# Transmembrane Movement of Diether Phospholipids in Human Erythrocytes and Human Fibroblasts†

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ABSTRACT: We have synthesized spin-labeled (SL) and fluorescently labeled diacyl, 1-alkyl-2-acyl-, and di-alkyl glycerophospholipids. The *sn*-2 chain was a short chain with either a nitroxide group or a 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD). After incorporation in the exoplasmic leaflet of human erythrocytes, we found that SL-phosphatidylcholine (PC) redistributed very slowly across the plasma membrane, less than 20% reaching the cytoplasmic leaflet in 3 h at 37 °C. In contrast, SL-phosphatidylserine (PS) accumulated on the cytoplasmic leaflet with the same plateau corresponding to 90% of the probes inside. The characteristic times for inward redistribution were different for the three PS analogues: at 37 °C, the $t_{1/2}$ for the diacyl, alkyl-acyl, and dialkyl compounds were 2.3, 3.5, and 41 min, respectively. ATP depletion or incubation with *N*-ethylmaleimide inhibited the rapid translocation of the PS derivatives. The diether PS bearing an NBD group translocated very slowly in human erythrocytes and no acceleration by ATP could be measured. On the other hand, in human fibroblasts, the diether NBD−PS and SL-PS were both transported from the exoplasmic to the cytoplasmic monolayer of the plasma membrane as it is the case for the transport of the respective diester PS analogues. These results prove that the ether bonds do not prevent completely PS binding and translocation by the aminophospholipid translocase despite a probable hindrance due to the ether linkage on the *sn*-2 chain. Because of the high stability of the ether linkage, SL and NBD diether analogues should be useful to investigate lipid traffic in cultured cells.

Apart from ester bonds, ether linkages of fatty acid residues are present in naturally occurring phospholipids. They are generally found in bacteria living in extreme conditions, particularly at high temperature, high salt, and unusual pH. Lipids of Archaebacteria are based on the ether linkage of glycerol with two alcanols chains or complex polyisoprenoid alcohols (*1*). Ether bonds are also found in phospholipids of eukaryotic cells (*2*). A significant fraction of ethanolamine derivatives in the plasma membrane of human red cell are plasmalogens which are alkenylacyl species (*2*). In addition to their occurrence as structural components of biomembranes, ether lipids are molecules with important biological functions. Platelet-activating factor (PAF) has many physiological effects which are elicited by very low concentrations of this molecule for example during inflammation, platelet aggregation, allergic response, etc.

Ether phospholipids may provide valuable tools to study phospholipid dynamics in cells due to their resistance toward hydrolytic cleavage of fatty acid residues. For investigation of lipid dynamics in biological membranes, several spin-labeled (SL) or NBD-labeled analogues of naturally occurring alkyl derivatives have been synthesized already. They generally include an ether bond in the *sn*-1 position and an ester bond in the *sn*-2 position. For example, a SL analogue of PAF-acether was synthesized with 4-doxylpentanoic acid replacing the acetyl normally present (*3*). 1-(NBD-alkyl)-2-acylglycero-3-phosphocholines were synthesized to assay for the specificity of phospholipase $A_2$ enzymes as well as to produce an analogue of PAF-acether (*4*). Analogues of ether lipids may be employed to study lipid-transporting membrane proteins as the aminophospholipid translocase in the plasma membrane of mammalian cells. This translocase has been shown to transport rapidly endogenous as well as SL and fluorescent diacyl aminophospholipids from the exo- to the cytoplasmic leaflet (*5*−*7*). Using ether analogues for studying the activity of those proteins, a crucial point is whether and how the ether bond affects the affinity of those analogues to the proteins.

To test the influence of an ether bond in the *sn*-1 position on the activity of the aminophospholipid translocase, we have

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[1] Abbreviations: BSA, bovine serum albumin; cmc, critical micelle concentration; DFP, diisopropylfluorophosphate; ESR, electron spin resonance; NBD, 7-nitrobenz-2-oxa-1,3 diazol-4-yl; NEM, *N*-ethyl maleimide; PAF, platelet-activating factor, (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PC, phosphatidylcholine; PS, phosphatidylserine; SL, spin-labeled at C2, 4-doxylpentanoyl; diester (diacyl), 1,2-diacyl-*sn*-glycero-3-; ether-ester, 1-alkyl-2-acyl-*sn* -glycero-3-; diether (di-alkyl), 1,2-dialkyl-*sn*-glycero-3- .

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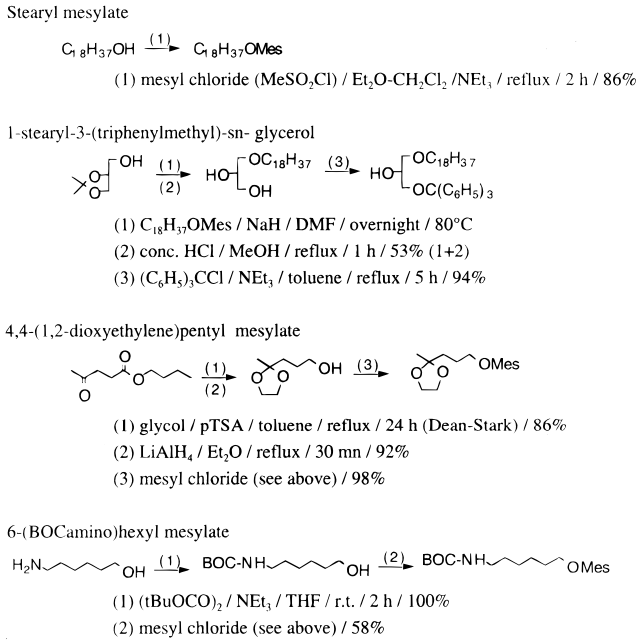
Scheme 1: Precursors

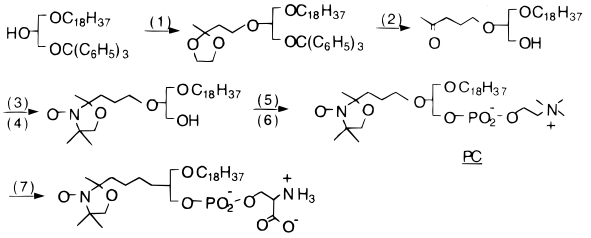



synthesized previously SL ethanolamine and choline plasmalogens and measured their transbilayer movements in human erythrocytes (*8*). We found that the SL plasmenylethanolamine analogue moves rapidly from the exoplasmic to the cytoplasmic leaflet with a half time at 37 °C of 30 min comparable to that of the corresponding SL diacylphosphatidylethanolamine (diester PE) in an ATP-requiring and NEM sensitive manner. By contrast, after 4 h of incubation, less than 20% of the SL plasmenylcholine reached the cytoplasmic leaflet. Thus, the plasmalogens behaved as the corresponding diacyl lipids. In the present work, we have synthesized SL and NBD-labeled analogues of diether phospholipids with either a choline or a serine headgroup and measured the outside-inside diffusion in the plasma membrane of human erythrocytes and human fibroblasts. We show that these lipid analogues which are not a substrate of endogenous phospholipase $A_2$ enzymes can be useful tools to investigate phospholipid trafficking in eukaryotic cells.

