Biochimica et Biophysica Acta 812 (1985) 277–285
Elsevier

BBA 72379

Spectroscopic characterization of vesicle formation on heated human erythrocytes and the influence of the antiviral agent amantadine

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(Received January 2nd, 1984)
(Revised manuscript received September 21st, 1984)

Key words: Vesicle formation; Heat treatment; Membrane composition; Amantadine; ESR; (Human erythrocyte)

EPR investigations on the vesiculation process of heated human erythrocytes were performed, using different fatty acid spin labels. Spectrin denaturation and vesiculation do not influence the fluidity of the lipid phase of the remaining membrane of human erythrocytes: Vesicles released differ in chemical composition as well as in the lipid fluidity of their membrane from the intact human erythrocyte membrane. A reduced cholesterol-to-phospholipid ratio and a depletion of spectrin was found. By changing the ionic concentration of the suspension medium an effect on membrane spectra and on vesicle release was established. The adamantane derivative amantadine causes fluidization of the human erythrocyte membrane and inhibits vesicle release. Based on these results, a model for the mechanism by which adamantane-like molecules could interact with membranes is proposed.

Introduction

The process of vesicle formation and the structure and properties of vesicle membranes are of growing interest [1,2].

1. Vesicle formation is a wide-spread event in natural systems. The triggering of vesicle release could be of therapeutical interest.

2. Vesicle formation represents a model system for investigating membrane fusion processes [3]. Vesicles are also involved in the fusion of myoblasts during embryonic skeletal-muscle development [4].

3. Because of their often simple and well characterized chemical composition vesicle membranes are used as a model for understanding membrane structure and properties, e.g. protein-protein interactions and transport activities [1].

Erythrocytes are a useful object for studying vesiculation processes. There are several ways to induce vesiculation of the human erythrocyte: mechanical or chemical stress [5], heating [2], incubation in the presence of lipid vesicles [6]. A scientific as well as practical problem is the formation of vesicles during blood preservation [7].

Coakley and his co-workers developed a macroscopic, hydrodynamic model of vesiculation of human erythrocytes by heating them up to about 50°C [2,8,9]. The wave formation observed on the cell rim is explained by assuming a membrane weakness due to spectrin denaturation followed by growing surface instabilities which can result in the formation of vesicles. The wave pattern and vesicle release were dependent on ionic concentration which could be explained in the framework of their model by an altered surface potential [9].

In the present paper we have started to characterize the formation of vesicles by heating human erythrocytes. The erythrocyte membrane as well as the vesicle membrane itself was studied on the molecular level by EPR using different fatty acid spin labels.

There are some indications that, besides a membrane weakness due to spectrin denaturation [9], alterations of the lipid phase structure could be involved in vesiculation. The lateral organisation of biological membranes is influenced by several factors, e.g. temperature [10], cholesterol [11], Ca^{2+} [12], specific protein-lipid interactions [13]. Especially in the case of microvesicles the geometrical shape of lipids has to be taken into account [14]. In highly bent regions of cell membranes the accumulation of membrane proteins was observed [15]. Als an altered transversal distribution of phospholipids in erythrocyte vesicles was established [16].

Adamantane derivatives are effective membrane perturbers of artificial lipid membranes as well as of biological membranes [17–19] probably due to their geometric shape. The effect on biological processes are well known. For instance, amantadine prevents the assembly of viruses which surround themselves with lipids of the host cell membrane by leaving the cell [18,19]. Also the blood platelet aggregation is inhibited in the presence of amantadine [19]. In both cases there is some experimental evidence that membrane perturbations are responsible for the effects observed. The mechanism by which adamantane-like molecules interact with membranes is quite unclear. We suppose that the observed inhibition of vesicles release of human erythrocytes in the presence of amantadine could serve as a model for understanding this mechanism.

Materials and Methods

Vesicle formation was investigated using the following isotonic NaCl-saccharose solutions (pH 7.4, phosphate buffer 5.8 mM) in accordance with Coakley et al. [9]: solution H, 154 mM NaCl; solution L, 8 mM NaCl.

Erythrocyte preparation. Human erythrocytes of several donors were used (Blood-bank Berlin-Lichtenberg). All experiments were performed not later than 48 h after blood sampling (ACD-storage medium, 4°C). After centrifugation at $500 \times g$ the erythrocytes were washed three times with 5 volumes of solution H at $2000 \times g$. Incubation in suspension L took 1 h at 21°C (hematocrit 10%).

