INTRODUCTION OF HYDROPHOBIC FLUORESCENT LIPID ANALOGUES INTO LIVING CULTURED CELLS AND THEIR IMAGING THEREIN

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Academic dissertation

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To my father Unto (1947 – 2000)
and to my son Kaarlo (2000 -)

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2. ABBREVIATIONS

10:0-Pyr_{10}PC	*sn*-1-decanoyl, 2-pyrenedecanoyl-phosphatidylcholine
16:0-Pyr_nPC	*sn*-1-palmitoyl,2-pyrenylacyl-phosphatidylcholine (*n* = number of aliphatic carbons in the pyrenylacyl chain)
BHK-21	Baby Hamster Kidney cells
$BODIBY_{12}$-PC	1-hexadecanoyl,2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-*sn*-glycero-3-phosphocholine
$BODIPY_{12}$-PE	1-hexadecanoyl,2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-*sn*-glycero-3-phosphoethanolamine
$BODIPY_{12}$-SM	s*n*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-sphingosylphosphorylcholine
CD	cyclodextrin
γ-CD	γ-cyclodextrin
CE-γ-CD	carboxyethyl-γ-cyclodextrin
$DiPyr_n$PC	*sn*-1,2-pyrenylacyl-phosphatidylcholine (*n* = number of aliphatic carbons in the pyrenylacyl chain)
DOPE	*sn*-1, 2-dioleyl-phophatidylethanolamine
ER	endoplasmic reticulum
FA	fatty acid
HF	Human fibroblast cells
LDH	lactate dehydrogenase
MEM	Minimum Essential Medium
$MonoPyr_n$PC	*sn*-1-acyl, 2-pyrenylacyl-phosphatidylcholine
NBD_{12}PS	2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero- 3-phosphoserine
NBD_6PC	2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero- 3-phosphocholine
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
POPA	*sn*-1-palmitoyl, 2-oleoyl-phosphatidic acid
POPC	*sn*-1-palmitoyl, 2-oleoyl-phosphatidylcholine
PS	phosphatidylserine
Pyr_nPC	16:0-pyrenylacyl-phosphatidylcholine (n = number of aliphatic carbons in the acyl chain)
PyrPC	pyrenylacyl-phosphatidylcholine
PyrPL	pyrene-labeled phospholipid
SM	sphingomyelin
TNP-LPE	*N*-trinitrophenyl-lysophosphatidylethanolamine
TNP-PE	*N*-trinitrophenyl-phosphatidylethanolamine

3. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals I - III

I Tanhuanpää, K., and P. Somerharju. 1999. γ-Cyclodextrins Greatly Enhance Translocation of Hydrophobic Fluorescent Phospholipids from Vesicles to Cells in Culture. *Journal of Biological Chemistry*. **274**:35359-35366.

II Tanhuanpää, K., K. Cheng,, J. Virtanen, A. Anttonen, P. Somerharju. 2001. Characteristics of Pyrene Phospholipid / γ-Cyclodextrin Complex. *Biophysical journal*. **81**(3)

III Tanhuanpää, K., J. Virtanen, and P. Somerharju. 2000. Fluorescence imaging of pyrene-labeled lipids in living cells. *Biochimica Biophysica Acta*. **1497**:308-320.

In addition some unpublished data is presented

4. INTRODUCTION

Eukaryotic cells contain multiple membrane-bound compartments whose specialized functions require distinct protein and lipid compositions. Assembly of a proper set of proteins and lipids into each compartment requires a three-step process involving (1) the biosynthesis of proteins and lipids, (2) selective transport of proteins and lipids across a bilayer, (3) selective transport of proteins and lipids between different membrane compartments. Experimental work over the last 15 years has brought us detailed knowledge about the molecular mechanisms responsible for intra- and intercompartmental protein transport, but knowledge concerning lipid trafficking is less detailed.

Most phospholipids are synthesized in the endoplasmic reticulum (ER), but are abundant in all organelle membranes (Dennis and Kennedy, 1972). Efficient transport of those phospholipids from ER to other organelles must take place. There are several mechanisms that could account for this transfer, i.e., vesicle traffic, spontaneous or protein-assisted diffusion via the cytoplasm, or diffusion via membrane (hemi)fusion sites (reviewed in Voelker, 1991; van Meer and van Genderen, 1994). The relative contributions of these various transport mechanisms have not been established. It is also unknown how the intracellular trafficking of phospholipids is controlled so that the different membranes can maintain their distinct lipid compositions. A major reason for this lack of knowledge is that it is very difficult to follow the trafficking of phospholipids inside the cell. For example, immunolocalization, which has played a key role in resolving the sorting and transport mechanisms of proteins, cannot be applied because phospholipids are generally poor antigens, most (glycero)phospholipids are abundant in all membranes, and because it is not possible to obtain stoichiometric complex formation due to the large size of the antibody molecule.

To circumvent the tracing problem, short-chain fluorescent (C_6-NBD) lipids were introduced by Pagano and co-workers (reviewed in Pagano and Sleight, 1985; Rosenwald and Pagano, 1993). However, due to their hydrophilicity these lipids can distribute spontaneously between all accessible membrane surfaces (Pagano et al., 1983). Thus their use is questionable when one studies intracellular trafficking of lipids having access to the cytoplasmic leaflet of organelle membranes, which is the case with most glycerophospholipids. Accordingly, fluorescent derivatives with hydrophobicity similar to that of endogenous phospholipids need to be employed to obtain relevant information on intracellular trafficking of the latter. Unfortunately, such derivatives are quite hard to introduce into cells be-

cause of their hydrophobicity. There are several, most often vesicle fusion based, methods to achieve this but none of them are fully satisfactory.

To follow trafficking of fluorescent lipids in cells, one must be able to quantitatively and selectively image organelle membranes. This is complicated by several factors like background fluorescence and scattering from the optics and the medium and photobleaching of the fluorophore. Also cellular autofluorescence deriving mainly from NAD/NADH+, flavins and lysosomal residues (Schnell et al., 1999) can be significant, particularly at low probe concentrations. Typically, an off-focus image or an image obtained from a cell-free area is subtracted to correct for the background signal (Wolf, 1998). These methods, however, are not always satisfactory, as they do not properly account for the uneven distribution of cellular autofluorescence.

The aim of this study was to address the aforementioned problems. Several methods allowing introduction and quantitative imaging of hydrophobic pyrene-labeled phospholipids in cells were developed.

5. REVIEW OF THE LITERATURE

5.1. The structure and lipid composition of biological membranes

5.1.1. Organization of biological membranes

Most biological membranes are extremely complex structures consisting of hundreds or thousands different molecules. The present view of organization of these membranes is based on the fluid-mosaic model from year 1972 (Singer and Nicolson, 1972). In this model phospholipids together with other lipids form a fluid bilayer where the incorporated proteins and the lipid themselves are free to diffuse laterally. Accordingly, the membrane components would be more or less randomly organized in the membrane. However, since then, experimental and theoretical data indicating that membranes are not laterally homogenous has been accumulated (Simons and Ikonen, 1997). Instead domains having distinct lipid and protein compositions exist. In particular, evidence for membrane domains enriched in sphingolipids, cholesterol and certain proteins has been obtained (Brown and Rose, 1992). The feasibility of domain segregation in biological membranes is strongly supported by the fact that domains have been detected in model membranes consisting of different phospholipid species or of a phospholipid and cholesterol (Sankaram and Thompson, 1991; Chong et al., 1994; Virtanen et al., 1995).

The factors driving domain formation in natural membranes are poorly understood at present. A commonly accepted model is that certain, more rigid lipids, like cholesterol and sphingolipids, prefer to associate with each other, thus resulting in formation of domains rich in these lipids (Simons and Ikonen, 1997; Rietveld and Simons, 1998; Brown and London, 1998). This model is largely based on the finding that treatment of cells with certain detergents at low temperature did not fully dissolve the membranes, but left fragments consisting mainly of cholesterol, sphingomyelin and glycosphingolipids as well as certain proteins (Brown and Rose, 1992). Later experiments have provided more direct evidence for the existence of cholesterol/sphingolipid-rich domains in cell membranes (e.g. Pralle et al., 2000). The formation of such domains is probably driven by the complementary geometrical shapes of the sphingolipid and cholesterol molecules as well as by intermolecular hydrogen bonding (Slotte, 1999).

On the other hand, there is now considerable evidence from model membrane studies that in multicomponent lipid bilayers the different components *tend* to adopt a regular, rather than random lateral distribution. This phenomenon (reviewed in Somerharju et al., 1999), also referred to as *superlattice* formation, is very intriguing because it means that there are a number of "critical" compositions which are predicted to be energetically more favorable than the other, intervening compositions. Because of this property, such critical compositions could play a central role in the regulation of the lipid composition of cellular membranes (Virtanen et al., 1998). Notably, the superlattice model also predicts that domains of different composition (i.e., different stoichiometry of the components) can be present in membranes.

5.1.2. Lipid composition of eucaryotic membranes

The main lipid components in eucaryotic cell membranes are glycerophospholipids, steroids and sphingolipids. The most abundant glycerophospholipids are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) which account for 50 to 70 % of the lipids in most membranes. Other common glycerophospholipids are phosphatidylserine (PS) and phosphatidylinositol (PI). The main sterol in mammalian cells is cholesterol. Sphingolipids are a group of lipids that have a ceramide backbone consisting of a sphingoid base and a fatty acid that is amide-linked to the 2-position of the base. The major sphingolipid is sphingomyelin (SM) and other sphingolipids include a large variety of glycosphingolipids like glucosylceramide and galactosylceramide (van Meer and van Genderen, 1994). Different cellular membranes have characteristic lipid compositions. The plasma membrane is rich in SM, cholesterol and PC, the ER is rich in PC and PI and all of cardiolipin is in mitochondria. In BHK cells about 27 % of total lipids and 17 % of phospholipids are in the plasma membrane. 67 % of total cellular SM and PS are in the plasma membrane, and of these most of the SM is in the outer leaflet and all of the PS is on the inner leaflet. In these cells the ER accounts for 28 %, mitochondria for 16 % and lysosomes for 14 % of total lipids (Allan, 1996).

5.1.3. Lipid synthesis and transport in eukaryotic cells

Glycerophospholipids are synthesized in the endoplasmic reticulum and SM synthesis occurs in the lumenal leaflet of the Golgi, but they are abundant in all organelle membranes (Dennis and Kennedy, 1972). In addition, lipids are not always synthesized in the

membranes where their concentration is highest, e.g. cholesterol synthesis occurs in the ER but cholesterol is concentrated in the plasma membrane. Accordingly, efficient transport of the lipids from their sites of synthesis to other organelles must take place. There are several mechanisms that could account for this transfer, i.e., vesicle traffic, spontaneous or protein-assisted diffusion via the cytoplasm, or diffusion via membrane (hemi)fusion sites (reviewed in Voelker, 1991; van Meer and van Genderen, 1994). Lipids are transported with proteins along the vesicle transport pathways from ER to Golgi to plasma membrane or endosomes. From plasma membrane they are transported to endosomes, back to Golgi and to lysosomes. There is a concentration gradient along the exocytotic transport pathway from the ER to plasma membrane where enrichment of SM, glycosphingolipids, and cholesterol is observed at the cost of PC and PI. Monomeric diffusion via aqueous phase is a slow process for most lipids, in the order of hours (cholesterol) to weeks (glycosphingolipids) between lipid vesicles. This transfer can be dramatically enhanced by close apposition of cellular membranes. In cells there are also lipid transfer proteins that enhance lipid transfer *in vitro*, but their role in lipid transport processes *in vivo* is unclear. Of the lipid transport processes, lipid diffusion in the plane of the membrane is the fastest process. It is relatively insensitive to lower temperatures. Some membranes, like the inner and outer mitochondrial membranes, may be connected through (temporary) membrane continuities, which would allow fast lipid diffusion between these membranes. The relative contributions of these various transport mechanisms have not been established. It is also unknown how the intracellular trafficking of phospholipids is controlled so that the different membranes can maintain their distinct lipid compositions.

