How Anesthetics, Neurotransmitters, and Antibiotics Influence the Relaxation Processes in Lipid Membranes

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We used pressure perturbation calorimetry to investigate the relaxation time scale after a jump into the melting transition regime of artificial lipid membranes. This time is equivalent to the characteristic rate of domain growth. The studies were performed on single-component large unilamellar and multilamellar vesicle systems with and without the addition of small molecules such as general anesthetics, neurotransmitters, and antibiotics. These drugs interact with membranes and affect melting points and profiles. In all systems, we found that heat capacity and relaxation times are linearly related to each other in a simple manner, and we outline the theoretical origin of this finding. Thus, the influence of a drug on the time scale of domain formation processes can be understood on the basis of their influence on the heat capacity profile. This allows estimations of the characteristic relaxation time scales in biological membranes.

Introduction

Artificial[1−3] as well biological membranes[1,4,5] display melting transitions. The physiological temperature is at the upper edge of the melting regime of biological membranes. It has been suggested that these transitions are related to nerve pulses[5] and to anesthesia.[6] Such transitions have been found for various bacterial membranes and lung surfactant. They seem to be absent in membranes with high cholesterol content as erythrocyte membranes.

In the melting regime, domains form due to the different hydrophobic length of the ordered and disordered lipids.[7,8] The melting events are obvious in calorimetric profiles where the heat capacity is recorded.[9] It is well-known that the heat capacity is proportional to the fluctuations in the enthalpy.[10] For membrane vesicles, this means practically that in a dispersion with a large number of vesicles each vesicle displays an enthalpy that slightly deviates from the average value. The mean square deviation of the enthalpy from the mean value is proportional to the heat capacity. Likewise, the fluctuations in membrane volume are proportional to the isothermal volume compressibility, and similar statements can be made for other elastic constants like the area compressibility that is proportional to the area fluctuations and the bending elasticity that is proportional to the fluctuations in curvature.[11,12,9] It has been proposed that the permeability of lipid membranes is proportional to the area compressibility because work has to be performed to generate a hole or pore.[13−16] Therefore, also the permeability of membranes is at a maximum close to the heat capacity maximum. The fluctuations in enthalpy are directly related to the amount of lipids in the disordered state and thereby to the total size of the disordered domains. Thus, the heat capacity is easily related to the fluctuations in the total domain size. These fluctuations possess a typical time scale that is the relaxation time. It has been shown that the relaxation time is also directly related to the magnitude of the fluctuations in the enthalpy and thereby to the fluctuations in domain sizes.[17]

A whole variety of molecules like proteins, peptides, or other small molecules influence melting transitions. The present view of biological membranes sees them as being rather heterogeneous[18] instead of being homogeneous fluids as suggested in the fluid-mosaic model.[19] Heterogeneities of biological membranes due to lipid domain formation have been postulated for many years.[20] Domain formation has gained a wider interest since the discussion about "rafts"[21,22] that have been suggested to consist of liquid-ordered clusters of sphingolipids, cholesterol, and some GPI-anchored proteins. Rafts are thought to be involved in trafficking processes.[23] Domain formation in general is believed to trigger biochemical reaction cascades and to influence enzyme activity.[24−28]

Domains can form as a consequence of melting transitions. Already in the 1970s, melting transitions were measured in artificial[2,3] and in biological membranes[4,29] using differential scanning calorimetry. The increased fluctuations in the melting regime of membranes are accompanied by changes in the timescales of the domain formation process. Studies on the kinetics of phase transitions in artificial membranes mainly use temperature and pressure jump techniques[17,30−36] but also volume perturbation,[37−41] pH or ion concentration changes,[42] ultrasonic measurements[43,44] and ac-calorimetry[45] were applied. The number of relaxation processes in different studies varies from one to five. Relaxation times in single-lipid membranes were reported to lie in the range from ns to min,[46] depending on the experiment and the detection method. Most studies agreed on a slowing down of relaxation processes in the transition regime.[17,30,32−34,38,39,43,44,46] It was also pointed out that maximum relaxation times were observed at the transition midpoint. In ref 17, relaxation processes were connected to macroscopical fluctuations, and it was shown that the relaxation times of the cooperative processes and the heat capacity are connected in a simple manner.

In earlier studies, an influence of cholesterol, dibucaine and peptides was found[17,36,41,46] but not systematically investigated. Molecules like general anesthetics, neurotransmitters, and

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antibiotics display a functional role in biological cells. The antibiotic gramicidin A is a hydrophobic, channel-forming peptide.[47] Dimers of this peptide induce channels of an outer diameter of about 5 Å that is mainly permeable for monovalent cations. Neurotransmitters are molecules which mainly occur in the nerve system but can also be found in other parts of the body. They influence nerve pulse propagation and are either inhibitory or excitatory. An example of an excitory neurotransmitter is serotonin (5-hydroxytryptamine). Neurotransmitters have been discussed to also act as anesthetics.[48] Anesthesia is the state when pain, consciousness, or other sensations are blocked. General anesthetics such as 1-octanol or halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) lead to a reversible complete loss of consciousness and sensation. The action of general anesthetics is still not fully known. Theories favoring a direct influence on protein function are at the moment favored,[49−51] but lipid membrane mediated mechanisms are also discussed. Ueda and Yoshida[52] claim that anesthetics action results from effects on both proteins and lipids and they consider the lipid/water interface. Cantor[53,54] relates anesthetic function to the influence of anesthetics on the lateral pressure profile of lipid membranes. Heimburg and Jackson[6,55] attribute anesthetic action to the influence of anesthetics on the lipid membrane state.

Peptides, neurotransmitters, and anesthetics all display a significant influence on lipid membrane melting transitions. This immediately raises questions about these molecules on the macroscopic membrane properties. In this paper, we focused on the influence of anesthetics, neurotransmitters, and antibiotics on the time scales of domain formation processes. This is an extension of a previous study by Grabitz and collaborators.[17] Relaxation processes are related to the cooperative fluctuations. More specifically, it was shown that heat capacity and relaxation times in pure lipid membranes are proportional functions. Here, the study is extended toward lipid systems with the incorporation of drugs. It will be shown that the addition of these molecules does not change the linear relation between heat capacity and relaxation times but systematically alters the details of the relaxation process.

