Degradation of pyrene-labelled phospholipids by lysosomal phospholipases *in vitro*

Dependence of degradation on the length and position of the labelled and unlabelled acyl chains

Sari LUSA, Marjukka MYLLÄRNIEMI, Kirsi VOLMONEN, Matti VAUHKONEN and Pentti SOMERHARJU*

Institute of Biomedicine, Department of Medical Chemistry, University of Helsinki, Helsinki, Finland

The hydrolysis of pyrenylacyl phosphatidylcholines (Pyr_nPCs) (*n* indicates the number of aliphatic carbons in the pyrene-chain) by crude lysosomal phospholipases *in vitro* was investigated. Pyr_nPCs consist of several sets in which the length of the pyrene-labelled or the unlabelled acyl chain, linked to the *sn*-1 or *sn*-2 position, was systematically varied. Lysophosphatidylcholine and fatty acid were the only fluorescent breakdown products detected, thus indicating that Pyr_nPCs were degraded by A-type phospholipases and lysophospholipases. Of these, mainly A₁-type phospholipases appear to be involved, as determined from the relative amounts of labelled fatty acid and lysolipid released from the positional isomers. Based on the effects of the length and position of the pyrene-labelled and unlabelled chains it is suggested that (1) the lysosomal A-type phospholipases acting on Pyr_nPCs recognize the carboxy-terminal part of the lipid

acyl chains and (2) the relevant part of the binding site is relatively narrow. Thus phospholipids with added bulk in the corresponding region, such as those that are peroxidized and polymerized, may not be good substrates for the lysosomal phospholipases mentioned. The impaired hydrolysis of the most hydrophobic Pyr_nPCs indicates that lysosomal phospholipases may not be able to penetrate significantly into the substrate interphase, but upward movement of the lipid may be required for efficient hydrolysis. Finally, the rate of hydrolysis of many pyrenyl derivatives was found to be comparable to that of a natural phosphatidylcholine species, both in micelles and in lipoprotein particles, indicating that these derivatives can be used as faithful reporters of lysosomal degradation of natural lipids *in vivo* and *in vitro*.

INTRODUCTION

Pyrene-labelled phospholipids have been used extensively *in vitro* to study a variety of membrane-related phenomena, including lateral [1,2] and transverse [3] diffusion of lipids, lateral organization of membranes [4–7], spontaneous and protein-mediated lipid transport [3,8,9], phospholipid conformation [10] and hydrolysis [11,12] or transverse distribution of membrane protein tryptophan residues [13]. A major advantage of pyrenyl lipids as compared with other fluorescent derivatives is their ability to form excimers, which provides a convenient tool to determine, for instance, local lipid concentrations either spectroscopically [4,5] or by employing quantitative fluorescence microscopy [14]. Furthermore, the pyrene fluorophore is hydrophobic and does not distort the conformation of the parent lipid [15], as do the more polar fluorophores [16,17].

Although used mainly in the test tube so far, pyrenyl lipids have many potentially important applications in cellular studies also. For instance, pyrenyl phospholipids could be used as reporters of lysosomal degradation of the phospholipids of endocytosed lipoproteins. However, for such studies to be successful, it is essential to establish that the lysosomal phospholipases degrade the pyrenyl lipids similarly to the endogenous lipids. This is important also when pyrenyl lipids are used to detect disorders of lipid degradation [18,19].

