Differences in intracellular transport of a fluorescent phosphatidylcholine analog in established cell lines

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Summary

The transport and metabolism of a fluorescent phosphatidylcholine analog, 1-palmitoyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl-phosphatidylcholine ((palmitoyl, C₆-NBD)-PC), in BHK, CHO-K1, CHO-15B, MDCK, VA-2, Vero, V79 and WI-38 cells has been investigated. When liposomes containing (palmitoyl, C₆-NBD)-PC were incubated with cells at 2°C, spontaneous transfer of the fluorescent lipid from the liposomes to the cells' plasma membranes occurred. Most of the lipid transferred to the cells could be removed by incubating the cells in the presence of nonfluorescent liposomes or media containing 10% serum, suggesting that the fluorescent probe resided exclusively in the outer leaflet of the plasma membrane at 2°C.

After insertion into the plasma membrane, internalization of (palmitoyl, C₆-NBD)-PC occurred when the cells were warmed to 37°C. This resulted in four different labeling patterns: (1) little or no internalization of (palmitoyl, C₆-NBD)-PC into punctate vesicles was observed in Vero cells. (2) Transport of (palmitoyl, C₆-NBD)-PC to the region of the Golgi apparatus and to a small number of intracellular vesicles was observed in both V79 and CHO-K1 cell lines. (3) A large number of fluorescently labeled intracellular vesicles with little or no labelling in the region of the Golgi apparatus appeared after the internalization of (palmitoyl, C₆-NBD)-PC in BHK, CHO-15B, MDCK and WI-38 cell lines. (4) Accumulation of (palmitoyl, C₆-NBD)-PC in small vesicles, mitochondria and the nuclear envelope was observed in VA-2 cells. In addition, cells having a defect in glycoprotein processing and those transformed with simian virus 40 (SV40) internalized the fluorescent lipid probe differently compared with parental lines. Neither differences in rates of endocytosis nor rates of (palmitoyl, C₆-NBD)-PC degradation between cell types appears to cause the observed dissimilarities in intracellular lipid transport. We suggest that these different cell types may have dissimilar pathways of intracellular lipid trafficking or differential regulation of a common transport pathway, and that the predominant pathway of lipid translocation can be altered in cells by changing the composition of their glycoproteins or by viral transformation.

Key words: phosphatidylcholine, fluorescence, transport.

Introduction

A series of fluorescent phospholipid analogs have been used to identify intracellular lipid transport pathways (Pagano et al. 1983; Lipsky & Pagano, 1983; Sleight & Pagano, 1984, 1985; Martin & Pagano, 1987). Several lines of evidence suggest that fluorescently labeled lipids faithfully mimic their native counterparts (see Pagano & Sleight, 1985, for review). For example: (1) after insertion into the plasma membrane, the analogs are mobile in the plane of the bilayer (diffusion constant = 2 x 10⁻⁹ cm²s⁻¹), suggesting that the fluorescent lipids are properly integrated into the membrane bilayer (Struck & Pagano, 1980); (2) both fluorescent analogs of phosphatidic acid (PA) and ceramide are metabolized to many of the fluorescent end products predicted from classical metabolic pathways (Lipsky & Pagano, 1983; Pagano & Sleight, 1985); (3) both fluorescently labeled phosphatidylethanolamine (PE) and de novo synthesized radioactively labeled PE appear to undergo transbilayer movement at the plasma membrane (Sleight & Pagano, 1985); and (4) both dansyl- and NBD-labeled phosphatidic acid and phosphatidylcholine derivatives behave the same way, suggesting that the fluorescent moiety affects neither transport nor metabolism (Pagano & Sleight, 1985).
A fluorescent phosphatidylcholine analog, 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine [(acyl,C\textsubscript{6}-NBD)-PC] has been used to follow the vesicle-mediated transport of lipid molecules from the outer leaflet of the plasma membrane to intracellular organelles (Sleight & Pagano, 1984). When cultured Chinese hamster lung fibroblasts (V79 cells) are incubated at 2°C with liposomes containing (acyl,C\textsubscript{6}-NBD)-PC, the fluorescent probe spontaneously inserts into the outer leaflet of the plasma membrane. Subsequent incubation of the cells at 37°C results in internalization of the fluorescent PC, with most of the internalized probe accumulating in a centrally located perinuclear region of the cells (Sleight & Pagano, 1984). In addition, a small amount of fluorescent lipid becomes associated with punctate intracellular vesicles. By co-localizing the internalized fluorescent phosphatidylcholine with organelle-specific stains, it was demonstrated that the perinuclear fluorescence corresponds to 'the region of the Golgi apparatus' (Sleight & Pagano, 1984). The small punctate intracellular vesicles have been assumed to be endosomes (Sleight, 1987). We have proposed that the internalization and transport of (acyl,C\textsubscript{6}-NBD)-PC is the result of endocytosis and the intracellular movement of vesicles that have the fluorescent lipid trapped in the luminal leaflet of their lipid bilayer (Sleight & Pagano, 1984; Sleight, 1987). Although native phosphatidylcholine species may be transported by additional mechanisms (e.g. via phospholipid exchange proteins), using (acyl, C\textsubscript{6}-NBD)-PC one is able to examine the vesicle-mediated movement of phospholipids both microscopically and biochemically.

