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Nonmediated Flip-Flop of Phospholipid Analogues in the Erythrocyte Membrane as Probed by Palmitoylcarnitine: Basic Properties and Influence of Membrane Modification

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Summary. The rules governing the transbilayer reorientation (flip-flop) of long-chain amphiphilic components in biological membranes were further elucidated by studying the flip-flop of palmitoylcarnitine in human erythrocytes. Flip rates were derived from the time-dependent decrease of extractability of palmitoylcarnitine by albumin after primary insertion of trace amounts of the labeled probe into the outer membrane layer. The flip rate (half time 2.6 hr at 37°C in human erythrocytes) is fast enough to be measurable also in membranes exhibiting low flip rates such as that of ox erythrocytes. Flip rate constants for the inward and outward reorientation are similar and the probe equilibrates at a 1 : 1 ratio between the two layers.

The flip is a simple, diffusion-like process. It is not inhibited but even enhanced by chemical modification of membrane proteins. It is also enhanced by insertion of channel-forming antibiotics into the membrane and by pre-exposure of the cells to temperatures exceeding 42°C. The extent of this enhancement increases with the duration and the temperature of the pre-exposure. Since spectrin is denatured in this range of temperatures, the finding constitutes a new piece of evidence that the membrane skeleton is involved in the maintenance of bilayer stability and that a decrease of bilayer stability goes along with the formation of local defects acting as flip sites for phospholipids and related compounds.

As a particularity, the flip is enhanced by lowering the pH and exhibits interindividual variability, phenomena not observed for the flip-flop of lysophosphatidylcholine. This suggests that generalizations on the kinetics of nonmediated flip-flop of membrane-intercalated amphiphiles may not be justified.

Key Words flip-flop · erythrocyte · membrane · palmitoylcarnitine · lysophosphatidylcholine

Introduction

Passive, noncarrier-mediated transport of small polar solutes across biological membranes has been the object of numerous studies for the last century (*see* e.g., Davson & Danielli, 1970; Deuticke, 1977; Stein, 1986). It has been the (almost invariable) common methodical approach in the investigation of these phenomena to derive transport rates from the time course of disappearance of the transport substrate from the aqueous compartment on the *cis* side of the membrane or from its appearance in the *trans* side aqueous compartment. This approach becomes increasingly less appropriate when more lipophilic compounds are considered. At least under nonsteady-state conditions such compounds will disappear from a *cis* side aqueous compartment, from which they are introduced much more rapidly than they appear in the *trans* side compartment, due to their high membrane/aqueous phase partition coefficient.

A class of particular interest among these solutes are long-chain amphiphilic compounds that are incorporated into the membrane in alignment with the head groups and the acyl chains of the membrane phospholipids, and are thought to reorient from one leaflet of the bilayer to the other by a process termed flip-flop (Kornberg & McConnell, 1971). This class comprises compounds such as fatty acids, long-chain alcohols, lysophospholipids, many detergents, acyl carnitines and, in principle, the native diacyl phospholipids.

The analysis of the kinetics and equilibria governing the process of transbilayer reorientation of such amphiphiles in cellular membranes is still in its beginning. In some instances, evidence for the involvement of carrier proteins is available, transporting, for instance, phospholipids (for a review *see* Devaux, 1988), acyl carnitines (Idell-Wenger, 1981; Murthy & Pande, 1984) and, possibly, fatty acids (Abumrad et al., 1981; Stremmel, 1988). In addition, passive nonmediated movement of such compounds has been demonstrated (Mohandas et al., 1982; Bergmann et al. 1984*b*; Zachowski, Fellman & Devaux, 1985; Haest et al., 1986; Schneider, Haest & Deuticke 1986*a*). The transbilayer reorientation of amphiphiles will usually become a complete transmembrane transport when

appropriate binding proteins in the adjacent aqueous phase are available, taking care of the charging and discharging of the adjacent leaflet of the bilayer.

Studies from others and from our own laboratory have recently provided first insights into the the properties of a membrane and of the transport probe that influence passive flip rates and steady-state distribution of lysophospholipids and other exogenously inserted phospholipid analogues (Lin, Macey & Mehlhorn, 1983; Bergmann et al., 1984*b*; Fujii, Tamura & Yamane, 1985; Mohandas et al., 1985; Haest et al., 1986; Middelkoop et al., 1986; Tamura et al., 1986; Devaux, 1988). These studies have not yet provided a final picture of the rules governing this particular type of transport process, in which a highly polar or even charged head group has to be translocated across, or to bypass somehow, the hydrophobic core of the membrane. In the work presented here, palmitoylcarnitine is introduced as a new probe to further elucidate the rules governing the process of flip-flop. We have used a methodical approach that has proven successful in earlier studies on slow flip processes (Mohandas et al., 1982, 1985; Bergmann et al., 1984*a*,*b*; Fujii et al., 1985; Schneider et al., 1986*a*). This approach takes advantage of the high affinity of many long-chain acyl compounds for serum albumin, which, therefore, can be used to extract such probes from cells as long as they are present in the outer membrane leaflet, provided their reorientation rate is slower than the rate of their extraction.