Amantadine treatment of erythrocytes. The cells were incubated with 5 mg/ml amantadine (suspension medium H) at a hematocrit of 5% for 1 h at room temperature. In the case of EPR measurements amantadine treatment was performed after labeling.

Microscopic observations of vesiculation. To observe wave pattern and vesicle formation of heated human erythrocytes an equipment according to Coakley and co-workers [8,9] was built up. Photographic registrations were performed and evaluated.

Vesicle enrichment. The erythrocyte suspension was adjusted to a hematocrit of 10% and, after preincubation at 42°C (5 min), the cells were incubated at 52°C for 1 h, using glasses with a thin vessel.

As pointed out by Crum et al. [8] a streaming stress on heated erythrocytes could induce the formation of beaded tethers. Therefore, in some cases after 120 s of incubation at 52°C glutaraldehyde was added to a final concentration of 5%. Light microscopic investigations yielded no evidence of the formation of tethers under our conditions.

After removal of heated erythrocytes the vesicles were enriched by stepwise centrifugation at 4°C, using a K 24 centrifuge (Janetzki, rotor 6×26): (1) 20 min at 5000 rev./min to remove larger fragments of erythrocytes and ghosts; (2) The supernatant was centrifuged at 16000 rev./min for 90 min to enrich vesicles.

Protein analysis and determination of cholesterol-to-phospholipid ratio (C/P ratio). The protein analysis of vesicle membranes was performed by polyacrylamide gel electrophoresis (5% acrylamide, pH 7.2) according to the procedure of Weber and Osborn [20]. Before starting electrophoresis the samples were incubated at 100 mM dithiothreitol (20 min, 37°C). Lower concentrations of dithiothreitol were not sufficient to reduce higher molecular weight complexes of vesicle membranes. Erythrocyte ghosts were prepared as a reference according to the procedure of Bodemann and Pas-

sow [21]. After extraction [22], cholesterol and organic phosphate were determined by the procedures of Zlatkis and Zak [23] and Bartlett [24], respectively. To avoid the possibility that chemical modifications of cholesterol Δ-5 bonding took place during heating, the C/P ratio of the whole heated suspension containing erythrocytes, ghosts and vesicles was determined. The result (C/P = 0.99) corresponds to the C/P ratio of human erythrocytes reported [11].

Spin labeling of erythrocytes and vesicles. Spin labeling of erythrocytes and vesicles. The spin labels I(10,3) (2-(3-carboxypropyl)-2-decyl-4,4-dimethyl-3-oxazolidinyloxyl; Reanal, Budapest), I(1,14) (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl; Syva, Palo Alto) and II (1-oxyl-2,2,6,6-tetramethyl-4-dimethylaminopiperidine-cetylbromide; Bulgarian Academy of Sciences) were used (Fig. 1). Spin labeling was performed as described previously [25].

Electron paramagnetic resonance measurements. EPR spectra were recorded on a Varian E-3 and an ESR 231 spectrometer (Center of Scientific Instruments, Academy of Sciences of G.D.R.) with a variable temperature equipment. Flat quartz cells for aqueous solutions were used. The temperature was measured by a small thermistor inserted into the sample cell.

The order parameter S of the membrane spectrum of I(10,3) was determined according to Griffith and Jost [26]. According to Suda et al. [11] an apparent correlation time τ was calculated for I(1,14) by adopting the equation of Butler and Smith [27].

$$\tau = 6.55 \cdot 10^{-10} \cdot \Delta H_0 \cdot \left((I_0/I_{+1})^{1/2} + (I_0/I_{-1})^{1/2} - 2 \right) \quad (1)$$

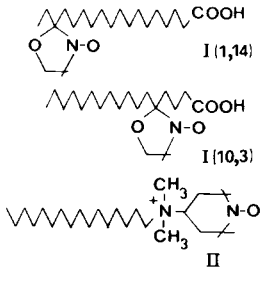


Fig. 1. Spin labels used (cf. Material and Methods).

