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Translocation of fluorescent ether phospholipid, but not its diacyl counterpart, after insertion in plasma membranes of control and plasmalogen-deficient fibroblasts

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Fluorescently labelled *ether* phospholipid (1-*O*-alkyl/alkenyl-2-acyl-glycerophosphocholine) readily internalizes at low temperatures (2°C) after insertion into the plasma membrane of cultured fibroblasts. This fate differs markedly from that of its *diacyl* phospholipid analogue, which remains associated with the plasma membrane under similar conditions. Analysis by thin-layer chromatography reveals that the translocation involves transfer of the intact ether phosphatidylcholine molecules. Relative to control cells, a 2-fold increase of ether phosphatidylcholine uptake was noted when plasmalogen deficient fibroblasts were used. Back-exchange experiments demonstrate that more than 60% of the cell-associated ether lipid is translocated within the cells, irrespective of the cell strain that was used. The potential mechanism by which the translocation process is accomplished is discussed.

Introduction

Ether phospholipids, i.e., 1-*O*-alkyl-2-acyl- and 1-*O*-alkenyl-2-acyl-glycerophospholipids (the latter are known as plasmalogens) have long been recognized to be widely distributed in mammalian cell membranes. Plasmalogens are the major end products of the ether lipid biosynthesis in mammals, comprising approx. 18% of total phospholipids in man [1,2]. Peroxisomes are involved in the biosynthesis of ether lipids [3,4], and the peroxisomal enzymes, responsible for the biosynthesis of the ether bond, are located at the inner surface of the peroxisomal membrane [5]. Tissues of Zellweger patients, deficient in peroxisomes, are also severely deficient in both types of ether lipids [2,6,7]. Indeed, it has been shown that the novo ether lipid biosynthesis, using [^{14}C]hexadecanol as precursor, is severely impaired in cultured skin fibroblasts of Zellweger patients [8–10].

In artificial membranes plasmalogens differ from the common diacyl glycerophospholipids with regard to physical properties and interaction with sterols and proteins. Not much is known, however, about the impact of plasmalogens on the properties of biological membranes. Ether lipid deficient membranes have been found to be consistently less fluid than diacyl glycerophospholipid membranes, although a discrepancy between intact cell membranes and extracted membrane lipids has been noted [11]. Furthermore, Loidl et al. [12] have recently demonstrated that plasmalogen-deficient cells exhibit a significantly increased tendency to take up exogenous choline or ethanolamine plasmalogens at 37°C.

A variety of studies, aimed at characterizing and revealing pathways and mechanisms of lipid traffic and lipid metabolism in animal cells, have shown the fruitful application of fluorescent lipid analogues for this purpose [13]. In fact, several of these studies have shown that different fluorescent species may be segregated from each other during lipid sorting and processed independently, a process that can be visualized in a fluorescence microscope [14].

In the present work we have followed a similar approach to study the intracellular processing of ether phospholipids. Therefore a fluorescent analogue of ether phosphatidylcholine was synthesized. Its fate was examined after inserting the lipid in the plasma membrane of fibroblasts of controls and of patients with

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Abbreviations: BHK, baby hamster kidney; C6-NBD, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminocaproyl; PC, phosphatidylcholine; RCDP, rhizomelic type of chondrodysplasia punctata.

plasmalogen-deficient membranes. In addition the influence of the type of chemical bonding (ether or ester) between glycerol and the aliphatic chain was studied by comparing the intracellular flow of ether phosphatidylcholine and diacyl phosphatidylcholine.

Materials and Methods

Materials

1-Acyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-aminocaproyl)-phosphatidylcholine (C6-NBD-PC) was purchased from Avanti Biochemicals, Pelham, AL (USA). Beef heart plasmalogen lysoglycerophosphocholine, (ether-lysoPC; see next section) was purchased from Serdary Research Laboratory (Canada). 6-[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)]aminohexanoic acid (C6-NBD) was purchased from Molecular Probes. All other chemicals used were of analytical grade.