## MATERIALS AND METHODS

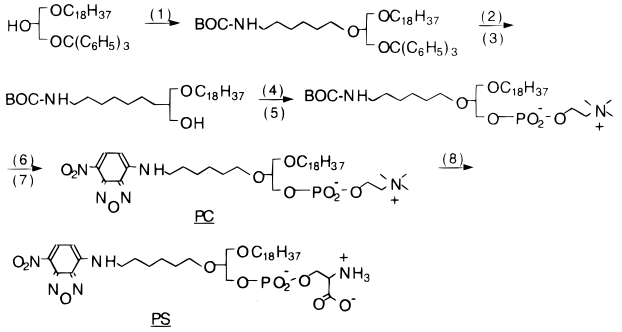
*Synthesis of Fluorescent and Paramagnetic 1, 2-Diether-sn-glycerophospholipids. (see Schemes 1, 2, and 3).* First steps (Scheme 1) led to a common intermediate, the 1-stearyl-3-(triphenyl methyl)-*sn*-glycerol: the hydroxyl group of 2,3-*O*-isopropylidene-*sn*-glycerol (from Aldrich Chemical) was alkylated with stearyl mesylate, and after deprotection with hydrochloric acid, the primary alcohol was tritylated (*9*, *10*) (Scheme 1). The second alkyl chain was introduced in a similar manner leading, after deprotection, to 1,2-dialkyl-*sn*-glycerol. In the case of paramagnetic lipids (Scheme 2), we used 4,4-(1,2-dioxyethylene)pentyl mesylate; trifluoroacetic acid removed both protective groups; the 4-doxyl derivative was then obtained by reaction of the carbonyl group with 2-amino-2-methyl propanol, followed by oxidation with *m*-chloro perbenzoic acid (*8*, *11*). For fluorescent lipids (Scheme 3), we used 6-(BOCamino)hexyl mesylate; during the second step, some deprotection of the amine took place, which needed a subsequent treatment with ditertiobutyl

Scheme 2: Paramagnetic Diether Phospholipids




(1) 4,4-(1,2-dioxyethylene)pentyl mesylate / NaH / DMF / reflux / 2 h / 62%
(2) $CF_3CO_2H$ / nBuOH / r.t. / 100%
(3) 2-amino-2-methylpropanol / pTSA / toluene / reflux / 24 h
(4) m-chloroperbenzoic acid / $Et_2O$ / r.t. / 38% (3+4)
(5) (2-bromoethyl) dichlorophosphate / $NEt_3$ / $CHCl_3$ / r.t. / 3 h / 42%
(6) (a) $NMe_3$ / $CHCl_3$- iPrOH-DMF / 55°C / 2 h - (b) $Ag_2CO_3$ / 1 h / 69% (a+b)
(7) L-serine / 0.2M acetic buffer, pH 5.6, 40mM $Ca^{2+}$ / phospholipase D (Streptomyces species) / AcOEt / 30°C / 24 h / 54%

Scheme 3: Fluorescent Diether Phospholipids




(1) 6-(BOCamino)hexyl mesylate / NaH / DMF / reflux / 2 h
(2) $BF_3$-MeOH / $CH_2Cl_2$ / r.t. / 4 h / 40% (1+2)
(3) reprotection of $NH_2$ / 80%
(4) (2-bromoethyl) dichlorophosphate / $NEt_3$ / $CHCl_3$ / r.t. / 3 h
(5) (a) $NMe_3$ / $CHCl_3$- iPrOH-DMF / 55°C / 2 h - (b) $Ag_2CO_3$ / 1 h / 43%
(6) $CF_3CO_2H$ / $CH_2Cl_2$ / 0°C / 30 mn
(7) NBD chloride / $NEt_3$ / EtOH / r.t. / 3 h / 23% (6+7)
(8) L-serine / 0.2M acetic buffer, pH 5.6, 40mM $Ca^{2+}$ / phospholipase D (Streptomyces species) / AcOEt / 30°C / 24 h / 36%

dicarbonate. After conversion to glycerophosphocholine, the BOC group was removed with trifluoroacetic acid and the amine alkylated with NBD chloride (*12*). Each 1,2-dialkyl-*sn*-glycerol was esterified with (2-bromoethyl) dichlorophosphate, and after treatment with trimethylamine and silver carbonate, the corresponding glycerophosphocholine was obtained (*9*, *13*). Transesterification with L-serine, mediated by phospholipase D (streptomyces species from Sigma, Paris) gave the PS (*14*). This reaction was at least 10 times slower for diether phospholipids than for the diester analogues, but gave comparable yields. The molecular weight of the 1-stearyl-2-(4-doxyl pentyl)-*sn*-glycerophosphocholine was assessed by FAB mass spectrometry using MNBA-glycerol-trichloracetic acid matrix. The paramagnetic diether PC gave a formula peak at *m*/*z* 681.6. $^1$H NMR spectra confirmed the introduction of the chains leading to the diether glycerol. The purity of the labeled phospholipids was checked by thin-layer chromatography on silica gel 60 which gave one spot with different eluting mixtures. $R_f$ values were 0.35 and 0.23 for fluorescent PC and PS, respectively, on plates developed in chloroform/methanol/water (65:25:4). Further characterization was performed by phosphate determination (*15*),

spectrophotometric assay for the fluorescent lipids ($\lambda_{max}$ = 471 nm, $\epsilon_{max}$ = 14 800 in $CHCl_3$) and ESR measurement for the spin-labeled ones (see below).

*Synthesis of Fluorescent and Paramagnetic 1,2-Diacyl and 1-alkyl-2-acyl Phospholipids.* 1,2-Diacyl and 1-alkyl-2-acyl phospholipid analogues were prepared as described previously for SL analogues (*8*) and fluorescent analogues (*16*). The *sn*-2 chain was a 4-doxylpentanoic acid in case of paramagnetic analogues, a (6-NBD)-hexanoic acid in case of fluorescent analogues. PC analogue was synthesized either from an ester or ether lyso-PC molecule which was reacylated by a probe-bearing fatty acid. PS analogues were obtained by an exchange reaction between choline and serine headgroups, catalyzed by phospholipase D.

*Preparation of Human Erythrocytes*. Freshly drawn adult human blood was obtained from a blood bank (Centre National de la Transfusion Sanguine). Blood was centrifuged at 3000 rpm for 5 min at 5 °C in buffer A (120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM EGTA, 10 mM glucose, 10 mM inosine, 10 mM adenosine, 10 mM sodium pyruvate, and 10 mM Hepes, pH 7.4). The buffy coat and supernatant were carefully removed by aspiration. The cells were washed four times with 20 vol of buffer A and used at a final hematocrit of 33%. Before adding the probes, unless otherwise mentioned, diisopropyl fluorophosphate (DFP) was added at a final concentration of 5 mM. In some instances, erythrocytes were treated with NEM to inhibit the aminophospholipid translocase. For that purpose, to 4 mL of red cell pellet 100 $\mu$L of NEM was added (1 mM in buffer A) and incubated 20 min at 37 °C before use. For ATP depletion, erythrocytes were incubated for 4 h at 37 °C in the following buffer: 70 mM NaCl, 70 mM KCl, 20 mM deoxyglucose, 10 mM Hepes, pH 7.4.

