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Uptake of fluorescent plasmalogen analogs by cultured human skin fibroblasts deficient in plasmalogen

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One of the consequences of hereditary peroxisomal dysfunction in the cerebro-hepato-renal (Zellweger) syndrome (CHRS) is a dramatic decrease in the biosynthesis and cellular content of ether lipids. In the present study effects of reduced cellular plasmalogen levels on membrane–membrane interactions were investigated. Cultured CHRS fibroblasts were incubated with unilamellar phospholipid vesicles consisting of 1-*O*-alkenyl-2-acyl- or 1,2-diacyl-*sn*-glycerophosphocholines and ethanolamines, carrying either the *trans*-parinaroyl or the 1,6-diphenyl-1,3,5-hexatriene propionyl group in position 2. Transfer of the fluorogenic phospholipids from vesicles to cells was followed by measuring the concomitant increase in fluorescence intensity. Transfer of phospholipids from cells to vesicles was monitored by incubating cells, prelabeled with [^{3}H]oleic acid, in the presence of phospholipid vesicles. Fibroblasts from healthy donors or CHRS fibroblasts supplemented with the plasmalogen precursor 1-*O*-hexadecylglycerol served as controls. Plasmalogen-deficient cells exhibited a significantly increased tendency to take up exogenous choline or ethanolamine plasmalogens. Cellular plasmalogens were transferred from control cells to vesicles at a higher rate if the acceptor vesicles consisted of plasmalogens as compared to diacylglycerophosphocholine. Thus, it appears as if mechanisms existed which preserve cellular plasmalogen levels during interaction with exogenous phospholipid pools. Preliminary experimental evidence suggests that the observed exchange of phospholipids between cultured fibroblasts and vesicles occurs by a protein-catalyzed process.

Introduction

Over the past years interest in ether lipids has centered mainly on biologically active species, among which the platelet activating factor is the most prominent. However, the abundance of ether lipids, especially of plasmalogens in animal cell membranes and in certain microorganisms suggests a specific role of this phospholipid subclass as a determinant of membrane properties and function. Thus far, it has not been possible to establish a correlation between plasmalogen content and function of biological membranes. A first insight into physicochemical properties of plasmalogens was gained from studies on artificial membrane systems (for reviews, see Refs. 1, 2). Previous experiments aimed at an alteration of the plasmalogen content of biological membranes in order to examine consequences on membrane properties led to ambiguous results, mainly because compensatory changes in other membrane components had masked possible effects of changes in plasmalogen levels. Recently, cultured human cells with an inherited deficiency in ether lipid biosynthesis were characterized [3–5]. Such cells can be obtained from patients affected with certain types of peroxisomal disorder, e.g., the cerebro-hepato-renal (CHRS) or Zellweger syndrome. In CHRS fibroblasts the plasmalogen content is significantly reduced [6,7]. Almost normal plasmalogen levels can be restored, however, by feeding CHRS cells hexadecylglycerol [7]. This ether lipid substrate is converted to plasmalogens by enzymes which are located in the endoplasmic reticulum and function normally in diseased cells [8].

Abbreviations: CHRS, cerebro-hepato-renal (Zellweger) syndrome; DMEM, Dulbecco's minimum essential medium; DPH, 1,6-diphenyl-1,3,5-hexatriene; HDG, 1-*O*-hexadecylglycerol; HOPC, 1-*O*-1′-hexadecenyl-2-oleoyl-*sn*-glycero-3-phosphocholine; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; MEM, minimum essential medium; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PnA, *trans*-parinaric acid (9,11,13,15-all-*trans*-octadecatetraenoic acid); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TMA-DPH, 1-[4′-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene.

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Studies from our laboratory have shown that membranes of plasmalogen-deficient cells are more 'fluid' than membranes of healthy control cells or of diseased cells supplemented with hexadecylglycerol [6,7]. Therefore, it was of considerable interest to examine possible consequences of plasmalogen deficiency by comparing membrane functions of diseased versus control cells. In a first attempt along these lines we tested the interaction of plasmalogen-depleted and control cells with externally presented artificial phospholipid vesicles consisting either of plasmalogens or of the corresponding diacyl analogs. The rationale for these experiments was to examine whether or not the plasmalogen content of donor and/or acceptor membranes would affect membrane–membrane interaction. Differences between plasmalogen-deficient and control cells could arise from the observed modulation of membrane lipid mobility by plasmalogens (see above), but other specific properties of plasmalogens observed previously in artificial membrane systems might also be of relevance in the in vivo system. For example, studies on monolayers at the air water interface had shown that plasmalogens exhibit significantly reduced dipolar membrane potentials. Differential scanning calorimetry in combination with optical birefringence and ^{31}P-NMR had revealed that ethanolamine plasmalogens have a higher tendency to undergo lamellar-to-hexagonal (H_{II}) phase transition as compared to the diacyl analog [9]. Any of these parameters might be relevant for membrane–membrane interactions, either by affecting membrane fusion, endocytosis, or lipid exchange. In the course of the present study we found that plasmalogen-depleted cell membranes have a significantly increased tendency to take up exogenous plasmalogens, most likely by a protein-mediated exchange process.

Materials and Methods

Dipalmitoylphosphatidylcholine was from Lukas Meyer (Hamburg, F.R.G.), diphenylhexatrienylpropanoic acid was from Lambda Probes & Diagnostics (Graz, Austria) and all-*trans*-parinaric acid was obtained from Molecular Probes (Eugene, OR, U.S.A.). 1-*O*-Hexadecyl-*sn*-glycerol was prepared from 2,3-isopropylidene-*sn*-glycerol and hexadecylmesylate [10]. Cytochalasin B was purchased from Sigma (St. Louis, U.S.A.). [9,10-$^3H_2$]Oleic acid was from Amersham International, U.K. Phospholipase A_2 from *Crotalus durissus* was purchased from Boehringer (Mannheim, F.R.G.). 1-*O*-Alkenyl-*sn*-glycero-3-phosphocholine was prepared from beef heart choline glycerophospholipids as described [11]. Approx. 70% of the alkenyl residues in position 1 of glycerol are of the hexadecenyl type [9].