Synthesis of C_6-NBD-etherPC

The following procedure was carried out under nitrogen. A mixture of 20 mg C6-NBD (68 μmol), 7 mg dicyclohexylcarbodiimide (34 μmol), 10 mg ether-lysoPC (20 μmol), and 2.5 mg dimethylaminopyridine (20 μmol) in 1 ml anhydrous chloroform/pyridine (4:1, v/v) was shaken at room temperature for 24 h under protection from light and atmospheric moisture. The formation of C6-NBD-etherPC was monitored by running samples on TLC and identifying the product by HCl treatment.

Purification on a preparative scale was carried out by thin-layer chromatography on silica gel 60 HPTLC plates, Merck, Darmstadt, Germany (solvent chloroform/methanol/NH_4OH (13:7:1, v/v); $R_f = 0.4$). Products were eluted with chloroform/methanol (1:4, v/v) and freed of residual silicic acid by partitioning between chloroform and methanol/water [15]. The product was stored under nitrogen at 4°C.

The product was characterized by determining its sensitivity towards HCl treatment, which hydrolyses the vinyl ether bond. When doing so after TLC in the first dimension, followed by elution in the second dimension, approx. 50% of the applied lipid was sensitive to this HCl treatment. The remaining spot was insensitive to alkaline hydrolysis. Essentially identical results were obtained when the starting material 'plasmalogen' lysoglycerophosphocholine (according to the manufacturer's claim consisting for more than 90% of the alkenyl species) was treated similarly. We conclude therefore that the C6-NBD-etherPC consists of roughly equal amounts of the alkyl and alkenyl species.

Cell culture and insertion of fluorescent phospholipids

The primary human fibroblast strains used in these studies are derived from a normal healthy person and from a patient with clinical and biochemical characteristics of rhyzomelic type of chondrodysplasia punctata, whose cell membranes are deficient in ether lipids, including plasmalogens [8]. The cells were grown in Ham's F10 medium supplemented with 7.5% foetal calf serum and antibiotics. To insert the fluorescent phospholipids into the plasma membrane the procedure was essentially as described elsewhere [16]. Briefly appropriate amounts of phospholipids in ethanol were injected into Hanks' buffer, pH 7.2 (< 1% ethanol v/v). Lipids thus dispersed, were incubated with the cells for 30 min at 2°C, unless indicated otherwise. Non-inserted lipids were removed by extensive washing (three or four times) with saline [16].

Back-exchange of membrane-inserted phospholipids

After insertion of the fluorescent phospholipids at 2°C, the monolayers were subjected to a so called 'back-exchange'. With this procedure exchangeable (fluorescent) lipids located in the outer leaflet of the plasma membrane can be removed, allowing quantitation of the intracellular fraction, i.e., the lipid fraction, which is not 'back'-exchanged. Back-exchange of the phospholipids was achieved by incubation of the cells with 5% BSA (w/v) for 10 min. This procedure was repeated twice, followed by extensive washing of the cells with saline. For biochemical analysis the cells were harvested from the plate with a rubber policeman.

Phospholipid extraction and analysis

Phospholipids were extracted by the procedure of Bligh and Dyer [17], and separated into their individual components by two-dimensional thin-layer chromatography [2]. The phospholipid spots were visualized by either fluorescence or iodine staining. Quantification of the fluorescence was performed as described previously [18]; to analyze the phosphorus content, the spots were scraped into glass-tubes and a phosphorus assay was carried out according to Rouser et al. [19]. The amount of fluorescent etherPC was estimated from the fluorescence intensity measured with a standard series of low concentrations of C6-NBD-PC.

Fluorescence microscopy

The fibroblasts were cultured on coverslips for 24 h and kept on ice in serum-free medium for 20 min. prior to addition of the fluorescent phospholipids. At indicated times after addition of C6-NBD-PC or C6-NBD-etherPC the photographs were taken with a Leitz orthoplan microscope equipped with a Leitz Vario Orthomat 2 photography system; filter set blue excitation, BP450 to 490/LP515; using Illford HP 5 films which were processed at 1600 ASA.