5.2. Short-chain fluorescent lipids

Short-chain fluorescent (6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl (NBD_6) labeled lipids have been shown to be very useful in studying sphingolipid trafficking (van Meer and Holthuis, 2000; Koval and Pagano, 1991; Hoekstra and Kok, 1992). To name some examples, NBD_6-GlcCer and NBD_6-SM labeling has been used to study segregation of glucosylceramide and sphingomyelin in the apical to basolateral transcytotic route in HepG2 cells (van IJzendoorn et al., 1997). NBD_6-ceramide has been used to study biogenesis of bile canaliculi in hepatic cells (Zaal et al., 1994) and trafficking of sphingolipids in differentiated versus undifferentiated HT29 cells (Babia et al., 1993).

NBD-lipids have been used more rarely to study glycerophospholipid trafficking. One reason for this is that at the physiological temperature short-chain NBD-phospholipids

are rapidly degraded in mammalian cells (Pagano et al., 1983; Sleight and Pagano, 1985; Sleight and Pagano, 1984). This might be due to the looping conformation of the labeled chain that renders NBD-lipids targets for some cellular phospholipases. This looping is caused by the NBD-moiety being so hydrophilic that regardless of the length of the connecting acyl chain, the NBD-group is located close to the hydrocarbon – water interface (Chattopadhyay and London, 1987; Wolf et al., 1992; Huster et al., 2001). An even more serious limitation of these lipids is that they can distribute spontaneously between all accessible membrane surfaces due to their hydrophilicity (Pagano et al., 1983).

Besides NBD also boron dipyrromethene difluoride (BODIPY) labeled lipids have been used in lipid trafficking studies. The BODIPY-fluorophore has a higher fluorescence yield and it is more photostable than NBD. It is also less polar than NBD and is therefore propably better anchored in the membrane bilayer (Bai and Pagano, 1997). BODIPY-labeled lipids have a molar density -dependent fluorescence emission maximum that makes it possible to differentiate membranes containing high and low concentrations of the fluorescent probe (Pagano et al., 1991). However, BODIPY-lipids are also hydrophilic enough to distribute spontaneously between all accessible membrane surfaces (see table 1 in I).

There are several other common fluorescent probes. 3,3'-dioctadecylidocarbocyanine (DiI) is hydrophobic but it also is positively charged, and does not resemble any natural lipid species (Pitas et al., 1981). 5-dimethylmethylaminonaphtene-1-sulfonyl (dansyl) is hydrophilic and like NBD it is located close to the hydrocarbon – water interface (Bartlett et al., 1997). Also antraoyloxy- and diphenylhexatriene fatty acids can be used but they mimic natural lipids less well than pyrene lipids (see below; Naylor et al., 1991).

5.3. Pyrene lipids

Pyrene labeled phospholipids (PyrPLs) represents another type of fluorescent lipid derivatives that can be used to study lipid traffic (Fig. 1). There are several advantages in using pyrene lipids. First, pyrene lipids form concentration-dependent excited-state pyrene dimers, i.e., excimers. At low concentrations, pyrene emits only monomer fluorescence at 370-420 nm. However, when the concentration of the fluorophore increases, the probability of collisions between these excited monomers and ground state pyrene molecules increases, which leads to formation of excimers that emit in the 430-550 nm region (Fig. 2). The excimer formation provides a convenient tool for monitoring many interesting phenomena (see below).

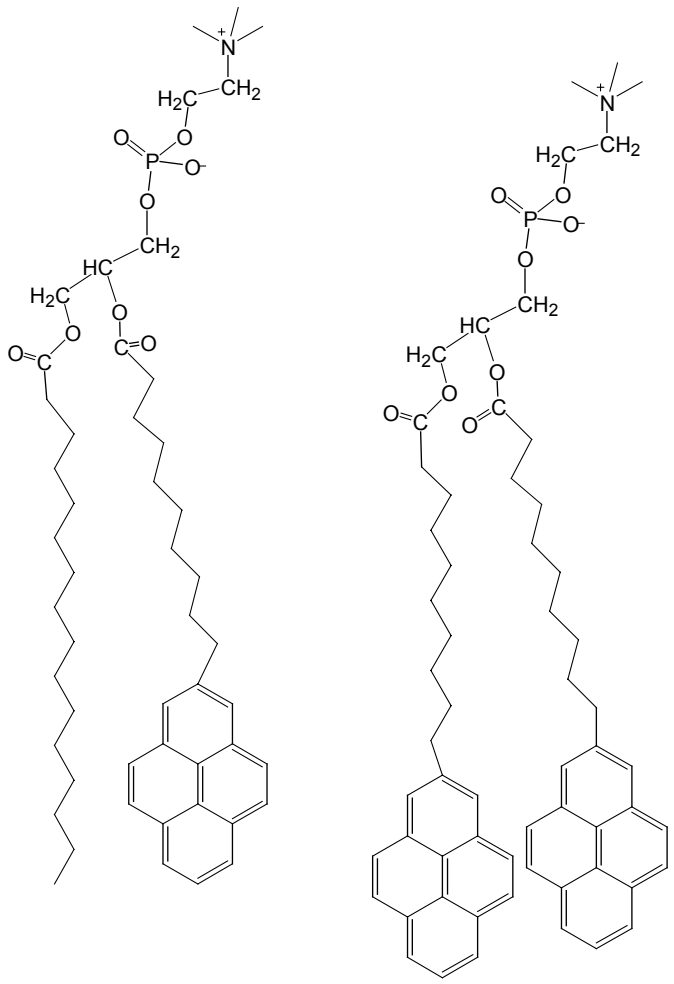

Fig. 1. Structures of 16:0-Pyr$_{10}$PC (left) and DiPyr$_{10}$PC (right). *In the monopyrene lipids used in this study (16:0-Pyr$_n$PXs and 10:0-Pyr$_n$PXs), only the sn-2 acyl chain contains the pyrene label, the sn-1 chain is saturated and contains either 16 or 10 carbons. The length of the pyrenylacyl chain (n) varied from 4 to 14 carbons with two methylene unit intervals. In the dipyrene lipids (DiPyr$_n$PXs) both the acyl chains contain a pyrene moiety linked to the terminal methylene unit. The length of the pyrenylacyl chains (n) varied from 4 to 14 carbons with two methylene unit intervals. Variation of the acyl chain length of DiPyr$_n$PXs by two methylene units has a much larger relative effect on the lipid hydrophobicity than in the case of 16:0-Pyr$_n$PXs, since both acyl chains are extended.*

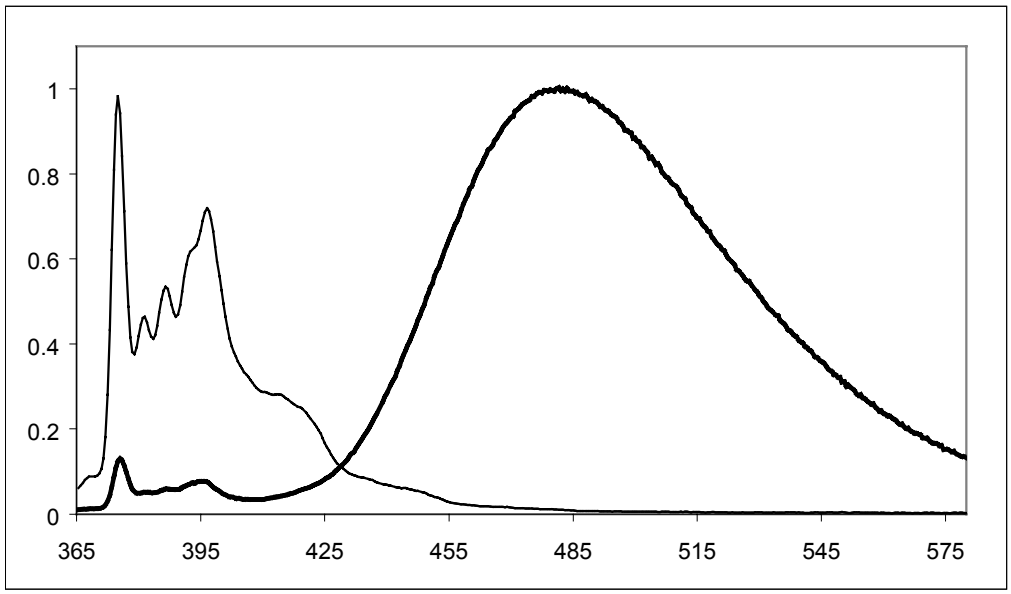

Fig. 2. Normalized emission spectra for a mono- and dipyrene lipid. *16:0-Pyr$_4$PC in γ-CD cavity emits mostly monomer fluorescence (thin line) and DiPyr$_4$PC in γ-CD cavity emits mostly excimer fluorescence (thick line). For details see II.*

Second, the pyrene moiety is hydrophobic and thus does not significantly alter the conformation of the parent lipid (Sassaroli et al., 1995) unlike more polar fluorophores like NBD (Wolf et al., 1992; Chattopadhyay and London, 1987; Huster et al., 2001) and Dansyl (Bartlett et al., 1997) do. It is also notable that pyrene does not significantly increase the effective size of a phospholipid molecule in a lipid monolayer (Somerharju et al., 1985).

Third, PyrPLs mimic well natural phospholipids. They are good substrates for intracellular lipid transfer proteins (Somerharju et al., 1987; van Paridon et al., 1988; van Amerongen et al., 1989; Szolderits et al., 1989). 1,2-dipyrenebutanoyl derivatives of phosphatidylcholine, -ethanolamine, and -serine and phosphatidic acid ($DiPyr_4PC$, -PE, -PS, and -PA, respectively) have similar metabolism and transbilayer movement as the corresponding natural lipids (Kasurinen and Somerharju, 1995). All of them are efficiently incorporated into plasma membrane via spontaneous diffusion from donor vesicles at 8 ^{o}C. At this temperature endocytosis is negligible and the PC-analogue is restricted to the outer leaflet of the plasma membrane. Conversely the PS- and PE-analogues move through plasma membrane via the aminophospholipid translocase and label intracellular structures. At 8 ^{o}C a considerable fraction of $DiPyr_4PA$ is metabolized to the corresponding diglyceride and triglyceride in one hour. At 37 ^{o}C, $DiPyr_4PA$ is metabolized to $DiPyr_4PC$, and after an 8-h incubation it represents 90% of total $DiPyr_4$ lipids. $DiPyr_4PS$ is converted to $DiPyr_4PE$ with an apparent half time of 3 h, by decarboxylation in the mitochondria as isolated mitochondria can decarboxylate all PyrPS species (Jasinska et al., 1993). In contrast to the PA and PS derivatives, no head group modification of either $DiPyr_4PC$ or $DiPyr_4PE$ has been reported. Pyrenyl fatty acids (Pyr_xFA) introduced to cell growth medium are incorporated to cellular lipids (Kasurinen and Somerharju, 1992). This incorporation is more efficient than what it is with antraoyloxy, dansyl, diphenylhexatriene or NBD fattyacids (Pownall and Smith, 1989; Naylor et al., 1991). The length of the acyl chain affects the efficiency of incorporation as well as distribution of the label between lipid species. Pyr_xFAs can be divided into two groups: 1) the short-chain ones including the pyrene butyrate and hexanoate derivatives, which show only modest incorporation to phospholipids and negligible labeling of triglycerides and cholesterol esters, and 2) the long-chain Pyr_xFAs including pyrene octanoate, decanoate, dodecanoate, and myristate derivatives. The longer ones incorporate efficiently to both phospholipids, mainly PC and PE, and neutral lipids, i.e. di- and triglycerides and cholesterol esters. The longer Pyr_xFAs are esterified preferentially to the *sn*-1 position of the glycerol moiety of PC and PE while the shorter ones are found exclusively in the *sn*-2 position indicating that the longer Pyr_xFAs mimic natural saturated fatty acids

whereas the shorter ones may be recognized as polyunsaturated fatty acids by the acylating enzymes. Pyr_xFAs are shortened, probably by β-oxidation in peroxisomes, down to pyrene butyrate.

