Transbilayer Movement of Phospholipids at the Main Phase Transition of Lipid Membranes: Implications for Rapid Flip-Flop in Biological Membranes

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ABSTRACT The transbilayer movement of fluorescent phospholipid analogs in liposomes was studied at the lipid phase transition of phospholipid membranes. Two NBD-labeled analogs were used, one bearing the fluorescent moiety at a short fatty acid chain in the *sn*-2 position (C_6-NBD-PC) and one headgroup-labeled analog having two long fatty acyl chains (N-NBD-PE). The transbilayer redistribution of the analogs was assessed by a dithionite-based assay. We observed a drastic increase of the transbilayer movement of both analogs at the lipid phase transition of DPPC (T_c = 41°C) and DMPC (T_c = 23°C). The flip-flop of analogs was fast at the T_c of DPPC with a half-time ($t_{1/2}$) of ~6–10 min and even faster at the T_c of DMPC with $t_{1/2}$ on the order of <2 min, as shown for C_6-NBD-PC. Suppressing the phase transition by the addition of cholesterol, the rapid transbilayer movement was abolished. Molecular packing defects at the phase transition are assumed to be responsible for the rapid transbilayer movement. The relevance of those defects for understanding of the activity of flippases is discussed.

INTRODUCTION

The bidirectional transbilayer movement of phospholipids in biological membranes can be either a passive diffusion (flip-flop) or a more complex protein-mediated process. In the lipid bulk phase, typically the former process is very slow due to the unfavorable passage of a hydrophilic headgroup across the hydrophobic membrane core. Therefore, passive diffusion is assumed to be of minor significance for transbilayer phospholipid dynamics in biological membranes. Indeed, it has been shown that biological membranes contain proteins, which facilitate a rapid transbilayer movement of phospholipids with a variable headgroup specificity. A rapid protein-mediated movement of phospholipids from the outer to the inner leaflet and vice versa with low lipid-specificity and independent of ATP (facilitated diffusion) has been observed in the inner membrane of *Bacillus megaterium* (Hrafnsdóttir et al., 1997), *Bacillus subtilis* (Hrafnsdóttir and Menon, 2000), *Escherichia coli* (Huijbregts et al., 1998; Kubelt et al., 2002), and in the rat liver endoplasmic reticulum (Bishop and Bell, 1985; Baker and Dawidowicz, 1987; Herrmann et al., 1990; Buton et al., 1996; Marx et al., 2000; Menon et al., 2000). The half-time of these flippase-mediated lipid transports is of the order of magnitude of a minute or even less. In red blood cells and other mammalian cells, a scramblase has been identified that mediates a fast bidirectional transbilayer movement of phospholipids between both leaflets (Bassé et al., 1996).

The molecular mechanism(s) of the rapid bidirectional transmembrane passage of phospholipids mediated by those flippases or scramblases is still unknown. However, it has been shown that the incorporation of proteins, peptides, or even synthetic compounds into a pure lipid membrane may accelerate transbilayer movement of phospholipids between both leaflets (Fattal et al., 1994; Matsuzaki et al., 1996; Boon and Smith, 1999; Kol et al., 2001). As an origin of such an enhanced lipid transbilayer movement, perturbations of the bilayer structure at the protein-lipid interface have been proposed. However, the nature of these defects is not known. To elucidate the effect of bilayer perturbations on the transbilayer movement of phospholipids, pure phospholipid membranes may provide a helpful tool, e.g., at lipid phase transitions, molecular packing defects in the bilayer structure may cause membrane perturbations, leading to an accelerated transbilayer phospholipid mobility.

In this study we investigated how the phase state of the membrane and the main lipid phase transition affects flip-flop rates of phospholipids in liposomes consisting of DPPC or DMPC. For that, we used two different fluorescent NBD-labeled phospholipid analogs, a phosphatidylcholine (PC) analog with a short chain in the *sn-2* position bearing the NBD group, and a long chain headgroup-labeled phosphatidylethanolamine (PE) analog. The transbilayer distribution of the analogs was determined using a dithionite-based assay (McIntyre and Sleight, 1991). Our results indicate that the analog transbilayer movement is tremendously enhanced at the main phase transition temperature (T_c). Above T_c, the behavior of

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Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; FRET, fluorescence resonance energy transfer; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; C_6-NBD-PC, 1-acyl-2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-*sn*-glycero-3-phosphocholine; eggPC, egg-yolk lecithin; N-NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine; *N*-Rh-PE, *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; PBS, phosphate buffered saline; SUV(s), small unilamellar vesicle(s); LUV(s), large unilamellar vesicle(s); T_c, phase transition temperature.

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the short chain analog was dependent on the phospholipid species: while the transbilayer movement in DMPC was very similar, it was reduced in DPPC membranes compared to that at the phase transition. For the long chain analog, flip-flop rates in the liquid-crystalline phase of DPPC were almost the same compared to those at the phase transition.

MATERIALS AND METHODS

Materials

The fluorescent phospholipids C_6-NBD-PC, N-NBD-PE, and N-Rh-PE were purchased from Molecular Probes (Leiden, the Netherlands). Sodium chloride, Tris, and Triton X-100 were from Fluka (Seelze, Germany). All other chemicals (at the highest quality available) as well as the lipids DPPC, DMPC, egg-yolk lecithin (eggPC), and cholesterol were purchased from Sigma (Deisenhofen, Germany).

Phosphate buffered saline (PBS) contained 150 mM NaCl, 5.8 mM Na_2HPO_4/NaH_2PO_4, and was set to pH 7.4. The stock solution of dithionite (1 M) was freshly prepared in 100 mM Tris (pH 10.0), stored on ice and used within 3 h after preparation.

Preparation of small unilamellar vesicles

SUVs were labeled with either C_6-NBD-PC or N-NBD-PE. With regard to the localization of the analog three different types of SUVs were prepared: vesicles containing the analog on the outer leaflet, on the inner leaflet, or symmetrically on both membrane leaflets. Lipid mixtures of the desired composition dissolved in chloroform were dried under a stream of nitrogen in a glass tube. The dried lipids were solubilized by a small quantity of ethanol and, subsequently, PBS was added, yielding a maximum final ethanol concentration of 1% and a total lipid concentration of 2 mM. To obtain multilamellar vesicles, suspensions were vortexed vigorously for 30 s. SUVs were prepared by sonification of multilamellar vesicles with a Branson sonifier 250 (Danbury, CT) on ice (duty cycle 90%, output control 2) until suspensions became opalescent, usually for 20 min.