Materials and Methods

Sample Preparation. Lipids were purchased from Avanti Polar Lipids (Birmingham/AL) and used without further purification. All lipid samples were dissolved in a 10 mM Hepes buffer with 1 mM EDTA at pH 7. Samples of multilamellar vesicles (MLVs) were prepared by adding buffer to the lipid powder and stirring the solution above the main phase transition temperature using a magnetic stirrer for at least 1 h. During this time the lipid solution was vortexed at least three times. Suspensions of large unilamellar vesicles (LUVs) were prepared using an Avestin extruder system (Avestin Europe GmbH, Mannheim, Germany). Suspensions of MLVs were extruded at 320.9 K using a filter with a pore size of 100 nm. DMPC/neurotransmitter systems were made by adding serotonin (hydrochloride; Sigma-Aldrich Inc., St. Louis/MO) to the buffer before dissolving the lipids in the buffer/neurotransmitter solution. In the case of DMPC/anesthetics solutions, 1-octanol (Sigma-Aldrich Inc., St. Louis/MO) or halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, Sigma-Aldrich Inc., St. Louis/MO) was added to the already prepared suspension of MLVs. The solution was stirred for another 30 min. DMPC/peptide membrane systems were prepared by dissolving the DMPC lipids and gramicidin A as a powder in organic solvent (dichloromethane:methanol 2:1). After mixing both solutions, the lipid/gramicidin A solution was dried through heating and a light nitrogen or air stream. The sample was kept in a high vacuum desiccator overnight. The rest of the preparation equaled the one of preparing suspensions of MLVs.

Calorimetry. Differential scanning calorimetry (DSC) measurements were performed with a VP−DSC from Microcal (Northhampton/MA) using high feedback mode at a scan rate of 5 K/h if not indicated otherwise. A concentration of 10 mM was used to measure the excess heat capacity profile of a sample directly in the calorimeter cell and determining the transition enthalpy. In other cases, sample solutions were filled into self-built pressure cells (see below). In these cases, concentrations of either 40 or 50 mM were prepared. The capillary volume was not exactly known, but with the knowledge of the transition enthalpy, we could determine absolute excess heat capacity values. Heat capacity profiles of DMPC/gramicidin A mixtures were determined with scan rates of 1 K/h. Due to hysteresis effects the scan rate has an influence on the temperature of the maximum heat capacity. Curves were corrected correspondingly. Sample solutions and buffer solutions were degassed for at least 15 min before the calorimetric experiments to avoid measurement artifacts arising from possibly evolving gas bubbles.

Pressure Perturbation Calorimetry. The same calorimeter was used for pressure perturbation calorimetry (PPC) experiments in the isothermal mode of the calorimeter. Measurements on solutions of MLVs were performed as previously described.[17] The sample solution was filled into a self-built pressure capillary which can hold pressures up to 500 bar. This capillary was inserted into the sample cell of the calorimeter. The melting transition regime shifts upon addition of hydrostatic pressure by about 1 K/40 bar (see refs 56 and 57 with citations therein). This is used to perturb the system. Pressure is controlled through addition or release of high-pressure nitrogen by opening and closing manual or solenoid pressure valves (Nova Swiss, Effretikon, Switzerland). The change of pressure occurs on a time scale of around 100 ms. Changing the pressure on the lipid dispersions by ±40 bar and an appropriate choice of temperature allowed us to jump to different points of the phase coexistence regime where domains started to form. Jumps into the transition regime were either from below the transition (−40 bar; negative pressure jump) or from above the transition (40 bar; positive pressure jump). In the first case, the signal is endothermic, whereas in the other case, it is exothermic. After a pressure change, the calorimeter needs to compensate the heat absorption or release to keep the sample temperature constant. This response contains information about the relaxation times of the domain formation process. Representative calorimetric responses after negative pressure jumps (−40 bar) are shown in the left panel of Figure 1 (top). The curves are results after jumps to different points of the transition defined by different sample temperatures. The area below the curves, i.e., the total heat absorption of the lipid membranes during the equilibration process, increases the further one jumps through the transition (here, increasing temperature). The normalized signals show that the relaxation process occurs on different time scales (Figure 1, bottom). A sample cell containing buffer solution also results in a characteristic response. This is the response function of the instrument. The observed calorimetric signal from the equilibration process is the convolution of the real signal (called the "sample response") with the instrument response function. The response after a pressure jump always contained one small component that contains the perturbation of the pressure cell itself and small contributions from lipid membrane processes faster than the resolution of the instrument. The dominant part of the signal consists of a single exponentially decaying heat