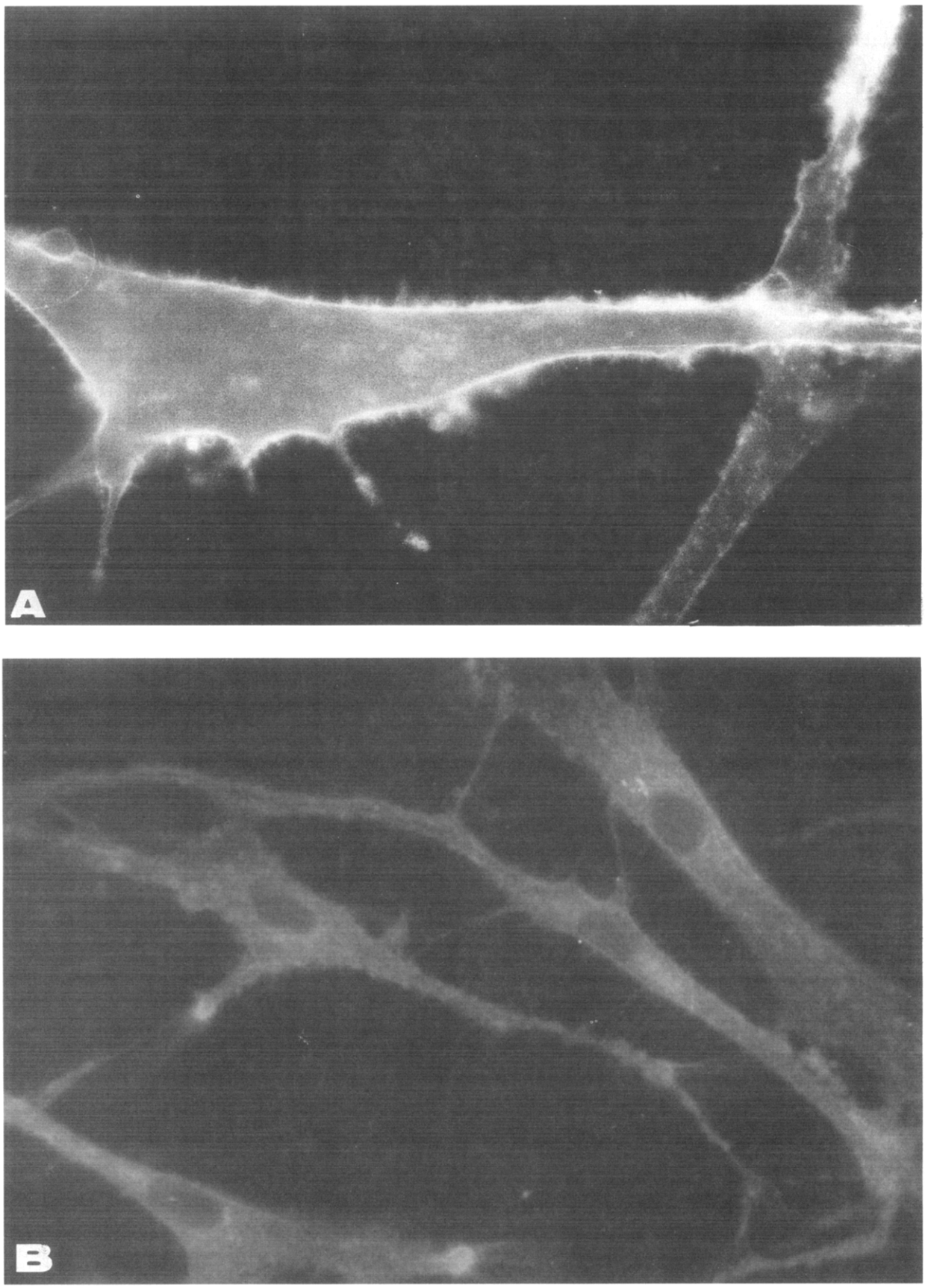
