Transport of a Fluorescent Phosphatidylcholine Analog from the Plasma Membrane to the Golgi Apparatus

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ABSTRACT We have examined the internalization and degradation of a fluorescent analog of phosphatidylcholine after its insertion into the plasma membrane of cultured Chinese hamster fibroblasts. 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine (C_6-NBD-PC) was incorporated into the cell surface by liposome–cell lipid transfer at 2°C. The fluorescent lipid remained localized at the plasma membrane as long as the cells were kept at 2°C; however, when the cells were warmed to 37°C, internalization of some of the fluorescent lipid occurred. Most of the internalized C_6-NBD-PC accumulated in the Golgi apparatus although a small amount was found randomly distributed throughout the cytoplasm in punctate fluorescent structures. Internalization of the fluorescent lipid at 37°C was blocked by the presence of inhibitors of endocytosis.

Incubation of cells containing C_6-NBD-PC at 37°C resulted in a rapid degradation of the fluorescent lipid. This degradation occurred predominantly at the plasma membrane. The degradation of C_6-NBD-PC resulted in the release of NBD-fatty acid into the medium.

We have compared the internalization of the fluorescent lipid with that of a fluorescent protein bound to the cell surface. Both fluorescent lipid and protein remained at the plasma membrane at 2°C and neither were internalized at 37°C in the presence of inhibitors of endocytosis. However, when incubated at 37°C under conditions that permit endocytosis, the two fluorescent species appeared at different intracellular sites.

Our data suggest that there is no transmembrane movement of C_6-NBD-PC and that the fluorescent probe reflects the internalization of the outer leaflet of the plasma membrane lipid bilayer. The results are consistent with the Golgi apparatus as being the primary delivery site of phospholipid by bulk membrane movement from the plasma membrane.

Although our knowledge of the synthesis, transport, and recycling of plasma membrane proteins has increased greatly (6, 7, 23), little is known about the assembly, transport, and recycling of plasma membrane lipids. This is because studies of the distribution and movement of lipid molecules cannot be performed using the methods available for the study of protein metabolism and translocation at the subcellular level. To begin to examine the movement of lipid molecules in vivo, our laboratory has followed the fate of fluorescent lipid analogs supplied to cultured cells (12, 16, 17, 22, 25).

To examine the movements of phosphatidylcholine located in the plasma membrane, we have used 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl phosphatidylcholine (C_6-NBD-PC)[1] as a probe. This fluorescent analog of phosphatidylcholine (PC) spontaneously transfers between liposomes and cells at 2°C (18, 25). Previous studies have demonstrated that C_6-NBD-PC properly integrates into the plasma membrane, and probably resides in the outer leaflet of the membrane's lipid bilayer (25). We report here that, when C_6-NBD-PC is inserted into the plasma membrane of Chinese hamster fibroblasts at 2°C and the cells are warmed to 37°C, some of the fluorescent lipid is internalized. To determine the

[1] *Abbreviations used in this paper*: C_6-NBD-PC, 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; HCMF, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid-buffered Puck's saline (without calcium and magnesium); HMEM, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid-buffered, Eagle's minimal essential medium, pH 7.4, without indicator; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine; PC, phosphatidylcholine; Rh-LCA, rhodamine-labeled *Lens culinaris* agglutinin; Rh-WGA, rhodamine-labeled wheat germ agglutinin.

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destination of the internalized lipid, we have optimized the quantity of C_6-NBD-PC inserted into the plasma membrane at 2°C and followed, both microscopically and biochemically, its fate in cells incubated at 37°C. Our results suggest that the fate of the fluorescent lipid is at least partially determined by its inability to undergo transmembrane movement (flip-flop) across the membrane bilayer.

In this report, we have also compared the internalization of C_6-NBD-PC with the internalization of a fluorescent lectin bound to the cell surface. Our results demonstrate that the two fluorescent molecules are separated during the internalization processes.

MATERIALS AND METHODS

Cell Culture: Monolayer cultures of Chinese hamster V79 lung fibroblasts (8) were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum in a water-saturated atmosphere of 5% CO_2 in air. For use in microscopy, cells were grown for 24 h on acid-washed glass coverslips; otherwise, cells were grown on plastic culture dishes. Cell suspensions were prepared from monolayer cultures by treatment with 0.05% trypsin in hydroxyethyl piperazine ethane sulfonic acid-buffered Puck's saline without calcium and magnesium (HCMF) for 12 min at 37°C.

Lipids and Lipid Vesicles: Dioleoylphosphatidylcholine (DOPC) and C_6-NBD-PC were obtained from Avanti Biochemicals (Birmingham, AL). The C_6-NBD-PC was prepared from egg PC having a mixture of 70% C16:0, 24% C18:0, and 5% C18:1 fatty acids in the 1 position. The lipids were periodically monitored for purity by thin-layer chromatography and were repurified as necessary. Lipid vesicles were prepared by ethanol injection as described by Kremer et al. (10). The concentration of lipid dissolved in ethanol was 14 mM. Vesicle preparations were dialyzed overnight against hydroxyethyl piperazine ethane 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 and Dyer (3), using 0.9% 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 $CHCl_3/CH_3OH/28\%$ NH_4OH (65:35:5). The amount of fluorescent lipid in the extracts was determined by reference to standard curves produced by analysis of known amounts of C_6-NBD-PC or *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (*N*-Rh-PE) in $CHCl_3/CH_3OH$ (2:1).

Transfer of Vesicle Lipid to Recipient Cells: Monolayer cultures were washed and then incubated at 2°C with HMEM for 10 min. Lipid vesicles were then added to the desired concentration. The incubations were then continued at 2°C for the desired amount of time with the culture dishes being swirled gently every 10 min.

Incubations were stopped by washing the cells three times with cold HCMF, followed by three washes with cold HMEM. In some experiments the cells were warmed to 37°C in an air incubator. After incubation at 37°C, the cells were washed with HMEM. To enhance the appearance of cytoplasmic fluorescence, the fluorescent lipid in the plasma membrane was removed by incubating the cells with nonfluorescent liposomes (back exchange) before photography (25). This was accomplished by incubating the cells at 2°C in HMEM that contained 0.2 mM DOPC vesicles for 10 min. The vesicles were then removed by washing with HCMF followed by three washes with HMEM at 2°C. In most instances, the 10-min back exchange was repeated three times before photography. Control experiments were performed in which cells incubated only at 2°C with C_6-NBD-PC were back-exchanged. In these control cells, no internal fluorescence was observed and only dim fluorescence at the plasma membrane was seen (Fig. 1).

Determination of Quantum Yield in Cells: To determine if quenching of the fluorophore occurred during the internalization of C_6-NBD-PC, 5×10^5 cells/ml were suspended in HMEM that contained 50 μM lipid vesicles composed of 40 mol % C_6-NBD-PC and 60 mol % DOPC. After incubation at 2°C for 30 min, the cells were washed three times in cold HCMF with the last wash carried out in a new tube. The cells were then incubated at 37°C for various times and washed three times with cold HCMF, and their NBD fluorescence was determined in an Aminco-Bowman spectrofluorometer (American Instrument Co., Silver Spring, MD) equipped with crossed polarizers to reduce light scattering. Fluorescence was determined in the presence and absence of 1% Triton X-100. Viability of the cells was monitored using Trypan Blue (20) and remained >95% throughout the experiment.