1-O-Radyl-2-trans-parinaroyl-sn-glycero-3-phosphocholines

1-Acyl-*sn*-glycero-3-phosphocholine was prepared from dipalmitoyl phosphatidylcholine by phospholipase A_2 treatment [12] and fatty acid was removed from the lysophospholipid by ether extraction at low temperatures [13].

The following procedures, including extractions and preparative thin-layer chromatography, were carried out strictly under argon. A mixture of 20 mg lysophospholipid (39 μmol), 20 mg *trans*-parinaric acid (72 μmol), 40 mg dicyclohexylcarbodiimide (194 μmol) and 25 mg dimethylaminopyridine (204 μmol) in 200 μl anhydrous and ethanol-free chloroform was stirred at room temperature for 24 h under protection from light and atmospheric moisture. The formation of product was 100% according to thin-layer chromatography on silica gel (solvent, chloroform/methanol/25% ammonia (65 : 35 : 5, v/v); $R_F = 0.3$). Spots were detected from the product's fluorescence by spraying with molybdic acid reagent or charring after spraying with sulphuric acid. The reaction was stopped by the addition of 15 ml chloroform/methanol (2 : 1, v/v). After washing with 3 ml methanol/water (1 : 1, v/v) and evaporation of the solvent, the crude product was purified by preparative thin-layer chromatography on silica gel H (Merck Darmstadt, F.R.G.) using as developing solvent chloroform/methanol/25% ammonia (65 : 35 : 5, v/v). Products were eluted with chloroform/methanol (1 : 4, v/v) and freed of residual silicic acid by partitioning between chloroform and methanol/water [14]. The yield was 20% based on lysophospholipid. The low yields after TLC purification are due to the extreme sensitivity of parinaroyl lipids toward light and oxygen. Products were stored under argon at −70 °C in chloroform/methanol (2 : 1, v/v) in the presence of 1% di-*tert*-butyl-*p*-kresol (Fluka, Switzerland).

1-O-Radyl-2-diphenylhexatrienylpropionyl-sn-glycero-3-phosphocholines

A mixture of 100 mg lysophospholipid (approx. 200 μmol), 76.5 mg DPH-propionic acid (250 μmol), 86 mg dicyclohexylcarbodiimide (416.5 μmol), and 102 mg dimethylaminopyridine (833.2 μmol) in 1 ml chloroform was reacted and worked up as described above. Preparative thin-layer chromatography (see above) provided the pure product (30% yield) exhibiting a single spot on thin-layer chromatograms; $R_F = 0.3$ (solvent: chloroform/methanol/25% ammonia, 65 : 35 : 5, v/v).

The fluorescent parinaroyl and DPH phospholipids were analyzed for the label to phosphorus ratio by measuring optical absorbances at 320 and 360 nm, respectively, and by phosphorus analysis [15]. Values of 0.9 and 0.7 were obtained for parinaroyl- and DPH-propionylphosphatidylcholines, respectively. Measured absorbances were lower compared with theoretical values, probably because fluorescent phospholipids aggregate to some extent also in organic solvents, which leads to self-quenching of the fluorophores. In addition, some

label degradation may occur during chemical synthesis and chromatographic purification. Extinction coefficients were obtained from the free fatty acids which are monomeric at concentrations (10^{-4} to 10^{-5} M) and in the solvents used for *A* measurements. In the case of diacylglycerophospholipids the palmitic acid (analyzed by gas-liquid chromatography) to phosphorus [15] ratio was also determined and found to be close to 1, which is additional proof for the identity of the fluorescent phospholipids.

Fluorescent ethanolamine phospholipids

1-*O*-Radyl-2-acyl-*sn*-glycero-3-phosphoethanolamines containing DPH-propionic acid in position 2 were prepared from the corresponding choline phospholipids by phospholipase-D-catalyzed *trans*-phosphatidylation in the presence of ethanolamine [16]. Compounds were purified by preparative thin-layer chromatography (see above) and showed single spots on analytical thin-layer chromatograms, $R_F = 0.5$ (solvent, chloroform/methanol/25% ammonia, 65 : 35 : 5, v/v).

Phospholipid vesicles

Unilamellar parinaroyl phospholipid vesicles were prepared by injection of 9 nmol lipid dissolved in 10 μl ethanol into 3 ml Hanks' solution with stirring at 37 °C under an atmosphere of argon [17]. Optical absorbances of the vesicle preparations were not greater than 0.14 at 320 nm. DPH-phospholipid vesicles were also prepared by sonication of 5 nmol lipid in 3 ml Hanks' solution for 5 min at 4 °C. A Brown-Labsonic 2000 sonicator equipped with a 4 mm soniprobe at 50 W input power was used.

Cell cultures

Human skin fibroblasts taken by biopsy from patients affected with the cerebro-hepato-renal syndrome (CHRS1, CHRS2) and from healthy donors (controls 1–3) were grown to confluency in 25 cm^2 culture flasks (Nunclon) containing MEM with 100 g/l fetal calf serum (from Flow Laboratories, Irwin, U.K.) under an atmosphere of air containing 5% carbon dioxide at 37 °C. Then the cells were washed with phosphate-buffered saline and trypsinized at 37 °C. Afterwards, the fibroblast suspensions were split into two 20 cm^2 culture dishes each containing two 4 × 1.1 cm glass coverslips. The cells were again grown to confluency. Several hours before the fluorescence measurements MEM was replaced by Hepes buffer providing sufficient buffer capacity for 24 h cultivation in the absence of carbon dioxide. Before use, glass coverslips were pretreated with the detergent 7X-O-Matic (Flow Laboratories), rinsed with water and sterilized for 30 min at 100 °C. Passage numbers were between 10 and 20. No difference in lipid uptake by the cells was observed within this range.

