Metabolism and Distribution of Intramolecular Excimer-Forming Dipyrenebutanoyl Glycerophospholipids in Human Fibroblasts. Marked Resistance to Metabolic Degradation†

Juha Kasurinen and Pentti Somerharju*

Institute of Biomedicine, Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, P.O. Box 8, 00014 Helsinki, Finland

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ABSTRACT: Metabolism and intracellular distribution of fluorescent 1,2-dipyrenebutanoyl derivatives of phosphatidylcholine, -ethanolamine, and -serine and phosphatidic acid ($diPyr_4PC$, $-PE$, $-PS$, and $-PA$, respectively) in human skin fibroblasts (HF) has been studied. When HF cells were co-incubated with phospholipid vesicles containing $diPyr_4PC$ at 8 °C, considerable amounts of the fluorescent lipid were incorporated into the cells. This incorporation occurred mainly by spontaneous diffusion, since 10-fold less of the vesicle marker, [^{3}H]cholesteryl oleate associated with the cells. Also $diPyr_4PE$, $-PS$, and $-PA$ were incorporated efficiently into the cells, probably by the same mechanism. HPLC analysis of the cells labeled with $diPyr_4PA$ at 8 °C for 1 h showed that a considerable fraction of the lipid had been metabolized to the corresponding diglyceride and triglyceride. No metabolism of the other dipyrenyl lipids was observed at this temperature. When the cells were shifted to 37 °C, $diPyr_4PA$ was further metabolized to $diPyr_4PC$, which represented 90% of total $diPyr_4$ lipids after 8 h of incubation. $DiPyr_4PS$ was converted to $diPyr_4PE$ with an apparent half-time of 3 h, probably by decarboxylation in the mitochondria. In contrast to the PA and PS derivatives, no head-group modification of either $diPyr_4PC$ or $diPyr_4PE$ was observed even at this temperature. Stability of dipyrenyl lipids toward phospholipase A degradation was investigated by labeling the cells simultaneously with $diPyr_4PC$ and NBD_6PC, a commonly used fluorescent glycerophospholipid derivative, followed by incubation at 37 °C. The half-times for cellular depletion were found to be approximately 2 h for NBD_6PC and >24 h for $diPyr_4PC$, showing that the dipyrenyl derivative is much more resistant to metabolic degradation. Also the other $diPyr_4$ derivatives appeared to be markedly stable. Intracellular distribution of the dipyrenyl lipids was studied by fluorescence imaging. At 8 °C, where endocytosis is negligible, $diPyr_4PC$ was restricted to the outer leaflet of the plasma membrane as revealed by back-exchange and selective quenching assays. In contrast, $diPyr_4PS$ and $-PE$ also labeled intracellular membranes at 8 °C. Such labeling by $diPyr_4PS$ was markedly reduced in cells treated with inhibitors of the aminophospholipid translocase, suggesting that this enzyme is responsible for the access of $diPyr_4PS$ into the cells. At 37 °C, a considerable fraction of $diPyr_4PC$ was internalized, probably via endocytosis, and after prolonged incubation prominent labeling of perinuclear structures, presumably secondary lysosomes, was observed. Initially, $DiPyr_4PS$ and $-PE$ labeled the Golgi apparatus and mitochondria most prominently, but they tended to accumulate in similar vesicular structures as the PC derivative upon extended incubation at 37 °C. In cells incubated with $diPyr_4PA$ at 37 °C intracellular lipid droplets became intensely labeled, probably by the triglyceride metabolite. We conclude that both the general metabolism and the transbilayer movement of $diPyr_4GPLs$ are similarly dependent on the head group as found previously for natural lipids. This, together with their remarkable resistance to cellular degradation and their environment-sensitive fluorescent properties, makes the dipyrenyl derivatives promising tools for studies on intracellular glycerophospholipid traffic and metabolism as well as for imaging of physical properties of membranes in living cells.

In recent years, the use of fluorescent lipid analogues has contributed considerably to our understanding of intracellular lipid traffic [reviewed in Pagano and Sleight (1985) and Simons and van Meer (1988)]. This is mainly because intracellular distribution and translocation of such analogues can be conveniently studied with a fluorescence microscope. Among the fluorescent derivatives used, short-chain NBD[1] lipids have been by far the most popular. Another major advantage of such NBD derivatives is that, due to their relatively low hydrophobicity, they readily transfer to the recipient cells from donor vesicles (Sleight & Pagano, 1984).

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* Address correspondence to this author. E-mail: Somerharju@Katk.Helsinki.Fi. Fax: 358-0-1918276.

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[1] Abbreviations: BSA, bovine serum albumin; BP, band-pass; $diPyr_4PC$, $diPyr_4PE$, $diPyr_4PS$, and $diPyr_4PA$, 1,2-dipyrenebutanoyl-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoserine, and -phosphate, respectively; $diPyr_4GPL$, 1,2-dipyrenebutanoyl-*sn*-3-glycero-3-phospholipid; $diPyr_4DG$, dipyrenebutanoyl diglyceride; $diPyr_4TG$, dipyrenebutanoyl triglyceride; DMEM, Dulbecco's modified Eagle's medium; GPL, glycerophospholipid; HPLC, high-performance liquid chromatography; I-DMEM, CO_2-independent DMEM; HF, human fibroblasts; NBD_6PC, 1-palmitoyl-2-(6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)phosphatidylcholine; NEM, *N*-ethylmaleimide; Pyr_4FA, pyrenebutyric acid; Tnp-LPE, (trinitrophenyl)lysophosphatidylethanolamine.

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Furthermore, they can be readily extracted by serum albumin, thus allowing the determination of the fraction of probe molecules residing on the plasma membrane (Van Meer et al., 1987). NBD_6-labeled sphingolipids, in particular, have been widely used, and much novel information on intracellular distribution and movement of sphingolipids has been obtained with these derivatives [reviewed in Koval and Pagano (1991) and Hoekstra and Kok (1992)]. In contrast, NBD glycerophospholipids (GPLs) have not gained equal popularity in lipid-transport studies. A major reason for this is that the NBD_6 derivatives are very rapidly degraded in cells at the physiological temperature (Sleight & Pagano, 1984, 1985; Sleight & Abanto, 1989). Thus, clearly, there is a marked need for more stable fluorescent GPL derivatives.