As will be shown, the flip-flop of palmitoylcarnitine is similar in a number of characteristics to the flip-flop of lysophospholipids, in spite of the different head group structure. On the other hand, there are differences, suggesting that generalizations on the kinetics (and mechanisms ?) of nonmediated flip-flop may not be justified.

Materials and Methods

Materials

Fresh human blood, anticoagulated with citrate, was obtained from the local blood bank, stored at 4°C and used within five days. Ox blood was from the local abbatoir. Erythrocytes were isolated by centrifugation and washed three times with 10 volumes of 0.9% NaCl after removal of the buffy coat. Bovine serum albumin (essentially fatty acid free) was from Boehringer Mannheim; palmitoylcarnitine and the buffers MES,[1] PIPES, HEPES and HEPPS from Sigma. L-[1-^{14}C]-palmitoylcarnitine (spec act 2.04 GBq/mmol) was from NEN, L-[1-^{14}C]-oleoyllysophosphatidylcholine (spec act 2.07 GBq/mmol) from Amersham.

Unless otherwise indicated incubations were carried out in a medium containing (concentrations in mmol · liter^{-1}) KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (12.5) (pH 7.4) and sucrose (44) (= medium A) to protect the cells against colloid-osmotic lysis.

Methods

Measurements of the Transbilayer Reorientation of Palmitoylcarnitine

The outer membrane layer of erythrocytes was loaded with [^{14}C]-labeled palmitoylcarnitine by incubation of 100 μl cell suspension in medium A (hematocrit 50%) for 2 min (22°C) in a 1.5 ml polypropylene snap cap tube containing 1 nmol of dry lipid probe. Following centrifugation, cells were resuspended in medium A (10% hematocrit). Reorientation of palmitoylcarnitine to the inner membrane layer was quantified by following the decrease of extractability of labeled palmitoylcarnitine by albumin as described before (Bergmann et al., 1984*b*) with some modifications.

Briefly, after different times of incubation (37°C) 50 μl aliquots of the cell suspension were transferred to small vials containing 400 μl ice-cold medium A. Two 200 μl aliquots of this suspension were centrifuged and the supernatants removed. One of the samples, serving as the control, was directly and completely transferred into scintillation fluid. Its radioactivity provided the total amount of radioactive probe in the membrane. The other sample was treated twice with 200 μl of a 1.5% albumin solution in medium A (2 min, 4°C) in order to extract the labeled lipid from the outer layer and then transferred into scintillation fluid to determine the inner layer radioactivity. The rate constants (k_{in}) and half times of the reorientation of palmitoylcarnitine from the outer to the inner layers were calculated using the mathematical model described (Bergmann et al., 1984*a*).

Effects of pH on Transbilayer Reorientation

In studies on the pH effect on flip rates, the phosphate buffer in medium A was exchanged for more effective buffers. MES was used for pH 6, PIPES for pH 6.5, HEPES for pH 7.1, 7.4 and 7.7, and HEPPS for pH 8.0 and 8.5. The desired pH of the cell suspension was adjusted by addition of NaOH or HCl,

ATP Depletion of Erythrocytes

A suspension of washed erythrocytes in nine volumes of medium A (pH 7.4), was treated with 5 mmol · liter^{-1} iodoacetate for 15 min at 37°C. The cells were washed twice with medium A and reincubated another 45 min in nine volumes of medium containing 10 mmol · liter^{-1} inosine. Flip measurements were then carried out as usual but in the presence of 10 mmol · liter^{-1} inosine, which by itself did not affect flip rates (*data not shown*). ATP levels in erythrocytes were determined by HPLC according to Jüngling and Kammermeier (1980).

Heat Treatment of Erythrocytes

Erythrocytes in medium A were incubated at temperatures between 42 and 48°C for varying times and then washed three times

[1] MES: 2-(N-morpholino)ethanesulfonic acid; PIPES: piperazine-N,N′-*bis*(2-ethanesulfonic acid); HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HEPPS: N-2-hydroxyethylpiperazine-N′-3-propanesulfonic acid; Diamide: diazenedicarboxylic acid *bis*-[N,N′-dimethylamide]; PAF: 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine.

with medium A. Subsequently, flip rates were measured at 37°C as usual.

Results

Basic Characteristics of the Flip-Flop of Palmitoylcarnitine

Palmitoylcarnitine, like lysophospholipids and platelet activating factor (PAF), becomes rapidly incorporated into the membrane of erythrocytes when a cell suspension is mixed with trace amounts of the dry labeled probe adsorbed to the wall of a polypropylene tube. At the low level of palmitoylcarnitine (1 nmol) used, about 70% of the lipid probe are inserted into the membrane; the rest remains adsorbed to the vessel wall. The membrane contents of palmitoylcarnitine thus amounts to 14 nmol per ml of packed cells. Assuming a phospholipid content of 4.25 μmol per ml of packed cells (Broekhuyse, 1974), this represents a ratio of palmitoylcarnitine to membrane phospholipid of 3 : 1000 (mol/mol). After incorporation of palmitoylcarnitine into the outer membrane layer, the cells were resuspended to measure the flip to the inner layer. During flip measurements less than 1.5% of the membrane-bound palmitoylcarnitine was lost to the medium.

