Metabolism and Intracellular Localization of a Fluorescently Labeled Intermediate in Lipid Biosynthesis within Cultured Fibroblasts

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ABSTRACT In this paper we report on the uptake and distribution of an exogenously supplied fluorescent phosphatidic acid analogue by Chinese hamster fibroblasts. Under appropriate in vitro incubation conditions, 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidic acid was rapidly and preferentially transferred from phospholipid vesicles to cells at 2°C. However, unlike similar fluorescent derivatives of phosphatidylcholine and phosphatidylethanolamine that remain restricted to the plasma membrane under such incubation conditions (Struck, D. K., and R. E. Pagano. 1980. *J. Biol. Chem.* 255: 5405–5410), most of the phosphatidic acid-derived fluorescence was localized at the nuclear membrane, endoplasmic reticulum, and mitochondria. This was shown by labeling cells with rhodamine-containing probes specific for mitochondria or endoplasmic reticulum, and comparing the patterns of intracellular NBD and rhodamine fluorescence. Extraction and analysis of the fluorescent lipids associated with the cells after treatment with vesicles at 2° or 37°C revealed that a large fraction of the fluorescent phosphatidic acid was converted to fluorescent diglyceride, phosphatidylcholine, and triglyceride. Our findings suggest that fluorescent phosphatidic acid may be useful in correlating biochemical studies of lipid metabolism in cultured cells and studies of the intracellular localization of the metabolites by fluorescence microscopy. In addition, this compound provides a unique method for visualizing the endoplasmic reticulum in living cells.

Phosphatidic acid (PA) is a key intermediate in lipid metabolism, functioning as a precursor in glycerolipid biosynthesis. Although the metabolic pathways of PA are well documented, the intracellular site(s) for conversion of PA to other lipids and the movement(s) of these molecules among intracellular membranes are essentially unknown. In addition to its role in *de novo* synthesis of glycerolipids, PA may have other physiologically important functions in cells. For example, in many tissues where hormones or neurotransmitters are postulated to increase Ca^{++} influx, stimulation of PA turnover has been found (10, 15). Again, the cellular localization of PA turnover is not known. To approach this problem, we have synthesized a fluorescent PA analogue having spectral properties similar to those of fluorescein and have introduced it into cells from an exogenous source, phospholipid vesicles. This approach allows us to carry out biochemical studies of PA metabolism and to determine the intracellular localization of lipid metabolites by fluorescence microscopy.

MATERIALS AND METHODS

Lipids and Lipid Vesicles

Dioleoylphosphatidylcholine (DOPC) and 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine (NBD-PC) were purchased from Avanti Biochemical Corp. (Birmingham, Ala.). *N*-(Lissamine) rhodamine B sulfonyl dioleoylphosphatidylethanolamine (*N*-Rh-PE) was synthesized as previously described (19). 1-Acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidic acid (NBD-PA) was prepared from NBD-PC using phospholipase D (2), and was purified by preparative thin-layer chromatography. 1-Acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl diglyceride (NBD-DG) was prepared from NBD-PC using phospholipase C (8). Lipids were stored in $CHCl_3$ at −20°C, periodically examined by thin-layer chromatography, and repurified when necessary. The concentrations of all lipid stock solutions were determined by a modified lipid phosphorus procedure (1).

Small unilamellar vesicles were formed by ethanol injection (9). In a typical preparation, ~1 μmol of lipids were mixed in the desired proportions, dried under a stream of argon gas, and further dried *in vacuo*. The lipids were dissolved in 0.2 ml of ethanol, then injected into 2.6 ml of HCMF[1] with stirring. The preparation was then dialyzed at 4°C overnight against several changes of buffer. In some experiments, small vesicles were also prepared by ultrasonication (6), giving identical results.

Cell Culture and Vesicle-Cell Incubations

Monolayer cultures of Chinese hamster V79 fibroblasts (4) were grown to confluence in Eagle's minimal essential medium (EMEM) supplemented with 12% horse serum in a water-saturated atmosphere of 5% CO_2 in air. Single-cell suspensions were prepared by trypsinization as described (20).

Cell pellets containing 10–20 × 10^6 cells were suspended in 1 ml of vesicle

[1] HCMF, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered, calcium- and magnesium-free Puck's saline, pH 7.4.