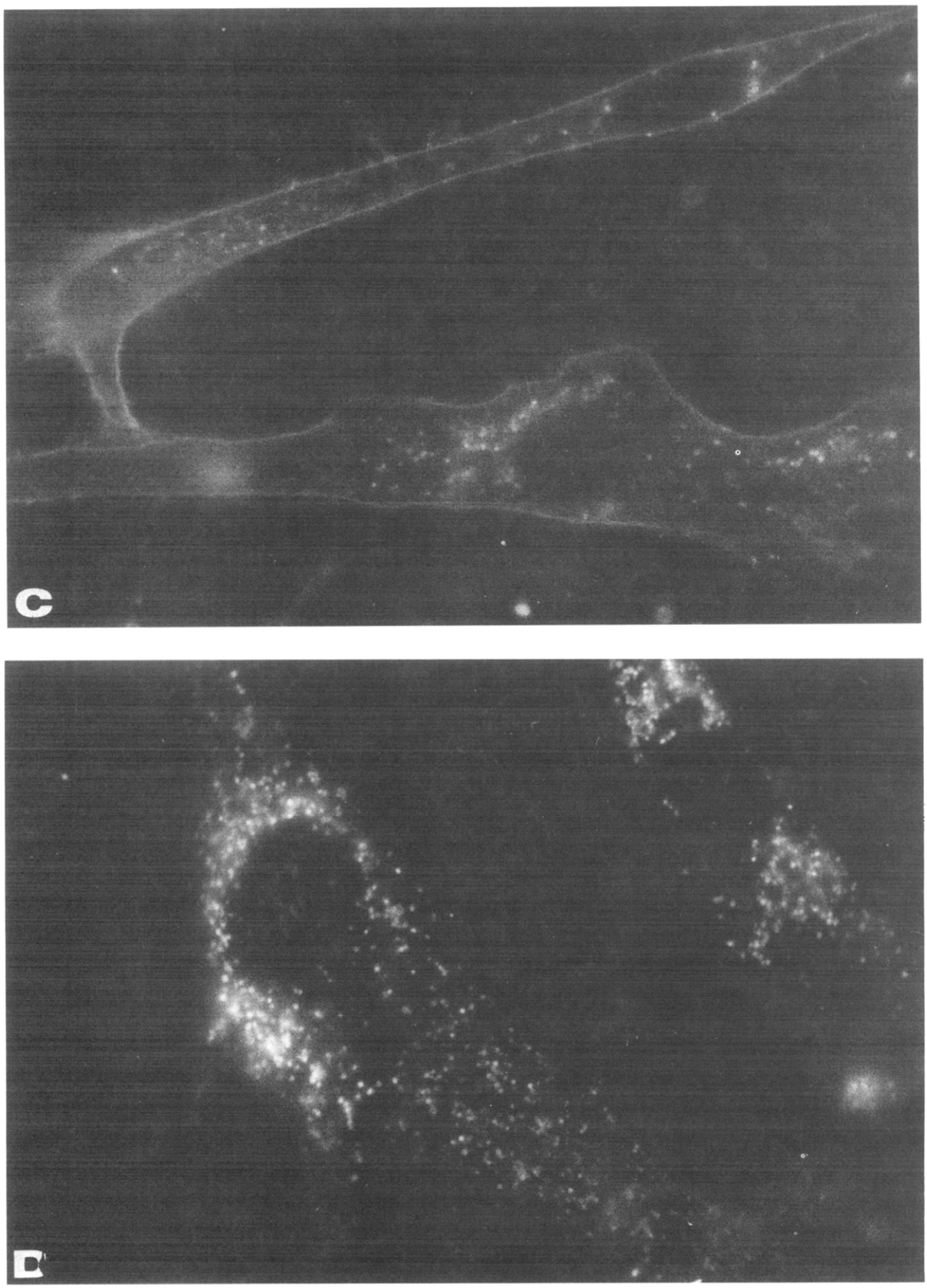