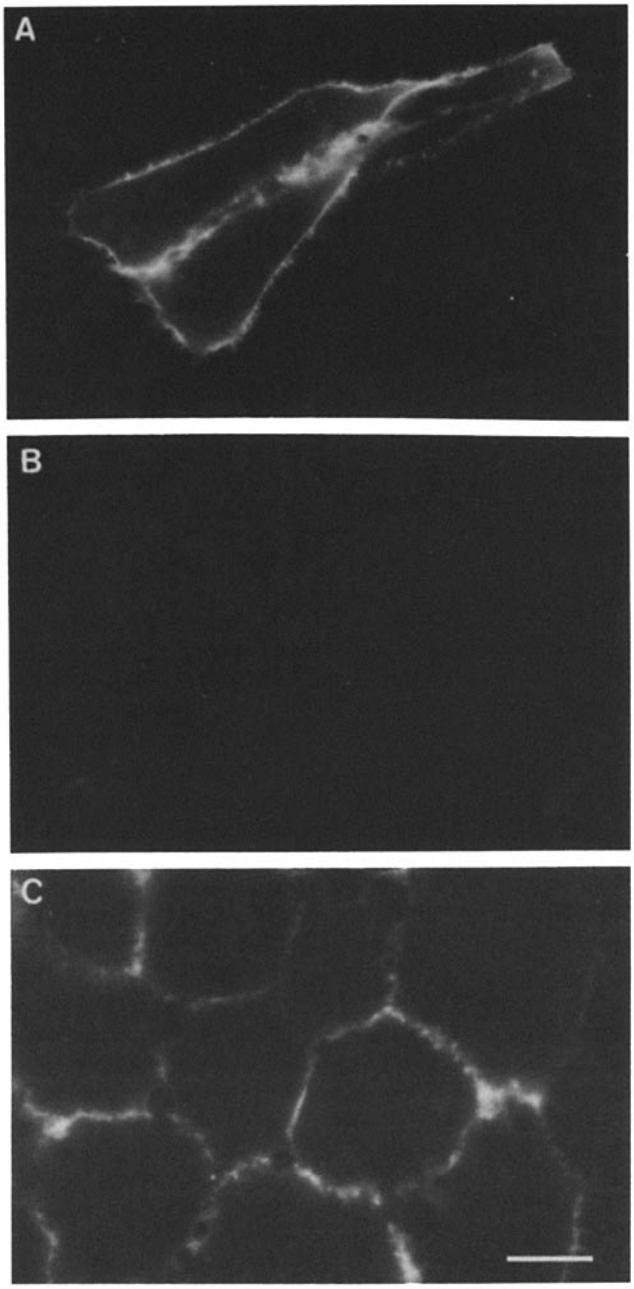


FIGURE 1 Reduction of plasma membrane fluorescence by back exchange. Cultures were incubated at 2°C with lipid vesicles containing 40 mol % C_6-NBD-PC. The cells were then washed with HCMF and either photographed or subjected to the back-exchange procedure at 2°C, as described under Materials and Methods, and then photographed. The appearance of cells before (*A*) and after (*B* and *C*) back exchange are presented. The photographs presented in *A* and *B* were taken and printed under exactly the same exposure conditions. In *C*, the same negative used in *B* was printed so as to enhance the appearance of the plasma membrane fluorescence (same exposure time with the lens opened 3 f-stops). Bar, 10 μm.

Miscellaneous Procedures: DNA was determined with diphenylamine (11) using salmon sperm DNA as a standard. Lipid concentrations were determined by the procedure of Rouser et al. (21). Staining of the Golgi apparatus was performed as described previously (12, 13, 28). Rabbit IgG directed specifically against the Golgi apparatus was kindly provided by Daniel Louvard (Pasteur Institute, Paris). Fluorescence microscopy was performed with a Zeiss inverted microscope (Carl Zeiss, Inc., New York) equipped with barrier filters that allowed no crossover of NBD and rhodamine fluorescence.

Fluorescent lectins were purchased from Vector Laboratories, Inc. (Burlingame, CA).

RESULTS

Insertion of C_6-NBD-PC into the Plasma Membrane

We have performed a series of experiments to optimize the insertion of fluorescent PC from liposomes into living cells. The effect of altering three parameters of C_6-NBD-PC insertion are presented in Fig. 2. Increasing the concentration of the liposomes, the proportion of C_6-NBD-PC in the liposomes, or the length of the 2°C incubation resulted in an increase in the amount of C_6-NBD-PC transferred to the cells. By incubating cells with 50 μM lipid vesicles composed of 40 mol % C_6-NBD-PC for 1 h, we were able to increase the amount of fluorescent PC delivered to the cells by >100-fold as compared with previous studies (25).

The fluorescence associated with cells incubated in the presence of lipid vesicles that contain C_6-NBD-PC may result from either the insertion of lipid monomers into the plasma membrane (12, 14, 16, 17, 19, 22, 25) or by the adsorption of intact liposomes to the cell surface (19, 24–26). To determine the amount of fluorescent PC associated with cells due to vesicle adsorption, all vesicle preparations contained 2 mol % *N*-Rh-PE, a nonexchangeable fluorescent lipid (18, 24, 25). Knowing the amount of *N*-Rh-PE associated with the cells and the initial ratio of fluorescence between C_6-NBD-PC and *N*-Rh-PE allowed us to calculate the maximal amount of C_6-NBD-PC associated with the cells by vesicle adsorption. Increasing the concentration of vesicles incubated with the cells or the length of incubation with vesicles increased the amount of adsorbed vesicles only slightly (data not shown). In contrast, increasing the proportion of C_6-NBD-PC in the vesicles resulted in a decrease in vesicle adsorption (Fig. 2*B*). The maximum amount of C_6-NBD-PC attributable to adsorption throughout this report was <0.65% of the total fluorescent PC present.

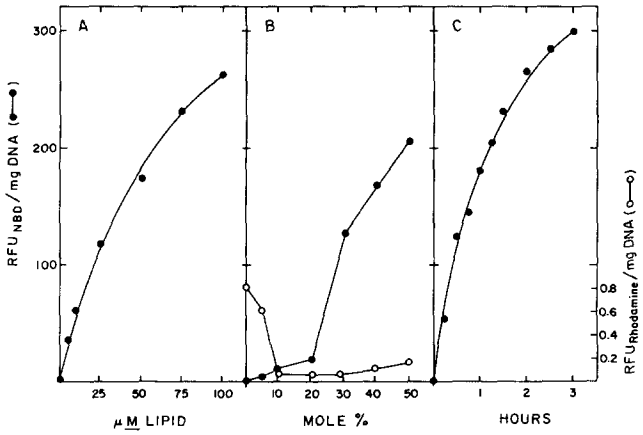


FIGURE 2 Transfer of C_6-NBD-PC from liposomes to cells. Cultures were incubated at 2°C with lipid vesicles containing C_6-NBD-PC, DOPC, and 2 mol % *N*-Rh-PE, and then the amount of fluorescent lipid transferred to the cells was determined. The abscissa represents the total amount of *N*-Rh-PE (○) or the amount of C_6-NBD-PC (●) transferred to the cells (i.e., the amount of C_6-NBD-PC associated with the cells due to vesicle adsorption has been subtracted). To normalize for variations in cell density, the amount of fluorescence in each dish was divided by the total amount of DNA from each dish. Data points represent the average of two determinations. In *A*, cells were incubated with vesicles containing 40 mol % C_6-NBD-PC for 1 h. In *B*, cells were incubated with 50 μM lipid vesicles for 1 h. In *C*, cells were incubated with 50 μM lipid vesicles containing 40 mol % C_6-NBD-PC.