The fourth advantage of using pyrene lipids is that several systematically constructed sets that cover the hydrophobicity range of natural lipids are available (Somerharju et al., 1987; van Paridon et al., 1988). This helps to draw conclusions regarding the mechanism of lipid transfer *in vivo*, (see Heikinheimo and Somerharju, 1998).

In microscopy the pyrene lipids are somewhat more problematic than NBD or BODIPY lipids because they require excitation in the UV range, i.e. at 345 nm. Many microscopes are not optimized for this wavelength. The pyrene monomer emission peaks at 378 and 395 nm are also not optimal for most microscopes, but this can be circumvented by using dipyrene lipids and monitoring excimer emission at 450-500 nm. In principle it is possible to microscopically map lipid concentration gradients in cellular membranes with excimer/monomer -ratio imaging (Dix and Verkman, 1990).

Pyrene lipids have been frequently used to study spontaneous or protein-mediated lipid transport (Ohnishi et al., 1994; Via et al., 1985; Roseman and Thompson, 1980; Pownall and Smith, 1989; Somerharju et al., 1987; Rao et al., 1997; Rajaram et al., 1994) and particle fusion (Stegmann et al., 1993). Also a variety of membrane-related phenomena including lateral (Galla et al., 1979; Eisinger et al., 1986; Sassaroli et al., 1990) and transverse (Pownall and Smith, 1989) diffusion of lipids, lateral organization of membranes (Galla and Sackmann, 1974; Hresko et al., 1986; Somerharju et al., 1985; Tang and Chong, 1992) and phospholipid conformation (Eklund et al., 1992) have been studied. Phospholipase activity has been studied with dipyrene lipids by measuring the excimer/monomer -ratio which decreases when the lipid is hydrolyzed (Hendrickson, 1994). Alternatively, pyrene lipids that have a covalently attached quencher group, e.g. trinitrophenyl have been used. Such a lipid has negligible fluorescence when intact, but hydrolysis of the lipid causes separation of the fluorophore and the quencher which causes an increase in the fluorescence intensity (Thuren et al., 1988; Zandonella et al., 1995; Duque et al., 1996).

5.4. Previously used cell labeling methods

Short-chain fluorescent lipids can be introduced into cells simply by incubating the cells with donor vesicles containing the fluorescent lipid. The lipid moves relatively rapidly to the cells via spontaneous diffusion. For example, when human fibroblast (HF) cells are co-incubated with phospholipid vesicles containing $DiPyr_4PC$ at 8 ^oC, a considerable amount of fluorescent lipid is incorporated into the cells, but 10-fold less of the vesicle marker, [^{3}H]cholesteryl oleate is associated with the cells (Kasurinen and Somerharju, 1995). On the other hand, long-chain fluorescent lipids cannot be introduced into cells in this way because of their hydrophobicity. Several methods for introduction of such lipids to cells have been reported. Van Meer and Simons (1983) developed a method where MDCK cells are infected with fowl plague or influenza N virus. After a 4 h incubation, the hemagglutinin spike glycoprotein of the virus is present on the plasmamembrane. Then lipid vesicles containing the fluorescent lipid, egg PC, cholesterol and hemagglutinin receptor are bound to the cells at 0 ^oC. The vesicles are then fused to the cells at pH 5.3, 37 ^oC for 30 s. This gives approximately 50 % fusion. The problem with this system is that the infection shuts off the host cell protein synthesis. This limits the time span of the experiment and such a drastic alteration of cellular metabolism may well disturb the normal structure of cellular membranes. The problems of infection can be circumvented by transfecting the hemagglutinin gene to the cells (Danieli et al., 1996). This, however, is quite tedious and the expression of a foreign protein on plasma membrane may also perturb the membrane organization. Another method by van Meer and co-workers (1980) uses phosphatidylcholine-specific exchange proteins from rat or beef liver that are used to transfer PC between vesicles and cells. This requires a relatively large amount of the precious transfer protein, which makes routine cell labeling with this method impractical.

Fusogenic vesicles consisting of a cationic amphiphile like dioleoyloxy-propyltrimethylammonium chloride (DOTMA) and DOPE can also be used to transfer nonexchangeable lipids to cultured cells (Leventis and Silvius, 1990). This method is more often used in cell transfection. The fusogenic vesicles unfortunately introduce a relatively large amount of cytotoxic lipids to cells and may cause pore formation in membranes (van der Woude et al., 1995).

As noted above, cells can be labeled by incubating them with a labeled fatty acid. This is a non-specific method, i.e., several lipid classes are labeled simultaneously (Kasurinen and Somerharju, 1995). A low concentration of Pluronic F127 detergent in the labeling medium has been shown to enhance cell labeling. Usually this method is used to

load calcium indicators to cells (Poenie et al., 1986), but it has also been used to introduce bis-pyrene-propane to cells (Dix and Verkman, 1990).

5.5. Cyclodextrins

Cyclodextrins (CDs) are doughnut-shaped cyclic polymers consisting of 6 (α-CD), 7 (β-CD), 8 (γ-CD) or more glucose (α-(1→4) glucopyranose) units. CDs originate from enzymatic degradation of starch by CD-glucano-transferase. They are capable of solubilizing hydrophobic molecules in aqueous media due to the hydrophobic character of their internal cavity. The cavity is hydrophobic because it is lined with skeletal carbons and ethereal oxygens of the glucose residues while all hydrophilic groups are on the outer surface of the molecule (Szejtli, 1988). Cyclodextrins have many pharmacological applications as they can be used to enhance drug solubility and delivery (Szejtli, 1988; Loftsson and Brewster, 1996). More recently cyclodextrins have become popular tools among cell biologist as well. β-CD and its derivatives have been widely used to manipulate the cholesterol content of cultured cells (Klein et al., 1995; Christian et al., 1997) as well as to assess cholesterol transport to the cell surface (Neufeld et al., 1996; Heino et al., 2000). β-Cyclodextrin derivatives mediate efficient transfer of cholesterol between lipid vesicles and cells in culture, but so far cyclodextrins have not been used to introduce hydrophobic fluorescent phospholipid derivatives into cultured cells.

5.6. Imaging of fluorescent lipids in cells

To accurately study trafficking of fluorescent lipids in cells one needs to perform quantitative imaging of the fluorescent lipid in cells. This is very difficult to achieve, for several reasons. First of all, most fluorophores photobleach rapidly under the intense excitation light of the microscope. The rate of photobleaching also varies from one organelle to another (Benson et al., 1985). These issues seriously hamper quantitative imaging. The problem is particularly serious when a series of images have to be obtained from the same field of view (Tsien and Waggoner, 1995). Although the rate of photobleaching can be significantly reduced by adding certain chemicals like *n*-propyl gallate (Giloh and Sedat, 1982) or commercial antifading agents (Florijn et al., 1995), this usually requires that the cells have been pretreated with an aldehyde fixative such as paraformaldehyde. This chemically modifies the head groups of the aminophospholipids PE and PS (Kenney, 1984). Since these lipids account for up to 80 % of the total phospholipids in the inner leaflet of

the plasma membrane (Allan, 1996), the head group modification is likely to lead to major rearrangements of the lipid bilayer. Accordingly, fixing should be avoided when studying lipid organization and transport in cellular membranes.

Secondly, it is difficult to properly correct for the background caused by cellular autofluorescence and light scattering. Simple subtraction of (i) an off-focus image, (ii) an image of a cell-free area of the coverslip or (iii) an image obtained by spatial averaging does not provide full correction since the spatial distribution of cellular autofluorescence is typically uneven. This is particularly problematic when the concentration of the fluorescent lipids is low. Low concentrations are desirable, however, to avoid fluorophore-induced membrane perturbations.

Thirdly, selective imaging of an organelle membrane is often impossible due to the contribution to the signal by the fluorophores located in another membrane in proximity. Confocal microscopy and computational methods available for wide-field microscopes (Agard et al., 1989; Brakenhoff et al., 1989) help here, but they do not fully resolve the problem because of the limited resolution of microscope objectives in the z-direction. The plasma membrane is particularly difficult to image selectively due to numerous endo- and exocytotic vesicles in its close proximity.

6. AIMS OF THE PRESENT STUDY

The main goal of this study was to develop methods allowing the use of hydrophobic pyrene labeled phospholipids and quantitative microscopic imaging to study intracellular phospholipid trafficking. More specifically, the aims were to elucidate:

1) Whether cyclodextrins can enhance interbilayer transfer of pyrene-labeled phospholipids and, if so, what is the mechanism of transfer?
2) Can cyclodextrins be used to transfer pyrene and other fluorescent phospholipids to living cells?
3) How can one optimize microscopic imaging of pyrene-labeled lipids in living cells?

The methods developed were then used to study how the hydrophobicity (acyl chain length) of pyrene labeled phosphatidylserine affects its intracellular location.

7. EXPERIMENTAL PROCEDURES

7.1. Lipids and other reagents.

All unlabeled lipids were obtained from Avanti Polar Lipids (Alabaster, AL). $BODIPY_{12}$-PC, -PE and -SM as well as $NBD_{12}PC$ were supplied by Molecular Probes Europe (Leiden, Netherlands). BODIPY-PC and -PE were repurified shortly before use by HPLC on silica gel and BODIPY-SM on a reverse phase column. The pyrenylacyl glycerophospholipids were synthesized as described previously (Somerharju and Wirtz, 1982; Somerharju et al., 1985). Pyrenyldecanoyl sphingomyelin was synthesized using the method of Via et al. (1985). All labeled lipids were at least 99.5 % pure as determined by HPLC. The CDs were obtained from Cyclolab (Budapest, Hungary). Trinitrophenyl-lysophosphatidylethanolamine was prepared according to (van Duijn et al., 1985) as described in [III]. All solvents used were of HPLC grade and were obtained from Merck (Espoo, Finland), the cell culture media were obtained from Gibco (Paisley, England) and all other chemicals from Sigma (St. Louis, MO).

7.2. Assay of cyclodextrin-mediated lipid transfer and binding.

The previously described fluorescence dequenching assays (Somerharju et al., 1987) were employed to study the transfer and binding of pyrenyl lipids. The donor vesicles consisted of a pyrenyl lipid, POPC and TNP-PE (0.2 : 45 : 5 nmol), while the acceptor vesicles consisted of POPC and POPA (480 : 20 nmol). TNP-PE is a nontransferable quencher of pyrene fluorescence. Transfer of the pyrenyl lipid molecules from quenched donor vesicles to acceptor vesicles results in dequenching of pyrene fluorescence which is recorded. To measure binding, quenched donor vesicles were titrated with a cyclodextrin solution and the pyrene fluorescence intensity was recorded. All measurements were carried out at 25 °C unless otherwise noted. The association constant K for the complex formation was obtained by fitting the following equation to the fluorescence data (II):

$$F = (F_0 + F_\infty * K * x^z) / (1 + K * x^z) \qquad (1)$$

F_0 is the pyrene fluorescence intensity in the absence of γ-CD, F_∞ is the fluorescence intensity at an infinite γ-CD concentration, x is the cyclodextrin concentration and z is the γ-CD to PyrPC ratio in the complex.