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

Results

Morphological characterization of vesicle formation

Light microscopic observations (not presented) of vesicle release by heating human erythrocytes up to 50°C agree with the findings of Coakley and co-workers [9]. Using suspension medium H (154 mM NaCl), the average of waves per cell was about 8 (extreme values were 3 and 15 waves per cell), which corresponds to the results reported [9]. Only a short delay of vesicle formation (normally about 3 s) after reaching 50°C was established. By lowering ionic concentration (solution L, 8 mM NaCl), a decreased number of waves per cell (cf. Ref. 9) and an inhibition of vesicle release during the first 20 s after reaching 50°C was found. However, longer incubation periods resulted also in the formation and release of vesicles. A higher yield of vesicles was achieved by incubating the erythrocytes at 52°C independently of the suspension medium used.

As shown by electron microscopy most of the vesicles are spherical (Fig. 2). No differences between the size of vesicles produced at 154 mM NaCl (solution H) or 8 mM NaCl (solution L) were observed. The diameter is about 240 nm: (238 ± 35) nm for solution H and (233 ± 58) nm, using solution L (mean ± S.D.). In both cases 150 vesicles were measured.

Chemical composition of vesicle membranes

The vesicles are depleted mainly of spectrin as shown by polyacrylamide gel electrophoresis (Fig. 3) whereas band 3 protein is not excluded from the vesicle membranes. High molecular weight complexes of proteins which do not pass the gel occur in vesicle membranes (cf. Materials and Methods). A decreased C/P ratio was found in comparison to the intact human erythrocyte membrane (C/P = 0.99, cf. also Materials and Methods). The C/P ratio of vesicles released in solution H (154 mM NaCl) was 1 : 4.1. The ratio obtained by using the solution L (8 mM NaCl) was 1 : 5.6 (number of experiments = 6).