Fig. 1. Localization of fluorescent lipid analogues in human fibroblasts at 2 °C. The cells were incubated with either C6-NBD-PC (A) or



C6-NBD-etherPC (B, C, and D) and examined by fluorescence microscopy. The photographs were taken 2 min (B), 25 min (A, C, and D) after starting the incubation. The photomicroscope is focused on the cell membrane (C) or on the inside of the cells (D).

Results

Plasma membrane insertion of C6-NBD-etherPC and C6-NBD-PC

When control and plasmalogen-deficient fibroblasts were incubated with C6-NBD-PC at 2°C (see Materials and Methods), the plasma membranes became intensely stained and showed up as bright fluorescent rings. An exclusive plasma membrane labelling pattern under these conditions is consistent with previous observations in BHK cells [16], and this pattern remained as long as the temperature of the incubation was kept at 2°C (Fig. 1A). An essentially identical procedure was then carried out, using C6-NBD-etherPC. Remarkably, as shown in Figs. 1B, C and D, membrane insertion of this lipid analogue was not restricted to the plasma membrane at the low temperature incubation. Rather, immediately after addition of the ether lipid analogue, intracellular fluorescence was apparent.

Intact C6-NBD-etherPC internalizes at 2°C

To investigate the molecular nature of the internalized fluorescence, i.e., to determine whether intact C6-NBD-etherPC had entered the cells at 2°C, the following experiment was carried out. The fibroblasts were labelled at 2°C for 20 min. Subsequently, the cells were extensively washed and the lipids were extracted and analyzed by thin-layer chromatography. As shown in Fig. 2, only one fluorescent spot was cell-associated after this incubation, showing R_f value identical to that of the starting material.

No preferential cellular association of either the alkenyl or alkyl ether species was found with mild acid hydrolysis. Thus, both species became cell-associated in a ratio identical to that of the starting material (not shown; see Materials and Methods).

Lipid internalization in control versus RCDP fibroblasts

To characterize the interaction of both lipid analogues (i.e., ether vs. acyl derivatives) with control and deficient cells further, some quantitative experiments were carried to determine the relative fractions of the added lipids that were inserted, and, in the case of the ether lipid analogue, the fraction that became internalized. The latter was determined by a so-called back-exchange reaction with BSA, which allows the quantitative extraction of lipid from the cell that is still present in (the outer leaflet of) the plasma membrane. The results, summarized in Table I, show that approx. 40% of the added C6-NBD-PC fraction becomes inserted into the plasma membrane of either control or deficient cells. Insertion of the etherPC is much more dependent on the acceptor membrane. In this case only 15% of the added fraction becomes inserted into the plasma membrane of control cells. In the plasmalogen deficient fibroblasts, this fraction increases to 27%.