Supplementation with 1-O-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 1-*O*-hexadecylglycerol per ml. This supplemented medium was prepared by addition of 1 μl of an ethanolic stock solution of HDG (20 mg/ml) per ml MEM under stirring at 37 °C. The cells were then incubated for an additional 24–30 h. The culture media were replaced by alkylglycerol-free Hepes-buffer and incubated for 2–6 h before the fluorescence measurements. Integrity of the cells was checked by the exclusion of Trypan blue (Gibco, U.K.). Cell numbers were determined in a Burker-Tuerk Kammer using a phase contrast microscope. The plasmalogen content of supplemented cells was 10% of total phospholipids as compared to 3–4% in untreated CHRS cells and 13–14% in control fibroblasts from healthy donors [7].

Labelling of cellular lipids with [^{3}H]oleic acid

Fibroblasts were grown in 25 cm^2 culture flasks (nunclon) to confluency as described above. Then the MEM medium was replaced by a DMEM medium containing 1.35 mg (4.8 μmol) [9,10-$^3H_2$]oleic acid (spec. act. 25 μCi/μmol) per ml DMEM. Cells were then incubated for about 15 h. The culture medium was replaced by oleic acid-free DMEM and cells were incubated for 2–6 h before phospholipid transfer experiments.

Transfer studies with [^{3}H]oleic acid supplemented cells

Cells grown in 25 cm^2 culture flasks (Nunclon) as indicated above were washed twice with Hanks' solution and then incubated with vesicles containing POPC or HOPC (4.5 mg phospholipid) at 37 °C for 30 min. vesicles were prepared by sonication using a Brown-Labsonic instrument equipped with a 10 mm Soniprobe at 70 W input power for 10 min. Phospholipids were extracted from culture media and from cells with chloroform/methanol (2 : 1, v/v) according to Folch et al. [18]. Phospholipids were analyzed by two-dimensional thin-layer chromatography on 20 × 20 cm silica-gel H plates. The developing solvent was chloroform/methanol/water (65 : 25 : 4, v/v) for both directions. For determination of plasmalogens the plates were exposed to HCl vapor for 12–15 min [19] after the first run. After development in the second direction the phospholipid patterns were visualized by iodine vapor. Phospholipid radioactivity was measured by liquid scintillation counting with a Kontron MR 300 using an HP-cocktail containing 5% water.

Fluorescence measurements

Fluorescence measurements were carried out on a Shimadzu RF 540 or a GREG 200 (I.S.S., La Spezia, Italy) spectrofluorometer. Excitation wavelengths for

DPH and PnA phospholipids were 360 nm and 320 nm, respectively. Fluorescence associated with cells grown on glass coverslips (40 × 11 mm) was measured in quartz cuvettes ($d = 1$ cm). The cover slips serving as a substratum for the cultured fibroblasts were oriented within the cuvette 30° relative to the excitation beam in order to avoid stray light, the cell monolayer being exposed directly to the excitation beam. The cover slip position was maintained by a self-made holder fitting precisely the cuvette interior. The cover slips were washed twice with Hanks' solution at 37° C and then incubated with vesicles consisting of fluorogenic phospholipids in 3 ml Hanks' solution. The cells in Hanks' solution were viable at least 2 h, as tested with Trypan blue and the absence of lactate dehydrogenase activity in the buffer solution as determined with a test kit from Boehringer-Mannheim, F.R.G. Phospholipid transfer between vesicles and cells was determined from the increase of fluorescence intensity of the DPH- or the PnA-fluorophores at 430 and 410 nm, respectively. The fluorescence intensity of the pure fluorogenic vesicles was low due to self-quenching.

The fluorescence technique as outlined above for lipid transfer measurements is clearly superior to methods using radioactively labeled lipids. The latter procedures imply comparison of different cell monolayers grown on different cover slips, separated from radioactively labeled donor vesicles after different incubation times. Using the fluorescence method, lipid transfer can be monitored continuously for a single cell monolayer.

Emission spectra of cells labeled with DPH or PnA phospholipids were determined after incubation of the cells with phospholipid vesicles (5 μM phospholipid) in 3 ml Hanks' solution for 30 min at 37° C, followed by washing the cells twice with Hanks' solution. Blank spectra of unlabeled cells were recorded immediately before incubation with the phospholipid vesicles.

Results

Spectral characteristics of fluorescent phospholipids and principle of phospholipid transfer measurement

Fluorescence excitation and emission spectra of fluorescently labeled choline and ethanolamine plasmalogens contained in egg yolk phosphatidylcholine vesicles correspond closely to spectra reported for membrane-associated diacylglycerophospholipids containing parinaric acid [20,21] or diphenylhexatrienylpropionic acid [22,23]. Emission maxima of parinaroyl and DPH-propionyl-labeled derivatives were at 410 and 430 nm,