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solution (0.2 μmol lipid/ml) and incubated for 60 min at 2°C or 30 min at 37°C. Cells were washed three times in cold HCMF, with the last wash carried out in a new tube. The cells were then resuspended in buffer and divided into aliquots for fluorescence microscopy and lipid extraction. In some experiments, subconfluent monolayer cultures were incubated directly with NBD-PA containing vesicles, washed several times with HCMF, and examined by fluorescence microscopy. To prevent detachment of cells from the substrate, all experiments with monolayer cultures were carried out in the presence of 1 mM Ca^{2+} and Mg^{2+}.

For lipid extraction, cell pellets were suspended in 0.9 ml of HCMF and acidified using 0.1 ml of 1N HCl. The cell lipids were extracted with 3 ml of ethyl acetate:acetone (2:1), followed by two additional extractions with 2.6 ml of ethyl acetate:acetone (2:0.6) (17). The organic phase was then taken to dryness under a stream of argon gas, and the lipids were redissolved in $CHCl_3$:CH_3OH (2:1). The amounts of fluorescent NBD—(λ_{ex} = 470 nm; λ_{em} = 530 nm) and rhodamine—(λ_{ex} = 555 nm; λ_{em} = 585 nm) labeled lipids present in the extracts were determined by reference to standard curves generated from known amounts of NBD-PA or *N*-Rh-PE in $CHCl_3$:CH_3OH (2:1).

Lipid extracts were analyzed qualitatively by thin-layer chromatography on Silica Gel 60 thin-layer plates (Merck & Co., Inc., Rahway, N. J.) in $CHCl_3$:CH_3OH:28% NH_4OH (65:35:5). Plates were air-dried and the fluorescent lipids visualized for photography by excitation with UV light. For quantitative analysis of the fluorescent lipids, extracts were subjected to thin-layer chromatography as above, using silica gel H thin-layer plates (Analtech, Inc., Newark, Del.). Fluorescent lipids were located with UV light, and the spots were scraped and the lipids extracted as described above, except that 1% Triton X-100 was present in the aqueous phase.

Microscopy

Microscopy was performed with a Zeiss Universal microscope equipped with Nomarski optics and epi-illumination for fluorescence. The light source for fluorescence was a tunable argon gas laser (Lexel Corp., Palo Alto, Calif.) that was defocussed and attenuated for observation and photography. For NBD fluorescence, the sample was excited at 457.9 nm and fluorescence observed with an FL 500 dichroic mirror and BF 500 barrier filter suitable for the 530-nm emission peak of this fluorophore. For rhodamine fluorescence, the sample was excited at 514.5 nm and fluorescence observed with an FL 580 dichroic mirror and BF 580 barrier filter combination. The microscope was equipped with a micrometer stage to permit relocation of a given field of cells after initial photography and subsequent fixation and staining with a second fluorophore.

Intracellular Distribution of NBD-PA-derived Lipids

Monolayer cultures were incubated for 60 min at 2°C with NBD-PA-containing vesicles, washed with HCMF, and selected fields of cells were photographed. For subsequent staining of the endoplasmic reticulum in NBD-PA-treated cells the method of Virtanen et al. (21) was used, with modifications. After selected fields of NBD-PA-treated cells were photographed, the monolayer cultures were incubated for 30–45 min at 2°C with 100 μg of nonfluorescent *Lens culinaris* agglutinin (Vector Laboratories, Burlingame, Calif.) per ml phosphate-buffered saline (PBS) (to block any surface receptors for the lectin) and washed with PBS. The cultures were fixed for 60 min at room temperature in 3% formaldehyde (3), washed with PBS containing 0.1 M glycine, and rinsed again with PBS. The cells were permeabilized by treatment with 0.1% Triton X-100 in PBS for 3 min (5) and thoroughly washed. Cells were then stained with 100 μg of rhodamine-conjugated *Lens culinaris* agglutinin (Rh-LcA; Vector Laboratories) per ml PBS for 20 min at room temperature, washed, and fixed for an additional 30 min. The stained cells were washed and photographed for rhodamine fluorescence. After this treatment, essentially no NBD fluorescence could be detected.

Mitochondria were visualized within cells using the fluorescent dye Rhodamine 3B (Eastman) as described by Johnson et al. (7). Monolayer cultures were incubated with 1 μg dye per ml at 2°C for 2 min, washed, and photographed using optics described above for rhodamine fluorescence.