Fig. 3*A* shows the results of incubating Chinese hamster fibroblasts grown on glass coverslips with lipid vesicles containing C_6-NBD-PC. Fluorescence was detected only at the plasma membrane of cells incubated at 2°C, consistent with previous findings using cells in suspension (25).

Internalization of C_6-NBD-PC from the Plasma Membrane

When cells previously incubated with C_6-NBD-PC-containing vesicles at 2°C were warmed to 37°C for 30 min, ~20% of the cellular C_6-NBD-PC was resistant to removal by extensive back exchange (see Materials and Methods), suggesting that this amount of the fluorescent lipid had been internalized. This internalized fluorescence was difficult to observe in the presence of the large amount of fluorescent lipid still at the cell surface (Fig. 3*B*). To enhance the appearance of the internalized lipid, cells were routinely back-exchanged before photography (Fig. 3*C*). The internalized fluorescence that became visible 10–15 min after warming the cells to 37°C appeared as a large spot located next to the nucleus and as several small punctate spots throughout the cell. Microscopically, it appeared that the total amount of fluorescence per cell decreased during incubations at 37°C.

To identify the internal fluorescent structures, we performed co-localization studies using stains specific for various organelles. Co-localization of the bright centrally located C_6-NBD-PC spot with rhodamine-labeled wheat germ agglutinin (Rh-WGA), a stain specific for the Golgi apparatus (12, 28), and co-localization of the fluorescent lipid with an antibody specifically directed against the Golgi apparatus (13), are shown in Fig. 4. No co-localization of C_6-NBD-PC with stains specific for mitochondria (9) (rhodamine 3B) or lysosomes (1, 4) (acid phosphatase or acridine orange) was seen (data not shown). Although most of the internalized fluorescence appeared to be located in the Golgi apparatus, we were unable to identify experimentally the smaller punctate fluorescent structures.

To determine the effect of temperature on the internalization of C_6-NBD-PC located at the plasma membrane, we incubated cells with C_6-NBD-PC-containing vesicles at 2°C for 30 min, washed, and then incubated for 60 min at temperatures between 2°C and 37°C. Fluorescence photomicrographs of cells after warming to 5°C, 16°C, and 21°C are presented in Fig. 5. At temperatures below 8–12°C, all of the fluorescence appeared to be located at the plasma membrane. If the cells were warmed above 18–20°C, then the fluorescent PC entered the cells and accumulated in the Golgi apparatus. When cells were warmed from 2°C to 12–18°C, there was no accumulation of fluorescence in the Golgi apparatus; however, there appeared to be a random accumulation of vesicles in the cells. Whether or not these fluorescent structures were actually intracellular vesicles or simply invaginations of the plasma membrane could not be absolutely determined. When these cells were warmed to room temperature, there was a rapid accumulation of fluorescence at the Golgi apparatus. In contrast, the appearance of fluorescent Golgi apparatus in cells that had been kept at 2°C and were warmed to room temperature was much slower. These observations suggest

that cells incubated between 10°C and 18°C are trapped at an intermediate stage of membrane internalization.

To determine the effect of blocking endocytosis on the internalization of the fluorescent lipid at 37°C, we incubated cells in the presence of 5 mM sodium azide and 50 mM 2-deoxyglucose, two energy poisons known to inhibit endocytosis. Under these conditions internalization of C_6-NBD-PC at 37°C was completely blocked (Fig. 6).

If a large amount of C_6-NBD-PC accumulated in a subcellular organelle, it is possible that self-quenching (19, 29) of the probe could occur. To ensure that our microscopic observations of the internalization of C_6-NBD-PC accurately reflected molecular events, we incubated cells in suspension at 2°C with C_6-NBD-PC-containing vesicles, washed, and warmed to 37°C for different times, and then the amount of fluorescence in the cells was determined in the presence or absence of 1% Triton X-100. The presence of Triton X-100 disrupts membranes and puts the lipid in a non-self-quenching environment (19). From these data the ratio of fluorescence in the absence/presence of Triton X-100 was calculated. If quenching had occurred, the ratio of fluorescence in the absence/presence would have changed as the lipids moved into and out of quenched lipid compartments; however, no change in the ratio was observed (Fig. 7), suggesting that little or no quenching occurred.

Degradation of C_6-NBD-PC

Our microscopic observations of the internalization of C_6-NBD-PC during incubations at 37°C suggested to us that a large amount of the fluorescence was disappearing during incubations at 37°C. To quantify this disappearance and to determine which fluorescent compounds were present in the cells during the incubations, we performed the following experiment: Cultures were incubated in the presence of C_6-NBD-PC-containing vesicles for 1.5 h at 2°C. The vesicles were then removed, and the cultures were washed and incubated further at 37°C. At various times, the identity and amount of fluorescent lipid present in the cells and incubation medium was determined (Fig. 8*A*). The only fluorescent lipid found in the cells was fluorescent PC (data not shown). The amount of fluorescent PC present in the cells decreased rapidly at 37°C, with a half-time of ~90 min. All of the fluorescent lipid lost from the cells could be accounted for by the appearance of fluorescent fatty acid in the incubation medium. When a similar experiment was performed, except that the cells were held at 2°C, very little degradation of the C_6-NBD-PC occurred (Fig. 8*B*).

To determine if lysosomal hydrolases were responsible for the degradation of C_6-NBD-PC, we examined the amount of degradation occurring in the presence of up to 20 mM ammonium chloride. Although the concentrations of ammonium chloride used were sufficient to inhibit the degradation of internalized protein ligands (2, 23, 27), no effect on C_6-NBD-PC degradation occurred (data not shown). We also examined the degradation of C_6-NBD-PC at 37°C in cells