To label SUVs exclusively on the outer leaflet an appropriate amount of C_6-NBD-PC dissolved in chloroform was placed in a glass tube and the solvent was evaporated under nitrogen. After solubilizing the analog with a small quantity of ethanol, PBS was added. The dispersion of the analog was added to unlabeled SUVs (final analog concentration 1 mol % of unlabeled lipids) and incubated for 30 min on ice (for DMPC-, DMPC/cholesterol- and eggPC-SUVs) or at 25°C (for DPPC-SUVs). To obtain symmetrically labeled SUVs, the analog was mixed with the lipid(s) in chloroform before sonification. SUVs labeled exclusively on the inner leaflet were obtained by preparing first symmetrically labeled SUVs (2 mol % analog) and subsequently reducing the NBD moieties located on the outer leaflet with dithionite (Dao et al., 1991). Twenty μl of a 1 M dithionite solution was added to 700 μl of an SUV suspension (lipid concentration 2 mM) and incubated for 30 min on ice. The reduction was terminated by separating dithionite and SUVs on two coupled Sephadex PD 10 columns.

Preparation of large unilamellar vesicles (LUVs)

Multilamellar vesicles prepared as described above were freeze-thawed five times. Subsequently, the suspension was extruded 10 times (extruder from Lipex Biomembranes Inc., Vancouver, Canada) through two stacked polycarbonate membranes (Millipore, Carrigtwohíll, Ireland) with 0.1 μm pore size.

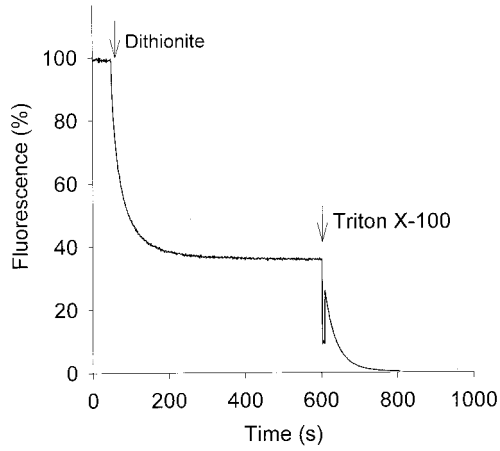

FIGURE 1 Dithionite-mediated reduction of C_6-NBD-PC located on the outer leaflet of symmetrically labeled DPPC-SUVs at 10°C. For details see Materials and Methods. Arrows indicate the addition of dithionite and Triton X-100 (excitation at 470 nm, emission at 530 nm, lipids 70 μM, C_6-NBD-PC 1 mol %, dithionite 25 mM, Triton X-100 0.6%).

Measurement of the analog transbilayer movement

To determine transbilayer rate and distribution of the analogs, labeled SUVs were incubated for varying times and at varying temperatures and, subsequently, a dithionite assay was performed (McIntyre and Sleight, 1991). Dithionite rapidly reduces the fluorescent NBD moiety to a non-fluorescent moiety with a half-time of ~20 s (Fig. 1). The determination of the transbilayer distribution of NBD analogs is based on the fact that dithionite does not penetrate membranes at low temperatures, thus reducing only NBD moieties located on the outer leaflet. One hundred μl of labeled SUV (see above) was mixed with 1 ml PBS prewarmed at the respective incubation temperature. After incubation at the respective conditions (temperature, time) the vesicle suspension was rapidly cooled by placing it on ice and adding 0.5 ml ice-cold PBS; 1.5 ml of the SUV suspension (lipid 70–100 μM) was placed in a fluorescence cuvette and fluorescence was recorded at 10°C with continuous stirring using a Shimadzu RF 5301-PC spectrometer (Duisburg, Germany). The excitation and emission wavelengths were 470 nm and 530 nm, respectively, and the slit widths were 5 nm/10 nm (for vesicles labeled on the inner leaflet) and 3 nm/5 nm (for all other vesicles). After 30 s, 50 μl of a 1 M dithionite solution was injected into the cuvette, resulting in a rapid decrease of the fluorescence intensity (Fig. 1, shown for symmetrically labeled vesicles). This fluorescence decline corresponds to the dithionite-mediated reduction of analogs localized in the outer membrane leaflet. Subsequently, fluorescence intensity adopted a new plateau representing the analogs on the inner leaflet. The very slow decrease of fluorescence indicates that the dithionite permeation across the membrane and/or the analog flip-flop were negligible under these conditions within the time scale of the experiment. From the fluorescence intensities before (F_0) and after (F_R) addition of dithionite, the relative fraction of the analog on the outer leaflet (P_o) was estimated according to Eq. 1.

$$P_o[\%] = \left(1 - \frac{F_R}{F_0}\right)100\% \quad (1)$$

Upon addition of Triton X-100 all analogs became accessible to dithionite, resulting in a complete loss of fluorescence. From the curve shown in Fig. 1 we estimated ~65% of the analog to be on the outer membrane leaflet of symmetrically labeled DPPC-SUVs. This is in good agreement with the

outer/inner surface ratio in small unilamellar vesicles prepared by sonification (de Kruijff and van Zoelen, 1978).