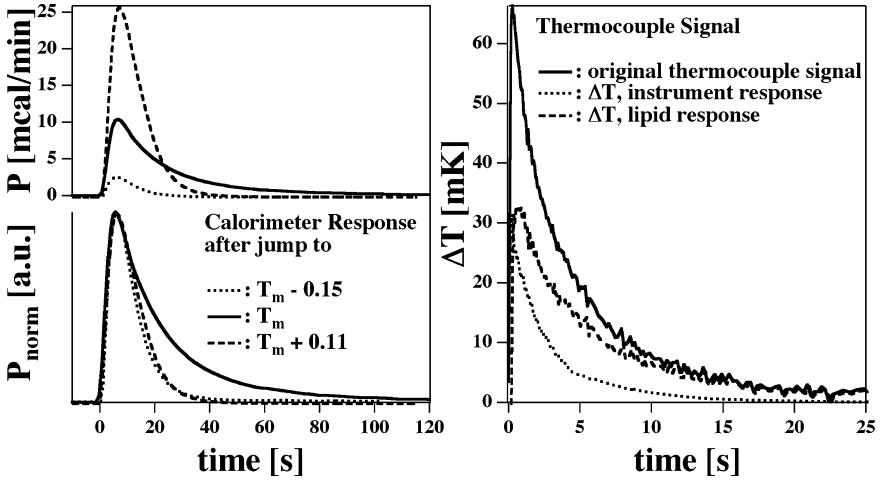

Figure 1. Left: Representative calorimeter responses after a negative pressure jump. The curves were obtained after jumps to different temperatures within the lipid melting transition of DMPC vesicles. The further one jumps through the transition (for negative pressure jumps the higher the temperature) the bigger is the integrated heat absorption of the lipid membrane (left, upper panel). After normalization of the curves it can be seen that they decay on different time scales (left, lower panel). Right: Representative temperature change after a positive pressure jump recorded by a thin thermocouple. The total response is the sum of the response of the water filled capillary (instrument response) and the response from the lipid sample (lipid response).

absorption or release from the lipid membranes after a perturbation. Faster relaxation processes only contribute to a minor degree to the overall signal (see the discussion concerning Figure 6). The above method allows a time resolution of about 3−4 s.

The relaxation times of lipid dispersions are related to the heat capacity. Broader melting profiles display smaller heat capacity values in the transition. Under these conditions, faster relaxation processes are found [17] for which the resolution of 3−4 s may not be good enough. For this reason, a refined experimental setup with increased time resolution was used especially for measurements on LUV dispersion that display melting peaks that are about 10 times broader than the MLV transitions. We only performed positive pressure jumps. The pressure release or addition was controlled by two computer controlled solenoid valves (Nova Swiss, Effretikon, Switzerland), and the time scale of the pressure relaxation was faster than 90 ms. The pressure cell consisted of two capillaries instead of only one. A K-thermocouple with a grounded hot junction of a diameter of 0.25 mm (Thermocoax, Stapelfeld, Germany) was put into the sample capillary of the pressure cell. Relaxation times were determined from a direct measurement of changes in temperature of the sample solution after the pressure jump. The thermocouple signal was amplified by a Nanovolt Preamp model 1801 (Keithley Instruments Inc., Cleveland, OH) and recorded by a Keithley Multimeter 2001 (Keithley Instruments Inc., Cleveland, OH).

As in the case analyzing the calorimeter response the total temperature change is seen as a convolution of the "instrument response" and the "sample response". This is displayed in the right panel of Figure 1. The total temperature change (solid curve) after a positive pressure jump can be divided into a change due to the "instrument response" (dotted curve) and the "lipid response" (dashed curve). The latter one is again modeled by a convolution of a single exponentially decaying heat release of the lipid membrane and the instrument response. For details we refer to refs 17 and 58. The contribution from the sample cell (only filled with water) was subtracted from the total temperature change, and only the remaining signal was analyzed.

Pressure perturbation calorimetry experiments were always conducted on a few successive days. Melting profiles were controlled after each measuring day. During these days heat capacity curves sometimes broadened slightly or shifted toward higher or lower temperatures. The shift could be easily corrected later. Below it will become obvious that a broadening of the curve means that relaxation times are influenced. The broadening, however, was small so that the determination of relaxation times was in the range of experimental error.

Theory

In an earlier paper from our laboratory, a theory of the relaxation times of lipids in the melting regime was derived on the grounds of nonequilibrium thermodynamics.[17] This theory made use of the experimental fact that enthalpy, volume and area are proportional functions in this temperature regime. Therefore, perturbation of the lipid samples by temperature, bulk or lateral pressure results in the same relaxation process. It was found that one expects a proportional relation between relaxation time and excess heat capacity.

The theory is based on the assumption that the distribution of enthalpy states at a given temperature can be described by a Gaussian distribution. This is correct when the system displays a continuous transition that is neither of first-order nature nor at a critical point

$$P(H - \langle H\rangle) = \frac{1}{\sigma\sqrt{2\pi}}\, e^{-(H-\langle H\rangle)^2/2\sigma^2} \qquad (1)$$

where H is the enthalpy, $\langle H\rangle$ is the mean enthalpy, and $\sigma^2 = \langle H^2\rangle - \langle H\rangle^2$ is the variance. The Gibb's free energy depends logarithmical on this distribution:[59] $G = RT \ln P$. Using this, one finds that the entropy S can be approximated as a harmonic potential

$$S(H - \langle H\rangle) \approx -\frac{R(H - \langle H\rangle)^2}{2\sigma^2} \qquad (2)$$

where R is the gas constant.

Throughout a melting transition, changes in area, volume, and enthalpy are proportional, and therefore, one only finds one independent fluctuation given by $\alpha = H - \langle H\rangle$. The thermodynamic force driving the lipid sample back to equilibrium (after a perturbation) is given by $X = dS/d(H - \langle H\rangle)$, and the flux of heat by $J = d(H - \langle H\rangle)/dt$. This is in fact the observable in our

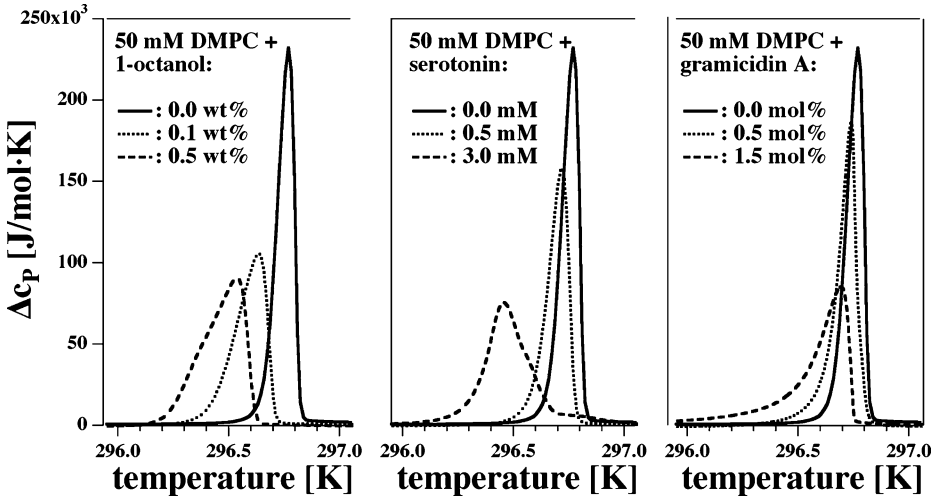

Figure 2. Heat capacity profiles of various multilamellar lipid dispersions in the presence of drugs. Left: DMPC plus various concentrations of 1-octanol. Center: DMPC plus different concentrations of serotonin Right: DMPC plus different concentrations of gramicidin A. In all three panels the drugs broaden the lipid melting profile and shift it to lower temperatures.

