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Molecular interactions between DPPC and morphine derivatives: a DSC and EPR study

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Abstract

The interaction between different morphine derivatives (morphine, codeine, *N*-methyl-morphine, *N*-methyl-codeine) and α-L-dipalmitoyl phosphatidylcholine (DPPC) liposomes was studied with differential scanning calorimetry (DSC) and electron paramagnetic resonance (EPR) spectroscopy. Small unilamellar DPPC-liposomes with the given morphine-derivative were prepared by sonication. The size distribution of liposomes was checked by dynamic light scattering (DLS). The amount of entrapped morphine was determined spectrophotometrically. Our results indicate that the morphine and its derivatives principally interact with the lipid head groups, and this interaction leads to a decrease in the mobility of the polar head groups, especially in case of codeine and *N*-methyl-codeine.

Keywords: DPPC-liposomes; Morphine; Codeine; DSC; EPR

1. Introduction

Liposomes are composed of relatively biocompatible and biodegradable material, and they consist of an aqueous volume entrapped by one or more bilayers of natural and/or synthetic lipids (Gregoriadis, 1995; Sharma and Sharma, 1997). Liposomes may be used as a vehicle for drug delivery, because drugs with varying lipophilic character can be encapsulated (a) into the phospholipid bilayer; (b) in the entrapped aqueous volume or; (c) at the bilayer interface (Allen and Moase, 1996; Gregoriadis, 1993). Liposomes are under extensive investigation for improving the delivery of various pharmacologically active agents such as anticancer (Lasic, 1996; Treat et al., 2001) and antimicrobial drugs, antibiotics (Fang et al., 2001; Kamath et al., 2000), enzymes (Muzykantov, 2001), vaccines and genetic material (Gregoriadis and Florence, 1993; Sharma and Sharma, 1997). Liposomes offer an excellent opportunity to selective targeting of drugs, thereby expected to optimize the pharmacokinetic parameters, the pharmacological effect and to reduce the toxicity of the entrapped drugs. In the treatment of pain the inadequate control of postoperative pain remains a mayor clinical problem

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(Bonica, 1987; Sinatra, 1991). A single intrathecal injection of morphine produces analgesia that has a duration of action limited by the clearance of the drug (Yaksh et al., 1999; Nishiyama et al., 2000). To achieve longer duration a continuous infusion by catether or use of higher doses can be applied (Stenseth et al., 1985). Larger doses, however, have an increased extraspinal side effect. The spinal delivery of high-dose morphine can evoke a dose-dependent blockade of the pinna reflex, the corneal reflex, a reduction in motor activity (catalepsy), motor disturbances (truncal rigidity, quadriceps flaccidity) and a depression of respiration (Yaksh and Noueihed, 1993). Liposomal morphine preparations administered systematically or spinally allow the delivery of larger absolute quantities of morphine which produces significant antinociception of extended duration, without an increase in the undesirable side effects (Grant et al., 1994, 1995; Kim et al., 1996; Planas et al., 2000). The half-life and the residency time of morphine increases in the lumbar cerebrospinal fluid by liposomal encapsulation of the analgesic agent (Yaksh et al., 1999). Less side effects were observed with morphine incorporated into DPPC compared with free drug (Nishiyama et al., 2000). Comparing the antinociceptive/anti-oedema effect of free and encapsulated morphine during inflammation, liposomal morphine had more pronounced anti-oedema effect and increased the duration of antinociceptive effect in the presence and absence of inflammation (Planas et al., 2000). These effects are explained by sustained release (Rathbun et al., 1997) of morphine from the liposomal depot and the ability of liposomal drugs to attenuate toxicity by their different pharmacokinetic profile and pattern of distribution compared with plain drugs.