To ascertain the applicability of the albumin extraction method for assessing the transbilayer movement of palmitoylcarnitine in erythrocytes, the efficiency of the extraction of palmitoylcarnitine from the outer layer by albumin was checked. Immediately after loading of the outer membrane layer with the lipid probe, a single extraction with albumin (1.5%) for 2 min at 4°C removed 95% and two sequential extractions >99% of the labeled palmitoylcarnitine. Two repetitive extractions (2 min each, 4°C) with albumin were therefore used in the flip experiments (*see* Methods).

Incubation of erythrocytes at 37°C following insertion of labeled palmitoylcarnitine into the outer membrane layer resulted in a time-dependent decrease of extractability of radioactive material by albumin (*cf.* Fig. 1), indicating a slow reorientation of palmitoylcarnitine from the outer to the inner membrane surface (about 15% · hr^{-1}). It was then checked whether metabolic conversion of palmitoylcarnitine contributes to the decrease of extractability by albumin. To this end, lipid extracts were prepared from cells after varying periods of [^{14}C]-palmitoylcarnitine flip, subjected to thin layer chromatography, and the chromatograms scanned for radioactivity as described by Bergmann et al., 1984*b*. After short incubation periods essentially all of the radioactivity was found in the [^{14}C]-

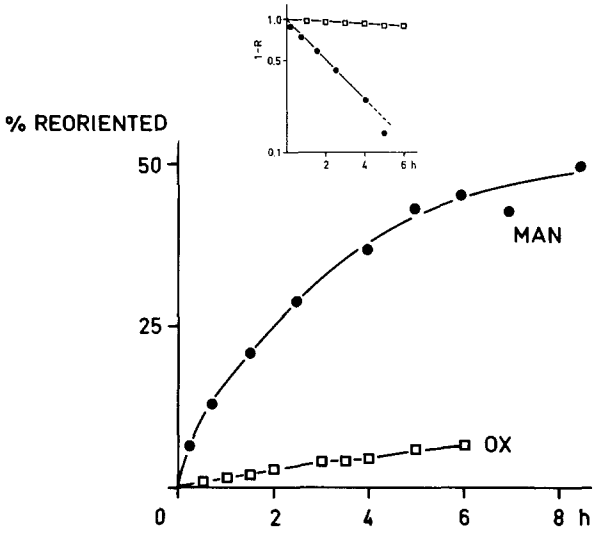

Fig. 1. Time-dependent reorientation of palmitoylcarnitine from the outer to the inner membrane layer of human and ox erythrocytes. Reorientation measured at 37°C by the increase of the fraction of [^{14}C]-palmitoylcarnitine nonextractable by albumin. The insert demonstrates the first order kinetics of the flip process. R = labeled palmitoylcarnitine in the inner layer after a given time of reorientation relative to that after infinite time

palmitoylcarnitine peak. After increasing time periods a small but progressive amount of radioactivity accumulated in additional peaks (probably palmitate and phospholipids), obviously as a result of hydrolysis of palmitoylcarnitine and subsequent incorporation of the labeled palmitate into phospholipids. From the data, a rate of metabolic conversion of palmitoylcarnitine of about 2% per hr at pH 7.4 was calculated. The hydrolytic cleavage of palmitoylcarnitine is thus slow compared to the rate of decrease of its extractability by albumin (Fig. 1). After 6 hr of incubation, palmitoylcarnitine still represented 90% of the radioactivity remaining in the cells after albumin extraction. The fraction of nonextractable radioactivity was, therefore, taken as the fraction of palmitoylcarnitine having reoriented to the inner membrane layer.

The kinetics of the increase of palmitoylcarnitine in the inner membrane layer are compatible with an equal distribution of palmitoylcarnitine between both membrane lipid layers after attainment of steady-state conditions. Furthermore, the inset of Fig. 1 demonstrates a first order process for the equilibration. On the basis of a final 1 : 1 distribution, a flip rate constant was calculated using the mathematical treatment of Bergmann et al. (1984*a*). The mean flip rate constant at 37°C for 38 donors was 0.13 hr^{-1} (±0.04, SD) and the half time of 2.6 hr. Thus, the flip of palmitoylcarnitine is faster than that of palmitoyl- and oleoyl-lysophosphatidylcholine as well as that of 1-O-alkyl-2-acetyl-sn-glyceryl-

Table 1. Interindividual variability of flip rate constants of palmitoylcarnitine[a]

Donor	Flip rate constant (hr^{-1} ± SD)	
	Palmitoylcarnitine	18 : 1 LysoPC
1	0.091 ± 0.002	0.059
2	0.099 ± 0.012	0.046
3	0.109 ± 0.005	0.056
4	0.139 ± 0.009	—
5	0.140 ± 0.004	0.054
6	0.166 ± 0.009	0.061

[a] Rate constants of palmitoylcarnitine flip were measured at three different times during a 10-week period. The mean pH of the suspension used in flip experiments was 7.39 with a difference of 0.03 between the highest and lowest pH value. For reasons of comparison, the flip rate constants for oleoyl-lysophosphatidylcholine (18 : 1 lysoPC) are given.