The kinetics of complex formation were studied by rapidly mixing γ-CD solution with quenched donor vesicles in a cuvette followed by recording the pyrene fluorescence intensity with time. The complex formation appeared to consist of two kinetic processes with different rate constants. The following equation was employed to obtain the rate constants (II):

$$F = F_0 + A_1 * (1 - e^{-t/t_1}) + A_2 * (1 - e^{-t/t_2}) \qquad (2)$$

F_0 is the initial fluorescence, t is the time, t_1 and t_2 are the half times of the two kinetic processes and A_1 and A_2 are fitting parameters.

7.3. Cell Culture

Normal Human fibroblasts (GM08333) and BHK-21 cells were grown as previously described (Kasurinen and Somerharju, 1995; Heikinheimo and Somerharju, 1998). For microscopy, the cells were plated on round 32-mm coverslips placed in home-built aluminum-Teflon chambers. Before use, the coverslips were cleaned by treating them for 1 h at 50 °C with 1 M NaOH and 1 M HCl each, followed by washing with water and ethanol. After 1-2 hours of incubation at 37 °C, the inoculum was removed and the attached cells were washed, covered with normal growth medium and placed in the incubator. Labeling and imaging were carried out the following day. This cultivation protocol considerably reduced the amount of cell debris on the coverslips.

7.4. Incubation of cells with cyclodextrins and donor vesicles.

Cell monolayers were washed twice with CO_2-independent minimal medium (I-MEM), twice with PBS and then incubated at room temperature or 37 °C in I-MEM containing the donor vesicles with or without cyclodextrin. The CE-γ-CD stock solution was prepared in I-MEM and adjusted to pH 7.4 to avoid acidification of the incubation medium. This adjustment somewhat reduced the efficiency of labeling but was necessary to avoid harmful effects to cells, particularly at higher CE-γ-CD concentrations. After the incubation, the cells were washed three times with I-MEM and then subjected to imaging or lipid

extraction. For the latter, the cells were scraped into PBS, washed twice by centrifugation and then extracted according to (Bligh and Dyer, 1959). The amount of cell-associated fluorescent lipid was determined by measuring the pyrene (excimer) or BODIPY fluorescence intensity of the extract. Extracts of non-incubated cells were used as blanks. Alternatively, the amount of cell-associated fluorescent lipid was determined by HPLC analysis using on-line fluorescence detection (Kasurinen and Somerharju, 1992). For quantification, an internal standard was added to the cell pellet before the extraction. To determine vesicle binding to cells, [^{3}H]-cholesterol oleate (50,000 cpm/dish) was included in the donor vesicles.

7.5. Fluorescence imaging

Imaging was carried out on a Zeiss Axiovert 10 microscope equipped with a Photometrics 200 CCD camera with a thermoelectrically cooled Kodak KAF 1440 chip. A 345 nm (BP 11 nm) excitation filter, a 480 nm (BP 80 nm) emission filter and a 395 nm dichroic mirror were used for PyrPL imaging. With the BODIPY lipids 480 nm (BP 30 nm) and 535 nm (BP 40 nm) excitation and emission filters were used, respectively.

Oxygen was depleted from the medium by adding glucose (0.6 %), glucose-oxidase (10 U) and catalase (6 U) (Englander et al., 1987). Catalase is present to consume the hydrogen peroxide produced upon oxidation of glucose. The proper concentration of glucose oxidase was determined by measuring the rate of oxygen depletion with an oxygen electrode.

To selectively quench the PyrPL fluorescence in the plasma membrane, 1 ml of TNP-LPE solution in PBS (30 mM) was added to the chamber containing 1 ml of CO_2-independent MEM.

7.6. Fluorescence and IR spectrometry.

Steady state fluorescence measurements were carried out with a PTI QuantaMaster spectrofluorometer equipped with a thermostated cuvette holder. The temperature was set to 25 °C unless otherwise indicated. Time resolved measurements were performed on an ISS GREG 200 (ISS, Champaign, IL) fluorometer, equipped with digital multi-frequency cross-correlation phase and modulation acquisition electronics. The infrared spectra were recorded with a Magna-IR 560 Fourier Transform Infrared Spectrometer (Nicolet Inc., Madison, WI) as described in (Cheng et al., 1994).

7.7. Other methods

Lactate dehydrogenase was determined essentially as described in (Absolom, 1986). Concentrations of unlabeled phospholipids were determined by a phosphate assay (Rouser et al., 1970) and protein concentrations by a fluorescamine assay (Storrie and Madden, 1990). The donor vesicles for cell labeling were prepared as described in I. Shortly, the appropriate lipids were mixed in chloroform / methanol, dried under a nitrogen stream, kept in vacuum for one hour to remove any residual solvent, dispersed in buffer and probe sonicated.

8. RESULTS

8.1. Interbilayer transfer of pyrene phospholipids by cyclodextrins

8.1.1. Transfer of pyrene phospholipids between vesicles

To test the ability of CD-derivatives to enhance intermembrane phospholipid transport, we employed a previously developed fluorescence dequenching assay (Fig. 3).

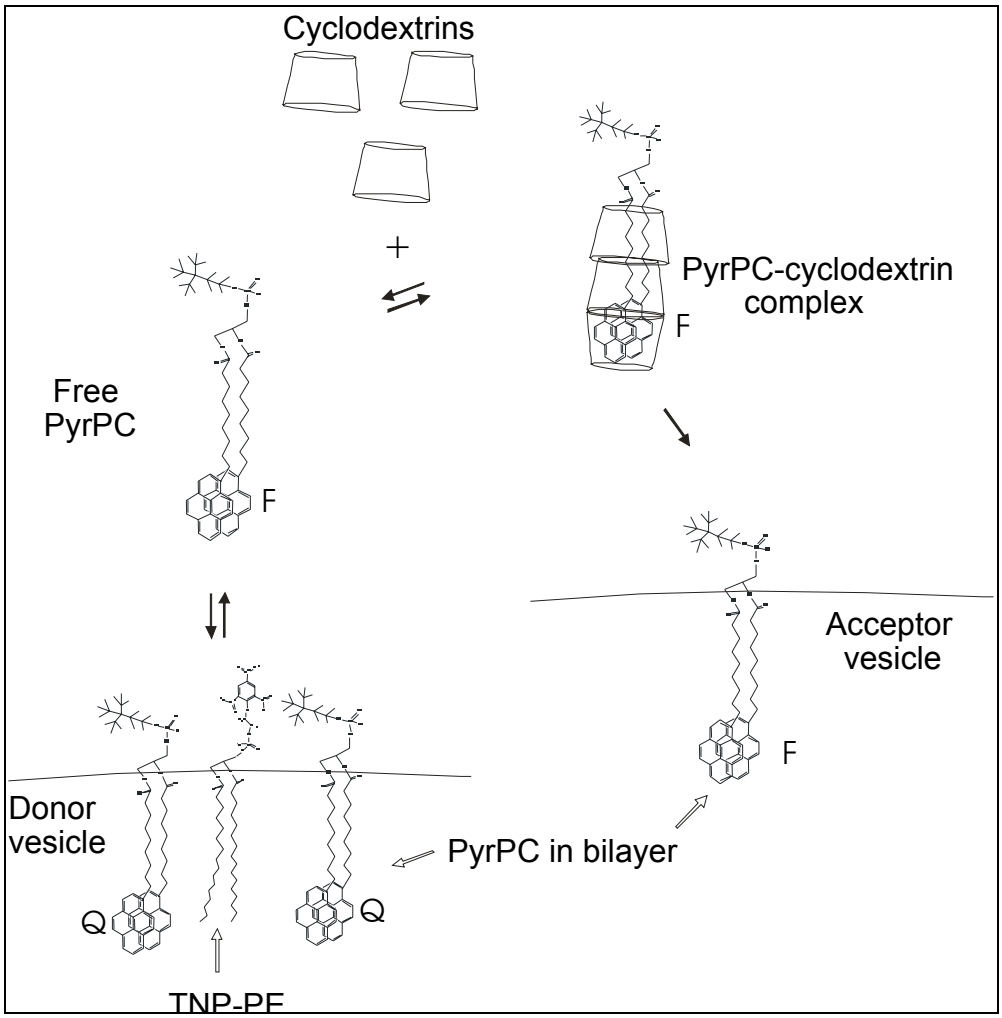

Fig 3. The assay for transfer of pyrenyl phospholipids between vesicles. *In this assay, the donor vesicles contain the pyrene lipid under study, TNP-PE as a quencher of pyrene fluorescence, and unlabeled phospholipids, while the acceptor vesicles consist of unlabeled lipids only (Somerharju et al., 1987). Transfer of the pyrene lipid molecules from the donor to the acceptor vesicles result in dequenching of pyrene fluorescence which is recorded. There is an excess of acceptor vesicles. Q beside a pyrene denotes a quenched, nonfluorescent pyrene, F a fluorescent pyrene.*

Both mono- and dipyrene species were used (Fig. 1) for the transfer studies. When 16:0-Pyr_{10}PC donors are incubated with the acceptor vesicles only, a very slow increase of pyrene fluorescence is observed, indicating that the spontaneous transfer of this particular pyrene lipid is a very slow process. Upon addition of 30 mM CE-γ-CD, a remarkable (≈ 350-fold) increase in the rate of fluorescence enhancement was observed (I, Fig. 1.), indicating that CE-γ-CD greatly accelerates intervesicle translocation of 16:0-Pyr_{10}PC. We found that the rate of transport is closely proportional to CD concentration.

8.1.2. Comparison of different cyclodextrins

Parallel experiments were carried out with a variety of other cyclodextrin derivatives, but none of them proved to be as efficient as CE-γ-CD. On later experiments we found that the rate of transfer with CE-γ-CD is quite dependent of pH and at pH 7.4 the transfer efficiency of unmodified γ-CD is close to that of CE-γ-CD (see Fig. 4). Because CE-γ-CD also remarkably enhanced intervesicle transfer of other pyrenyl phospholipids as well as those labeled with the NBD_{12}- or $BODIPY_{12}$- fatty acids, CE-γ-CD was initially chosen for further experiments.

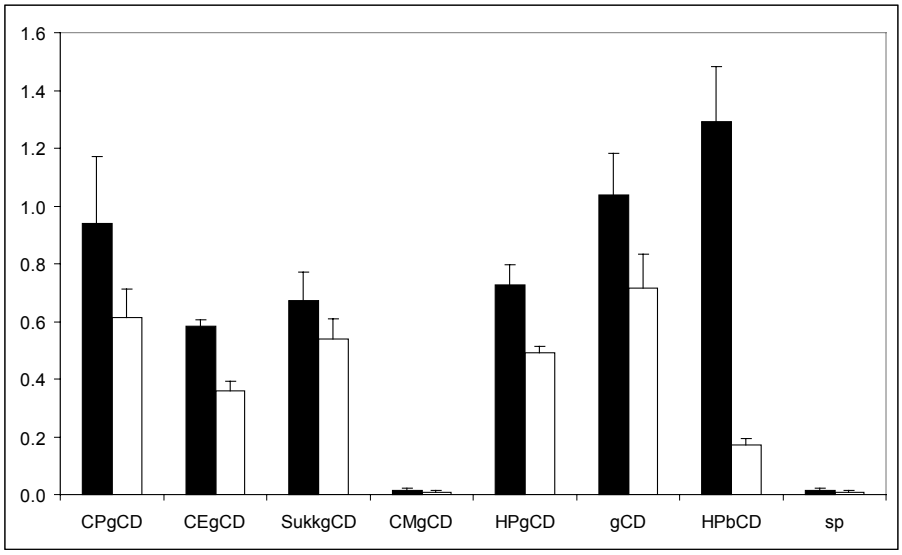

Fig. 4. Relative efficiencies of PyrPC transfer between vesicles for different cyclodextrin derivatives at pH 7.4. *All the studied γ-CD derivatives have similar, lipid hydrophobicity -dependent, difference in transfer efficiency for 16:0-pyr_{10} (black) and di-pyr_{10}PC (white). Unlike the γ-CDs hydroxypropyl-β-CD has very different transfer efficiency for the two lipids. Apparently the β-CD cavity is not large enough for two pyrenes, but one pyrene and an acyl chain fit well in the cavity. This was not explored further because β-CD complicates cell labeling by depleting cellular cholesterol. Legend keys: CPgCD, carboxypropyl-γ-CD, CEgCD carboxyethyl-γ-CD, SukkCD, succinylated γ-CD, CMgCD carboxymethyl-γ-CD, HPgCD hydroxypropyl-γ-CD, gCD, γ-CD, HPbCD, hydroxypropyl-β-CD, sp, spontaneous transfer.*

8.1.3. Effect of pyrene phospholipid acyl chain length

To obtain information on the mechanism of cyclodextrin-mediated phospholipid transfer, the effect of the length of the pyrene labeled acyl chain (4 to 14 carbons) on the rate of CE-γ-CD mediated transfer was investigated (I, Fig. 2.). The rate decreases strongly (exponentially) with increasing length of the pyrene labeled chain. Analogous results were obtained for lipids that contained two pyrene labeled chains of identical length (n = 4 – 14) and with other γ-CDs. Such a strong chain length -dependency on the rate of fluorescence increase (probe dilution) clearly indicates that CE-γ-CD enhances monomeric transport of the labeled lipid molecules, rather than causes fusion or hemifusion between the donor and acceptor vesicles. Would either of the latter mechanisms dominate, the rate of fluorescence increase should be independent on the chain length of the pyrene phospholipid, which clearly is not the case. In addition, the fact that hardly any increase in fluorescence was observed for the pyrene PC with the longest chain (14 carbons) excludes the possibility that transfer of the quencher molecules, if occurring, would significantly contribute to the observed enhancement of pyrene fluorescence in this assay.