RESULTS

When DOPC vesicles containing NBD-PA or NBD-PC and the nonexchangeable lipid, *N*-Rh-PE (12, 19, 20) were incubated with Chinese hamster fibroblasts at 2°C, significant amounts of the NBD-lipids were transferred to the cells with relatively little uptake of *N*-Rh-PE (Table I). In every experiment, the ratio of NBD to rhodamine fluorescence in the washed, vesicle-treated cells was significantly greater than in the applied vesicle suspension. This demonstrates that the uptake of vesicle lipids was due to preferential exchange or transfer of lipids (13, 14, 16, 20) and was not due to association of intact vesicles with cells. Uptake of intact vesicles would cause the ratio of NBD to rhodamine fluorescence in the cell extracts to be identical to that found in the starting vesicles. Whereas the amount of NBD-PC that became cell-associated was approximately linearly dependent on the mole fraction of NBD-PC present in the vesicles, a fourfold increase in NBD-PA concentration in the vesicles resulted in approximately a 14-fold enhancement in uptake of NBD-fluorescence by the cells. Vesicle-cell incubations at 37°C also resulted in preferential uptake of NBD-PA relative to *N*-Rh-PE (data not shown). Finally, in control experiments it was found that the presence of *N*-Rh-PE had no effect on the transfer of NBD-PA or NBD-PC from vesicles to cells.

Fig. 1 and Table II present a thin-layer chromatogram and quantitative analysis of the fluorescent lipids extracted from cells treated at 2° or 37°C with exogenous NBD-PC or NBD-PA. When NBD-PC was used, no metabolism of this fluorescent lipid could be detected. However, in cells incubated with NBD-PA at 2° or 37°C, substantial conversion of the PA analogue to other fluorescent lipids occurred. At 2°C, ~90% of

TABLE I

Cellular Uptake of NBD-Phospholipid Analogues at 2°C

Analogue	ng NBD-lipid	ng N-Rh-PE	ng NBD-lipid / ng N-Rh-PE	
mol %	*10^7 cells*		*vesicles*	*cells*
NBD-PA (5)	160	16	1.7	10
NBD-PA (20)	2,320	20	6.7	116
NBD-PC (5)	95	22	1.7	4.3
NBD-PC (20)	437	7	6.7	6.2

Cells were incubated with DOPC vesicles containing 3 mol % *N*-Rh-PE and 5 or 20 mol % NBD-lipid for 60 min at 2°C (0.2 μmol lipid and 10^7 cells/ml). The uptake of each of the fluorescent lipids was determined as described in Materials and Methods.

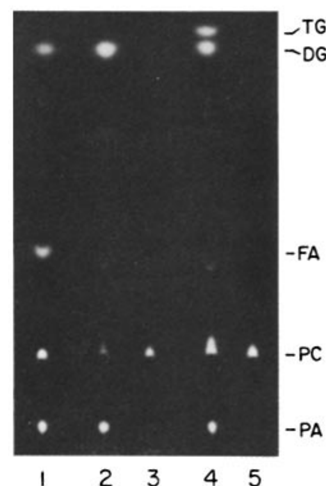

FIGURE 1 Thin-layer chromatogram of lipid extracts obtained from Chinese hamster fibroblasts treated with DOPC vesicles containing 5 mol% NBD-PA or NBD-PC. Approximately 2 × 10^7 cells were incubated with vesicles for 30 min at 37°C or 60 min at 2°C, washed, and extracted. The lipid extracts were subjected to thin-layer chromatography and the chromatogram was photographed under UV light. Vesicle-cell incubations were: Lane *2*, NBD-PA, 2°C; Lane *3*, NBD-PC, 2°C; Lane *4*, NBD-PA, 37°C; Lane *5*, NBD-PC, 37°C; Lane *1*, fluorescent lipid standards: NBD-*PA*, -*PC*, -fatty acid (*FA*), -*DG*.

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Table II
Distribution of Fluorescence in Lipids from Cells Treated with NBD-PA

Lipid	Relative Fluorescence 2°C	37°C
	%	
NBD-PA	10	9
NBD-PC	2	34
NBD-Fatty acid	2	4
NBD-Monoglyceride	5	1
NBD-Diglyceride	78	16
NBD-Triglyceride	3	36

Cells were incubated with DOPC vesicles containing 10% NBD-PA for 60 min at 2°C or 30 min at 37°C. Lipids were extracted with ethyl acetate:acetone, separated by thin-layer chromatography, and quantified as described in Materials and Methods.