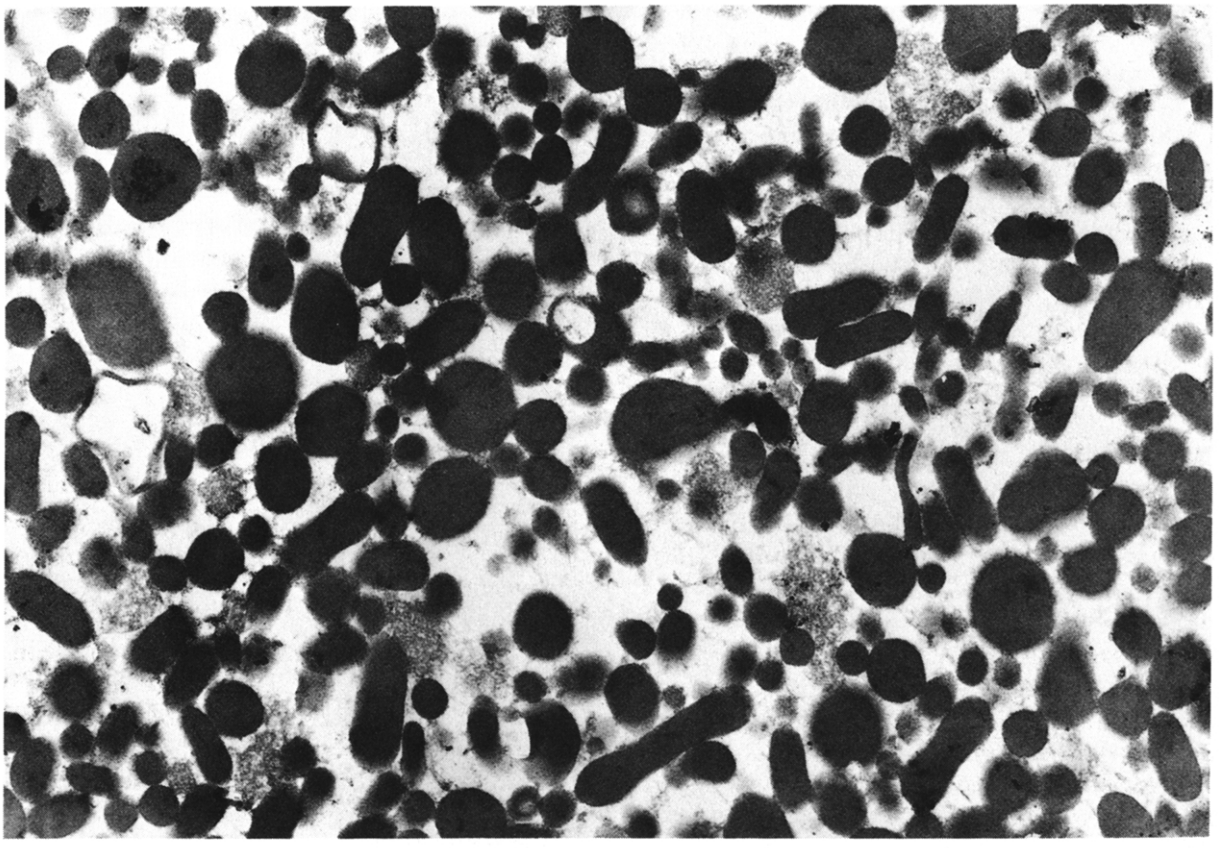


Fig. 2. Electron micrograph of vesicles released by heated human erythrocytes (suspension medium L, 8 mM NaCl). Vesicles were prefixed with 2.5% glutaraldehyde, 1% formaldehyde in 0.1 M cacodylate buffer (pH 7.4). To replace the fixative the samples were centrifuged at each step until the membranes were embedded in 3% gelatine. The samples were then rinsed with the buffer and postfixed with 1% OsO_4 in the same buffer. After being rinsed in buffer the fixed membranes were embedded in 1% agar stained en bloc with 0.5% aqueous uranyl acetate, dehydrated in graded concentrations of ethanol and embedded in Vestopal. Magnification: 30000×.

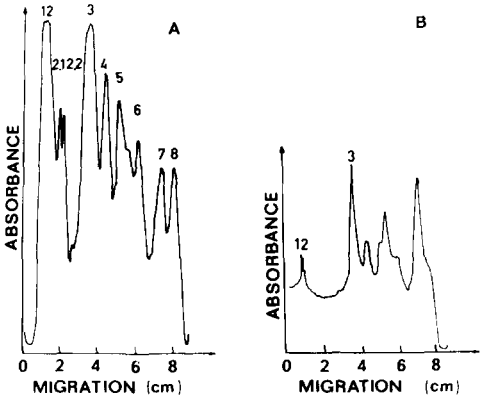
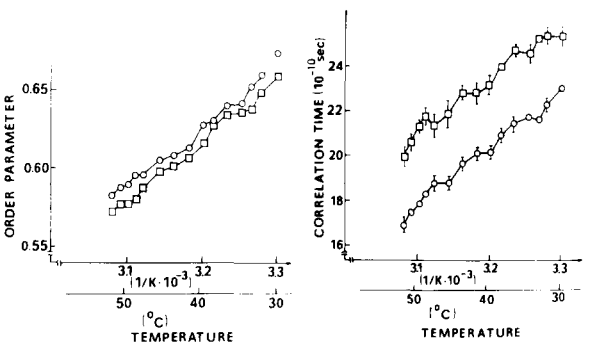


Fig. 3. Densitometric scan at 550 nm of polyacrylamide gels of membrane proteins (A, ghosts; B, vesicles). The nomenclature of Fairbanks et al. [28] was used.

Characterization of erythrocyte membrane during vesiculation by EPR

In Figs. 4 and 5 the temperature dependence of the order parameter of I(10,3) and of the apparent correlation time τ of I(1,14) of erythrocyte membrane spectra are shown. The order parameter of I(10,3) depends linearly on temperature, also in the temperature range of the vesiculation process. This seems to be also the case for the temperature dependence of τ of I(1,14). Only slight discontinuities of the correlation time were observed at about 32°C and 48°C. However, no significant change of the slope of τ by changing the temperature was detected suggesting that no drastic alterations of the lipid phase fluidity occur. (After EPR measurements the samples were checked on vesicle



Figs. 4 and 5. Order parameter of I(10,3) (Fig. 4, left-hand) and apparent correlation time of I(1,14) (Fig. 5, right-hand) of erythrocyte membrane spectra as a function of temperature at different NaCl concentrations of the suspension medium (●———●, 154 mM NaCl and □———□, 8 mM NaCl). The standard error of estimate is presented. In the case of the order parameter, the error is in the order of symbols. Each point represents at least four independent measurements.