8.1.4. Effect of lipid head group and backbone structure

The effect of the head group on the rate of cyclodextrin-enhanced transfer of pyrene-labeled phospholipids was next studied (I, Fig. 3). Among glycerophospholipids, transfer of PG, PE, PS and PC is similarly enhanced while that of PI is enhanced about twice as much by 5 mM CE-γ-CD. This more rapid transfer of PI is probably due to the considerable polarity of the head group. Supporting this, transfer of diglyceride, which has a very small polar moiety, is much less efficient than that of the phospholipids. $Pyr_{10}SM$ is transported almost 3 times more rapidly than $Pyr_{10}PC$. The additional hydroxyl group and double bond in the sphingosine moiety obviously make SM somewhat less hydrophobic than PC with similar acyl chains. We conclude that the overall molecular hydrophobicity, rather than some structural details, determines the rate of CE-γ-CD-mediated transport of the pyrenyl lipids.

8.2. Binding of pyrene lipids by γ-cyclodextrins

To study whether γ-CD enhances PyrPL transport by forming soluble complexes in the aqueous phase or by some other mechanism, binding studies were carried out. Unmodi-

fied γ-CD was used instead of the carboxyethyl derivative so that interpretation of the data would not be complicated by the charged carboxyethyl-moieties. The affinity of pyrene lipids to γ-CD is very similar to that of the carboxyethyl analogue at neutral pH (c.f Fig. 4). Binding of pyrene PC species to γ-CD was studied by titrating quenched vesicles containing a dipyrenylacyl (DiPyr$_n$PC) or a monopyrenylacyl PC (16:0-Pyr$_n$PC) with γ-CD in buffer, followed by reading the fluorescence intensity (Fig. 5; II, Fig. 1).

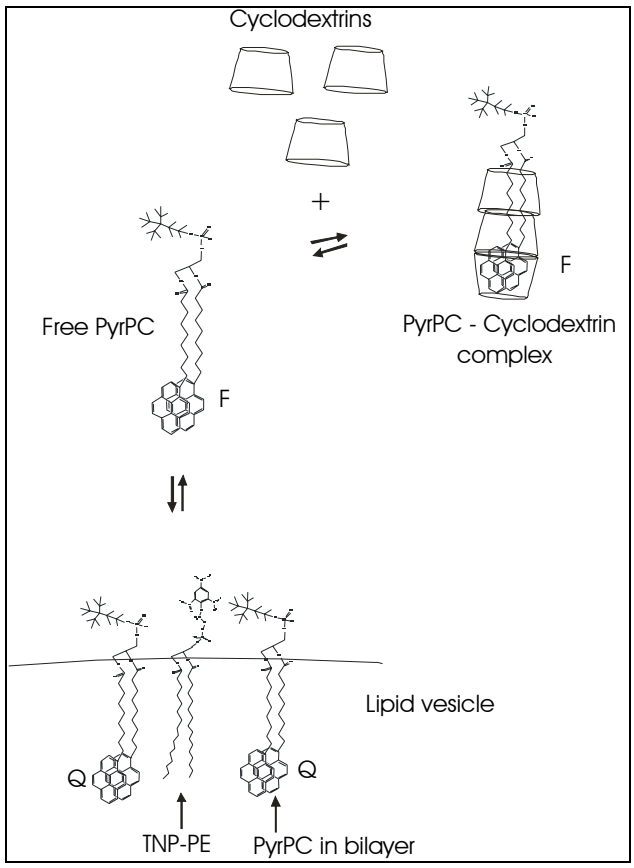

Fig. 5. *To study the binding of pyrene PC species to γ-CD, quenched vesicles containing a dipyrenylacyl (DiPyr$_n$PC) or a monopyrenylacyl PC (16:0-Pyr$_n$PC) were mixed with varying concentrations of γ-CD in buffer and the fluorescence intensity was recorded after the equilibrium had been reached. There is an increase in pyrene fluorescence intensity due to removal of the PyrPC molecules from the lipid bilayer containing the quencher molecules (trinitrophenyl-PE). Q beside a pyrene denotes a quenched, nonfluorescent pyrene, F a fluorescent pyrene.*

Equation 1 was then fitted to the fluorescence titration data. For DiPyr$_4$-, DiPyr$_6$- and DiPyr$_8$PC the best fits were obtained when assuming the stoichiometry of two γ-CD molecules for each PC, while for DiPyr$_{10}$-, DiPyr$_{12}$- and DiPyr$_{14}$PC best fits were obtained when assuming a 3:1 stoichiometry. Parallel studies with 16:0-Pyr$_n$PCs showed that the binding stoichiometry was 2:1 when n was 4, 6, 8 or 10 but changed to 3:1 for n = 12 or 14 (II, Fig. 1).

8.2.1. Thermodynamics of the complex formation

The thermodynamic parameters ΔG, ΔH and ΔS for complex formation were determined from the temperature-dependence of the association constant K for 10:0-Pyr_{10}PC/γ-CD complex (II, Fig. 3). The following values were obtained: ΔG = 0.34 kJ/mol; ΔH = 0.475 kJ /mol; T_{298} * ΔS = 0.13 kJ/mol (ΔS = 0.43 kJ/mol*K). These numbers indicate that transfer of 10:0-Pyr_{10}PC form the bilayer to the γ-CD cavity is a nearly energy-neutral process. This is not an unexpected result, since the acyl chains of a PyrPC are transferred from one hydrophobic environment (i.e., the bilayer interior) to another one (i.e., the γ-CD cavity) and the head group is located in the aqueous phase in both cases.

An activation energy of +92 kJ/mol was determined from the temperature-dependence of $DiPyr_{10}$PC binding to γ-CD. This high value indicates that the efflux of the PC molecule from the bilayer is the rate-limiting step of the binding process.

8.2.2. Spectroscopic characterization of pyrene PC/γ-cyclodextrin complex

The structure of the PyrPC/γ-CD complex was studied with several physical methods: absorbance, steady state and time-resolved fluorescence, and FTIR spectrometries. The absorption as well as the excimer excitation spectra obtained for $DiPyr_n$PC/γ-CD complexes are strongly broadened. This is a clear sign of ground state dimer formation (Winnik, 1993). Steady state emission spectra show that complex formation greatly increases the probability of excimer formation of $DiPyr_4$PC and $DiPyr_{10}$PC, as compared to bilayer associated lipids. This indicates that the pyrenes are constrained to lie close to each other in the complex. Time-resolved studies, showing a greatly enhanced excimer formation rate, strongly support this view. FTIR-studies indicate marked suppression of certain deformation modes of the pyrene ring in the $DiPyr_4$PC– γ-CD complex. This is compatible with the idea that pyrenes are forced to lie close together in the complex; the pyrene ring is quite rigid and thus close apposition of two pyrenes is expected to limit ring deformations. Neither broadening of the excitation peaks nor excimer formation was observed for mono-PyrPCs. Collectively these data indicate that the pyrene moiety of a pyrene lipid is inside the cyclodextrin cavity, the pyrene moieties of a dipyrene lipid are located very close to each other in the cavity and that both chains of a monopyrene lipid are in the same cavity (see II).

8.3. γ-cyclodextrin-mediated transfer of fluorescent lipids from vesicles to cultured cells

8.3.1. Transfer assays

We next studied whether CE-γ-CD catalyzes transport of fluorescent phospholipids from vesicles to cells in culture. Vesicles containing a pyrene- or BODIPY-labeled phospholipid were incubated with cell monolayers at RT or 37 °C and the amount of cell-associated fluorescent lipid was determined using either fluorescence imaging or spectroscopy. Donor vesicles labeled additionally with [^{3}H]-cholesterol ester were used to assess the amount of donor vesicles adhering to or fusing with the cells (for details see Experimental procedures). For the imaging studies dipyrenyl phospholipids were used because excimer fluorescence is more readily visualized with the imaging system used than the monomer fluorescence. Furthermore, the wide separation of the excitation (345 nm) and emission peaks (480 nm) avoids excitation/emission crossover as well as allows efficient correction for background fluorescence (see below). Another important advantage of the dipyrenyl derivatives is that the presence of fatty acid and/or lysolipid impurities in the donor vesicles does not interfere with the interpretation of images, as these compounds do not display detectable excimer fluorescence in cells. Such selective imaging of the intact phospholipid is of course impossible with the monopyrenyl- or BODIPY-derivatives.

8.3.2. Mechanism of cell labeling

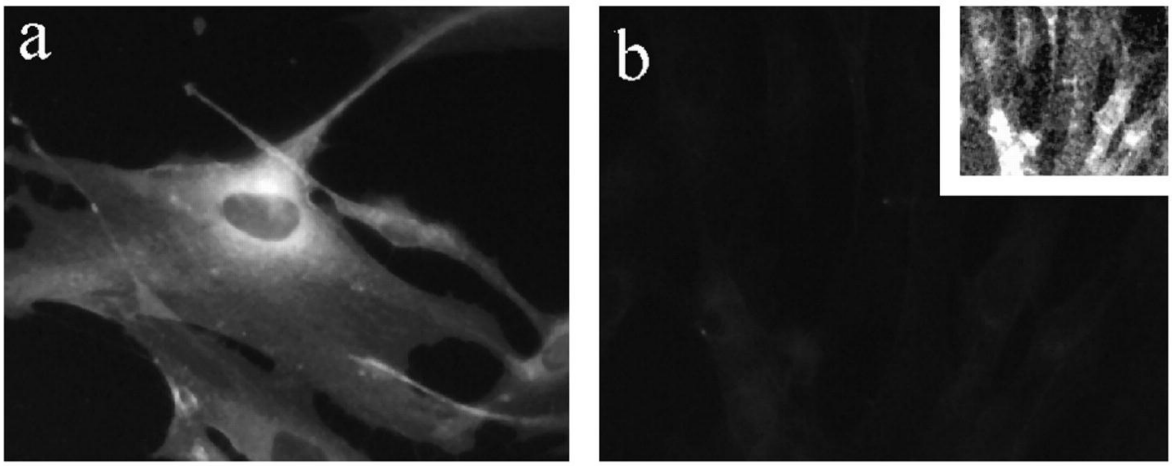

Fig. 6. γ-CD greatly enhances transfer of DiPyr$_{10}$PC from donor vesicles to cells. *Monolayers of human fibroblasts on coverslips were incubated with DiPyr$_{10}$PC containing donor vesicles for 30 min at 25 °C, washed and imaged as detailed under "Experimental Procedures." In panel a 30 mM CE-γ-CD was present during the incubation while in panel b there was no CD present. The camera settings and image adjustments are identical in both images. The inset in panel b shows the original image with the pixel intensities multiplied by a factor of 20.*

Fig. 6 shows human fibroblast (HF) cells incubated with donor vesicles containing DiPyr$_{10}$PC in the presence or absence of 30 mM CE-γ-CD. Clearly visible excimer fluorescence, mainly on the plasma membrane, is observed in the presence of γ-CD (panel *a*), while practically none is detectable in its absence (panel *b*). γ-CD also strongly enhanced transfer of the BODIPY-derivatives from the donor vesicles to cells. However, significant labeling of cells by BODIPY-SM occurred also in the absence of cyclodextrin, probably because of the relatively low hydrophobicity of the BODIPY-derivatives.