*Preparation of Resealed Ghosts*. Resealed ghosts were prepared by hypotonic lysis as described by Schwoch and Passow (*17*). Briefly, lysis was performed at 4 °C by dilution of 4 mL of ice-cold suspension of erythrocytes (50% packed) with 60 mL of ice-cold lysis buffer containing 1.2 mM acetic acid, 4 mM $MgSO_4$, and 0.5 mM EGTA (pH 3.2). After 5 min, 1 mM ATP and an ATP-regenerating system consisting of 10 mM creatine phosphate and 100 IU creatine kinase/mL were added (all from Calbiochem, Bad Soden, Germany). Isotonicity (150 mM NaCl and 5.8 mM $Na_2HPO_4/NaH_2PO_4$) was established by addition of 10-fold concentrated salt solution. The pH was adjusted to 7.4 by addition of NaOH. After 5 min, cells were resealed by incubation at 37 °C for 45 min. Resealed ghosts were collected by centrifugation at 25000*g* for 10 min and washed twice with ice-cold PBS. The pellet was stored at 4 °C.

*Cell Culture Fibroblasts.* Gingival human fibroblasts, originally from B. Zimmermann (Freie Universität Berlin, Germany), were maintained at 37 °C in DMEM/F 12 medium supplemented with 2 mM glutamine, 50 mg/L ascorbic acid, 105 units/L of penicillin, 100 mg/L of streptomycin, 2.5 mg/L amphotericin B, and 10% (vol/vol) fetal calf serum (all from Biochrom KG, Berlin, Germany). Cells were grown on TC Chamber Slides with two chambers (Nunc, Biochrom KG, Berlin, Germany) for microscopy and in 35-mm-diameter tissue culture dishes for other analyses. For ESR experiments, cells were detached using trypsin and resuspended in Hank's balanced salt solution without bicarbonate, supplemented with 10 mM Hepes (pH 7.4), 20 mM glucose, and 1 mM sodium pyruvate to prevent a decline in cellular ATP (*18*).

*ESR Experiments*. Labeling of the erythrocytes and fibroblasts with SL phospholipids and determination of analogue redistribution have been described in detail (*8*, *18*, *19*). The back-exchange procedure was modified because of the higher lipophilic character of the diether SL molecules: 6% (w/v) fatty acid free bovine serum albumin (BSA, final concentration) was used and each aliquot was incubated at 0 °C for 4 min to allow an efficient extraction of the spin labels present in the exoplasmic leaflet. After centrifugation of the suspension, supernatant was removed and generally frozen before ESR analysis. The amount of spin label in the supernatant which corresponds to lipid analogues originally present in the exoplasmic leaflet was determined by ESR spectroscopy after thawing the sample and addition of ferricyanide (10 mM final concentration) using a Bruker ESR 9 GHz spectrometer (200D SRC or ECS 106). The amplitude of the central line was used to determine the percentage of probes on the cytoplasmic leaflet. In the case of partial hydrolysis of the *sn*-2 chain, a correction has to be made (*8*). However, this correction was barely necessary for the diacyl or alkyl-acyl probes if DFP has been used. In some cases, to obtain a better sensitivity, an equal amount of SDS (final concentration 5%) was added to the supernatant. The latter procedure which is applicable only in the absence of hydrolysis or in the case of negligeable hydrolysis is well adapted to diether SL lipids.

*Fluorescence Spectroscopy and Microscopy*. Fluorescent analogues were inserted into the plasma membrane of ghosts and fibroblasts grown as a monolayer as described in Pomorski et al. (*18*, *20*), respectively. After labeling, the cells were washed and subsequently incubated for different periods of time as described in the figure legends. The determination of analogue transbilayer distribution in ghost membranes using the selective destruction of NBD fluorescence by dithionite has been described in detail (*20*). For the determination of analogue redistribution in fibroblasts, cell monolayers were incubated with Hank's balanced salt solution without bicarbonate, 25 mM Hepes (pH 7.4) on ice, and 25 mM dithionite (final concentration) was added for 5 min. After extensive washing, the lipids were extracted according to Bligh and Dyer (*21*). All aqueous solutions were acidified to 20 mM acetic acid and extracted twice with chloroform. Control cells not treated with dithionite were subjected to lipid extraction together with their incubation medium allowing thereby analysis of fluorescent lipid metabolism. After two-dimensional separation (I, chloroform/methanol/ammonium hydroxide/water (90:54:5.5:5.5, vol/vol); II, chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, vol/vol)] on Silica Gel 60 thin-layer plates (Merck, Darmstadt, Germany), the fluorescent lipid spots were visualized under a ultraviolet lamp, scraped, and quantitated as described before (*18*). The relative amount of NBD−lipid present in the dithionite-treated sample (% protected) was calculated as a percentage of the total amount present in a control sample not treated with dithionite. When internalization of analogues was followed by microscopy, analogues oriented to the exoplasmic leaflet were removed by incubation of cells for 10 min on ice with 6% BSA in Hank's solution. This procedure was repeated once. In addition, to destroy fluorescence of residual analogues still confined to the exoplasmic leaflet, cells were incubated for

5 min with dithionite (25 mM, final concentration) (*22*). Fluorescence microscopy was performed with an inverse standard Zeiss microscope (Oberkochen, Germany) equipped with fluorescence facility, Planapo 100/1.3 numerical aperture, and the following barrier filter set: BP 450 to 490 excitation filter, FT 510 dichroic mirror, and LP 515 emission filter (Carl Zeiss, Oberkochen, Germany). Photographs were taken using Kodak Ektachrome Panther P1600 films which were push-processed to 3200 ASA.

*Analysis of the Data*. Data of redistribution kinetics were recalculated and plotted according to

$$y(t) = \frac{h_0 - h(t)}{h_0}$$

where $h_0$ is the amplitude of the ESR signal or the fluorescence intensity at time zero and $h$ the amplitude at time $t$. The experimental curves were fitted to the following equation:

$$y(t) = y_x[1 - \exp(-kt)]$$

This implies two opposite first-order reactions with $k = k_i + k_o$ ($k_i$ and $k_o$ are the rate constants of the inward and outward movement, respectively) and

$$\frac{k_0}{k_i} = \frac{100 - y_x}{y_x}$$

with $y_x$ being the percentage of analogue on the cytoplasmic side at equilibrium.

Thus, $k_i = y_x k/100$ and $t_{1/2} = (\ln 2)/k$, while $t_{50\%} = 1/k \ln y_x/y_x - 50$.

*Miscellaneous Procedures*. Small unilamellar egg phosphatidylcholine vesicles (Sigma, Deisenhofen, Germany) containing 1 mol % NBD−lipid were prepared in PBS by sonication for 30 min at 2 °C with a Branson sonifier 250 (Danbury; duty cycle 70%, output control 2) equipped with a microtip. The accessibility of dithionite to the NBD−lipids located in both leaflets of the vesicle membrane was determined as described for ghosts by Pomorski et al. (*20*).

