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Effect of inhalation anaesthetics on the phase behaviour, permeability and order of phosphatidylcholine bilayers

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Abstract

We have used differential scanning calorimetry and fluorescence anisotropy measurements to investigate the effect of five inhalation anaesthetics of diverse chemical structure (halothane, enflurane, n-pentane, chloroform and diethylether) on the phase behaviour of liposomes prepared from dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC), respectively. The incorporation of these anaesthetics induced a decrease of the phase transition temperature and/or a broadening of the phase transition peak depending on the transverse localisation of the investigated anaesthetic. At high anaesthetic concentrations we observed the disappearance of the pretransition peak and the appearance of a shoulder on the main phase transition peak due to the domain formation of the anaesthetics. An anaesthetic induced carboxyfluorescein efflux from the vesicle lumen was completed within a few minutes after the addition of the anaesthetics, probably resulting from a transient formation of membrane holes. All results are discussed with regard to the physicochemical properties of the anaesthetics applied. © 1997 Elsevier Science B.V.

Keywords: Inhalation anaesthetics; Phase transition; Bilayer order; Carboxyfluorescein permeability; Differential scanning calorimetry; Fluorescence anisotropy

1. Introduction

The molecular mechanisms of the anaesthetic action still are a question of fundamental importance in molecular pharmacology. Up to now no generally accepted theory exists to explain the effect of general anaesthetics on nerve membranes capable to induce the reversible loss of consciousness. Among the various classes of general anaesthetics the inhalation anaesthetics constitute a large class of lipophilic compounds of diverse molecular structures. To understand the molecular mechanism of general anaesthesia, two main controversial ideas have been followed. One of them regards the membrane proteins as the primary target site of the anaesthetic action [1,2], whereas the other focuses on the non-specific interaction with the surrounding lipid matrix [3–8]. The discovery that hydrostatic pressure antagonises the anaesthetic action of inhalation anaesthetics [9,10] initiated several studies on the physical properties of the pure lipid matrix. The physical property that correlates best with anaesthetic potency is the lipid solubility termed the Meyer–Overton rule [6]. De-

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spite the close correlation between lipid solubility and anaesthetic potency, deviations from this correlation do exist [3]. The difference in the anaesthetic potency of agents having nearly identical oil/water partition coefficients suggests that the potency of an agent depends also on factors other than solubility. Considering the lipid membrane theory, there is evidence that the lateral as well as the transverse organisation of the membrane components, characterized by static and dynamic heterogeneity in biological membranes, is of importance for the biological functioning of membrane associated processes. In order to explain the varying efficiency of different inhalation anaesthetics at identical bilayer concentrations, we have investigated their effects on the gel-to-fluid lipid phase transition, on the membrane order and on the membrane permeability. Physical perturbations of the heterogeneous lipid membrane may help to reveal the molecular mechanisms of anaesthesia. Out of the large group of inhalation anaesthetics, we have chosen halothane and enflurane which are of clinical use today, chloroform and diethylether which had widespread use in the past and n-pentane as an experimental anaesthetic.

The phase transitions (gel-to-liquid crystalline $P_{\beta'}$–$L_{\alpha'}$ and lamellar gel-to-gel $L_{\beta'}$–$P_{\beta'}$–pretransition) of pure DMPC and DPPC, and how they are affected by the incorporation of anaesthetic molecules interacting with the lipid bilayer, are of considerable interest, because they reflect the molecular involvement of the basic lipid membrane structures. Two theories have been based on the assumption that the phase transition of phospholipids induces an anaesthetic effect. According to Lee [11] and Lee et al. [12], a fluidizing effect of incorporated anaesthetics on the lipid surrounding of membrane proteins may simulate this phase transition. The hypothesis of a phase separation [13] is based on the coexistence of several lipids in their gel- or liquid-crystalline state. The opening of transmembrane channel proteins is correlated to the gel to liquid transition, which might be inhibited in case of anaesthetic incorporation. Investigations have shown that the protein surrounding lipid boundary layer is in a state between gel- and fluid-crystalline [14–16].

In this paper, we report on calorimetric and fluorometric measurements of the temperature dependent phase behaviour of model membranes prepared from DMPC and DPPC which are incubated with various anaesthetics in the concentration range of 10–50 mol%. At clinical relevant concentrations (4 mol%), the physico-chemical effects are negligible. This results from the fact that the lipid distribution is inhomogeneous in biological membranes. Jørgensen et al. [28] have shown that anaesthetics accumulate at interfaces of lipid clusters and at lipid–protein interfaces, which leads to an inhomogeneous distribution of anaesthetics with high local concentrations, especially in the protein surrounding, because the protein–lipid boundary layer is in a state between gel- and fluid-crystalline. It has been shown [28] that the lipid–water partition coefficient is maximum at the phase transition, because the interfacial regions are maximum. Consequently, high local concentrations may exist at a low overall concentration.

Another aim of this study is directed to the differentiation of anaesthetic effects on bilayer order in the lipid–water interface of the phospholipid headgroup region and in the acyl chain region of the bilayer centre. The depth-dependent perturbation of the bilayer order is monitored on egg phosphatidylcholine vesicles (EPC) with the fluorescence anisotropy method using DPH and TMA–DPH as the fluorescent probes.

An anaesthetic induced increase in the cation and proton permeability [17,18] is observed as a result of a disturbance of the structural integrity of the bilayer. The permeability increase is paralleled by an increased efflux of low molecular weight molecules like carboxyfluorescein (CF) [19]. The release of CF, which is encapsulated in liposomes at a high concentration (150 mM) results in relief of its fluorescence selfquenching [20]. We have measured the increase of the CF fluorescence intensity as a function of the concentration of the inhalation anaesthetics in order to elucidate a relation between bilayer disruption and lateral or transverse accumulation of the anaesthetics.

