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REORIENTATION RATES AND ASYMMETRY OF DISTRIBUTION OF LYSOPHOSPHOLIPIDS BETWEEN THE INNER AND OUTER LEAFLET OF THE ERYTHROCYTE MEMBRANE

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Labelled lysophospholipids were inserted into the outer layer of the erythrocyte membrane and their reorientation (flip) to the inner layer quantified by following the increase of the fraction of lysophospholipids not extractable by albumin. Flip rate constants were calculated from the kinetics of equilibration of the lysophospholipids between two compartments, the outer and the inner leaf of the bilayer, in the early phase of the flip kinetics where correction for non-enzymatic hydrolysis and acylation could be omitted. The distribution of a lysophospholipid finally attained reflects its affinity for the two layers. Whereas lysophosphatidylcholine has a slight preference for the outer layer of the membrane, lysophosphatidylserine spontaneously concentrates in the inner layer up to a ratio of 4:1. This asymmetry mimics the distribution of phosphatidylserine in the native membrane. Flip rates depend on membrane lipid compositions. They are enhanced by cholesterol depletion. Comparison of various mammalian species demonstrates that erythrocytes with a higher phosphatidylcholine/sphingomyelin ratio and high content of polyunsaturated fatty acids (mouse and rat) have a high transbilayer mobility, in contrast to erythrocytes with a low phosphatidylcholine/sphingomyelin ratio and a low content of polyunsaturated fatty acids (ox). Molecular properties of lysophospholipids influence their transbilayer mobility. Flip rates of lysophospholipids are enhanced not only by unsaturation of their fatty acid, but also by a negative net charge on the headgroup. This indicates that the strongly asymmetric distribution of phosphatidylserine in the native erythrocyte membrane, which is maintained for the lifespan of the cell, does not result from a lack of transbilayer mobility.

Introduction

In pure lipid bilayer systems, even in the liquid-crystalline state, the transverse mobility of phospholipids between the two lipid leaflets (flip-flop) is very low with half-times of up to several days [1–3]. Mobility becomes enhanced by phase separations occurring in mixed lipid systems containing gel or hexagonal lipid phases in addition to a liquid-crystalline lipid bilayer [4–7]. Moreover, incorporation of erythrocyte membrane proteins into artificial lipid bilayers has been shown to increase the transverse mobility of phospholipids [8,9]. The extent of this increase was found to depend on the nature of the lipids used, which suggested a causal role of a 'mismatch' [10] between lipids and proteins [9]. In line with these observations the transbilayer reorientation of phosphatidylcholine in the erythrocyte membrane proceeds with half-times in the range of hours [11–15], much faster than in a pure lipid membrane.

The detailed analysis of transversal motions of lipids in complex biological membranes has been hampered by the lack of simple techniques suited (a) to incorporate labelled diacylphospholipids (in

particular other than phosphatidylcholines) into the membrane or to label native phospholipids with some specificity, and (b) to quantify the reorientation of such diacylphospholipids between the two lipid layers. These problems have to some extent been overcome by the use of lysophospholipids [16–18]. Such monoacylphospholipids are easily incorporated into membranes and their reorientation to the inner lipid leaflet can be estimated by a procedure taking advantage of the high affinity of lysophospholipids for serum albumin [19]. Albumin can thus be used to extract selectively lysophospholipids present in the outer leaflet of the bilayer, while lysophospholipids of the inner leaflet are protected from extraction by their low transbilayer mobility.

This strategy of measuring the process of transbilayer reorientation was introduced by Mohandas et al. [18] and applied to investigate some factors influencing the transbilayer reorientation of lysolecithin between the two lipid leaflets of the erythrocyte membrane. We have used a modification of their method and omitted albumin from the incubation medium. Essentially all of the lysophosphatidylcholine in the suspension is present in the membrane under our conditions [20,21] and the transport data can, to a first approximation, be evaluated in terms of a two-compartment system [21]. The rates of transbilayer movement of lysophospholipids are shown to depend on the structure of the acyl chain and the polar headgroup of the lysophospholipid, but also on the phospholipid composition and the cholesterol content of the membrane. Consequences of the observed relatively fast transbilayer reorientation of phospholipids for the stability of the asymmetric arrangement during the life span of the erythrocyte are discussed.