## RESULTS

*Spin-Labeled Analogues in Erythrocytes.* As reported previously SL diester PS and PC molecules possessing a short *sn*-2 chain with a nitroxide and a long *sn*-1 chain form micelles with a cmc in the range $10^{-7}$−$10^{-6}$ M (*19*, *23*). This property enables one to label cell membranes with those probes without requiring a phospholipid exchange protein or BSA. In particular, they spontaneously incorporate into the exoplasmic leaflet of human erythrocytes. This can be demonstrated by ESR spectroscopy. In water, these spin labels give rise to a single broad line due to the spin−spin interactions within the micelles. Three very narrow lines corresponding to the monomers in the aqueous solution are superimposed over the broad line (see Figure 1a). In the case of the diether spin labels, the water solubility is diminished. As a result, the concentration of monomers is too weak to see the spectral component due to monomers (Figure 1b). This indicates that the diether probes with a stearyl chain in the *sn*-1 position have a cmc probably below $10^{-7}$ M. Nevertheless, these probes, and the corresponding NBD derivatives, can be used to label the exoplasmic leaflet of biological membranes without requiring the help of a transfer protein. Figure 1c shows the line shape change in the case of the diether SL-PC molecule incorporated into human red blood cells. The spectrum is very close to that obtained with the diester analogue (not shown). If BSA is added to the labeled erythrocytes, the probes can be extracted, and the line shape of the probe attached to BSA is rather similar to that of the probe in the membrane as is the case with the diacyl PC and PS (*24*). On the other hand, the more hydrophobic character of the diether analogues makes it necessary to use higher concentration of BSA to efficiently extract the probes from the erythrocyte exoplasmic leaflet. We found that for a membrane suspension corresponding to approximately 1 mM of lipids a 4 min incubation on ice in the presence of 6% (w/v) BSA was necessary. By comparison, 1% BSA is sufficient to extract the diester analogues completely from the exoplasmic leaflet of RBC within 1 min on ice.

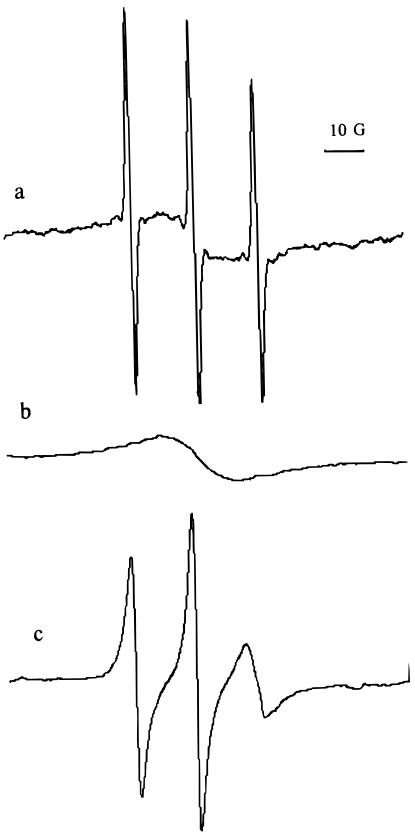



FIGURE 1: ESR spectra of SL diester PC (a) and diether PC (b) at a concentration of 50 $\mu$M in buffer. The narrow peaks in spectrum a are due to probes tumbling freely in water, while the broad component underneath corresponds to micelles. In the case of diether spin-labels (spectrum b) the monomers are at a concentration too low to be visible by ESR which is indicative of a cmc much below 1 $\mu$M. Spectrum c was obtained after incorporation of diether PC in human red blood cells. The spectrum of the diester PC in red blood cells (not shown) is very close to that of the diether PC.

Figure 2A shows the kinetics of outside−inside movement at 37 °C of the three analogues of PS, diacyl, alkyl−acyl, and dialkyl, after incorporation in human erythrocytes. The three molecules reach the same plateau corresponding to approximately 90% of analogues on the cytoplasmic leaflet. However, while the diacyl and the alkyl−acyl flip practically with the same rate [$t_{1/2}$ around 3 min (see Table 1)], the

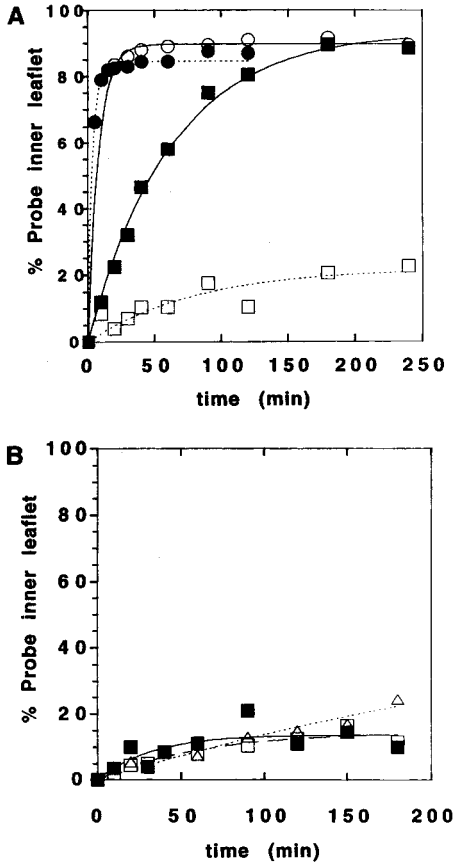



FIGURE 2: Transmembrane redistribution of SL analogues in human red blood cells at 37 °C. (A), SL-PS analogues: diester PS (filled circles); ether-ester PS (hollow circles); diether PS (filled squares); diether PS in ATP-depleted cells (hollow squares). Redistribution was measured also in cells pretreated with NEM (1 mM), and gave the same curve than in the absence of ATP. (B) SL-PC analogues: diether PC (filled squares); cells preincubated with 1 mM NEM (hollow triangles); ATP depleted cells (hollow squares). Data are plotted as percentage of analogues on the cytoplasmic leaflet. The curves are fitted to an exponential function by least-squares nonlinear regression. The results shown are for one representative series of experiments.

Table 1: Parameters of Spin-Labeled Phospholipids Translocation in Human Red Blood Cells at 37 °C

| | $n^a$ | % cytoplasmic leaflet, at equilibrium | $k$ ($min^{-1}$)$^b$ | $t_{1/2}$ (min) | $t_{50\%}$ (min) |
|---|---|---|---|---|---|
| diacyl PS | 3 | 85 ± 4 | 0.30 ± 0.03 | 2.3 | 3.0 |
| alkyl-acyl PS | 2 | 90 ± 3 | 0.20 ± 0.03 | 3.5 | 4.1 |
| dialkyl PS | 4 | 90 ± 2 | 0.017 ± 0.001 | 41 | 48 |
| dialkyl PC$^c$ | 2 | 17 ± 2 | | | |
| dialkyl PS +NEM$^c$ | 1 | 18 | | | |
| dialkyl PS − ATP$^c$ | 1 | 22 | | | |

$^a$ *n*, number of experiments. $^b$Simulations assuming first-order kinetics. Data are given as average ± sd for *n* experiments. $^c$Results obtained after 4 h incubation.

dialkyl PS has a much slower transbilayer movement ($t_{1/2}$ around 40 min). The incorporation of the diether PS is nevertheless ATP dependent, and its translocation is inhibited by NEM, indicating that the aminophospholipid translocase is very likely to be involved. The flip of diether PS after ATP depletion (Figure 2A) or NEM treatment (not shown) is characterized by the same slow speed as that of the diether PC analogue (Figure 2B). Incubating the cells with NEM or depleting them from ATP has no significant effect on the flip of diether PC (only shown for ATP depletion in Figure 2B). In the experiments involving diacyl or alkyl-acyl spin-labeled lipids presented in Figure 2, cells were incubated with DFP, an inhibitor of the endogenous phospholipase $A_2$ which cleaves off the short SL fatty acid in the *sn*-2 position (*19*). As a result, only a very small fraction of the probes were hydrolyzed: less than 3% of the spin-labeled PS after 1 h.