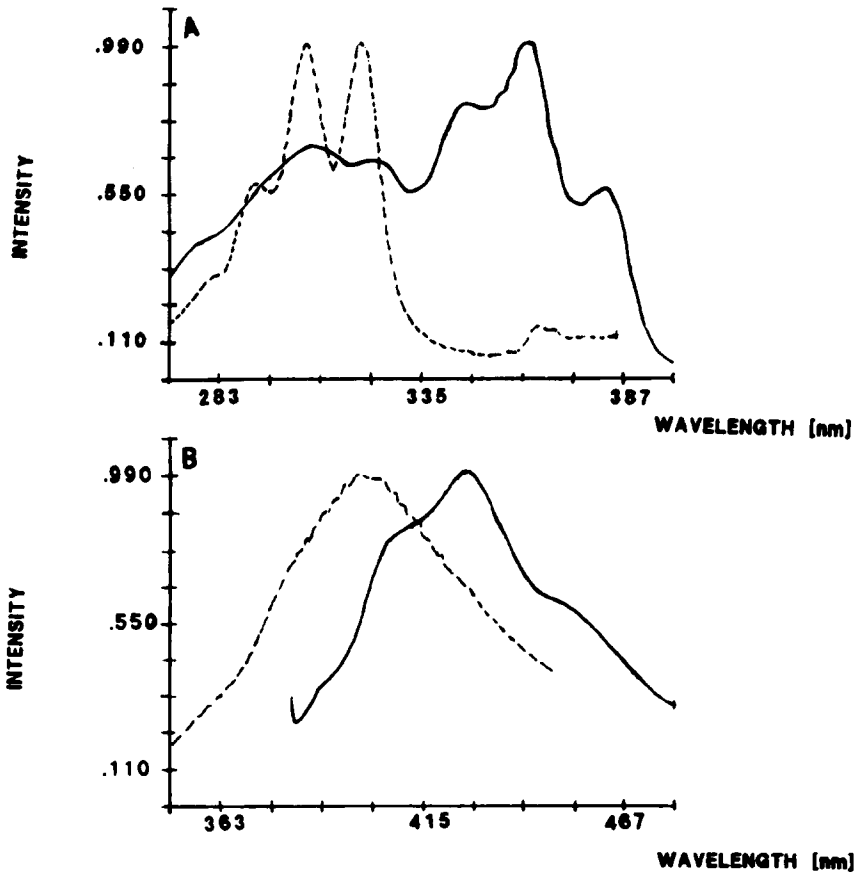

Fig. 1. Excitation (A) and emission (B) spectra of DPH-propionic (———) and parinaroyl (------) choline plasmalogens in vesicles of unlabeled choline plasmalogen. Spectra are normalized. Concentration of labeled and unlabeled phospholipids were 3 μM and 900 μM, respectively.

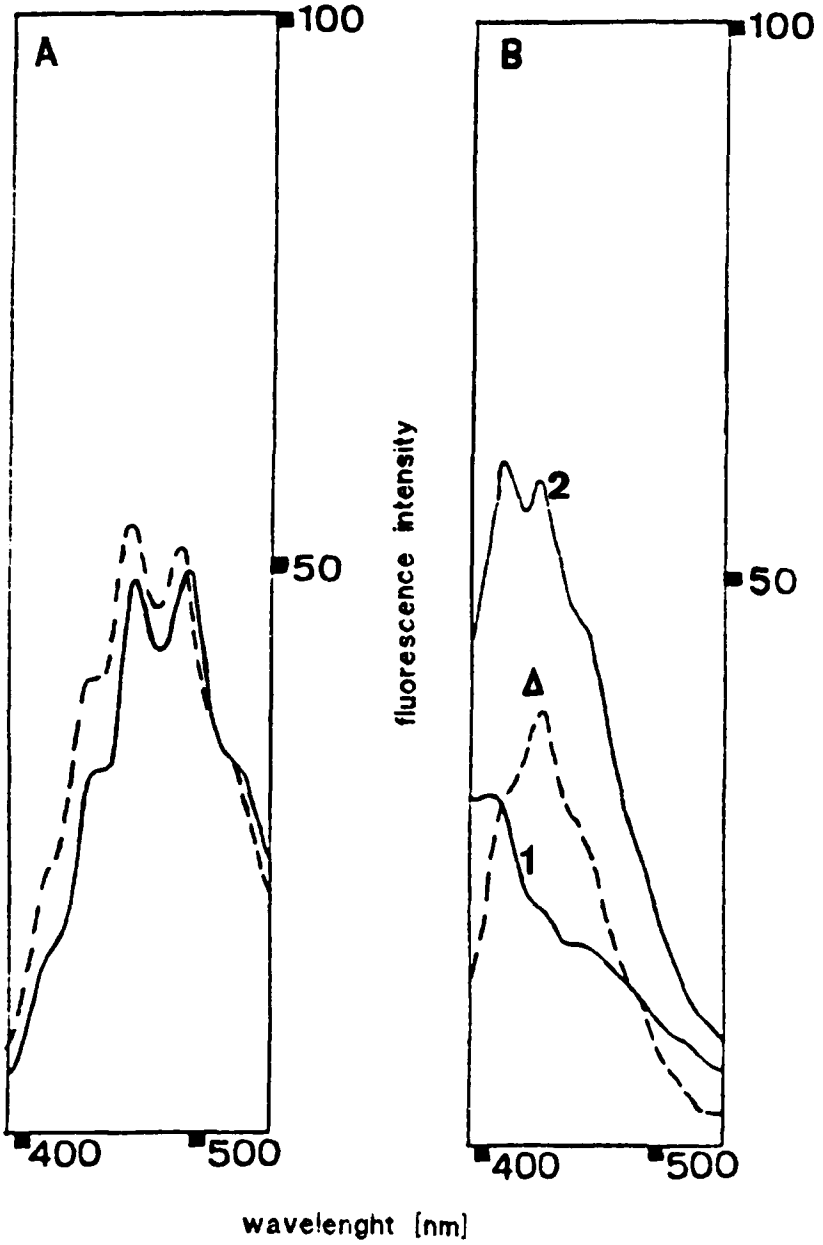

Fig. 2. (A) Emission spectra of vesicles containing DPH-labeled choline plasmalogen before (———) and after (------) incubation with cultured human CHRS skin fibroblasts. (B) Emission spectra of cultured human CHRS skin fibroblasts before (1) and after (2) incubation with DPH-labeled plasmalogen. Δ is the difference spectrum obtained from spectra 2 minus 1. Vesicles consisting of 5 nmol DPH-propionyl phospholipid in 3 ml Hanks' solution were made by sonication. Cell monolayers (4.4 cm^2, corresponding to 22 μg phospholipid) were grown to confluency.

