showing the therapeutic effect on several nervous disorders (Tilley 1981). Not surprisingly, most of these disorders are also caused by changes in neurotransmitter release involving complex membrane interaction processes as well.

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