Previous works have been carried out in animal models to determine the pharmacodynamic behavior and pharmacokinetic properties of liposomal morphine. Also in human cases, it would be very useful to provide long-lasting (postoperative) analgesia with a single dose of liposomal morphine formulation instead of intermittent doses. It is known that morphine and its derivatives act at opioid receptors. Opioid receptor multiplicity (μ-, κ- and δ-receptors) is well established and compounds with agonist and/or antagonist properties can interact with a variety of receptor types (Fürst et al., 1995). Many studies were devoted to the properties of interactions between opioid molecules and their receptors, but investigation of the nature of interactions between opioid molecules and membrane lipids is in its early state. Studies of this latter interaction have also importance: because in the biological membranes the membrane receptor proteins are situated in a lipid environment, and all kind of changes in the physicochemical parameters of the lipids due to various drugs and chemicals can be accompanied by the conformational changes of the receptors too. This conformational change can also occur at the allosteric site of the receptor modifying the binding of agonist or antagonist molecules and their pharmacological effect. By this way the opioids might have an indirect effect on their own receptors. From pharmacological and technological aspects our purpose is similar: to get a better insight into the possible interactions between the drug and the compounds of its delivery system. Rational design of new drug-delivery systems, therefore, requires knowledge of the physicochemical properties of the lipid bilayer membranes and their changes due to various interactions with drug molecules. Many drugs can adsorb/bind to the liposomal membrane because of the energetic, covalent binding, as well as entropic, hydrophobic effects. The interactions often appear to be, 'specific', i.e. are sensitive to the choice of the host membrane phospholipid. It was realized that molecules bound interfacial affect the properties of the lipid bilayer appreciably (Cevc, 1990). The interactions between the drug and lipid molecules could have influence both on entrapment of drugs, redistribution of liposomes and release of enclosed molecules in vitro and in vivo in the biological milieu after administration (Grant et al., 2001; Stadnichuk and Kozhemiakina, 1976). All pharmacokinetic and pharmacodynamic advantages of liposomal morphine compared with free drug are mainly explained by sustained release of morphine from liposomal depot, which release can mainly be influenced by the molecular interactions between morphine and lipid compounds. Over the last decade some results indicated the importance of

such researches. It was shown that the ability of opiates to make the membrane permeable is highly dependent on the hydrophobicity of the opiate molecules (Reig et al., 1992). Increasing lipid content of morphine containing liposomes increased the proportion of drug reaching the intrathecal space in pig model in vivo. The degree of interaction between opioids and lipid bilayer in liposomal formulations of opioids dictates the rates at which epidurally administered drugs distribute into the intrathecal compartment and blood (Bethune et al., 2001).

Our goal was to design a liposomal opioid formulation and to investigate the molecular changes due to interaction of the lipid with some morphine derivatives. We addressed also to the question if it were possible to correlate fluidity changes detected by EPR spectroscopy with alteration in pre- or main transition temperatures observed by differential scanning calorimetry (DSC). As morphine derivative morphine, codeine, *N*-methyl-morphine, *N*-methyl-codeine was chosen. As the most simple and most generally used lipid molecule the α-L-dipalmitoyl phosphatidylcholine (DPPC) was our choice among the possible lipids.

2. Materials and methods

2.1. Preparation of liposomal morphine and codeine

Liposomes were prepared using the thin-film hydration method. Two hundred milligrams of α-L-DPPC (Sigma Chemicals Co.) was dissolved in 3 ml pure ethanol (Merck). The mixture was dried to thin film under nitrogen stream. In all cases, the concentration of the morphine derivatives was 0.1 M, which corresponds to 37.6 and 37.2 mg/ml for morphine chlorate and codeine chlorate, respectively (all morphine derivatives were from Alkaloida Pharmaceutical Co., Tiszavasvári, Hungary). The aqueous phase contained the given concentration of the morphine or codeine derivative dissolved in distilled water. Thin films of lipid were hydrated with 3 ml of aqueous phase at 50 °C, above the phase-transition of the DPPC, resulting a lipid concentration of 67 mg/ml. The heterogeneous population of multilamellar vesicles (MLV) formed by the hydration was sonicated at 50 °C, with a frequency of 20 kHz, and a wave amplitude of 8 μm, two times 10 min with a sonicator of type Soniprep 150 MSE, with 10 min pause between each sonication to produce small and uniformly sized population of small unilamellar vesicles (SUV). pH of the liposomal formulations was measured after sonication and found to be 6.9. Storing the samples at ambient temperature, 24 h later the mean diameter and size distribution of liposomes were monitored by dynamic light scattering (DLS) (ALV Goniometer). The goniometer was equipped with a Spectraphysics 124B He–Ne laser operating with 10 mW and at a wavelength of 632.8 nm. The size analysis was done at lipid concentration 1.0 mg/ml.