Several different pathways of protein transport from the plasma membrane to intracellular organelles have been reported (Farquhar, 1983; Steinman et al., 1983; Wall & Maack, 1985). These pathways may be the result of sorting in response to signals encoded within the proteins, or they may be caused by differential targeting of intracellular vesicles to organelles (Pfeffer & Rothman, 1987; Robinson, 1987; van Loon et al., 1988; Eilers & Schatz, 1988; Lodish, 1988). It has been demonstrated that in some instances the sorting of plasma membrane proteins and lipids occurs by distinct pathways (Sleight & Pagano, 1984; Pagano & Sleight, 1985). Since most organelles contain the same phospholipids it is unlikely that phospholipids are sorted in response to signals encoded within their structure. Investigators, using different techniques and unrelated cell models, have obtained conflicting results concerning the internalization pathway of plasma membrane lipids (see Sleight, 1987, for review). To determine whether the intracellular vesicular transport of a single lipid species occurs similarly in all cells, we have examined the movement of 1-palmitoyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl-phosphatidylcholine ((palmitoyl, C\textsubscript{6}-NBD)-PC) in eight cultured cell lines.

In this paper we report that (palmitoyl, C\textsubscript{6}-NBD)-PC exhibits strikingly different patterns of internalization in a variety of cell types. Our results suggest that these inconsistencies may have resulted from different cell types having dissimilar pathways of intracellular lipid trafficking or differential regulation of a common transport pathway. In addition, after transformation a transmembrane pathway for the internalization of lipids from the plasma membrane is expressed in WI-38 cells. We suggest that previously reported inconsistencies concerning the pathways of plasma membrane lipid internalization from the plasma membrane may have resulted from the use of different cultured cell lines as model systems.

**Materials and methods**

**Cell culture**

Baby hamster kidney (BHK), normal and N-acetylgalcosamine transferase-I-deficient Chinese hamster ovary (CHO-K1, CHO-15B), Vero (African Green monkey), Madin-Darby canine kidney (MDCK) and WI-38 (human lung fibroblast) cells were obtained from the American Type Culture Collection. A strain of WI-38 cells transformed with simian virus 40 (SV40) (VA-2 cells) was graciously provided by Dr M. Ediden, Johns Hopkins University.

BHK, MDCK and Vero cells were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum. CHO-K1, CHO-15B, VA-2 and WI-38 cells were grown in alpha plus minimal essential medium supplemented with 5% fetal calf serum. V79 cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. All tissue culture media and fetal calf serum was obtained from Gibco Laboratories (Grand Island, NY). For use in microscopy, cells were grown for 24-48 h on acid-washed glass coverslips; otherwise, cells were grown on plastic culture dishes.

**Lipids and lipid vesicles**

In previous studies we have used the fluorescent probe (acyl, C\textsubscript{6}-NBD)-PC to follow the bulk movement of lipids from the plasma membrane in V79 cells (Sleight & Pagano, 1984; Pagano & Sleight, 1985). This probe is prepared starting with egg phosphatidylcholine. Since egg PC has approximately 70% palmitic, 24% stearic and 5% oleic acid in the 1-position, (acyl, C\textsubscript{6}-NBD)-PC is actually a mixture of different lipid species. For this study we have used a single fluorescent PC species, (palmitoyl, C\textsubscript{6}-NBD)-PC. To ensure that (palmitoyl, C\textsubscript{6}-NBD)-PC had the same properties as (acyl, C\textsubscript{6}-NBD)-PC, control experiments using V79 cells were performed. The results of these control experiments were in every instance identical to those obtained using (acyl, C\textsubscript{6}-NBD)-PC (compare Results, with those of Sleight & Pagano, 1984).

Initial experiments were performed using (palmitoyl, C\textsubscript{6}-NBD)-PC prepared by the method of Longmuir et al. (1985). For later experiments, (palmitoyl, C\textsubscript{6}-NBD)-PC was purchased from Avanti Biochemicals (Birmingham, AL). Identical results were obtained using both preparations. Dioleoylphosphatidylcholine (DOPC) and (acyl, C\textsubscript{6}-NBD)-PC were obtained from Avanti Biochemicals. Small unilamellar lipid vesicles were prepared by ethanol injection as described (Kremer et al. 1977). The concentration of lipid dissolved in ethanol was 14 mM. Vesicle preparations were dialyzed overnight against hydroxethyl piperazineethane sulfonic acid-buffered, Eagle's minimal essential medium (HMEM) before use.

Cells were harvested for lipid extraction using a rubber policeman and then extracted by the procedure of Bligh & Dyer (1959), using 0.9-9% NaCl, 10 mM-HCl in the aqueous phase. Lipid extracts were analyzed qualitatively by thin-layer chromatography on Silica Gel 60 thin-layer plates (Merck & Co., Inc., Rahway, NJ) in CHCl\textsubscript{3}/CH\textsubscript{3}OH/28% NH\textsubscript{4}OH (65:35:5, by
The amount of (palmitoyl, C₆-NBD)-PC in the extracts was determined by reference to standard curves produced by analysis of known amounts of the compound (excitation maximum = 470 nm; emission maximum = 530 nm) in CHCl₃/CH₃OH (2:1, v/v).

**Vesicle–cell incubations**

For microscopy, monolayer cultures grown on glass coverslips were washed three times with HMEM and chilled for 5 min on an ice-water bath. Lipid vesicles were then added to the dishes at a final concentration of 50 μm (with respect to lipid phosphorus) and the incubation at 2°C was continued for 30 min. The cells were then washed three times with HMEM. In some experiments the washed cells were further incubated at 37°C for 45 min and then washed three times with HMEM.

