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Influence of plasmalogen deficiency on membrane fluidity of human skin fibroblasts: a fluorescence anisotropy study

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The influence of plasmalogen deficiency on membrane lipid mobility was determined by measuring fluorescence anisotropy of trimethylammoniumdiphenylhexatriene (TMA-DPH) and diphenylhexatrienylpropanoylhydrazylstachyose (glyco-DPH) inserted in the plasma membranes of human skin fibroblasts deficient in plasmalogens. The cells used were from patients affected with cerebrohepatorenal (Zellweger) syndrome (CHRS) or rhizomelic chondrodysplasia punctata. Their plasmalogen content (0–5% of total phospholipid) is significantly reduced compared with that of control cells from healthy donors (13–15% of total phospholipid) or of CHRS fibroblasts supplemented with the plasmalogen precursor, hexadecylglycerol. Plasmalogen-deficient cells consistently showed lower fluorescence anisotropies of membrane-bound DPH fluorophores corresponding to higher membrane lipid mobilities as compared to controls. However, very similar lipid mobilities were found for sonicated aqueous dispersions of phospholipids extracted either from CHRS or control cells. Therefore, the differences observed with living cells are not due to differences in the overall physical properties of the membrane lipid constituents. Other phenomena such as lipid asymmetry and/or plasmalogen–protein interactions may be responsible for the effects observed in the biomembranes.

Introduction

Plasmalogens (1-O-(*Z*-1′-alkenyl)-2-acylglycerophospholipids) are major constituents of most animal cell membranes and of some microorganisms [1]. Some human and animal tissues are especially rich in this lipid subclass, e.g., nervous tissue and muscle cells. Information on the membrane properties of alkenylether glycerophospholipids has become available mainly from studies with chemically defined phospholipid species in artificial membranes [2]. However, knowledge of their behavior in biological membranes is still very limited. Recently, ^{2}H-NMR studies on whole cells, isolated cell membranes and phospholipids from *Clostridium butyricum*, a microorganism rich in plasmalogens, have been reported. The order profiles had the same shape as those found for liquid crystalline diacylphospholipid bilayers [3]. A few years ago, a suitable system was recognized which permits study of the impact of plasmalogens on the properties of biomembranes. Cultured fibroblasts from patients affected with a certain type of peroxisomal disorder [4–6], e.g., the cerebrohepatorenal (Zellweger) syndrome – herein referred to as CHRS cells – or the rhizomelic chondrodysplasia punctata (RCP cells) show a greatly reduced content of ethanolamine plasmalogens. This is the consequence of a biosynthetic block in ether lipid synthesis due to a dysfunction of peroxisomes where enzymes catalyzing the first steps of ether lipid biosynthesis are located. Nonperoxisomal enzymes involved in plasmalogen biosynthesis such as alkylacyl-Gro*P* Etn desaturase are still active [6,7]. A comparison of plasmalogen-deficient cells with control cells containing normal plasmalogen levels should reveal the influence of alkenylether lipids on properties of biomembranes.

Abbreviations: CHRS, cerebrohepatorenal syndrome; Gro*P*Etn, *sn*-glycero-3-phosphoethanolamine; Gro*P*Cho, *sn*-glycero-3-phosphocholine; HDG, 1-*O*-hexadecyl-*sn*-glycerol; RCP, rhizomelic type of chondrodysplasia punctata; TMA-DPH, trimethylammoniumdiphenylhexatriene; Z, Zellweger syndrome; glyco-DPH, diphenylhexatrienylpropanoylhydrazylstachyose; MEM, minimal essential medium.

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In the present study we compared the relative membrane lipid mobilities, as determined from fluorescence anisotropies of diphenylhexatrienyl derivatives, for CHRS, RCP and control cells. Controls were fibroblasts from healthy donors or CHRS cells with close to normal plasmalogen levels after supplementation with 1-*O*-hexadecyl-*sn*-glycerol, a plasmalogen precursor which bypasses the block of peroxisomal ether lipid biosynthesis in diseased cells. We found that CHRS and RCP membranes deficient in plasmalogen were more fluid than membranes of both types of control cell.

Materials and Methods

DPH and TMA-DPH were from Molecular Probes, Eugene, OR, U.S.A. The synthesis of the new probe, glyco-DPH, will be described elsewhere.