The anaesthetic to lipid ratio was calculated from the octanol/water partition coefficient, because of the good correlation between octanol–water and lipid–water partition coefficients [3,37]. We did not take into account the temperature dependence of the lipid partition coefficient which is maximum at the phase transition temperature, because we expect that the relative differences between the investigated anaesthetics are constant in this temperature range.

2. Materials and methods

Egg yolk phosphatidylcholine (EPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosporylcholine (DPPC) were purchased from Lipoid KG (Ludwigshafen, Germany). Lipid purity was greater than 99% and all lipids were used without further purification. Dichloromethane, methanol, ethanol and EDTA were from Merck (Darmstadt, Germany). Chloroform, n-pentane and diethylether were from Riedel de Haen (Seelze, Germany), halothane from Sigma (Geisenhofen, Germany), while enflurane was a gift from Dr. H. Jung (St. Jürgens Hospital, Bremen, Germany). All anaesthetics investigated were analytical grade. The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-[trimethylammonium)phenyl]-6-phenylhexa-1,3,5-triene (TMA–DPH) and 5,6-carboxyfluorescein (CF) were purchased from Molecular Probes (Eugene, OR, USA). Triton X-100 was from Serva (Heidelberg, Germany), α-tocopherol from Aldrich (Steinheim, Germany) and dimethylformamide (DMF) from Janssen (Geel, Belgium).

2.1. Liposome preparations

For the phase transition and steady state membrane fluidity measurements EPC, DMPC or DPPC dissolved in a mixture of dichloromethane–methanol (2:1) were evaporated to deposit a thin lipid film on the wall of a glass tube. The final traces of residual solvent were removed under vacuum at 50°C overnight. Lipids were dissolved in an appropriate amount of TRIS buffer (150 mM TRIS, pH 7.4) to give a lipid concentration of about 50 mg ml^{-1} and vigorously vortexed at room temperature. The final lipid concentration was determined according to Stewart [21]. The liposomal suspension was stored under nitrogen in darkness at 4°C to avoid lipid peroxidation [22]. All liposomal preparations were used within 2 weeks.

For the membrane permeability measurements liposomes were prepared as described above, but EPC containing 18 mol% α-tocopherol and additionally 20 or 40 mol% cholesterol were used. These liposomes were dissolved in a 150 mM CF-solution (5 mM MOPS, 50 mM NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4) and vortexed for 60 min. The nonencapsulated CF was separated from the liposomes by chromatography on a Sephadex G 50 column at 4°C. Liposomes were diluted in MOPS buffer and stored in small glass tubes at 4°C under nitrogen in darkness.

The liposomes obtained are heterogeneous in size and lamellarity. With respect to membrane fluidity and phase transition measurements this heterogeneity is of no relevance. To measure the relative membrane permeability changes no homogeneous liposomes are necessary, as well. Sonification and ultracentrifugation would cause leakage of encapsulated carboxyfluorescein from the liposomes.

2.2. Calculation of the molar anaesthetic-to-lipid ratio

The appropriate amount of anaesthetic for a given molar anaesthetic-to-lipid ratio was calculated according to following equation:

$$m_A = \left(1 + \nu_{B/L}/\gamma\right) x_{A/L} n_L M_A \qquad (1)$$

where m_A (ml) = amount of added anaesthetic; $\nu_{B/L}$ = buffer to lipid volume ratio; γ = octanol–water partition coefficient of the investigated anaesthetic [23,24]; $x_{A/L}$ = molar anaesthetic to lipid ratio; n_L = molar lipid concentration of the assay; M_A = molecular weight of the investigated anaesthetic.

All anaesthetics were added at 4°C and the sample tubes were closed immediately to avoid solvent evaporation.

In case of low molar anaesthetic to lipid ratio or low molecular weight, the investigated anaesthetics were diluted in ethanol before they were added to the lipid sample. Control measurements have shown that ethanol in the applied concentration range (< 5 mol%) has no effect on the phase transition.

2.3. Differential scanning calorimetry (DSC)

DSC measurements were performed in a DSC 20 scanning calorimeter (Mettler, Gießen, Germany) with the original data acquisition and analysis software. The temperature difference between sample and reference was measured. Samples were heated in the temperature range 10–35°C and 27–52°C at a scanning rate of 0.5°C min^{-1}.

The appropriate amount of the investigated anaesthetic was added to 1 ml of the liposomal preparation in a glass tube at 4°C, which was closed to avoid evaporation and vortexed for 10 min to ensure a homogeneous distribution of the anaesthetics in the bilayer. For the measurements aliquots of 100 μl were transferred to the sample compartment of the DSC. All measurements were performed in triple.

2.4. Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were carried out on a SFM 25 Kontron-spectrofluorometer (Kontron Instruments, Eching, Germany) equipped with a L-format anisotropy inset and a Julabo HC thermostat (±0.5°C) (Julabo Labortechnik, Seelbach, Germany). Fixed wavelengths of 360 nm for the excitation and 425 nm for the emission of both DPH and TMA–DPH were used at a slitwidth of 2.5 nm for both, excitation and emission wavelength. In case of phase transition measurements, the temperature of the 1 cm square cross-section quartz cuvettes were scanned in the same temperature range as the DSC measurements. For the determination of membrane fluidity, the temperature was kept at 25°C. All samples were thermostated for 10 min before each measurement. Fluorescence intensity was measured every 0.5°C near the phase transition. All measurements were performed in triple and corrected for light scattering.

The degree of fluorescence anisotropy was calculated according to Eq. (2) [25,26]

$$r = I_{\parallel} - I_{\perp} / I_{\parallel} + 2 I_{\perp} \qquad (2)$$

where r is the steady state fluorescence anisotropy and $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities at 425 nm parallel and perpendicular to the anisotropy plane of the excitation light, respectively.