3-phosphocholine (platelet activating factor), which reorient with half times of 11, 4 and 17 hr, respectively (Bergmann et al., 1984*b*; Schneider et al., 1986*a*). Transbilayer reorientation of palmitoylcarnitine can, therefore, also be assessed in membranes having unmeasurably low flip rates for lysophospholipids, e.g., those of ox erythrocytes (Bergmann et al., 1984*b*). For ox erythrocytes, the rate constant of the palmitoylcarnitine flip derived from the curve in Fig. 1 is 0.012 hr^{-1}, 10 times lower than in human erythrocytes. These results substantiate the concept (Bergmann et al., 1984*b*; Fujii et al., 1985; Haest et al., 1986; Middelkoop et al., 1986; Tamura et al., 1986) that the flip process is determined by the properties of the lipid probe as well as those of the membrane.

Interindividual Variability

In experiments on erythrocytes from different individuals, the flip rates for palmitoylcarnitine appeared to vary to a greater extent than those for oleoyl-lysophosphatidylcholine. For this reason, the question of interindividual differences of the flip rates of such lipid probes was addressed. Flip rate constants of palmitoylcarnitine for six healthy donors (three male, three female) revealed a remarkable constancy over a 10-week period for each of the donors (Table 1). Between the donors, however, the flip rates varied up to 100%. This interindividual variability of flip rates may not be a common property of all flip probes, since the flip rates of oleoyl-lysophosphatidylcholine were almost the same for the six individuals (Table 1). No reasonable explanation for this difference between the probes is presently at hand.

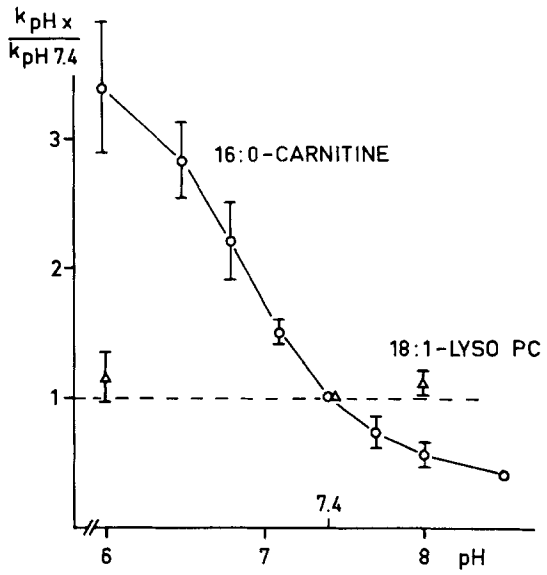

Fig. 2. pH dependence of the transbilayer reorientation of palmitoylcarnitine. The pH of erythrocyte suspensions was adjusted to different values between 6.0 and 8.5 and the outer membrane layer of cells loaded with [^{14}C]-palmitoylcarnitine. Rate constants for the time-dependent transbilayer reorientation of palmitoylcarnitine were calculated as described (Bergmann et al., 1984*b*). Rates obtained at different pH values were normalized to the rate at pH 7.4. Data represent mean values of 2–16 experiments (±SD). For reasons of comparison, the pH independence of the flip rate of oleoyl-lysophosphatidylcholine (18 : 1-lysoPC) is also shown

pH Dependence and Symmetry of Flip Rates

Palmitoylcarnitine has a positively charged quaternary nitrogen and a negatively charged carboxylate group (pK′ ~ 4). Lowering of pH converts the zwitterionic form of the lipid into a positively charged one. It seemed of interest, therefore, to study the pH dependence of the flip rate. As shown in Fig. 2, the flip rate is enhanced 3.5-fold by lowering the pH from 7.4 to 6 and diminished to half the value at pH 7.4 upon an increase of pH to 8. The increase of the flip rate, upon a decrease of pH from 8.5 to 6, describes a sigmoidal curve similar to that of the protonation of a dissociable group with a pK ≤ 7. This pH dependence is specific for palmitoylcarnitine. The flip rate of oleoyl-lysophosphatidylcholine does not vary between pH 6 and 8 (Fig. 2).

Since the flip rate of palmitoylcarnitine is particularly high at pH 6, it is possible to rapidly load the inner membrane layer with palmitoylcarnitine, extract the outer layer palmitoylcarnitine with albumin and subsequently measure the backward reorientation of the lipid probe to the outer membrane layer by quantification of the time-dependent increase of its extractability by albumin. After a 3 hr