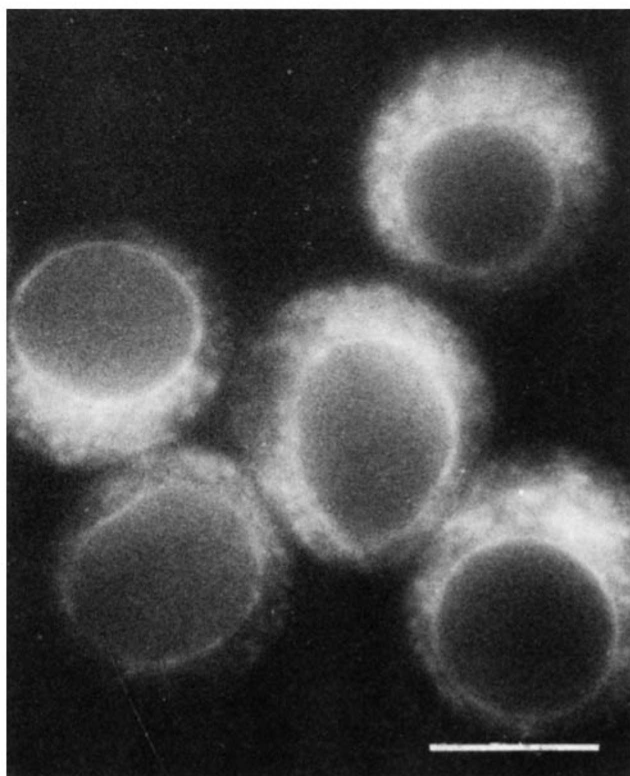

Figure 2 Fluorescence micrograph of Chinese hamster fibroblasts in suspension after incubation with NBD-PA containing vesicles for 60 min at 2°C. Bar, 10 μm.

the NBD-PA was metabolized, with the majority being converted to NBD-DG, but also with small amounts of NBD-PC and 1,3-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl triglyceride (NBD-TG) being formed. At 37°C, the qualitative pattern of fluorescent products was unchanged but, quantitatively, significantly more fluorescent phosphatidylcholine and triglyceride were made. Vesicle-cell incubations were also carried out at 2° and 37°C using isotopically labeled phosphatidic acid in place of NBD-PA. As with NBD-PA, this compound was also hydrolyzed to diglyceride and further metabolized to phosphatidylcholine and triglyceride (unpublished observations).

Distribution of Cell-associated NBD-PA-derived Lipids

Cells incubated with NBD-PA-containing vesicles at 2°C (Figs. 2 and 3) had little or no fluorescence at the cell surface. Instead, they had a bright fluorescence around the nucleus and substantial cytoplasmic fluorescence. Similar fluorescence was seen in both cell suspensions (Fig. 2) and monolayer cultures. In the monolayer cultures this fluorescence could be resolved into bright dots and a fine reticular network particularly visible at the cell periphery (Fig. 3). This pattern of fluorescence persisted for hours as long as the cells were maintained at 2°C. When incubations were carried out at 37°C with NBD-PA, essentially identical patterns of labeling were seen except that the specimen was more intensely fluorescent because of the enhanced uptake at 37°C.

To identify the locations of NBD fluorescence, we first treated monolayer cultures of V79 cells with NBD-PA vesicles at 2°C and photographed selected fields of cells. Then, the endoplasmic reticulum in the same cells was visualized by labeling with Rh-LcA (21) (Fig. 4). Comparison of the NBD fluorescence (Fig. 4*a*, *c*) with the Rh-LcA fluorescence (Fig. 4*b*, *d*) demonstrated that the reticular network visualized by NBD-fluorescent lipids appears coincident with the endoplasmic reticulum.

We attempted to carry out similar colocalization experiments on NBD-PA-treated cells using the mitochondria-specific dye. Rhodamine 3B (7) but were unsuccessful. Apparently, photodamage that occurred during photography of the NBD-fluorescence inhibited subsequent uptake of the rhodamine dye by the photographed (but not by the surrounding) cells. Therefore, to determine whether mitochondria were labeled by treatment of cells with NBD-PA, we conducted separate experiments in which the fluorescence and Nomarski images of a given cell were compared. In Fig. 5*a* it is seen that the Rhodamine 3B-labeled mitochondria correspond to cytoplasmic images that appear as bumps with Nomarski optics. In Fig. 5*b* the fluorescent dots seen in NBD-PA-treated cells also correspond to such Nomarski images. We thus concluded that NBD-PA treatment of cells also leads to labeling of mitochondria.