calorimetric experiments. According to Onsager,[60,61] the flux is proportional to the thermodynamics force. Therefore

$$\frac{\mathrm{d}(H - \langle H \rangle)}{\mathrm{d}t} = -L\frac{R(H - \langle H \rangle)}{\sigma^2} \tag{3}$$

using the phenomenological constant L. This leads to a simple differential equation that is solved by an exponential decay

$$(H - \langle H \rangle) = (H - \langle H \rangle)_0 \exp\left(-\frac{t}{\tau}\right) \tag{4}$$

introducing the relaxation time τ. Using the identity $c_\mathrm{p} = (H - \langle H \rangle)^2/RT^2 \equiv \sigma^2/RT^2$ (fluctuation theorem), one arrives at

$$\tau = \frac{T^2}{L}c_\mathrm{p} \tag{5}$$

where T is the temperature. The constant L has to be obtained from experiments. For the details of the derivation, we refer to ref 17. Note, however, that in the cited paper a factor of RT was omitted in the derivation so that an unit error occurred. This, however, does not change the message of the previous paper that relaxation times and heat capacity are proportional functions.

Results

The nonequilibrium thermodynamics theory briefly outlined above predicts a proportionality between heat capacity and relaxation times for the cooperative processes with large excess heat capacity. Important for this prediction is the cooperative nature of the process rather than the chemical composition of the membrane. In ref 17, it is shown for different one component lipid systems (MLV and LUV) and a DMPC/cholesterol mixture (MLV) that one finds a linear relationship between heat capacity and relaxation times in pressure perturbation calorimetry. Maximum relaxation times are up to about a minute and they are decreased by the addition of cholesterol in a manner closely related to the effect of cholesterol on the heat capacity profile. In the present study, we extended the range of the membrane systems. In particular, we investigated the influence of small drugs on domain formation processes and the related relaxation times. For this it was necessary to improve the time resolution of our calorimetric relaxation measurements. The latter part is important for large unilamellar membrane systems that display smaller heat capacities and broader transitions than multilamellar preparations.

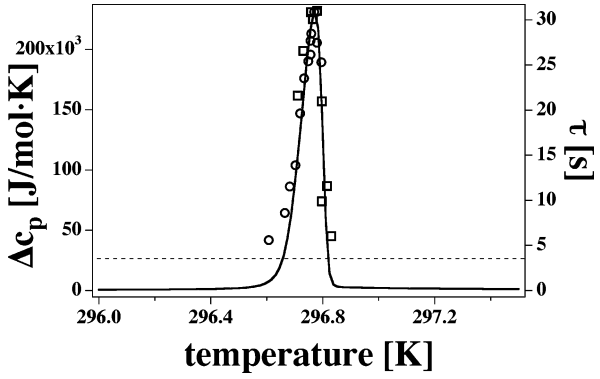

Figure 3. Heat capacity curve (solid curve) of a 50 mM multilamellar DMPC sample and relaxation times (open circles and squares) obtained from pressure perturbation experiments on the same sample as measured by the calorimeter. The different markers refer to different experimental situations where pressure is released (open circles) or added (open squares). The change in relaxation times correlates nicely with the heat capacity profile. The dashed line indicates the lower resolution limit of the experiment (3−4 s).

First, we determined the influence of molecules such as general anesthetics, neurotransmitters and antibiotics on the melting behavior of lipid membranes by differential scanning calorimetry. In Figure 2, the heat capacity profiles of 50 mM multilamellar DMPC vesicles with different concentrations of 1-octanol (left panel), serotonin (center panel), and gramicidin A (right panel) are displayed. The transition enthalpy of these systems was determined to be 21.5 kJ/mol. The general finding is that the addition of the respective molecules broadened the transition profile and shifted it to lower temperatures. The total melting enthalpy remains unaltered. In the following, we wanted to understand in which way relaxation processes were influenced in the respective systems.

In Figure 3, heat capacity profiles and relaxation times of a 50 mM DMPC (MLV) solution are displayed. The heat capacity curve shown is the same as in Figure 2. Relaxation times were determined from measurements using the same lipid dispersion as for the DSC scans. The melting transition profile of lipid membranes with increased pressure was shifted to higher temperatures, whereas the shape of the profile was not influ-

enced. Therefore, the relaxation time profiles obtained from positive pressure jumps were temperature corrected with $\Delta T = -0.9$ *K* (open squares). Results from negative pressure jumps are given by open circles. The time resolution of the setup is indicated by a dashed line. Relaxation times faster than this experimental response time cannot be determined accurately.

Comparison of heat capacity and relaxation times shows the linear relation of these two functions. In the proximity of the melting transition, relaxation processes slow down. The maximum relaxation time measured is about 31.0 ± 1.5 s. With a maximum heat capacity value of 232 kJ/mol K and the temperature at the transition midpoint of 296.77 K, we found a phenomenological constant (see eq 5) of $(6.6 \pm 0.5) \times 10^8$ JK/mol s. The previous experiment was performed while recording the heat compensation of the calorimeter (with a time resolution of about 4 s). Performing experiments on a new suspension of 40 mM DMPC (MLV) and evaluating the temperature change with the thermocouple inserted into the pressure cell (with a time resolution of about 0.3 s) after a positive pressure jump of +15 bar yielded $L = (6.8 \pm 0.3) \times 10^8$ JK/mol s. Both *L*'s obtained agree within error with each other. This demonstrates that our two different setups record the same time scales.