*Fluorescent Analogues in Liposomes and Erythrocyte Ghosts.* The redistribution of fluorescent phospholipid analogues initially incorporated into the exoplasmic leaflet of resealed ghosts was determined by the dithionite assay. In this assay, the NBD moiety of those NBD−phospholipid analogues present in the outer membrane leaflet is reduced and its fluorescence destroyed. As the analogues flip to the cytoplasmic monolayer, an increasing fraction of analogue becomes protected to dithionite. The applicability of the dithionite assay was confirmed in experiments on symmetrically labeled lipid vesicles containing either diacyl or diether NBD−lipids (data not shown). No difference in the kinetics of fluorescence reduction between both analogues was observed. This suggests a similar orientation of the NBD group in the membrane. Since reduction of the NBD moiety by dithionite is rather slow at 3 °C, all further experiments using dithionite were done at 10 °C.

ATP-containing ghosts were labeled with NBD−phospholipid analogues and incubated at 37 °C. Analogue redistribution was assessed by the dithionite assay. Again, kinetics of NBD reduction by dithionite was similar for diester and diether analogues exposed on the outer layer (Figure 3) providing additional indication for an identical orientation of the NBD group in membranes. A slow redistribution of the various NBD−PC analogues to the cytoplasmic leaflet was found and is shown only for diether NBD−PC in Figure 4. We observed no significant difference of the transbilayer movement between the diether and the diester NBD−PC (not shown). However, in agreement with previous results, a fast inward translocation of diester NBD−PS was found with a steady-state distribution in the cytoplasmic monolayer of about 70% for diester NBD−PS (Figure 4). As we have previously shown, in ghosts resealed without ATP, the fast inward translocation of diester NBD−PS disappeared and its redistribution was similar to that of diester NBD−PC (*20*). Remarkably, we did not observe such a fast translocation of the diether NBD−PS in ATP-containing ghosts; its redistribution was only slightly faster than that of the diether NBD−PC (Figure 4) or diester NBD−PC (not shown).

Since the diether NBD−PS is relatively poorly transported by the aminophospholipid translocase, one may wonder if it is a potent inhibitor for spin-labeled diester or diether PS transport in erythrocytes. If such were the case, it would be a usful tool to characterize this protein in various native and reconstituted membranes. Therefore, we have carried out experiments to look at the influence of diether-NBD−PS on the translocation of SL-PS in RBC. Experiments carried out at 25 °C, a temperature which was selected in order to slow the inward directed transport of SL-PS, we have not been able to detect any difference in the kinetics of SL-PS internalization in the presence of 3 or 6% diether-NBD−PS or in the absence of diether NBD−PS (data not shown). These results suggest that diether NBD−PS binds poorly to

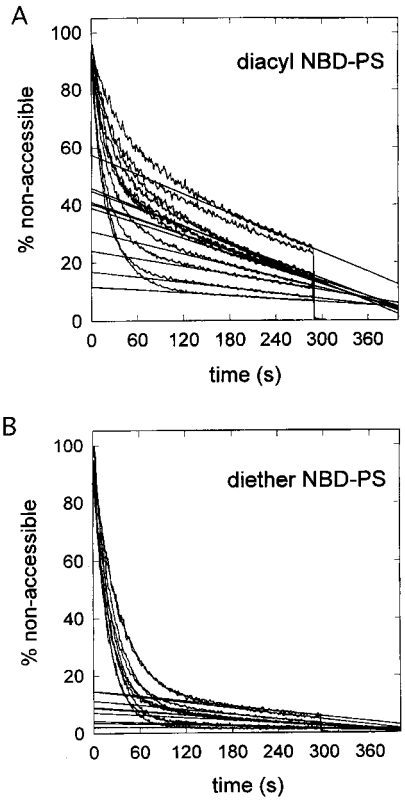



FIGURE 3: Reduction of the NBD moiety of the diacyl (A) and diether (B) NBD−PS analogues in human erythrocyte ghosts after various times of labeling. Resealed ghosts with 1 mM ATP were labeled with fluorescent analogues and incubated at 37 °C. After various time aliquots were taken and incubated at 4 °C in the presence of 50 mM dithionite. To determine the amount of analogue residing on the cytoplasmic leaflet the slow decline of the fluorescence was extrapolated to time $t = 0$ graphically. Those data can be replotted as a function of time (see Figure 4 in the case of erythrocyte ghosts). For details, see Materials and Methods.

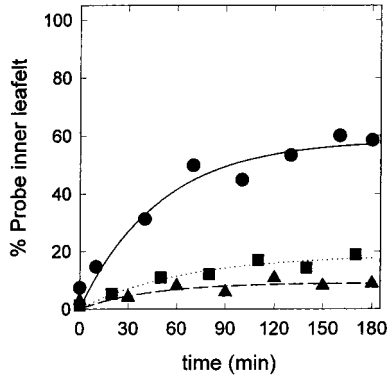



FIGURE 4: Transmembrane redistribution of diester NBD−PS (circles), diether NBD−PS (squares), and diether NBD−PC (triangles) in resealed ghosts containing ATP and an ATP regenerating system at 37 °C (see Materials and Methods). Before labelling, DFP was added at a final concentration of 5 mM. Redistribution was measured by selective destruction of NBD fluorescence on the exoplasmic leaflet using dithionite. The curves are fitted to an exponential function by least-squares nonlinear regression. Note that at time-zero a small fraction of fluorescent analogues is already located on the cytoplasmic leaflet due to labeling and washing procedure before starting the incubation at 37 °C. The results shown are for a representative experiment of two independent experiments.

the aminophospholipid translocase. In conclusion, an ether bond can affect recognition and/or translocation of the respective PS analogue by the aminophospholipid translocase

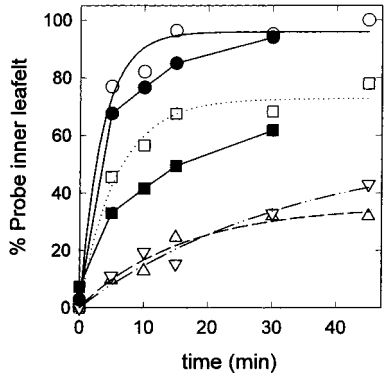



FIGURE 5: Internalization of SL lipid analogues into human fibroblasts. Cells in suspension were labeled with diester SL-PS (circles), diether SL-PS (squares), diester SL-PC (triangles down) and diether SL-PC (triangles up) in human fibroblasts at 37 °C (hollow symbols). For the two PS analogues redistribution was also measured at 20 °C (filled symbols). Experiments were done in the absence of DFP (for hydrolysis, see Results). Analogues on the exoplasmic leaflet were extracted by BSA and their amount measured by ESR (see Materials and Methods). Data are plotted as percentage of analogues on the cytoplasmic leaflet. Data corresponding to 37 °C are fitted to an exponential function by least-squares nonlinear regression. The results shown are for a representative experiment of two independent experiments.

in erythrocytes. From the ESR data with the ether−ester SL analogue, we know that the *sn*-2 position is likely to be the crucial position.

