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ARTICLE

Marta Zuvic-Butorac · Peter Müller
Thomas Pomorski · Jeanette Libera
Andreas Herrmann · Milan Schara

Lipid domains in the exoplasmic and cytoplasmic leaflet of the human erythrocyte membrane: a spin label approach

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Abstract The existence of different lipid domains in the monolayers of the human erythrocyte membrane was investigated at 4 °C by employing spin-labelled phospholipid analogues. Spectra of analogues located exclusively either in the exoplasmic or in the cytoplasmic leaflet of erythrocyte membranes were recorded. Spectra were simulated by variation of order parameter describing the average amplitude of motion of the long molecular axis of the nitrogen $2p\pi$ orbital of the spin label and of the respective correlation times. For both leaflets at least three components were required to fit the experimental spectra, differing mainly in the order parameter. While the parameters of each component are not very different between both membrane halves, the relative contribution of each component to the spectrum is different between the exoplasmic and cytoplasmic leaflet. The order parameter of the most fluid component, presumably resembling the lipid bulk phase, is smaller in the cytoplasmic leaflet in comparison to the exoplasmic one. The lateral coexistence of different lipid domains in the human red blood cell membrane is concluded. The molecular nature of those domains is discussed.

Key words Erythrocyte · Plasma membrane · Leaflet · Lipid domains · Spin label

Abbreviations *ATP* Adenosine triphosphate · *BSA* Bovine serum albumin · *DFP* Diisopropyl fluorophosphate · *ESR* Electron spin resonance · *HEPES* *N*-2-Hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid · *PBS* Phosphate buffered saline · *PC* Phosphatidylcholine · *PL* Phospholipid · *PS* Phosphatidylserine · *SM* Sphingomyelin · *SL* 4-Doxylpentanoyl · *(0,2)PC, (0,2)PE and (0,2)PS* 1-Palmitoyl-2-(SL)-PC, -PE and -PS, respectively · *(0,2)SM* *N*-(SL)-*trans*-sphingosyl-1-phosphocholine

M. Zuvic-Butorac
University of Rijeka, Croatia

P. Müller · T. Pomorski · J. Libera · A. Herrmann (✉)
Humboldt-Universität zu Berlin, Math.-Nat. Fak. I,
Institut für Biologie/Biophysik,
Invalidenstrasse 42, D-10115 Berlin, Germany
e-mail: Andreas=Herrmann@rz.hu-berlin.de

M. Schara
J. Stefan Institute, University of Ljubljana,
SI-1000 Ljubljana, Slovenia

Introduction

The inhomogeneous lateral and transbilayer arrangement of lipids in biological membranes and its physiological relevance is a subject of continuous interest. Most of our knowledge in this field has been derived from the plasma membranes of mammalian cells (for a review, see Welti and Glaser 1994). It is now well accepted that an asymmetric distribution of phospholipids between the exoplasmic and cytoplasmic leaflet of the plasma membrane is typical for mammalian cells (Op den Kamp 1979; Devaux 1991; Zachowski 1993; Roelofsen and Op den Kamp 1994): the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are preferentially localized in the cytoplasmic leaflet, while the choline-containing lipids phosphatidylcholine (PC) and sphingomyelin (SM) are enriched in the exoplasmic leaflet.

The asymmetric arrangement of the phospholipids may be accompanied by differences of the physico-chemical properties between both monolayers of the plasma membrane, e.g. the membrane fluidity. For example, the exoplasmic leaflet of human red blood cells is more rigid than the cytoplasmic one (Tanaka and Ohnishi 1976; Seigneuret et al. 1984; Morrot et al. 1986) owing to the enrichment of saturated fatty acid residues of the phospholipids in the exoplasmic layer (Cribier et al. 1990; Hullin et al. 1991). However, the existence of distinct lateral lipid domains in mammalian plasma membranes, not determined by morphologically distinguishable and physically separable cellular structures as, for example, tight junctions, are less well characterized (Welti and Glaser 1994). Cholesterol and sphingolipid rich membrane fractions which are insoluble in non-ionic detergents may provide some indications for phase separation and the existence of microdomains in

plasma membranes (Simons and Ikonen 1997; Brown and London 1998). In contrast to biological membranes, experimental and theoretical approaches on artificial membranes offer a detailed description of macroscopic membrane properties determined by the lateral heterogeneity of different size scales (Mouritsen and Jorgensen 1994, 1995).

Using spin-labelled lipid analogues, electron spin resonance (ESR) enables one to characterize the "fluidity" of the lipid phase by measuring distinct dynamical properties of its components. The ESR spectrum of the nitroxide (NO) moiety is sensitive to spatial and temporal restrictions of analogue motion. By this technique, coexisting lipid domains can be distinguished provided they affect differently the motion of those analogues and, thus, give rise to a spectrum of superimposed components (McConnell 1976). In principle, simulation of ESR spectra allows us to deconvolute a composite spectrum and to specify the domains.

In the present study we have asked whether different lipid domains exist in the outer and inner leaflet, respectively, of human red blood cells. For that purpose, we have investigated the motion of spin-labelled phospholipid analogues. These analogues, bearing the NO moiety on a short β-chain of the glycerol backbone, rapidly incorporate into the exoplasmic leaflet of human red blood cell membranes (Seigneuret et al. 1984; Marx et al. 1997). It has been shown that the transbilayer distribution of these analogues across the red blood cell membrane is similar to that of endogenous phospholipids (Tilley et al. 1986, Roelofsen et al. 1987; Morrot et al. 1989). The exclusive orientation of the analogues to only one leaflet of the plasma membrane can be easily achieved. For characterizing the exoplasmic membrane leaflet we used the spin-labelled analogues of PC [(0,2)PC] and SM [(0,2)SM] since they remained within the experimental time course essentially in this layer. Spin-labelled PS [(0,2)PS] was employed for characterization of the cytoplasmic leaflet. This analogue becomes rapidly enriched in that layer by the action of the aminophospholipid translocase (Seigneuret and Devaux 1984; Morrot et al. 1989).