For biochemical experiments, incubation conditions were identical to those described above, except that monolayer cultures grown on plastic culture dishes were used. After the appropriate incubation, cells were harvested using a rubber policeman and samples were removed for determination of DNA content (Leyva & Kelly, 1974) and for lipid extraction.

**Back exchange**

To facilitate the detection of cytoplasmic fluorescence, the fluorescent lipid in the plasma membrane was removed by back exchange (Struck & Pagano, 1980; Sleight & Pagano, 1984). Initially this procedure was performed as described (Sleight & Pagano, 1984). Cells were incubated at 2°C in HMEM containing 0.2 mM-DOPC vesicles for 10 min. The vesicles were then removed by three washes with HMEM. The 10-min incubation and washing procedure was repeated a total of three times. In later experiments the incubation with liposomes was replaced by an incubation with HMEM supplemented with 10% calf serum.

**Miscellaneous procedures**

Phospholipid concentrations were determined by the procedure of Rouser et al. (1966). DNA was determined with diphenylamine (Leyva & Kelly, 1974) using salmon sperm DNA as a standard. Fluorescence microscopy was performed with a Zeiss IM-35 inverted microscope equipped with a planapochromatic 100× (1-3 n.a.) objective and barrier filters that allowed no crossover of NBD and rhodamine fluorescence.

**Results**

**Insertion of (palmitoyl, C₆-NBD)-PC into the plasma membrane**

When liposomes composed of 40 mole% (palmitoyl, C₆-NBD)-PC and 60 mole% DOPC were incubated at 2°C with eight cell types, some of the fluorescent probe spontaneously transferred to the cells. The amount of (palmitoyl, C₆-NBD)-PC inserted into the various cell lines after a 30-min incubation with the fluorescent liposomes differed by as much as 3-8-fold (VA-2 versus MDCK, Table 1). We have observed that neither the shape (rounded versus flattened) nor the amount of native phospholipid per cell is directly related to the rate of (palmitoyl, C₆-NBD)-PC insertion.

With all cell types tested, the insertion of (palmitoyl, C₆-NBD)-PC at 2°C occurred at the plasma membrane. Photomicrographs of fluorescently labeled VA-2, WI-38 and CHO-K1 cells are presented in Figs 1A, 1B and 6A, respectively. These photomicrographs are representative of the results obtained with all cell types. As long as the cells were maintained at 2°C, none of the fluorescent PC entered the cells.

After inserting the (palmitoyl, C₆-NBD)-PC at 2°C, greater than 95% of the fluorescent lipid could be removed from all cell types, except Vero cells, by the back-exchange procedure. That is, when the cells were incubated in the presence of nonfluorescent liposomes or medium containing 10% calf serum, (palmitoyl, C₆-NBD)-PC spontaneously dissociated from the plasma membrane and became associated with the acceptor liposomes or serum. When Vero cells were subjected to the back-exchange procedure, between 8 and 11% of the (palmitoyl, C₆-NBD)-PC could not be removed from the cells.

The fluorescent lipid that becomes associated with cells during incubations with lipid vesicles containing (palmitoyl, C₆-NBD)-PC is the result of either the insertion of lipid monomers into the plasma membrane or the adsorption of intact liposomes to the cell surface (Pagano et al. 1983; Sleight & Pagano, 1984). To determine the amount of fluorescent PC associated with cells due to vesicle adsorption, donor vesicles were prepared containing 1–4 mole% N-Rh-PE or N-sh-Rh-PE (Rh, rhodamine), nonexchangeable fluorescent lipids (Struck & Pagano, 1980). Knowing the amount of rhodamine-labeled PE associated with the cells and the initial ratio of fluorescence between (palmitoyl, C₆-NBD)-PC and rhodamine-labeled PE, one can calculate the maximal amount of (palmitoyl, C₆-NBD)-PC associated with the cells by vesicle adsorption (Pagano et al. 1983; Sleight & Pagano, 1984). The

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**Table 1. Insertion, degradation and internalization of (palmitoyl, C₆-NBD)-PC**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>% Degraded after 45 min at 37°C†</th>
<th>% Resistant to back exchange after 45 min at 37°C†</th>
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<tbody>
<tr>
<td>BHK</td>
<td>27</td>
<td>39</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>57</td>
<td>27</td>
</tr>
<tr>
<td>CHO-15B</td>
<td>52</td>
<td>43</td>
</tr>
<tr>
<td>MDCK</td>
<td>40</td>
<td>71</td>
</tr>
<tr>
<td>V79</td>
<td>36</td>
<td>43</td>
</tr>
<tr>
<td>VA-2</td>
<td>41</td>
<td>61</td>
</tr>
<tr>
<td>Vero</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>WI-38</td>
<td>33</td>
<td>32</td>
</tr>
</tbody>
</table>

* Cultures were incubated for 30 min at 2°C with lipid vesicles containing 40 mole% (palmitoyl, C₆-NBD)-PC and then the amount of fluorescent lipid transferred to the cells was determined. To normalize for variation in cell density, the amount of fluorescence in each dish was divided by the total amount of DNA from each dish. The data represent the mean ± s.d. of the number of assays listed in parenthesis.
† To assess the cells' ability to degrade the fluorescent lipid, the amount of fluorescent lipid remaining in the cells after a 45-min incubation at 37°C was determined. In some instances, all incubations were carried out with nonfluorescent liposomes or serum. When Vero cells were subjected to the back-exchange procedure, between 8 and 11% of the fluorescent PC present after back exchange divided by the amount of fluorescent PC present prior to back exchange, multiplied by 100%.