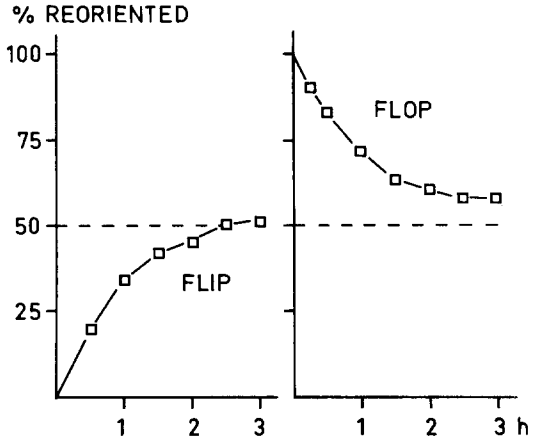

Fig. 3. Inward (*flip*) and outward (*flop*) reorientation of palmitoylcarnitine between the outer and the inner erythrocyte membrane layer. The outer membrane layer of erythrocytes was loaded with [^{14}C]-pamitoylcarnitine and its reorientation to the inner layer measured at pH 6.0 and 37°C (left panel). Subsequently, palmitoylcarnitine in the outer layer was removed by extraction with albumin and the outward reorientation of palmitoylcarnitine measured by following the time-dependent decrease of the fraction nonextractable by albumin (right panel)

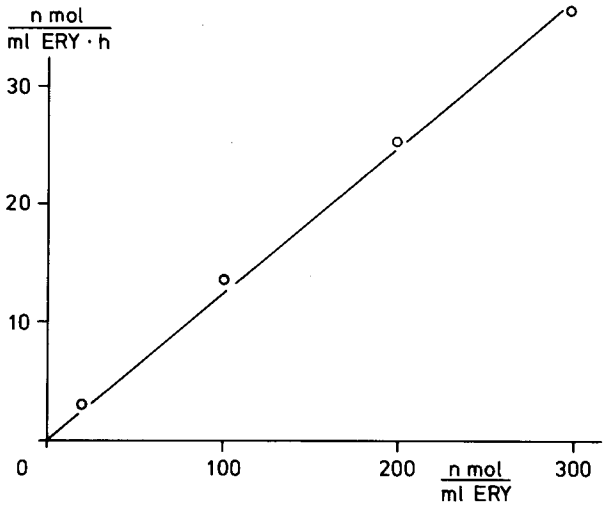

Fig. 4. Concentration dependence of the flip of palmitoylcarnitine. Erythrocytes suspended in medium A were loaded with mixtures of labeled and nonlabeled palmitoylcarnitine in amounts of 17–300 nmol/ml packed cells. Flip rates were measured as described in the Methods section

flip period at pH 6, a stationary 1 : 1 distribution of the probe was attained and about 50% of the membrane palmitoylcarnitine could be removed by extraction with albumin (Fig. 3). All the remaining palmitoylcarnitine now resided in the inner membrane layer. Further incubation of these albumin-extracted cells at 37°C resulted in a time-dependent reappearance of palmitoylcarnitine in the outer membrane layer as measured by the increase of the albumin-extractable fraction (Fig. 3). The half time of this "flop" was about the same as that of the flip, indicating that the transbilayer movement of palmitoylcarnitine is a symmetrical process with half times for the flip and flop process of about 0.7 hr at pH 6 and 37°C.

Concentration, Temperature and ATP Dependence

In further experiments, we checked the possible carrier mediation of the palmitoylcarnitine reorientation. To this end, the dependence of the flip process on substrate concentration, temperature and ATP were studied. Between 17 and 300 nmol of palmitoylcarnitine per ml of packed erythrocytes (palmitoylcarnitine to membrane phospholipid ratio's 1 : 250 to 1 : 14), the translocated amount per hr increases linearly with the amount inserted initially into the outer membrane leaflet (Fig. 4). From the temperature dependence of the flip rate constant between 25 and 41°C, an activation energy of 108 ± 2 kJ/mol (±SD; $n = 4$) could be calculated (Fig. 5). This activation energy is in between the values for the flip rates of palmitoyl-lysophosphatidylcholine (134 kJ/mol), and for oleoyl-lysophosphatidylcholine (71 kJ/mol) (Bergmann et al., 1984*b*). High activation energy and lack of saturation kinetics provide evidence for a nonmediated translocation of palmitoylcarnitine.

This view is further substantiated by a lack of ATP dependence. ATP depletion of erythrocytes by a pretreatment with iodoacetate and inosine (*see* Methods), which decreased the intracellular ATP to <5% of the original level, had no significant inhibitory effect on the flip rate of palmitoylcarnitine (*data not shown*). Moreover, treatment of the cells with the SH-reagent diamide, which suppresses glycolytic metabolism (*data not shown*), did not inhibit, but even accelerated the flip rate (Table 2). It is, therefore, likely that the flip of palmitoylcarnitine across the erythrocyte membrane is not dependent on the availability of metabolic energy.