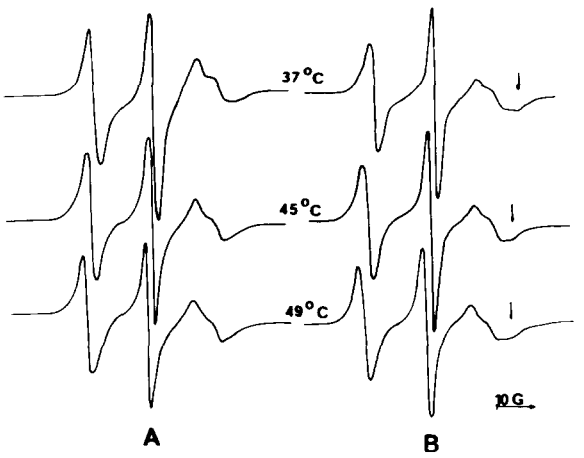


Fig. 6. Spectra of I(1,14) incorporated into the erythrocyte membrane at different temperatures and ionic concentrations of the suspension medium (A, 154 mM and B, 8 mM NaCl). A more immobilized component is observed at lower ionic concentration (arrow).

release by light microscopy. No differences to the observations mentioned above were established.) After heating, the samples were cooled down to different temperatures (35°C and 30°C) and the spectra were recorded again (not shown). Apart from a reduced intensity (see below) no significant differences of parameters used were observed in comparison to spectra recorded before heating the sample.

In the case of I(1,14) differences of the correlation time between both suspension media (H, 154 mM and L, 8 mM NaCl) are obvious (cf. Fig. 5). At lower ionic concentration τ is increased, suggesting an enhanced restriction of label motion. In view of the difficulties of calculating correlation times from the spectra of anisotropic motion in ordered systems we evaluated the differences $\Delta\tau$ of two correlation times as a qualitative measure of lipid order as proposed by Schreier et al. [29]. In all cases conclusions drawn from τ (Eqn. 1) are similar to those drawn from $\Delta\tau$. Also the composite shape of membrane spectra of I(1,14) reflects a dependence on ionic concentration (Fig. 6), especially the high-field line. At low ionic concentration are more immobilized component can be seen (arrow in Fig. 6). At temperatures lower than 37°C the differences disappear in contrast with the correlation time calculated.

The differences of the order parameter of I(10,3) membrane spectra measured at various ionic concentrations are much smaller and the fluidity seems to be enhanced slightly by lowering the NaCl concentration. The shape of the spectra of I(10,3) were independent of the suspension medium. The differences between the parameters calculated and the shape of the spectra of I(1,14) are not caused by an increased saccharose concentration in solution L (8 mM NaCl), for instance any viscosity effect. This was checked by using spin label II sensitive to alterations immediately on the membrane surface [30]. No influence on spectra (not shown) were established at different suspension media. A further insight into membrane behaviour during vesiculation is given by observing the linewidth ΔH_0 of the central resonance line of the spectrum of I(1,14). Between 30°C and 45°C ΔH_0 decreases by about 10% by enhancing the temperature. At temperatures higher than 48°C a rise of the linewidth was observed (ΔH_0(52°C) : ΔH_0(30°C) = 1.1). By cooling the sample the effect of linewidth change is reversible, suggesting that the increase of the linewidth at higher temperature is due to spin-spin interactions in agreement with results of Yamaguchi et al. [31]. These effects are independent on the suspension medium used.

Characterization of vesicle membranes by EPR

Although no drastic alterations of the lipid

phase fluidity of the erythrocyte membrane were caused by vesiculation the membrane spectra of vesicles released are clearly distinct from those measured on intact human erythrocyte membranes. The order parameter of I(10,3) spectra, the apparent correlation time and the shape of the high field line of I(1,14) spectra (Fig. 7) indicate an altered structure and/or dynamic of vesicle membranes. Vesicle membrane spectra were recorded at different temperatures (21, 30 and 37°C). The order parameter and the correlation time of these spectra are lowered by about 5% and 15%, respectively, in comparison to the values calculated from membrane spectra of intact human erythrocytes. Using the *t*-test statistics for in pair arranged measured values the difference between the values of erythrocyte and vesicle membranes are significant ($\alpha = 0.01$, three or four independent measurements). Differences between membrane spectra of vesicles produced under various conditions (solution H and L) were not detected.

Influence of amantadine on vesiculation and membrane structure

The formation of heat-induced microvesicles is inhibited by the treatment of erythrocytes with amantadine. Only a few large vesicles are released. The heating process in the presence of amantadine is characterized by the formation of long tails which do not detach from the cell membrane (Fig. 8). The EPR measurements performed on amantadine-treated erythrocytes at different temperatures (30, 37 and 49°C) indicate a membrane altered towards a higher disturbed structure, resulting in an enhanced membrane fluidity (Fig. 7). The apparent correlation time and the order parameter are decreased by about 10% and 5%, respectively, in comparison to the values measured on control cells. Using the *t*-test mentioned above the differences between the values of untreated and amantadine-treated cells are significant ($\alpha = 0.01$, three independent measurements). Also the high-field line shape of I(1,14) suggests a membrane disturbance due to adding amantadine. A more immobilized component can be seen in the case of untreated cells (Fig. 7).