Analogous labeling experiments with other DiPyr$_n$PC species were carried out in order to establish the limits of the method, as well as to confirm that the enhanced labeling in the presence of cyclodextrin is due to monomeric transfer of the fluorescent lipid rather than fusion or adherence/endocytosis of the donor vesicles (I, Fig. 5). As expected, most efficient γ-CD-mediated labeling was obtained with DiPyr$_4$PC. The efficiency decreased systematically with increasing length of the acyl chains and with DiPyr$_{14}$PC virtually no labeling was observed. For DiPyr$_6$PC significant and for DiPyr$_8$PC some labeling was observed also in the absence of γ-CD, but not for the long-chain derivatives. The fact that

practically no labeling with the most hydrophobic pyrene lipids was observed in the absence of γ-CD provides strong evidence that γ-CD enhances monomeric transfer of the pyrene derivatives rather than induces vesicle-cell fusion, adherence or endocytotic uptake of vesicles. These data also show that $DiPyr_{10}PC$ is the most hydrophobic dipyrenyl derivative for which reasonable levels of labeling were achieved under the present labeling conditions.

To confirm that γ-CD indeed mediates monomeric transfer of the fluorescent lipids rather than causes fusion or association of the donor vesicles with the cells, BHK cell monolayers were incubated with donor vesicles containing BODIPY-SM and [^{3}H]-cholesterol oleate, a non-transferable liposomal marker, in the presence of 0 - 30 mM CE-γ-CD for 30 min at RT (I, Fig 6). The amount of cell-associated BODIPY-SM increased strongly with the CE-γ-CD concentration up to 15 mM CE-γ-CD. At this concentration, about 5 % of the liposomal BODIPY-SM become cell-associated. In contrast, only about 0.3 % of the [^{3}H]-cholesterol ester, the liposomal marker, was associated with the cells independently of the CE-γ-CD concentration used. Parallel results were obtained for $DiPyr_6PC$ except that somewhat less fluorescent PC was transferred to the cells (I, Fig. 6). These data are consistent with the proposition that γ-CD enhances monomeric transport of the fluorescent phospholipids from donor vesicles to cells.

To study the initial site of introduction of fluorescent lipids to cells we compared cell labeling with pyrene PC to labeling with pyrene PS. In BHK cells labeled with $DiPyr_8PC$ and then chased for 5 hours at 37 °C, the plasma membrane fluorescence was strongly reduced as compared to situation before the chase, while there is a marked increase in the labeling of intracellular structures, including punctate pericellular and more diffuse perinuclear structures (I, Fig. 7a). These may represent secondary endosomes and the endosytic recycling compartment, respectively (Mukherjee et al., 1999). On the other hand, no significant labeling of the mitochondria or the nuclear membrane was apparent. These findings indicate that dipyrene PCs are initially introduced as monomers to the outer leaflet of the plasma membrane and are later incorporated to endosomes and related organelles. If fusion of the vesicles with cell membrane would have occurred, labeling of the mitochondria and the nuclear membrane would be expected (see below and cf. Pagano and Sleight, 1985).

When BHK cells were analogously labeled with $DiPyr_8PS$ for 15 min at 37 °C and then chased for 5 hours at 37 °C, we saw prominent labeling of mitochondria (I, Fig. 7b). Labeling of this organelle is in agreement with previous data obtained for NBD_6PS (Martin and Pagano, 1987), $NBD_{12}PS$ (Kobayashi and Arakawa, 1991) and $DiPyr_4PS$ (Kasurinen

and Somerharju, 1995). Apparently the PS molecules are initially introduced to the outer leaflet of the plasma membrane from where they move rapidly to the inner leaflet with the assistance of the aminophospholipid translocase (Devaux, 1992). From the inner leaflet the fluorescent PS molecules move further, possibly via spontaneous diffusion (see below), to various organelles, including mitochondria, where they are decarboxylated to PE (Kobayashi and Arakawa, 1991; Jasinska et al., 1993). In conclusion, the intracellular distribution modes of $DiPyr_8PC$ and -PS are compatible with the proposition that γ-CD mediates monomeric incorporation of the fluorescent lipids initially to the outer leaflet of the plasma membrane, rather than induces fusion or endocytosis of the donor vesicles.

8.3.3. Effect of γ-cyclodextrin on cell viability

Incubation with γ-CD does not compromise plasma membrane intactness as measured by lactate dehydrogenase and Trypan Blue leakage or long term cell viability measured as protein mass per dish (data not shown). Unlike β-CD (Kilsdonk et al., 1995), γ-CD incubation does not seem to affect cellular cholesterol content. To study whether incubation with γ-CD and donor vesicles alters the phospholipid composition of the cells, mass spectroscopic measurements (Han and Gross, 1994; Brügger et al., 1997) of the medium and the cells were carried out. Our analyses of cellular lipid composition indicated that γ-CD does not extract cellular phospholipids like α-CD does (Ohtani et al., 1989). Unfortunately, the phospholipid composition of the labeling medium could not be determined because of compounds present interfering with the mass-spectroscopic analysis. The analysis of the total cellular lipid composition does not fully exclude the possibility that some changes in the lipid composition of the plasma membrane outer leaflet might occur, since this compartment contains only a fairly small fraction of the total cellular phospholipid.

8.4. Fluorescence imaging of pyrene lipids in cells

8.4.1. Prevention of photobleaching

Photobleaching is a major problem in quantitative fluorescence imaging. Oxygen often plays a central role in this process (Tsien and Waggoner, 1995). The lifetime of excited pyrene is in the order of 100 ns (Morrison, 1988) and therefore oxygen bleaches pyrene fluorescence very effectively at normal atmospheric pressure (Xu et al., 1995). This

led us to test whether deoxygenation of the medium would counteract photobleaching of pyrene lipids in cells. The enzymatic deoxygenation protocol described by Englander et al. (1987) was used.

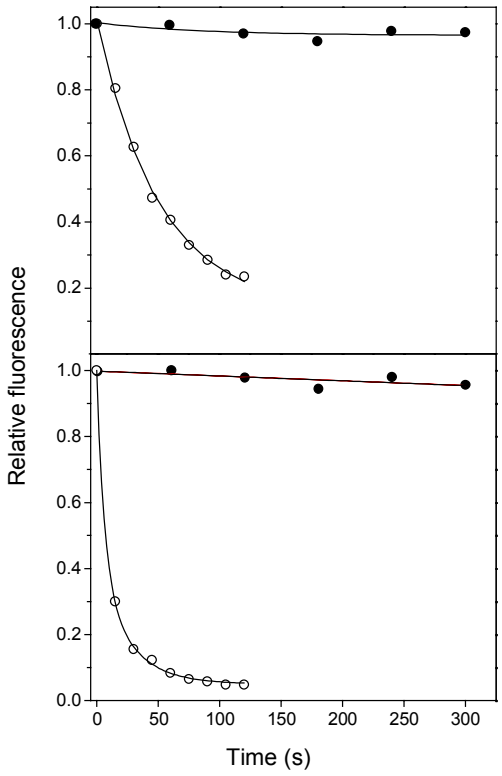

Figure 7. Kinetics of pyrene photobleaching in cells. *Fibroblasts were labeled with pyrenedodecanoate for one hour as described in (Kasurinen et al., 1990) and then subjected to imaging with continuous excitation through a 345 nm (11 nm BP) filter either in the presence (closed symbols) or absence (open symbols) of the oxygen-depleting reagents. The upper panel shows bleaching of the pyrene label in membranes. The half times of bleaching in the presence or absence of deoxygenation were determined to be 9400 and 71 s, respectively. The lower panel shows the bleaching of pyrene fluorescence in brightly-labeled neutral lipid droplets. The half-times of bleaching with or without deoxygenation were 5400 and 8.5 s, respectively. In the absence of deoxygenation also another (minor) bleaching component with half-time of 72 s was observed. This component may relate to photobleaching of pyrene phospholipids in membranes below and above the lipid droplets in focus.*

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Deoxygenation has a very strong effect on the photobleaching of lipid-bound pyrene fatty acid in BHK cells. In the absence of the oxygen depleting system, the rate of photobleaching was rapid, i.e. the pyrene fluorescence virtually disappeared after 5 min of continuous excitation. Instead, when oxygen had been depleted, photobleaching was negligible. Quantitative image analysis (Fig. 7) showed that deoxygenation diminished pyrene photobleaching about 130-fold (III). The effect of deoxygenation was even more dramatic for the cytoplasmic neutral lipid droplets where the rate of photobleaching decreased approximately 1000-fold.

8.4.2. Effect of oxygen on cell viability

Depletion of oxygen could potentially damage the cells by preventing aerobic metabolism and thereby reducing cellular energy levels. However a 30 minute deoxygenation did not affect the cellular energy status (III, Fig. 3) or protein synthesis. Furthermore, deoxygenation was not observed to cause any visually detectable damage to the cells during the first 20 min. Later, however, plasma membrane blebbing was observed, possibly due to depletion of cellular energy levels (Scott, 1976; Johnson et al., 1994).

8.4.3. Elimination of background fluorescence

Pyrene has a sharp excitation maximum close to 345 nm but is hardly excited at 360 nm, while the cellular autofluorescence/scattering shows little wavelength-dependency in this region. Thus, it should be possible to largely eliminate the cellular background fluorescence/scatter by subtracting an image obtained with an 360-nm excitation filter from one recorded with an 345-nm filter. To test this, images of *unlabeled* cells were obtained with 345 and 360 nm excitation and the latter image was subtracted from the former, which had been multiplied by an empirically determined factor to correct for wavelength-dependency of the optical transmission and lamp output. An essentially "black" image resulted indicating that the method properly corrects for the background (III, Fig 4). We tested the usefulness of this protocol further by labeling BHK and mucolipidosis IV cells with a low concentration of $DiPyr_4PC$ (III, Fig 5). When BHK-cells were imaged with a 345-nm pyrene filter most of the fluorescence was at the plasma membrane. When a 360-nm filter was used most of the fluorescence was internal, as expected for cellular autofluorescence. When the 360 nm image was subtracted from the 345 nm image, the overall image contrast improved remarkably. However, besides this contrast enhancement, which is largely due to elimination of the cell-independent signal, the effect of background subtraction was not obvious from these images. This is largely due to the fact that the autofluorescence of BHK cells with 345-nm excitation is quite low. For the mucolipidosis-IV cells, which typically have rather intense autofluorescence (Goldin et al., 1995; Chen et al., 1998), the subtraction of the 360-nm image from the 345-nm image also provided a reasonable background correction.