Fluorescence measurements

Steady-state fluorescence measurements were carried out on a Shimadzu RF-540 spectrofluorometer or a GREG 200 fluorometer from I.S.S. (La Spezia, Italy). Cells grown on glass cover-slips or in suspension were measured in quartz cuvettes ($d = 1$ cm). The cover-slips serving as the substratum for cultured fibroblasts were oriented within the cuvette 30° relative to the excitation beam in order to minimize stray light, the cell monolayer being exposed directly to the excitation beam [8]. This configuration was found to be superior to other orientations of the cover-slip within the cuvette. The cover slip position was maintained by a self-made holder fitting precisely the cuvette interior. Before labeling, the cells on cover-slips were washed twice with Hanks' solution at 37°C. After transferring the cover slips into the cuvette containing 3 ml Hanks' solution, 9–15 μl of 1 mM stock solutions of DPH (in tetrahydrofuran), TMA-DPH (in ethanol) or glyco-DPH (in ethanol) were added, corresponding to a final label concentration of 3–5 μM each. Incubation times did not exceed 35 min if not otherwise indicated.

After incubation, the cells were washed with Hanks' solution at 37°C and transferred into cuvettes containing Hanks' solution. For recording the emission spectra, the excitation wavelength was 360 nm; monochromator slits were 10 and 5 nm for the excitation and the emission path, respectively. For recording the excitation spectra, the emission wavelength was 430 nm; monochromator slits were 5 and 10 nm, respectively. Fluorescence anisotropies, r, were determined according to

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \quad G = \frac{I_{HV}}{I_{HH}}$$

I_{VV} and I_{VH} being the fluorescence intensities parallel and normal, relative to the vertically oriented excitation polarizers. G is the g-factor. I_{HV} and I_{HH} are the fluorescence intensities determined with the emission polarizer oriented vertically and horizontally when the excitation polarizer was set in the horizontal position [9]. Excitation wavelength was 360 nm. Excitation and emission slits were 5 and 20 nm, respectively. The fluorescence background of a blank sample (unlabeled cells on cover slips) never exceeded 20% of the fluorescence signal of cells labeled with DPH or its derivatives. Blanks were subtracted from the final fluorescence signal. For fluorescence measurements on extracted phospholipid, vesicle preparations were incubated for 2 h after addition of 1 mM stock solution of TMA-DPH in ethanol (final lipid and label concentrations were 100 and 0.1 μM, respectively).

Fluorescence lifetimes were determined with a multifrequency phase fluorometer [10] GREG 200 (frequency range of 1–200 MHz) from I.S.S., La Spezia, Italy, using POPOP in ethanol ($\tau = 1.35$ ns) as a lifetime reference [11]. A helium-cadmium laser was used as an excitation light source at 325 nm. A Schott KV 370 filter was used in the emission light path. Phase and modulation data were collected for 12 modulation frequencies from 2 to 200 MHz. Data were analyzed by a computer program from I.S.S. according to a least-squares fitting procedure for a multiexponential decay [12]. Cells in suspension were labeled and the fluorescence was measured according to the same procedure as outlined for the cover slip system. In addition, the suspended cells had to be centrifuged at $100 \times g$ for 2 min and resuspended after each washing step.

Cell cultures.

Human skin fibroblasts taken by biopsy from patients with CHRS (CHRS strains AS, GM and HA) or with RCP (RCP strain NE), and from healthy donors (control cells SA, KO, MO) were grown in 25-cm^2 culture flasks (Nunclon) containing minimal essential medium (MEM) under a CO_2 atmosphere (5% CO_2, 95% air) at 37°C to confluency. Then the cells were washed with phosphate-buffered saline, trypsinized at 37°C and split into two portions, one 20 cm^2 culture dish containing two 4×1.1 cm glass cover slips. The cells were again grown to confluency. Several hours before the fluorescence measurements, the MEM was replaced by Hepes in the culture dish, providing sufficient buffer capacity for 24 h cultivation in the absence of CO_2. Before use, glass cover-slips were pretreated with the detergent 7-X-O-Matic (Flow-laboratories), rinsed with water and sterilized for 30 min at 100°C.

Supplementation with hexadecylglycerol

Fibroblasts were grown in 20-cm^2 culture dishes to confluency as indicated above. Then the MEM was replaced by 3 ml MEM containing 20 μg HDG/ml, or 3 ml MEM for control cells. The cells were then incubated for additional 24–30 h. The culture media were replaced by alkylglycerol-free Hepes medium and incubated 2–6 h before the fluorescence measurement.