Prior to the fluorescence anisotropy experiments DPH or TMA–DPH from a $0.5 \cdot 10^{-3}$ M stock solution in DMF were added to the liposomal suspension of 0.2 mg ml^{-1} lipid concentration to give a final lipid to fluorescent probe ratio of 500. All samples were incubated at room temperature for 1 h before measurement to allow the fluorescent probe to incorporate into the lipid bilayer.

2.5. Membrane permeability measurements

Experiments were done with a Perkin–Elmer MPF-3 spectrofluorometer using fixed wavelengths of 492 nm for excitation and 518 nm for emission. The temperature in the quartz cuvette of 1 cm square cross section in the fluorometer was kept at 25°C. Both, excitation and emission slits were set to 3 nm spectral bandwidth. Prior to the measurements the liposomal suspension was diluted to give a final lipid concentration of 5.5 μg ml^{-1} in buffer. Different amounts of the investigated anaesthetics were added directly to the cuvettes. Since the absolute amount of CF varied in each sample, the 100% reference point was determined separately for each sample by solubilizing the vesicles by addition of 100 μl of a solution of 10% Triton X-100.

The CF leakage was determined according to following equation:

$$\text{CF} - \text{leakage}\,[\%] = (F_b - F_a / F_t - F_a)\,100 \qquad (3)$$

where F_a = fluorescence intensity before the addition of anaesthetics; F_b = fluorescence intensity after addition of anaesthetics; F_t = fluorescence intensity after addition of Triton X-100.

The leakage was corrected for the leakage of a reference probe without anaesthetics.

3. Results

3.1. DSC measurements

Figs. 1–5 exhibit the calorimetric scans of DMPC- (Fig. 1a, 2a, 3a, 4a, 5a) and DPPC-liposomes (Fig. 1b, 2b, 3b, 4b, 5b) as a function of inhalation anaesthetic concentration, given in mol% anaesthetic to lipid. Without anaesthetics the phase transition of the pure lipid bilayer of DPPC is at higher temperatures and narrower than for DMPC and a pretransition was found at 35°C, which disappears in the presence of anaesthetics.

For halothane (Fig. 1a,b) the decrease in phase transition temperature (T_m) with increasing halothane concentration in the bilayer is paralleled by a broadening of the phase transition especially for DMPC. In presence of 25 mol% halothane a shoulder appears at the low temperature side of the main transi-

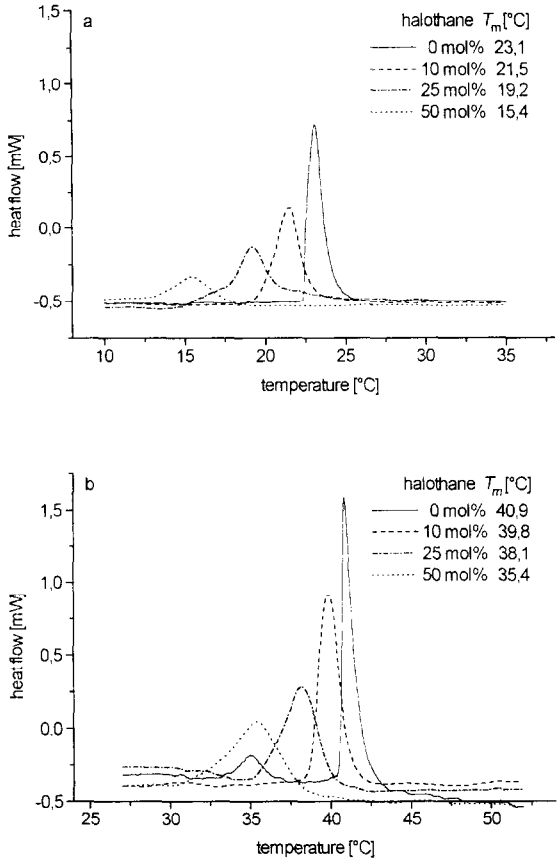

Fig. 1. Original DSC scans with (a) DMPC- and (b) DPPC-liposomes at increasing halothane concentration.

tion peak of DMPC, which probably results from the superposition of two individual transitions. The main transition temperature T_m becomes continuously depressed with the addition of higher halothane concentrations (Fig. 1a,b).

Enflurane affects the phase transition of both DMPC (Fig. 2a) and DPPC (Fig. 2b) in a similar way as halothane. As for halothane a stronger increase was found for DMPC than for DPPC-liposomes.

The effect of chloroform (Fig. 3a,b) on T_m is stronger than from enflurane, but weaker than from halothane in both DMPC and DPPC bilayers. Like for halothane and for enflurane the pretransition observed for DPPC disappears in presence of chloroform and also the shoulder in the phase transition of DMPC after addition of at least 25 mol% chloroform appears.

Other results were obtained for diethylether (Fig. 4a,b): In contrast to enflurane, halothane and chloroform, increasing concentrations of this anaesthetic obviously do not change the shape or heights of the transition peak in the concentration range investigated and the extent of the phase transition decrease is comparable in DMPC and DPPC. The pretransition in DPPC remains even at the highest concentration of diethylether and no superposition of two transitions could be observed for DMPC with increasing anaesthetic concentration.

Out of all the investigated anaesthetics n-pentane has the smallest effect on the decrease of T_m, but causes a marked broadening of the phase transition peak (Fig. 5a,b). The pretransition peak of DPPC also disappears at n-pentane concentrations higher than 25 mol%.