Materials and methods

All substances were obtained from Sigma (Deisenhofen, Germany).

Suspension medium

Phosphate buffered saline (PBS) contained 150 mM NaCl and 5.8 mM Na_2HPO_4/NaH_2PO_4, pH 7.4.

Preparation of erythrocytes

Citrate-stabilized blood samples of healthy donors were purchased from the local blood bank (Berlin, Germany).

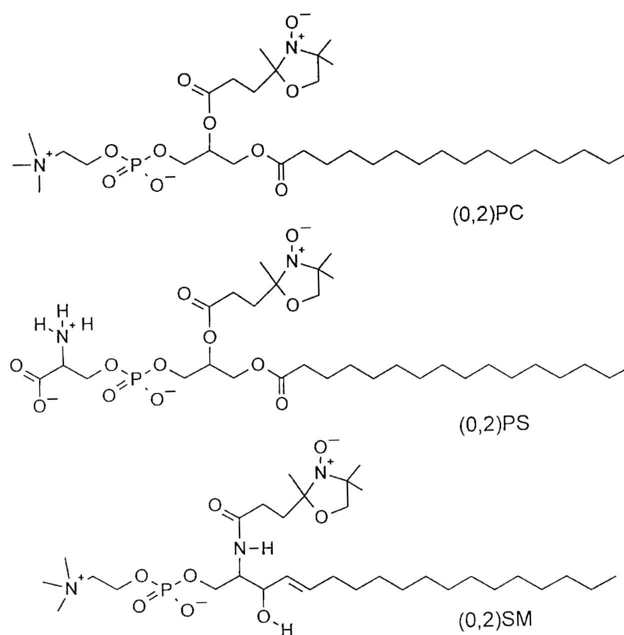

Fig. 1 Spin-labelled lipid analogues of phosphatidylcholine [(0,2)PC], phosphatidylserine [(0,2)PS] and sphingomyelin [(0,2)SM]

Red blood cells were washed once in PBS to remove plasma and buffy coat. Subsequently, two additional washes in PBS followed (2000 g, 10 min at 4 °C). Before labelling, cells were preincubated for 5 min in the presence of 5 mM diisopropyl fluorophosphate (DFP) at 4 °C in order to prevent significant hydrolysis of the label (Calvez et al. 1988; Morrot et al. 1989).

Spin-labelling of red blood cells

Spin-labelled phospholipids (Fig. 1) were synthesized according to Fellmann et al. (1994). Suitable amounts of analogues (corresponding to 1–2 mol% of endogenous phospholipids) in chloroform were dried under nitrogen and resuspended in 1 volume of PBS. For labelling, 4 volumes of erythrocyte suspension (hematocrit 25%) were added to 1 volume of label dispersion at 4 °C. The analogues rapidly incorporated into the outer membrane leaflet within less than 1 min (Seigneuret et al. 1984; Marx et al. 1997). Subsequently, (0,2)PS was translocated to the inner leaflet, whereas (0,2)PC and (0,2)SM remained almost exclusively on the outer leaflet under our conditions (Seigneuret and Devaux 1984). To assess the transbilayer distribution of analogues, at regular intervals, 80 μl of the suspension were drawn and mixed with 20 μl of ice-cold PBS containing 10% fatty-acid free bovine serum albumin (BSA) (Calvez et al. 1988; Morrot et al. 1989). After 1 min incubation on ice, the suspension was centrifuged (13 000 g, 2 min). Fifty μl of the supernatant were drawn and mixed with 5 μl of 100 mM potassium ferricyanide in order to reoxidize reduced lipid analogues. The amount of probe present in the supernatant corresponding to lipid analogues

in the exoplasmic leaflet was estimated from the intensity of the ESR spectrum measured on a Bruker ECS 106 (Bruker, Karlsruhe, Germany). Despite the presence of DFP, a small portion of the spin-labelled analogues were hydrolysed into *lyso* derivatives. This became obvious from the appearance of three narrow peaks corresponding to the spectrum of the cleaved short-chain, spin-labelled fatty acid which is water soluble. In those cases, intensities were corrected for hydrolysis as described previously (Morrot et al. 1989).

Measurement of membrane spectra

ESR membrane spectra were recorded at 4 °C with a modulation amplitude of 2.8 G (power 20 mW; accumulation: 16 scans with 167 s/scan). To improve the signal-to-noise ratio, the densely packed pellet of labelled erythrocytes after centrifugation was used for recording of spectra.

Cytoplasmic leaflet

Erythrocytes were labelled with the PS analogue. After 4 h on ice, about 80% of (0,2)PS had redistributed to the cytoplasmic leaflet (see above). The (0,2)PS remaining on the exoplasmic half was removed by back-exchange to BSA. After back-exchange, the pellet was washed three times in PBS to remove BSA and the spectrum of (0,2)PS in the cytoplasmic leaflet was measured. Although the inward transport of the analogue is much faster at higher temperatures like 37 °C (half time of redistribution is about 3 min; Morrot et al. 1989), those conditions are not recommended for our purpose since (1) the NO moiety of analogues oriented to the inner leaflet is rapidly reduced by cellular redox systems (Seigneuret et al. 1984) and (2) significant hydrolysis of (0,2)PS occurs (Calvez et al. 1988).

Exoplasmic leaflet

After labelling of erythrocytes with (0,2)PC or (0,2)SM the respective membrane spectrum was recorded. The spectra corresponded to analogues of the exoplasmic half, since during the experimental time course both analogues did not redistribute significantly to the cytoplasmic leaflet as probed by the back-exchange assay (data not shown). Membrane spectra of both analogues were not affected when cells were preincubated for 4 h on ice before labelling (not shown).