respectively (Fig. 1). Vesicles consisting only of the fluorescent phospholipids exhibit very low fluorescence intensities due to self-quenching of the fluorophore. In this system two emission maxima of the DPH derivatives appear at 480 and 495 nm (Fig. 2A). Dilution of the fluorophore, e.g., from vesicles into a cell membrane after a transfer event, results in a blue shift of emission maxima to 430 nm with a concomitant several-fold increase in fluorescence intensity (Fig. 2B). An increase of fluorescence intensity at 410 nm is observed when parinaroyl phospholipids are taken up by cells. Recording of the time-course of fluorescence intensity reflects the time course of phospholipid transfer from vesicles to cells. As outlined in more detail in Materials and Methods, cells adhered to their substratum during the fluorescence measurement. Fluorescence intensities of fibroblasts after transfer of phospholipids from vesicles to the cells were determined by subtracting the background fluorescence of unlabeled cells (recorded before the uptake experiment) from total fluorescence determined after separating the cells from excess vesicles. Extraction of cellular lipids with chloroform/methanol

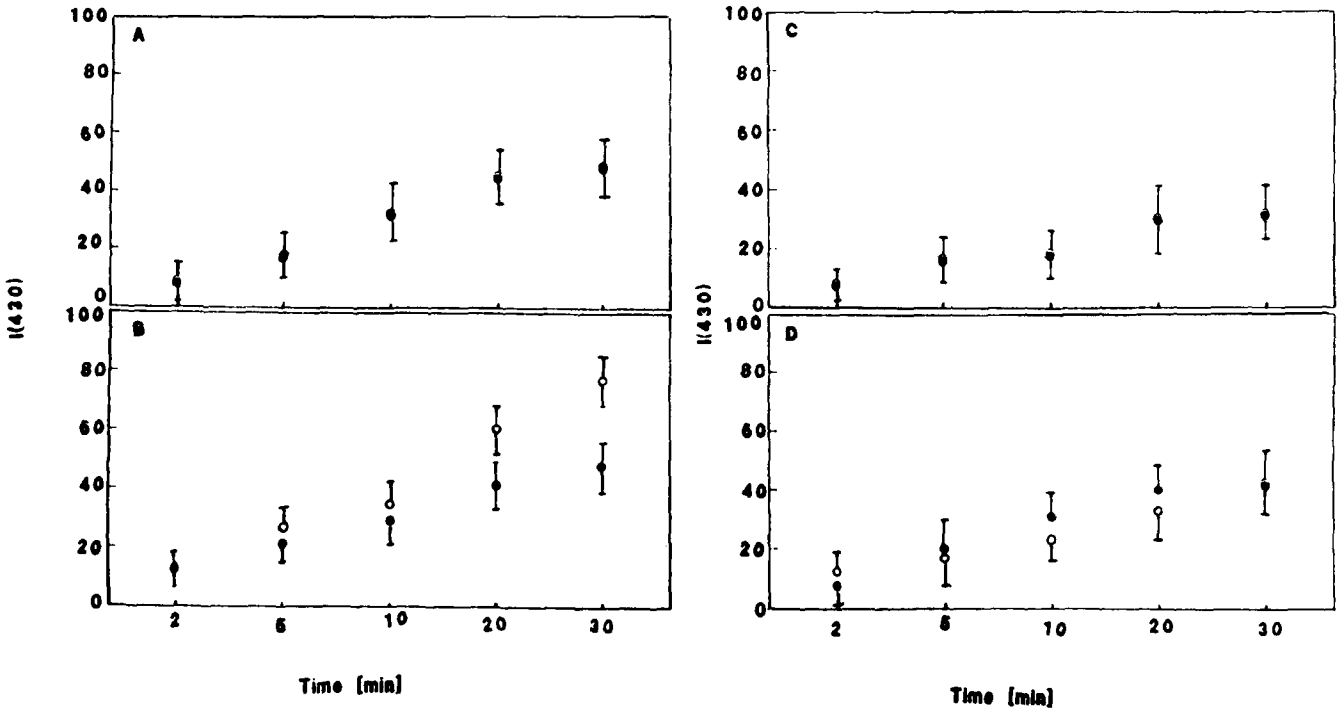

Fig. 3. Transfer of 1-palmitoyl-2-DPH-propionyl-GPC to CHRS (A) and control (C) skin fibroblasts and of 1-*O*-hexadecenyl-2-DPH-propionyl-GPC to CHRS (B) and control (D) skin fibroblasts grown in the presence (●) and absence (○) of 1-*O*-hexadecylglycerol. *I*(430) = relative increase of fluorescence intensity at 430 nm (see Materials and Methods). Vesicles consisting of 5 nmol DPH-propionyl phospholipid in 3 ml Hanks' solution were made by sonication. Cell monolayers (4.4 cm^2, corresponding to 22 μg phospholipid) were grown to confluency.