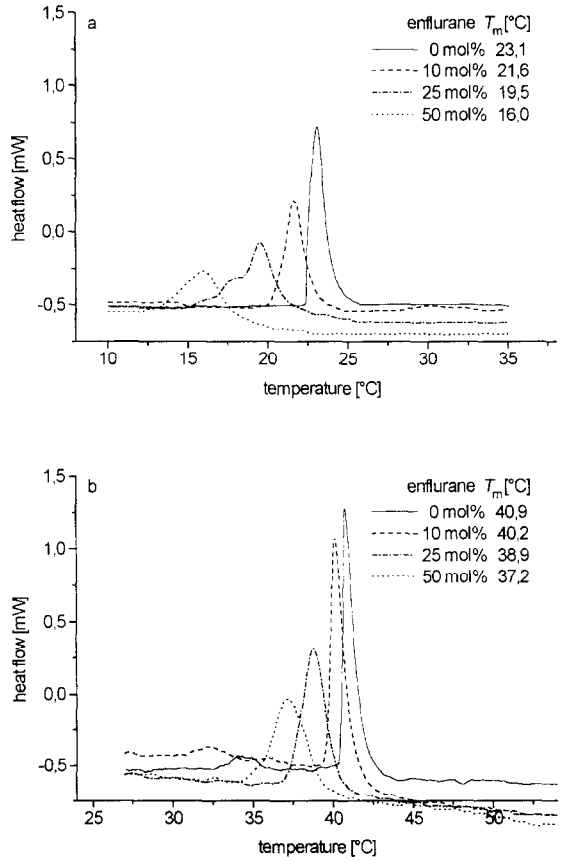

Fig. 2. Original DSC scans with (a) DMPC- and (b) DPPC-liposomes at increasing enflurane concentration.

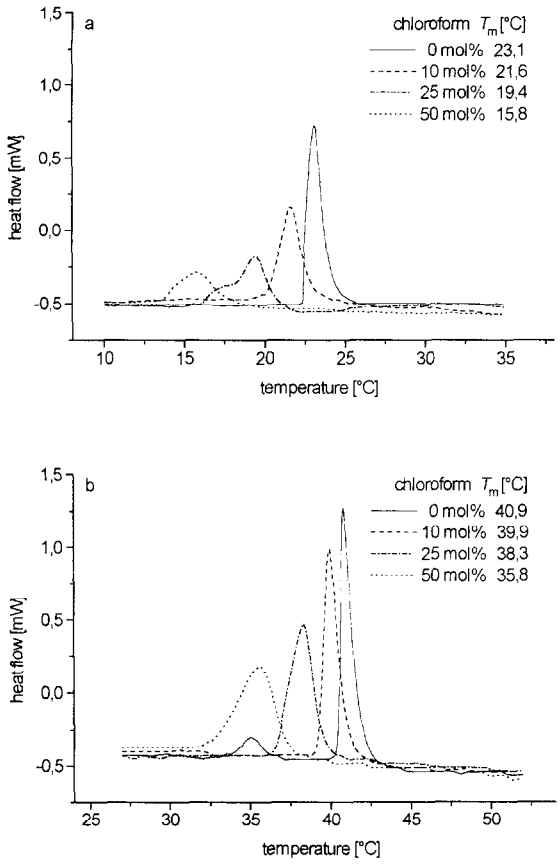

Fig. 3. Original DSC scans with (a) DMPC- and (b) DPPC-liposomes at increasing chloroform concentration.

A comparison of the anaesthetic induced decrease of T_m in DMPC- and DPPC-liposomes is shown in Fig. 6a,b for all the investigated anaesthetics. T_m decreases linearly with increasing anaesthetic concentration for both lipids, but the T_m decrease is stronger in DMPC than in DPPC for halothane, enflurane and chloroform.

3.2. Temperature dependent fluorescence anisotropy measurements

We have also determined the change of the phase transition at increasing concentrations of the selected inhalation anaesthetics with the fluorescence anisotropy method using DPH as the fluorescent probe. As the presence of the anaesthetics in the investigated concentration range did not lead to fluorescence quenching, we concluded that the fluorescence lifetimes of DPH and TMA–DPH are constant.

The results from all the investigated anaesthetics have in common that the phase transition temperature decreases with increasing concentration of the anaesthetic to a comparable extent for both DMPC (Fig. 7a) and DPPC (Fig. 7b) lipid bilayers. The anaesthetic induced effect on T_m is of the same order (halothane > chloroform > enflurane > diethylether > n-pentane) as revealed with the DSC method (Fig. 6a,b).

3.3. Carboxyfluorescein-efflux

First of all we made sure that none of the investigated anaesthetics has any effect on the intensity or spectral distribution of the CF fluorescence due to

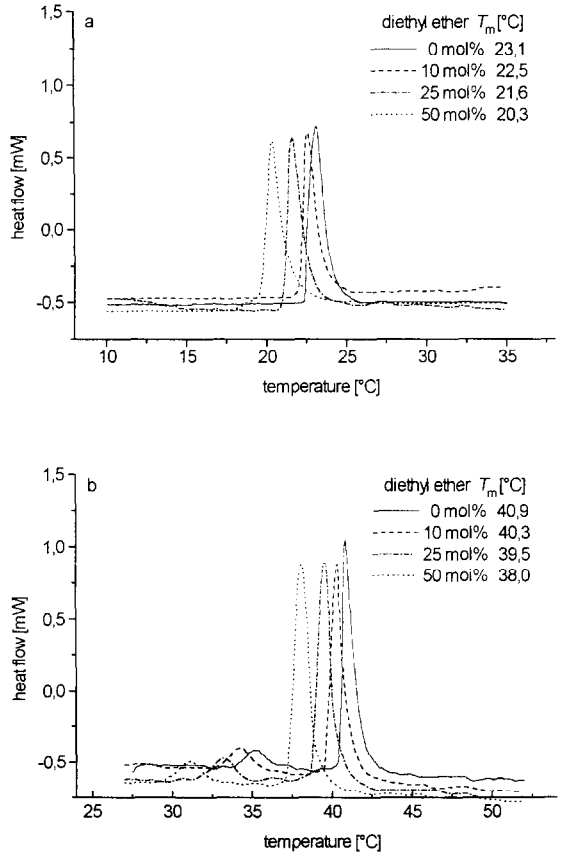

Fig. 4. Original DSC scans with (a) DMPC- and (b) DPPC-liposomes at increasing diethylether concentration.