We noted recently that a phosphatidylcholine containing two short pyreneacyl chains ($diPyr_4PC$) was quite resistant to degradation by phospholipases *in vitro*.[2] This suggested that such lipids might be resistant also to degradation in cells. Accordingly, $diPyr_4$ derivatives of phosphatidylcholine ($diPyr_4$-PC), phosphatidylethanolamine ($diPyr_4PE$), phosphatidylserine ($diPyr_4PS$), and phosphatidic acid ($diPyr_4PA$) were synthesized and their metabolism and intracellular distribution in human skin fibroblast (HF) cells was studied. Indeed, $diPyr_4$-GPLs were found to be remarkably resistant to metabolic degradation. Furthermore, their metabolism followed the general pattern observed previously for their natural counterparts, and their intracellular distribution was dependent on the head group in an expected manner. Thus the use of $diPyr_4$GPLs opens up new possibilities to investigate intracellular trafficking and sorting of glycerophospholipids, both poorly understood phenomena at present.

EXPERIMENTAL PROCEDURES

Reagents. Phospholipases D (*Streptomyces* species) and C (*Clostridium welchii*) were obtained from Sigma Chemical Co. (St. Louis, MO); nonyl acridine orange, nile red, and BODIPY-ceramide were from Molecular Probes (Eugene, OR); and fatty acid-free bovine serum albumin (BSA) was from Boehringer-Mannheim (Germany). All solvents were of either reagent or HPLC grade and were supplied by Merck (Germany).

Lipids. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1-lauroyllysophosphatidylethanolamine were from Avanti Polar Lipids (Alabaster, AL). $DiPyr_4PC$ was synthesized as described previously (Vauhkonen et al., 1990) or was obtained from Molecular Probes, as was NBD_6PC. $DiPyr_4$-PE and $diPyr_4PS$ were synthesized from $diPyr_4PC$ by phospholipase D catalyzed transesterification (Comfurius et al., 1990). $DiPyr_4PA$ was obtained by phospholipase D catalyzed hydrolysis of $diPyr_4PC$. $DiPyr_4DG$ was prepared from $diPyr_4PC$ by phospholipase C treatment using standard protocols. The fluorescent lipids were purified by HPLC on a silica gel column as described previously (Kasurinen & Somerharju, 1992) and stored at −70 °C. [^{3}H]Cholesteryl oleate was from Amersham (England) and was purified by reverse-phase HPLC before use. Trinitrophenylated lysophosphatidylethanolamine (Tnp-LPE) was obtained by reacting 1-lauroyllysophosphatidylethanolamine with (trinitrophenyl)sulfonic acid (van Duijn et al., 1985) and was purified by HPLC on a silica column eluted with a gradient of methanol into chloroform.

[2] S. Lusa and P. Somerharju, unpublished data.

Cell Culture. Normal human fibroblasts (GM08333) were obtained from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). The cells were grown on tissue culture dishes (Nunc, Denmark) in DMEM supplemented with 15% fetal calf serum, 2 mM L-glutamine, 0.1 mM each nonessential amino acids, and penicillin (100 units/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL) at 37 °C in an atmosphere of 5% CO_2 in air. For microscopy the cells were grown on 32-mm round coverslips placed in culture dishes. All cell culture media were from Gibco (England).

Incorporation of the Fluorescent Lipids into HF Cells. Donor vesicles containing a dipyrenyl phospholipid, POPC, and trace amounts of [^{3}H]cholesteryl oleate were prepared as follows. Appropriate amounts of the lipids were mixed in chloroform, dried under nitrogen followed by vacuum, and dispersed in CO_2-independent DMEM (I-DMEM) by a 3-min sonication (50-W output) at room temperature with a Branson sonicator equipped with a microtip. HF cell monolayers were washed twice with PBS and incubated with the donor vesicles in I-DMEM at 8 °C for 1 h, washed five times with PBS and 0.9% NaCl each, and subjected to fluorescence imaging or lipid extraction directly or after further incubation at 37 °C. In back-exchange experiments labeled cells were treated three times with 1% BSA in DMEM for 10 min at 4 °C prior to imaging or lipid extraction and quantitation.

Lipid Analysis. Cells were scraped from culture dishes into PBS using a rubber policeman, washed by centrifugation, suspended in 0.05 N HCl, and then extracted according to Bligh and Dyer (1959). Extracted lipids were separated by HPLC on a silica gel column according to Christie (1985) with minor modifications (Kasurinen & Somerharju, 1992). The eluent was passed through two coupled fluorescence detectors (Hitachi F-1150), one set to the monomer (395 nm) and the other to the excimer emission wavelength (480 nm) of pyrene. Excitation was at 345 nm. The signals were recorded on a Merck-Hitachi D-2000 two-channel integrator, and the peak areas were corrected for the differences in the pyrene quantum yield due to changes in the eluent composition (Homan & Pownall, 1989) by running known amounts of mono- and dipyreneacyl lipid standards under identical conditions. The amount of fluorescent lipid in cell lipid extracts was determined by measuring the fluorescence intensity with a Hitachi F-4000 fluorometer calibrated with appropriate standards.

Fluorescence Imaging. A cover slip containing HF cells labeled with a $diPyr_4GPL$ was placed in a home-built aluminum/Teflon chamber, covered with I-DMEM, and cooled on ice. After depletion of oxygen (see the caption to Figure 6), the cells were imaged on a system consisting of a Zeiss Axiovert 10 microscope equipped with a Neofluar 100× objective and a Photometrics 250 CCD camera (Photometrics, Tucson, AZ) with a thermoelectrically cooled Kodak KAF-1440 chip operated at 517 × 658 pixel resolution. The camera and excitation shutters were controlled and the images were analyzed with the IP-Lab software (Signal Analytics, Vienna, VA) running on a Macintosh Centris 650 computer. Hardcopies were produced with a Kodak sublimation printer. For pyrenyl lipid imaging, a 340-nm (BP 10 or 40 nm) excitation filter, a 480-nm (BP 80 nm) emission filter, and a 375-nm dichroic mirror were used. For BODIPY-ceramide, nonyl acridine orange, acridine orange,