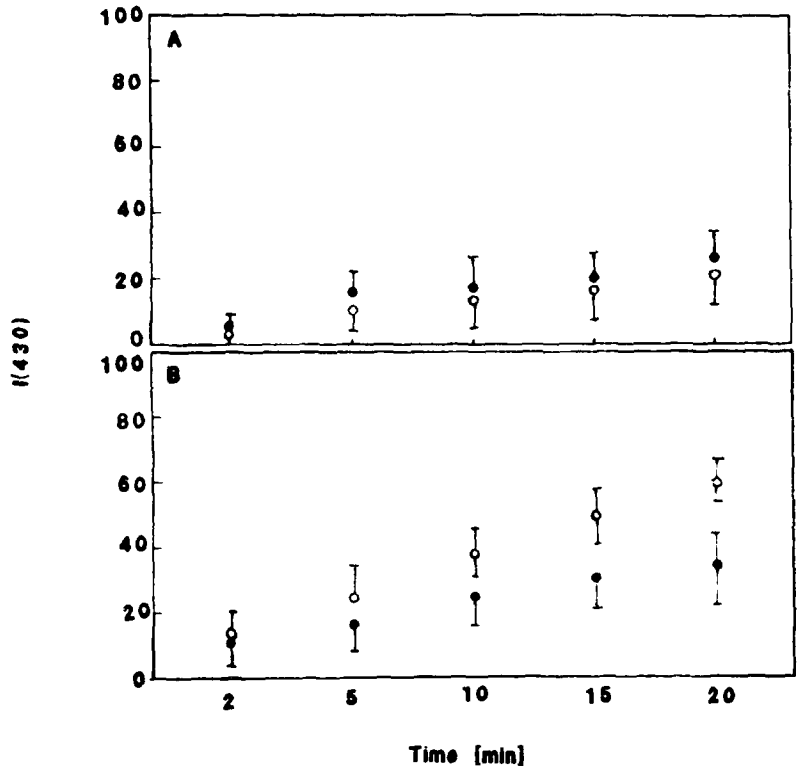

Fig. 4. Transfer of 1-palmitoyl-2-DPH-propionyl-GPE (●) and of 1-*O*-hexadecenyl-2-DPH-propionyl-GPE (○) to CHRS (B) and control (A) skin fibroblasts. *I*(430) = relative increase of fluorescence intensity at 430 nm (see Materials and Methods). Vesicles consisting of 5 nmol DPH-propionyl phospholipid in 3 ml Hanks' solution were made by sonication. Cell monolayers (4.4 cm^2, corresponding to 22 μg phospholipid) were grown to confluency.

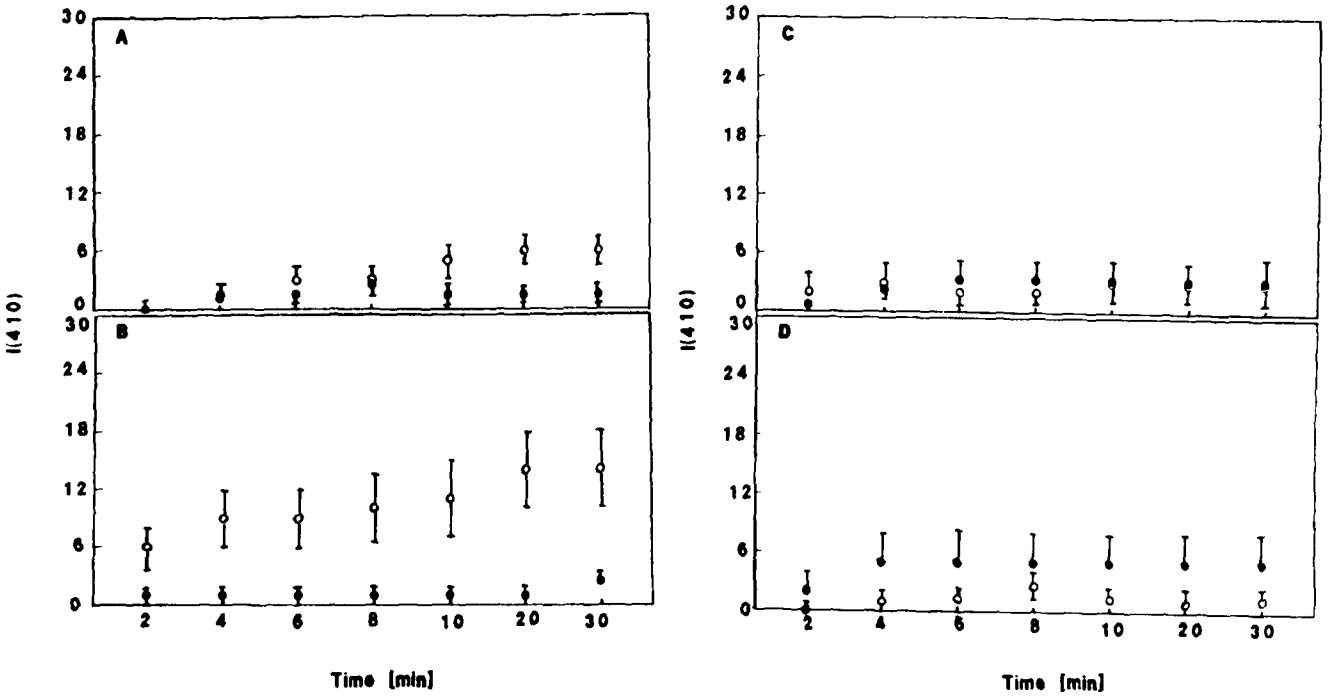

Fig. 5. Transfer of 1-palmitoyl-2-*trans*-parinaroyl-GPC to CHRS (A) and control (C) skin fibroblasts and of 1-*O*-hexadecenyl-2-*trans*-parinaroyl-GPC to CHRS (B) and control (D) skin fibroblasts in the presence (●) and absence (○) of 1-*O*-hexadecylglycerol. *I*(410) = relative increase of fluorescence intensity at 410 nm (see Materials and Methods). Vesicles consisting of 9 nmol parinaroyl phospholipid in 3 ml Hanks' solution were made by ethanol injection. Cell monolayers (4.4 cm^2, corresponding to 22 μg phospholipid) were grown to confluency.

followed by TLC analysis on silica gel (see Materials and Methods) showed that more than 90% of the fluorescence was in the choline phospholipid fraction. Phospholipid vesicles were prepared by ethanol injection [17] rather than by sonication in order to avoid oxidative degradation of the fluorophor, and to produce vesicles of a size large enough to circumvent problems that might arise from differences in vesicle curvature. It had been shown previously [24] that, upon sonication, choline plasmalogens form larger vesicles than do diacyl glycerophosphocholines.