Free morphine or codeine derivatives were removed by gel filtration through a Sephadex® G-50 (Sigma Chemicals Co.) column (1.5×40 cm). The column was equilibrated with distilled water. Each 2 ml of the eluted fractions was collected. In the fractions without liposomes the amount of non-encapsulated morphine or codeine derivative was directly determined by spectrophotometer (Perkin–Elmer Lambda 15 UV–Vis) at the wavelength of 207.2 or 208.4 nm, respectively. To measure the amount of encapsulated opiate, the liposomes were solubilized with a great concentration of polyethylene glycol 1540 (PEG 1540; Sigma Chemicals Co.; 2 mg PEG 1540/1.00 ml lipid dispersion; Jones, 1999). The amount of opiate released from solubilized liposomes was determined spectrophotometrically following a dialysis. Solubilized liposome (1.0 ml) was sealed in a dialysis membrane with a 20 kD molecular-weight cut-off. After 96 h dialysis in 5 ml of distilled water at 4 °C, the amount of morphine or codeine was spectrophotometrically determined.

2.2. Sample preparation and DSC measurements

For DSC measurements, dispersions prepared from 10 mg of DPPC/40 μl distilled water were used. Ten milligrams DPPC was solved in 200 μl of pure alcohol. The organic solvent was removed by nitrogen stream, continuously stirring the tilted

vessel with the lipid solution, forming a thin film of the lipid on the wall of the vessel. Multilamellar liposomes were prepared by hydrating the lipid film with 40 μl distilled water at 50 °C and further dispersed by 20 min shaking in a water bath at 50 °C. In case of the samples with morphine derivatives, the hydrating solution contained morphine chlorate (FW: 376 g/mol) or codeine chlorate (FW: 372 g/mol) or *N*-methyl-morphine chlorate (FW: 391 g/mol) or *N*-methyl-codeine chlorate (FW: 387 g/mol) in a 10:1 final molar concentration of lipid to morphine derivative. Thus, in case of the DSC measurements, the lipid concentration is 250 mg/ml, with an opioid concentration of 10 mg/ml (please note the small differences in formula weights of the opioid used). About 5.0–8.0 mg amounts of samples were encapsulated in hermetically sealed standard aluminum DSC pans of 40 μl, without removing the free morphine derivatives. The phase transitions parameters of the samples were determined by a Du-Pont 990 differential scanning calorimeter, in a temperature range from 20 to 50 °C. The scan rate and the sensitivity were 5 °C/min and 5 mV/cm, respectively. Each morphine derivative formulation was measured in ten separate experiments. Due to the small sample volume, homogeneity was ensured as follows. Each preparation allowed to take three parallels. These parallels were measured; the pre- and main transition temperatures were determined. If the deviations between the three parallels were greater than the usual errors in our DSC measurements, the given charge was discarded from the evaluation.

2.3. Sample preparation and EPR measurements

In our EPR measurements 5-doxyl-stearic acid (Sigma Chemicals Co), 7-doxyl-stearic acid (ICN), 12-doxyl-stearic acid (Sigma Chemicals Co) and 16-doxyl-stearic acid (Sigma Chemicals Co) probes were used. Samples were prepared like those for DSC with the difference that after the MLV were formed samples were sonicated during two times 10 min with the above sonicator. A DPPC concentration of 10 mg/ml was used, and similarly to the DSC measurements, a 10:1 lipid to opiate molar ratio was maintained; with an opioid concentration of about 0.4 mg/ml. Similarly to the DSC measurements, samples containing the free morphine derivatives were used throughout the EPR measurements. The molar ratio of the spin probe to lipid molecules was 1 to 100. Spectra were registered with an EMX6 Bruker X-band on-line spectrometer. Temperature dependence of the spectra was measured by controlling the temperature within the sample with a precision of ± 0.1 °C. To characterize the fluidity of the lipid membrane the hyperfine coupling constants were determined in the temperature range from 2 to 50 °C. Modulation frequency (100 kHz) and maximally 2 G modulation amplitude were used throughout the measurements. The applied microwave power was 15 mW; scan-speeds of 167.77 or 335.5 s with 2048 points on 100 G field interval were used.

3. Results and discussion

3.1. DSC measurements

The phase transition parameters of the hydrated DPPC dispersions with and without the morphine derivatives are collected in Table 1. As characteristics, we use the absence or presence of pretransition, the temperature of the pre- and main transitions, T_{pre} and T_m, respectively. The half-width and the enthalpy of the main transition ($\Delta T_{1/2}$ and ΔH_m, respectively) were also determined.