Materials

Fresh human blood, anticoagulated by heparin, was obtained from the local blood bank, Blood from ox, pig, rabbit, rat and mouse was freshly collected and anticoagulated with heparin, or in case of ox and pig blood with citrate. Camel blood was a kind gift of the University of Beer-Sheva, Israel. Erythrocytes were isolated by centrifugation, the buffy coat removed and cells washed three times with 10 vol. of 145 mmol · l^{-1} NaCl. Bovine serum albumin (essentially fatty-acid-free) was obtained from Sigma. L-1-[l-^{14}C]Palmitoyllysophosphatidylcholine (spec. act. 47.5 mCi/mmol) and D-α-dioleoylphosphatidyl-L-[U-^{14}C]serine (specific activity 60 mCi/mmol) were purchased from Amersham Buchler and L-1-[l-^{14}C]oleoyllysophosphatidylcholine (spec. act. 57 mCi/mmol) from New England Nuclear. Dipalmitoylphosphatidylcholine was obtained from Sigma, and cholesterol (analytical grade) from Merck. The scintillation fluid Quickscint R 212 was purchased from Zinsser (Frankfurt). All other chemicals used were of analytical grade. ^{14}C-labelled lysophosphatidylserine was prepared by enzymatic cleavage of dioleoylphosphatidyl-l-[U-^{14}C]serine according to Dennis [22], followed by purification by thin-layer chromatography.

Methods

Measurements of the flip rate of lysophospholipids. Flip measurements were carried out as described in Ref. 21. Routinely 100 μl of a suspension of erythrocytes (hct. 50%) were loaded with 30 nCi of ^{14}C-labelled lysophospholipids, representing 12 nmol/ml of packed cells or about 1 molecule of lysophospholipid per 350 molecules (= 0.3%) of membrane phospholipids. For reasons of comparison, the lysophosphatidylcholine content in the native erythrocyte membrane comprises 0.5–1% of total phospholipids. After incorporation of labelled lysophospholipids cells were isolated by centrifugation and 1 vol. of cells resuspended in 10 vol. of medium A [21] (pH 7.4), containing gentamycin (0.1 mg/ml) to prevent bacterial growth during prolonged incubations.

Measurements of hydrolysis and acylation of lysophospholipids. To quantify the products of the spontaneous hydrolysis of labelled lysophosphatidylcholine into glycerylphosphorylcholine and fatty acids (see Results) and the extent of acylation of lysophospholipids to their diacyl analogues [21] during incubation of cells with lysophospholipids, 100 μl of the cell suspension were centrifuged and the erythrocytes hemolysed with 2 vol. of H_2O. The lipids were extracted with chloroform/methanol [23] and separated by thin-layer chromatography on Silica gel 60 plates from

Merck, Art. No. 5715, with chloroform/methanol/acetic acid/water (60 : 30 : 12 : 2, v/v). The spots containing labelled lysophospholipid, diacylphospholipid and fatty acids were scraped from the plates, radioactivities quantified by liquid scintillation counting and fractions calculated.

In case of lysophosphatidylserine the situation is somewhat different. Hydrolysis of this phospholipid, which was labelled at the serine group, will result in the formation of water-soluble radioactive material. Thus hydrolysis could be quantified by the loss of radioactivity from the cells (see Results).

Results and Interpretation

To a first approximation the reorientation of lysophospholipids from the outer to the inner membrane layer can be regarded as a process of equilibration between two pools. A mathematical description of such a process has been given elsewhere (Ref. 21, see also Ref. 41). A set of experiments was now designed to investigate difficulties that interfere with the evaluation of the kinetics of reorientation during prolonged incubations. Such difficulties arise (a) from a spontaneous hydrolysis of lysophospholipids producing fatty acids and water-soluble glyceryl-phosphoryl derivatives, and (b) from acylation of lysophospholipids to diacylphospholipids.