Integrity of cells was checked from the exclusion of Trypan-blue (Gibco, U.K.). Cell numbers were determined in a Bürker-Türk Kammer using a phase contrast microscope.

Vesicles from cellular lipid extracts

Lipids were extracted from cells according to the procedure of Folch et al. [13]. Neutral lipids were separated from the phospholipid fraction by preparative thin-layer chromatography on silica gel plates with diethyl ether as a solvent. Phospholipids were eluted with chloroform/methanol (1 : 4, v/v). Aliquots of the phospholipid solution were mixed with adequate amounts of cholesterol dissolved in chloroform to adjust a final sterol concentration of 40 mol% of total lipid. After removing the solvent under nitrogen, the phospholipids were sonicated in 10 mM Tris-HCl (pH 7.0) using a sonicator Brown Labsonic 200 equipped with a 4 mm soniprobe at 50 W input power for 10 min. Titanium particles were removed by centrifugation.

Analytical methods

Total lipids were extracted from the cells with chloroform/methanol (2 : 1, v/v) according to Folch et al. [13]. The different phospholipid classes and, in particular, the plasmalogens were quantitated by phosphorus analysis [14] after two-dimensional thin-layer chromatography of the lipid extract on silica gel H plates (20 × 20 cm). The solvent was chloroform/methanol/water (65 : 25 : 4, v/v) for the first and the second direction. For determination of plasmalogens, the thin-layer plate was exposed, after the first run, to gaseous HCl for 10 min which converts the alkenylacylglycerophosphoethanolamine into lysophosphatidylethanolamine [15]. After development in the second direction, the phospholipid pattern was visualized by iodine vapor.

Total phospholipids were interesterified with boron trifluoride/methanol and the resulting fatty acid methyl esters were analyzed by gas-liquid chromatography [16]. Cholesterol was determined using a commercially available cholesterol oxidase assay from Boehringer, Mannheim, F.R.G. Protein was assayed according to the method of Lowry et al. [17].

Results

Characterization of the fluorescence of DPH derivatives incorporated in fibroblast cells

DPH or TMA-DPH when incorporated in fibroblast cells exhibits spectra characteristic for these fluorophores in membranes (Fig. 1). There is no difference between the spectra obtained with cells grown on glass cover-slips and in suspension. In accordance with data from the literature [18], the rate of uptake by the cells is different for DPH and TMA-DPH (Fig. 2). Incorporation of TMA-DPH and glyco-DPH as determined from the extent of fluorescence dequenching is almost complete after a few minutes, whereas DPH is incorporated at a much lower rate. Results from fluorescence mi-

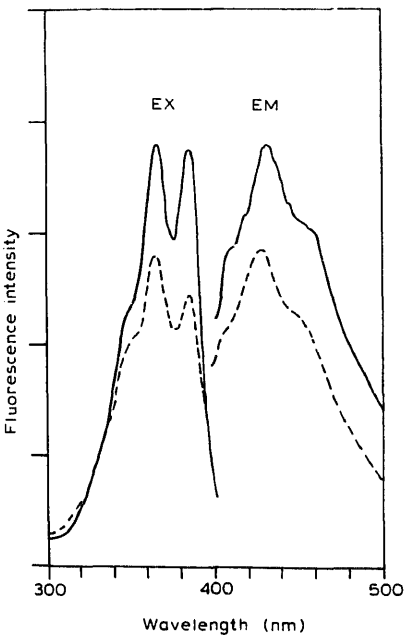

Fig. 1. Excitation (EX) and emission (EM) spectra of glyco-DPH (———) and TMA-DPH (— — —) in fibroblast monolayers (cell strain GM) attached to glass cover-slips. Cells were labeled with 1.3 μM glyco-DPH or 3 μM TMA-DPH in 3 ml of Hanks' solution at 37 °C.