Model for the transbilayer movement of fluorescent lipids

To fit the experimental data for the kinetics of analog translocation, a simple two-compartment model was applied. Briefly, the outward (flop) and inward (flip) movement of the analog are regarded as mono-molecular reactions, with the rate constants k_1 and k_2, respectively. The corresponding differential equation is:

$$\frac{dP_o}{dt} = k_1 P_i - k_2 P_o \tag{2}$$

where the analog concentrations on the outer and inner leaflet, P_o and P_i, respectively, obey the conservation law $P_o + P_i = P = 100\%$. Solving Eq. 2 yields the solution:

$$P_o(t) = \left[P_o(t=0) - \frac{k_1}{k_1 + k_2} P \right] e^{-(k_1+k_2)t} + \frac{k_1}{k_1 + k_2} P \tag{3}$$

To estimate the transbilayer distribution of analogs from the experiments, a linear relationship between analog concentration in the membrane and measured fluorescence is required, which is valid for low label concentration as used here. Fitting the experimental kinetics according to Eq. 3 one obtains the relative amount of the analog in either leaflet at time 0 and in the steady state, as well as the rate constants k_1 and k_2. The half-time of the transbilayer movement ($t_{1/2}$) follows from the rate constants according to Eq. 4:

$$t_{1/2} = \frac{\ln 2}{k_1 + k_2} \tag{4}$$

All calculations were performed by using the software SigmaPlot (Jandel Scientific, Ercrath, Germany).

Fluorescence resonance energy transfer assay

Flip-flop of C_6-NBD-PC in DMPC vesicles was assessed by an independent assay using the rapid exchange of C_6-NBD-PC, initially incorporated into donor vesicles, to acceptor vesicles containing the nonexchangeable fluorescent lipid analog N-Rh-PE vesicles (Nichols and Pagano, 1982, 1983; Hrafnsdóttir et al., 1997). The transfer of C_6-NBD-PC to the acceptor vesicle leads to a decrease of its fluorescence due to fluorescence resonance energy transfer (FRET) between C_6-NBD-PC and N-Rh-PE. To this end, SUVs of DMPC symmetrically labeled with 1 mol % C_6-NBD-PC (donor vesicles), and LUVs containing eggPC and 3 mol % N-Rh-PE (acceptor vesicles) were prepared as described above. The final lipid concentration of all vesicle preparations was 2 mM. Twenty-five μl of the donor vesicle suspension were added to prewarmed 1.475 ml buffer in a fluorescence cuvette, and incubated for 5 min to equilibrate at the selected temperature. After the initial fluorescence intensity of C_6-NBD-PC (excitation wavelength 470 nm, emission wavelength 540 nm) was monitored for 30 s, prewarmed acceptor vesicles (200 μl) were added in excess with respect to donor vesicles, and the time-dependent emission of C_6-NBD-PC was monitored. Typically, initially a fast decline of fluorescence intensity was observed, corresponding to the transfer of C_6-NBD-PC from the outer leaflet of the donor vesicle to the acceptor vesicles, followed by a slower decrease corresponding to a flop of C_6-NBD-PC from the inner to the outer monolayer of the donor vesicles, with subsequent transfer to the acceptor vesicles. For calibrating the extent of transfer, eggPC-LUV containing 3 mol % N-Rh-PE and 0.125 mol % C_6-NBD-PC were prepared to mimic the complete transfer of C_6-NBD-PC to the acceptor vesicles. Subsequently, by normalization of the fluorescence intensity the percentage of C_6-NBD-PC remaining in the donor vesicle population at a given time is obtained. For further details see Nichols and Pagano (1982, 1983) and Hrafnsdóttir et al. (1997).

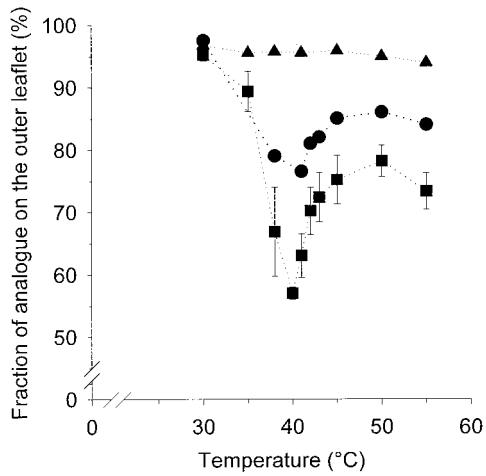

FIGURE 2 Influence of temperature on the transbilayer movement of C_6-NBD-PC in DPPC-SUVs and DPPC-SUVs containing 30 mol % cholesterol. Vesicles were labeled on the outer leaflet and incubated at different temperatures for 8 min (*circles*) or 20 min (*squares*) in the case of DPPC-SUVs, and for 8 min in the case of DPPC/cholesterol-SUVs (*triangles*). Subsequently, vesicles were cooled and the relative amount of analog in the outer leaflet was estimated by performing the dithionite assay at 10°C (see Materials and Methods and Fig. 1). Data points represent the mean of two (DPPC/cholesterol- and DPPC-SUVs, 8 min incubation) and the mean and standard error of three experiments (DPPC-SUVs, 20 min incubation).

RESULTS

Transbilayer movement of C_6-NBD-PC in DPPC-SUVs

DPPC-SUVs labeled with C_6-NBD-PC exclusively on the outer leaflet were incubated for 8 min or 20 min at different temperatures. Subsequently, the transbilayer distribution of analogs was determined by the dithionite assay at 10°C (see Materials and Methods). The dependence of the fraction of analogs localized in the outer leaflet on the incubation temperature is shown in Fig. 2. As can be seen, the transbilayer movement of C_6-NBD-PC was slow at temperatures below the phase transition of DPPC (T_c = 41°C; Silver, 1985), i.e., only a small fraction of the analog had moved to the inner leaflet at 30 and 35°C. In the temperature range of the phase transition the extent of the analog translocation was significantly enhanced, being largest at T_c. At temperatures above T_c, flip-flop rates of C_6-NBD-PC were decreased compared to those in the temperature range of the phase transition, but were still higher than below the phase transition. For both incubation periods a similar dependence

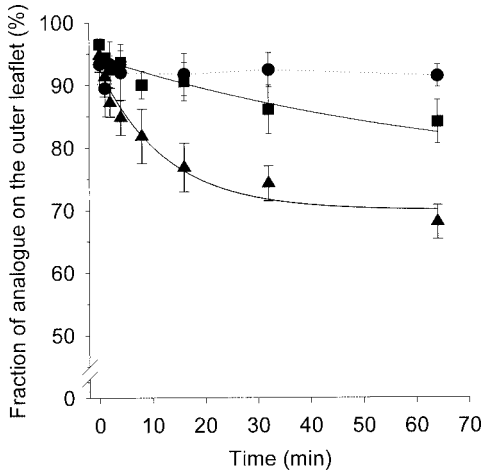

FIGURE 3 Transbilayer movement of C_6-NBD-PC in DPPC-SUVs. SUVs were labeled on the outer leaflet and incubated at 30°C (*circles*), 41°C (*triangles*), and at 50°C (*squares*). After various times an aliquot was withdrawn and the relative amount of analog in the outer leaflet was estimated by performing the dithionite assay at 10°C. Data points and error bars represent the mean and standard error of three (30°C), five (41°C), and of four (50°C) experiments. The solid lines represent the fit of the experimental data according to the model described in Materials and Methods. Kinetic parameters of the regression are given in Table 1.