Flip Enhancement Upon Membrane Perturbation

Flip rates of palmitoylcarnitine can be accelerated by various types of membrane modification. This is shown in Table 2 for cells treated with diamide, which selectively oxidizes SH-groups (Haest et al.,

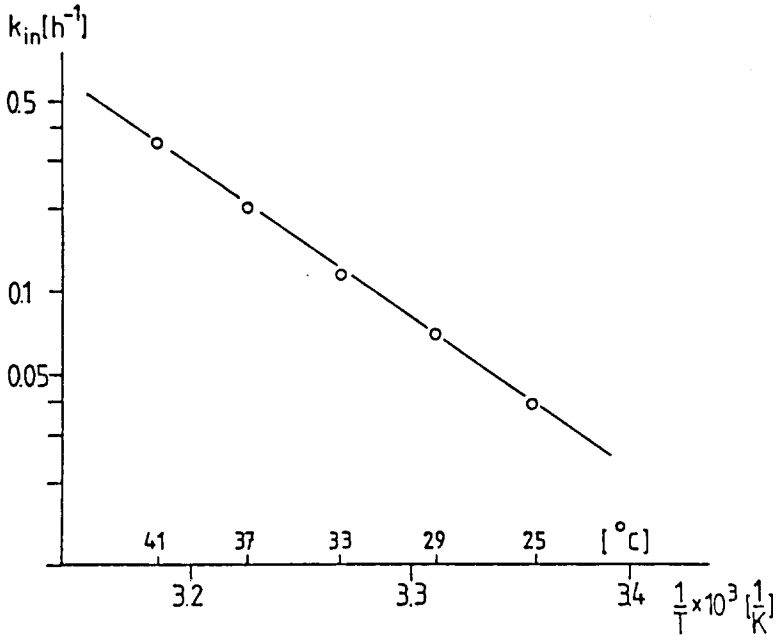

Fig. 5. Temperature dependence of the flip of palmitoylcarnitine. Flip rates were measured at temperatures between 25 and 41°C. The apparent activation energy was calculated from a semilogarithmic plot of the rate constant against the inverse of temperature

1978), but also for cells subjected to peroxidative damage by the O_2-derived reactive species that are formed when erythrocytes are treated with t-butylhydroperoxide (Deuticke, Heller & Haest, 1986) or with iodoacetate/vanadate/ferricyanide, as discovered recently (Heller, Jahn & Deuticke, 1987). Moreover, noncovalent perturbation of the membrane by channel-formers, such as gramicidin A and amphotericin B, and by local anaesthetics enhances the flip of palmitoylcarnitine. Since the latter type of perturbation as well as diamide treatment were previously shown also to stimulate the flip of lysophospholipids (Schneider et al., 1986*b*; Classen et al., 1987), it seems safe to conclude that the hypothetic flip sites induced by such membrane perturbations do not discriminate between various probes. This is, however, not generally the case, as will be demonstrated in the following paragraph.

Effect of Thermal Denaturation of Spectrin on the Flip Rate

Spectrin and the membrane skeleton have been claimed to keep the nonmediated transbilayer mobility of phospholipids at a low level and to be involved in the control of their transmembrane distribution (Haest et al., 1978; Bergmann et al., 1984*a*; Dressler et al., 1984; Franck et al., 1985*a*,*b*; Mohandas et al., 1985). Spectrin is also known to denature at temperatures above 42°C (Brandts et al., 1977; Tomaselli, John & Lux, 1981; Minetti et al., 1986). Heat exposure of erythrocytes might therefore be expected to alter the transbilayer mobility of phospholipids and analogous membrane-intercalated amphiphilic probes. Earlier measurements of the effect of temperature on flip rates of diacylphosphatidylcholine demonstrated an unexpected increase of the flip rates at temperatures above 44°C (Franck et al., 1985*a*), which exceeded the extent extrapolated from the activation energy of the flip process. In those studies, however, flip rates were measured while the cells were kept at the elevated temperatures. Thus, flip enhancement related to the denaturation of spectrin could not be distinguished from the temperature dependence of the flip process reflecting its activating energy. In the present study we investigated the effects of a heat pretreatment on the flip rate subsequently measured at a fixed temperature of 37°C.

Heating of erythrocytes to 42°C for 3 hr resulted in a significant increase of the flip rate of palmitoylcarnitine as measured subsequently at 37°C (Fig. 6). Flip rates became even more enhanced upon heating the cells to higher temperatures. The higher the temperature of the heat treatment, the shorter was the time required to obtain a measurable flip acceleration. Flip enhancement after a heat pretreatment was also observed for oleoyl-lysophosphatidylcholine but was much less pronounced. A heat pretreatment at 50°C for short time periods causes fragmentation of erythrocytes (Ham et al., 1948; Minetti et al., 1986). In the present experiments, no fragmentation occurred when cells were incubated for 2 hr at 46°C. At 48°C, fragmentation only started after 1–2 hr of incubation (*data not shown*).