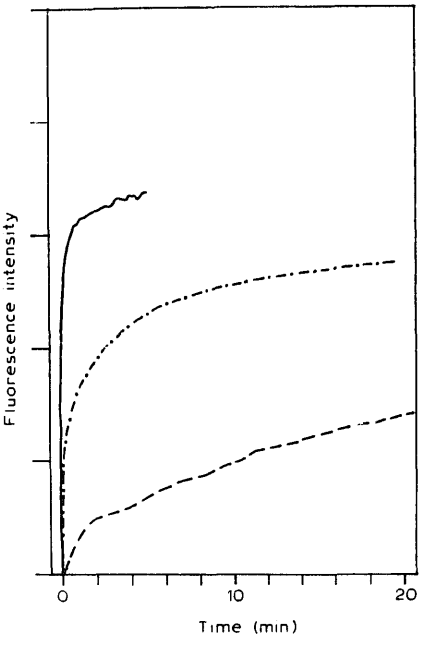

Fig. 2. Uptake of glyco-DPH (———), TMA-DPH (·-·-·) and DPH (— — —) by fibroblast monolayers (cell strain GM) from aqueous label suspensions at 37 °C (in Hanks' solution) (1.8, 3.0 and 5.0 μM label concentrations, respectively). The increase of fluorescence intensity at 430 nm is representative of the incorporation of the fluorophore into the cells.

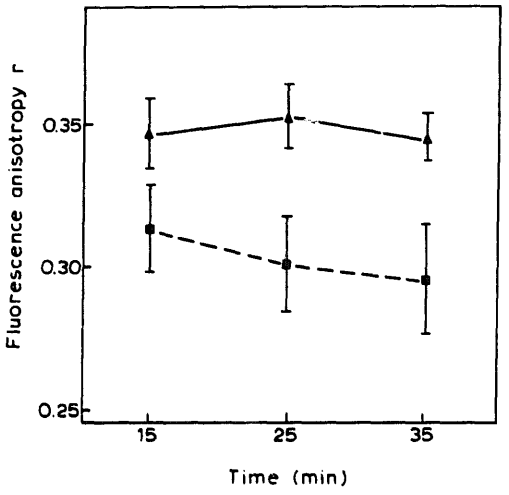

Fig. 3. Time-profiles of fluorescence anisotropy of TMA-DPH in fibroblast monolayers attached to glass cover-slips. Measurements were carried out after labeling the cells in the presence of 3 ml of 3 μM TMA-DPH suspensions in Hanks' solution at 37 °C. Bars represent standard deviations from mean values. Cell strains: MO (C) (▲), GM (Z) (■).

croscopy studies [18] suggest that TMA-DPH is located in the plasma membrane at least within the first 30 min of labeling, whereas DPH is equally distributed among plasma and organelle membranes even after short labeling times. Therefore, we suppose that the effects we observed after labeling the cells with TMA-DPH for 30 min were in the plasma membrane of fibroblasts. This assumption is also in accordance with the time-dependent profiles of fluorescence anisotropy during 35 min incubation of the cells with TMA-DPH (Fig. 3). Two representative cell strains, GM (plasmalogen-deficient) and MO (control), were investigated. The anisotropy of control cells was almost invariant over the period of 35 min, whereas the CHRS cells exhibited a slight decrease of anisotropy. The latter might be a consequence of minor label penetration through the less rigid CHRS cell membrane which showed consistently lower fluorescence anisotropies compared to the control (see below). Fluorescence anisotropies of DPH in cell membranes decrease with time (data not shown). This effect might be due to migration of the label into intracellular membranes [18].

The anisotropy values are significantly smaller for cells in suspension as compared to cells on cover-slips (Tables I and II). This difference might be a consequence of membrane alterations caused by trypsinization of the cell monolayers and/or centrifugation of the cells [20]. Throughout fluorescence measurements on cultured cells attached to cover-slips, we found that the determination of the *G*-factor, which is, in principle, a property of the instrument [9], was of crucial importance for the estimation of anisotropies. Depending on the cell number, this factor varied considerably from about 0.5 to 0.7. So far, no information has been

TABLE I

Fluorescence anisotropies of different DPH probes in monolayers of cultured human skin fibroblasts from patients [a] and healthy controls [b] at 37 °C in Hanks' solution

Measurements were carried out after labeling the cells attached to glass cover slips in the presence of 3 ml Hanks' solution containing 5 μM DPH, 3 μM TMA-DPH or 1.8 μM glyco-DPH for 35 min at 37 °C. Pretreatment of cells with hexadecylglycerol was carried out for 24–30 h as described in the Materials and Methods section. Data obtained with TMA-DPH and glyco-DPH are expressed as the means of five experiments ± S.E.; * refers to P less than 0.01 between deficient and control cells (except for HA vs. SA) by Student's t-test. DPH anisotropies are the results from single experiments.