The anisotropic membrane spectra were, if necessary, corrected for the hydrolysed short spin-labelled fatty acid (<2% of total phospholipid analogues) seen by the three narrow peaks (see above). To this end, the corresponding spectrum of the free fatty acid recorded separately in the pellet of red blood cells was subtracted.

Erythrocyte lipids

Lipids of erythrocyte plasma membrane were isolated according to the procedure of Bligh and Dyer (1959). Lipids dissolved in chloroform-methanol (2:1 v/v) were mixed with spin-labelled phospholipid analogues (1.5 mol%) and dried under a stream of nitrogen. Subsequently, small unilamellar vesicles were prepared by sonification in PBS using a Branson Sonifier W250 (Carouge-Geneve, Switzerland) at an output control setting of 2 and 50% duty cycle in a glass tube cooled in an ice bath. Membrane spectra were recorded as described above.

Simulation and deconvolution of ESR spectra

Assuming a fast rotational motion of the nitroxide due to conformational changes of the phospholipid acyl chains hindered by the alignment of the molecules in the membrane bilayer, membrane spectra of the analogues were simulated according to the model of Schindler and Seelig (1973) for bilayers and liquid crystals. As deduced from a comparison between spectra of membranes of intact erythrocytes and of vesicles of erythrocyte lipids, lateral heterogeneous, coexisting domains occur on both sides of the membrane of red blood cells. Those domains are assumed as long lived in relation to the nitroxide anisotropy expressed in the time scale. Thus, spectra $\mathscr{S}(B)$ are a superimposition of components $s_i(B)$ related to the domains with different order parameters S_i and rotational correlation times τ_i in a magnetic field B:

$$\mathscr{S}(B)=\sum W_i\, s_i(B) \tag{1}$$

$$s_i(B)=s_i(S_{1i},\, S_{3i}\, \tau_{20i},\, \tau_{22i},\, B) \tag{2}$$

$$\sum W_i = 1 \tag{3}$$

The weighting factor W_i of a component $s_i(B)$ expresses its relative contribution to the whole membrane spectrum. The correlation times τ_{22} and τ_{20} refer to the rotation around and perpendicular to the long molecular axis of the spin label attached to the fatty acid residue. The order parameters describe the average amplitude of motion; S_3 describes the time averaged position of the long molecular axis of the nitrogen $2p\pi$ orbital relative to the bilayer normal, while S_1 is perpendicular to the bilayer normal. The goodness of fit was deduced from the χ^2-value. Simulated spectra shown are selected by the minimum of the χ^2-value.

Results

Experimental membrane spectra of red blood cells

The specific transbilayer localization of spin-labelled phospholipids enabled us to measure membrane spectra reflecting the microenvironment either of the exoplasmic or of the cytoplasmic leaflet. To support spectral deconvolution, spectra were recorded at low temperature (4 °C) to

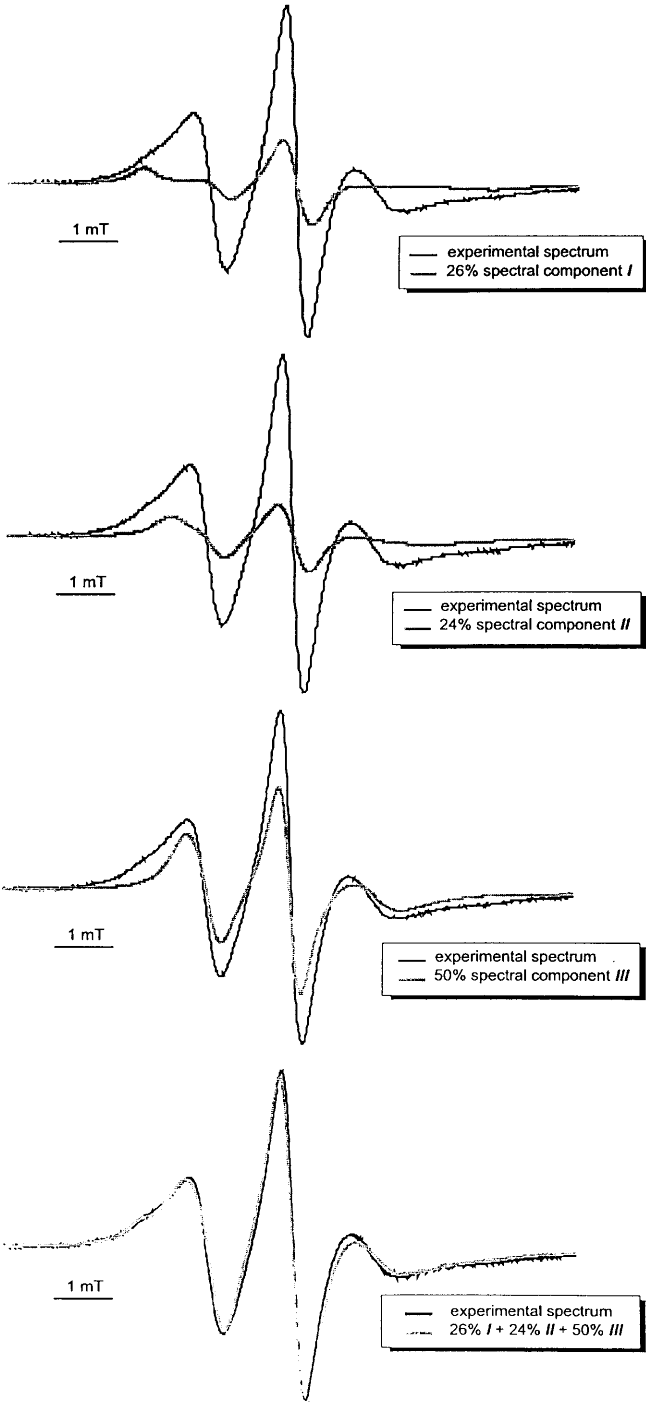

Fig. 2 Experimental and simulated membrane spectra of (0,2)PC localized in the exoplasmic leaflet of human red blood cells. The membrane spectrum was measured at 4 °C. Comparison of the experimental spectrum of (0,2)PC with the three calculated characteristic spectra assigned to specific domains I, II, and III and with the superimposed spectrum (domains I, II and III). The weighting factor W_i reflecting the contribution of each domain to the total spectrum is given. Spectra of the domains are scaled to the weighting factor. The parameters of the simulated spectra are summarized in Table 1 (see Results)