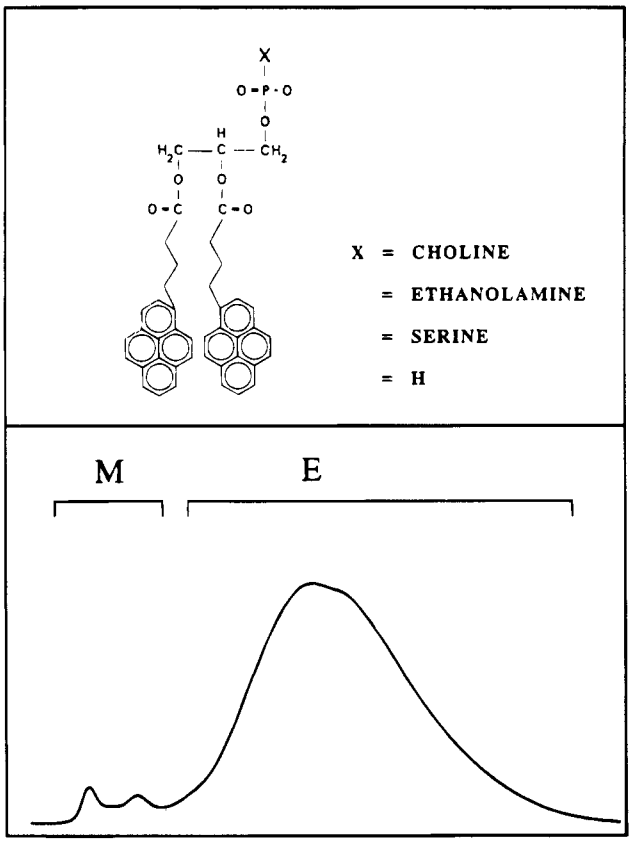

FIGURE 1: Schematic structure and fluorescence emission spectrum of diPyr$_4$PLs. Structures of the diPyr$_4$PLs used. The lower panel shows the emission spectrum recorded for diPyr$_4$PC (1 μM) in 2-propanol/chloroform (8:2, v/v). Excitation was at 342 nm, and the excitation and emission band passes were 5 nm. The monomer (M) and excimer (E) emission regions are indicated.

and Nile red imaging a 480-nm (BP 20 nm) excitation filter, a 540-nm (BP 45 nm) emission filter, and a 490-nm dichroic mirror were used. All filters and dichroics were from Omega Optical (Brattleboro, VT). For further details, see the caption to Figure 6.

Other Procedures. Protein was measured according to Markwell et al. (1981) using fatty acid-free bovine serum albumin as the standard. Concentrations of diPyr$_4$GPLs and NBD$_6$PC were determined photometrically in chloroform on the basis of the molar extinction coefficients 8×10^4 and 2×10^{-4} M^{-1} cm^{-1}, respectively. Radioactivity was measured with a Wallac liquid scintillation counter (Turku, Finland). Lipid phosphorus was determined according to Rouser et al. (1970).

RESULTS

Structure and Fluorescence Properties of diPyr$_4$GPLs. The structures of the diPyr$_4$GPLs used in the present study are depicted in Figure 1. These lipids contain two pyrenebutanoyl chains, one in the *sn*-1 and the other in the *sn*-2 position of the glycerol moiety. Upon excitation, a pyrenyl residue may emit directly so-called monomer fluorescence (M) at 375−410 nm, or it can collide with another pyrene in the ground state to form an excited complex which emits s.c. excimer fluorescence (E) centered at 475 nm. At low dipyrenyl lipid concentrations, which are typical for cellular lipid extracts and also for the membranes of labeled cells, only *intramolecular* excimer is formed (Eklund et al., 1992). Because the two pyrene moieties of intact diPyr$_4$GPLs are

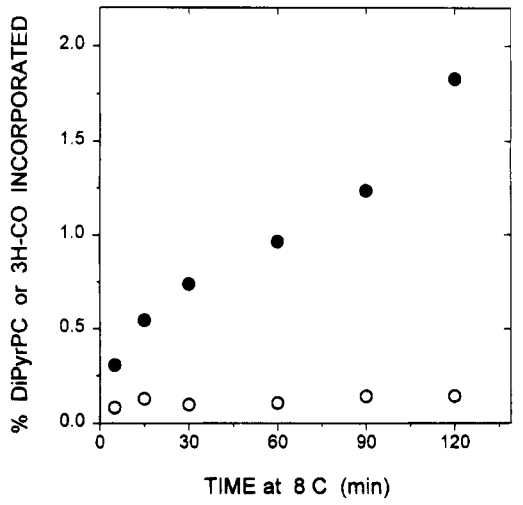

FIGURE 2: DiPyr$_4$PC incorporates into HF cells mainly by spontaneous monomer diffusion. HF cells growing on dishes were incubated with 40 nmol of diPyr$_4$PC/POPC (1:3 molar ratio) vesicles containing a trace amount of [^{3}H]cholesteryl oleate ([^{3}H]CO) for the indicated time periods at 8 °C in DMEM, detached with a rubber policeman, washed three times with PBS, and assayed for diPyr$_4$PC (●) and [^{3}H]CO (○) as described under Experimental Procedures.

bound to be very close to each other, the probability of excimer formation is high and thus the excimer component dominates the emission spectrum of these derivatives in both membranes and solvents (Figure 1). However, if one of the fatty acids is cleaved off, by phospholipase A, for instance, the fatty acid and the lysolipid display only monomer fluorescence at low concentrations. These structure-dependent emission characteristics are very useful, since they allow distinction of metabolic products that have experienced only head-group modification (which retain a high excimer to monomer emission ratio) from those cleaved by a deacylating enzyme (which display only monomer fluorescence). Such structure-sensitive detection can be applied both to HPLC analysis and microscopic imaging (see below).

DiPyr$_4$GPLs Are Incorporated into HF Cells Mainly by Spontaneous Monomer Diffusion. The incorporation of diPyr$_4$GPLs into HF cells was studied by co-incubating the cells with vesicles containing diPyr$_4$PC and a nonexchangeable vesicle marker, [^{3}H]cholesteryl oleate ([^{3}H]CO) up to 2 h at 8 °C. As shown in Figure 2, the fraction of either diPyr$_4$PC or [^{3}H]CO in cells increased with time, but much more (>10-fold) of the former lipid was associated with the cells at all time points, strongly suggesting that spontaneous diffusion, rather than vesicle adhesion, is the prevailing mechanism of diPyr$_4$PC incorporation into the cells. Also the other diPyr$_4$GPLs became cell associated much more efficiently than the liposomal marker (not shown), indicating that they, too, are capable of fast spontaneous diffusion at 8 °C. DiPyr$_4$PE was incorporated more slowly than the other diPyr$_4$GPLs, which further supports spontaneous diffusion as the incorporation mechanism, since PE is known to translocate between membranes more slowly than other GPLs (Massey et al., 1982).