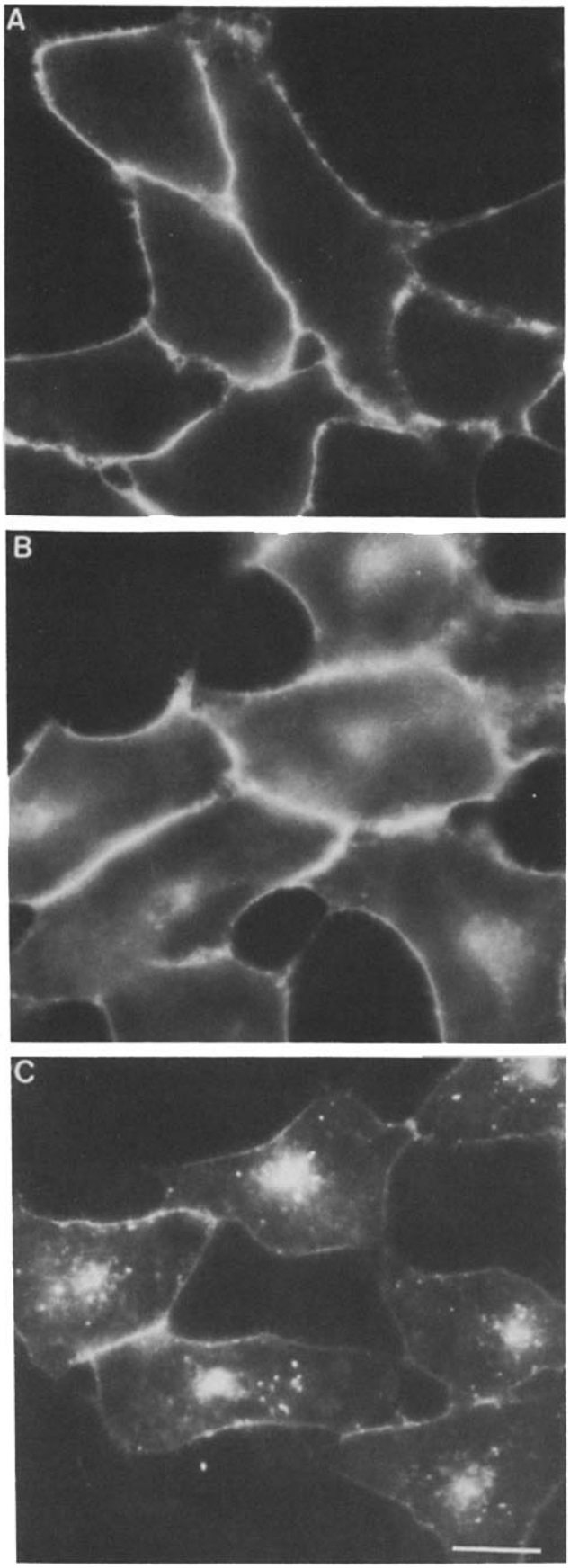


FIGURE 3 Insertion and internalization of C_6-NBD-PC. In *A*, cells were incubated with 50 μM lipid vesicles containing 40 mol % C_6-NBD-PC for 30 min at 2°C. The cultures were then washed with HCMF to remove the vesicles and photographed using optics appropriate for visualizing NBD fluorescence. In *B* and *C*, the cells were treated as described in *A*, except that before photography, the cultures were incubated at 37°C for 30 min in HMEM. In *C*, the cells were treated as described in *B*, except that before photography, the cells were back-exchanged three times as described under Materials and Methods. Bar, 10 μm.

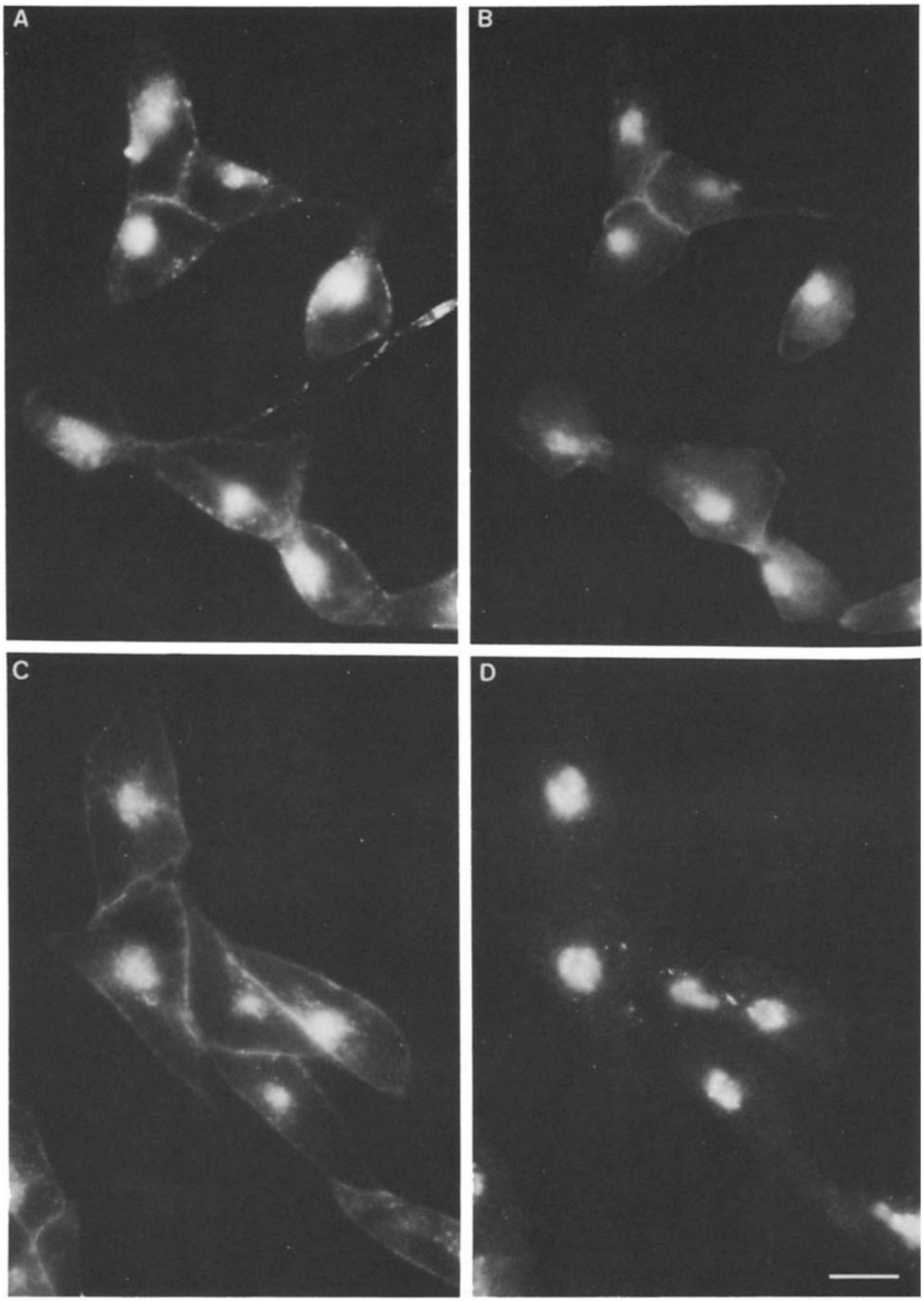
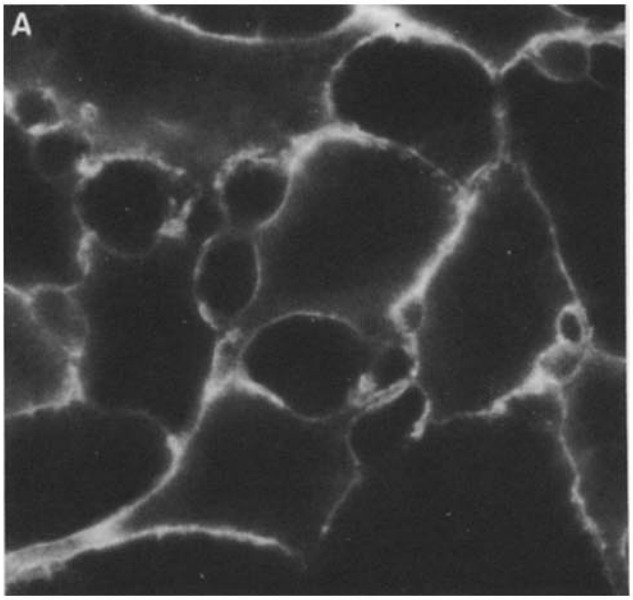
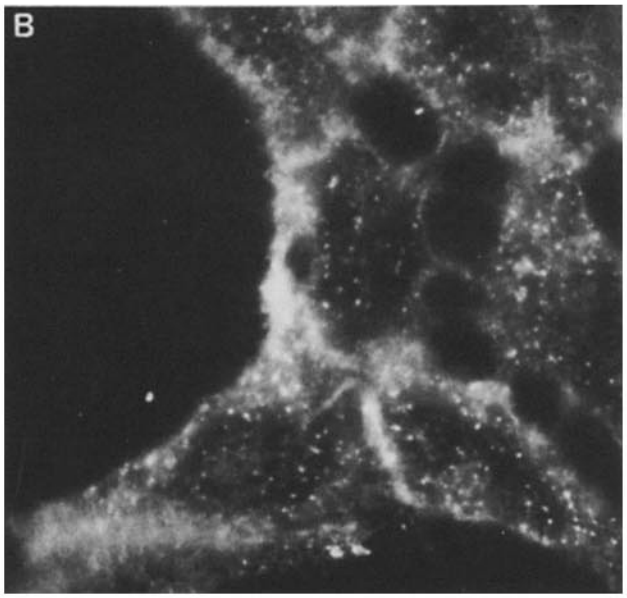