Pretransition found in case of some lipids is a consequence of the formation of periodic ripples on the membrane surface (Janiak et al., 1976), which below the pretransition and above the main transition disappear. Pretransition was found to be pronounced and clearly distinguishable from the main transition in case of highly packed liposome structures, i.e. at MLVs, while it becomes less pronounced, but detectable for unilamellar structures, SUV or LUV. Moreover, Heimburg (1998) reported differences in the DSC signal between samples of LUVs extruded and LUVs sonicated, which were attributed to differences in cooperativity of melting processes, reflected by the half-width of the pre- and main transitions. The ripple

Table 1
Phase transitions parameters determined for multilamellar DPPC liposomes with and without morphine derivatives

Morphine derivative	Pretransition	T_{pre} (°C)	T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH_m (mJ/mg)
None	+	35.8±0.1	41.7±0.1	1.5±0.0	42.2±1.6
Morphine	+	36.3±0.3	41.5±0.2	1.6±0.1	36.5±1.7
N-Methyl-morphine	+	32.7±0.4	41.4±0.1	1.5±0.1	44.8±1.7
Codeine	–	–	40.3±0.2	1.5±0.1	42.9±1.6
N-Methyl-codeine	–	–	40.7±0.3	1.8±0.2	42.3±1.8

Each value given in the table was calculated from $n = 10$ parallels, and are given by the mean±S.D.

phase formation and stability, thus the shape and the exact temperature of the pretransition is influenced by the type of vesicle (MLV, LUV or SUV) and also by the molecules added, e.g. gramicidin S (Prenner et al., 1999), anestethics (Engelke et al., 1997), anticancer taxol derivative (Ali et al., 2000). Interbilayer forces acting between bilayers of an MLV increase the cooperativity of melting processes, thus decreasing the temperature range, the half-width of the phase-transition signals, be it pre- or main transition. In a recent theoretical model (Heimburg, 2000), coupling of the ripple formation with the chain-melting transition was worked out. It is assumed that the pretransition can be treated as the consequence of fluid lipid line defects. Thus, if the periodic ripple structure on the lipid head part changes, due to an interaction between a molecule and the head-groups, it can be detected either as a change in the pretransition width or in its exact temperature.

The endothermic pretransition of DPPC liposomes is characterized by great changes in the appearance/lack of the pretransition due to possible different interactions between the DPPC and the morphine derivatives at a molar ratio of 10:1 lipid to opioid molecules. While it is pronounced in case of pure DPPC, it disappears in presence of codeine and *N*-methyl-codeine. A statistical evaluation of the changes in the pretransition temperature for liposomes entrapping *N*-methyl-morphine shows a significant ($P < 0.05$) decrease in comparison to control DPPC's. Remarkable differences between control and codeine or *N*-methyl-codeine treated samples were found: the pretransition disappears, and there is a corresponding significant ($P < 0.05$) decrease in T_m. Thus, codeine depresses the T_m by as much as 1.4 °C and *N*-methyl-codeine by as much as 1.0 °C. Concerning the morphine and *N*-methyl-morphine no significant ($P > 0.05$) effect on the T_m of the DPPC was observed.

From the fact that the half-width, $\Delta T_{1/2}$, of the main transition increases significantly ($P < 0.05$) only in presence of *N*-methyl-codeine we can conclude that a detectable decrease of the cooperativity between the apolar lipid chains occurs only in case of *N*-methyl-codeine. On the contrary, it is only the morphine, which causes a small decrease ($P < 0.05$) in the enthalpy of the endothermic main transition. Such findings indicate the existence of stronger interactions with lipid head groups and slighter ones with lipid chains in case of codeine and *N*-methyl-codeine. So we conclude that these agents could be situated (a) in the lipid bilayer but close to the upper regions of hydrophobic chains; (b) close to the polar head groups or; (c) can be adsorbed to the polar heads of the lipid molecules, remaining, however, in the aqueous phase. Since both the methoxy- and the hydroxyl groups are substituents at the C_6-position of the morphine ring, the above findings provide support for the role of the methoxy group (codeine, *N*-methyl-codeine) versus the hydroxyl group (morphine, *N*-methyl-morphine) in the interaction of these molecules with the lipid. These results also suggest that at the pH used in our experiments, pH 6.9, a change in the C_6-connected group can affect to slightly higher extent the molecular interactions, between the DPPC and the drug molecules, than the existence or absence of quaternary nitrogen (*N*-methyl-derivatives) providing a permanent positive charge. Further supports to the above idea come from our EPR

measurements, detailed in the subsequent parts. They showed that spin labeled fatty acids, used to monitor the local changes along the hydrocarbon chain, give evidence of a modified interaction between the lipid-tails, close to the head group, at the fifth, seventh carbon atom position, while it is not detectable in the depth of the 12th or 16th position. Thus, the existence and formation of the ripple structures is modified due to an interaction between the head groups or the lipid chains, not deeper than about the ninth carbon atom, and the morphine derivative.