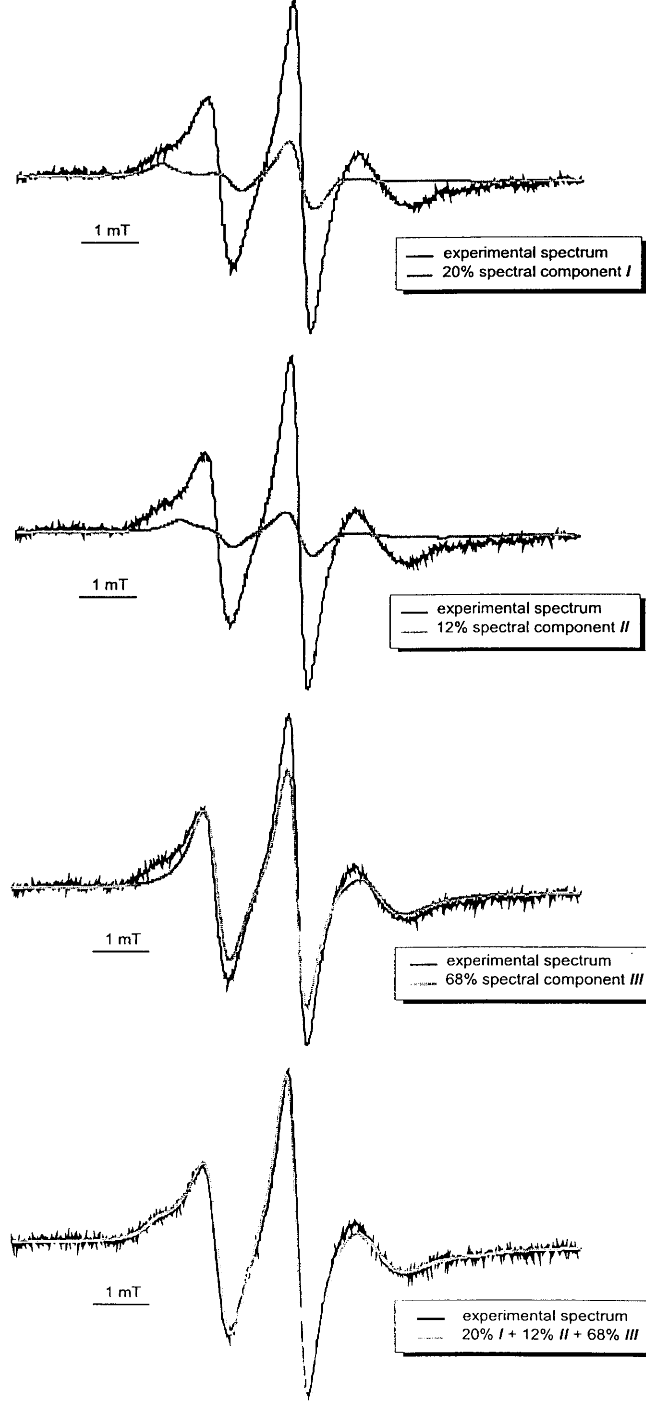

Fig. 3 Experimental and simulated membrane spectra of (0,2)PS localized in the cytoplasmic leaflet of human red blood cells. The membrane spectrum was measured at 4 °C. Comparison of the experimental spectrum of (0,2)PS with the three calculated characteristic spectra assigned to specific domains I, II, and III and with the superimposed spectrum (domains I, II and III). The weighting factor W_i reflecting the contribution of each domain to the total spectrum is given. Spectra of the domains are scaled to the weighting factor. The parameters of the simulated spectra are summarized in Table 1 (see Results)

slow down the exchange of spin-labelled probes between different domains. In Figs. 2 and 3 are shown the corresponding membrane spectra of (0,2)PC and (0,2)PS localized in the exoplasmic and cytoplasmic leaflet, respectively. As is obvious, in particular from the low field line, the spectrum of (0,2)PC reflects a more hindered motion than that of (0,2)PS. This confirms previous results in that the membrane fluidity in the cytoplasmic leaflet is higher than in the exoplasmic one (Tanaka and Ohnishi 1976; Seigneuret et al. 1984; Morrot et al. 1986).

One may wonder whether the differences are due to a head-group specific partition of the analogues into domains rather than to differences of the membrane fluidity between both leaflets. However, this seems very unlikely, since the spectrum of (0,2)PS in the exoplasmic leaflet which was recorded immediately after labelling of red blood cells (not shown) is identical to that of (0,2)PC (Fig. 2). Additional support for differences in the overall membrane fluidity between both membranes halves is given by the membrane spectrum of (0,2)SM (exoplasmic leaflet) (Fig. 4), which reflects also a more hindered motion than that of (0,2)PS oriented to the cytoplasmic leaflet (see Fig. 3). Remarkably, motional hindrance of the SM analogue is even more pronounced than that of the PC analogue.

Experimental membrane spectra of erythrocyte lipid vesicles

To elucidate to what extent the motion of spin-labelled analogues in erythrocyte membranes is affected by membrane proteins, we have recorded membrane spectra in vesicles made of erythrocyte lipids (see Materials and methods). All spin-labelled analogues used are much more mobile in these vesicle membranes in comparison to erythrocyte membranes (Fig. 5; compare with Figs. 2–4). While the spectra of (0,2)PC and (0,2)PS are identical, we note again a higher motional restriction of (0,2)SM. A disadvantage of the vesicle membranes is that an asymmetric transbilayer distribution of lipids as known for the intact erythrocyte membrane cannot be established. Thus, the membrane fluidity in those vesicles will presumably correspond to an average of the fluidity between the lipid phase of both plasma membrane leaflets.