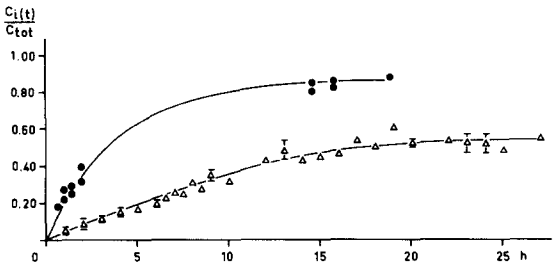

Fig. 1. Time-dependent reorientation of lysophospholipids from the outer to the inner membrane layer. Erythrocytes were loaded with ^{14}C-labelled lysophospholipid, washed and incubated at 37°C. After different intervals of time, the amount of radioactivity non-extractable by albumin ($C_{i(t)}$) was determined as described in the Methods and related to the total radioactivity in the membrane (C_{tot}). In case of oleoyllysophosphatidylserine the curve was fitted according to Eqn. 3 of Ref. 21, △, oleoyllysophosphatidylcholine; ●, oleoyllysophosphatidylserine.

Difficulties interfering with the evaluation of the data

(a) Hydrolysis of lysophospholipid. In the course of preliminary studies on the reorientation of lysophosphatidylcholine we observed spontaneous hydrolysis of palmitoyllysophosphatidylcholine at rates of the same order of magnitude as the reorientation of palmitoyllysophosphatidylcholine. We could demonstrate that the hydrolysis occurs in the aqueous phase and results from the non-enzymatic cleavage of the small fraction (< 0.2%) of the lysophosphatidylcholine not incorporated into the membrane due to the finite membrane/medium partition coefficient of this phospholipid. In case of the labelled lysophosphatidylcholine used in our study, hydrolysis produces labelled fatty acids which partition into the membrane,

TABLE I

CALCULATION OF THE FRACTION OF OLEOYLLYSOPHOSPHATIDYLCHOLINE IN THE INNER LAYER AT EQUILIBRIUM

Erythrocytes were loaded with [^{14}C]oleoyllysophosphatidylcholine and incubated for 19–24 h at 37°C for transbilayer equilibration. The distribution of radioactivity non-extractable by albumin was determined as described in Methods. Data represent a typical example. Since the labelled phosphatidylcholine (*b*) is not extractable by albumin, this fraction has to be subtracted from both, the non-extractable as well as the total radioactivity. The radioactivity of the fatty acid fraction (*c*) has to be subtracted from the total radioactivity. Since fatty acids of the outer layer can be extracted by albumin, only the nonextractable fraction of labelled fatty acids (*e*) has to be subtracted from the non-extractable fraction of radioactivity (*d*) to obtain the inner-layer lysophosphatidylcholine fraction (*f*).

	% of total radioactivity in the membrane
(*a*) Total lysophosphatidylcholine	60
(*b*) Total phosphatidylcholine	31
(*c*) Total fatty acids	9
(*d*) Radioactivity non-extractable by albumin	53
(*e*) Non-extractable fatty acids (= inner-layer fatty acids)	3
(*f*) Inner-layer lysophosphatidylcholine ($d - b - e$)	19

Fraction of lysophosphatidylcholine in the inner layer at equilibrium $q = f/a = 0.32$

while lysophosphatidylcholine leaves the membrane in order to maintain the partition coefficient of lysophosphatidylcholine between cells and medium. A chain reaction comprising hydrolysis and re-partition is thus set up resulting in a slow but progressive degradation of lysophosphatidylcholine. Most of our studies were carried out at 37°C and a hematocrit of 10%. Under these conditions the rate of hydrolysis of palmitoyllysophosphatidylcholine was about 2% per h. Although, at this rate, hydrolysis does not affect the estimation of the reorientation of lysophosphatidylcholine during the first hours, it interferes significantly after prolonged incubation. Oleoyllysophosphatidylcholine hydrolyses at a lower rate (0.5% per h, Table I) which can be explained by its faster reorientation from the outer to the inner layer.