3.2. Liposomal morphine and codeine

The amount of the entrapped drug was determined from the absorbencies of morphine or codeine containing fractions. To determine the amount of the morphine or codeine derivatives incorporated into the liposomes a solubilization of the samples is necessary. In preliminary experiments we have determined that among the possible surfactants, it was the PEG 1540, which did not interfere with the absorbance of the morphine at the chosen wavelength of 207.2, or 208.4 nm for the codeine. According to our measurements, relatively good encapsulation efficiency was achieved, 49 ± 4 or $51 \pm 8\%$ of the aqueous morphine or codeine was trapped inside the liposomes, respectively. In comparison to our encapsulation method, previous studies reported about 70% entrapment efficiency, but using alkalinized water to dissolve the morphine (Planas et al., 2000). As it is known, at alkaline pH there is a higher possibility of morphine-oxidation, which can reduce the stability of the formulation (Vermeire and Remon, 1999). Thus, by lowering the pH, but maintaining a relatively high entrapment efficiency we can arrive to an increase in the stability of the formulation, which was one of our aims in the present study.

DLS is a versatile tool in determination of the size distribution of the macromolecular systems (Chu, 1991; Hallett et al., 1991). In a certain range of size, where the radius of gyration of the particles under study is much smaller than the wavelength of the applied laser beam, determination of the size distribution is possible using the autocorrelation function obtained by DLS measurements. The best method for evaluation of the autocorrelation functions, nowadays available, is the so-called maximum entropy method (MEM; Chu, 1991) which we also used in our practice.

Under the preparation conditions given in the Section 2, we arrived to produce liposomes with an average hydrodynamic radius of 40 nm showing a distribution of radius between 28 and 52 nm. The distribution is narrow in this case, the thin layer hydration method was found to be very useful for producing liposomes of controllable particle size. Vesicles prepared by us are comparable in size to those of similar composition but different method of preparation described by, e.g. Sheetz and Chan (1972), De Kruijff et al. (1976). Fig. 1 shows a typical plot of the DLS results. To characterize the polydispersity of a liposome preparation the half-width of the peak belonging to the main particle's component can be used. The half-width is usually defined as half of the width at the half maximum of the peak. The overall average of the half-width of the hydrodynamic radius for the liposome preparations used in our experiments provided a value of about 12 nm. Another useful characteristic is the integral contribution of the main particle, which has been found to be about 85% for the component centered at the hydrodynamic radius of about 40 nm. Inspecting Fig. 1, one can detect a bimodal distribution, which is generally present on our 'experimental' curves calculated for liposome preparations from the measured DLS data by MEM. The feature present at smaller radii indicates a liposome population of about 15 nm radius with a contribution not more than 11%. At the first glance a contribution of about 11% may seem to be high, however, it is a contribution calculated on the basis of the number of liposomes with the given radius. Since, in the EPR and the DSC techniques, the measured physical property is proportional to the number of molecules, which is on its part proportional to the surface, thus to the square of the radius of a liposome, the resulting contribution of the lipid molecules present in the larger liposomes should overwhelm the smaller ones.

Addition of morphine, codeine, and *N*-methyl-codein did not change the particle distribution