FIGURE 4 Co-localization of internalized C_6-NBD-PC with the Golgi apparatus. After photographing the C_6-NBD-PC-containing cells that had been warmed to 37°C for 30 min (*A* and *C*; see legend for Fig. 3*C*), we fixed the cells, permeabilized them, stained them with Rh-WGA (*B*) or with rabbit anti-Golgi apparatus IgG followed by rhodamine-labeled goat anti-rabbit IgG (*D*) to label the Golgi apparatus and then rephotographed them by use of optics appropriate for visualization of rhodamine fluorescence. The internal distribution of C_6-NBD-PC fluorescence is shown in *A* and *C* and the fluorescent rhodamine staining of the Golgi apparatus is shown in *B* and *D*. Bar, 10 μm.





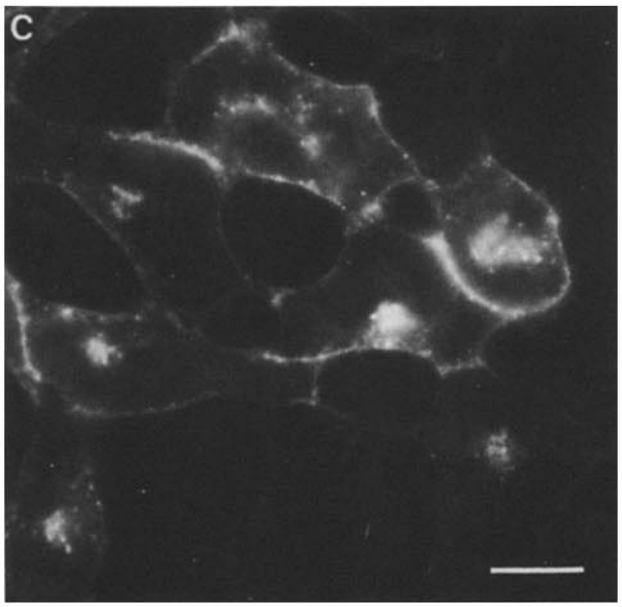
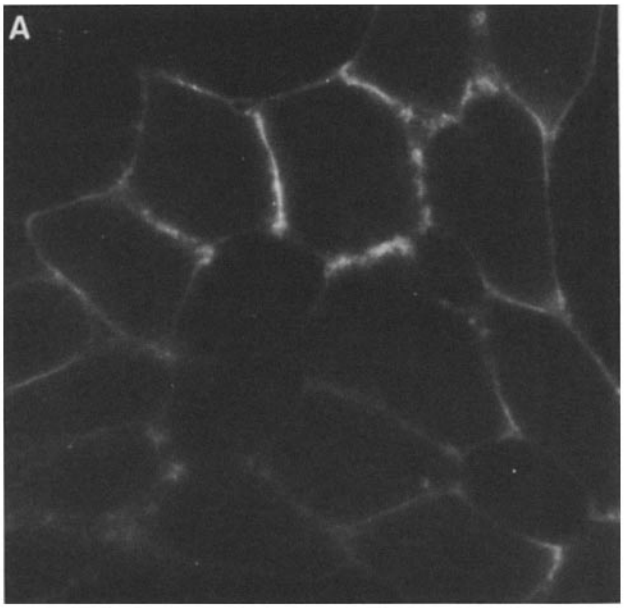


FIGURE 5 Effect of temperature on C_6-NBD-PC internalization. Cells were incubated for 30 min at 2°C in the presence of 50 μM lipid vesicles containing 40 mol % C_6-NBD-PC. The cultures were then washed and incubated at either 5°C (*A*), 16°C (*B*), or 20°C (*C*) for 1 h. After this incubation, the cells were back-exchanged at 2°C and photographed using optics appropriate for visualization of NBD. Bar, 10 μm.



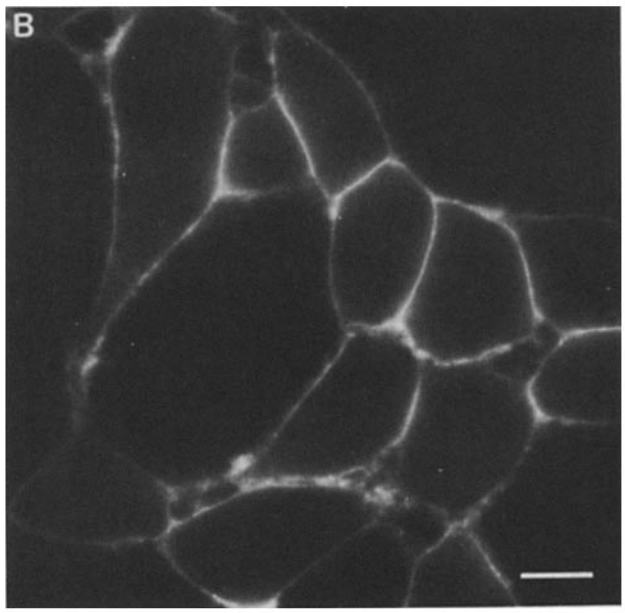


FIGURE 6 Insertion and internalization of C_6-NBD-PC in the presence of inhibitors of endocytosis. In *A*, cells were incubated for 5 min at room temperature in HMEM containing 5 mM sodium azide and 50 mM 2-deoxyglucose. The cells were then chilled to 2°C and incubated for 30 min in the presence of 50 μM lipid vesicles containing 40 mol % C_6-NBD-PC. After the 2°C incubation, the cells were washed with HCMF and photographed with optics appropriate for visualization of NBD fluorescence. In *B*, the cells were treated as described in *A*, except before photography, the cultures were incubated at 37°C for 30 min in HMEM containing the inhibitors and then back-exchanged three times as described under Materials and Methods. Bar, 10 μm.

treated with sodium azide and 2-deoxyglucose. Under these conditions all of the fluorescent lipid remained at the plasma membrane, but there was no significant difference in the rate of C_6-NBD-PC degradation in cells treated with or without the inhibitors (Table I). It therefore appeared that the majority of C_6-NBD-PC was degraded at the cell surface.