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maximum amount of \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) attributable to adsorption in all cell types was less than 1%, except for Vero cells. Typically, 16–20% of the fluorescent PC associated with Vero cells at 2°C could be attributed to vesicle adsorption. After back exchange, less than 1% of the \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) that remained associated with Vero cells could be attributed to vesicle adsorption.

**Internalization of \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) from the plasma membrane**

After inserting the \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) into the cells at 2°C and warming the cells to 37°C for 45 min, several different patterns of intracellular fluorescent labeling were observed (Figs 2, 3). For comparison, the appearance of the various cell types when incubated with a fluorescent analog of ceramide, a vital stain for the Golgi apparatus (Lipsky & Pagano, 1983, 1985), is also shown. The pattern of internal \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) fluorescence varied greatly among cell types, with the following four patterns of labeling observed: (1) little or no internalization of \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) into punctate vesicles was observed in Vero cells (Fig. 2). (2) Transport of \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) to the region of the Golgi apparatus and a small number of intracellular vesicles was observed in both V79 and CHO-K1 cell lines (Fig. 2). (3) A large number of fluorescently labeled intracellular vesicles with little or no labeling in the region of the Golgi apparatus appeared after the internalization of \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) in BHK, CHO-15B, MDCK and WI-38 cell lines (Figs 2, 3). (4) Accumulation of \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) in small vesicles, mitochondria and the nuclear envelope was observed in VA-2 cells (Fig. 3).

Different patterns of intracellular labeling were often observed in cells treated with \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) compared with those treated with \(C_6\text{-NBD-ceramide}\) (Figs 2, 3), suggesting that the fluorescent phosphatidylcholine was not delivered to the Golgi apparatus in all cell types. Similar labeling by the two fluorescent compounds occurred only in the V79 and CHO-K1 cell lines (Fig. 2). Co-localization of internalized \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) and the Golgi apparatus has been shown in V79 cells (Sleight & Pagano, 1984). Experiments co-localizing internalized fluorescent lipid and the Golgi apparatus experiments in CHO-K1 and V79 cells were performed side-by-side using rhodamine-labeled wheat germ agglutinin to label the Golgi apparatus. As shown in Fig. 4, although the internalized fluorescent PC in CHO-K1 cells is located in a region of the cell rich in Golgi membranes, there are at least some components of the Golgi apparatus that do not become fluorescently labeled. As seen previously (Sleight & Pagano, 1984), this differential labeling was not apparent in V79 cells (data not shown). When similar co-localization experiments were performed using Vero, CHO-15B, MDCK, BHK, WI-38 and VA-2 cells, little or no co-localization of the Golgi apparatus and fluorescent lipid was observed (data not shown).

**Mechanism of \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC internalization}**

To determine the effect of blocking endocytosis on the internalization of the fluorescent lipid at 37°C, cells were incubated in the presence of 5 mM-sodium azide and 50 mM-2-deoxyglucose, two energy poisons known to inhibit endocytosis (Sleight & Pagano, 1983). Under these conditions internalization of \((\text{palmitoyl, } C_6\text{-NBD})\text{-PC}\) was completely blocked in all cell types except VA-2 cells (Fig. 5). The inability of the fluorescent PC to enter the cells at low temperature and under conditions that...
Fig. 2. Fluorescence photomicrographs of cells treated with either (palmitoyl, C₆-NBD)-PC or C₆-NBD-ceramide at 2°C, washed and warmed to 37°C. V79, Vero, CHO-K1 and CHO-15B cells were grown on glass coverslips. Cells were incubated with liposomes containing either 40 mole% (palmitoyl, C₆-NBD)-PC or 20 mole% C₆-NBD-ceramide at 2°C for 30–60 min, washed to remove the liposomes, and then incubated further at 37°C for 45 min. Cells treated with (palmitoyl, C₆-NBD)-PC were then incubated with DOPC vesicles at 2°C prior to photography to reduce the amount of fluorescence at the plasma membrane (Struck & Pagano, 1980). Bar, 10 μm.

Table 2. Endocytic rates of cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Fluid uptake (nl min⁻¹ (mg DNA)⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>BHK</td>
<td>7.5 ± 1.7 (12)</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>8.6 ± 1.9 (48)</td>
</tr>
<tr>
<td>CHO-15B</td>
<td>12.9 ± 1.4 (7)</td>
</tr>
<tr>
<td>MDCK</td>
<td>5.0 ± 0.6 (4)</td>
</tr>
<tr>
<td>V79</td>
<td>2.9 ± 0.2 (12)</td>
</tr>
<tr>
<td>VA-2</td>
<td>5.4 ± 1.6 (12)</td>
</tr>
<tr>
<td>Vero</td>
<td>1.4 ± 0.2 (4)</td>
</tr>
<tr>
<td>WI-38</td>
<td>3.7 ± 1.4 (12)</td>
</tr>
</tbody>
</table>

Endocytic rates were determined by measuring the uptake of horseradish peroxidase as described (Steinman & Cohn, 1972; Adams et al. 1982). The data represent the mean ± s.d. of the number of assays listed in parenthesis.

lower ATP levels 19% (Martin & Pagano, 1987) suggests that the lipid enters cells via endocytic vesicles.