8.4.4. Selective imaging of pyrene lipids in the plasma membrane.

A previous study indicated that *N*-trinitrophenyl-lysophosphatidylethanolamine (TNP-LPE), when added to the medium, selectively quenches the pyrene lipids located in the plasma membrane of fibroblasts (Kasurinen and Somerharju, 1995). This finding suggested that one could obtain plasma membrane images free from contribution by internal membranes by taking an image before and after the addition of the quencher followed by subtraction of the latter from the former. To study this issue, BHK cells were incubated with donor vesicles containing $DiPyr_4PC$ and then chased for 2 h at 37 °C. This protocol guarantees that the probe is present in both the plasma membrane and various intracellular structures, mainly endosomes and lysosomes (Kasurinen and Somerharju, 1995). The cells were imaged, TNP-LPE was then added to the medium and the cells were imaged again. The plasma membrane fluorescence virtually disappeared, but the intracellular fluorescence appeared to remain unaffected (III, Fig. 6). These findings indicate that TNP-LPE indeed selectively quenches $DiPyr_4PC$ in the plasma membrane. Supporting this conclusion, only the plasma membrane was visible in the difference image. The quencher seems to penetrate the plasma membrane only very slowly, since no apparent changes in the internal fluorescence were observed during several minutes of follow-up. An image that predominantly represents the outer leaflet of the plasma membrane is acquired, since $DiPyr_4PC$ is largely restricted to this leaflet (Kasurinen and Somerharju, 1995). Control experiments (III, Figs. 7-8) indicated that TNP-LPE also quenches the pyrene lipids in the inner leaflet, even if it itself is probably restricted to the outer leaflet. If TNP-LPE had access to the inner leaflet of the plasma membrane it would rapidly partition to all the cytosolic membranes due to its high hydrophilicity as a lysolipid. If this happens, also fluorescence inside the cell would be quenched which it not the case. Accordingly, one can also obtain specific images of the inner leaflet, by labeling the cells with a pyrene lipid that is present in the inner leaflet only, such as PS (III, Fig. 7).

8.4.5. Pyrene fluorescence quenching in model membranes

Quenching of pyrene lipid fluorescence by TNP-LPE was also studied in unilamellar vesicles. The vesicles consisted of POPC, POPA, cholesterol and either 16:0/Pyr_8PC, 16:0/$Pyr_{10}PC$ or 16:0/$Pyr_{12}PC$. The pyrene lipid was present either in both leaflets of the vesicles (symmetric vesicles) or in the outer leaflet only (asymmetric vesicles). When the symmetric vesicles were titrated with TNP-LPE the length of the pyrene-labeled chain had little effect on the quenching efficiency (III, Fig. 8). In these vesicles the pyrenes in the in-

ner leaflet come closer to the quencher in the outer leaflet as the acyl chain length of the PyrPC increases. This seems to negate the effect of the pyrenes moving farther from the quencher in the outer leaflet with increasing chain lenght. In the asymmetric vesicles the quenching efficiency diminished with increasing length of the pyrene-labeled acyl chain as all the pyrenes in the vesicle move farther from the quencher (III, Fig. 8). We used previously derived equations to determine the effective R_0 value for this energy transfer - dependent quenching (Wolf et al., 1992). Fitting these equations to the data gave R_0 the value of 15 Å for both the symmetric and asymmetric vesicles. This value of R_0 is valid for a single donor-acceptor pair, but in the membrane there are, except for very low acceptor (quencher) concentrations, several acceptor molecules at a distance from the donor which allows energy transfer to take place. It was calculated that at moderate quencher concentrations the effective R_0 is close to 20 Å, i.e., 50 % of pyrenes at this distance are quenched. Since the chromophore of TNP-LPE is probably located at or has access to the bilayer/water interface (van Duijn et al., 1985), and since the centroid of a pyrene attached to an acyl chain of 12 carbons is about 14.5 Å from the interface (Sassaroli et al., 1995), it is clear that any pyrene phospholipid present in the same leaflet as the quencher should be efficiently quenched. Also the pyrenes in the opposite leaflet should be quite efficiently quenched, at least when attached to an acyl chain of moderate length. For example, the centroid of a pyrene attached to a 10 carbon chain is about 13.5 Å from the bilayer/water interface (Sassaroli et al., 1995). Since the thickness of the hydrocarbon region of a PC/cholesterol bilayer is approximately 30 Å (Nezil and Bloom, 1992) the pyrenes would be only 16 - 17 Å away from the TNP-groups in the opposite leaflet. Since the distance dependency of quenching efficiency is a 6^{th} power equation, it is feasible that TNP-LPE in the outer leaflet of the plasma membrane can efficiently quench the fluorescence of $DiPyr_{10}$ - lipids in the inner leaflet as the distance between the pyrene and the quencher is under the R_0. When multilamellar liposomes containing 16:0/Pyr_{10}PC were titrated with TNP-LPE, the quenching was much less efficient than with unilamellar vesicles. This indicates, in accordance with the cell experiments, that TNP-LPE does not readily penetrate thorough the membrane.

8.5. Hydrophobicity-dependent transfer of dipyrenyl PS from plasma membrane to mitochondria.

Previous studies have shown that fluorescent PS species are rapidly transported from plasma membrane to mitochondria (Kobayashi and Arakawa, 1991; Kasurinen and Somerharju, 1995). To study whether this rapid transport is an artifact due to the low hydrophobicity of the PS species used, we compared intracellular distribution of short- and long-chain $DiPyr_nPS$ species. After $DiPyr_6PS$ or $DiPyr_8PS$ labeling the plasma membrane is the most prominently labeled structure. After three to five hours' chase the mitochondria were typically the most strongly labeled structure (I, Fig 7). After $DiPyr_{10}PS$ labeling most of the label is also in the plasma membrane (I, Fig. 8). After 3 hours chase, the juxtanuclear region, possibly the endosomal recycling compartment (cf. Mukherjee et al., 1999) is prominently labeled, along with the plasma membrane. At 5 hours, the probe distribution remained similar except that juxtanuclear labeling was somewhat diminished and punctate fluorescence was observed. After 23 hours of chase, the overall fluorescence intensity was somewhat diminished and, in most cells, the punctate structures were more prominent than before. In none of these images $DiPyr_{10}PS$ was seen to be present in mitochondria. This is in striking contrast with what we observed here for $DiPyr_{6\text{-}8}PS$ and what has been previously observed for $DiPyr_4PS$ and $NBD_{12}PS$ (Kasurinen and Somerharju, 1995; Kobayashi and Arakawa, 1991). It is concluded that short-chain (hydrophilic) PS species do not properly mimic trafficking of natural, typically long-chain PS species.

9. DISCUSSION

9.1. Transfer of pyrene phospholipids by cyclodextrins

9.1.1. Mechanism of γ-CD -mediated phospholipid transfer

The most probable model for γ-CD -mediated PyrPL transfer is that the lipid molecule first fully detaches from the bilayer and then associates with 2-3 γ-CD molecules in the aqueous phase. Subsequently the lipid partitions from the complex to any available membrane. Two findings strongly support this view: First, the activation energy of the formation of a DiPyr10PC/γ-CD complex (+92 kJ/mol) is very high and similar to that obtained previously for the efflux of a pyrene-labeled and other phospholipid molecules from a phospholipid surface to the aqueous phase (Massey et al., 1982b; McLean and Phillips, 1984; Wimley and Thompson, 1990; Pownall et al., 1982). Secondly, the rate of complex formation decreases exponentially with increasing acyl chain length of PyrPC. Analogous behavior has been observed for spontaneous interparticle transfer of PyrPCs (Massey et al., 1982b) and unlabeled PCs (Ferrell et al., 1985), where the efflux from the donor surface to the aqueous phase was considered to be the rate-limiting step. So the rate of lipid efflux to the aqueous phase, which is determined by its hydrophobicity (Massey et al., 1982b; McLean and Phillips, 1984; Massey et al., 1985; Wimley and Thompson, 1990; Reinl and Bayerl, 1994) most propably sets the ultimate limit for CD-mediated interbilayer lipid transfer. This has already been shown with sterols: at constant surface pressure the desorption rate of different sterols from a lipid monolayer to β-CD is largely determined by their hydrophobicity (Ohvo and Slotte, 1996).

The thermodynamic data obtained for the transfer of 10:0-Pyr_{10}PC from a bilayer to the γ-CD complex indicate that this process is nearly energy-neutral. The very small value of ΔG derives from the PyrPC molecule being transferred from one amphipathic environment to another, i.e., from a lipid bilayer to the CD cavity. Both the acyl chains and the head group experience a relatively insignificant change in their environment.

The entropy of the system is a complicated issue because there are many factors involved. The rotational entropy of the PyrPC molecule may increase upon complex formation because the PyrPC can tumble isotropically with the complex, while this is not possible

in the anisotropic bilayer environment. The removal of a PyrPC molecule from the bilayer is likely to increase the entropy of the latter due to increased conformational freedom of the matrix lipid acyl chains that were proximal to the relatively bulky and rigid pyrene moiety. The cavity on the CD-molecule contains water molecules that have a lower entropy than the bulk water molecules (Tabushi et al., 1978). The release of these water molecules upon formation of the PyrPC/γ-CD complex is an entropically favorable process. The entropy of the γ-CD molecules participating in the complexes should decrease due to their diminished rotational freedom as compared to uncomplexed molecules. Apparently, all these factors together cause the total entropy of the system to be nearly constant. Also ΔH of PyrPC transfer from the bilayer to the γ-CD complex is very low. This is most probably a result of many compensating factors (see III) one of which is again the water molecules residing inside the uncomplexed cyclodextrin molecules

9.1.2. Characteristics of the pyrene PC/γ-cyclodextrin-complex

DiPyr$_n$PCs with short acyl chains form a 1:2 complex with γ-CD, while those with longer chains seem to form a 1:3 complex. The shift from a 1:2 to a 1:3 complex occurs when the length of the pyrene labeled chains is ≥ 10 carbons. With 16:0-Pyr$_n$PC species the shift from a 1:2 to 1:3 complex occurs when the length of the labeled chain (n) is ≥ 12 carbons. This may be because the overall cross-sectional area of monoPyrPCs (in its all-trans conformation) is smaller than that of the γ-CD cavity (as demonstrated by experimenting with space-filling models, see below), the acyl chains kink to maximize the van der Waals interactions with the γ-CD molecules. Such kinking would make the effective acyl chain length shorter than what is the case with DiPyr$_n$PCs. Tests with space-filling models indicate that the labeled chains of a DiPyr$_n$PC are almost fully included inside the combined cavity of 2 γ-CD molecules when $n \leq 10$ (when placing the carbonyl carbons just outside the cavity), but a cavity consisting of 3 γ-CD molecules is needed to shield longer pyrenyl chain from the aqueous medium. In conclusion, the spectroscopy results indicated that the two acyl chains of a pyrene lipid molecule share the same γ-CD cavity, consisting of 2-3 γ-CD molecules.

9.1.3. Cyclodextrins as carriers for other lipids.

Knowledge on factors that determine the rate of cyclodextrin-mediated PyrPL transfer should help to develop more efficient transfer methods also for other lipids. While γ-CD

and its derivatives seem to enhance the transport of pyrene-labeled phospholipids more efficiently that β- or α-cyclodextrins, the latter may actually be more efficient carriers of natural phospholipids. This is because the cross-sectional area of the acyl chains of the natural lipids more closely corresponds to the dimensions of the cavity of β- or α-CD. Experiments with space-filling models indicate that the cavity of β-CD can accommodate two normal acyl chains while that of α-CD only one. Previous studies have indicated that α-CD interacts more strongly with unlabeled phospholipids than the β- or γ-derivatives (Irie et al., 1982; Nishijo et al., 2000; Fauvelle et al., 1997; Debouzy et al., 1998). According to the present study hydrophobicity-dependent efflux from the bilayer is the rate-limiting step in CD-mediated lipid transfer. However, this may not be applicable to all lipids and cyclodextrins because the activation energy for cholesterol transfer from a model membrane to β-CD is only 8.4 kJ/mol while to apo-HDL-phospholipid acceptor particles it is 84 kJ/mol. In this case cholesterol molecules desorbing from a membrane surface can possibly diffuse directly into the hydrophobic core of a cyclodextrin molecule without having to desorb completely into the aqueous phase before being sequestered by the acceptor (Yancey et al., 1996). On the other hand, it has been shown that sterol desorption from a monolayer is dependent on sterol hydrophobicity and that β-CD does not penetrate into the membrane (Ohvo and Slotte, 1996). The low value for activation energy of cholesterol transfer to β-CD is most probably due to the fact that this particular CD species represents a very specific, non-physiological, type of cholesterol acceptor.