Internalization of Fluorescence in Cells Labeled with C_6-NBD-PC and Rhodamine-labeled Lens culinaris Agglutinin

To determine if the proteins and lipids of the plasma membrane co-migrate during and after internalization, cells were incubated at 2°C in the presence of C_6-NBD-PC-containing vesicles and rhodamine-labeled *Lens culinaris* agglutinin (Rh-LCA) for 30 min and then washed. Upon fluorescent microscopic examination, both the C_6-NBD-PC and Rh-LCA fluorescence appeared to be localized exclusively at the plasma membrane (data not shown). The cells were then incubated at 37°C for 30 min and photographed using the appropriate optics for either NBD or rhodamine fluorescence (Fig. 9). There was no evidence of either patching or capping of either the C_6-NBD-PC or Rh-LCA during the incubation at 37°C. Although internalization of both NBD and rhodamine fluorescence occurred at the same time, the fluorescent lipid and protein did not co-migrate. Very little of the Rh-LCA was located in the Golgi apparatus, but was located instead in a number of punctate spots located throughout the cytoplasm. Although every punctate spot containing NBD fluorescence also contained rhodamine fluorescence, there were a number of rhodamine-containing spots that lacked NBD.

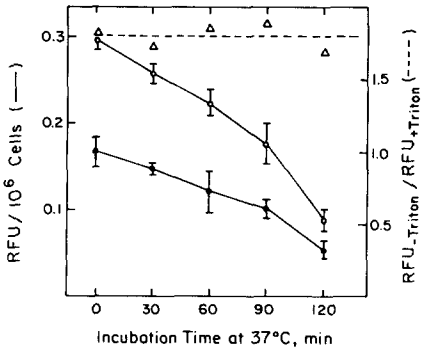


FIGURE 7 Absence of C_6-NBD-PC quenching during cell incubations at 37°C. Cells in suspension were incubated at 2°C for 30 min with 50 μM lipid vesicles containing 40 mol % C_6-NBD-PC, washed and then warmed to 37°C. At the indicated times, the cells were washed three times, and the amount of C_6-NBD-PC fluorescence was determined in the presence (●) and absence (○) of 1% Triton X-100. The ratio of fluorescence detected in the absence/presence of Triton X-100 (△) remained at a constant value of ~1.8 throughout the experiment.

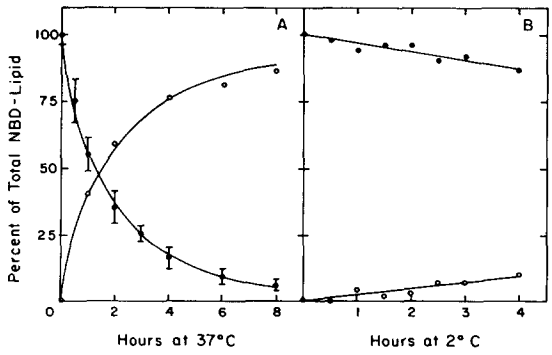


FIGURE 8 Time course of C_6-NBD-PC degradation at 2°C and 37°C. Cells were incubated in HMEM containing 50 μM lipid vesicles composed of 40 mol % C_6-NBD-PC for 90 min at 2°C. The cultures were then washed and incubated further at either 37°C (*A*) or 2°C (*B*) in HMEM. At the indicated times, the incubation medium was removed and the cells harvested. The amount of fluorescent lipid present in the cells (●) and the medium (○) was then determined. Data points with error bars represent the mean ± SD of at least six determinations. All other data points represent the average of two to three determinations.

TABLE I

Degradation of C_6-NBD-PC in the Presence and Absence of Sodium Azide and 2-Deoxyglucose

Inhibitors	Incubation time at 37°C	RFU/mg of DNA
	min	*n ± SD*
−	0	97 ± 6
+	0	97 ± 7
−	30	52 ± 4
+	30	53 ± 5

Cells were incubated in the presence (+) or absence (−) of 5 mM sodium azide and 50 mM 2-deoxyglucose during the insertion of the fluorescent PC at 2°C. The cultures were then washed to remove the lipid vesicles and incubated in the presence or absence of the inhibitors for the indicated times at 37°C. The amount of fluorescent lipid in the cells was then determined. To normalize for variations 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 ± standard deviation of four determinations. RFU, relative fluorescence unit.

DISCUSSION

In this report, we have presented evidence demonstrating that a fluorescent analog of PC inserted into the plasma membrane at 2°C is internalized and accumulates at the Golgi apparatus when warmed to 37°C. The internalization process has been shown to be both temperature and energy dependent. In addition, when both the internalization and sorting of the fluorescent lipid and a membrane-bound fluorescent-labeled protein were observed in the same cell, a partial sorting of the lipid and protein occurred.

Our results lead us to propose the following working model (Roman numerals in brackets refer to steps in Fig. 10): When cells are incubated with vesicles containing C_6-NBD-PC, the fluorescent lipid spontaneously inserts into the outer leaflet of the plasma membrane lipid bilayer (I). The lipid probe apparently remains trapped in the outer leaflet (15) and does not cross the membrane (flip-flop) because of some as yet unidentified property of the membrane or the lipid. When the cells are warmed to 37°C, two processes begin to occur (or are greatly accelerated). Some of the fluorescent lipid from the plasma membrane is internalized by endocytosis (II), whereas the fluorescent lipid remaining at the plasma membrane is degraded resulting in the release of NBD-fatty acid into the incubation medium (VI). The fluorescent lipid that enters the cells initially resides in endocytic vesicles. Transmembrane movement of the C_6-NBD-PC does not occur in these vesicles and the lipid is trapped in the inner leaflet of the vesicles' lipid bilayer. Eventually most of the endocytic vesicles containing the fluorescent lipid fuse with the Golgi apparatus (III), where the lipid remains trapped again in the inner leaflet of the membrane's bilayer. With continued in-