DISCUSSION

In this report we have shown that an exogenously supplied fluorescent phosphatidic acid derivative, NBD-PA, was incorporated into mammalian fibroblasts in a distinctive manner. Whereas fluorescent analogues such as NBD-PC and NBD-phosphatidylethanolamine label predominantly the cell surface during vesicle-cell lipid transfer at 2°C (20), under identical incubation conditions NBD-PA labeled the nuclear membrane, mitochondria, and endoplasmic reticulum. Furthermore, unlike NBD-PC or NBD-phosphatidylethanolamine, NBD-PA was converted to the fluorescent products expected from the established lipid biosynthetic pathways in mammalian cells. This metabolism of NBD-PA suggests that the presence of the fluorescent group on the acyl chain of the PA molecule does not inhibit the activity of enzymes involved in PA metabolism.

The intracellular localization of the NBD-lipids derived from exogenously supplied NBD-PA is of considerable interest. For determining the distribution of the fluorescent lipids within cells, we did not use cell fractionation methods because the NBD-lipid molecules might redistribute between membranes (11, 12) during the fractionation procedure, and because pure subcellular membrane fractions are difficult to obtain. Instead,

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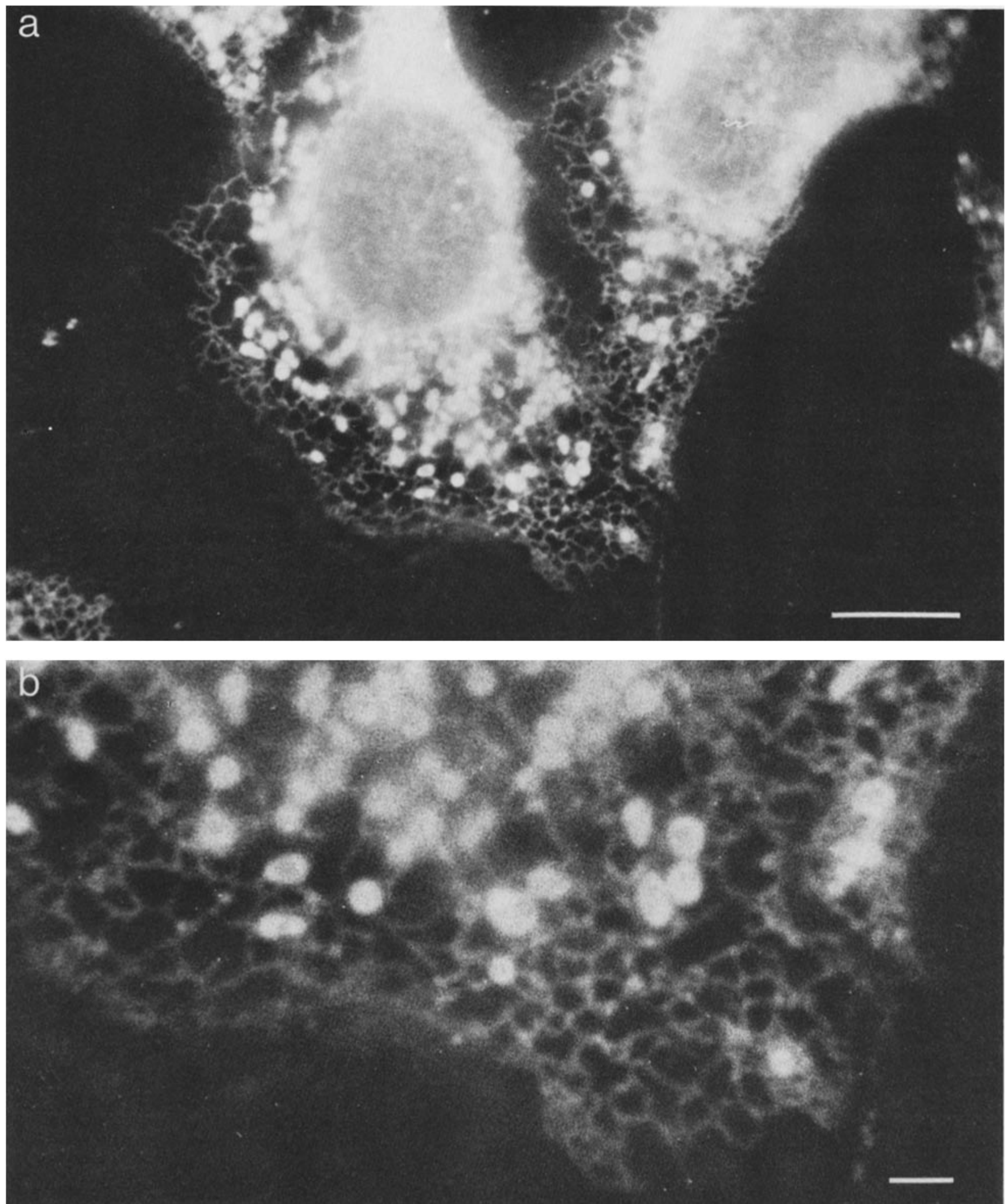