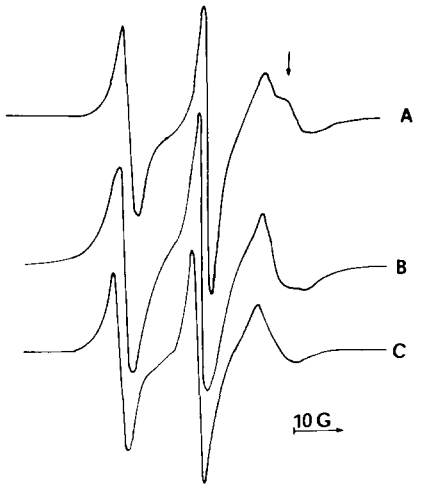


Fig. 7. Membrane spectra of I(1,14) incorporated into (A) erythrocyte membranes, (B) vesicle membranes and (C) amantadine-treated erythrocyte membranes (37°C, 154 mM NaCl).

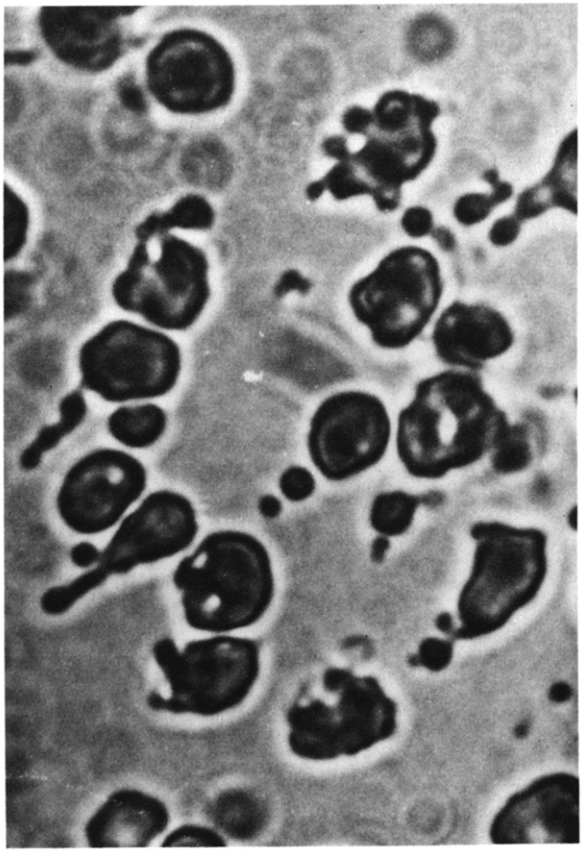


Fig. 8. Light microscopic observations of tail formation of amantadine-treated human erythrocytes heated for 20 s at 50°C. Magnification: 1500×.

Discussion

In the present paper alterations of membrane structure during the formation of waves and vesicles by heating human erythrocytes were investigated by means of EPR. Our experimental conditions are comparable to those of Coakley and co-workers which was confirmed by light microscopic observations. Considering the age and the storage conditions of blood samples used vesicle formation induced by ATP or glutathione depletion can be excluded.

It is obvious from the temperature dependence of the order parameter of I(10,3) and the apparent correlation time of I(1,14) that spectrin denaturation [30] and vesiculation at 49°C does not result in dramatic alterations of the lipid phase fluidity of the erythrocyte membrane. Taking into account that the spectra present an average signal of the labeled lipid phase it can be concluded that spectrin denaturation does not influence the fluidity at least of the whole erythrocyte membrane. This is supported by the identity of spectra measured before and after heating the sample of about 50°C. If at all, there seems to be only a minor association between spectrin and lipid motion in the bilayer which is in agreement with recent reports [33–35]. These results corroborate also the hypothesis [36] that the reduced membrane deformability of heated erythrocytes is mainly caused by the altered cytoskeleton.