DiPyr$_4$PC Is Not Subject to Head-Group Modification at 37 °C. The metabolism of diPyr$_4$PC at 37 °C was monitored by HPLC using two-channel detection (see Experimental Procedures). Even after 12 h of incubation, the diPyr$_4$PC peak was the only significant one detectable on the excimer

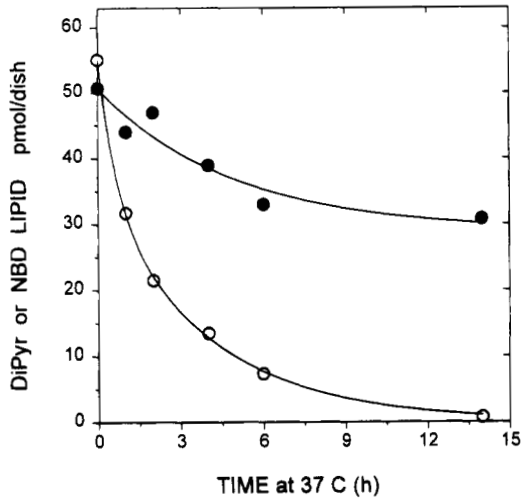

FIGURE 3: DiPyr$_4$PC is degraded much more slowly than NBD$_6$PC in HF cells. HF cells were labeled with sonicated liposomes (21.3 μM total lipid) composed of NBD$_6$-PC/diPyr$_4$PC/POPC (1:1:2 molar ratio) for 60 min at 8 °C, washed, and further incubated at 37 °C in DMEM. The cells were then washed and extracted, and their NBD$_6$PC (○) and diPyr$_4$PC (●) contents were determined as described under Experimental Procedures. The experiment was repeated once with similar results.

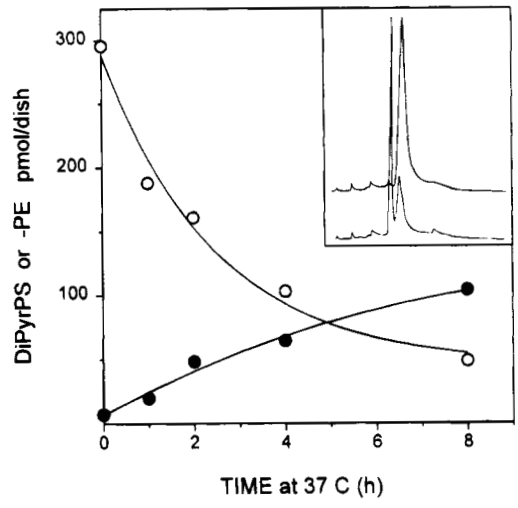

FIGURE 4: DiPyr$_4$PS is converted to diPyr$_4$PE at 37 °C. Confluent HF monolayers were labeled with diPyr$_4$PS/POPC (1:3 molar ratio, 21.3 μM total lipid) liposomes for 60 min at 8 °C, washed, further incubated at 37 °C in DMEM, and analyzed for diPyr$_4$PS (○) and diPyr$_4$PE (●) content. Lipid extraction and HPLC analysis were done as described under Experimental Procedures. The inset shows chromatograms of cellular lipids after labeling at 8 °C (upper trace) or after further incubation at 37 °C for 8 h (lower trace). The first major peak from the left is diPyr$_4$PE, and the second one is diPyr$_4$-PS. The small peak to the right of the diPyr$_4$PS peak in the lower chromatogram corresponds to the position where diPyr$_4$PC elutes. Detection was at 480 nm. The experiment was repeated four times with similar results.

channel (not shown), indicating that diPyr$_4$PC was not subjected to polar head group modification in HF cells. Since the amount of diPyr$_4$PC in cells decreased slowly during this period (see below), some acyl-chain cleavage probably had occurred. However, significant amounts of Pyr$_4$FA, the lyso compound, or reacylation products, which all should be detectable on the monomer channel, were not observed, indicating that no reincorporation of the released fatty acid into cellular lipids occurred and, secondly, that the degradation products were probably excreted from the cells. Indeed, analysis of the incubation medium revealed increasing amounts of Pyr$_4$FA with time, indicating that the released Pyr$_4$FA is excreted from the cells (not shown). The absence of Pyr$_4$FA reacylation products in cells is in agreement with our previous finding that exogenous Pyr$_4$FA incorporates very poorly into the lipids of hamster kidney fibroblasts (Kasurinen & Somerharju, 1992). As in the case of Pyr$_4$-PC, no head-group modification of diPyr$_4$PE was detected in extracts of cells incubated at 37 °C.

DiPyr$_4$PC Is Degraded Much More Slowly than NBD$_6$PC in HF Cells. To compare the stability of diPyr$_4$PC with that of the commonly used NBD$_6$PC, HF cells were labeled simultaneously with NBD$_6$PC and diPyr$_4$PC by co-incubation with vesicles containing both derivatives at 8 °C. Similar amounts of the two lipids were incorporated into the cells (Figure 3). However, when the temperature was shifted to 37 °C, NBD$_6$PC rapidly disappeared from the cells with a half-time of about 2 h. In contrast, diPyr$_4$PC disappeared much more slowly. Even after 14 h of incubation, over 60% of incorporated diPyr$_4$PC was still retained in cells (Figure 3). Thus the pyrenyl lipid appears to be far more stable toward metabolic degradation than the NBD$_6$ derivative. The stability of the other diPyr$_4$GPLs was not studied in similar detail, but they also appeared to be markedly stable toward degradation by cellular phospholipases as indicated by the relatively slow depletion of cellular excimer fluorescence (see Figures 5 and 6).

DiPyr$_4$PS Is Converted Efficiently to diPyr$_4$PE at 37 °C. When HF cells were incubated with diPyr$_4$PS-containing vesicles for 1 h at 8 °C, no metabolism of the fluorescent lipid was observed (Figure 4, inset). However, when the cells were shifted to 37 °C, efficient conversion to diPyr$_4$PE was observed (Figure 4). After 8 h, >30% of the diPyr$_4$PS introduced into the cells had been converted to the corresponding PE derivative. Small amounts of diPyr$_4$PC was also detected after 8 h of incubation (Figure 4, inset). It is very likely that the conversion of diPyr$_4$PS to diPyr$_4$PE is due to decarboxylation by the mitochondrial decarboxylase, since diPyr$_4$PS is efficiently decarboxylated by mitochondria *in vitro* (Jasinska et al., 1993), and second, prominent labeling of mitochondria was observed in cells incubated with diPyr$_4$PS (see below). However, we cannot exclude that the base-exchange mechanism (Kuge et al., 1986) also contributes to the formation of diPyr$_4$PE. On the basis of the assumption that degradation (i.e., conversion to nonexcimer-forming metabolites) of diPyr$_4$PS and diPyr$_4$PE occurred at similar rates, the half-time of decarboxylation was calculated to be approximately 3 h. This corresponds to a rate that is about twice that measured for the decarboxylation of exogenous NBD$_{12}$-labeled phosphatidylserine (Kobayashi & Arakawa, 1991) in HF cells or endogenous, newly formed PS in hamster kidney cells (Voelker, 1984).