Simulation of experimental membrane spectra

The spectra of analogues in the exoplasmic or cytoplasmic leaflet of red blood cells suggest a superimposition of spectral components originating from different domains. We were not able to simulate those spectra of the erythrocyte membrane by a single component, neither for spectra of the outer nor of the inner leaflet. Therefore, we fitted the experimental spectra by a superimposition of different simulated spectra in the following way (compare with Figs. 2–4). First, a spectrum (component I) was simulated to fit the outer hyperfine splitting. This component corre-

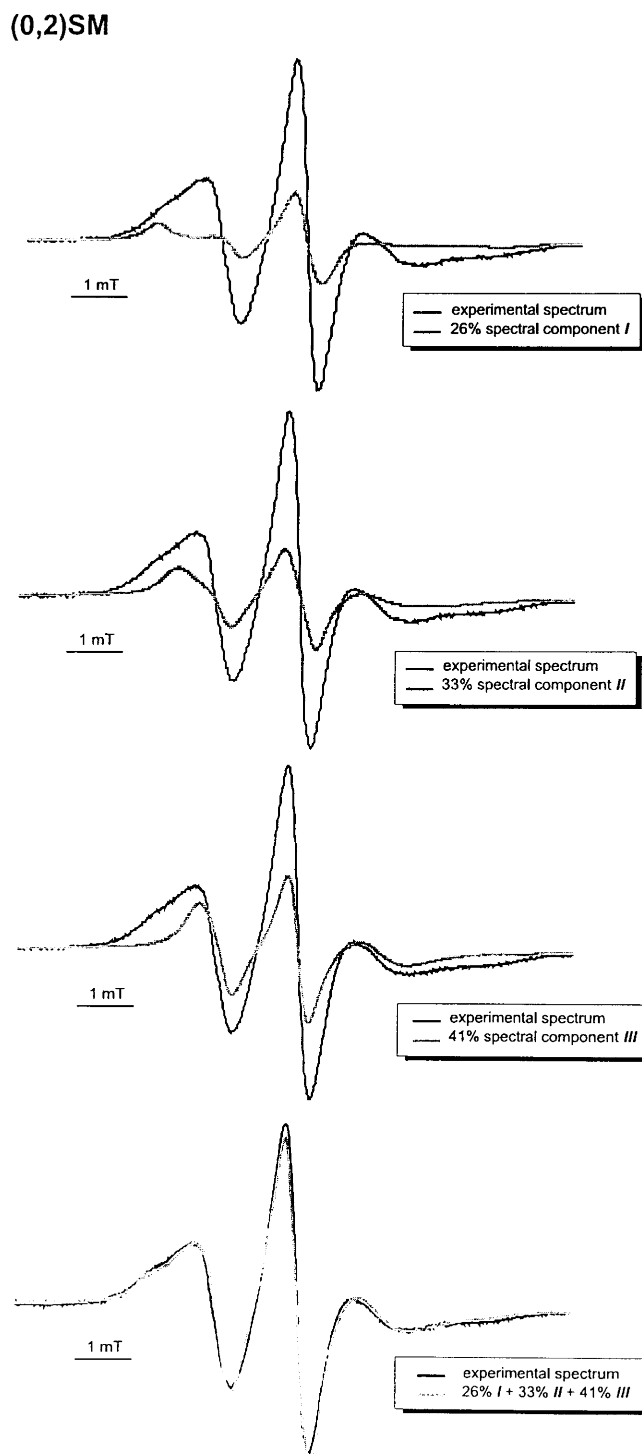

Fig. 4 Experimental and simulated membrane spectra of (0,2)SM localized in the exoplasmic leaflet of human red blood cells. The membrane spectrum was measured at 4 °C. Comparison of the experimental spectrum of (0,2)SM with the three calculated characteristic spectra assigned to specific domains I, II, and III and with the superimposed spectrum (domains I, II and III). The weighting factor W_i reflecting the contribution of each domain to the total spectrum is given. Spectra of the domains are scaled to the weighting factor. The parameters of the simulated spectra are summarized in Table 1 (see Results)