FIGURE 3 Fluorescence micrographs of Chinese hamster fibroblast monolayer cultures incubated with NBD-PA-containing vesicles for 60 min at 2°C. (*a*) Bar, 10 μm. (*b*) The same cell at higher magnification. Bar, 2 μm.

we used separate rhodamine-containing probes for mitochondria (7) and endoplasmic reticulum (21) and showed that the intracellular distributions of rhodamine and NBD fluorescence were highly correlated. Although correlation of NBD-fluorescence and the Rh-LcA-stained endoplasmic reticulum was not perfect, it should be noted that the rhodamine-fluorescence photomicrographs were made using fixed and permeabilized cells. Apparently, these procedures, which are necessary for staining of the endoplasmic reticulum with Rh-LcA, damaged the reticular network to some extent.

Although the presence of the NBD-lipids on the endoplasmic reticulum and nuclear membrane (an extension of the endoplasmic reticulum) might be expected because most of the enzymes involved in lipid biosynthesis are found there, the

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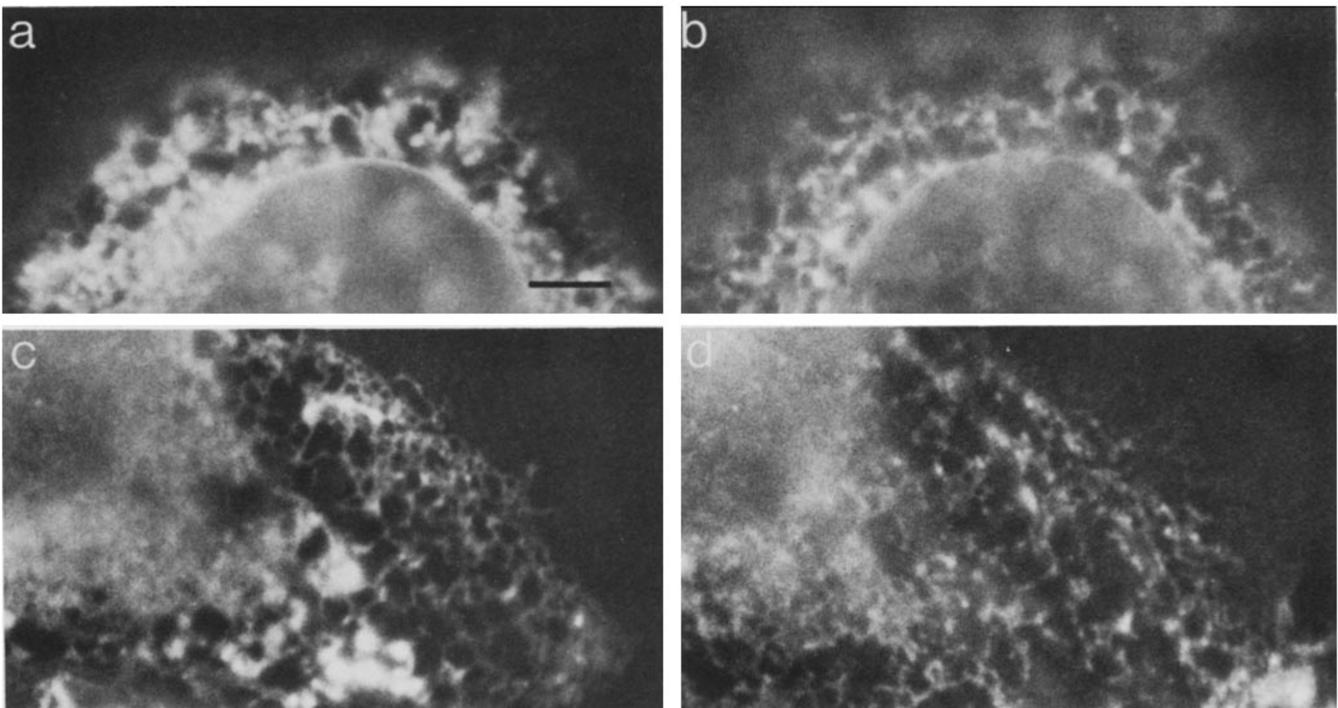