Endocytic rates for the different cell types were measured using horseradish peroxidase (Table 2). Except for Vero and CHO-15B cells, the endocytic rates of the various cell lines were found to be somewhat similar. Therefore, rates of endocytosis cannot, in most instances, explain the differences observed in the internalization of lipids from the plasma membrane. The exception to this rule is exemplified by Vero cells, where the extremely slow rate of endocytosis corresponds to the cells’ inability to internalize much of the fluorescent probe.

The fraction of fluorescent lipid present inside cells following 45 min of internalization at 37°C was measured by the back-exchange procedure. After inserting the lipid at 2°C and allowing the lipid to be internalized at 37°C, the amount of fluorescence present per μg DNA was determined before and after performing the back-exchange procedure. As displayed in Table 1, the fraction of fluorescent PC associated with intracellular organelles ranged from a low value of 16% to a high value of 71%. As stated previously, the back-exchange procedure was at least 95% efficient in removing the fluorescent PC from the plasma membranes of all cell types except Vero cells. In Vero cells, 16% of the fluorescent lipid was resistant to back exchange after incubating the cells for

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45 min at 37°C. This amount of internalized (palmitoyl, C₆-NBD)-PC may appear high when compared with the small amount of internalized lipid observed microscopically (Fig. 2). However, these data are consistent. As stated earlier, 8–11% of the fluorescent PC located in the plasma membrane of Vero cells at 2°C is resistant to removal by the back-exchange procedure.

Metabolism of (palmitoyl, C₆-NBD)-PC
When cells previously incubated with (palmitoyl, C₆-NBD)-PC-containing vesicles at 2°C were warmed to 37°C for 45 min, some degradation of the fluorescent lipid occurred (Table 1). The amount of degradation was dependent on cell type, and did not appear to be related to the amount of fluorescent probe initially inserted. In all cell types, the only fluorescent lipid present in the cells after the 37°C incubation was (palmitoyl, C₆-NBD)-PC. Cellular degradation of this lipid resulted in the release of C₆-NBD-fatty acid into the medium (data not shown). The fluorescent fatty acid was not reutilized by the cells for the synthesis of new fluorescent lipids or in the acylation of proteins.

To determine if endocytosis affected (palmitoyl, C₆-NBD)-PC degradation, cells were incubated at 37°C in the presence or absence of 2-deoxyglucose plus sodium azide to inhibit endocytosis, and the rate of fluorescent PC degradation was measured. As presented in detail in Table 1, the rate of degradation of (palmitoyl, C₆-NBD)-PC was not significantly reduced by the presence of these inhibitors.

Comparison of (palmitoyl, C₆-NBD)-PC internalization in wild-type versus glycosylation-defective and transformed cell lines
The CHO-15B cell line, a mutant line derived from CHO-K1 cells, has a low level of N-acetylglucosamine transferase I activity and incompletely processes the carbohydrate moieties of glycoproteins to GlcNAc₂Man₅ (Gottlieb et al. 1975). We found that both CHO-K1 and CHO-15B cells degraded (palmitoyl, C₆-NBD)-PC at similar rates (Table 1), but their patterns of intracellular transport of the fluorescent lipid differed (Fig. 2). Although both cell lines internalized the fluorescent phosphatidylcholine, most of the internalized lipid in CHO-K1 cells accumulated in the region of the Golgi apparatus, while most of the fluorescent lipid remained in small vesicles in CHO-15B cells. When CHO-1021 cells, a mutant line with decreased levels of terminal sialic acid
residues (Briles et al. 1977), were examined, they were observed to internalize (palmitoyl, C₆-NBD)-PC in the same manner as wild-type cells (data not shown).

When CHO-K1 cells were incubated at 16°C, fluorescent phosphatidylcholine located at the plasma membrane was internalized and accumulated in a large number of small intracellular vesicles (Fig. 6B), resembling CHO-15B cells incubated at 37°C (Fig. 2). As long as the CHO-K1 cells were maintained at 16°C the fluorescent lipid remained associated with these vesicles and did not accumulate in the region of the Golgi apparatus. Blocking of (palmitoyl, C₆-NBD)-PC delivery to the region of the Golgi apparatus by incubation at 16°C may be analogous to the inhibition of endosome fusion with lysosomes at 16–18°C (Dunn et al. 1980; Steinman et al. 1983).

VA-2 cells internalized the fluorescent phosphatidylcholine analog in a unique manner (Fig. 3). This pattern of internal fluorescence resembles that reported previously using 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl-phosphatidylethanolamine ((acyl, C₆-NBD)-PE). This fluorescent analog of PE enters cells by undergoing transmembrane movement (flip-flop) at the plasma membrane, followed by the movement of lipid monomers from the inner leaflet of the plasma membrane to intracellular organelles (Sleight & Pagano, 1985). When fluorescent lipid monomers are allowed to move freely across the cytoplasm, they tend to accumulate in the mitochondria and nuclear envelope (Sleight & Pagano, 1985; Pagano & Sleight, 1985). The accumulation of (palmitoyl, C₆-NBD)-PC in the mitochondria of VA-2 cells was confirmed by colocalization with rhodamine-3b, a vital stain for mitochondria (data not shown).
Fig. 5. Effect of NEM treatment and lowered ATP levels on the internalization of (palmitoyl, C\textsubscript{6}-NBD)-PC in VA-2 cells. ATP-depletion (A) was performed by pretreating cells with 5 mM-NaN\textsubscript{3} and 50 mM-2-deoxyglucose in glucose-free HMMEM for 30 min at 37°C, and carrying out all subsequent incubations in glucose-free HMMEM containing the inhibitors. NEM treatment (B) was performed by pretreating cells with 0.5 mM-NEM in HMMEM at 2°C for 30 min. The cells were then washed and incubated with 0.5 mM-dithiothreitol in HMMEM at 2°C for 5 min prior to incubating with the fluorescent liposomes (Martin & Pagano, 1987). After insertion of (palmitoyl, C\textsubscript{6}-NBD)-PC, the cultures were then warmed to 7°C for 30 min, back-exchanged and photographed. This incubation was performed at 7°C to decrease the amount of lipid internalized via endocytosis. Bar, 20 μm.