*Spin-Labeled Analogues in Fibroblasts.* We have measured the inward redistribution of SL diester as well as diether phospholipids in the plasma membrane of fibroblasts at 37 °C (Figure 5). To this end, fibroblasts were suspended and labeled as described in the Materials and Methods. Both PS analogues disappeared rapidly from the exoplasmic leaflet of the plasma membrane. However, the inward movement of the diester PS analogue was more efficient in comparison to that of the diether PS analogue. After 15 min, more than 90% of the diacyl PS analogue and about 70% of the diether analogue redistributed to the cytoplasmic leaflet.

We can preclude by two reasons that the rapid inward movement of both analogues is mainly caused by the endocytic route. First, different to the PS analogues, the PC analogues were internalized at a much slower rate with no significant difference between the diester and the diether analogues. Presumably, a main pathway of internalization of the PC analogues at 37 °C is endocytosis (*18*). If the endocytic route would be responsible also for the internalization of PS analogues, we should not expect a difference between PC and PS analogues. Second, we found also rapid internalization of the PS analogues at 20 °C, which was only slightly slower in comparison to that at 37 °C. This argues against an internalization via endocytosis, since the endocytic activity at 20 °C of fibroblasts is significantly reduced (*25*).

Experiments presented in Figure 5 were done in the absence of DFP. After 30 min, about 9 and 11% of SL diester PS became hydrolzyed at 20 and 37 °C, respectively (not shown). Hydrolysis of SL diacyl PC was lower. After 30 min only 4% became hydrolyzed at 37 °C. As expected, no destruction of the diether analogues was found. Notably, we have already shown previously that DFP did not affect translocation of diester phospholipid analogues in human fibroblasts (*18*).

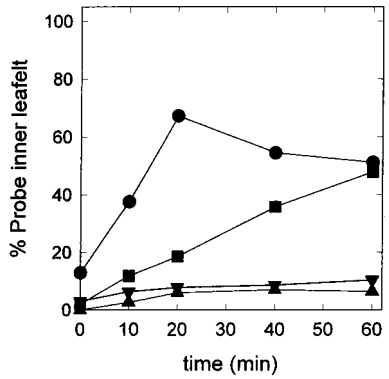



FIGURE 6: Internalization of NBD−lipid analogues into human fibroblasts at 20 °C. Cells on tissue culture plates were labeled with diester NBD−PS (circles), diether NBD−PS (squares), diester NBD−PC (triangles down), and diether NBD−PC (triangles up), washed at 2 °C and incubated for the indicated times at 20 °C. Experiments were done in the absence of DFP (for hydrolysis, see Results). The fraction of internalized analogues was assessed by extraction of analogues from cells upon treating them with dithionite to reduce the NBD moiety of analogues in the exoplasmic leaflet (see Materials and Methods). The results shown are for a representative experiment of two independent experiments.

*Fluorescent Analogues in Fibroblasts*. Figure 6 shows the internalization of the fluorescent phospholipid analogues of PS and PC in human fibroblasts as a function of time at 20 °C. To assess the redistribution of the analogues, aliquots of labeled cells were incubated at various times with dithionite at 2 °C for 5 min, and the lipids were extracted and separated by two-dimensional TLC followed by quantification as described in the Materials and Methods. The analogues of diester and diether PS became rapidly protected against dithionite, although the diether NBD−PS was internalized at a slower rate than diacyl NBD−PS. After 20 min at 20 °C, about 70% of the diester PS and 20% of diether PS was not accessible to dithionite. In contrast to the aminophospholipid analogues, the bulk of diester and diether NBD−PC was accessible to dithionite even after prolonged incubation. After 40 min at 20 °C, only about 7% of both diester and diether NBD−PC were internalized.

For incubation times above 20 min, we observed a slow decrease of the amount of internalized diester NBD−PS. Thin-layer chromatography revealed that, in the absence of DFP, the diacyl analogues were hydrolyzed into lyso derivates and free NBD−fatty acid, consistent with previous results (*16*, *18*, *26*). The amount of liberated free fluorescent fatty acid from diester NBD−PS and NBD−PC was up to 20 and 1%, respectively, after 60 min at 20 °C which, in the case of diester NBD−PS, accounts for the observed decrease of the amount of internalized analogue. Indeed, such a decrease was not found if fibroblasts were pretreated with DFP (5 mM). In that case, the amount of internalized analogue reached a plateau of about 80% after 40 min of labeling (not shown). In that case, hydrolysis was almost neglibile (<5%) for both diacyl analogues even 60 min after labeling. Lipid analysis also revealed that during the incubation, up to 7% of the diacyl NBD−PS had been converted to the corresponding PE derivative while the diacyl PC analogue was not subjected to headgroup modifications. Diether analogues were subjected neither to hydrolysis nor to headgroup modifications during the incubation at 20 °C.

In conclusion, the efficient internalization of the PS analogues compared to the slow internalization of the PC analogues suggests that the diether PS, similar to its diacyl counterpart, is internalized by the action of the aminophospholipid translocase, the activity of which has been already demonstrated in fibroblasts (*18*, *27*).

*Fluorescence Microscopy of Fibroblast Monolayer Cultures.* Microscopic studies were carried out to characterize on a qualitative basis the internalization of the phospholipid analogues (NBD−phospholipids) after their insertion into the exoplasmic leaflet of the plasma membrane. This approach also enables one to differentiate between transbilayer movements and uptake by the endocytic route. When human fibroblasts were incubated for 30 min at 2 °C with the diester or diether fluorescent analogues of PS or PC, their plasma membrane became highly fluorescent. This labeling pattern remained unchanged when the cells were maintained at that low temperature. Back-exchange to BSA resulted in a complete removal of both diester analogues (PS, PC), while for the diether probes, significant fluorescence remained associated to the plasma membrane. Apparently, BSA has a lower efficiency to extract the diether species from the exoplasmic leaflet of the plasma membrane (see Materials and Methods). Indeed, when dithionite was added, which cannot easily permeate membranes of fibroblasts at 2 °C (*18*, *28*), and destroys NBD fluorescence (*22*), no remaining fluorescence was found at the plasma membrane nor in the cell interior, indicating that all analogues were confined to the exoplasmic leaflet of the plasma membrane at 2 °C.

When labeled analogues were incubated at 20 °C, all different NBD analogues were internalized with pattern of intracellular fluorescence depending on the nature of the phospholipid headgroup. The aminophospholipid analogues appeared rapidly in the cytosol and spread over the whole cell interior within 30 min. After back-exchange to BSA and treatment with dithionite at 2 °C, a homogeneous distribution of diacyl and diether NBD−PS through the cytosol was observed (Figure 7). Indeed, once the analogue has moved to the cytoplasmic leaflet of the plasma membrane, it can redistribute to intracellular membranes as found for diester NBD−aminophospholipids by diffusion and/or other, perhaps protein-mediated mechanisms (*18*, *27*).

In contrast, for fibroblasts labeled with diester or diether NBD−PC and incubated at 20 °C, most of the analogues remained confined to the plasma membrane. After back-exchange to BSA and treatment with dithionite, both PC analogues were only detected in intracellular fluorescent spots indicative of an internalization by the endocytic pathway. No bright labeling of the cytoplasm by PC analogues as found for the PS analogues was detected (Figure 7).