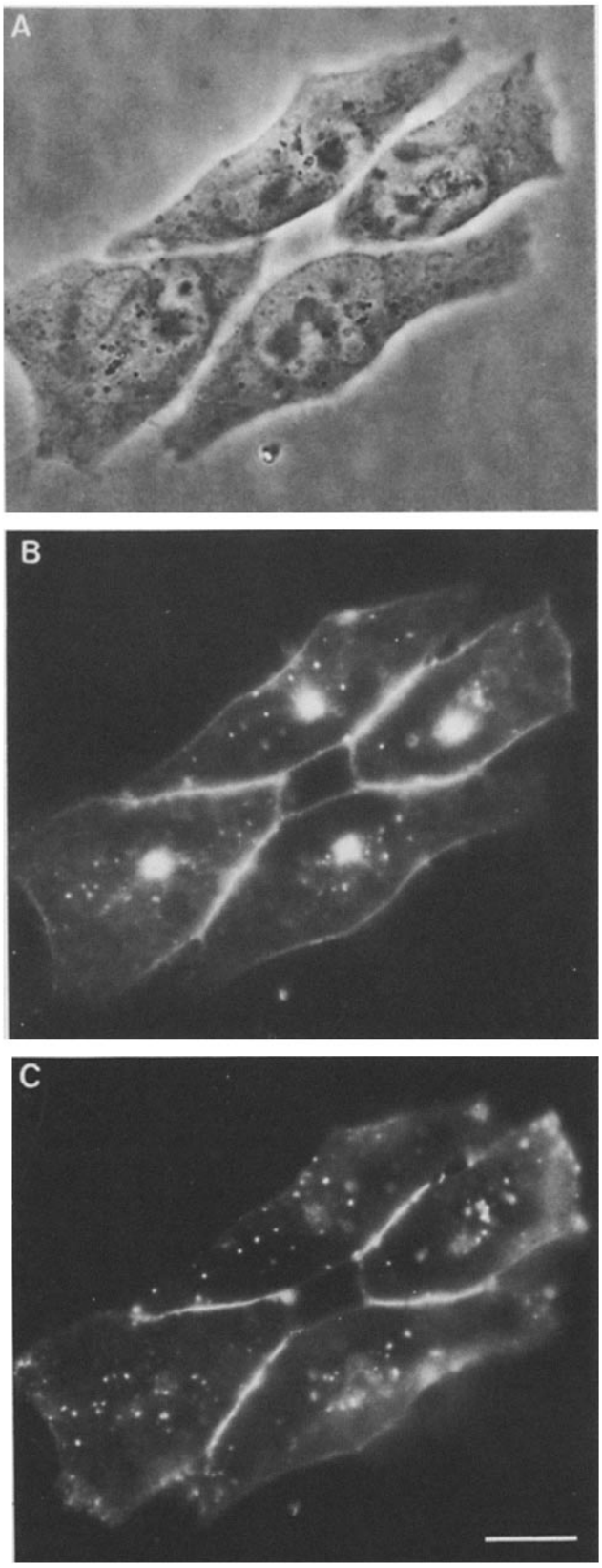


FIGURE 9 Internalization of inserted C_6-NBD-PC and Rh-LCA-bound to the plasma membrane. Cells were incubated for 30 min at 2°C in the presence of both 50 μM lipid vesicles containing 40 mol % C_6-NBD-PC and 5 μg/ml Rh-LCA. The cells were then washed and incubated for 30 min at 37°C in HMEM. After the 37°C incubation, the cells were back-exchanged (see Materials and Methods) at 2°C and then photographed. Phase-contrast optics were used in *A*. Optics appropriate for visualization of NBD (*B*) or rhodamine fluorescence (*C*) were used. Bar, 10 μm.

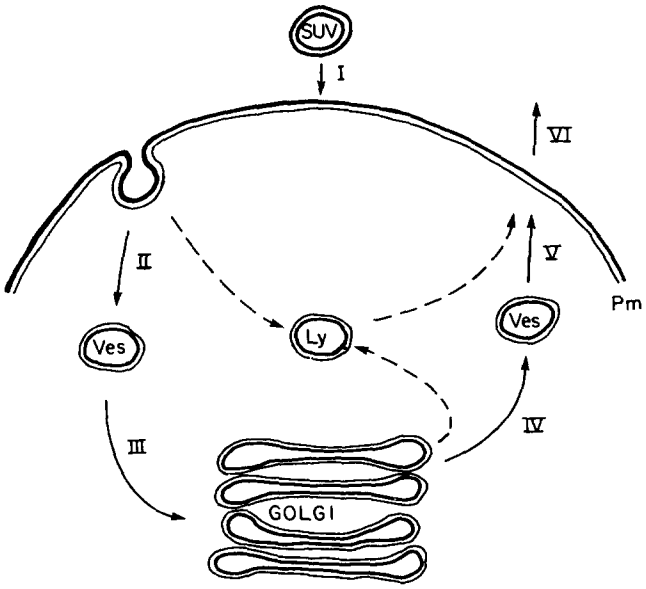


FIGURE 10 Simple working model for the movement of C_6-NBD-PC in cells incubated at 37°C. Solid arrows indicate steps supported by direct observations. Dashed lines indicate possible alternate minor pathways. Membrane lipid bilayers are represented by parallel lines. The dark lines represent the leaflet of the membrane containing the fluorescent phosphatidylcholine. *SUV*, small unilamellar vesicle (liposome) containing C_6-NBD-PC. Ly, lysosome; *Ves*, intracellular transport vesicle; *Pm*, plasma membrane.

cubation at 37°C, the fluorescent lipid is recycled from the Golgi apparatus back to the outer leaflet of the plasma membrane bilayer (IV and V). This process continues until all of the lipid is degraded. Several lines of evidence support the model described above:

(I) C_6-NBD-PC spontaneously inserts into the outer leaflet of the plasma membrane. Previous studies have demonstrated that, when cells or lipid vesicles are incubated with liposomes containing C_6-NBD-PC, the fluorescent lipid spontaneously transfers between membranes (25). The mechanism for this transfer has been demonstrated to be diffusion of soluble fluorescent lipid monomers (14). As shown in Fig. 2*A*, when this transfer is performed by use of liposomes and Chinese hamster fibroblasts at 2°C and the cells are kept at 2°C, the fluorescent lipid is limited to the plasma membrane. If the C_6-NBD-PC molecules are able to spontaneously transfer between membranes at 2°C, why don't the molecules located at the plasma membrane transfer to intracellular membranes at 2°C? We believe that the molecules cannot enter the cell because they are located exclusively in the outer leaflet of the plasma membrane and are unable to cross the membrane. Therefore, because no fluorescent lipid is located in the inner leaflet of the plasma membrane, monomers are not released into the cytosol. This idea is supported by three pieces of evidence. (*a*) C_6-NBD-phosphatidylethanolamine, when transferred to cells at 2°C appears microscopically identical to C_6-NBD-PC (25).[2] The transbilayer distribution of the fluorescent phosphatidylethanolamine, which can be determined chemically by reaction with a nonpermeable reagent, has been shown to be located on the outer leaflet of the plasma membrane lipid bilayer after transfer at 2°C (25). (*b*) C_6-NBD-PC inserted into the plasma membrane is able to move back out of the cell into nonfluorescent recipient DOPC vesicles (back

[2] Sleight, R. G., and R. E. Pagano, manuscript in preparation.

exchange). (*c*) When vesicles containing C_6-NBD-PC are microinjected into the cells, rapid translocation of the fluorescent lipid into intracellular membranes occurs,[3] indicating that there is no special property inside the cells that blocks spontaneous transfer of the fluorescent lipid.

In addition to being unable to cross the plasma membrane at 2°C, our data suggest that no transmembrane movement of C_6-NBD-PC occurs at 37°C. As shown in Fig. 6, when cells containing C_6-NBD-PC in their plasma membranes are warmed to 37°C in the presence of 2-deoxyglucose and azide, no internalization of the fluorescent lipid occurs. If transmembrane movement of the lipid had occurred, we would have expected all internal membranes to become labeled by the monomer diffusion process described above. Two observations indicate to us that 2-deoxyglucose and azide are not blocking a specific transport process for transmembrane movement of C_6-NBD-PC. First, we have observed that other species of fluorescent lipid are able to cross the plasma membrane and label intracellular structures under the same conditions.[2] Second, when cells containing C_6-NBD-PC in their plasma membranes are warmed to 37°C in the absence of any inhibitors, only specific structures became labeled. This pattern of labeling is much different than that observed either after microinjection of C_6-NBD-PC or after warm-up experiments performed with fluorescent lipids believed to be capable of transmembrane movement.[2]

(II) C_6-NBD-PC is internalized by endocytosis and is trapped in the inner leaflet of endocytic vesicles. Two lines of evidence support the idea that entry of C_6-NBD-PC occurs by endocytosis. (*a*) When cells are incubated in the presence of agents known to inhibit endocytosis, internalization of the fluorescent lipid is blocked. (*b*) Both the known characteristics of endocytosis and of C_6-NBD-PC entry into the cells appear to be similar with respect to temperature. Although the existence of C_6-NBD-PC containing intracellular vesicles that exist only briefly is consistent with all of our data, in this report we have not positively identified the location or the nature of these vesicles. When cells containing C_6-NBD-PC in their plasma membranes are warmed to 16°C for 1 h, an accumulation of vesicle-like structures appears in the cells (Fig. 5). It was not possible at our level of microscopic resolution to determine if these structures were actually free vesicles, invaginations of the plasma membrane, or other similar structures. At 16°C no fluorescence was present in the Golgi apparatus, and warming the cells above 20°C resulted in the disappearance of the vesicle-like structures and the appearance of a fluorescent Golgi apparatus. These observations are similar to those made previously during examinations of receptor mediated endocytosis (5, 23). Several investigators have observed that, although endosomes are formed during incubations at ~16°C, these vesicles do not fuse with lysosomes until the temperature is raised to ~20°C. Thus it is conceivable that during our experiments, fusion of plasma membrane-derived, C_6-NBD-PC-containing vesicles with the Golgi apparatus was blocked below 20°C.