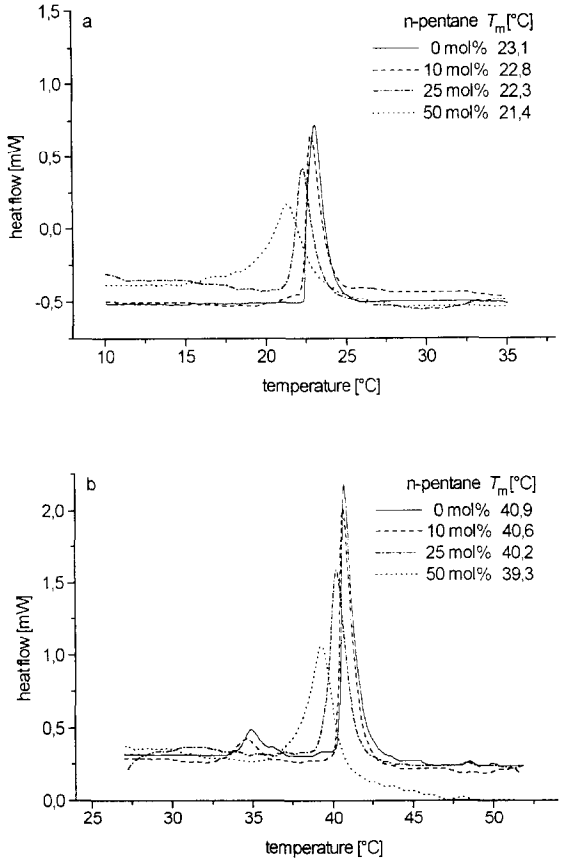

Fig. 5. Original DSC scans with (a) DMPC- and (b) DPPC-liposomes at increasing n-pentane concentration.

anaesthetic-CF interaction. CF-leakage was measured in presence of 10, 20, 30, 40 and 50 mol% anaesthetics in EPC-liposomes without and with 20 or 40 mol% cholesterol. The fluorescence intensity was always measured before and 5 min after the addition of the anaesthetics. Measurements for time periods of several h revealed no further change in the CF fluorescence compared to the control.

In Fig. 8, the CF-leakage at increasing concentrations of the anaesthetics are compared for pure EPC-liposomes (Fig. 8a) and liposomes which contain 20 (Fig. 8b) or 40 mol% (Fig. 8c) cholesterol. Our results show that halothane, chloroform and diethylether have the strongest effect on the CF permeability of the liposomes independently from the cholesterol concentrations in the lipid bilayer. Enflurane and n-pentane are less effective. These differences are more pronounced in liposomes without and with low cholesterol concentration. All the investigated anaesthetics have in common that their effect on CF permeability decreases with the amount of cholesterol incorporated in the lipid bilayer.

3.4. Membrane order

Anaesthetic induced disorder in the lipid bilayer of EPC-liposomes was determined from fluorescence anisotropy of DPH (Fig. 9a) and TMA–DPH (Fig. 9b). Unfortunately, the bromine containing halothane quenches the DPH fluorescence lifetime by more than 50% at the concentrations applied. According to

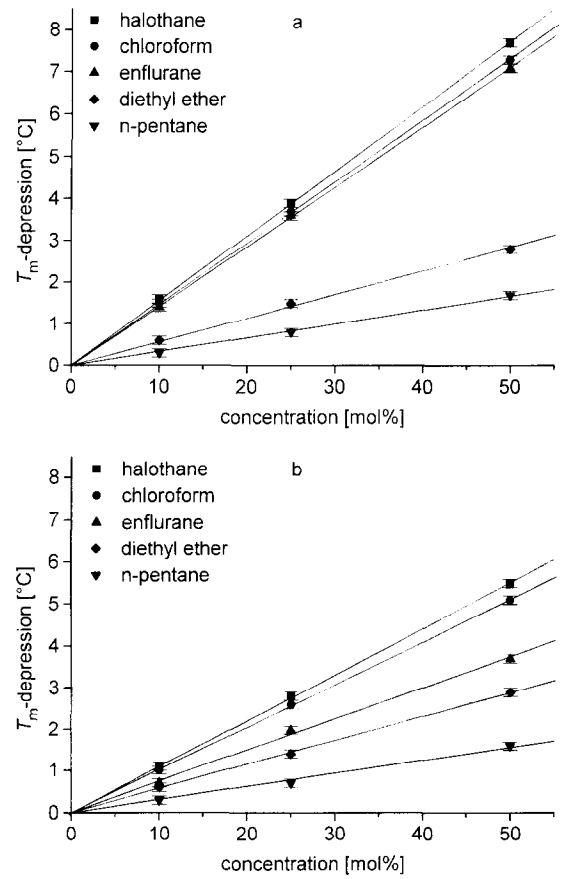

Fig. 6. Comparison of the decrease of T_m in (a) DMPC- and (b) DPPC-liposomes at increasing concentrations of inhalation anaesthetics from the DSC measurements.

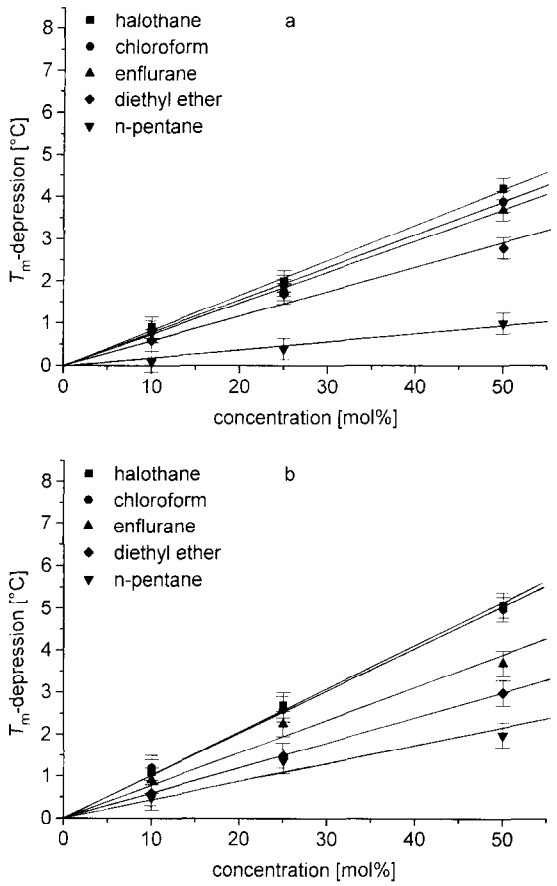

Fig. 7. Comparison of the decrease of T_m in (a) DMPC- and (b) DPPC-liposomes at increasing concentrations of inhalation anaesthetics from the fluorescence anisotropy measurements.