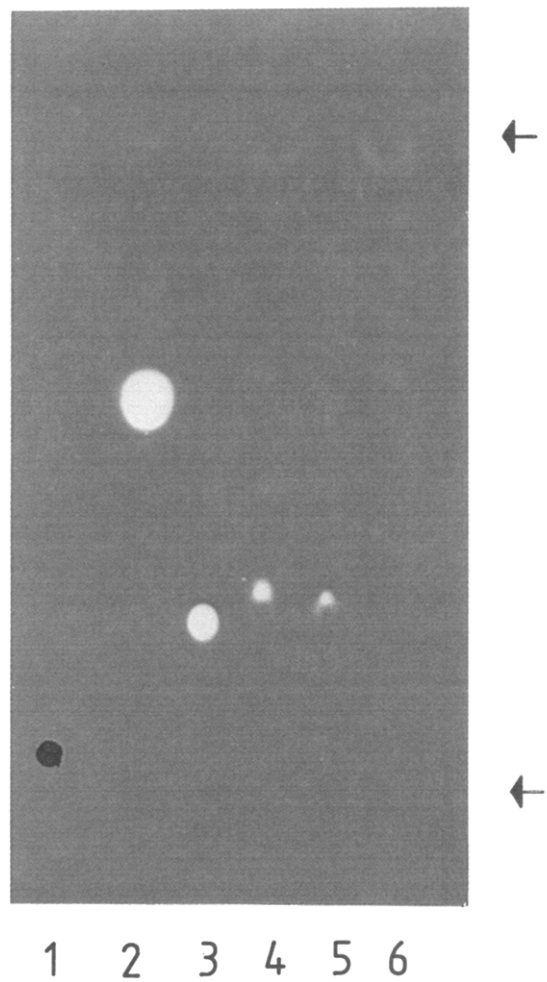


Fig. 2. Thin-layer chromatogram of phospholipid extracts. The control fibroblasts were incubated for 20 min at 2°C with C6-NBD-PC (lane 5) or C6-NBD-etherPC (lane 4), or used as controls (lane 6). The position of authentic fluorescent lipid standards include C6-NBD-PC (lane 3) and C6-NBD (lane 2). Lyso-etherPC could only be visualized after iodine staining (position is marked, lane 1). Arrows indicate place of origin and front, respectively.

As referred to above, essentially all C6-NBD-PC remains in the plasma membrane, provided that the temperature is kept at 2°C. Consequently, by BSA back-exchange essentially the total cell-associated C6-NBD-PC fraction could be recovered from the cells. In contrast, less than 40% of the cell associated C6-NBD-etherPC fraction could be recovered from the cells, at similar back-exchange conditions. Thus, more than 60% of the ether lipid internalizes during the indicated incubation time. The internalization appears to be independent of the cell type, which would imply that translocation appears independent of the ether lipid content of the plasma membrane.

Similar as found for the total cell-associated ether lipid fraction, no preferential translocation of either ether lipid species was observed. As revealed by mild acid hydrolysis of the back-exchanged lipid fraction

TABLE I

Insertion and translocation of NBD-labeled diacylPC and etherPC in fibroblasts at low temperature

Cells, control or plasmalogen deficient (plg-def) fibroblasts, were labeled with fluorescently tagged diacyl- or ether phosphatidylcholine (PC or etherPC, resp.) as described, at a lipid concentration as indicated (row 3). The amount of lipid associated with the cells was determined after 30 minutes (row 4; percentage of total fluorescence between brackets) and the fraction of the cell-associated lipid that could not be removed with 5% BSA (row 5).

Donor phospholipids	Fibroblast	Fluorescence			
		medium (pmol/ml)	cell-associated lipid		non-exchangeable lipid (rel %)
			pmol/ml	%	
PC	control	129	76	(37)	9
	plg-def	127	108	(46)	10
EtherPC	control	149	26	(15)	73
	plg-def	174	67	(27)	63

(plasma membrane fraction) and of the residual cell-associated fraction, representing the translocated ether lipid fraction, the ratio of alkyl to alkenyl species was equal to that of the starting material (1 : 1; see Materials and Methods).

Discussion

In this work evidence is presented that fluorescently tagged ether phosphatidylcholine (1-*O*-alkyl/alkenyl-2-acyl-glycerophosphocholine), when inserted into the plasma membrane of cultured fibroblasts at low temperatures (2°C), does not remain associated with the plasma membrane like the diacyl phospholipid analogue, but readily internalizes. By back-exchange experiments it could be demonstrated that within 30 min more than 60% of the cell-associated ether lipid can be translocated within the cell. Moreover, lipid analysis reveals that the translocation most likely involves transfer of the intact C6-NBD-etherPC molecules, without displaying a particular preference for either the alkyl or alkenyl derivative. It appears that, given the higher fraction that becomes associated with the plasmalogen deficient cell line, these cells are more translocation active than control cells. In this context it is interesting to note that Loidl et al. [12] recently observed a significant increase in the fraction of exogenously added choline plasmalogens that was taken up by a plasmalogen deficient cell strain, relative to that taken up by control cells. These experiments were done at 37°C, but in agreement with these results we observed at 2°C, that the uptake of fluorescently tagged etherPC increases about 2-fold, when deficient and control cells are compared, respectively. In the present work we could not confirm their observation that uptake of the etherPC is enhanced relative to the uptake of diacylPC. It should be noted, however, that both studies differ in temperature at which the experiments were carried out, and which may affect the relative rate of exchange. It is equally possible that distinctions in rate are obscured by the relative high 'aqueous' solubility of NBD-lipids. Indeed Loidl et al. [12] noted far less significant differences when lipid analogues more closely resembling natural lipids were employed.