Since the cellular excimer fluorescence decreased somewhat during the incubation at 37 °C (Figure 4), part of the diPyr$_4$PS and/or the diPyr$_4$PE produced thereof was obviously degraded to monopyrenyl derivatives. As with the PC derivative, a time-dependent increase of Pyr$_4$FA in the medium was detected (not shown).

DiPyr$_4$PA Is Metabolized to DiPyr$_4$DG, -TG and -PC. DiPyr$_4$PA differed from the other derivatives in that extensive metabolism was observed already at 8 °C (Figure 5). The major metabolite at this temperature was diPyr$_4$DG, which represented almost half of the total cellular dipyrenyl lipids

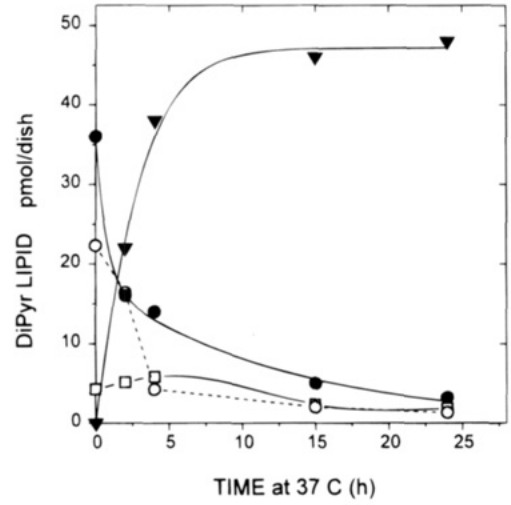

FIGURE 5: $DiPyr_4PA$ is metabolized to $DiPyr_4DG$, -TG and -PC. Confluent HF monolayers were labeled with $diPyr_4PA$/POPC liposomes (1:3 molar ratio, 21.3 μM total lipid) for 60 min at 8 °C, washed, and incubated at 37 °C in DMEM. HPLC analysis and quantitation of the lipids were done as described under Experimental Procedures. A representative experiment is shown. Some variation in the DG/PA ratio after labeling at 8 °C was observed between experiments, apparently due to variation in the number of adhering donor vesicles. $DiPyr_4PA$ (●), $diPyr_4PC$ (▼), $diPyr_4DG$ (○), and $diPyr_4TG$ (□).

at the end of the labeling period. Previously NBD_6-phosphatidic acid was shown to metabolize efficiently to NBD_6 diglyceride in Chinese hamster fibroblasts at 2 °C (Pagano et al., 1983). This process is probably catalyzed by a phosphatidate phosphohydrolase located in the outer leaflet of the plasma membrane (Florin-Christensen et al., 1992). After the cells were shifted to 37 °C, $diPyr_4PA$ and -DG decreased rapidly and were replaced by $diPyr_4TG$ and -PC. After extended incubation $diPyr_4PC$ was the main $diPyr_4GPL$ present, representing >90% of the cellular dipyrenyl lipids after 24 h at 37 °C (Figure 5). The accumulation of $diPyr_4PC$ is consistent with the stability of *exogenous* $diPyr_4PC$ (see above). As with other $diPyr_4GPLs$, HPLC analysis showed a time-dependent increase of Pyr_4-FA in the medium.

Intracellular Distribution of $diPyr_4GPLs$. A fluorescence microscope equipped with a digital imaging system was used to determine the intracellular distribution of the various $diPyr_4GPLs$ in HF cells after labeling at 8 °C, i.e., under conditions where endocytosis is inhibited (Weigel & Oka, 1981), or after further incubation at 37 °C. The cellular compartments labeled by $diPyr_4GPLs$ were identified on the basis of their characteristic appearance and/or by colocalization of organelle-specific fluorescent markers (see the captions to Figures 6−8 for details).

At 8 °C, $diPyr_4PC$ labeled the plasma membrane prominently (Figure 6A). Because labeling of the intracellular membranes could not be detected, the restriction of $diPyr_4$-PC to the outer leaflet of the plasma membrane was indicated. This localization is supported by the fact that repeated incubation in BSA-containing medium extracted 88% (n = 2) of $diPyr_4PC$ according to a fluorometric determination, or 76 ± 18% (n = 5) as determined by quantitative image analysis. Furthermore, addition of Tnp-LPE, a nonpenetrating quencher, to the medium abolished the cellular fluorescence almost completely, thus supporting the restriction of $diPyr_4PC$ to the plasma membrane (Figure 6B). That Tnp-LPE did not penetrate into the cells and thus indeed quenched only the fluorescent lipid molecules present in the plasma membrane is demonstrated by the finding that the fluorescence of intracellular membranes was not significantly quenched upon addition of Tnp-LPE (see below).