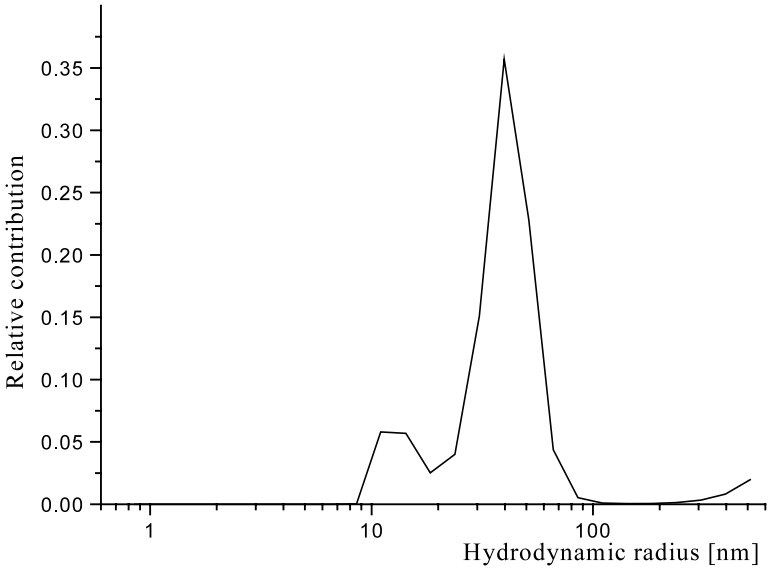

Fig. 1. Size distribution of liposomes. Samples of liposomes prepared from 1 mg/ml lipids were measured in each case to check the hydrodynamic radius of the sonicated liposomes. Contributions of the liposomes were normalized to unity in the interval from about 1 to 500 nm. Relative contributions from the particles of greater or smaller radii has been found to be negligible ($< 1\%$).

within the limit of experimental errors: the average hydrodynamic radius ($\pm$S.E.M.) for the three different derivatives was found to be, 32 ± 11; 38 ± 15; and 43 ± 14 nm, respectively. This indicates that there is no change in the size distribution, or in the type of the vesicle-structure upon interaction with the opioids. This expected finding is, however, an important verification for the EPR measurements, because there are indications that the EPR signal is sensitive also against the structure and the size of the lipid vesicles (Ge et al., 1994).

3.3. EPR spectroscopy

Samples of small unilamellar DPPC vesicles were prepared and spin-labeled as given above. In order to avoid any interference due to sample aging, samples were freshly prepared and measured on the subsequent 1–2 days. To minimize possible deviations of any source, control and treated samples were measured always on the same day. Hyperfine splitting between the outermost maxima on the EPR spectra of 5-doxyl or 7-doxyl spin-labels incorporated into the liposomes can be used to parameterize changes in the fluidity of the lipid membrane (Marsh, 1981). A decrease in the hyperfine splitting can be an indication of an increased fluidity. To check the possible sensitive range along the lipid molecule, we used four different spin labels in a series of preliminary experiments. Spin labeled stearic acids, labeled at the 5th, 7th, 12th and the 16th positions were used. According to our preliminary measurements we observed that changes in the molecular interactions between the lipid and the opioid molecules could be expected only close to the head group of the DPPC molecules, when the monitoring doxyl-group was situated at the 5th or 7th position. This is the reason why we focused our study to the closest neighborhood of the polar head group. Figs. 2 and 3 contain typical temperature dependence of the hyperfine splitting constant ($2A_{zz}$) for pure and treated liposomes. Fig. 2 shows all the four morphine derivatives and the case control with the given experimental errors (indicated, however, only for *N*-methyl-morphine treated DPPC to avoid confusion). Regarding the level of experimental uncertainty we concluded that using the 5-doxyl spin-label, which monitors the closest neighborhood of the polar region, there is a remarkable, significant difference between the

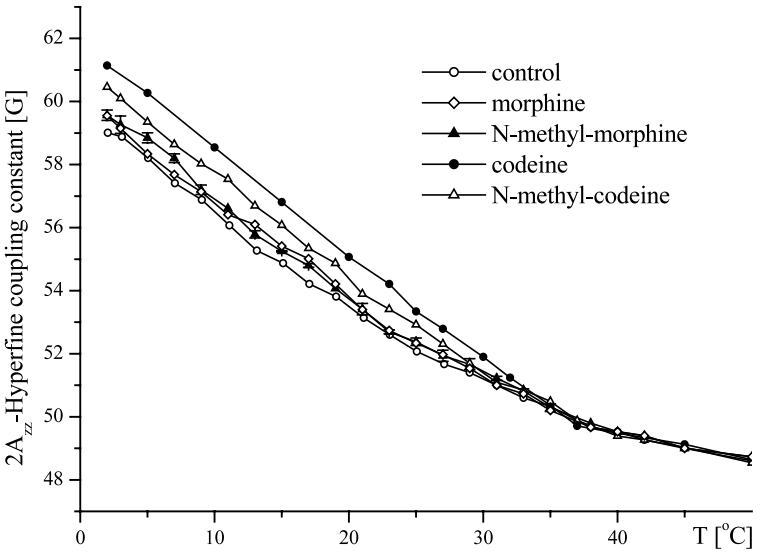

Fig. 2. Hyperfine coupling constant vs. temperature. Coupling constants were measured between the outermost extrema from the EPR spectra of liposomes spin-labeled by 5-doxyl-stearic acid for all derivatives studied. Experimental errors (S.E.M.) are given only for the *N*-methyl-morphine, and in all the other cases it is within the limits given for this case. Significant deviation from the control was specified for codeine and *N*-methyl-codeine.