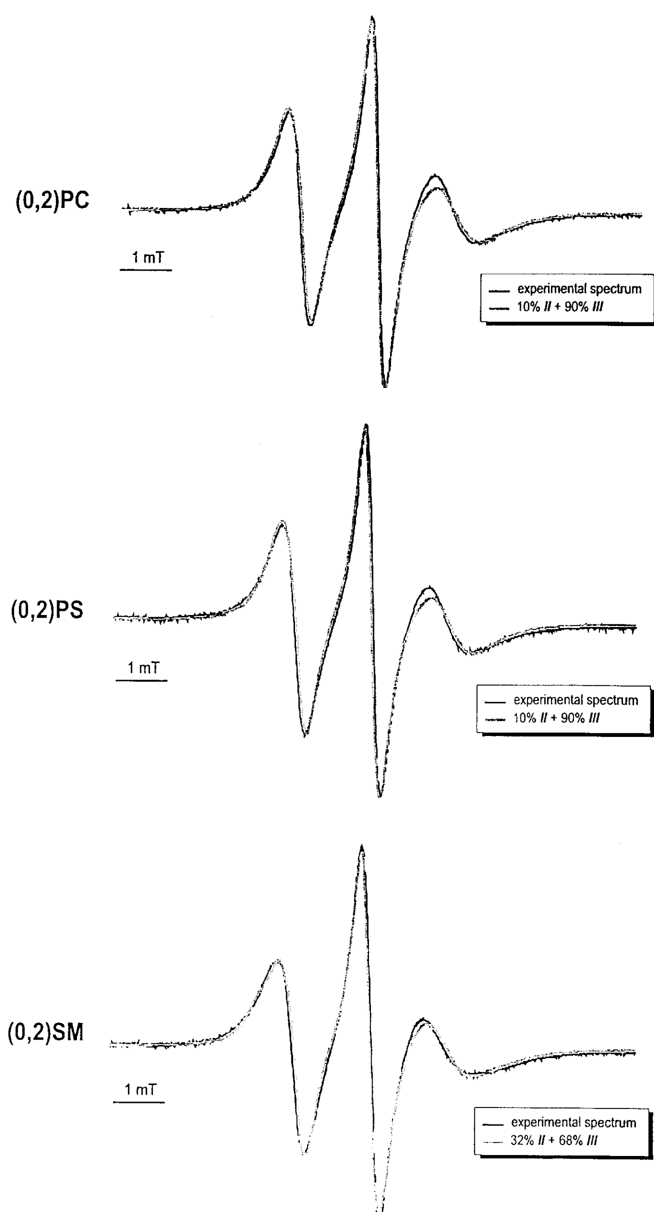

Fig. 5 Experimental and simulated membrane spectra of (0,2)PC, (0,2)PS and (0,2)SM, in vesicles of erythrocyte lipids at 4 °C. The weighting factor W_i reflecting the contribution of each domain (II and III) to the total spectrum is given. The parameters of the simulated spectra are given in Table 2 (see Results)

sponds to the most rigid one (with the highest S_3). Next, a spectrum was calculated (component III) to match the most fluid component (with the lowest S_3). With only those two superimposed components (I and III) we could not obtain a satisfying fit of the spectra measured in erythrocyte membranes. Thus, an additional component (II) was simulated (with intermediate S_3). Of course, by employing more intermediate components we could still improve the fit of the experimental spectra. However, for reasons of comparison between measured spectra and the interpretation of different components, we limited the fitting procedure to three weighted components resembling the original spectra well enough. The various spectra of spin-labelled lipids were fitted independently. Interestingly, it turned out that the set of order parameters and correlation times for each component was not very different between the spectra of various lipid analogues (see below).

Table 1 Parameters of the simulation of experimental spectra of different spin-labelled lipid analogues measured in the plasma membrane of intact human erythrocytes. Lipid analogues were localized either in the exoplasmic [(0,2)PC and (0,2)SM] or in the cytoplasmic leaflet [(0,2)PS]. Measured spectra were fitted by a superimposition of three different spectra reflecting domains I, II and III. For optimization of fitting, a non-resolved broadening (*SS*) which could be assigned perhaps to nonidentified hyperfine splitting was taken into account

Domain	Order parameter		Rotat. correl. time		Non-res. broad. *SS* (G)	Weight. factor *W*
	S_3	S_1	τ_{20} (ns)	τ_{22} (ns)		
Outer leaflet (0,2)PC						
I	0.62	–0.22	2.6	2.6	0.7	0.26
II	0.44	–0.20	2.5	2.5	0.2	0.24
III	0.25	0	2.4	2.4	0.0	0.50
Outer leaflet (0,2)SM						
I	0.62	–0.25	2.4	2.4	0.50	0.26
II	0.44	–0.20	2.4	2.4	0.0	0.33
III	0.25	–0.10	2.1	2.1	0.0	0.41
Inner leaflet (0,2)PS						
I	0.62	–0.20	2.2	2.2	0.5	0.20
II	0.44	–0.20	2.2	2.2	0.0	0.12
III	0.15	0.0	2.1	5.0	0.2	0.68

In Figs. 2–4 the results of simulation of the experimental membrane spectra of (0,2)PC, (0,2)SM and (0,2)PS, respectively, are shown. The parameters of the simulation are summarized in Table 1. In all cases a minimum of three different components (I, II and III, respectively) was required to obtain a reasonable fit of the measured spectra corresponding either to the exoplasmic (PC and SM analogue) or to the cytoplasmic leaflet (PS analogue). From this we conclude that in the lipid phase on either side of the erythrocyte plasma membrane at least three different domains exist and are sensed by different motional restrictions of our analogues.

The spectral components are mostly distinguished by the order parameter S_3. We established two less mobile components on both membrane layers with S_3 being 0.62 (domain I) and 0.44 (domain II), respectively. It is S_3 of the most fluid component (domain III) which differs between both layers. While a satisfying fit for the exoplasmic leaflet was obtained with S_3 being 0.25, it was 0.15 for the cytoplasmic leaflet, reflecting an even more fluid environment. With the exception of the weighting factor, the other parameters (S_1, τ_{20} and τ_{22}) of the different special domains are almost identical. We note that for matching the fluid component (III) of the experimental spectrum of (0,2)PS a higher correlation time τ_{22} had to be assumed.

The contribution of each spectral domain (I, II and III) to the total spectrum depends on the transbilayer localiza-

Table 2 Parameters of simulation of experimental ESR spectra of different spin-labelled lipid analogues incorporated into vesicle membranes made of erythrocyte lipids (see Fig. 5) (for details see Materials and methods and Results). Spectra were measured at 4 °C

Domain	Order parameter		Rotat. correl. time		Weight. factor W
	S_3	S_1	τ_{20} (ns)	τ_{22} (ns)	
(0,2)PC					
II	0.44	–0.20	1.6	5.0	0.10
III	0.20	0	2.0	1.0	0.90
(0,2)SM					
II	0.44	–0.20	1.6	5.0	0.32
III	0.20	0	2.0	1.0	0.68
(0,2)PS					
II	0.44	–0.20	1.6	1.6	0.10
III	0.20	0	1.9	1.0	0.90

tion of the analogue and also on its head-group structure. Thus, we found quantitative differences of the weighting factors between spectra for (0,2)PC and (0,2)SM representative of the exoplasmic layer. The partition of (0,2)SM into the most fluid domain (III) is lower, but higher into the more rigid domain II in comparison to (0,2)PC. Since the membrane spectra of (0,2)PC and (0,2)PS in the exoplasmic leaflet are identical (see above), the partition behaviour of both analogues is similar in this layer.