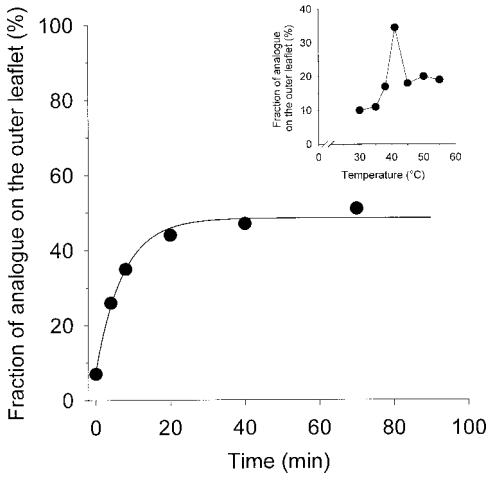

FIGURE 4 Transbilayer movement of C_6-NBD-PC in DPPC-SUVs. SUVs labeled on the inner leaflet were incubated for varying incubation periods at 41°C. Subsequently, vesicles were cooled and the relative amount of analog in the outer leaflet was estimated by performing the dithionite assay at 10°C. The solid line represents the fit of the experimental data according to the model described in Materials and Methods. Kinetic parameters of the regression are given in Table 1. The inset shows the temperature dependence of the analog flip-flop. SUVs were incubated for 8 min at varying temperatures and then the dithionite assay was performed at 10°C to estimate the transbilayer analog distribution.

of the analog translocation on temperature was observed, but with a lower extent of analog translocation for 8 min compared with the values obtained for 20-min incubation.

To quantify rates of transbilayer movement of C_6-NBD-PC, kinetics of the analog redistribution from the outer to the inner leaflet were measured at selected temperatures (30°C, 41°C, and 50°C) (Fig. 3). At the phase transition temperature a rapid flip-flop of C_6-NBD-PC was observed. After ~30 min a stable plateau was reached with ~70% of analogs on the outer leaflet. Fitting the kinetics to a single exponential function (Eq. 3) yielded a half-time of 8 ± 2 min. Below the phase transition temperature (30°C) only a very low fraction of C_6-NBD-PC had moved to the inner leaflet compared with the analog flip-flop at the T_c. At 50°C the transbilayer redistribution of the analog was rapid but did not reach the rates measured at the phase transition.

A similar observation was made when DPPC-SUVs were labeled on the inner leaflet with C_6-NBD-PC and the outward redistribution of analog was measured after incubation for 8 min at various temperatures. As expected, the extent of this redistribution was dependent on the incubation temperature of the vesicles being largest at the T_c of DPPC (Fig. 4, *inset*). The half-time of the outward movement at T_c (Fig. 4) was 10 ± 4 min, which is of the same order of magnitude as the half-time for SUVs labeled on the outer leaflet (see above). Thus, the analog flip-flop is independent of the labeling protocol and shows the same behavior for vesicles labeled initially either on the inner or on the outer leaflet.

One may argue that the decreased amount of analogs in the outer or inner leaflet at the phase transition is not caused by a translocation of C_6-NBD-PC in intact vesicles, but by a lipid scrambling due to vesicle fusion at that temperature. To test this notion, we prepared symmetrically labeled DPPC-SUVs that have ~65% of the analog on the outer membrane leaflet (Fig. 1). Any vesicle fusion would lead to larger vesicles with a outer/inner leaflet surface ratio close to 50:50. However, incubation of symmetrically labeled DPPC-SUVs up to 2 h at 41°C did not lead to such a ratio, the fraction of analogs in the outer leaflet remained larger and was 62% (mean of two experiments). Thus, fusion-mediated scrambling of lipids could not be the reason for the increased transbilayer movement of C_6-NBD-PC at the phase transition.

According to the experimental protocol, vesicles incubated at temperatures above T_c sensed the phase transition temperature twice, first while heating and second while cooling the vesicles to perform the dithionite assay (see Materials and Methods). An increased lipid flip-flop while passing the temperature range of T_c could mimic an accelerated lipid translocation in the measurements performed above T_c. However, this can be precluded. As can be seen in Fig. 3, the amount of analogs redistributed during the first minute at T_c is <10%. Cooling of vesicle suspension was achieved in <20 s. Thus the amount of analogs redistributed while passing the phase transition can be neglected. Nevertheless, to provide independent evidence, the following experiments were done. Unlabeled SUVs were preincubated at different temperatures above T_c. Subsequently, an aqueous dispersion of prewarmed C_6-NBD-PC was added to

TABLE 1 Parameters for the kinetics of transbilayer movement of C_6-NBD-PC and N-NBD-PE in lipid membranes at various conditions

Sample	n	P_∞ (%)	k_2 (min^{-1})	k_1 (min^{-1})	$t_{1/2}$ (min)
DPPC-SUV o					
$T = 41°C$	5	70 ± 8	0.06 ± 0.02	0.024 ± 0.006	9 ± 2
DPPC-SUV i					
$T = 41°C$	3	46 ± 3	0.04 ± 0.01	0.05 ± 0.01	10 ± 4
DPPC-SUV o					
$T = 50°C$	4	70 ± 10	0.011 ± 0.006	0.004 ± 0.001	50 ± 20
DPPC-SUV o (not shown)					
$T = 50°C$ (labeled at 50°C)	3	80 ± 10	0.020 ± 0.020	0.003 ± 0.002	30 ± 20
DMPC-SUV o					
$T = 23°C$	2	67 ± 4	0.30 ± 0.01	0.15 ± 0.03	1.6 ± 0.2
DMPC-LUV o					
$T = 23°C$	2	42 ± 20	0.15 ± 0.09	0.2 ± 0.1	1.9 ± 0.7
DPPC-SUV o					
(N-NBD-PE)	2	62 ± 1	0.09 ± 0.04	0.06 ± 0.03	6 ± 3
$T = 41°C$					