temperature dependence of the control and codeine-treated samples (please note also that the level of the experimental errors can be judged from the high-temperature end of the experiments, where the measured points coincide to almost one curve). The difference can be observed in the range between 2 °C and 35 °C, while beginning from 37 °C all the curves coincide. A similar but

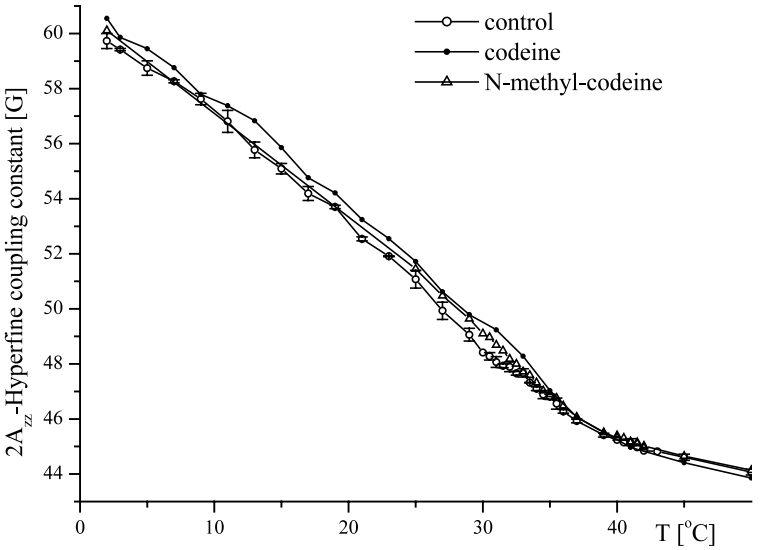

Fig. 3. Hyperfine coupling constant vs. temperature: 7-doxyl-stearic acid. Coupling constants were measured as in case of 5-doxyl-label. Experimental errors (S.E.M.) are given for case control. Finer temperature step was used around the expected pretransition temperature in the interval from 28 to 35 °C.

smaller difference can be accepted for the *N*-methyl-codeine treated samples. Apart from some temperatures, e.g. 7 and 17 °C for the *N*-methyl-morphine, both morphine and *N*-methyl-morphine show quite similar temperature dependence as the case control. The changes in the hyperfine-splitting constant indicate a decrease of the fluidity, which is the most pronounced in the case of codeine, while it is detectable, but smaller for the *N*-methyl-codeine. No significant deviation between the control and the morphine or *N*-methyl-morphine treated samples can be identified.

Among the available doxyl-stearic acid spin labels, it is the 5-doxyl-stearic acid which situates closest to the polar head groups, while the next one in the series is the 7-doxyl-stearic acid. Thus, this spin labeled compounds can be the most sensitive in detecting any change, which occurs in the attractive–repulsive, forces between the head groups of the DPPC molecules. The pretransition is mainly determined by a change in the mobility of the polar head groups. Thus, the decrease in fluidity detected by changes in the temperature dependence of the hyperfine splitting indicates also an altered pretransition behavior in case of codeine and *N*-methyl-codeine, which finding coincides with those found by our DSC measurements. We also addressed to the question why could we not observe such a remarkable break point on the EPR parameter as could be detected by the DSC measurements (Biltonen and Lichtenberg, 1993). Our explanation comes from two sides. (1) DSC measures an overall thermic property, while the EPR spectra are more sensitive to molecular motions/forces in a given range, around the monitoring group of the spin labeled molecule (Mader, 1998). (2) Samples prepared for DSC measurements consist of multilamellar DPPC vesicles, where the outermost two layers, possessing almost the same curvatures, are built up from the highest amount of lipid molecules. In case of SUV used in our EPR measurements, the difference between the outer and the inner layer is relatively greater, compared with those can be thought to exist between the outermost two layers of a MLV. Thus, in this respect, the MLV are the more homogeneous ones, which can lead to a more remarkable change at the pretransition temperature. We prepared multilamellar samples of the untreated DPPC liposomes and measured with EPR spectroscopy. In agreement with the result found also in the earlier references (Marsh, 1998), the temperature dependence of such samples (figure not shown here) possesses an abrupt change near the pretransition temperature. Since a couple of decades it became clear that membrane fluidity, thus also the compressibility and elasticity are modulated by the structure of the liposomes, especially in the gel phase and in the gel–fluid phase transition region. Bilayer–bilayer interactions enhance the cooperativity, leading to a relatively sharp pre- and main transition in the case of MLVs, while it becomes broader for LUVs and SUVs. This concerns, however, only such physical characteristics, which reflects macroscopic fluctuations, but may be less important in other properties modifying the membrane's lipids locally. It has been concluded, therefore, that as far as the MLV remains fully hydrated, the interbilayer interactions have negligible effect on the intrinsic structure of the lipid bilayer (Nagle and Tristram-Nagle, 2000). In our present study we accepted the above finding, and due to technical reasons (our DSC device has not satisfying sensitivity to study unilamellar vesicles in the concentration range as that used for EPR measurements), MLVs were used for DSC measurements, while the greatest sensitivity of the EPR technique allowed the measurements of SUVs in a smaller concentration too. Thus, because our aim was to detect changes in liposome formulation with SUV, in the followings we continued to study the opioids' effect only on such systems.