The spectra of analogues incorporated into vesicle membranes of erythrocyte lipids could be fitted by two components (Table 2, Fig. 5). These are almost identical to components II and III, respectively, obtained from fitting of the spectra of intact erythrocytes (see Table 1). For (0,2)PS and (0,2)PC, the contribution of component II to the whole spectrum is low (about 10%, see Table 2). However, by including component II the fit of the measured spectra could be significantly improved. This was also confirmed by the χ^2-value which was about 20% lower in comparison to a one-component fit (only component III). The weighting factor of component II was much higher for the fit of the experimental spectrum of (0,2)SM. This component amounts to about 32% of the total spectrum. Thus, in comparison to (0,2)PS and (0,2)PC, a larger part of the SM analogues experienced a more restricted movement in vesicles made from erythrocyte lipids.

Discussion

Substantial indications have been given that lipids are not randomly arranged in the leaflets of cellular membranes. Even in the absence of physically and functionally distinct membrane surfaces as basolateral and apical domains of epithelial plasma membranes, lipids can be organized in distinct domains which may differ by several orders of magnitude in size (see, for a review, Welti and Glaser 1994). Lipid-lipid as well as protein-lipid interactions are mainly responsible for distinct lipid domains. Much of our knowledge on those interactions has been derived from (reconstituted) model membranes, but not from intact biological membranes. To characterize lipid domains in plasma membranes is often rather difficult. Even the investigation of lateral specific lipid domains in the plasma membrane of the comparatively simple organized human red blood cells might be faced with distinct technical and preparative problems: (1) the efficient and specific labelling of membrane leaflets by lipid analogues which organize like their andogenous counterparts is mandatory; (2) the characterization of lateral lipid domains by fluorescence lipid analogues may require the use of ghost membranes to avoid fluorescence quenching by haemoglobin. However, ghost preparation may affect membrane structure and, by that, the size and lifetime of domains or even their existence. Since reconstitution of a specific leaflet in model systems as vesicles is difficult, an experimental approach allowing the detection of domains in the two monolayers of an intact biological membrane would be desirable.

Here, we have shown that spin-labelled phospholipids mimicking essentially the transbilayer orientation of endogenous lipids (Tilley et al. 1986; Roelofsen et al. 1987; Morrot et al. 1989) provide a tool to investigate the existence and characteristics of lateral lipid domains specifically in the exoplasmic and cytoplasmic leaflet of an intact, non-modified plasma membrane. Taking advantage of the different, lipid-specific mechanisms of transbilayer movement of phospholipids (Devaux 1991; Zachowski 1993), an exclusive labelling either of the outer or of the inner leaflet was possible (Seigneuret et al. 1984). The combination (1) of specific leaflet labelling with spin-labelled analogues, which permitted the recording of the respective membrane (leaflet) spectra, and (2) of the subsequent simulation of the spectra by an appropriate, well-established model for the motion of spin probes in an anisotropic environment such as a membrane (Schindler and Seelig 1973), enabled us to elucidate the lipid domain structure of both leaflets. However, our approach does not allow us to conclude on the size and number of the domains. For example, we cannot state whether a domain is organized as a single domain or as several smaller domains with similar physico-chemical properties.

A prerequisite of our approach was that the motion of the spin-labelled phospholipids is sensitive towards their molecular environment but did not mainly reflect an inherent property of the label structure. While that sensitivity has been proven already in previous studies of protein-lipid interaction (Zachowski and Devaux 1983; Dreger et al. 1997), we have presented here additional evidence:

1. The spectra of (0,2)PS on the outer and inner leaflet are different.
2. The membrane spectra of analogues in vesicles of erythrocyte lipids are different from those in the human red blood cell membrane.
3. Membrane spectra of (0,2)PC and (0,2)PS in vesicles made of egg-PC are identical (unpublished results).

A comparison between spectra recorded for vesicles and for red blood cells disclosed the coexistence of lipid domains with different molecular ordering and dynamics in both leaflets of the erythrocyte membrane. Simulation of the membrane spectra revealed that at least three different lipid domains have to be assumed in red blood cell membranes. In contrast, experimental spectra of analogues in lipid vesicles could be well modelled with two components. The different domains were characterized (1) by parameters describing the motion of the analogues and (2) by a weighting factor providing an estimate for the relative contribution of each spectral component to the whole spectrum. This factor is determined by the total amount of the given lipid domain in the leaflet as well as the concentration of the analogue within the domain. In principle, we have also to consider that, for a given domain, the motion of various analogues may be different owing to the molecular structure of the analogues and their intramolecular interaction within the domain. If that is the case, we would expect differences in the values of the order parameter and/or of the correlation times between spectra of (0,2)SM and (0,2)PC. From our simulation we have no indication for that. Simulated spectra differ only in the weighting factor of the domains, but not in respective order parameters and correlation times. However, we cannot preclude that our approach is not sensitive enough to detect differences in the NO tumbling of analogues in a given domain. We note that we found a more hindered motion of (0,2)SM in comparison to (0,2)PC in egg-PC vesicles at 4 °C. No difference was found between (0,2)PC and (0,2)PS (unpublished observations).

Unfortunately, those domains could be characterized by our approach only at low temperature (4 °C) when exchange between different microenvironments become slow, giving rise to separate spectra. At higher temperatures, which would reflect more closely physiological conditions, exchange between domains did not allow us to resolve them. As it turned out from simulation, axial rotation was still high at low temperature. This could be rationalized by the fact that the NO moiety which is attached to the distal terminus of a short chain fatty acid residue of the analogues can still experience a high flexibility at low temperature.