The shape of the spectra and the apparent correlation time of I(1,14) as well as the order parameter of I(10,3) of vesicle membranes are clearly distinct from those measured on intact human erythrocytes, suggesting an enhanced fluidity of vesicle membranes. These results give evidence that local alterations of the erythrocyte membrane exist during the formation of vesicles not detected by spin labeling the whole cell membrane (see above). This is not surprising as shown by a rough estimate. Let us assume that about 10 vesicles per erythrocyte are released, which corresponds to a membrane surface of about 3 μm^2 (the surface of one vesicle is about 0.3 μm^2, using the values obtained by electron microscopy). Keeping in mind that the surface of erythrocytes is about 130 μm^2 [37], only 2.3% of membrane are released in form of vesicles.

The alterations of vesicle membranes seen by EPR correlate with a reduced C/P ratio and a depletion of spectrin. The increase of membrane fluidity by lowering the cholesterol concentration is well known [11,38] and the results presented are in agreement. Because of its geometric shape, cholesterol is excluded from membrane regions of high bendings [14,39]. We conclude that vesicle formation induced by heating erythrocytes is accompanied by a segregation in the lipid phase at least of cholesterol. A lateral reorganisation of the lipid phase is also indicated by the increased spin-spin interactions of I(1,14) at temperatures higher than 48°C. In view of these results it can be assumed that local alterations of the lateral compressibility as well as the elastic module of the erythrocyte membrane could be involved in vesicle formation [14,40].

As shown, the NaCl concentration of the suspension medium influences membrane fluidity. As indicated by the I(1,14) membrane spectra (Figs. 5 and 6), the lowered membrane fluidity of erythrocytes corresponds to a reduced formation of wave on the cell rim as well as to the observed delay of vesicle release. Considering this effect of ionic concentration, the influence of surface and transmembrane potential on membrane structure and fluidity [41] has to be taken into account. A correlation between erythrocyte shape and transmembrane potential as well as between erythrocyte shape and membrane fluidity was established [42–44].

In conclusion, the chemical composition of vesicle membranes, the differences of membrane spectra between erythrocytes and vesicles and the influence of NaCl concentration on vesicle release as well as on membrane spectra of erythrocytes clearly demonstrate that, besides a membrane weakness due to spectrin [9], modifications of the lipid phase of the erythrocyte membrane have to be considered when explaining vesiculation.

As indicated by the EPR results, the incorporation of amantadine into erythrocyte membranes increases membrane fluidity, which is consistent with the findings obtained for phospholipid bilayers [17] and other biological membranes [19]. Because of the opposite charges of amantadine and labeled fatty acids interactions between both substances could be expected. Electrophoretic

measurements on amantadine-treated erythrocytes [45] and EPR measurements on phospholipid bilayers with labeled adamantone [46] indicate that amantadine is incorporated into the membrane itself in the head group region. It strongly influences the mechanical bending properties of the erythrocyte membrane. It induces the transformation of discocytes as well as of echinocytes to stomatocytes [45]. In both cases the positive curvature of the membrane increases consistently with the localisation in the membrane and the shape of this substance. In cell membrane regions of vesicle release immediately before the vesicle is cut off, a high negative curvature exists which is not favoured by the addition of amantadine. Vesicle release is suppressed. These results could be important to some extent for understanding the effect of amantadine on different biological processes at the level of membranes. Amantadine can influence the curvature of biological membranes, which is an important parameter in membrane-membrane interactions [47], for instance adhesion, fusion, exo- and endocytosis. This is underlined by the observed inhibition of vesicle release. Vesiculation reflects to some extent related membrane properties important for the process of virus penetration, uncoating, and the virus budding from the cell membrane. Erythrocytes are not a typical target cell. However, adamantane-like derivatives are not simply specific antiviral substances in considering the large scale of therapeutic effects on nervous disorders [18], which are caused by alterations in neurotransmitter release involving membrane-membrane interactions too.

Acknowledgements

The authors would like to thank the Professors J. Tigyi, K. Hideg, Biophysical Institute of the Medical University Pecs, Hungary, and U. Zimmermann, KFA Jülich, F.R.G., for providing spin labels and Dr. V.A. Tverdislov, Moscow State University, Dept. Physics, Lab Biophysics, USSR for providing amantadine. We are indebted for helpful discussion to Dr. L. Pratsch and for technical assistance to Mrs. B. Hillebrecht, Mrs. G. Seidler and Mr. P. Müller, Humboldt-University, Div. Biology, Dept. Biophysics, Berlin, G.D.R.

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