The experimental data from Figs. 3, 4, 6, and 7 were fitted according to the model described in Materials and Methods (see lines in the respective figures). From the fitting, P_∞, k_1, and k_2 were calculated, which are the relative amounts of the analog in the outer leaflet in the equilibrium and the rate constants of the outward (flop) and inward (flip) movement, respectively. $t_{1/2}$ is the half-time of transbilayer movement (see Eq. 4). n is the number of experiments; o and i indicate labeling on the outer leaflet and the inner leaflet, respectively.

incorporate the label into the outer membrane leaflet and to allow lipid flip-flop at that temperature. After 8 or 20 min the vesicles were rapidly cooled to 10°C and the dithionite assay was performed. Using this protocol vesicles passed the phase transition only once. However, the fraction of analogs which had redistributed to the inner leaflet (data not shown) was similar to that found when the vesicles passed twice the phase transition. Next, we labeled DPPC-SUVs and followed the whole kinetics of analog redistribution at 50°C. The kinetics was essentially the same as that found for SUVs labeled below the phase transition (see Fig. 3) as confirmed by the respective fits of the kinetics (Table 1). We conclude that passing of the vesicles through the phase transition is of minor relevance for the results of those experiments performed above T_c.

Transbilayer movement of C_6-NBD-PC in DMPC-SUVs

To further support an enhancement of the analog flip-flop at the phase transition temperature we used DMPC-SUVs that were labeled on the outer leaflet with C_6-NBD-PC. The main phase transition of DMPC is at 23°C (Silver, 1985). The experimental protocol was the same as for DPPC-SUVs, but vesicles were labeled at 4°C and subsequently incubated at various temperatures for 4 min or 8 min. Similar to what we have found for DPPC-SUVs, at temperatures below T_c only a small fraction of the analog had moved to the inner leaflet (Fig. 5, *closed circles*, only shown for 8 min incubation). At temperatures around T_c this fraction increased becoming maximal at 23°C. At higher temperatures this fraction remained almost similar to that at T_c, which is different from the behavior observed for DPPC. Fig. 6 shows the kinetics of the transbilayer redistribution

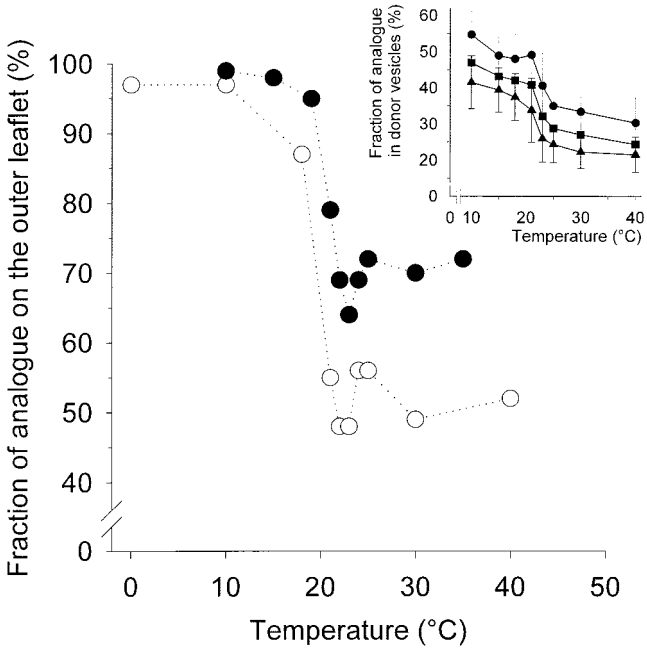

FIGURE 5 Influence of temperature on the transbilayer movement of C_6-NBD-PC in DMPC-SUVs (*closed circles*) and LUVs (*open circles*). Vesicles were labeled on the outer leaflet and incubated for 8 min (SUV) or 4 min (LUV) at varying temperatures as described (see Materials and Methods). Subsequently, the analog transbilayer distribution was estimated by performing a dithionite assay at 10°C. *Inset:* Fraction of C_6-NBD-PC remaining in DMPC-SUVs (donor vesicles) after incubation with eggPC-LUVs containing the nonexchangeable N-Rh-PE (acceptor vesicles) at different temperatures for 4 min (*circles*), 8 min (*squares*), and 15 min (*triangles*). The rapid transfer of C_6-NBD-PC, initially incorporated into donor vesicles, to acceptor vesicles leads to a decrease of the NBD fluorescence due to fluorescence resonance energy transfer (FRET) between C_6-NBD-PC and N-Rh-PE. For details see Materials and Methods. Data points and error bars (for the sake of clarity only shown for circles and triangles) represent the mean and standard error of three experiments.

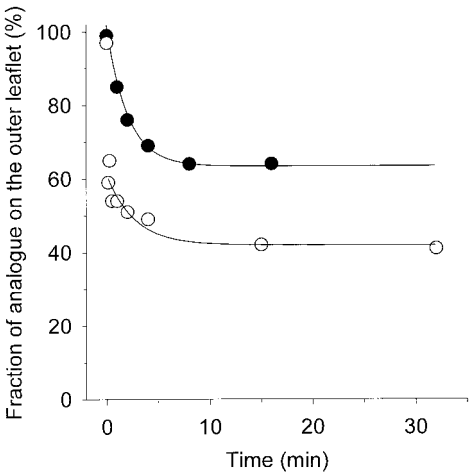

FIGURE 6 Transbilayer movement of C_6-NBD-PC in DMPC-SUVs (*closed circles*) and LUVs (*open circles*). Vesicles were labeled on the outer leaflet and incubated at 23°C. After various times an aliquot was withdrawn and the relative amount of analog in the outer leaflet was estimated by performing the dithionite assay. The solid lines represent the fit of the experimental data according to the model described in Materials and Methods. Kinetic parameters of the regression are given in Table 1.

of C_6-NBD-PC in DMPC-SUVs at 23°C (*closed circles*). After ~10 min a steady-state distribution was reached with ~65% of the analog in the outer membrane leaflet. The kinetics of analog inward motion was faster ($t_{1/2}$ = 1.6 ± 0.2 min) than that found in DPPC-SUVs at 41°C (7 ± 3 min).