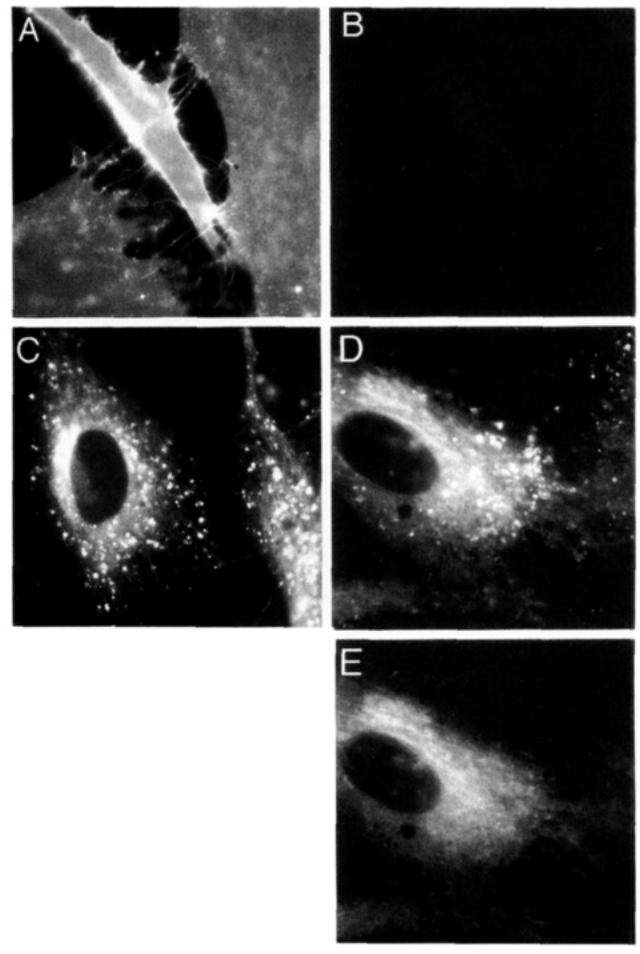

FIGURE 6: $DiPyr_4PC$ remains on the plasma membrane of HF cells at 8 °C but is internalized at 37 °C. (A) HF cells on glass cover slips were labeled with $diPyr_4PC$/POPC liposomes (1:3 molar ratio, 64 μM total lipid) for 60 min at 8 °C and imaged. In panel B the same cell was imaged 1 min after addition of a nonpenetrating quencher of pyrene fluorescence, Tnp-LPE (10 μM). In panel C the cells were incubated at 37 °C in DMEM for 2 h and imaged after addition of Tnp-LPE to improve contrast. Tnp-LPE did not have any detectable effect on the fluorescence of the internal membranes. In panel D, the cells were incubated at 37 °C in DMEM for 24 h before imaging. In panel E, the same field was imaged again after addition of acridine orange, which accumulates in lysosomes and endosomes and thus quenches pyrene lipid fluorescence in these structures. All images were obtained with the pyrene filter set, and in each case the medium was deoxygenated (Englander et al., 1987) by adding glucose (0.6%), glucose oxidase (10 units), and catalase (0.06 unit) to precooled chambers 1 min before imaging. Such deoxygenation of the medium, which virtually eliminates photobleaching of pyrene (unpublished results), had no detectable effect on fluorophore distribution or on cell morphology during the period required for imaging.

When the $diPyr_4PC$-labeled cells were shifted to 37 °C for 2 h before imaging, internalization of the lipid occurred as indicated by labeling of intracellular membranes, including those of mitochondria and the endoplasmic reticulum (Figure 6C). However, the most prominently labeled structures were vesicles scattered throughout the cytoplasm. With time these vesicular structures increased in size and tended to accumulate in the perinuclear region (Figure 6D). Most

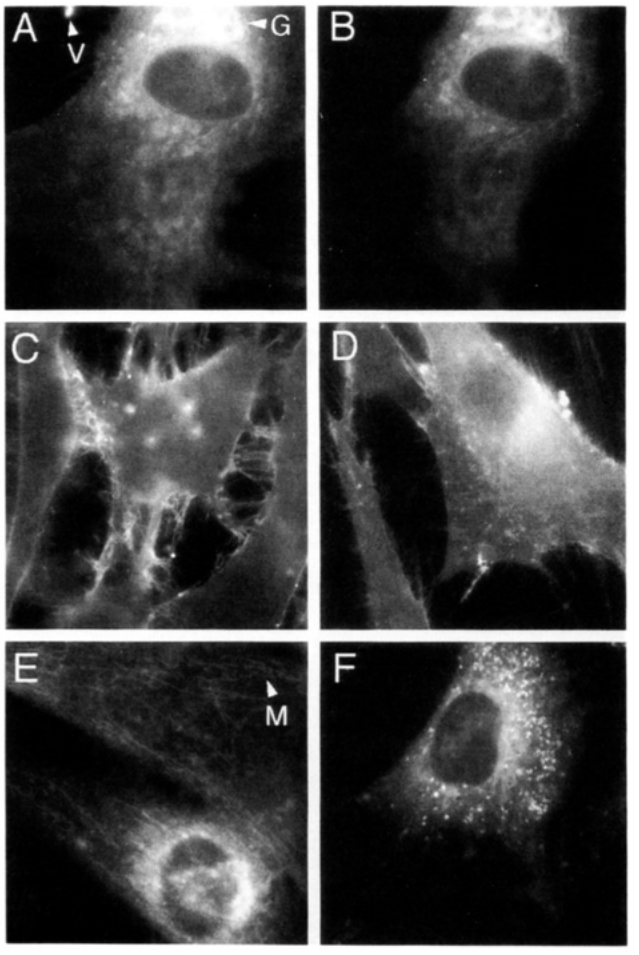

FIGURE 7: DiPyr$_4$PS is translocated through plasma membrane at 8 °C and labels mitochondria and the Golgi apparatus at 37 °C. HF cells on glass cover slips were incubated with diPyr$_4$PS/POPC (1:3 molar ratio, 21.3 μM total lipid) vesicles for 60 min at 8 °C and further treated as specified in the caption to Figure 6. (A) Cells after labeling at 8 °C. (B) Cells of panel A treated with Tnp-LPE. (C) As in panel A, but the cells were pretreated with NEM (Martin & Pagano, 1987). (D) As in panel A, but the cells were incubated in the presence of 100 μM orthovanadate. (E) Cells incubated at 37 °C in DMEM for 2 h. (F) Cells incubated at 37 °C in DMEM for 24 h. Other details are given in the caption to Figure 6. Mitochondria (M) (panel E) were identified on the basis of their characteristic shape and colocalization of a mitochondria-specific dye, nonyl acridine orange (Septinius et al., 1985) added after pyrene imaging (not shown). The Golgi apparatus (G) (panel A) was recognized on the basis of its characteristic tubular network appearance or by labeling of parallel samples (not shown) with BODIPY-ceramide, a Golgi marker (Pagano et al., 1991). An adhering donor vesicle (V) (panel A) is also indicated.

probably, they are secondary endosomes and/or lysosomes since their fluorescence was specifically quenched upon addition of acridine orange (Figure 6E), which is known to accumulate in acidic organelles (Matteoni & Kreis, 1987). The quenching probably occurs by the resonance energy transfer mechanism, since acridine orange absorption overlaps extensively with the pyrene excimer emission spectrum.

DiPyr$_4$PS behaved completely differently from the PC derivative, as a major fraction of this lipid was internalized already at 8 °C (Figure 7A). The internalization of diPyr$_4$-PS was rapid and extensive since after 1 h at 8 °C a major fraction of the probe appeared to be present in intracellular membranes, including those of the endoplasmic reticulum, the Golgi complex, and the nucleus. Addition of Tnp-LPE quenched only the (diffuse) plasma membrane fluorescence (Figure 7B), demonstrating that (i) the internalization diPyr$_4$-PS was a consequence of physiological processes rather than permeabilization of the plasma membrane upon incubation with the fluorescent lipid and (ii) Tnp-LPE does not penetrate the plasma membrane. The rapid internalization of diPyr$_4$-PS and the lack of internalization of diPyr$_4$PC at 8 °C suggest that the former lipid was internalized through the action of the aminophospholipid translocase, which is present in the plasma membrane of various cell types (Devaux, 1991) and tranlocates PS and PE, but not PC, from the outer to the inner leaflet of the plasma membrane. Treatment of the cells with NEM or orthovanadate, both inhibitors of the translocase (Devaux, 1991), greatly inhibited labeling of the internal membranes by diPyr$_4$PS (Figure 7C,D), strongly supporting the involvement of the translocase.