Table 2. Enhancement of flip rates of palmitoylcarnitine and lysophosphatidylcholines by various membrane modifications[a]

Membrane modification	Flip rate (hr^{-1})	
	16:0 Carnitine	16:0 LysoPC
None	0.13	0.02
Diamide (5 mmol/liter, 37°C, pH 8, 90 min)	1.4	1.1
t-BuOOH + azide (2 + 2.5 mmol/liter, 37°C, 30 min)	2.0	—
Vanadate + iodoacetate + ferricyanide (0.2 + 0.5 + 5 mmol/liter, 45 min, 37°C)	1.7	—
Gramicidin A (5 μmol/liter)	2.0	1.2
Amphotericin B (40 μmol/liter)	0.38	0.41
Tetracaine (3.5 mmol/liter)	1.0	~0.8

[a] Erythrocytes were chemically modified by diamide, by *t*-butylhydroperoxide (*t*-BuOOH) in presence of azide, and by the simultaneous exposure to vanadate, iodoacetate and ferricyanide for the time periods indicated in the Table according to Bergmann et al. (1984*b*), Deuticke, Heller and Haest (1987) and Heller et al. (1987). After washing of the cells, flip rates of palmitoylcarnitine and palmitoyllysophosphatidylcholine (16:0 lysoPC) were determined. Tetracaine, amphotericin and gramicidin were introduced into the membrane and flip rates measured in the presence of the agents (Schneider et al., 1986*b*; Classen et al., 1987). Flip rate constants were calculated according to Bergmann et al. (1984*b*).

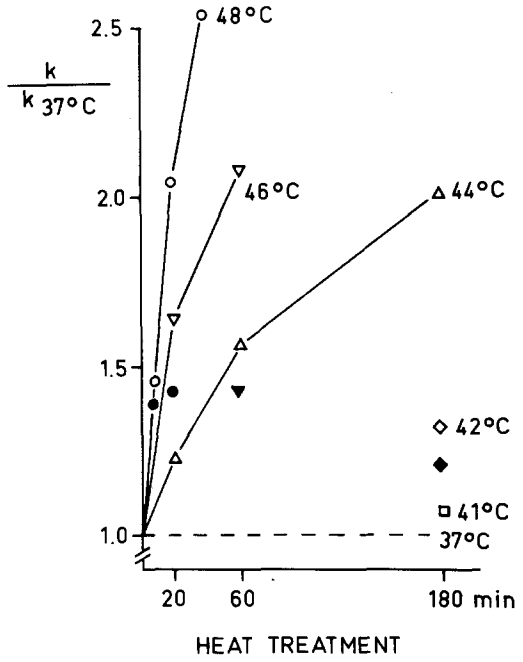

Fig. 6. Acceleration of the transbilayer reorientation of palmitoylcarnitine and oleoyl-lysophosphatidylcholine after thermal denaturation of spectrin. Erythrocytes were pre-exposed to different temperatures for the time periods given on the abscissa. Subsequently, the transbilayer reorientation rates of [^{14}C]-palmitoylcarnitine (open symbols) or [^{14}C]-oleoylphosphatidylcholine (closed symbols) were measured at 37°C and pH 7.4. Data normalized to rates measured after exposure of the cells to 37°C

Discussion

As observed earlier for lysophospholipids, platelet activating factor and fatty acids (Bergmann et al., 1984*b*; Dressler et al., 1984; Schneider et al., 1986*a*; Bröring, 1988), palmitoylcarnitine added to an erythrocyte suspension (hematocrit 10%) is almost exclusively bound to the membrane in our method of loading of the cells with lipid probes. The ease of extractability of palmitoylcarnitine is also comparable to that of lysophospholipids (Bergmann et al., 1984*b*) and fatty acids (Deuticke et al., 1981). This compound therefore fulfills two major prerequisites for being used as a probe in our technique. The characteristics of the transbilayer reorientation of palmitoylcarnitine reported here may even make it a very suitable probe for exploring the influence of membrane parameters on the rate of nonmediated flip.

First, the flip rate of palmitoylcarnitine in human erythrocytes (half time 2.6 hr, pH 7.4, 37°C) is high enough to allow its quantification within a few hours at 37°C. Palmitoylcarnitine therefore can be used to measure flip rates in membranes in which the transbilayer mobility of lysophospholipids is too low to be quantified (e.g., membranes of ox erythrocytes) (Bergmann et al., 1984*b*). Second, palmitoylcarnitine is more stable than lysophospholipids. No disturbing metabolic conversions were observed during the short incubation periods required to quantify flip rates. Hydrolysis of the probe in the presence of erythrocytes is slow at physiological pH and is essentially absent at pH 6. While diacyl- and lysophospholipids exhibit preferences for one of the lipid layers of the membrane (Seigneuret & Devaux, 1984; Bergmann et al., 1984*b*; Tilley et al., 1986; Haest et al., 1986; Schneider et al., 1986*a*), palmitoylcarnitine does not. From the kinetics and the symmetry of the flip-flop process, a nearly equal distribution of palmitoylcarnitine under stationary conditions can be derived, which simplifies the calculation of rate constants. For all these reasons, palmitoylcarnitine may also be a promising probe to study the simple nonmediated transbilayer mobility of lipids in plasma membranes of other cell types, which will not stand prolonged incubation procedures.