In case of the [^{14}C]serine labelled lysophosphatidylserine used in our study, hydrolysis occurs at a rate comparable to that of lysophosphatidylcholine, but produces a water-soluble labelled degradation product (glycerylphosphorylserine) which will not enter the membrane but accumulate in the aqueous phase. Due to re-partition the labelled moiety of lysophosphatidylserine will thus be lost progressively from the cell membrane. Hydrolysis could in part be suppressed in experiments with prolonged incubation periods (up to 20 h) by increasing the hematocrit from 10 to 30%. This increased the ratio of membrane compartment to aqueous compartment thereby decreasing the extracellular lysophospholipid fraction. Spontaneous hydrolysis of lysophosphatidylserine was thus diminished to about 0.5% per h.

(b) Acylation of lysophospholipid. A second process interfering with the quantification of reorientation rates of lysophospholipids is their acylation to diacylphospholipids at the cytoplasmic surface of the erythrocyte membrane [24]. Since acylation is an energy dependent process (reviewed in Ref. 24), requiring stoichiometric amounts of acyl-CoA and lysophospholipid (but see Ref. 25), it should be diminished by metabolic depletion of the cells. Therefore our measurements were done on cells incubated without energy supply. This procedure reduced the rate of acylation by about 80%. The flip process per se was not affected by metabolic depletion, in agreement with the results of Mohandas et al. [18].

In case of the slowly reorienting palmitoyllysophosphatidylcholine, acylation was found, under such conditions, to amount only to about 3% of the incorporated lysophosphatidylcholine after 23 h of incubation at 37°C. This small fraction of diacylphosphatidylcholine does not significantly affect the data. In case of faster reorientation processes considerable amounts of the lysophospholipid will accumulate in the inner layer before the acyl-CoA required for acylation has disappeared as a consequence of the lack of energy supply. Therefore, a rapidly reorienting lysophospholipid such as oleoyllysophosphatidylcholine (see Fig. 1) will be acylated to a considerable extent (see also Table I).

In studies with lysophosphatidylserine 60% of the radioactivity not extractable out of the cells by albumin after 12 h of incubation were present as the diacyl analogue. In such studies, acylation of labelled lysophospholipid could be reduced by preincubating the cells for 5 h with 70 nmol of nonradioactive lysophosphatidylserine per ml of cells, which is equivalent to 2% of the membrane phospholipid. This procedure does not affect the kinetics of reorientation (unpublished data) in agreement with results obtained with lysophosphatidylcholine [18], but reduces the fraction of acylated [^{14}C]lysophospholipid (after 20 h incubation) from 60 to less than 5%.

Evaluation of the experimental data

(a) Estimation of the equilibrium distribution of the lysophospholipid. The value of the equilibrium distribution of the lysophospholipid (q), which is essential for calculating the rate constant of the unidirectional movement of lysophospholipid from the outer to the inner layer [21], can be obtained by plotting the increase of the fraction of the lysophospholipid in the inner layer against time. Fig. 1 gives examples for the time-course of equilibration of oleoyllysophosphatidylcholine and oleoyllysophosphatidylserine. After 20 to 27 h of incubation the fraction of non-extractable radioactivity saturates, in case of oleoyllysophosphatidylcholine, at 0.53 ± 0.04 ($n = 6$). In order to obtain the 'true' fraction of lysophosphatidylcholine in the inner layer (q) from this value, the total and the non-extractable radioactivity have to be corrected for acylation of lysophosphatidylcholine (=

formation of labelled phosphatidylcholine) and for its hydrolysis (= formation of labelled fatty acids). A typical example for such a correction is demonstrated in Table I. From all experiments carried out, we obtained, after correction for acylation and hydrolysis, a fraction (q) of oleoyllysophosphatidylcholine in the inner layer at equilibrium of 0.3 ± 0.1 (S.D.; $n = 9$) of the total oleoyllysophosphatidylcholine present in the membrane (C_{tot}) at the end of the experiment. This number may indicate a slight preference of lysophosphatidylcholine for the outer membrane layer, although in view of the considerable corrections required to obtain this value, its reliability is only limited.