To assess by an independent assay the rapid flip-flop at and above T_c we measured the transfer of C_6-NBD-PC initially incorporated symmetrically in DMPC-SUVs (donor vesicles) to eggPC-LUVs (acceptor vesicles) containing the nonexchangeable lipid fluorophore N-Rh-PE by FRET (see Materials and Methods). C_6-NBD-PC has been shown to rapidly exchange between vesicles (Nichols and Pagano, 1982, 1983; Hrafnsdóttir et al., 1997). This assay allows measuring the flip-flop at the selected temperature directly, thus circumventing the cooling of the sample for measurement of transbilayer redistribution of C_6-NBD-PC as required for the dithionite assay. Upon mixing of donor and acceptor vesicles a first rapid decline of the NBD fluorescence was observed (not shown). This phase corresponds to the analogs on the outer leaflet of the donor vesicles transferred to the acceptor vesicle. At and above T_c a second slower decline was found corresponding to flip-flop of C_6-NBD-PC from the inner to the outer monolayer of the donor vesicles, with subsequent transfer to the acceptor vesicles. Below T_c essentially only the first phase was observed suggesting that the flip-flop of C_6-NBD-PC in DMPC-SUVs was very slow. In Fig. 5 (*inset*) the fraction of C_6-NBD-PC remaining in DMPC-SUVs after 4, 8, and 15 min, respectively, is shown as a function of temperature. Even 15 min after mixing of donor and acceptor vesicles at $T < T_c$, ~40% of analogs remained in DMPC-SUVs (Fig. 5). This fraction of analogs corresponds mainly to those on the inner monolayer of DMPC-SUVs, keeping in mind the area difference between both monolayers for SUVs. Due to the mixing ratio between donor and acceptor vesicles, a nonnegligible fraction of analogs remained in the outer leaflet of the donor vesicles (see also below). However, due to the enhanced flip-flop at and above T_c, the fraction of analogs in DMPC-SUVs reduced drastically. After 15 min of incubation, ~15% of the analogs were associated with DMPC-SUVs. By taking into account the mixing ratio between donor and acceptor vesicles, this value corresponds almost to the equilibrium of analog exchange between vesicles. Confirming the experiments using dithionite, the exchangeable fraction of analogs above T_c was similar to that found at T_c. In summary, the results of this assay are in accordance with those of the dithionite assay.

Transbilayer movement of C_6-NBD-PC in DPPC/Cholesterol-SUVs and eggPC-SUVs

Suppressing the gel-to-fluid phase transition in vesicle membranes should prevent the increased lipid flip-flop at T_c as found above. To test this hypothesis we prepared SUVs consisting of DPPC and cholesterol. Increasing concentrations of cholesterol in a pure phospholipid membrane causes a broadening and, finally, a suppression of the gel-to-fluid phase transition (Vist and Davis, 1990). DPPC-SUVs containing 30 mol % cholesterol were labeled on the outer leaflet with C_6-NBD-PC and incubated for 8 min at varying temperatures between 30 and 55°C. Subsequently, the analog distribution was determined by the dithionite assay at 10°C. We found no enhanced transbilayer movement of the analog in the region of the DPPC phase transition (Fig. 2, *triangles*).

EggPC membranes do not exhibit a gel-to-fluid phase transition due to the mixture of various fatty acyl chains. EggPC-SUVs labeled on the outer membrane leaflet with C_6-NBD-PC were incubated for 8 min at varying temperatures in the range between 30 and 50°C. The transbilayer movement of the analog was slightly enhanced with increasing temperatures: at 30 and 50°C ~1% and 6%, respectively, of the analog had moved to the inner leaflet within 8 min. Thus no pronounced temperature dependence of the analog movement in eggPC-SUVs was detectable.

Transbilayer movement of C_6-NBD-PC in DMPC-LUVs

Membranes of SUVs are characterized by a high surface curvature that might influence the movement of lipids across the membrane. To elucidate whether membrane curvature is a determinant of lipid flip-flop, we performed the same experimental protocols as described above for SUVs on large unilamellar vesicles (LUVs) prepared of DMPC. LUVs symmetri-

cally labeled with C_6-NBD-PC contain 50% of the analog in each leaflet, as was verified by the dithionite assay (data not shown). Similar to the results obtained with SUVs, C_6-NBD-PC redistributed rapidly between both membrane leaflets of outer leaflet-labeled DMPC-LUVs at the main phase transition temperature of 23°C (Fig. 6, *open circles*). While in the initial phase the rapid change of the analog concentration in the outer leaflet could not be well resolved, values could be measured accurately for time points >15 s. From fitting those data to a single exponential function, it can be concluded that half-times for flip-flop of C_6-NBD-PC at T_c are of the same order of magnitude in SUVs and LUVs (Table 1). As it becomes obvious from Fig. 5 (*open circles*), the transbilayer movement of the analog in DMPC-LUVs was increased considerably at T_c compared to that in the gel phase. Above T_c the transbilayer movement was almost as rapid as that at T_c, similar to the behavior observed for SUVs (Fig. 5, *closed circles*). Thus, the increase of lipid flip-flop at T_c does not depend essentially on membrane curvature.

Transbilayer movement of N-NBD-PE in DPPC-SUVs

Short chain NBD analogs might display different flip-flop rates compared to endogenous lipids due to the short fatty acid residue in the *sn-2* position and/or the NBD moiety, which might perturb the bilayer organization. To this end we used a phospholipid analog with two long chain fatty acids, the headgroup labeled N-NBD-PE. Since long chain analogs are very hydrophobic, they cannot be incorporated into membranes rapidly in a quantitative manner by adding from an aqueous solution, as done for short chain analogs. Therefore, we used SUVs labeled exclusively on the inner leaflet with fluorescent N-NBD-PE (see Materials and Methods).