When the temperature was shifted to 37 °C, diPyr$_4$PS was almost completely internalized and labeled prominently the mitochondria and the Golgi complex (Figure 7E). After 24 h of incubation perinuclear vesicular structures became brightly labeled (Figure 7F). These structures resembled closely those seen in diPyr$_4$PC-labeled cells after similar incubation and may thus represent secondary lysosomes. It is noted that after such a long incubation diPyr$_4$PS should be almost completely converted to diPyr$_4$PE (see Figure 4), and thus fluorescence of the vesicular structures may originate mainly from the latter lipid.

The intracellular distribution of diPyr$_4$PE was similar to that observed for the PS derivative at both 8 and 37 °C (not shown) with the exception that this lipid was less effectively internalized at 8 °C, in line with the lower activity of the translocase toward PE (Devaux, 1991).

In cells incubated with diPyr$_4$PA at 8 °C only the plasma membrane was significantly labeled (Figure 8A). Although considerable amounts of diPyr$_4$DG are formed during such an incubation (see Figure 5) and this lipid should be able to penetrate the plasma membrane readily (Ganong & Bell, 1984; Pagano & Longmuir, 1985), slow interbilayer transfer, which is typical for diglycerides (Massey et al., 1982; Nichols & Pagano, 1983), probably inhibits translocation to the internal membranes. When the cells were shifted to 37 °C, almost all of the fluorescence was intracellular (Figure 8B). The endoplasmic reticulum, the nuclear membrane, and the Golgi apparatus became labeled. However, the most characteristic feature was the presence of numerous, brightly fluorescent bodies scattered throughout the cytoplasm (Figure 8B). These structures, which were not seen in cells incubated similarly with other diPyr$_4$GPLs, most likely represent neutral lipid droplets which are typically present in the cytoplasm of cultured cells. To confirm this, diPyr$_4$PA-labeled cells were first incubated for several hours at 37 °C and then imaged with the pyrene filter set (Figure 8C). Nile red, a marker for neutral lipid droplets (Greenspan et al., 1985), was then added to the medium, and the same cells were imaged again, this time with the nile red filter set (Figure 8D). Clearly, the droplets displayed prominent fluorescence when imaged with either filter set, thus strongly supporting the identification of the spherical bodies as neutral lipid droplets. Pagano et al. (1983) have shown that in Chinese hamster cells labeled with NBD$_6$-phosphatidic acid, NBD$_6$ triglyceride is the major fluorescent compound of such lipid droplets. Therefore, it is likely that the diPyr$_4$TG metabolite is the major fluorescent lipid of the lipid droplets in HF cells incubated with diPyr$_4$PA.

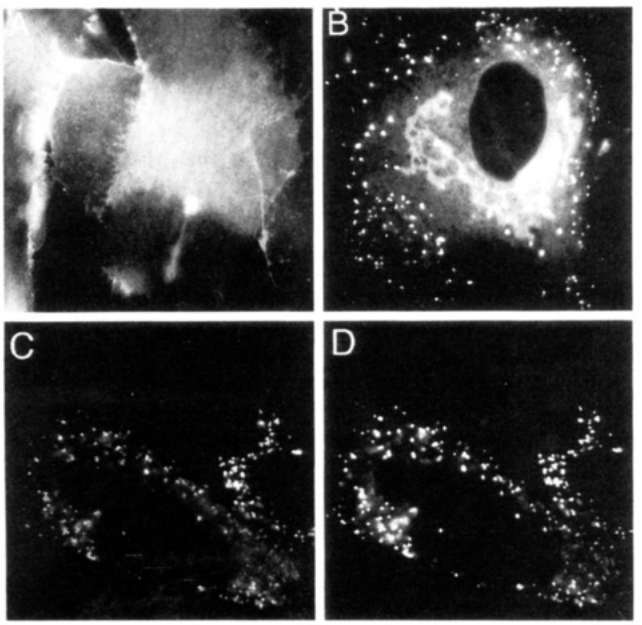

FIGURE 8: Lipid droplets are prominently labeled in cells incubated with diPyr$_4$PA at 37 °C. HF cells grown on cover slips were incubated with diPyr$_4$PA/POPC (1:3 molar ratio, 21.3 μM total lipid) vesicles for 60 min at 8 °C, washed, and imaged immediately (A) or after further incubation at 37 °C in DMEM for 2 h (B). In panel A the cells partially overlap, thus creating areas of brighter fluorescence. In panel C the cells were labeled with diPyr$_4$PA, further incubated at 37 °C for 5 h, and then imaged with the pyrene filter set. In panel D the same cells were additionally labeled with nile red, a lipid droplet stain, for 1 min, washed, and imaged again with the nile red filter set. Extensive overlap between the pyrene and the nile red images is obvious. Other details were as described in the caption to Figure 6.

DISCUSSION

The present study demonstrates that the general, head group dependent metabolic behavior of diPyr$_4$GPLs is similar to that of natural lipids (Vance, 1989); i.e., diPyr$_4$PA is converted to the corresponding diglyceride, triglyceride, and phosphatidylcholine derivatives, and diPyr$_4$PS is decarboxylated to diPyr$_4$PE, while the PC and PE derivatives are not subject to head-group modification. In many respects the metabolism of diPyr$_4$GPLs also resembles that found for the corresponding NBD$_6$ derivatives (Pagano & Sleight, 1985). Thus it appears that many head group related metabolic events are surprisingly insensitive to the structure of the GPL hydrocarbons. This is an important result, since it adds confidence to the extrapolation of data obtained with fluorescent lipid analogues to the behavior of their natural counterparts. Nevertheless, such extrapolation has to be done with proper caution, as there are phenomena where the structure of the acyl chains is of importance (see below).

The present data also demonstrates a major, important difference between the diPyr$_4$- and NBD$_6$GPLs: the former are much more resistant to cellular degradation. This is well demonstrated by the finding that the half-life of diPyr$_4$PC in HF cells at 37 °C was >24 h, while the corresponding value for NBD$_6$PC was ~2 h (Figure 3). Previously, even faster degradation of NBD$_6$PC, -PE, and -PA has been observed in other cell types (Pagano et al., 1983; Sleight & Pagano, 1984, 1985; Sleight & Abanto, 1989). What is the reason for such a remarkable difference in metabolic stability? It has been demonstrated that the NBD moiety attached to an acyl chain of a phospholipid "loops up" to the bilayer−water interface (Chattopadhyay & London, 1988; Wolf et al., 1992). This may alter the conformation and/or the depth of the phospholipid molecule in the membrane so that the ester bonds become more exposed and thus available for phospholipases. In this respect, the NBD derivatives may resemble, as suggested by Steinbrecher and Pritchard (1989), peroxidixed GPLs, which also have a polar fatty acid residue and are susceptible to hydrolysis by certain cellular phospholipases (van Kuijk et al., 1987). It is also possible that the serum phospholipase A that degrades both NBD$_6$PC and oxidized, but not unoxidized, phosphatidylcholines (Steinbrecher & Pritchard, 1989) is retained on the plasma membrane of the cells and is responsible for the degradation of the NBD$_6$ lipids. Supporting this possibility, Sleight and Pagano (1984) have shown that degradation of NBD$_6$PC occurs mainly on the plasma membrane of Chinese hamster fibroblasts.