While the main objective of our work was to present an approach to investigate lipid domains specific for each membrane half in vivo, we are also challenged to enlighten the putative molecular nature of and the specific interactions within those domains affecting the motion of the analogues. While being aware that for a conclusive statement on this topic additional studies are required, we would like to put forward some suggestions.

By comparison with the simulation of membrane spectra derived from vesicle membranes we can assign the most fluid domain (domain III) of the erythrocyte membrane to the lipid bulk phase. The order parameter S_3 of vesicle membranes is close to that obtained for domain III of the exoplasmic leaflet [(0,2)PC and (0.2)SM] and of the cytoplasmic layer [(0,2)PS]. Differences of S_3 between vesicle membranes and the various leaflets of the erythrocyte membrane are not unexpected since the lipid composition of both layers of the vesicle membrane reflects a mixture of that of the cytoplasmic and exoplasmic leaflet of the erythrocyte membrane. The lower S_3 of domain III of the cytoplasmic leaflet in comparison to that of the exoplasmic leaflet reflects the already described difference of the membrane fluidity between both leaflets (Seigneuret et al. 1984), presumably as a result of higher levels of unsaturated fatty acids in the aminophospholipids which are concentrated on the cytoplasmic leaflet (Cribier et al. 1990; Hullin et al. 1991). This gives rise to a different lipid packing of the erythrocyte bilayer, with a more loosely packed inner leaflet (Tanaka and Ohnishi 1976; Tullius et al. 1989; Herrmann et al. 1990) enabling a less restricted motion of analogues (Herrmann et al. 1990).

Presumably, the most motional hindered component (domain I) represents analogues motionally restricted in the shell of the membrane proteins. It is known that protein-lipid interactions exert a prominent influence on the rate and, perhaps, on the spatial spin label motion in biological membranes (Devaux and Seigneuret 1985; Marsh 1995). The contribution of this domain to the membrane spectra is almost similar irrespective of the analogues used. This does not necessarily contradict the existence of specific protein-lipid interactions because we described here only the overall interaction of an analogue with various membrane proteins.

Domain II is characterized by an order parameter S_3 intermediate between that of domains I and III. One may wonder whether this intermediate component is caused by a slow exchange of analogues between domains I and III, instead of representing a membrane domain distinct from I and III. However, the existence of component II in spectra obtained for analogues in vesicle membranes eliminates this possibility. For red blood cells, the weighting factor of domain II was rather small for the cytoplasmic leaflet [(0,2)PS], $W=0.12$, while for labels situated on the exoplasmic layer it was 0.24 [(0,2)PC] and 0.33 [(0.2)SM]. In the light of the known differences in phospholipid composition between both leaflets of the erythrocyte membrane (Op den Kamp 1979), it may be tempting to assume that domain II is represented by lipids involved in specific lipid-lipid interactions which are absent in domain III. About 80% of the endogenous SM are localized in the exoplasmic leaflet, which may give rise to a lipid domain more rigidified in comparison to the most fluid domain (III) for various reasons:

1. The phase transition of SM occurs generally at higher temperatures than that of many other phospholipids (Boggs 1980).
2. Intermolecular interaction between SM molecules via hydrogen bonds (Boggs 1980, 1987) as well as the formation of SM-cholesterol complexes (Grönland et al. 1991; Kan et al. 1991) may lead to an enhanced microviscosity of the exoplasmic membrane leaflet.

The specific involvement of SM in domain II is supported by the fact that the SM analogue has the highest weighting factor for this domain. Moreover, the pronounced or-

ientation of SM to the exoplasmic leaflet would also explain why this domain is depopulated on the cytoplasmic leaflet. Remarkably, the contribution of domain II to the total spectrum of (0,2)SM in vesicle membranes was similar to that found for the exoplasmic leaflet of intact red blood cells. This suggests the preservation of domains by lipid-lipid interaction even in the absence of membrane proteins. Very recently, it has been shown that physiological concentrations of cholesterol and sphingomyelin can induce the formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes even in the absence of proteins (Ahmed et al. 1997). The dynamical properties of lipids in the lipid-ordered phase are intermediate between those of the gel and the liquid-disordered phase (Mouritsen 1991). Although detergent-insoluble fractions of membrane lipids have been found for the plasma membrane of various eukaryotic cells, the existence of a liquid-ordered phase in those membranes, in particular at physiological temperature, has not been unequivocally demonstrated (for a review, see Brown and London 1998). Thus, further studies are warranted to identify the origin and molecular identity of component II. Finally, we cannot preclude that domain II, at least for (0,2)PC and (0,2)PS, may also reflect some weaker lipid-protein interaction.

Recently, employing fluorescent phospholipid analogues, Glaser and co-workers observed by fluorescence digital imaging microscopy large domains of fluorescent PC analogues in the exoplasmic leaflet of human erythrocyte ghost membranes (Rodgers and Glaser 1993). Those domains, which were not seen for the PS analogue, were caused presumably by a specific interaction of PC with the integral membrane protein band-3. This observation is distinct from our results. We found no difference in the partition of (0,2)PC and (0,2)PS between the various domains of the exoplasmic leaflet of human red blood cells. However, at least two essential differences between both approaches exist:

1. In our study, domains were defined by rotational characteristics but not by lateral distribution of analogues seen by microscopy.
2. We used intact human erythrocytes but not ghost membranes.

In conclusion, we have shown that spin-labelled phospholipid analogues can be used to study specific, motional distinct lipid domains in the two leaflets of the intact plasma membrane of erythrocytes and, perhaps, of other mammalian cells. This approach offers a technique to characterize the interaction between both leaflets of cellular membranes. For example, it can be investigated whether alterations of the exoplasmic layer, e.g. triggered by extracellular ligands, affect the cytoplasmic leaflet by specific labelling of the latter.

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