The most remarkable observation in the present work is the ability of intact C6-NBD-etherPC to be translocated across the plasma membrane at a temperature (2°C) where C6-NBD-diacylPC is not internalized by the cell. Once C6-NBD-etherPC has been translocated to the inner leaflet of the plasma membrane, the analogue will diffuse through the aqueous phase as a monomer, thus also allowing insertion in intracellularly accessible membranes. The intriguing question is obviously the mechanism by which the translocation process is accomplished. In principle three mechanisms might be taken into account to explain the present observations. These mechanisms include (i) metabolic conversion followed by flip-flop; (ii) protein-mediated translocation ('translocase activity'); or (iii) 'spontaneously' induced flip-flop.

Previously, it has been demonstrated that the NBD-analogue of phosphatidic acid (C6-NBD-PA) can undergo a similar fate at the same conditions as shown in the present work for the etherPC analogue [14]. However, in those experiments C6-NBD-PA turned out to be converted to NBD-diglyceride at the level of the plasma membrane, after which the diglyceride was translocated at 2°C. In the present work this possibility can be excluded in that the entire cell-associated fraction, both in the plasma membrane as well as intracellularly was not converted into metabolic products. This observation is not surprising given the low incubation temperature and the very short incubation period. In fact, the intracellular localization is already apparent after 2 min. Also at those conditions only one fluorescent spot, co-localizing with reference C6-NBD-diacylPC is detectable. Evidently this time is a priori too short to account for significant metabolism and/or reutilization of metabolic products. For example, incorporation of the fatty acids in lipids usually

takes several hours, when occurring at elevated temperatures (see, for example Ref. 12). In this context it should be noted that when cleaved, C6-NBD cannot be reused for synthesis.

Aminophospholipids and their fluorescent analogues are known to be actively translocated across cellular membranes, mediated by a specific translocase ('flippase'). In fact, translocation of these lipids already occurs at temperatures as low as 7°C [20]. Although our experiments were done at 2°C, we cannot rigorously exclude that a translocase activity may exist for the etherPC. It remains quite remarkable then that diacyl phosphatidylcholine is not transferred by this translocase and that specificity should therefore be provided by the ether linkage. Related to the latter, translocation is seen for both the alkyl and alkenyl species, indicating that the ether linkage per se is an important parameter in the phenomenon. In the context of an active translocation process it should be noted that an endocytic internalization can be excluded. Endocytosis is effectively inhibited at temperatures below 10°C.

The final option which we tend to consider likely at present, involves a spontaneous flip-flop of the ether lipid analogue, immediately after insertion into the plasma membrane. A spontaneous flip-flop has also been noted for fluorescent C6-NBD-ceramide, a precursor for sphingolipid synthesis [21]. This probe can also be inserted into the plasma membrane of cultured cells at 2°C. As observed for the ether lipid analogue, C6-NBD-ceramide rapidly translocates after insertion at 2°C. Also in this case, a fraction of the lipid remains associated with the plasma membrane. It is possible that the molecular properties of the probe, in conjunction with molecular and physical properties of the acceptor membrane, may determine whether and to what extent spontaneous transfer may occur. In the case of ceramide, it has been observed that this lipid analogue eventually accumulates in the Golgi complex, partly because of metabolic conversion to sphingolipid analogues. However, the strong capacity of the sphingosine backbone to engage in hydrogen bonding with adjacent molecules may in addition facilitate a more or less tight localization in an acceptor membrane [22]. Conversely, it is therefore possible that the association of the ether lipid after insertion into the plasma membrane is such that a stable interaction with adjacent membrane molecules does not occur, which would then allow its passage across the membrane. From a molecular and structural point of view it is, however, quite remarkable that the ether linkage than suffices to bring about this 'spontaneous' translocation effect. Evidently, these notions warrant further investigations aimed at clarifying the mechanism and physiological significance of translocation of etherPC in general, and plasmalogen PC in particular.

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