Cell strain	Fluorescence anisotropy (r)		
	DPH	TMA-DPH	Glyco-DPH
HA (Z)	0.208	0.307 ± 0.015 *	0.315 ± 0.017
GM (Z)		0.289 ± 0.006 *	
GM + HDG [c]		0.324 ± 0.026	
AS (Z)		0.294 ± 0.015	
AS + HDG		0.325 ± 0.014	
NE (RCP)		0.294 ± 0.004	
SA (C)	0.221	0.337 ± 0.033 *	0.338 ± 0.014
MO (C)		0.346 ± 0.017 *	
KO (C)		0.317 ± 0.009	0.331 ± 0.009
KO + HDG		0.317 ± 0.008	

[a] Zellweger syndrome (Z) or rhizomelic chondrodysplasia punctata (RCP).
[b] C, control cells from healthy donors.
[c] HDG, 1-*O*-hexadecyl-*sn*-glycerol.

available about the relevance of *G*-factors for the determination of anisotropies of DPH labels in cell monolayers. We believe that the different *G*-factors observed may be considered as empirical characteristics of the cell monolayers, as these are highly anisotropic systems by themselves when attached to a surface.

For cell suspensions, we consistently found *G*-factors of 1.0, irrespective of the cell strain or DPH derivative. Therefore, we believe that the anisotropy data obtained with different cell strains on glass cover-slips have to be corrected for the respective *G*-factors, which most likely reflect differences in the optical properties of the cells arising from differences in cell density or shape.

TABLE II

Fluorescence anisotropies (r) of DPH and TMA-DPH in suspensions of cultured human skin fibroblasts

Measurements were carried out after labeling the cell suspensions in 3 ml Hanks' solution containing 5 μM DPH or 3 μM TMA-DPH for 35 min at 37 °C. Anisotropies were obtained from single experiments. Cell strains: HA (Zellweger); MO, SA (controls).

Cell strain	Fluorescence anisotropy (r)	
	DPH	TMA-DPH
HA (Z)	0.120	0.215
MO (C)		0.230
SA (C)	0.140	0.230

TABLE III

Membrane lipid composition of cultured human skin fibroblasts

Cells were grown to confluency and constituents were analyzed as described in the Materials and Methods section. Ratios (except for plasmalogens) are given on a weight basis. The ratio saturated/unsaturated fatty acids was determined for total cellular phospholipids. The fatty acid pattern was also determined for phosphatidylcholine, phosphatidylethanolamine and ethanolamine plasmalogen from CHRS cells and controls. No significant differences were found.

Cell strain	mol% plasmalogen per total phospholipid	Cholesterol / Phospholipid	Protein / Phospholipid	Saturated fatty acids / Unsaturated fatty acids
HA (Z)	2.5	0.49	10.8	0.88
GM (Z)	3.1	0.32	12.3	
GM + HDG	10.1			
AS (Z)	5.1			
NE (RCP)	0			
SA (C)	13.9	0.30	12.8	
SA + HDG	14.5			
MO (C)	11.7	0.32	11.7	
KO (C)	13.4			0.88

Membrane fluidity and membrane lipid composition

Fluorescence anisotropies of TMA-DPH incorporated in cell membranes of cultured fibroblasts attached to cover slips were determined for various cell strains differing in their plasmalogen content. Data summarized in Tables I, II and III show a clear correlation between fluorescence anisotropy (Tables I and II) and cellular plasmalogen levels (Table III). Fluorescence anisotropy was significantly lower for plasmalogen-deficient CHRS cells (AS, GM and HA) and for RCP cells (NE) than for control cells (SA, MO and KO) which had normal plasmalogen levels.

Glyco-DPH was incorporated into the plasma membrane of cultured fibroblasts at a faster rate than TMA-DPH (Fig. 2). In addition, the new probe, glyco-DPH, revealed lower anisotropies in plasmalogen-deficient CHRS cells compared with controls (Table I).

Other factors influencing membrane fluidity [19], such as the cholesterol/phospholipid ratio, the protein/phospholipid ratio and fatty acid composition are very similar for the cells under investigation, except for the rather high cholesterol/phospholipid ratio of the HA cells (Table III). This might explain the somewhat higher anisotropies measured with this CHRS cell line as compared to the others (Table I).