A linear increase of the flip with the concentra-

tion of palmitoylcarnitine as well as the lack of inhibitory effects of ATP depletion and of treatment with SH-reagents suggest that the transbilayer reorientation of palmitoylcarnitine is a simple process comparable to diffusion, like that of lysophospholipids, and not mediated by an ATP-dependent carrier as in the case of the translocation of diacylaminophospholipids in blood cells (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986; Martin & Pagano, 1987; Connor & Schroit, 1988). This is also supported by the observed high activation energy of the palmitoylcarnitine flip (108 kJ/mol), which is comparable to that of nonmediated reorientation of lysophospholipids in erythrocytes and of diacylphospholipids in lipid vesicles (Bergmann et al., 1984*b*; Haest et al., 1986; Homan & Pownall, 1988), but is considerably higher than that of the mediated translocation of phosphatidylserine in erythrocytes (30 kJ/mol, Zachowski et al., 1986). A further similarity between the flip of palmitoylcarnitine and that of lysophosphatidylcholine consists of its enhancement by chemical and noncovalent modification of the membrane (Table 2). The lack of ATP dependence and of inhibition by SH-reagents as well as of saturation kinetics also excludes the involvement of a specifc transfer system for acylcarnitines comparable to that present in mitochondria (Idell-Wenger, 1981; Murthy & Pande, 1984). Since the human erythrocyte has a membrane-bound carnitine-palmitoyltransferase activity (Wittels & Hochstein, 1967), and contains carnitine and acetylcarnitine (Cooper, Forte & Jones, 1988) the presence of such a transfer system as a relict might have seemed possible.

The flip of palmitoylcarnitine differs from the flip of lysophosphatidylcholines in a dependence on pH, in its interindividual variability and in a pronounced sensitivity to thermal denaturation of spectrin. Principally, a pH dependence of palmitoylcarnitine flip could either result from changes of the ionization of its carboxyl group or from a pH dependence of flip-site formation. The difference between the inflection point of the sigmoidal flip rate *vs.* pH curve and the free solution pK value of palmitoylcarnitine does not contradict the former interpretation. In analogy to the known increase of the apparent pK value of the carboxyl group of fatty acids upon their insertion into lipid bilayers (Von Tscharner & Radda, 1981; Rooney et al., 1983; Hamilton & Cistola, 1986), the protonation of the carboxyl group of membrane-intercalated palmitoylcarnitine will probably already start below pH 8. Protonation of the carboxyl group produces a positively charged species. Consequently, the positively charged form of palmitoylcarnitine would appear to have a higher flip rate than the zwitterionic form. Such a faster flip of the charged species would be in line with the higher flip rate of negatively charged lysophosphatidylserine as compared to that of zwitterionic lysophosphatidylcholine (Bergmann et al., 1984*a*). On the other hand, true cationic amphiphiles have been reported to reorient at best very slowly from the outer to the inner layer of the erythrocyte membrane (Lin et al., 1983).

Interestingly, the pH dependence of the flip of palmitoylcarnitine is no longer observed after enhancement of the flip rates of diamide (Classen, 1989). This may indicate that the native flip sites for palmitoylcarnitine differ in their selectivity from the flip sites created by membrane perturbation. This is also supported by the observation that the diamide-induced flip sites in the erythrocyte membrane discriminate between lysophophatidylcholine and lysophosphatidylserine to a lesser extent than the native flip sites (Bergmann et al., 1984*a*).

The flip of palmitoylcarnitine also contrasts from that of lysophospholipids in its marked stimulation by a heat pretreatment of the erythrocytes, a feature much less pronounced in the case of the flip of lysophospholipids. Heat treatment of erythrocytes above 42°C results in a highly selective denaturation of spectrin (Brandts et al., 1977; Tomaselli et al., 1981; Minetti et al., 1986). Intrinsic membrane proteins only begin to denature at higher temperatures (>60°C). The extent of spectrin denaturation increases with increasing temperature and incubation time. A treatment of erythrocytes at 49–50°C for 15 min also results in destabilization of the membrane indicated by the formation of exocytotic vesicles (Ham et al., 1948; Tomaselli et al., 1981). Due to our choice of experimental conditions, only irreversible or slowly reversible alterations of membrane structure will be detectable. Our results may be compared with data reported by Mohandas et al. (1982). These authors also observed only a slight increase of the flip of palmitoyl-lysophosphatidylcholine after a short heat pretreatment of cells.

A causal relationship between the enhancement of the flip of palmitoylcarnitine upon the mild heating of the cells and the denaturation of spectrin seems rather likely. This finding is a further piece of evidence for the importance of an intact membrane skeleton in preventing the formation of transient structural defects in the hydrophobic membrane domain acting as flip sites (Bergmann et al., 1984*a*; Dressler et al., 1984; Franck et al., 1985*a*,*b*; Mohandas et al., 1985). On the other hand, the different responses of the two probes, palmitoylcarnitine and palmitoyl-lysophosphatidylcholine to a spectrin denaturation indicate that different amphiphiles may not always respond to the occurrence of structural defect in an uniform way. The situation seems

to be even more complex, since structural defects induced by other types of modification of the skeleton enhance the transbilayer mobility of our two probes to almost the same extent (*compare* Table 2).

In conclusion, our results on the transbilayer reorientation of palmitoylcarnitine indicate that concepts of indiscriminate flip sites in biological membranes equally well accessible to various exogenous amphiphiles may be an oversimplification. Further studies with different types of probes, particularly ones differing in the structure of their polar head group will be needed before we can formulate molecular models for this special type of membrane transport.

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