The temperature dependence of the transbilayer movement of N-NBD-PE in DPPC-SUVs is shown in Fig. 7 (*inset*). Similar to C_6-NBD-PC, in the gel phase flip-flop rates of the analog were very low as compared to those at the phase transition. Differences became apparent in the fluid phase, where flip-flop of N-NBD-PE was faster as at the phase transition and not reduced, as was observed for C_6-NBD-PC (see above). The half-time of N-NBD-PE transbilayer movement at the main phase transition was 6 ± 3 min, and thus of the same order of magnitude as that of C_6-NBD-PC (Fig. 7, Table 1).

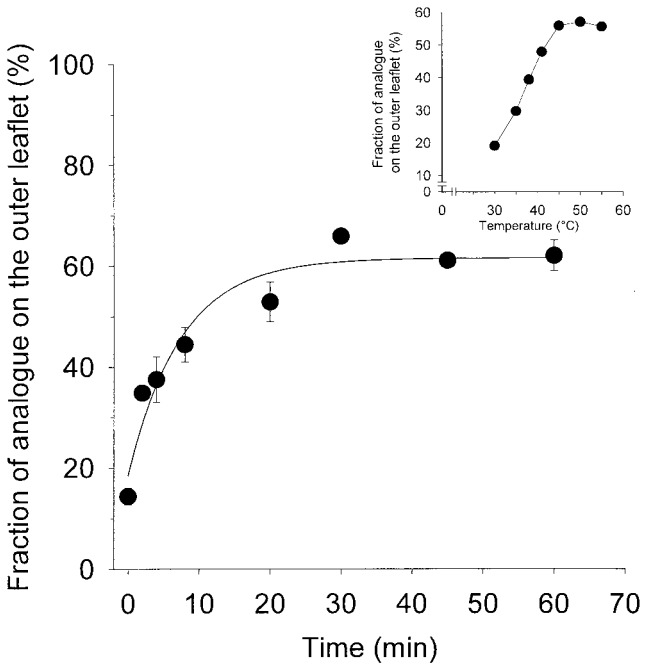

FIGURE 7 Transbilayer movement of N-NBD-PE in DPPC-SUVs. SUVs labeled on the inner leaflet were incubated for varying incubation periods at 41°C. Subsequently, vesicles were cooled and the relative amount of analog in the outer leaflet was estimated by performing a dithionite assay at 10°C. The solid line represents the fit of the experimental data according to the model described in Materials and Methods. Kinetic parameters of the regression are given in Table 1. The inset shows the temperature dependence of the analog flip-flop. SUVs were incubated for 8 min at varying temperatures and then the dithionite assay was performed at 10°C to estimate the transbilayer analog distribution.

DISCUSSION

The purpose of this study was to investigate the transbilayer movement of phospholipids across membranes at the phase transition between the lamellar gel and liquid-crystalline phases. For measuring lipid flip-flop, fluorescent NBD phospholipids were incorporated into liposomes and their transbilayer (re-)distribution was measured by a dithionite assay (McIntyre and Sleight, 1991). We found that the flip-flop rates of the short chain analog C_6-NBD-PC were highly sensitive to the phase state of the lipid membrane, becoming very large at the phase transition temperature. First, in DPPC membranes which exhibit a main phase transition from the gel to the liquid-crystalline phase at 41°C we observed a tremendous acceleration of the C_6-NBD-PC flip-flop compared to that in the gel phase and in the liquid-crystalline phase. The half-time of the analog flip-flop at the phase transition was ~8 min. In the liquid-crystalline phase the transbilayer movement was still enhanced compared to the gel state, but slower than at the phase transition. Addition of cholesterol known to abandon main phase transitions at concentrations of 25 mol % or higher (Vist and Davis, 1990) abolished the strong enhancement of analog flip-flop at the phase transition temperature of DPPC. Second, the flip-flop rates of the analog in DMPC membranes were also dependent on the lipid phase state with a strongly enhanced flip-flop at the phase transition temperature of 23°C. Interestingly, the flip-flop rate of C_6-NBD-PC in DMPC membranes at the phase transition of 23°C (half-time 1.6 min) was faster than that found in DPPC membranes at the T_c of this lipid (half-time 8 min). Third, in contrast to DPPC and DMPC, the analog flip-flop was slow over a wide temperature range in

vesicles composed of eggPC. Since eggPC does not exhibit a distinct phase behavior, this supports the conclusion that the enhanced flip-flop rate at the T_c of pure lipid membranes is indeed associated with the phase transition of the lipid, and not merely temperature-related.

To assess whether the short fatty acyl chain and the chain-linked NBD moiety of C_6-NBD-PC determine enhanced flip-flop at the T_c, we used the analog N-NBD-PE having two long fatty acyl chains and the NBD moiety attached to the headgroup. Despite those differences, both analogs were very similar with respect to transbilayer movement. Flip-flop was slow in the gel phase and increased steeply in the temperature range of the phase transition. At 41°C the half-time of N-NBD-PE flip-flop in DPPC-SUVs was 6 min, similar to that of C_6-NBD-PC with ~8 min. Different from C_6-NBD-PC, transbilayer movement of N-NBD-PE did not decrease in the liquid-crystalline phase, but remained almost on the same high level as that observed at the phase transition. However, since C_6-NBD-PC showed only a moderate decrease of flip-flop rates in the liquid-crystalline phase and both analogs had virtually the same half-time of transbilayer movement at the phase transition, it can be concluded that the short chain analog resembles quite accurately the transbilayer movement of phospholipids with two long fatty acyl chains. Differences in the transbilayer redistribution could be related to the localization of the NBD moiety. For C_6-NBD-PC, it seems reasonable to assume an increase in activation energy for flip-flop due to the additional hydrophilic moiety linked to the fatty acid chain. Indeed, due to its polarity this moiety is not solely confined to the hydrophobic phase of the bilayer. It undergoes a rapid reorientation between the hydrophobic part of the membrane and the headgroup interface with a preference for the latter, as we have shown very recently (Huster et al., 2001). In comparison, attachment of the NBD moiety to the phospholipid headgroup increases only the size of an already existing hydrophilic domain. Therefore, the change in activation energy might be smaller than that for attaching an NBD moiety to the acyl chain. This may account for the moderate differences in the flip-flop between C_6-NBD-PC and N-NBD-PE in the liquid-crystalline phase of DPPC.