The plasmalogen content of CHRS cells increases to almost normal levels after addition of alkylglycerol to the culture medium. Under similar conditions, the relative amount of plasmalogens in control cells was only slightly affected. After labeling with TMA-DPH or glyco-DPH, CHRS cells supplemented with alkylglycerol are characterized by fluorescence anisotropies that are significantly higher than those of untreated CHRS cells, and are very close to fluorescence anisotropies measured with control cells from healthy donors (Table I). It is important to mention that unmetabolized hexadecylglycerol acts as a detergent and has a fluidizing effect on fibroblast membranes. Therefore, after incubation in the presence of the glycerol ether, cells must be kept in a hexadecylglycerol-free medium for at least 2 h.

Sonicated dispersions of phospholipid extracts from CHRS or control cells were also labeled with TMA-DPH. In contrast to the intact cells, the plasmalogen content of the lipid dispersions, either in the presence of absence of cholesterol, had no effect on fluorescence anisotropies (Table IV). The corresponding fluorescence decays were double-exponential and were also very similar for plasmalogen-deficient and control samples (Table IV).

TABLE IV

Fluorescence anisotropies r, lifetimes τ (ns), and the respective fractional intensities, f, of TMA-DPH in vesicles of a cellular phospholipid extract from cultured human skin fibroblasts in the presence or absence of the natural content of cholesterol

Dispersions of phospholipid extracts from human skin fibroblasts (100 μM phospholipid) in 3 ml of Hanks' solution were prepared by sonication. After addition of an ethanolic TMA-DPH stock solution (final label concentration 0.1 μM), samples were incubated at 37°C for 2 h. Cell strains: HA, GM (Zellweger), MO, SA (controls)

Cell strain	r	τ_1 (f_1)	τ_2(f_2)
HA (Z)	0.220	4.6 (0.84)	1.5 (0.16)
HA (Z) + 40 mol% cholesterol	0.280	7.9 (0.89)	3.1 (0.11)
GM (Z)	0.217	4.4 (0.90)	1.1 (0.10)
GM (Z) + 40 mol% cholesterol	0.286	8.4 (0.81)	4.3 (0.19)
KO (C)	0.215	4.6 (0.79)	1.1 (0.21)
KO (C) + 40 mol% cholesterol	0.285	7.7 (0.85)	3.4 (0.15)
SA (C)	0.213	4.6 (0.84)	1.6 (0.16)
SA (C) + 40 mol% cholesterol	0.286	7.7 (0.89)	3.3 (0.11)

Discussion

The possible role of plasmalogens as membrane constituents has been a puzzling problem ever since the 'unusual' chemical structure of this subclass of glycerophospholipids was elucidated 30 years ago [21]. Studies on biophysical properties of plasmalogens have so far been restricted mostly to model membrane systems. Although model membranes have always been and still are considered suitable systems to investigate membrane-forming properties of lipids, it is obvious that data obtained with such simple systems do not necessarily allow one to draw conclusions concerning the behavior and role of a particular lipid in a much more complex biological membrane. Therefore, whenever possible, it is desirable to confirm that properties of a particular lipid observed in model membranes do in fact contribute to the properties of biomembranes in which this lipid occurs. It has been shown previously that the gel-to-liquid phase transition T_c of 1-*O*-alkenyl-2-acyl-Gro*P*Etn and 1-*O*-alkyl-2-acyl-Gro*P*Etn are somewhat lower compared with that of 1,2-diacyl-Gro*P*Etn [22,23]. On the other hand, it has been found that choline plasmalogens pack more densely than diacyl Gro*P*Cho in monolayers at the air–water interface [24]. Are these (or other) differences between plasmalogens and their diacyl analogs as observed in model systems relevant in determining properties of biological membranes, e.g., by affecting the packing or mobility of membrane lipids? To answer this specific question, or to approach the general problem of the effects of plasmalogens on biophysical properties of biomembranes, one needs a suitable biological system. 'Suitable' means that the system should allow significant alterations of the cellular plasmalogen level without compensatory changes in the pattern of other membrane components. Such a system has become available recently in the form of cultured skin fibroblasts obtained from patients affected with peroxisomal disorders, e.g., the Zellweger syndrome or the rhizomelic chondrodysplasia punctata [5]. In such cells, the plasmalogen content is reduced to approx. 25% and zero relative to control cells in the case of CHRS and RCP, respectively. Plasmalogen deficiency is due to a block of peroxisomal steps of ether lipid biosynthesis [4–6]. This block can be bypassed by feeding cells an ether lipid precursor, e.g., 1-*O*-hexadecyl-*sn*-glycerol [6]. Supplemented cells, after 24 h incubation in the presence of hexadecylglycerol, reach almost normal plasmalogen levels. In addition, fibroblasts obtained from healthy donors can be used as a control. It is noteworthy that the phospholipid class composition, fatty acid pattern, phospholipid-to-protein ratio and cholesterol-to-phospholipid ratio, all of which are determinants of biophysical membrane properties [19], are essentially identical for diseased cells and controls.