the Perrin equation [27], the calculated anisotropy data increase with decreasing fluorescence lifetime and the measurements reveal misleading results in case of halothane. We therefore omitted the anisotropy measurements without halothane.

The anaesthetic induced decrease of the fluorescence anisotropy is of the same order as the anaesthetic induced CF-efflux. The linear decrease of the fluorescence anisotropy with increasing anaesthetic concentrations is much stronger in DPH-labelled than in TMA–DPH-labelled liposomes. TMA–DPH specifically labels the phosphorus head group region of the bilayer. Our results reveal that the perturbation of the bilayer in the membrane headgroup region is much smaller than in the hydrophobic centre of the fatty acid acyl chains for all investigated anaesthetics. While the diethylether induced disorder in the acyl chain region is only moderate compared to

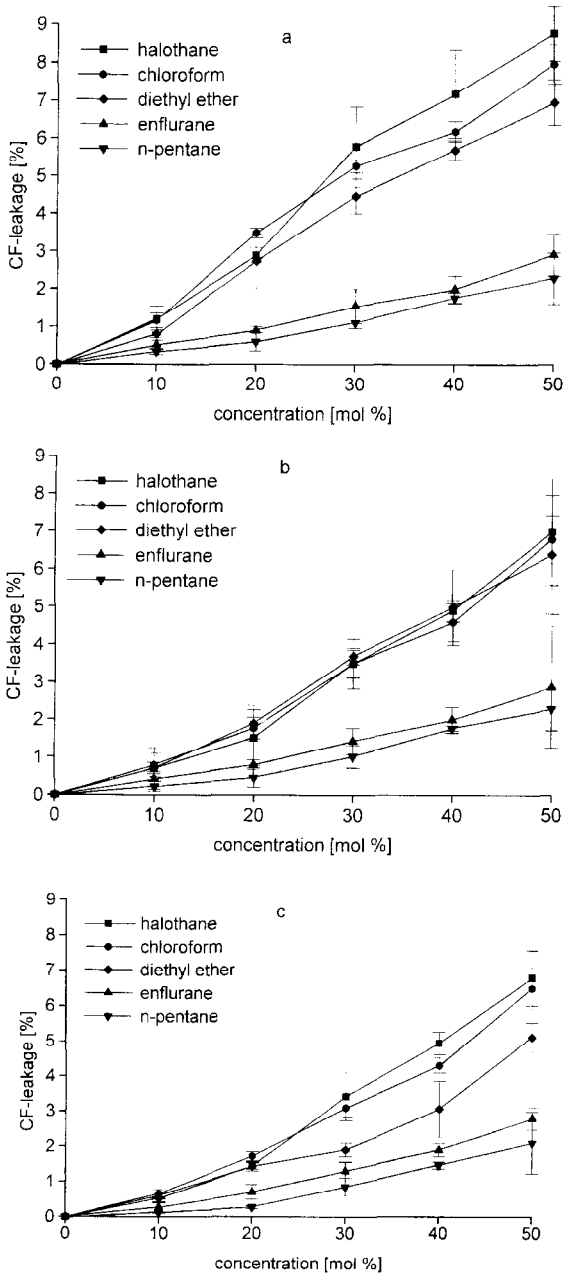

Fig. 8. CF-leakage from liposomes prepared from (a) pure EPC, (b) EPC + 20 mol% cholesterol and (c) EPC + 40 mol% cholesterol in presence of increasing concentrations of inhalation anaesthetics.

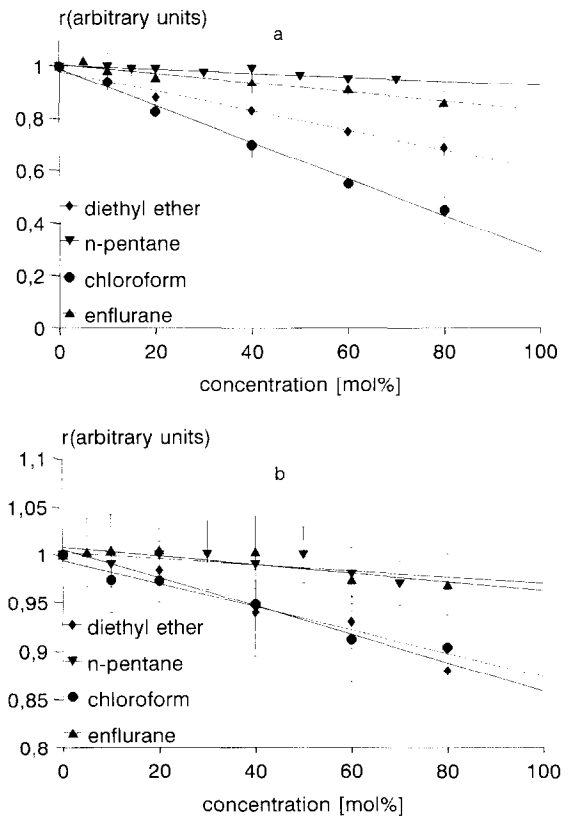

Fig. 9. Fluorescence anisotropy (arbitrary units) measurements in EPC-liposomes at increasing molar anaesthetic to lipid ratio using (a) DPH or (b) TMA–DPH as the fluorescence label.

chloroform, its effect in the headgroup region is of a comparable extent.