Typical natural phospho- and glycolipid species (c.f. table 1 in I) have quite high hydrophobicity and accordingly low off-rate from a membrane. It is thus unlikely that even the optimal CD-derivatives could mediate efficient transfer of these lipids, unless donors providing very high off-rates can be developed. Previous studies have shown that the rate of lipid efflux is strongly dependent on properties of the donor membrane. Factors increasing the off-rates include high surface curvature (Massey et al., 1984; Lund-Katz et al., 1982) and lipid composition that leads to looser lateral packing of the membrane (Massey et al., 1982a). Also bilayer perturbing molecules, like bile salts (Nichols, 1986), hydrophobic peptides or solvents could be of use.

9.2. γ-cyclodextrin-mediated labeling of cells with hydrophobic fluorescent phospholipids.

γ-CD greatly accelerates translocation of hydrophobic pyrene-labeled phospholipids from vesicles to cells. The transfer of $BODIPY_{12}$- and NBD_{12}-phospholipids was also remarkably enhanced by γ-CD, thus demonstrating that this is not a phenomenon spesific for the pyrene derivatives. Several lines of evidence indicate that γ-CD enhances intermembrane phospholipid transfer by increasing the effective concentration of the fluorescent phospholipid in the aqueous phase, rather than by inducing membrane fusion or hemifusion.

Several lines of evidence indicate that incubation with the donor vesicles and γ-CD is not harmful to the cells. Cell growth was not detectably affected by the labeling procedure. Plasma membrane remained intact as the incubation did not induce leakage of lactate dehydrogenase, a cytoplasmic enzyme, to the medium. Further, γ-CD did not extract cholesterol from cells like β-CD does and cellular cholesterol levels remained constant throughout the labeling. Extraction of phospholipids from cells was not observed. It has been shown previously that incubation of red blood cells with α-cyclodextrin results in significant extraction of cellular phospholipids, while β- and γ-cyclodextrins were much less efficient in this respect (Ohtani et al., 1989). The larger hydrophobic cavity of γ-cyclodextrins apparently favors complex formation with fluorescent lipids having bulky groups in the acyl chain(s), but disfavors complexation of both phospholipids with natural acyl chains and cholesterol.

Short-chain, hydrophilic lipids can be efficiently extracted from exposed compartments, like the outer leaflet of the plasma membrane by using phospholipid vesicles or albumin as acceptors (van Meer et al., 1987). This allows one to assess the amount of the fluorescent lipid in the outer leaflet of the plasma membrane, which is useful when monitoring recycling of lipids, for example. Incubation with γ-CD (± acceptor vesicles) does not remove medium or long chain dipyrenyl phospholipids efficiently from the outer leaflet of the plasma membrane. Curvature of the plasma membrane is quite low and the outer leaflet of the membrane is rich in tightly packed saturated sphingolipids and cholesterol (Allan, 1996; van Meer and van Genderen, 1994). Accordingly, lipids leaving the plasma membrane should have quite low off-rates, which helps to explain this. It is notable that selective quenching techniques can be used to determine the fraction of a pyrene lipid located in the plasma membrane (see below).

Currently $DiPyr_{10}$ –lipids are the most hydrophobic ones that can be introduced to cell with a high enough efficiency for lipid trafficking studies. Many normal phospholipids are even more hydrophobic than these, and accordingly, the labeling method should be refined to include $DiPyr_{12-14}$ –lipids in the usable hydrophobicity range. This may be possible by modifying the donor particles to give higher off-rates (see above).

9.3. Improved imaging of pyrene labeled lipids in living cells

9.3.1. Prevention of photobleaching of pyrene lipids in cells

Deoxygenation of the medium virtually eliminates photobleaching of pyrene lipids in cells (Fig. 7). Deoxygenation was achieved simply by adding glucose oxidase and glucose into the medium. Oxidation of glucose consumes molecular oxygen (Englander et al., 1987). This method is very simple and rapid as adequate deoxygenation is obtained within 1-2 minutes. Furthermore, the cells do not seem to suffer detectably, at least during the first 20-30 min of deoxygenation, as indicated by the maintenance of ATP levels and by the lack of inhibition of protein synthesis. This period is more than sufficient for various types of manipulations and sequential imaging. Similar deoxygenation methods have been tested previously for other fluorophores (Bloom and Webb, 1984). However, either no beneficial effect was observed or the effect was far less dramatic than what was observed for pyrene in this study. The mechanism of oxygen-dependent pyrene photobleaching is not clear, but reaction of oxygen with the triplet state of the fluorophore is an obvious possibility (Tsien and Waggoner, 1995).

9.3.2. Correction for autofluorescence and light scattering

In addition to photobleaching, cellular autofluorescence and cell-independent signal (i.e., fluorescence or scatter from the optics, coverslip or the medium) can severely impede quantitative imaging (Jericevic et al., 1989). These can be efficiently eliminated from pyrene excimer images by taking images with 345 and 360 nm excitation followed by subtraction of the latter image from the former. This is simplified by the large (≈ 130 nm) Stokes shift of the pyrene excimer fluorescence. In principle, a similar background correction can be done for pyrene monomer images (as well as for those obtained with other fluorophores). Because the pyrene monomer has a much smaller (30-40 nm) Stokes shift this is technically much more demanding and was not attempted here. Another problem with py-

rene monomer imaging is that most microscope objectives are not well corrected at less than 400 nm where the monomer fluorescence is observed. This tends to result in blurred images.

9.3.3. Selective imaging of the plasma membrane and its leaflets

Fluorescent lipid analogues are potentially useful tools for imaging of the plasma membrane, but their use is hampered by the fact that it is virtually impossible to restrict the probes only to the plasma membrane, since a significant fraction is very rapidly incorporated to endocytotic vesicles (Chen et al., 1997). Because some of these vesicles are very close to the plasma membrane, they can, due to the limited z-resolution of microscope objectives (Young, 1989), obscure the plasma membrane images. Although pre-fixation of the cells could prevent probe internalization, it is likely to lead to other serious artifacts as discussed above. This study provides evidence that the pyrene lipids in the plasma membrane can be selectively imaged in the presence of strong intracellular labeling by using a plasma membrane specific quencher, TNP-LPE, and image subtraction. Experiments with cells and liposomes indicated that TNP-LPE efficiently quenches the pyrene lipids present in the same leaflet. Also the pyrenes in the opposite leaflet are effectively quenched, at least when attached to a longer acyl chain. From the determined R_0-value it is obvious that pyrene lipids present in endocytotic vesicles cannot be significantly quenched and should not contribute to the plasma membrane images. It is possible to selectively image either the outer or inner leaflet of the plasma membrane by labeling the cells with pyrene phosphatidylcholine or -serine, respectively. This offers a possibility to probe the physical properties of either leaflet independently of each other. Selective quenching also allows one to determine the fraction of cell-associated fluorescent lipid in the plasma membrane, which is very helpful when studying lipid endocytosis and trafficking. However, it must be kept in mind that TNP-LPE as an unnatural lysolipid may perturb normal function of the plasma membrane and so quenching should be done only as an end point for an experiment. It is also possible that the quencher might interfere with the lateral organization of the plasma membrane, which might affect the results if one attempts to image rafts or caveolae. It should also be noted that fixation of cells seems to allow fairly rapid permeation of TNP-LPE through the plasma membrane, as indicated by quenching of intracellular structures as well. The reasons for this permeability increase are not clear but could result from modification of the head groups of PE and PS by the aldehyde fixative (see above). Quenching of the plasma membrane fluorescence can also be used to improve the visibility of intracellular structures.

9.4. Advantages of hydrophobic lipid analogues in lipid trafficking studies

As noted above, it is important to use hydrophobic analogues when studying intracellular trafficking of lipids. γ-CD provides a simple way to introduce hydrophobic fluorescent phospholipids to cells in culture, thereby making it feasible to study many unresolved aspects of intracellular phospholipid trafficking. For example, intracellular trafficking of plasma membrane PS can now be studied more readily than before. PS comprises as much as 30 mole% of the phospholipids of the inner leaflet of the plasma membrane (Allan, 1996), and it is unclear how the cell can maintain such a high concentration of PS in the inner leaflet. One possibility is that PS in the plasma membrane consist predominantly of saturated, hydrophobic species (Keenan and Morre, 1970), and the hydrophobicity could prevent their spontaneous diffusion from the plasma membrane to other organelles and eventually to mitochondria where they would be decarboxylated to PE (Voelker, 1997; Vance and Shiao, 1996). Hydrophobic PS species might become enriched in the plasma membrane because of preferential transfer of the less hydrophobic species to mitochondria after their synthesis in the ER (Heikinheimo and Somerharju, 1998), or due to their preferential association with membrane domains or "rafts" destined to the plasma membrane (van Meer, 1998). The present results strongly support this model by showing that the hydrophobic fluorescent derivative, $DiPyr_{10}PS$, labels the plasma membrane and various endocytic compartments but not the Golgi apparatus or mitochondria.

Caveolae and "rafts" are another example of a phenomenon that can be studied more readily using the present technology. These domains may play a crucial role e.g. in signal transduction and lipid and protein trafficking. (Simons and Ikonen, 2000; Bretscher and Munro, 1993; Simons and Ikonen, 1997; Anderson, 1998). The actual lipid compositions as well as the physical properties of these domains are largely unknown. By using the present method one could introduce to cells fluorescent analogues of the putative domain lipids, i.e., SM and glycosphingolipids, and then use fluorescence imaging and/or spectroscopy to obtain information on the physical properties of the domains. In addition, one could make use of the fact that cholesterol has a marked effect on the intramolecular excimer formation by dipyrenyl phospholipids (Sassaroli et al., 1995; Virtanen et al., 1995) to microscopically map the concentration of cholesterol in different membrane domains.

It has been previously shown that $NBD_{12}PS$ moves rapidly from the plasma membrane to the Golgi apparatus and mitochondria (Kobayashi and Arakawa, 1991). Since the authors considered this fluorescent species to be “relatively nonexchangeable”, and since

inhibition of vesicular transport had no effect on labeling of the Golgi apparatus and mitochondria, they proposed that the transport of $NBD_{12}PS$ from the plasma membrane to these organelles is probably mediated by a lipid transfer protein. However, since the $NBD_{12}PS$ is far less hydrophobic than typical native species (see table 1 in I), it can certainly move from the PM to mitochondria by spontaneous diffusion via the cytoplasm and no transfer protein is needed. Results like this underline the importance of using hydrophobic analogues when studying intracellular trafficking of lipids that have access to the cytoplasmic leaflet of organelle membranes.

10. CONCLUSIONS

1. γ-CD forms complexes with several fluorescent phospholipid analogues and transfers these to cells. Even relatively hydrophobic pyrene lipids can be used to label cells.

2. The transfer of these lipids is limited by their efflux rate from the donor membrane. This information should help to develop even more efficient lipid transfer methods.

3. The developed imaging methods significantly facilitate quantitative imaging of pyrene lipids in living cells.

4. The use of lipid analogues having similar hydrophobicity as the natural phospholipids is important in phospholipid trafficking studies. Otherwise there is a danger of obtaining misleading data.

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