In the following, we investigated in which way other small molecules and peptides influence the relaxation behavior of lipid membranes. Therefore, we performed experiments adding different concentrations of the anesthetic 1-octanol, the neurotransmitter serotonin and the antibiotic gramicidin A to the DMPC lipid membranes. As seen in Figure 2 these molecules broadened the melting transition regime and shifted it to lower temperatures. This has also an influence on the relaxation behavior of the lipid membranes. In Figure 4, measurements on the three different systems are displayed. Heat capacity profiles are taken from Figure 2. Results were obtained from analyzing the calorimetric response after jumps of ±40 bar. Results from negative and positive pressure jumps are not indicated by different symbols. The dashed line always indicates the time resolution of the setup. Both heat capacity and relaxation time scale of the various panels are different. In all cases, we found a proportionality between heat capacity and relaxation times. Phenomenological constants, *L*, were calculated to be $(6.3 \pm 0.3) \times 10^8$ JK/mol s (DMPC plus 0.1 wt % 1-octanol), $(6.5 \pm 0.5) \times 10^8$ JK/mol s (DMPC plus 0.5 mM serotonin), and $(5.8 \pm 0.5) \times 10^8$ JK/mol s (DMPC plus 1.5 mol % gramicidin A); that is, within error, *L* is independent of the system. Note that deviations close to 3−4 s are most likely a consequence of the finite time resolution of the experiment.

A series of PPC experiments were performed using different concentrations of the respective molecules (data not shown). The maximum relaxation times obtained and the phenomenological constants determined are displayed in Table 1. As it is seen in Figure 2, the addition of the chosen small drugs led to a broadening of the heat capacity curves and to a decrease in the maximum heat capacity value. The maximum relaxation time was influenced in the same way as is the maximum heat capacity value. The whole relaxation time profile behaves the same as does the heat capacity curve. The addition of molecules alters the relaxation behavior of lipid domain formation in a systematic way.

Further experiments were conducted on solutions with large unilamellar vesicles. We investigated LUVs of DMPC lipids with and without addition of the anesthetic halothane. We also studied LUVs of DPPC. The respective heat capacity values and relaxation times are plotted in Figure 5. Relaxation times were obtained using a thermocouple inserted into the pressure cell using positive pressure jumps. The dashed line represents the time resolution of the setup. In all cases, we found again that heat capacity and relaxation times are proportional to each other. Halothane has a similar effect on the melting behavior as the anesthetic octanol. It broadened and shifted the heat capacity curve to lower temperatures. The maximum relaxation time was again decreased compared to that of the DMPC LUVs without halothane. The phenomenological constants for the LUV systems are somewhat higher than those of the MLVs (see Table 1). The L s obtained from measurements on MLVs suggest that there is a constant independent of the lipid membrane composition. The same is true for LUVs, while we calculated different

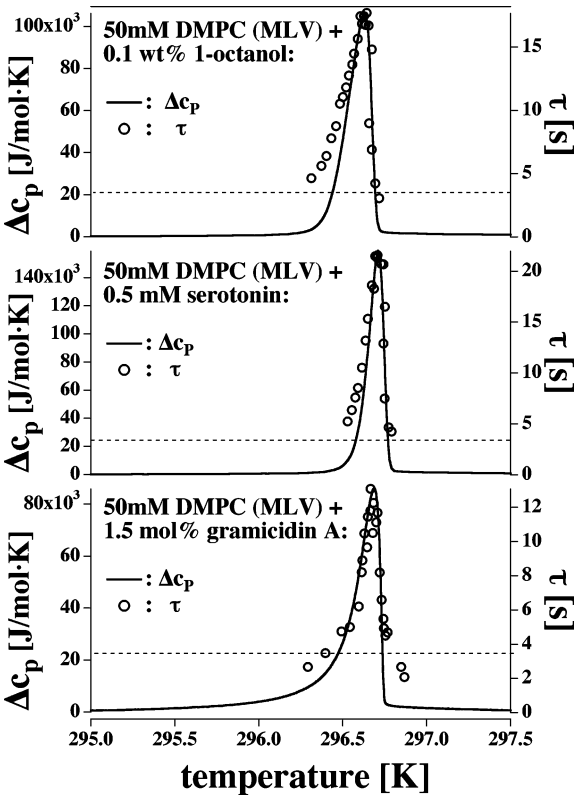

Figure 4. Comparisons between heat capacity profiles (solid curves) and relaxation times (open circles) of different multilamellar DMPC systems. Top: 50 mM DMPC plus 0.1 wt % 1-octanol. Center: 50 mM DMPC plus 0.5 mM serotonin. Bottom: 50 mM DMPC plus 1.5 mM gramicidin. The drugs alter the temperature dependence of the heat capacity and the magnitude and temperature dependence of the relaxation times. The dashed line indicates the resolution limit of the experiment (3−4 s).

TABLE 1: Relaxation Times and Phenomenological Constants at the Transition Midpoint of the Studied Systems[a]

lipid membrane system	τ_m/s	$L/(10^8$ (JK/mol s))
DMPC (MLV)	31.0 ± 1.5	6.6 ± 0.5
+ 0.1 wt% 1-octanol (MLV)	17.7 ± 0.4	6.3 ± 0.3
+ 0.5 wt% 1-octanol (MLV)	14.8 ± 0.2	6.3 ± 0.3
+ 0.5 mM serotonin (MLV)	21.6 ± 1.2	6.5 ± 0.5
+ 3.0 mM serotonin (MLV)	9.0 ± 0.9	7.5 ± 0.8
+ 0.5 mol% gramicidin A (MLV)	20.8 ± 0.8	7.9 ± 0.5
+ 1.5 mol% gramicidin A (MLV)	13.1 ± 0.8	5.8 ± 0.5
DMPC (MLV)	25.2 ± 0.1	6.8 ± 0.3
DMPC (LUV)	1.5 ± 0.2	15.7 ± 3.0
+ 20 wt% halothane (LUV)	1.0 ± 0.1	20.0 ± 1.0
DPPC (LUV)	2.0 ± 0.1	18.4 ± 1.0

[a] In the upper parts relaxation times were determined from the calorimeter response. In the lower part they were obtained from the temperature change curve in the sample capillary.