4. Discussion

The potency of anaesthetics and other chemical compounds that are used to modify the membrane-associated physiological function is related to their capacity of altering the structure and the dynamics of the lipid bilayer, in particular the degree of order. The alteration of the lipid order, and domain formation due to the presence of drugs in the bilayer is met by a number of dramatic consequences for the macroscopic properties of the bilayer, e.g., to an increased membrane permeability or a shift in the main phase transition temperature. Our results demonstrate that the effects of inhalation anaesthetics on liposomes depend on structural features of the anaesthetics.

The effect of the anaesthetics to decrease T_m at identical molar anaesthetic to lipid ratios is of the following order: halothane, chloroform, enflurane, diethylether and n-pentane. We assume that the extent of T_m depression and the broadening of the phase transition peak can be explained by the structure of these chemicals: Halothane and chloroform are most effective due to their molecular tetraeder structure. Both anaesthetics accumulate in the unpolar region of the acyl chains due to their unpolar character and decrease the lipid–lipid interaction as a result of a free volume expansion. Despite of its higher molecular volume enflurane has a lower effect on T_m than halothane and chloroform. This can be explained by the higher polarity of enflurane, which results in a preferred localisation closer to the phosphorous head group region so that the acyl chain interaction is disturbed to a lower extent. This interpretation is confirmed by the fact that the pretransition observed for DPPC at 35°C vanishes at 10 mol% of halothane or chloroform, but not until 25 mol% enflurane.

After the addition of 25 mol% halothane, chloroform or enflurane a shoulder appears in the phase transition. This result may be explained by a superposition of two phase transitions due to a non-random distribution of the anaesthetic molecules in the lateral plane of the membrane. Jørgensen et al. [28] have analyzed the effect of membrane perturbing drugs like anaesthetics theoretically on the basis of a general microscopic interaction model of the gel-to-fluid chain melting transition of one-component phospholipid membranes and phospholipid membranes with low content of cholesterol. They have shown that the gel-to-fluid transition of the lipid bilayer is strongly influenced by the presence of anaesthetics. Their calculations revealed that anaesthetics have a high affinity to the fluctuating domain interfaces that are dominated by kink-like lipid-chain conformations. This leads to the formation of more interfaces and to locally high concentrations of anaesthetics in the interfacial regions, which is much higher than the global concentration in the membrane.

The depression of T_m is much weaker in presence of both, diethylether and n-pentane (Fig. 6a,b). While

the shape of the phase transition peak is almost unaffected in the presence of diethylether (Fig. 4a,b), n-pentane (Fig. 5a,b) broadens the peak to a comparable extent as enflurane. Because of its polar character diethylether is supposed to accumulate in the headgroup region of the lipid bilayer, where its presence leads to a weakening of the attractive headgroup interaction. Therefore, diethylether has only little effect on the fatty acid acyl chain interaction forces, which are responsible for the phase transition. This interpretation is validated by the fact that the pretransition in DPPC-liposomes remains unaffected even at 50 mol% diethylether (Fig. 4b) and by the membrane order measurements (Fig. 9a,b): The anisotropy data using TMA–DPH as the fluorescent probe show that diethylether disorders the phospholipid headgroup region to the same extent as chloroform does (Fig. 9b), but only moderate disturbance of the bilayer centre is observed (Fig. 9a).

Because of its unpolar character, n-pentane locates around the hydrophobic bilayer centre. The structure of n-pentane resembles that of the fatty acid acyl chains. Due to its flexibility, it intercalates between the acyl chains in the bilayer centre without increasing the free volume of the bilayer to a great extent [29]. Only little decrease of T_m is observed but a broadening of the phase transition resulting from a disturbance of acyl chain interactions.

The influence of the structurally diverse anaesthetics on the main phase transition can be explained by the 'excluded volume interaction' theory [30]: The phase transition of lipid bilayers results from a 'melting' or disordering due to the *trans–gauche*-isomerisation of acyl chains of the lipids [31]. The longer the acyl chain length, the higher the melting temperature because of a smaller relative influence of the phospholipid headgroups. Lipid molecules that are packed into a lipid bilayer are not free to disorder gradually, but because of the close packing the increasing chain rotation with increasing temperature is a cooperative process giving rise to a sharp anomaly, the phase transition. The phase transition of DPPC is narrower and higher than that of DMPC, because the two additional methyl groups lead to an increased rotational cone with a trans-gauche isomerisation and an increased fatty acid interaction. The insertion of the anaesthetic molecules in the hydrocarbon region disturbs this cooperative process of the acyl chains, the lipids exhibit a more gradual disordering, which results in a broadening of the phase transition. In case of an anaesthetic impact in or near to the phospholipid head group region, the decrease in T_m results from a weaker interaction within the different headgroup moieties.

From the depression of T_m coincident with the broadening of the phase transition peak at the same time, we conclude that halothane, chloroform and enflurane distribute in the acyl chain region from C2 to C14 or C16, respectively. This assumption is confirmed for halothane by Koehler et al. [32] and Simon et al. [33]. n-Pentane mainly accumulates in the bilayer centre leading to a broadening of the phase transition peak with little depression of T_m (this paper) in correspondence to the results for n-hexane, which also accumulates in the bilayer centre [34]. In contrast to these unipolar anaesthetics, diethylether preferentially enriches in the phospholipid headgroup region, where it leads to the depression of T_m with no change in the shape of the phase transition peak.