Transfer of fluorescently labeled phospholipids from unilamellar vesicles to cells

Data in Figs. 3–5 show that rates of phospholipid transfer from vesicles to cells depend both on the type of donor phospholipid (plasmalogen or diacyl subclasses) and on the plasmalogen content of the acceptor cell membranes. Highest uptake rates were observed when plasmalogen-deficient CHRS cells were incubated with plasmalogen vesicles (Figs. 3–5, panels B). Uptake rates for plasmalogens were nearly identical with control cells from healthy donors (Figs. 3D and 5D)) with CHRS cells supplemented with hexadecylglycerol (Figs. 3B and 5B). Cellular uptake of fluorescently labeled diacyl analogs was always significantly lower than uptake of the corresponding plasmalogens. Hexadecylglycerol supplementation had no effect on the transfer of DPH propionyl-labeled diacylglycerophosphocholine to CHRS cells (Fig. 3A), but in the case of parinaroyl-labeled diacylglycerophosphocholine the rate of uptake was slightly reduced (Fig. 5A). In this context it should be noted that transfer rates were in any case higher for DPH-propionyl than for parinaroyl phospholipids. This difference is paralleled by a higher rate of spontaneous transfer of the DPH derivative between artificial phospholipid vesicles (data not shown). Considering the closer resemblence of parinaric acid to 'normal' fatty acids data obtained with parinaroyl-labeled phospholipids more closely reflect the behavior of natural phospholipids. Results obtained with 2-pyrenedecanoyl glycerophospholipids (data not shown) were similar to those reported here for the parinaroyl-derivatives.

Ethanolamine glycerophospholipids behaved very similarly to the choline derivatives: ethanolamine plasmalogens were transferred from vesicles to CHRS cells at considerably enhanced rates as compared to the diacyl analogs (Fig. 4).

The absolute amounts of parinaroyl phospholipid transferred from vesicles to cells was estimated from the increase of fluorescence intensity which was standardized using a series of phosphatidylcholine vesicles containing different low concentrations of parinaroyl phospholipid. The amount of fluorescent phospholipid incorporated into CHRS and control cells corresponds to approx. 10 and 2% of the plasma membrane phospho-

TABLE I

Absolute amounts of fluorescent choline plasmalogen transferred to cultured human skin fibroblasts

Incorporation of fluorescent plasmalogen by cultured cells was determined from the increase of fluorescence intensity at 410 nm (see Materials and Methods). In a typical experiment, 4.4 cm^2 of a cell monolayer containing 22 μg (approx. 29 nmol) phospholipid were incubated with 15 nmol parinaroyl plasmalogen (vesicles) in 3 ml Hanks' solution at 37 °C for 30 min. CHRS + HDG and control + HDG denote CHRS and control cells, respectively, supplemented with 1-*O*-hexadecylglycerol (see Materials and Methods).

Cell strains	Cellular plasmalogen content (mol% of total phospholipid)	Transferred phospholipid (pmol/cm^2 cell layer)
CHRS	3	100
CHRS + HDG	10	20
Control	14	14
Control + HDG	14	18

lipids (Table I). This estimation is based on the assumption that 10% of total cellular phospholipid is contained in the plasma membrane; the latter value has to be considered, however, as a lower limit. It was recently reported that approx. 50% of cellular phospholipid accounts for the surface membrane (Ref. 25 and references cited therein), which seems to be a rather surprisingly high value.

Separation of cells and vesicle-containing media after incubation and subsequent fluorescence measurements of cells and vesicles revealed that not only was cell-associated fluorescence intensity increased (Fig. 2B), but also vesicles showed enhanced fluorescence, although at higher wavelength (Fig. 2A). This latter observation can be explained by the assumption that interaction of vesicles and cells involves phospholipid exchange between the two membrane systems. Dilution of fluorescently labeled vesicle phospholipids with unlabeled phospholipids originating from the plasma membrane would alleviate self-quenching of vesicle fluorescence. The notion that cell–vesicle interaction involved bidirectional phospholipid transport was corroborated by more direct evidence obtained with radioactively labeled cells as described in the subsequent section.

When cultured human skin fibroblasts (CHRS cells) were incubated with phospholipid vesicles containing the maximum amount of cholesterol (50 mol%) a 25% increase in fluorescence anisotropy was observed using TMA-DPH as a probe which inserts primarily into the cell surface [7]. This effect is in agreement with the known effect of cholesterol as a membrane rigidifying component [26]. The observed increase in cell membrane rigidity did not affect the extent of plasmalogen transfer from vesicles to cells (data not shown).

Transfer of radioactively labeled phospholipids from fibroblasts to phospholipid vesicles

Cellular lipids of CHRS and control fibroblasts were radioactively labeled by incubating cells with [^{3}H]oleic acid for 15 h. Upon incubation of prelabeled cells with

TABLE II

Transfer of [^{3}H]oleoyl-labeled choline and/or ethanolamine glycerophospholipids from cells to vesicles

For experimental conditions see Materials and Methods. acc. = acceptor.

Cell strain	% of cellular phospholipid radioactivity in plasmalogen	% of transferred radioactivity in choline plasmalogen
Choline glycerophospholipids		
CHRS	4.6 ± 0.4	5.4 ± 0.4 (acc.: plasmalogen) 3.4 ± 0.2 (acc.: POPC)
Control	8.4 ± 0.6	8.9 ± 0.6 (acc.: plasmalogen) 3.5 ± 0.3 (acc.: POPC)
Ethanolamine glycerophospholipids		
CHRS	3.8 ± 1.0	3.0 ± 0.2 (acc.: plasmalogen) 2.5 ± 0.2 (acc.: POPC)
Control	13.7 ± 1.0	15.0 ± 0.6 (acc.: plasmalogen) 6.0 ± 0.4 (acc.: POPC)
	Ratio of choline to ethanolamine phospholipids in total cellular phospholipids	Ratio of choline to ethanolamine cellular phospholipids transferred to acceptor vesicles
Choline and ethanolamine glycerophospholipids		
CHRS	3.7 ± 0.3	1.4 ± 0.1 (acc.: plasmalogen) 1.7 ± 0.1 (acc.: POPC)
Control	4.3 ± 0.3	2.0 ± 0.1 (acc.: plasmalogen) 2.1 ± 0.1 (acc.: POPC)

phospholipid vesicles consisting of either choline plasmalogen or diacylglycerophosphocholine, vesicles incorporated radioactively labeled phospholipids originating from the cells (Table II). By thin-layer chromatographic analysis of vesicle lipids it was found that radioactivity was associated mainly with choline and ethanolamine phospholipid fractions. Only trace amounts of radioactivity were detected in other phospholipids or in neutral lipids. With control cells as donors, relatively more [^{3}H]plasmalogen was transferred to plasmalogen-containing acceptor vesicles than to diacylglycerophosphocholine vesicles. With CHRS cells as donors, the type of acceptor vesicle (plasmalogen or diacylglycerophosphocholine) had a marginal effect on the percentage of [^{3}H]plasmalogen transferred. [^{3}H] Phosphatidylethanolamine was preferentially transported from cells to acceptor vesicles, irrespective of the type of vesicle phospholipid.