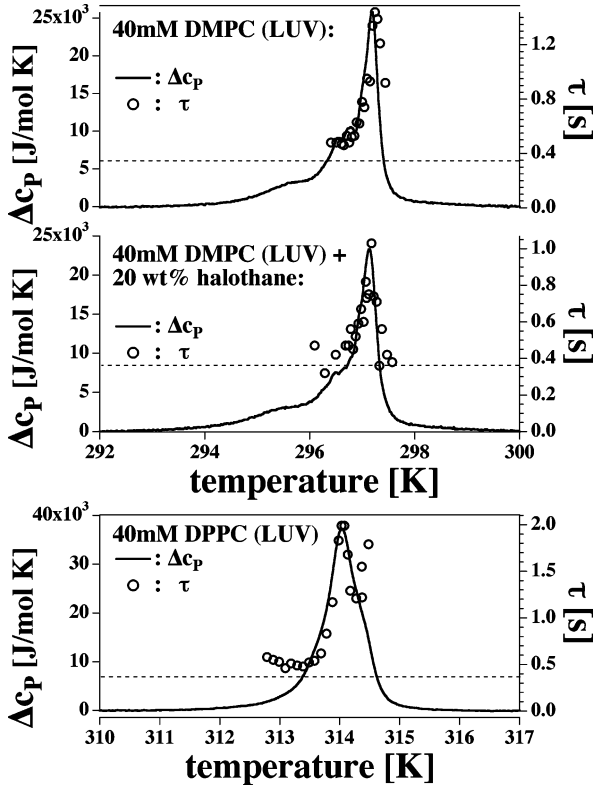

Figure 5. Heat capacity profiles (solid lines) and relaxation times (symbols) of three different large unilamellar vesicle systems. Relaxation times were recorded with a thin thermocouple. Top 40 mM DMPC. Center: 40 mM DMPC plus 20 wt% halothane. Bottom: 40 mM DPPC. Again a proportionality between heat capacity and relaxation times was found. Halothane acts similar on the lipid membrane as 1-octanol. The dashed line indicates the resolution limit of the experiment (around 350 milliseconds).

phenomenological constants for MLVs and LUVs. From our results, we calculated averaged L's of $(6.7 \pm 0.7) \times 10^8$ JK/mol s (MLVs) and $(18.0 \pm 2.2) \times 10^8$ JK/mol s (LUVs). The reason for this is unknown. One can speculate on whether relaxation processes are influenced by volume changes of the vesicles in the case of LUV or by bilayer−bilayer interactions in the case of MLV.

The linear relation between heat capacity can also nicely be seen when plotting the relaxation time versus the heat capacity for all experiments shown here (graph not shown).

In the literature, up to five relaxation processes in the time range from ns to s were reported for one lipid component systems.[46] In this study, we assumed only one relaxation time, which describes domain formation processes. In order to test whether there are contributions from faster relaxation processes, we analyzed the heat absorption after negative pressure jumps as a function of temperature in comparison to the transition enthalpy obtained from scanning calorimetry. The transition enthalpy of the DMPC (MLV) solution (see the heat capacity profile in Figure 2) is shown as a solid curve in the left and right panel of Figure 6. The total heat absorption is displayed in the left panel of Figure 6. It is proportional to the transition enthalpy. All relaxation data contained contributions of a fast component with a time scale corresponding to the instrument response time. The heat absorption due to this component also showed a proportionality to the transition enthalpy (right panel of Figure 6). This means that at least one faster relaxation process is present which occurs on a time scale faster than our experimental time resolution. Analyzing all measurements, we found that the contribution of faster relaxation processes is lower than 10% of the total heat absorption. Using the thermocouple signal as a detection method we did not detect faster relaxation processes.

In total, we can state that domain formation processes are related to fluctuations in enthalpy at large excess heat capacities. Relaxation times and heat capacity are linear functions (within error). This linear relation is still true in the presence of drugs. However, the temperature dependence of both heat capacity and relaxation times are altered by drugs in a simple systematic manner. Fast relaxation processes contribute less than 10% to the overall heat. Thus, they probably do not represent the fluctuations in domain size linked to the lipid chain state.

Discussion

The melting of lipid membranes is a cooperative event involving collective changes of many lipid molecules. Within the melting transition one finds domain formation, but also changes in the elastic constants. If pressure, temperature, or any other relevant intensive thermodynamic variable are slightly changed, the lipid system changes its state and domains grow or shrink while absorbing or releasing significant amounts of heat. Thus, the domain formation process can be followed by monitoring the heat change. If we talk about relaxation times within the lipid melting transitions, we therefore basically refer to the time scale of domain growth. These processes have been studied by some groups before using calorimetry.[38,39,40,41,17] One typically finds slow relaxation close transitions. For the case of critical points, this phenomenon is known as "critical slowing-down".