The results of the fluorescence anisotropy measurements correspond to the DSC measurements. In both DPPC- and DMPC-liposomes the addition of the inhalation anaesthetics induce a decrease of the phase transition in the same order (Fig. 7a,b) as determined with the DSC method, but the extent of the T_m depression in presence of halothane, enflurane and chloroform revealed from the anisotropy measurements in DMPC appears to be lower than obtained from the DSC measurements (Figs. 6a, 7a). The difference in both methods can be ascribed to the dependence of the partition coefficients of the anaesthetics from the acyl chain length of the fatty acids. We could not take into account the dependence of the partition coefficient on the fatty acid acyl chain length, because no reliable data do exist. Therefore we have always used the octanol–water partition coefficients [23,24] to calculate the anaesthetic to lipid ratio according to Eq. (1), which gives satisfactory results in case of DPPC. Our results are consistent with the fact, that the partition coefficient decreases with decreasing acyl chain length. The temperature dependence of the partition coefficient does not explain the different results in the DMPC and DPPC liposomes, because the temperature dependences of the partition coefficients are rather

small and of opposite signs. For the DSC measurements, the buffer to lipid ratio is very low. Therefore most of the added anaesthetics accumulate in the lipids and inaccurate partition coefficients reveal little mistake in the calculation of the amount of added anaesthetics. In contrast, little deviations from the correct partition coefficient leads to big mistakes in the calculation of anaesthetic concentration in case of the fluorescence measurements where the assays are diluted to avoid turbidity effects. The coincidence of the results obtained from both methods in case of DPPC reveal that the used octanol–water partition coefficients are in good agreement with the real lipid–water distribution of the anaesthetics, but they are too low for DMPC. The calculation of the real DMPC–buffer partition coefficient from the regression in Figs. 6a, 7a and Eq. (1) revealed, that the partition coefficient is about half the partition coefficient for DPPC in case of halothane, enflurane, chloroform and n-pentane. Diethylether seems to have the same partition coefficient for DPPC and DMPC as we expected from its localisation in the headgroup region of the membrane.

It has been shown that inhalation anaesthetics increase the membrane permeability for protons and ions [18]. Our studies indicate that the anaesthetics also effect the permeability for larger molecules like CF. At very high concentrated CF solutions, the CF fluorescence is selfquenched. In our experiments highly concentrated CF solutions (150 mM) were entrapped in liposomes. The increase of CF fluorescence was therefore diagnostic for the CF-efflux from the EPC vesicle lumen into the buffer. In our experiments, the abrupt release of encapsulated CF was observed within minutes after adding the investigated anaesthetics to the lipid suspension. No further increase of the fluorescence intensity was observed within the next 3 h after the anaesthetic injection. The abrupt release of CF results either from transient membrane holes of morphologically integer bilayers, when the anaesthetics penetrate into the lipid layer from the buffer, or from a partly vesicle fusion or vesicle destruction. Our explanation for the steep release of entrapped molecules is that the asymmetric intrusion of these anaesthetics to the outer monolayer of the lipid membrane results in a different number of anaesthetic molecules in the two half layers generating a membrane tension, which causes a membrane foldover until the anaesthetic molecules have equilibrated across the membrane. It has been shown [35] for various detergents that membrane holes that form during the redistribution lead to a short lived release of entrapped substances. To exclude the possibility of an anaesthetic induced vesicle fusion as the reason for an increased CF fluorescence intensity, we have checked the vesicle fusion kinetics (data not shown). An anaesthetic induced increase of vesicle fusion was measurable only hours after the addition of the anaesthetics.

Cholesterol enrichment in liposomes at 20 and 40 mol% concentration inhibits the CF-efflux to a considerable extent in case of halothane, chloroform and diethylether. Cholesterol is anchored in the phospholipid headgroup region with its sterol rings extended to C10 of the acyl chain region. The antagonistic influence of cholesterol may either result from a decreased partition of the anaesthetics in the bilayer [36,37] or from a mechanical stiffening and increased flexibility of the lipid bilayer, which decreases the number or the lifetime of transient holes.

n-Pentane, enflurane, chloroform and halothane in this order induce an increasing depression of T_m, an increasing CF-efflux and an increasing membrane fluidity. Diethylether, due to its polar character accumulates in the bilayer headgroup region. Therefore, its disordering effect mainly occurs in this region as can be seen from the anisotropy measurements using TMA–DPH, which is anchored in the phospholipid headgroup region. As diethylether leads to a strong effect in both CF-leakage and membrane fluidity experiments, but not in the depression and broadening of the phase transition, we conclude that permeability changes are determined by the degree of order in the headgroup region, while phase transition behaviour strongly depends on the acyl chain region of the bilayer centre.

In summary, our results show that inhalation anaesthetics interacting with the lipid bilayer enhance the dynamic fluctuations in the membrane, which is manifested by a depression of the phase transition temperature and the broadening of the phase transition peak because of a reduced acyl chain cooperation. These observations are in agreement with a concentration dependent increase of the bilayer fluidity and the increase of the passive permeability for CF. A non-random distribution of the

anaesthetic molecules in the lateral plane of the bilayer observed for higher concentrations of chloroform, halothane and enflurane, leads to the assumption of a local concentration that is much higher than the global concentration in the bilayer.

Besides the inhomogeneity of the lateral distribution of the anaesthetic molecules, their transverse distribution and ability to disorder the lipid molecules has to be considered. The present study clearly reveals that the perturbing effect of anaesthetic molecules of different structure on the bilayer depends both on their transverse distribution and the ability to fit between the acyl chains. Such a relation between membrane perturbing effect and chemical structure has also been shown for organic solvents of aromatic, aliphatic and alicyclic structure [38–41].

We have also shown that a membrane component like cholesterol acts as a natural antagonist to decrease the perturbing effect of anaesthetics on the physical properties of the bilayer. The lateral and transverse accumulation of the anaesthetic molecules and the antagonistic influence of natural membrane components must be taken into account when drug induced physiological membrane processes are related to the drug induced modulation of physical membrane parameters like membrane lipid order.

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