In experiments with oleoyllysophosphatidylserine, extrapolation to infinite time gives a non-extractable fraction of radioactivity of 0.84 ± 0.06 (Fig. 1). After correction, a fraction of 0.81 ± 0.07 ($n = 4$) of the oleoyllysophosphatidylserine is obtained for the inner layer. This value is much more reliable than that for lysophosphatidylcholine, since only minor corrections were necessary (acylation 5%, hydrolysis 5–10% of the total radioactivity incorporated initially).

(b) Determination of rate coefficients of lysophospholipid reorientation. Since errors in the determination of acylation and hydrolysis of the lysophospholipid are rather high as a result of its quantification by thin-layer chromatography, values of q have a rather high standard deviation (see above). Fortunately, this does not greatly affect the calculation [21] of the rate constants for unidirectional inward reorientation (k_{in}). For example, in case of oleoyllysophosphatidylcholine a range for q between 0.20–0.40 results, for the data from a typical experiment, in k_{in} values ranging only from 0.054 to 0.044 h^{-1}.

On the basis of the above considerations we decided to use a mean q value of 0.3 for calculating k_{in} values in experiments with various lysophosphatidylcholines and on erythrocytes of different species and with different cholesterol contents. Moreover, in order to simplify the procedure the data points for $C_{i(t)}/C_{tot}$ (see legend of Fig. 1 and Ref. 21) used for the calculation of k_{in} values were obtained in the early phase of the experiments where acylation and hydrolysis could still be neglected.

Influence of fatty acid composition and polar headgroup structure of lysophospholipids on their reorientation rate

For human erythrocytes the rate coefficient (k_{in}) for unidirectional inward reorientation of palmitoyllysophosphatidylcholine (saturated) amounts to 0.019 h^{-1}, while for oleoyllysophosphatidylcholine , its mono-unsaturated analogue, it is three-times higher (0.057 h^{-1}, Table II).

The temperature dependence of reorientation also varies with the structure of the fatty acid chain of the lysophospholipid (Fig. 2). Between 28°C and 42°C a linear relationship between $\log k_{in}$ and $1/T$ was obtained. From the Arrhenius plot an apparent activation energy of 134 kJ/mol (32 kcal/mol) can be calculated for the reorientation of palmitoyllysophosphatidylcholine, whereas for oleoyllysophosphatidylcholine a lower value is obtained (71 kJ/mol or 17 kcal/mol, Table II).

In further studies we investigated the effect of a negative charge of the polar head on the reorientation process. The rate of reorientation of oleoyllysophosphatidylserine was considerably faster than that of its zwitter-ionic analogue, oleoyllysophosphatidylcholine (Fig. 1; Table II). The energy barrier for the reorientation process, however, was not affected by the negative charge of the polar head. The activation energy for oleoyllysophosphatidylserine was quite similar to that of oleoyllysophosphatidylcholine (Table II).

TABLE II

RATE CONSTANTS AND APPARENT ACTIVATION ENERGIES FOR THE REORIENTATION OF DIFFERENT LYSOPHOSPHOLIPIDS

Erythrocytes were loaded with ^{14}C-labelled lysophospholipids, washed and incubated at different temperatures. Reorientation of lysophospholipids was measured as described in the Methods.