Studies of relaxation processes in artificial membranes by use of pressure perturbation calorimetry have been performed by our group before.[17] We used the same and a refined version of the setup as described in ref 17 to extend the previously published studies. Relaxation processes in LUVs and the influence of small drugs on the relaxation behavior of lipid domain formation in LUVs and MLVs were investigated. The typical time scales of domain formation processes are related to cooperative fluctuations in enthalpy, and in all systems, we found a proportionality between heat capacity and relaxation times. The relaxation processes depend on the final state rather than on the initial state. A remarkable finding is that the drugs used modulate the time-scale of domain formation processes in a similar manner as they influence the heat capacity profiles. They tend to broaden both heat capacity profiles and relaxation time profiles. After addition of anesthetics, neurotransmitters, and antibiotics, the maximum relaxation time is decreased in the same way as is the maximum heat capacity value. Therefore, these drugs alter static and kinetic aspects of domain formation.

Our findings support the theoretical prediction from Grabitz et al.[17] that heat capacity and relaxation time display a linear relation not only for pure lipid membranes but also for membrane mixtures and mixtures with drugs that associate with membranes. The proportional constant between relaxation time and heat capacity takes the form T^2/L, where L is a phenomenological coefficient originating from Onsager's phenomenological equations. We calculated average L's to be $(6.7 \pm 0.7) \times 10^8$ JK/mol s for MLVs and $(18.0 \pm 2.2) \times 10^8$ JK/mol s for LUVs made of DMPC and DPPC. The constant L is different for MLV and LUV by about a factor of 2.5. Surprisingly, however, the addition of drugs does not change the L for a given vesicular system even though they have significant influence on the heat capacity profiles. With measurements on suspensions of multilamellar DMPC vesicles we obtained L s of 6.6×10^8 and 6.8×10^8 JK/mol s using the two different detection methods. They agree within error. Thus, the difference in result

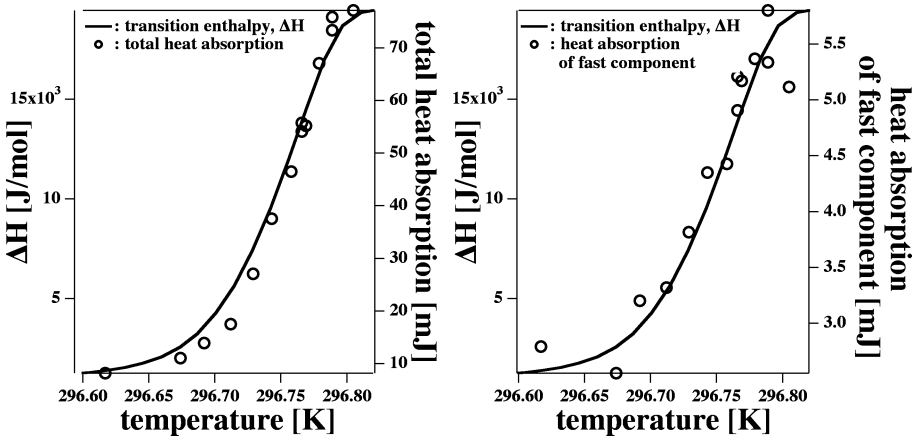

Figure 6. Solid curve in the left and right panel show the transition enthalpy of DMPC multilamellar vesicles as obtained from integrating the excess heat capacity profile. The total heat compensation of the calorimeter after a pressure jump from below the transition to a given point within the transition is proportional to the transition enthalpy (circles, left panel). The signal typically contains one single-exponential slow component and a minor contribution from fast processes that show up as a signal with the characteristic time constant of the calorimetric setup. The contribution from the fast processes (circles, right panel) also displays a proportionality to the integrated heat. This means that there is at least one relaxation processes faster than the time resolution of our experiment. Its contribution, however, is less than 8% of the overall signal. Thus, the relaxation process of the cooperative events is well approximated by a single relaxation process.

does not seem to be related to the method of detection. We do not know the origin the difference between MLV and LUV. However, MLVs display much more cooperative transitions because of coupling between adjacent membranes. It has been shown in the literature that in MLVs one finds spacial correlations of domains not only in the bilayer planes but also in the third dimension between adjacent bilayers.[62] The fluctuations therefore contain a coupling in the third dimension that might cause small differences in the relaxation behavior. The difference may also be related to the change of vesicle size while changing the lipid membrane state and area. This may involve permeation of water into the vesicles that influence the rates. It may be interesting for future studies to investigate vesicles with different diameters.

Anesthetics, neurotransmitters, and antibiotics influence the melting behavior of lipid membranes. The anesthetic octanol shifts profiles to lower temperatures. Long chain alcohols (with chain length larger than 10) shift them to higher temperatures.[63] The latter ones do not display anesthetic potency. Other anesthetics like halothane (this study and unpublished data) or methoxyflurane[64] also lead to a decrease of the melting temperature. In fact, a depression of the melting point has well been documented for all general anesthetics[6] and also for local anesthetics like tetracaine (e.g., ref 65). It has been stated that clinically relevant anesthetics concentrations show effects on the shift of melting temperatures.[64] The effective potency of an anesthetic is correlated to its ability to deplete melting transition temperatures.[66] Serotonin possibly also acts as an anesthetic[48] and influences the phase melting transition. This has also been shown for other neurotransmitters, like GABA (γ-aminobutyric acid), dopamine and fluoxetin,[67] or similar drugs like caffeine that interact with neuroreceptors. The interactions with peptides or proteins and other small molecules like anesthetics and neurotransmitters influence the domain structuring of lipid membranes in the phase melting transition regime.[68] All these molecules influence relaxation processes in a systematical way.

In previous studies kinetic aspects of melting transitions in artificial single-component lipid membranes have been studied with a whole variety of different techniques. This study, however, is the first one systematically investigating the effects of various small drugs on relaxation processes in single lipid membranes and relating it to the enhancement of fluctuations in the transition regime. Van Osdol and co-workers[41] studied the effect of the anesthetics dibucaine on relaxation processes. They claimed that even though dibucaine clearly shows an influence on the melting behavior only a small alteration of relaxation processes has been found. This finding does not agree with ours, where we have found that the drugs influence relaxation processes similar to their influence on the heat capacity.