Our results are qualitatively consistent with a study by de Kruijff and van Zoelen (1978). In this study ^{13}C-labeled DMPC was incorporated from donor to DMPC acceptor vesicles containing 15 mol % phosphatidic acid by incubation in the presence of phosphatidylcholine exchange protein at 37°C. The transbilayer distribution of ^{13}C-labeled DMPC after incubation for various times at different temperatures was measured by NMR, taking advantage of a nonpermeable shift reagent. At the phase transition of DMPC a relative maximum for the flip-flop rates was found. However, the half-times of transbilayer movement obtained in this study are at least two orders of magnitude higher than those determined with our method. Several reasons have to be considered for this quantitative difference. First, it might be that membranes consisting of various lipids as DMPC/phosphatidic acid may behave different in comparison to membranes composed of only one lipid species (see below, discussion of eggPC membranes). Second, the nature of the fluorescent lipids used in our study accounts for the difference. However, we consider this as very unlikely since we found a similar flip-flop for two NBD phospholipid analogs of very different structure.

How can the strong increase of transbilayer movement of lipids at the lipid phase transition be rationalized? Langner and Hui (1993) have shown an enhanced dithionite permeability at the main phase transition in vesicles of various lipid species which was related to molecular packing defects in the bilayer. Note that we have performed the dithionite assay after incubation of vesicles at the respective temperatures at 10°C (see Materials and Methods). At the main phase transition domains of lipids in the gel phase and of lipids in the liquid crystalline phase are known to coexist. At the phase boundaries molecular packing defects are likely to occur and can facilitate ion diffusion. We propose that those molecular packing defects at the main phase transition can also mediate a rapid transbilayer lipid diffusion. This is confirmed by our results with C_6-NBD-PC in eggPC liposomes and in liposomes consisting of DPPC and cholesterol, where no phase separation can be assumed.

Compared with the gel state, we observed a faster transbilayer lipid movement in the liquid-crystalline phase. This state is characterized by highly flexible fatty acyl chains and a lower lipid packing density. Additionally, the thickness of the hydrophobic core of the bilayer (D_C) is reduced above T_c, i.e., D_C of DPPC bilayers is 34.3 Å and 28.5 Å at 20°C and 50°C, respectively (Nagle and Tristram-Nagle, 2000). Very likely, a larger D_C value decreases transbilayer movement rates of lipids (de Kruijff and van Zoelen, 1978) by impeding the passage of the polar headgroup through the hydrocarbon core. The relevance of D_C might be supported by the observation that the transbilayer movement of C_6-NBD-PC at the T_c was faster for DMPC than for DPPC. Notably, the higher-order parameter of fatty acyl chains of phospholipids upon addition of cholesterol is accompanied by an increase of the hydrophobic core thickness (Barry and Gawrisch, 1995).

Strikingly, the transbilayer movement of analogs in DPPC and DMPC membranes above T_c is much faster in comparison to that in eggPC at similar temperatures. Although eggPC membranes are considered to be in the liquid-crystalline phase under those conditions, this state seems to be different from that of the liquid-crystalline state of DMPC and DPPC. The slow flip-flop in eggPC membranes is at least in qualitative agreement with the slow transbilayer movement of phospholipids in the bulk lipid phase of biological membranes. Due to its mixture of PC molecules varying in the degree of saturation and chain length, eggPC membranes accommodate much better the bulk lipid phase of biological membranes than DPPC and DMPC.

Recent studies on rat liver microsomal membranes and inner membranes of *B. megaterium* and of *E. coli*, respectively, indicate a rapid translocation of short chain phospholipid an-

alogs across the membrane, but also of long chain endogenous phospholipids (Herrmann et al., 1990; Buton et al., 1996; Huijbregts et al., 1998; Hrafnsdóttir et al., 1997; Hrafnsdóttir and Menon, 2000; Marx et al., 2000). These rapid translocations seem to be protein-mediated, but ATP-independent and unspecific concerning the nature of the headgroup of the phospholipid. A rapid transbilayer movement was not observed in vesicles consisting of lipids isolated from those biological membranes, implying that structural features of lipid membranes at the phase transition leading to an enhanced flip-flop do not exist in membranes of isolated lipids. The mechanism(s) of how putative flippases trigger lipid flip-flop is/are not clear. Possible mechanisms could be that flippases induce the formation of lipid domains which exhibit typical properties of the lipid phase at the phase transition, or the protein itself can cause packing defects at the protein-lipid interface. Indeed, for example, the half-time of transbilayer movement of C_6-NBD-PC in the rat microsomal membrane at room temperature is ~2 min (Marx et al., 2000). This value is of the same order as that found for DMPC at the phase transition ($t_{1/2} = 1.6$ min). Very recently, it was proposed that membrane-spanning peptides induce a rapid flip-flop of C_6-NBD-phospholipid analogs in membranes of lipid from *E. coli* by local perturbations of the bilayer structure in the vicinity of peptides (Kol et al., 2001). Furthermore, flippases may accelerate the flip-flop by exposing an interface of a reduced hydrophobic thickness to the lipid phase. Although it has been known for a long time that insertion of membrane proteins as, e.g., glycophorin (van Zoelen et al., 1978) into lipid membranes causes an enhanced lipid flip-flop, its rate was still much slower than movement rates of phospholipids observed in the rat endoplasmic reticulum (Buton et al., 1996; Marx et al., 2000; Menon et al., 2000) or in the inner membrane of bacteria (Hrafnsdóttir et al., 1997; Huijbregts et al., 1998; Hrafnsdóttir and Menon, 2000; Kubelt et al., 2002). Thus, whatever the mechanism, it is evident that flippases must be specifically adapted to the function of a rapid transbilayer phospholipid movement.

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