To get further insight into the molecular dynamics of the lipid–morphine interaction we used the 7-doxyl-stearic acid (Fig. 3). In this case the doxyl-ring lies by two carbon atoms deeper inside the vesicles than the 5-doxyl ring. Thus, according to the fluidity map along the hydrocarbon chain of the lipid molecules, a higher fluidity can be expected for the untreated liposomes (Hoffmann et al., 2000). If the morphine derivatives interact not only with the polar head groups, but also deeper with the hydrocarbon chain, then a change in the membrane-fluidity can be expected also at

these sites. This change, however, can result in either an increase or decrease of the fluidity, depending on the ordering–disordering effects of the opioid molecules. According to our results, the 7-doxyl-stearic acid reflects a slight decrease in the fluidity for codeine and *N*-methyl-codeine, but no change was found for the other two derivatives. The difference between the control and the treated samples is, however, smaller than that found closer to the head group. Thus, we concluded that morphine derivatives may be localized rather close to the outer surface of the liposomes, modifying significantly the membrane properties not deeper than the environment of the seventh/ninth carbon atoms along the hydrocarbon chains. In addition, using this latter spin-label we followed more detailed also the temperature range close to expected pretransition, which is in this case at a lower temperature than that can be found by DSC measurements. In a temperature step of 0.5 °C, between 28 ° and 35 °C, a detailed map of the temperature dependence for the control and *N*-methyl-codeine treated DPPC samples were studied. Our observation resulted in a greater change in the slopes of the temperature dependence for the control than for the treated samples, which is in agreement with the DSC measurements, where a disappearance of the pretransition for the codeine derivatives was obtained.

3.4. Membrane fluidity: ordering and rotational freedom

During the evaluation of the fluidity changes observed in our experiments, we were faced to the question if the decrease in fluidity was a result of (1) a higher ordering due to the constrain exerted by the morphine derivatives; or (2) an increase in the rotational correlation time which has the same origin. In both cases we can expect a similar change in the hyperfine splitting. In principle, interaction of the lipid molecule with incorporated molecules can result in disordering of the lipid molecules, decreasing the ordering potential, which would diminish the observed hyperfine splitting. In parallel, a decrease in the molecular order parameter, S_{mol}, could have been observed (Jost and Griffith, 1978). A constrain which results in an increase of the attractive forces, can decrease the rotational freedom, thus increasing the rotational correlation time. Such an effect would cause an increase of the hyperfine splitting, which we have also observed. By the simple evaluation we applied in the present work, however, an increase of the ordering and a parallel decrease of the rotational correlation time cannot be excluded. It is also possible, that a disordering effect of the morphine derivative is compensated by a higher increase in the rotational correlation time, which would lead to a decrease of the membrane fluidity. To answer these assumptions a detailed dynamical simulation of the EPR spectra is required (Budil et al., 1996) which is, however, beyond the scope of the present work.

4. Conclusions

Results of our DSC and EPR measurements show evidences that some of the morphine derivatives interact with the closest environment of the polar head group of the DPPC molecules. This impact modifies the molecular forces between the adjacent head groups increasing the rigidity at this level of the bilayer. The range of this modification does not expand, however, deeper than about the 7th–9th carbon atom along the hydrocarbon chain. Our observations may direct attention to investigate how lipid molecules of different headgroups can modulate this interaction and to what extent it would modify the encapsulation or the delivery of the specific opioids used.

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