Discussion

Vesicle–cell interaction can occur by at least four different mechanisms: adhesion of vesicles to cells, uptake of vesicles by endocytosis, fusion of vesicle and cell membranes, or exchange of phospholipids between the two membrane systems [27]. Adhesion of fluorescently labeled vesicles to cells would escape detection in the type of experiments described here, because fluorescence intensity would not change in the case of vesicle adsorption without membrane lipid mixing. Uptake of vesicles by endocytosis might eventually lead to membrane mixing within the cells; however, endocytosis can also be excluded because the fluorescence increase observed during the 30 min period of incubation was insensitive to the addition of cytochalasin B, an inhibitor of endocytosis. Membrane fusion would result in an increased fluorescence intensity. However, using the non-transferable fluorescence marker octadecyl Rhodamine B [28] it could be established that fusion between phospholipid vesicles and fibroblast membranes did not occur under the experimental conditions employed (Hermetter, A., unpublished results). The following experimental evidence supports the notion that phospholipid exchange between vesicles and cells is the prevailing mechanism. Upon incubation of fibroblasts in the presence of fluorescently labeled phospholipid vesicles the increase in fluorescence intensity occurs in both cellular and vesicle membranes. This means that fluorescent phospholipids are transferred from vesicles to cells, but at the same time unlabeled phospholipids originating from cells are incorporated into vesicles. More direct evidence for reciprocal translocation of phospholipids between the two membrane systems was obtained from experiments by which transfer of radioactively labeled phospholipids from cells to vesicles could be demonstrated (Table II, a–c). Exchange of choline and ethanolamine glycerophospholipids between cells and phospholipid vesicles had been demonstrated by Pagano et al. [29], who studied the uptake of fluorescent phospholipids by cultured Chinese hamster V79 fibroblasts [29]. Apart from possible effects of membrane composition and physical properties of the exchanging species on lipid uptake by cells, exchange of phospholipids in this system was supposed to be catalyzed by (a) membrane bound protein(s) [27]. Circumstantial evidence suggests a role for proteins also in the system described here. The buffer collected after incubating CHRS cells for 15–30 min with Hanks' solution (in the absence of Ca^{2+} and Mg^{2+}) contained a heat-labile factor capable of catalyzing intervesicular phospholipid transport (unpublished results). This factor did not originate from nonspecific leaking of intracellular contents because cells remained viable as judged from Trypan blue-staining and supernatants of cells were free of lactate dehydrogenase activity. The putative exchange protein, which we believe to be located primarily at the cell surface, catalyses plasmalogen exchange several-fold better than diacylglycerophosphocholine exchange when tested in an in vitro system consisting of small unilamellar vesicles as donor and acceptor membranes. Cytosolic proteins obtained after lysis of CHRS or control cells also promote phospholipid transfer between vesicles but do not show any preference for plasmalogen (Loidl, J., et al., unpublished observations). Transfer activity due to residual calf serum proteins (from the culture medium) which may bind to the cell surface even after extensive washing with Hanks' solution can be excluded, too. Such effects should be observed not only with CHRS cells but also with control cells.

Preferred intermembrane exchange of plasmalogens as compared to the diacyl analogs is not restricted to the examples described here. It was also observed with other biomembrane systems such as erythrocyte ghosts [32] or platelets (unpublished observations); however, the phenomenon we report in this paper is a highly selective and directed uptake of plasmalogen into the plasma membrane of the plasmalogen-deficient fibroblasts.

The same effect was observed with fibroblasts obtained from patients affected with the rhizomelic type of *Chondrodysplasia punctata*. In these cells plasmalogen deficiency is caused by a different genetic defect [30]. In contrast to CHRS cells, RCDP cells have structurally normal peroxisomes and do not accumulate very-long-chain fatty acids [31]. Thus it appears that, indeed, plasmalogen deficiency of cellular membranes rather than pleiotropic effects of peroxisomal dysfunction is responsible for increased ether lipid uptake by CHRS and RCDP cells.

It appears as if mechanisms existed that guarantee maintenance of cellular plasmalogen levels during inter-

action of cells with exogenous phospholipid pools. How can plasmalogen deficiency be sensed by the components of the exchange process? First of all one might consider effects due to the physical properties of the plasma membrane which is involved in lipid exchange. Plasmalogen deficiency of cultured human skin fibroblasts is associated with increased lipid fluidity [7]. The increase of cellular plasmalogen after treatment of the cells with HDG restores higher membrane rigidity [7]. However, lipid mobility per se is presumably not the only important parameter in this respect. When membrane rigidity of CHRS fibroblasts was increased by supplementation with cholesterol via incubation with phospholipid-cholesterol vesicles plasmalogen uptake by the cells remained unchanged. CHRS cells enriched with cholesterol still exhibited high preference for the incorporation of plasmalogens as compared with diacyl lipids. Therefore, influences of plasmalogens on optimum membrane organization must exist which are different from simple effects on lipid fluidity.

Finally, the preference of the putative surface-anchored exchange protein for plasmalogens is in line with similar properties of other phospholipid transfer proteins [32,33]. However, it is unclear why the respective transfer activity is much higher in plasmalogen-deficient cells. Further studies are under way to investigate the molecular characteristics and origin of this factor as well as the mechanism of its preferential transfer activity toward enol ether phospholipids.

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