Lysophospholipid used as test molecule	$k_{in}(h^{-1})$ mean ± S.D.	E_a (kJ/mol)
Palmitoyllysophosphatidylcholine	0.019 ± 0.003 ($n = 17$)	134
Oleoyllysophosphatidylcholine	0.057 ± 0.009 ($n = 8$)	71
Oleoyllysophosphatidylserine	0.192 ± 0.063 ($n = 5$)	64

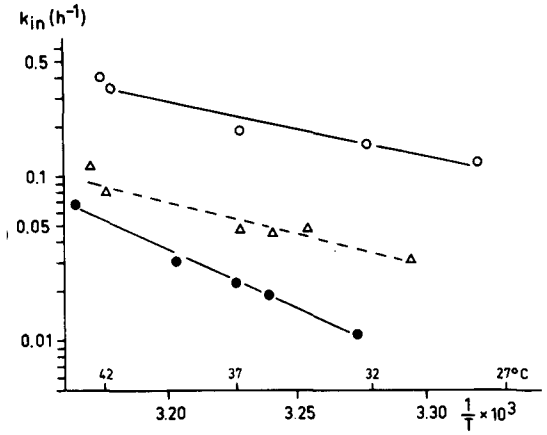

Fig. 2. Temperature dependence of the reorientation of lysophospholipids, plotted as an Arrhenius reciprocal plot. Erythrocytes were loaded with ^{14}C-labelled lysophospholipid, washed and incubated at different temperatures. Reorientation of lysophospholipid was determined as described in Methods. ○, oleoyllysophosphatidylserine; △, oleoyllysophosphatidylcholine; ●, palmitoyllysophosphatidylcholine.

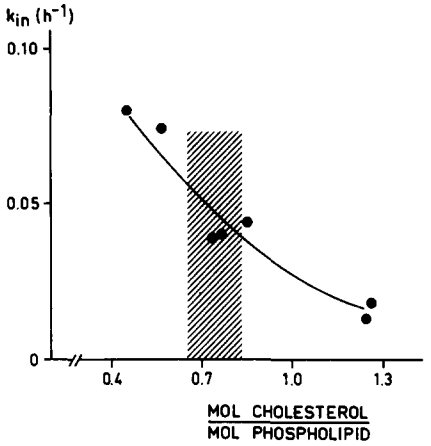

Fig. 3. Effect of membrane cholesterol content on lysophosphatidylcholine reorientation. Erythrocytes were depleted of, or enriched with cholesterol by incubation with phosphatidylcholine/cholesterol dispersions [45]. After three washings with Medium A cells were loaded with lysophosphatidylcholine, washed and reorientation of lysophosphatidylcholine determined. The shaded area represents the range of the cholesterol/phospholipid ratio in native erythrocytes.

Influence of membrane lipid composition on reorientation

Erythrocytes of various mammalian species differ in their phospholipid composition. They have a variable ratio of sphingomyelin to phosphatidylcholine and a variable mean number of double bonds in the fatty acids of the glycerophospholipids [26,27]. Correlations between rates of various transport processes and the lipid composition of the erythrocyte have been obtained [28,29]. It was therefore of interest to check correlations between flip rates in the erythrocytes of various species and the lipid composition of their membranes. In addition, variation of the membrane cholesterol content, known to affect transport processes, too [30–32], was checked.

Enrichment of the membrane with cholesterol decreased the rate of reorientation of lysophosphatidylcholine, as recently also reported by Mohandas et al. [18], while cholesterol depletion of the membrane increased the reorientation rate. A curvilinear relationship between the cholesterol content of the membrane and the reorientation rate was obtained (Fig. 3). Since according to Cooper et al. [33] membrane microviscosity, as derived from fluorescence polarisation, is linearily dependent on membrane cholesterol content and