## DISCUSSION

In this article, we have investigated whether the substitution of the ester linkage between fatty acids and the glycerol backbone by ether bonds affects the transbilayer redistribution of labeled PS and PC molecules in the plasma membrane of human erythrocytes and fibroblasts. The purpose is to obtain more information on what part of PS is recognized by the aminophospholipid translocase and, additionally, to determine if the more stable diether derivatives can be used as probes in studies dealing with lipid trafficking within eukaryotic cells.

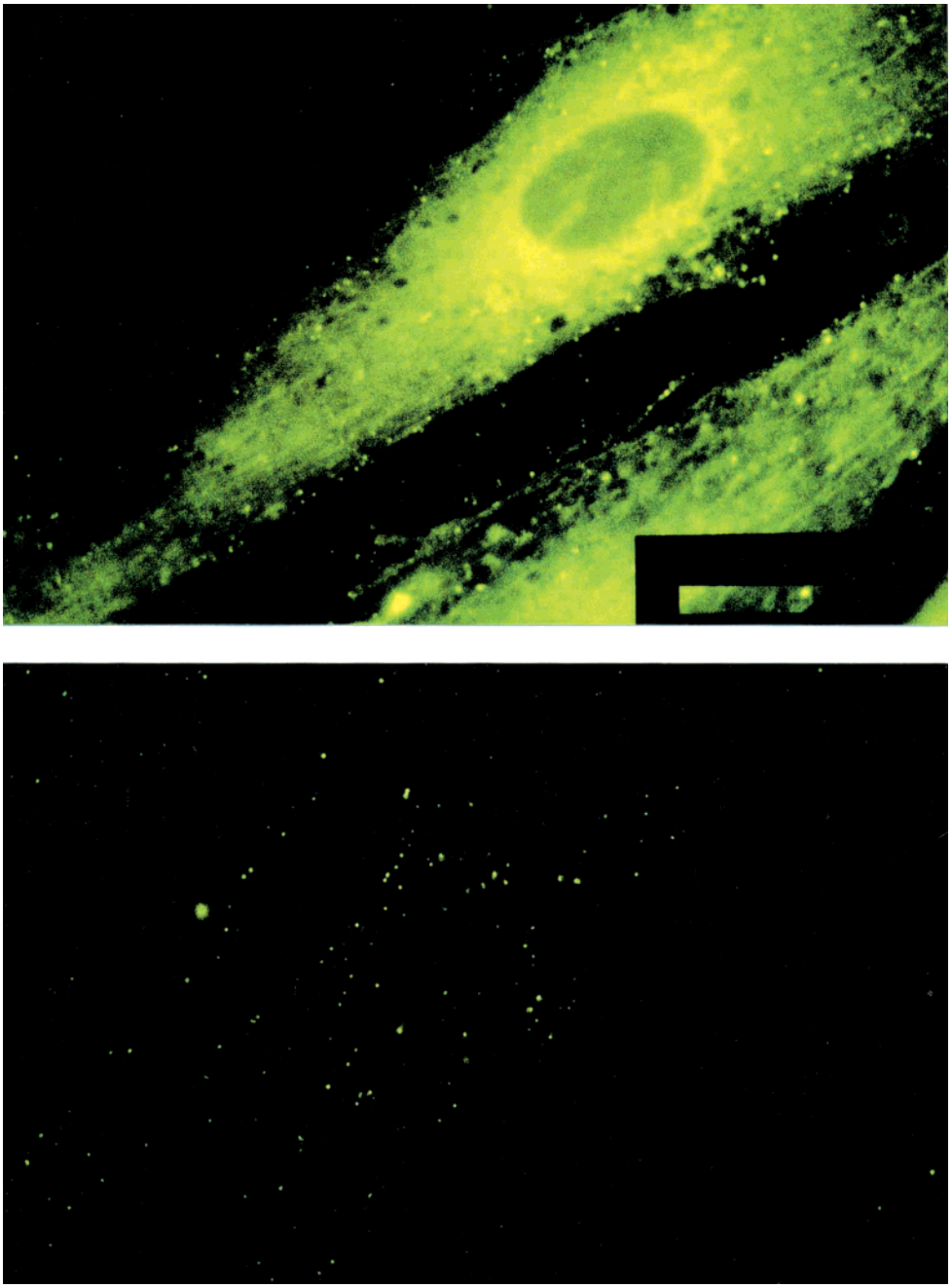


FIGURE 7: Comparison of the internalization of diether NBD−PS (upper photograph) and diether NBD−PC (lower photograph) from the plasma membrane of human fibroblasts at 20 °C. Cells on chamber slides were labeled for 30 min at 2 °C. Cells were then washed and incubated for 30 min at 20 °C to allow internalization of the lipid. To reduce the amount of fluorescence at the plasma membrane, the cells were back exchanged and treated with dithionite at 2 °C (see Materials and Methods). Bar corresponds to 20 $\mu$m.

We have found that the presence of the ether bond on the *sn*-1 position has no major influence on the rate of translocation as shown for erythrocytes. This is in agreement with former studies where plasmalogen transport was tested in human erythrocytes (*29*). However, substitution of the acyl chain by an alkyl one on the *sn*-2 position does slow the transport of the SL derivative of PS and blocks the transport of the NBD derivative in erythrocytes. Nevertheless, the fluorescent diether PS derivative is selectively transported in fibroblasts indicating that diether probes can be used to investigate translocase activity in nucleated cells.

Both spin labels and NBD analogues of naturally occurring phospholipids have been used successfully in erythrocytes as well as in various nucleated cells to elucidate lipid flip-flop as well as lipid trafficking (*5*, *6*, *27*, *30*). Yet, these results raise the question of the relevance of labeled phospholipids with one short chain. Obviously, these molecules have severe chemical modifications associated with the probe itself (an oxazolidine ring or a bulky NBD group). Furthermore, the water solubility is enhanced compared to that of normal long-chain phospholipids due to the short *sn*-2 chain. However, by modifying stepwise different parts of the substrat, it is possible to evaluate which structural features are crucial (*19*). In fact, there is ample evidence to show that the part of the molecule recognized by the aminophospholipid translocase is primarily the phospholipid headgroup, while the nature of the fatty acid chains has only little effect on the selectivity. Thus, providing one is interested first in

comparisons between different headgroups, such probes can give valuable information. This does not imply that rates of translocation of naturally occurring long-chain phospholipids are identical to those measured here. That an NBD group causes a less efficient transport of aminophospholipid analogues by the aminophospholipid translocase than a nitroxide ring approximately at the same position of the *sn2*-chain is not surprising. We have demonstrated already that the presence of the bulky NBD slows down the translocation of the diester PS and that diester NBD−PE is not transported in erythrocytes (*16*). On the other hand, we showed also that in nucleated cells, such as fibroblasts, the same NBD−lipids are transported in an ATP-dependent fashion from the exoplasmic to the cytoplasmic monolayer of the plasma membrane, revealing that the aminophospholipid translocase activity is less efficient in erythrocytes than in nucleated cells (*18*, *31*, *32*).