In the present study we investigated the effects of plasmalogens on a fundamental membrane parameter, namely membrane fluidity, by measuring the fluorescence anisotropy of suitable fluorescent probes inserted in the plasma membrane of plasmalogen-depleted CHRS or RCP cells and of control cells. At the beginning, several methodological problems had to be solved. First, it appeared to be essential to measure anisotropy with cells in a native state. For cultured fibroblasts, 'native' means that the cells adhere to the substrate. We therefore adopted the method described by Sumbilla and Lakowicz [8] and used cells grown on glass cover slips that could directly be mounted in a fluorescence cuvette. Next we had to choose a fluorescent probe that inserted specifically into the plasma membrane without significant internalization during the time of the experiment. One such probe, TMA-DPH, has been widely used to monitor membrane fluidity and was employed also in this study. In addition, we synthesized a new probe, DPH-propionylhydrazylstachyose, whose large hydrophilic tetrasaccharide headgroup should make it even less prone to transmembrane translocation than TMA-DPH. The concentrations of probes used in the present study were much lower than those used by others [8]. Low concentrations and short incubation times were chosen in order to avoid cell damage and internalization of the fluorescent label.

Both fluorescent probes gave essentially the same results, namely, that fluorescence anisotropy correlates with cellular plasmalogen levels (Tables I, III). In other words, the higher the plasmalogen content of cellular membranes, the lower is their 'fluidity'. In principle, this result correlates with analogous results obtained by measuring fluorescence anisotropy of the same fluorophores in phospholipid vesicles made either of choline or ethanolamine plasmalogen, or the corresponding diacyl analogs. Plasmalogen membranes were consistently less fluid than diacylglycerophospholipid membranes (Hermetter et al., unpublished results). However, considering that ethanolamine plasmalogen makes up no more than 15% of total cellular phospholipids (Table III), it had to be tested whether the lipid phase of cellular membranes alone was responsible for the observed fluidity changes. Anisotropy measurements with aqueous dispersions of phospholipids extracted from the respective cells gave a clear answer: the phospholipids, either as such or in admixture with cholesterol, did not show the differences observed with whole cells (Table IV). Several possibilities can be discussed to explain the discrepancy between intact cell membranes and extracted membrane lipids. For instance, the formation of plasmalogen-rich domains, which may form spontaneously or due to specific lipid–protein interactions, could provide an environment rigid enough to significantly enhance fluorescence anisotropy of, e.g., TMA-DPH, if the probe preferentially partitions into

these domains. Alternatively, the transbilayer distribution may be different for plasmalogens and the diacyl analogs in a biological membrane. The possibility that very long chain fatty acids (VLCFA), which are increased in CHRS cells [5], might contribute to the effects observed can be excluded, since in RCP cells VLCFA levels are normal [25]. Whatever the underlying mechanism might be, the increased membrane fluidity observed in plasmalogen-depleted CHRS cells might have implications for the functioning of membrane-linked processes and thus for the proper functioning of the cells. It has to be noted that in various organs of patients affected with peroxisomal disorders, plasmalogen levels drop to almost zero, e.g., in nervous tissue, where in healthy individuals plasmalogens make up a significant proportion (up to 35%) of membrane phospholipids [26]. Thus, changes in fluidity might be much more pronounced in such cells than in cultured fibroblasts as reported here. It is well-established that proper functioning of most membrane-associated proteins, e.g., enzymes, transport proteins or receptors, requires an optimal membrane fluidity [19]. A decrease in 'viscosity' might be as deleterious as the more often discussed increase in 'viscosity'.

We are hesitant in claiming that the severe clinical symptoms observed with patients affected with a peroxisomal disorder, e.g., CHRS or RCP, are causally linked to aberrations of membrane fluidity. However, results from ongoing experiments aimed at correlating membrane fluidity of diseased versus control cells to the structure and function of their membranes should provide more insight into the effects of fluidity changes caused by the absence of plasmalogens, and will thus augment our understanding of the pathophysiology of peroxisomal dysfunction.

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