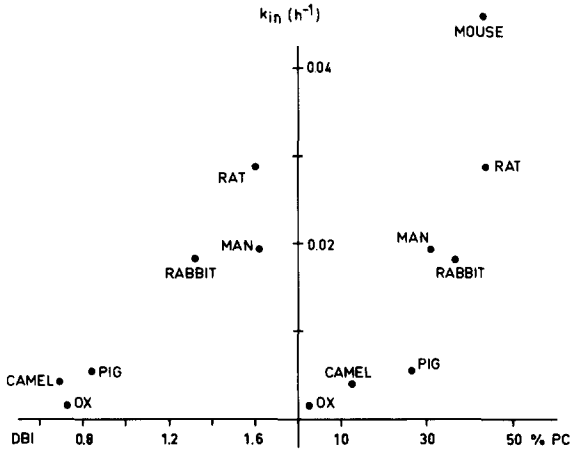

Fig. 4. Correlation between the transbilayer reorientation rates of palmitoyllysophosphatidylcholine in erythrocytes of various mammalian species and the lipid composition of their membranes. The phosphatidylcholine (PC) contents and fatty acid composition of the lipids were taken from Refs. 26, 27 and 46–49. The double bond index (DBI) of the fatty acids of the phospholipids represents the mean number of double bonds per hydrophobic chain. Sphingomyelin was considered to contain only saturated chains. Results represent mean values from three experiments.

membrane fluidity is related inversely to microviscosity, an almost linear relationship between membrane fluidity and the rate of reorientation of lysophosphatidylcholine may be postulated.

A dependence of the reorientation process on membrane lipid composition is further suggested by a relationship between reorientation rates of lysophosphatidylcholine in erythrocytes from various mammalian species (Fig. 4) and their phosphatidylcholine content or the mean saturation of their fatty acids, as quantified by the double bond index. Erythrocytes of mice and rats, with a high content of phosphatidylcholine and unsaturated fatty acids, exhibit high reorientation rates whereas rates are slow in cells with a lower phosphatidylcholine content (ox, camel) corresponding to a high sphingomyelin content, or with a lower content of unsaturated fatty acids (pig).

Discussion

The method

In this paper a simple method is presented to quantify the reorientation of lysophospholipids between the two lipid layers of a biological membrane. The technique differs from a similar approach described by Mohandas et al. [18] in that albumin is omitted from the incubation medium. Due to this difference all of the radioactivity present in the cell suspension during the flip measurement is localized in the membrane from the beginning and is essentially constant with time. The incubation medium contains only very little of the total radioactivity of the system ($< 0.2\%$) in contrast to the technique of Mohandas et al. [18]. Therefore, intensive washings of the cells, going along with a loss of radioactivity from the membrane are dispensible in our technique.

As a further advantage, the rate of reorientation and the equilibrium distribution of lysophospholipids may be obtained simultaneously, although at the expense of somewhat cumbersome correction procedures for obtaining the value of q, the equilibrium ratio of inner-layer lysophosphatidylcholine : total lysophosphatidylcholine. As a final advantage of our method only small suspension volumes (< 1 ml), are required from which cells can rapidly be isolated by centrifugation. Since all of the radioactive material is localized in the membrane, only small amounts of labelled lysophospholipid and of cells are required. Liquid scintillation counting of small amounts of erythrocytes will avoid quenching problems. These advantages of our method make it easy to handle and thereby promising for routine studies.

Mechanism of flip site formation

In general, flip-flop of phospholipids may be envisaged as a statistical event occurring at fluctuating defects in the lipid bilayer. These defects may either be enclosed by the polar heads or by the alkyl chains of the phospholipids [34,35]. Evidence has been obtained, that the defects may also act as aqueous pores, permeable to ions and hydrophilic non-electrolytes [36,37,50]. Alternatively, non-bilayer phases [38] or the interface between lipids and intrinsic proteins may, in principle, act as flip sites [8,9].

The decrease of flip rates following cholesterol enrichment of the lipid bilayer, as observed in the present work, may result from a suppression, by cholesterol, of the formation of structural defects in the lipid bilayer. In addition, cholesterol might even suppress formation of structural defects at the interface of lipids and intrinsic proteins [9]. Alternatively, cholesterol may bind lysophosphatidylcholine, thereby suppressing its access to the reorientation sites.