In V79 cells, the transmembrane movement of fluorescent analogs of PE and PS (phosphatidylserine) (but not PC) appears to be dependent on the activity of a 'flipase' located in the plasma membrane (Martin & Pagano, 1987). This transmembrane movement has been demonstrated to be energy dependent and can be inhibited by N-ethylmaleimide (NEM) treatment. When V79 cells are treated with agents that lower intracellular ATP levels 83% or with NEM, movement of (palmitoyl, C\textsubscript{6}-NBD)-PE to the mitochondria and nuclear envelope is blocked (Martin & Pagano, 1987). To determine if a similar flipase, able to transport (palmitoyl, C\textsubscript{6}-NBD)-PC, was present in VA-2 cells, the effect of NEM treatment and lowered ATP levels on the internalization of the fluorescent probe was examined. As shown in Fig. 5, transmembrane movement and subsequent movement of the fluorescent PC to the mitochondria and nuclear envelope were not affected either by NEM treatment or by lowering ATP levels. However, lowering ATP levels did block the appearance of punctate fluorescent vesicles in the cells (Fig. 5A).

Discussion

Three general mechanisms are believed to be responsible for the intracellular transport of phospholipids between membranes: (1) vesicular transport; (2) monomer transport; and (3) lateral diffusion (see Sleight, 1987, for review). Microscopic visualization of these processes has been made possible by the development of fluorescent phospholipid analogs. In this study we have examined the transport of a PC analog having a fluorescently labeled acyl chain. We have observed that (palmitoyl, C\textsubscript{6}-NBD)-PC spontaneously transfers from liposomes to the plasma membranes of all eight cell types tested. It seems likely that in most instances this transfer was the result of diffusion of soluble (palmitoyl, C\textsubscript{6}-NBD)-PC monomers between the donor liposomes and acceptor plasma membranes. This mode of transport has been documented for the movement of (acyl, C\textsubscript{6}-NBD)-PC between liposome populations (Nichols & Pagano, 1981).

With the exception of Vero cells, we observed that very little of the cell-associated fluorescent PC was present as a result of the adsorption of liposomes to the cell surface. We are unable to explain why fluorescently labeled liposomes tend to adsorb to the surface of Vero cells at relatively high levels. However, most of the liposomes adsorbed to Vero cells could be removed by incubating the cells in serum-containing medium, suggesting a weak binding of the liposomes to the cells. Our inability to remove (palmitoyl, C\textsubscript{6}-NBD)-PC efficiently from the plasma membranes of Vero cells by the back-exchange procedure indicates that the fluorescent PC was either transported to the inner leaflet of the plasma membrane and was therefore not available for back exchange, or it was tenaciously bound to something evenly distributed within the plasma membrane. In this regard, the characteristics of (palmitoyl, C\textsubscript{6}-NBD)-PC insertion into the plasma membranes of Vero cells resembles that of (acyl, C\textsubscript{6}-NBD)-PS insertion into red blood cells (Schroit et al.)
Fig. 6. Effect of temperature on (palmitoyl, C₆-NBD)-PC internalization. CHO-K1 cells were incubated for 30 min at 2°C in the presence of 50 μM lipid vesicles containing 40 mole% (palmitoyl, C₆-NBD)-PC. The cultures were then washed and incubated for 1 h at either 2°C (A) or 16°C (B). After this incubation, the cells were incubated with DOPC vesicles at 2°C prior to photography to reduce the amount of fluorescence at the plasma membrane (Struck & Pagano, 1980). Bar, 10 μm.

1985). After insertion into the cell membrane, (acyl, C₆-NBD)-PS can neither be completely removed from red blood cell ghosts by back exchange nor react to completion with amino-reactive reagents (Schroit et al. 1985).

Two pieces of evidence suggest that (palmitoyl, C₆-NBD)-PC remained exclusively localized in the outer leaflet of the cells' plasma membrane after insertion at 2°C: (1) labeling at 2°C resulted in a homogeneous labeling of the plasma membrane. All of our previous experience using acyl-chain-labeled fluorescent lipid analogs suggests that when these lipids are located in membrane leaflets that face the cytosol they are able to transfer spontaneously to organelles (see Pagano & Sleight, 1985, for review). Since no internal labeling was observed, we believe that (palmitoyl, C₆-NBD)-PC did not undergo transmembrane movement at 2°C in any of the cell types tested. To determine whether some special property of any of the cell types used blocked this spontaneous transfer, the movement of lipid probes known to undergo transmembrane movement was examined. In all cases, (palmitoyl, C₆-NBD)-PE and (acyl, C₆-NBD)-PS were unable to transfer and brightly label the nuclear envelope and mitochondria (Sleight, unpublished observation). (2) The fluorescent PC could be quantitatively transferred from most cell types to recipient liposomes by the back-exchange procedure. Since this procedure depends on transfer via aqueous monomers, all the lipid leaving the cells must face the bathing medium before transfer.