In other studies relaxation times faster than the ones we measured and up to five relaxation processes were reported.[30−32,34,35,43,44,46,69] Different spectroscopic detection methods were used to investigate relaxation phenomena. In our calorimetric experiments the temperature was controlled with 1mK accuracy. Since MLV display a transition half width on the order of 0.05 K, such an accuracy is needed. In most of the optical experiments, such an accuracy is nearly impossible to obtain, due to the necessity of using glass cuvettes that are exposed to air and the likely existence of temperature gradients. These might cause the presence of life time distributions, because every local temperature in the transition displays a different relaxation time. The appearance of different time scales must therefore not necessarily be due to the physical nature of the relaxation process if the temperature is not controlled much better than the half width of the transition. In general, it is not easy to assign the various timescales found in experiments. However, it is clearly known that trans−gauche isomerizations happen on the ns time scale[70] and that diffusion processes (nearest-neighbor lipid exchange) happen on the 100 ns time scale.[71] The relaxation process found is also related to the time sensitivity of the instrumentation. X-ray methods typically detect very fast changes, whereas calorimetric measurements are slow. While optical measurements detect local geometry and dynamics changes close to a fluorescent dye or a spin label that cannot easily be translated into a meaningful thermodynamic change, the big advantage of calorimetric relaxation measurements is that they detect the change in an important thermodynamic variable with an unambiguous meaning, namely the enthalpy. Several studies are available in the literature that make use of calorimetric measurements. In favor of longer relaxation times are estimations using ac-calorimetry.[45] The authors found relaxation times faster than 120 s (MLV of DMPC) and 260 s (MLV of DPPC). They, however, did not give relaxation times in dependence of temperature. As mentioned, relaxation processes have been reported from the ns to min regime. One reason

might lie in an inaccurate temperature control in many optical methods. However, another reason, which seems likely to us is that the different detection methods probe different relaxation processes. Our study does not exclude the existence of several faster relaxation processes, but the focus was only on one relaxation process, namely the formation of domains which is responsible for nearly all of the heat absorption. We found evidence that there is at least one faster relaxation process which has a minor contribution to the total heat absorption (less than 10%). Most previous studies agreed on a slowing down of relaxation processes in the melting transition regime.[30−39]

Biological membranes also display melting transitions[4,29] (see the Introduction). Typically, these transitions are slightly below physiological temperatures. Therefore, the concepts described here will be of interest if the transition is shifted into the physiological regimes. This is for example possible by lowering the ambient pH close to the membrane.[6] From the knowledge of the *L* and the excess heat capacity profile were estimated relaxation times in the melting transition regime of biological membranes.[17] The authors used a DSC measurement of bovine lung surfactant from Ebel et al.[57] and an *L* similar to the one for DMPC and DPPC MLV vesicles. They estimated that relaxation times in biological membranes would lie in a time regime of up to 120 ms in the transition regime. We have calculated two different *L*'s for MLVs and LUVs but the order of magnitude is unaffected. The time regime of these domain formation processes is in a regime which seems to be important in biology. These relaxation times do not only give the typical time scales of domain formation processes but also the life time of domains. Domains are subject to fluctuations[68] and the smaller they are the shorter their lifetime.

It has been shown before that the concepts described here are also valid for membranes containing cholesterol.[72,17] In the ongoing discussion on "rafts", this may be of putative relevance. Rafts are thought to be domains rich in sphingolipids and cholesterol. Rafts are notoriously difficult to detect in microscopic experiments. Their size is often smaller than 100 *nm*[22] and therefore they should display short life times. Additional to their small size the short life time may makes it difficult to really detect them. It should be added that some in biological systems like macrophages and fibroblasts large domains are found that are visible in optical microscopy, i.e., they are on the micrometer scale.[73] One may therefore want to be careful when linking domain sizes with the broadness of melting profiles, a correlation that must obviously exist between the broadness of the melting profile and the domain size fluctuations.

The action of general anesthetics is still not fully understood. Many groups presently favor a picture where anesthetics directly influence the function of proteins.[49−51] Theories about the action of general anesthetics mediated by the lipid membrane are put forward in several other papers.[53−55,6] The later models are in our opinion more convincing because the action of anesthetics is known to be linearly related to their solubility in lipid membranes, including the noble gas Xenon that is inert and therefore unlikely to have specific interactions with macromolecules. Thinking about lipid-mediated anesthesia, a possible implication of our findings might be related to ion permeation through lipid bilayers. The predicted relaxation time of biological membranes in the several 10 ms range is just the typical time scale of the opening and closing of ion channel proteins. Recently, several groups started to argue that the lipid environment has a direct influence on the channel opening statistics.[74−76] For this reason anesthetics and neurotransmitters may display an indirect influence on these proteins via their action on the lipid membrane. This implies that the channel lifetimes may be coupled to the relaxation time scales of the lipid membranes, in particular since they are obviously of right order. It was also reported that permeation through pure lipid bilayers in the absence of proteins is enhanced in the melting transition regime and reaches a maximum at a temperature of maximum heat capacity. This finding was related to the magnitude of fluctuations.[13−15] Measurements on black lipid membranes in the melting transition regime yielded characteristic fluctuations of the transmembrane ion currents.[77,16,78] No such currents were found for lipid membranes at temperatures well above or below the transition midpoint temperature of one component lipid membranes.[16] The life times of these lipid pores within the phase transition regime were found to be as long as several seconds, in agreement with the relaxation time scales found by us.

In this study, we have shown that relaxation times of domain formation processes in simple model systems are proportional to the excess heat capacity. Relaxation processes slow down in the melting regime. Several drugs studied by us broaden the melting transition profiles and shift them to lower temperatures. This influences the relaxation behavior in a simple manner. This is presumably true for all drugs, peptides and proteins influencing the melting behavior of lipid membranes. Clearly, the free energy of membranes includes the chemical potentials of all membrane associated molecules and it is therefore just a consequence of thermodynamics that the functions of states change in a coherent manner due to the change in one of these variables. This is likely to be of great importance for the general understanding of the function of biological membranes.

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