Thus, it can be inferred that, with the exception of erythrocytes where no endocytosis is taking place anyway, the diether lipids should be valuable tools to follow phospholipid trafficking within eukaryotic cells. Figure 7 shows the typical pattern of fluorescent spots revealing the endocytic pathway followed by the PC derivatives. As indicated originally by Martin and Pagano (*27*) a very different fluorescence distribution is obtained with the PS analogue. In the latter case, subcellular membranes are labeled. Such distribution can be explained by the translocation of the PS from the exoplasmic to the cytoplasmic monolayer followed by a spontaneous partitioning between all organelles due to the partial water solubility of these lipids with a short *sn-2*-chain. Recently, Van der Veer et al. (*33*) reported on a rapid inward redistribution of a NBD-labeled ether phospholipid, 1-*O*-alkyl/alkenyl-2-acyl-glycerophosphocholine, into a primary human fibroblast cell line even at low temperature. As determined by the back exchange assay, about 70% of analogues incorporated initially into the exoplasmic leaflet redistributed to the cytoplasmic leaflet within 30 min at 2 °C while about 10% of the diester analogue C6−NBD−PC became nonexchangeable to BSA. We did not observe such a rapid redistribution of the diether NBD−PC in our experiments even at 37 °C. We have no explanation for this different behavior. Notably, we found that the back-exchange to BSA is not efficient to remove diether NBD−PC analogues from the exoplasmic leaflet of human fibroblasts. This became evident by the treatment of cells with dithionite subsequently to the back-exchange (see Results). Thus, to our experience, determination of transbilayer distribution of diether NBD−PC analogues by the back exchange significantly overestimates the amount of inward redistributed analogues.

A major difficulty in the experiment on phospholipid analogue redistribution originally described by Martin and Pagano (*27*) is that the short chain diacyl PS molecules are rapidly hydrolyzed in vivo due to the ubiquitous presence of a phospholipase $A_2$ activity which seems to cleave off preferentially phospholipids partially water soluble. For that reason, fluorescent analogues of sphingomyelin have been more frequently used in cultured cells than PS analogues (*30*, *34*). The chemical instability of diester derivatives with one short chain is encountered with spin labels as well and is only partially circumvented by addition of DFP (*8*, *35*). Diether PS (or PC) on the other hand is perfectly stable and does not suffer from this drawback.

The lower solubility of the diether lipids used in the present study, i.e., with a stearyl chain in the *sn*-1 position, makes it slightly more difficult to label a membrane and significantly more difficult to extract the probes from a membrane with BSA and, thus, to perform a back exchange experiment (see Materials and Methods section). This is particularly true in the case of the NBD derivatives as indicated in the Results. However, the dithionite technique can be used, which allows a quantification of the distribution of fluorescence between the two leaflets of a plasma membrane. Furthermore, the low water solubility can be circumvented by using slightly shorter fatty acid in the *sn*-1 position. We are presently synthesizing other diether analogues with *sn*-1 chains of variable length in order to determine the optimum length for technical applications.

## ACKNOWLEDGMENT

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## REFERENCES

1. Ourisson, G., Rohmer, M., and Poralla, K. (1987) *Annu. Rev. Microbiol. 41*, 301−333.
2. Paltauf, F. (1994) *Chem. Phys. Lipids 74*, 101−139.
3. Bette-Bobillo, P., Luxembourg, A., and Bienvenue A. (1986) *Biochim. Biophys. Acta 860*, 194−200.
4. Kleuser, B., Meister A., Sternfeld, L., and Gercken, G. (1996) *Chem. Phys. Lipids 79*, 29−37.
5. Devaux, P. F. (1991) *Biochemistry 30*, 1163−1173.
6. Schroit A. J., and Zwaal R. F. A. (1991) *Biochim. Biophys. Acta 1071*, 313−329.
7. Menon, A. K. (1995) *Trends Cell Biol. 5*, 355−360.
8. Fellmann, P., Zachowski A., and Devaux P. F. (1994) Biomembrane Protocols: II. Architecture and Function. In *Methods in Molecular Biology* (Graham, J. M., and Higgins, J. A., Eds.) vol. 27, pp 161−175, Humana Press.
9. Heymans F. (1984) *Thèse d'état*, Paris.
10. Prashad, M., Tomesch, J. C., and Wareing, J. R. (1990) *Chem. Phys. Lipids 53*, 121−126.
11. Joseph, J. C., Shih, C.-Y., and Lai C.-S. (1991) *Chem. Phys. Lipids 58*, 19−26.
12. Kleuser, B., Schmidt, N., and Gercken G. (1993) *Chem. Phys. Lipids 66*, 111−122.
13. Duclos, R. J., Jr. (1993) *Chem. Phys. Lipids 66*, 161−170.
14. Juneja, L. R., Kazuoka, T., Goto, N., Yamane, T., and Shimizu, S. (1989) *Biochim. Biophys. Acta 1003*, 277−283.
15. Rouser, G., Siakotos, A. N., and Fleischer, S. (1966) *Lipids 1*, 85−86.
16. Colleau, M., Hervé, P., Fellmann, P., and Devaux, P. F. (1991) *Chem. Phys. Lipids 57*, 29−37.
17. Schwoch, G., and Passow, H. (1973) *Mol. Cell Biochem. 2*, 197−218.
18. Pomorski, T., Müller P., Zimmermann, B., Burger, K., Devaux, P. F., and Herrmann, A. (1996) *J. Cell Sci. 109*, 687−698.
19. Morrot, G., Herve, P., Zachowski, A., Fellmann, P., and Devaux, P. F. (1989) *Biochemistry 28*, 3456−3462.
20. Pomorski, T., Herrmann, A., Zachowski, A., Devaux, P. F., and Müller, P. (1994) *Mol. Membr. Biol. 11*, 39−44.
21. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.
22. McIntyre, J. C., and Sleight, R. G. (1991) *Biochemistry 30*, 11819−11827.

23. King, M. D., and Marsh, D. (1987) *Biochemistry 26*, 1224−1231.
24. Herrmann, A., Zachowski, A., and Devaux P. F. (1990) *Biochemistry 29*, 2023−2027.
25. van Genderen, I., and van Meer, G. (1995) *J. Cell Biol. 131*, 645−654.
26. Kasurinen, J., and Somerharju, P. (1995) *Biochemistry 34*, 2049−2057.
27. Martin O. C., and Pagano R. E. (1987) *J. Biol. Chem. 262*, 5890−5898.
28. Pomorski, T., Herrmann, A., Zimmermann, B., Zachowski, A., and Müller, P. (1995) *Chem. Phys. Lipids. 77*, 139−146.
29. Fellmann, P., Hervé, P., and Devaux, P. F. (1993) *Chem. Phys. Lipids 66*, 225−230.
30. van Helvoort, A., Giudici, M. L., Thielemans, M., and van Meer, G. (1997) *J. Cell Sci. 110*, 75−83.
31. Cribier, S., Sainte-Marie J., and Devaux P. F. (1993) *Biochim. Biophys. Acta 1148*, 85−90.
32. Müller, P., Pomorski, T., Porwoli, S., Tauber, R., and Herrmann, A. (1996) *Hepatology 24*, 497−503.
33. van der Veer, E., Vanderweide, D., Heijmans, H. S. A., and Hoekstra, D. (1993*) Biochim. Biophys. Acta 14*, 294−300.
34. Koval, M., and Pagano, R. E. (1989) *J. Cell Biol. 111*, 429−442.
35. Calvez, J.-Y., Zachowski, A., Herrmann, A., Morrot, G., and Devaux, P. F. (1988) *Biochemistry 27*, 5666−5670.

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