The amount of (palmitoyl, C₆-NBD)-PC inserted into the plasma membranes of the six cell lines tested varied by as much as 3-8-fold. Neither cell size, shape, nor the amount of phospholipid per cell correlated directly with the observed differences in fluorescent PC uptake. It may be that the rate of the probe's insertion is dependent on the lipid composition of the cells' plasma membranes. This idea is supported by the finding that the rate of spontaneous (acyl, C₆-NBD)-PC transfer between donor and acceptor liposomes is dependent on the lipid composition of the acceptor (Nichols & Pagano, 1981). Since the relationship between the rate of fluorescent PC transfer between liposomes and proteoliposomes is unknown, it is difficult to assess what role membrane proteins might play in the insertion of (palmitoyl, C₆-NBD)-PC.

Two different mechanisms, endocytosis and transmembrane movement followed by monomer transport, appear to shuttle (palmitoyl, C₆-NBD)-PC into cells at 37°C. All eight of the cell lines examined appeared to internalize the fluorescent lipid via endocytosis, resulting in the appearance of labeled punctate vesicles and/or an accumulation of the probe in the region of the Golgi apparatus. This process could be blocked by incubating the cells at low temperature or by lowering intracellular ATP levels. Previously it was reported (Sleight & Pagano, 1984) that after being endocytosed into V79
cells, (acyl, C6-NBD)-PC was transferred to the Golgi apparatus. As shown in Fig. 4, although most of the internalized (palmitoyl, C6-NBD)-PC accumulated near the Golgi apparatus of CHO-K1 cells, there are portions of the stained Golgi apparatus that did not contain the fluorescent PC, and (palmitoyl, C6-NBD)-PC labeled membranes that were not labeled with the Golgi-specific stain. This suggests that some or all the fluorescent PC accumulated near, but not within, Golgi membranes. To determine the exact location of the fluorescent probe in relation to the Golgi apparatus, a technique for visualizing the probe at the level of the electron microscope must be developed.

Except for V79 and CHO-K1 cells, which accumulated most (palmitoyl, C6-NBD)-PC in the region of the Golgi apparatus, endocytic internalization of the fluorescent PC in the cell types tested resulted in the appearance of randomly located fluorescently labeled punctate vesicles. In BHK, CHO-15B, MDCK, Vero and WI-38 cells there does not appear to be an appreciable accumulation of the fluorescent lipid at the Golgi apparatus. There are several possible mechanisms that might explain why the different cell types endocytosed the fluorescent probe to different locations, these include: (1) intracellular degradation of the fluorescent lipids may occur at different organelles in different cell types. (2) The same endocytic pathways may exist in all cell types; however, various branches of this pathway may operate at different rates. (3) Completely different endocytic pathways may exist in the different cell types. (4) The same endocytic pathways may exist in all cells; however, different pathways may predominate in the different cells.

Of these possibilities, it does not appear that differential rates of lipid degradation play an important role in determining the intracellular distribution of internalized plasma membrane lipids. Although the rate of (palmitoyl, C6-NBD)-PC degradation varied between cell types (Table 1), no correlation between the rate of lipid degradation and the distribution of the internalized fluorescent lipid was discernible. In addition, the amount of (palmitoyl, C6-NBD)-PC internalized does not appear to be related to the intracellular distribution of the fluorescent lipid. We have demonstrated that the rate of (palmitoyl, C6-NBD)-PC degradation is not affected by incubation conditions that block endocytosis (Table 1). Therefore, if in addition to the plasma membrane, intracellular sites of (palmitoyl, C6-NBD)-PC degradation exist, they must either be: (1) sites that are never visualized because they rapidly degrade the fluorescent lipid that is delivered to them; or (2) sites that degrade (palmitoyl, C6-NBD)-PC at the same rate as the plasma membrane. If intracellular sites exist that rapidly degrade (palmitoyl, C6-NBD)-PC, only very small amounts of the fluorescent lipid must be delivered to these sites. Otherwise we would expect blocking endocytosis to decrease the rate of (palmitoyl, C6-NBD)-PC degradation. Thus it seems unlikely that a major internal site of (palmitoyl, C6-NBD)-PC delivery was masked.

The second possible explanation for differences observed in (palmitoyl, C6-NBD)-PC internalization is that, although the same endocytic pathways exist in all cell types, they operate at different rates. We have presented photographs demonstrating the intracellular location of internalized fluorescent PC after a 45-min incubation at 37°C (Figs 2, 3). If endocytosis and/or intracellular sorting processes occurred at unequal rates and steady-state conditions had not been reached, the photographs could represent different stages of a single pathway. In fact, this does not appear to be true. We have examined the internalization process in each cell type for up to 3h, and have observed that within 20min a characteristic pattern of internal fluorescence became apparent. Photographs were taken after 45-min incubations at 37°C, because the intensity of the fluorescent lipid present in the region of the Golgi apparatus in V79 and CHO-K1 cells was most intense. After this time there was a gradual decrease in the fluorescent intensity of all labeled membranes. These findings suggest that the photomicrographs were taken under steady-state conditions. The only relationship we have observed between rates of endocytosis and internalization of (palmitoyl, C6-NBD)-PC was quantitative rather than temporal. As stated above, the very low rate of endocytosis in Vero cells corresponded to these cells' inability to internalize much of the fluorescent PC.