FIGURE 4 Photomicrographs showing co-localization of intracellular NBD-lipids with endoplasmic reticulum in Chinese hamster fibroblasts. Cells were incubated for 60 min at 2°C with exogenous NBD-PA, washed, and selected fields photographed. The NBD-PA-treated cells were then fixed, permeabilized, and stained with Rh-LcA, and the same cells rephotographed under conditions appropriate for visualizing rhodamine fluorescence. (*a*) and (*c*): NBD fluorescence; (*b*) and (*d*): rhodamine fluorescence in corresponding cells. Bar, 5 μm.

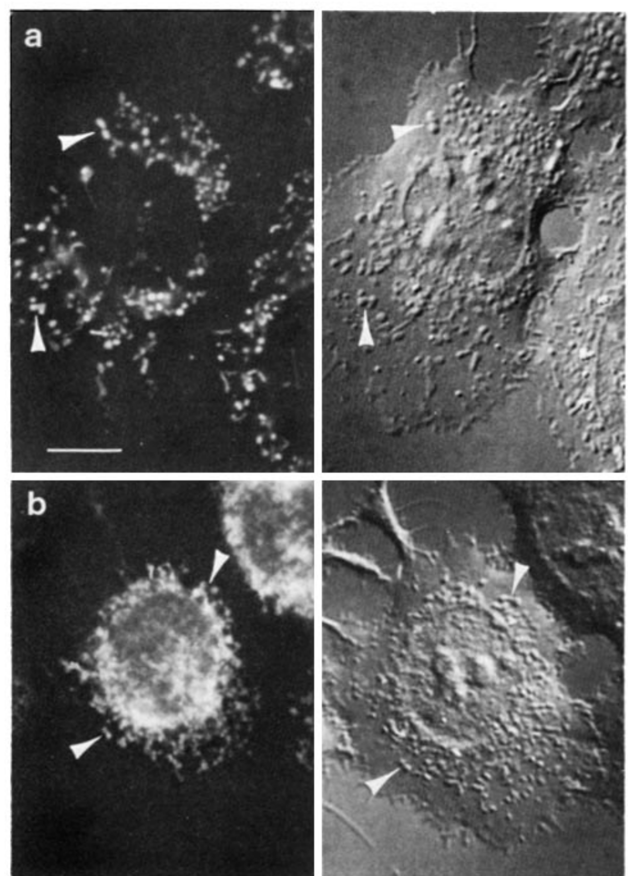

FIGURE 5 Fluorescence and corresponding Nomarski photomicrographs comparing intracellular distribution of mitochondria and NBD fluorescence. Cells were treated with (*a*) Rhodamine 3B as described in Materials and Methods or (*b*) NBD-PA for 60 min at 2°C. Note (e.g. at arrows) the correlation of both the Rhodamine 3B and punctate NBD fluorescence with cytoplasmic images seen by Nomarski optics. Bar, 10 μm.

apparent staining of the mitochondria is somewhat surprising. This finding cannot be explained at present but may be due to rapid translocation of newly synthesized lipids from the endoplasmic reticulum to mitochondria (18), or to selective uptake of a particular fluorescent lipid species by this organelle.

In summary, we have shown that a fluorescent compound, NBD-PA, behaves as an analogue for phosphatidic acid, an important intermediate in lipid biosynthesis. Because both biochemical studies of lipid metabolism and fluorescence microscopy on the intracellular location of the probe are possible, we hope this approach will prove useful in examining the pathways and mechanism(s) of lipid metabolism and lipid transport within cells. In addition, this compound provides a useful method for visualizing the endoplasmic reticulum in living cells.

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