The resistance of diPyr$_4$GPLs to cellular degradation may result (1) from a more natural conformation of these lipids or (2) from the bulkiness of the hydrocarbon region interfering with binding to the active site of the (phospho)lipases. Relative to the first point, the conformation of the glycerol moiety of dipyrenyl GPLs appears to be similar to that of their natural counterparts (Eklund et al., 1992), and there seems to be no tendency for the pyrene residue to loop up toward the bilayer−water interface (Virtanen et al., 1994). These results are not unexpected in view of the low polarity of the pyrenyl moiety. As to the second point, it has been demonstrated that bulky residues in the acyl chain close to the ester bond effectively inhibit the action of certain A-type phospholipases and related enzymes (DeBose et al., 1985; Pownall et al., 1987).

A very important consequence of the stability of the diPyr$_4$-GPLs is that these lipids offer the possibility to study, at the physiological temperature, lipid transport routes that involve several cellular compartments and may thus have half-times of several hours. One example of such a complex route is the transport of PS from the plasma membrane to the mitochondrial inner membrane (Kobayashi & Arakawa, 1991) and the subsequent transport of the decarboxylation product, PE, from there to other organelles. Another example is the recycling of plasma membrane lipids through various intracellular compartments, including endocytotic vesicles, endosomes, and the Golgi apparatus (Hoekstra & Kok, 1992). Furthermore, the nearly quantitative conversion of diPyr$_4$-PA to diPyr$_4$PC (Figure 5), along with the apparent stability of the latter, makes it now possible to investigate intracellular trafficking of endogenous fluorescent PC. However, it is important to note that due to their low hydrophobicity diPyr$_4$-GPLs, when present in the cytoplasmic leaflet of organelle membranes, can bypass certain interorganelle transport mechanisms (e.g., protein- or vesicle-mediated transport) that may be necessary for translocation of the more hydrophobic natural lipids. Accordingly, due care should be exercised when diPyr$_4$GPLs and other relatively water soluble derivatives, such as the NBD$_6$ lipids, are used to investigate interorganelle lipid traffic. On the other hand, low hydrophobicity does not seem to interfere with protein-mediated transmembrane movement of GPLs (Bishop & Bell, 1985; Devaux, 1991), and thus diPyr$_4$GPLs could be used to study this process without complications arising from probe degradation (Colleau et al., 1991).

The intracellular distribution of diPyr$_4$GPLs resembles that of the corresponding NBD$_6$ lipids (Pagano & Sleight, 1985), indicating that the fluorophore does not significantly affect the distribution. However, there are some exceptions to this similarity. First, after prolonged incubation at 37 °C, diPyr$_4$-GPLs accumulated in vesicular structures which probably are secondary lysosomes (and/or endosomes) on the basis of selective quenching of the pyrene fluorescence of these structures by acridine orange, a stain that accumulates in acidic compartments (Matteoni & Kreis, 1987). Accumulation of diPyr$_4$GPLs in lysosomes is not unexpected since (i) these lipids, like their natural counterparts, should eventually enter secondary lysosomes; (ii) they are poorly degraded by lysosomal phospholipases *in vitro*;[2] and (iii) it is generally held that phospholipids cannot escape from lysosomes without hydrolysis. Similar accumulation of NBD$_6$ glycerophospholipids has not been observed, probably because they are rapidly degraded even before they enter the lysosomes (see above). If accumulation of diPyr$_4$GPLs in secondary lysosomes is confirmed by future experiments, these lipids could serve as useful model compounds for studies on lysosomal transport and accumulation of glycerophospholipids or as fluorescent markers for lipids in secondary lysosomes. Another difference between the intracellular distributions of diPyr$_4$GPLs and the NBD$_6$ derivatives is that little if any staining of the intracellular membranes was observed in cells incubated with diPyr$_4$PA at 8 °C (Figure 8A), while extensive labeling of those membranes was observed in NBD$_6$PA-labeled cells under similar conditions (Pagano et al., 1983). The reason for this difference is not clear, but the difference could be due to less efficient conversion of diPyr$_4$PA to the diglyceride (see Figure 5), which is the penetrating species, in our cells. Alternatively, albeit less likely, spontaneous translocation of the diPyr$_4$DG product from the inner leaflet of the plasma membrane to the intracellular membranes could be considerably slower than that of NBD$_6$DG.

As mentioned above, intramolecular excimer formation is an important feature of the diPyrGPLs since its allows structure-sensitive detection of metabolites upon HPLC analysis, i.e. direct distinction of intact lipids from those that have experienced deacylation. However, an even more exciting application of the intramolecular excimer formation is E/M ratio imaging, which could be used to map local fluidity (Dix & Verkman, 1990) or to detect segregated domains in membranes of living cells (Rodgers & Glaser, 1991). Dipyrenyl derivatives are most suitable for such studies because (i) at low concentrations their E/M ratio becomes independent of probe concentration, (ii) their E/M ratio is sensitive to physical properties of the membrane (Eklund et al., 1992), and (iii) they are markedly resistant to metabolic degradation. E/M ratio imaging and diPyrGPLs also could be used to monitor local phospholipase activities in cells. For this purpose, however, the long-chain homologues may be preferred, as they appear to be more susceptible to hydrolysis by cellular phospolipases than diPyr$_4$GPLs.[2]

In conclusion, diPyr$_4$GPLs are easily incorporated into target cells by spontaneous diffusion from donor vesicles. They are remarkably resistant to metabolic degradation, and their head group dependent metabolism and transbilayer movement are similar to those of their natural counterparts. Because of these properties, important aspects of intracellular traffic, such as recycling of glycerophospholipids between various compartments, become accessible to experimentation with fluorescent derivatives. Furthermore, diPyrGPLs should allow direct microscopic monitoring of physical properties of membranes and lipolytic activities in living cells based on environment- and structure-sensitive intramolecular excimer formation.

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