We speculate that any endocytic vesicle containing C_6-NBD-PC has the lipid located exclusively in the inner leaflet of the membrane lipid bilayer because, as described above, the pattern of membrane fluorescence in cells warmed to either 16°C or 37°C is not consistent with monomer diffusion of the fluorescent lipid.

(III) C_6-NBD-PC-containing endocytic vesicles fuse with the Golgi apparatus and the molecule resides in the inner leaflet of the membrane lipid bilayer. As shown in Fig. 4, internalization of C_6-NBD-PC co-localizes with stains for the Golgi apparatus. At the resolution of the fluorescence microscope, it was impossible to determine if all of the C_6-NBD-PC at the Golgi apparatus represented lipid that was integrated into the membranes, as would occur by fusion of fluorescent lipid-containing endocytic vesicles, or if the lipid was associated with some other structure that closely associates with the Golgi apparatus. Although we believe that our co-localization experiments are strong evidence that the internalized fluorescence accumulates in the Golgi apparatus, definitive proof of its location will depend on the development of techniques to visualize the fluorescent lipid at the level of the electron microscope.

(IV and V) C_6-NBD-PC is recycled from the Golgi apparatus to the plasma membrane. We have no direct evidence to support this part of the pathway proposed in our model. The circumstantial evidence that suggests that this pathway occurs comes from our observation of the degradation of C_6-NBD-PC. As described in Results and below, degradation of C_6-NBD-PC occurs in the plasma membrane. In addition, we have observed microscopically that both the plasma membrane and the Golgi apparatus appear to decrease in fluorescence intensity similarly during incubation at 37°C (data not shown). Thus, it seems likely that, as lipid molecules cycle between the Golgi apparatus and the plasma membrane, some of the fluorescent lipid is degraded during the lipid's residence in the plasma membrane.

(VI) C_6-NBD-PC is degraded at the plasma membrane. We have demonstrated that the rate of C_6-NBD-PC degradation is not affected by incubation conditions that block the entry of the fluorescent lipid into the cell (Table I). Although this clearly proves that C_6-NBD-PC is degraded at the plasma membrane, it does not rule out the possibility that C_6-NBD-PC degradation also occurs at some other site(s) in the cells. If other cellular sites of C_6-NBD-PC degradation do exist, they must fall into one of two classes: (*a*) sites that are exposed to little or no C_6-NBD-PC during our experimental incubations and which degrade the fluorescent lipid very quickly. These sites could exist and would not be seen microscopically because of the rapid degradation of the fluorescent lipid. The amount of lipid degraded at these sites would have to be small enough so as not to affect the results of determining the rates of lipid degradation in the presence and absence of agents that block C_6-NBD-PC internalization. (*b*) Sites that degrade C_6-NBD-PC at the same rate as the plasma membrane—if these sites exist they might include endocytic vesicles and the Golgi apparatus.

The simple model we have proposed for the internalization and recycling of membrane lipid raises many questions, most of which will have to be examined by further experimental work. Undoubtedly the actual mechanism involves many more processes than we have proposed. Our model suggests three important questions which are discussed below. It is believed that the Golgi apparatus supplies the cell membranes found in several organelles. Why do these organelles not appear fluorescent in the C_6-NBD-PC-treated cells? As seen in Figs. 3*C* and 9*B*, in addition to the Golgi apparatus, several small punctate spots appear fluorescent in cells warmed to 37°C. These structures may come directly from the plasma membrane or be derived from the Golgi apparatus. Their appearance and disappearance coincide closely with that of the fluorescent Golgi apparatus, making it impossible to de-

[3] Pagano, R. E., manuscript in preparation.

termine if a precursor–product relationship exists.

Why are C_6-NBD-PC-containing lysosomes not present? One reason might be that only a small amount of C_6-NBD-PC reaches the lysosomes and is rapidly degraded there. This occurrence would explain why neither newly synthesized fluorescent lysosomes or secondary lysosomes formed by fusion with endocytic vesicles are observed. A second and seemingly less likely possibility is that during the formation of some intracellular vesicles C_6-NBD-PC is actively excluded. We hope that further studies using the fluorescent PC and labeled proteins known specifically to move from the plasma membrane to lysosomes will provide a clear answer to this question.

Does the internalization of C_6-NBD-PC mimic the movement of native lipids? The fluorescent NBD group of the lipid analog certainly gives the molecules properties not typically associated with native phospholipids. In fact, were it not for the special property of the C_6-NBD-PC to insert in the plasma membrane of living cells spontaneously, the experiments reported here could not have been performed. One important advantage of using the fluorescent lipid is that the free C_6-NBD-fatty acid can not be reutilized by the cells (16); thus any ambiguities introduced by fatty acid remodeling and *de novo* synthesis are eliminated. Our laboratory's experience in using fluorescent lipids has indicated that fluorescent analogs of several different lipid species are taken up, metabolized, and translocated in different ways (12, 15–19, 22, 25). We believe that the apparent inability of C_6-NBD-PC to cross membranes (flip-flop) makes it a valuable probe for examining the flow of membrane lipids in the outer leaflet of the plasma membrane bilayer. Whether or not transmembrane movement of native phospholipids occurs, the C_6-NBD-PC still appears to be a marker for membrane movement.

Perhaps the most intriguing finding reported here is that when both the fluorescent lipid located in the plasma membrane and a fluorescently labeled lectin bound to the cell surface are internalized, they are segregated. As shown in Fig. 9, the majority of the internalized C_6-NBD-PC accumulates in the Golgi apparatus. Whereas some of the rhodamine-labeled lectin can be found in the Golgi apparatus, most of it is present in randomly distributed punctate spots. Using the present techniques, it was not possible to determine whether the separation of the rhodamine-labeled protein and NBD-labeled lipid occurred at the plasma membrane or after the formation of endocytic vesicles.

Our findings on the internalization of the fluorescent lipid are striking and much different from what one might have anticipated knowing the internalization pathways of proteins. Using our working model as a guide, we hope to explore the mechanisms involved further in the internalization, segregation, and recycling of plasma membrane lipids and proteins.

The authors thank Ona Martin for expert technical assistance and Dr. A. Hubbard, Dr. M. Snider, and Dr. J. Schwartz for reading the manuscript.

This work was supported by grant GM-22942 from the U.S. Public Health Service. Dr. Sleight was supported by a postdoctoral fellowship (GM-08866) from the National Institutes of Health.

Received for publication 30 January 1984.

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