At present we are unable to determine unambiguously whether the same pathway(s) of endocytosis and/or intracellular sorting exist in all cells and different branches of the pathway(s) predominate, or whether completely different pathways exist. To distinguish between these possibilities, it will be necessary to differentiate between individual linear pathways and the branches of complex pathways present in a single cell type. Towards this end, we have sought means of altering the normal internalization of plasma membrane lipids. We have observed significant differences in the internalization of (palmitoyl, C6-NBD)-PC in wild-type cells compared with cells having a defect in N-acetylglycosamine transferase I (Fig. 2). The distribution of the fluorescent PC in wild-type cells resembled the distribution of (palmitoyl, C6-NBD)-PC in the glycosylation-defective CHO-15B cells only when intracellular transport in CHO-K1 cells was allowed to occur at 16°C (compare Fig. 2 and Fig. 1B). Since incubation at 16°C blocked the internalized fluorescent PC from accumulating near the Golgi apparatus, it may be that the altered glycoproteins of CHO-15B cells caused an inefficient delivery of internalized plasma membrane to the region of the Golgi apparatus. When CHO-1021 cells, a mutant line having decreased levels of terminal sialic acid residues (Briles et al. 1977), were examined, they were observed to internalize (palmitoyl, C6-NBD)-PC in the same manner as wild-type cells. Taken together, these data suggest that glycosylation somehow influences transport.

In addition to the glycosylation mutant, we have identified a second cell line that internalized (palmitoyl, C6-NBD)-PC in a manner unlike its wild-type parent. VA-2 cells are a strain of WI-38 cells that have been transformed with SV40. When incubated at 37°C, the fluorescent PC moved from the plasma membranes of VA-2 to punctate intracellular vesicles, mitochondria and...
the nuclear envelope (Fig. 3). This pattern of internalization mimics exactly that observed previously using (acyl, C₆-NBD)-PE (Sleight & Pagano, 1985). It has been demonstrated (Sleight & Pagano, 1985) that (acyl, C₆-NBD)-PE enters cells by two processes: (1) the endocytic internalization of fluorescently labeled plasma membrane; (2) transmembrane movement (flip-flop) of the fluorescent lipid across the plasma membrane, followed by movement of lipid monomers from the outer leaflet of the plasma membrane to intracellular organelles. Transmembrane movement of the fluorescent PE is thought to be catalyzed by a flipase located at the plasma membrane (Martin & Pagano, 1987). It should be noted that neither (palmitoyl, C₆-NBD)-PC nor (acyl, C₆-NBD)-PE have been observed to undergo transmembrane movement in artificial vesicles.

The transport of (palmitoyl, C₆-NBD)-PC into VA-2 cells and (acyl, C₆-NBD)-PE into other cell types appears very similar in some respects: (1) both lipids label the same organelles after transport. (2) Both lipids appear to undergo transmembrane movement followed by monomer movement to mitochondria and the nuclear envelope under conditions that block endocytosis (e.g. low ATP levels, Fig. 5). A noticeable difference in the transport of the two lipid probes is their mechanisms for movement across the plasma membrane. The imputed flipase that transports (acyl, C₆-NBD)-PE across the plasma membrane can be inhibited by drastic lowering of cellular ATP or by treating living cells with NEM (Martin & Pagano, 1987). We have observed that under conditions that block the transmembrane movement of (acyl, C₆-NBD)-PE, no inhibition of (palmitoyl, C₆-NBD)-PC across the plasma membrane and into intracellular organelles occurs (Fig. 5). Several laboratories have reported changes in membrane lipid composition and cell membrane function after cell transformation (de Laat et al., 1984; van Blitterswijk, 1984). Whether an altered physical/chemical property of the plasma membrane or a change in membrane function after cell transformation (de Laat et al., 1984; van Blitterswijk, 1984). Whether an altered physical/chemical property of the plasma membrane or a different flipase permits the transmembrane movement of the fluorescent PC remains unknown.

Our finding that cells defective in glycosylation and transformed cells have different patterns of intracellular (palmitoyl, C₆-NBD)-PC labeling from their parental lines suggests that it may be possible to isolate and study specific portions of the pathway(s) of lipid transport in a given cell line. However, it is likely that these investigations will be extremely complex because the parental and mutant cell lines undoubtedly have numerous differences.

In summary, we have demonstrated that the internalization of (palmitoyl, C₆-NBD)-PC from the plasma membrane resulted in different labeling patterns, depending on the cell type tested. Although in VA-2 cells it appears that (palmitoyl, C₆-NBD)-PC undergoes transmembrane movement, we believe that, in general, (palmitoyl, C₆-NBD)-PC acts as a marker of bulk lipid movement from the outer leaflet of the plasma membrane. Since it is unlikely that phospholipids are sorted in response to signals encoded within their structure, it has been difficult to explain the seemingly contradictory descriptions of lipid transport reported from several laboratories (reviewed by Sleight, 1987). We suggest that these inconsistencies may have resulted from different cell types having dissimilar pathways of intracellular lipid trafficking or differential regulation of a common transport pathway. Since few investigations have been performed examining intracellular lipid transport, different model systems have often been compared. This study suggests that postulates made from these comparisons may be invalid, and that great caution must be used in both designing and interpreting studies of intracellular lipid transport.

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References


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