The lower rate of flip-flop in membranes rich in sphingomyelin and containing little unsaturated fatty acids (ox) as compared to membranes with a low sphingomyelin content and a high content of unsaturated fatty acids (rat) can be explained in a similar way. Sphingomyelin and saturated fatty acids may be expected to decrease the frequency of formation of structural defects [38,39]. Sphingomyelin also stabilizes the interaction between headgroups of lipids [40].

Surprisingly, the activation energy of the flip-flop process depends on the acyl chain of the lysophospholipids, but does not seem to depend on their head group. In view of this finding, but also of the comparatively low value of the activation energy it seems unlikely that the polar head group has to cross the unmodified hydrophobic barrier during the flip process. Interestingly, lysophospholipids demonstrate the same dependence of their rate of reorientation on their acyl

composition as their diacyl analogues [12,41]. In addition, the half-times of transbilayer reorientation of the lysophosphatidylcholines ($t_{1/2}$ for oleoyl- and palmitoyllysophosphatidylcholine, 4 and 11 h, respectively) calculated from the k_{in} values in Table II using the equation $t_{1/2} = q \cdot \ln(2/k_{in})$ and of diacylphosphatidylcholines as measured in human and rat erythrocytes ($t_{1/2} = 4 - 27$ h, Refs. 11–15 and 41) lie in the same range. Comparison of half-times of transbilayer reorientation of palmitoyllysophosphatidylcholine ($t_{1/2} =$ 11 h) with those of its diacyl analogues shows that although the introduction of a second saturated palmitoyl chain into the palmitoyllysophosphatidylcholine molecule somewhat reduces flip ($t_{1/2}$ of dipalmitoylphosphatidylcholine 27 h, Ref. 41), introduction of an unsaturated fatty acid, as in case of phosphatidylcholines from egg yolk and from soya bean, does not greatly affect flip ($t_{1/2}$ 12.8 and 8.1 h according to Table II of Ref. 41). It is concluded therefore that exogenous lysophospholipids represent a very useful tool to study the properties of the flip process of native phospholipids.

Correlation between the asymmetric intramembrane distribution of lysophosphatidylserine and the native asymmetry of its analogue, phosphatidylserine

In contrast to the slight preference of neutral lysophosphatidylcholine for the outer layer, negatively charged lysophosphatidylserine accumulates in the inner layer (Fig. 1) of the erythrocyte membrane. This behaviour simulates the preferential orientation of endogenous phosphatidylserine in the inner layer of the native membrane and provides a new piece of information on possible mechanisms responsible for the asymmetry of phospholipids in biological membranes. In the first place it demonstrates that a negatively charged phospholipid is able to move from the outer layer of the native membrane to the inner layer and excludes the possibility that the asymmetric distribution of phosphatidylserine in the native erythrocyte membrane originates from its intrinsic inability to reorient between the two lipid layers. In the second place, the result demonstrates, that attractive forces in the inner layer act on negatively charged lysophosphatidylserine. Such forces could be interactions of phosphatidylserine with other lipids or with proteins. Several years ago we presented evidence for a crucial role of the membrane skeletal protein, spectrin, in the maintenance of the phospholipid asymmetry of erythrocyte membranes [23]. Such a stabilization of asymmetry, in spite of a high intrinsic mobility of the phospholipid could result from a direct interaction between negatively charged phosphatidylserine and spectrin, which prevents the actual reorientation of the phospholipid without affecting its intrinsic mobility. Evidence for such interactions between spectrin and phosphatidylserine has meanwhile been obtained in model systems [42–44].

Whether asymmetric insertion is a common feature of negatively charged lysophospholipids is presently under investigation. The significance of the membrane skeleton as the stabilizing mechanism responsible for the preferential orientation of negatively charged phosphatidylserine to the inner membrane surface will be further dealt with in a following paper.

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