Pyrenyl lipids are also promising tools for studies on lipid

transfer *in vivo*. We have recently found that glycerophospholipids (GPLs) containing two short pyrene acyl chains [di-pyrenebutanoyl glycerophospholipids (diPyr₄GPLs)] are quite resistant to degradation in cultured cells [20], unlike the previously used *N*-nitrobenzo-2-oxa-1,3-diazole-labelled GPLs which are very rapidly degraded in cells at physiological temperatures [21–23], thus complicating their use for studies on intracellular lipid traffic. DiPyr₄GPLs are, however, relatively hydrophilic, which may allow them to bypass certain transport routes (like vesicle or protein mediated translocation) that could be essential for the inter-organellar transport of the natural, more hydrophobic lipids. Thus it would be desirable to use more hydrophobic pyrenyl lipids for transport studies. Such hydrophobic pyrenyl derivatives can be introduced into cells either via metabolic incorporation of long-chain pyrenyl fatty acids (Pyr_nFA; *n* indicates the number of aliphatic carbons in the acyl chain) into cellular lipids [18,19,24], via cellular acylation of exogenous pyrenylacyl lysophosphatidylcholine (Pyr_nLPC; where *n* indicates the number of aliphatic carbons in the acyl chain) [25] or via fusion of lipid vesicles with the plasma membrane [26]. However, it is necessary to check if and how these fluorescent derivatives are degraded by cellular phospholipases; without such information meaningful interpretation of the transport data is difficult because of reincorporation of the labelled fatty acid into various cellular lipids (see above).

Monitoring the effects of systematic changes in pyrenyl lipid

Abbreviations used: C_xPyr_nPC, 1-acyl-2-pyrenylacylphosphatidylcholine (*x* and *n* indicate the number of aliphatic carbons in the saturated acyl chain and the pyrene-labelled chain respectively); diPyr₄GPL, di-pyrenebutanoyl glycerophospholipid; ¹⁴C-DPPC, 1-palmitoyl-2-[1-¹⁴C]-palmitoyl phosphatidylcholine; GPL, glycerophospholipid; NBD, *N*-nitrobenzo-2-oxa-1,3-diazole; PLA₁, phospholipase A₁ (EC 3.1.1.32); PLA₂, phospholipase A₂ (EC 3.1.1.4); Pyr_nFA, pyrenyl fatty acid (*n* indicates the number of aliphatic carbons in the acyl chain); Pyr_nLPC, pyrenylacyl lysophosphatidylcholine; Pyr_nPC, pyrenylacyl phosphatidylcholine; rHDL, reconstituted high-density lipoprotein particles.

* To whom correspondence should be addressed at, Siltavuorenpenger 10, P.O. Box 8, 00014 University of Helsinki, Finland.

structure on their degradation could also provide important information on the substrate-binding sites of cellular phospholipases as well as on their surface-binding properties, both poorly known aspects at present. Apart from its own intrinsic value, such information could be very useful in order to understand whether impaired degradation is an important reason for the accumulation of altered lipids, such as peroxidized and polymerized lipids, in lysosomes upon ageing of cells [27].

Accordingly, we have investigated the effects of systematic variation of the length and position (*sn*-1/*sn*-2) of the pyrene-labelled and unlabelled acyl chains on the rate of hydrolysis of pyrenylacyl phosphatidylcholine species (Pyr_{*n*}PCs; *n* indicates the number of aliphatic carbons in the pyrene-labelled chain) by acidic phospholipases of human fibroblasts.

EXPERIMENTAL

Lipids

The di- and mono-Pyr_{*n*}PCs were synthesized and purified by HPLC as described previously [6,28,29]. The positional purity of these lipids is typically higher than 95%, based on HPLC analysis of the phospholipase A₂ (PLA₂; EC 3.1.1.4) degradation products. The lyso derivatives were prepared from the di-acyl species by PLA₂ degradation followed by purification with HPLC. 1-Palmitoyl-2-oleoylphosphatidylcholine and 1-palmitoyl-2-oleoylphosphatidic acid were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). 1-Palmitoyl-2-[1-¹⁴C]-palmitoyl phosphatidylcholine (¹⁴C-DPPC; specific activity 55 mCi/mmol) was supplied by Amersham, UK.

Reagents

All solvents used were of HPLC grade and were obtained from Merck (Espoo, Finland). Other chemicals were from Sigma.

Cell-free fibroblast extract

Human fibroblast cells were grown on plastic tissue-culture dishes in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (Gibco), 10 mM glutamine, penicillin (100 units/ml), streptomycin (100 mg/ml) and non-essential amino acids (0.01 ml/ml) under 5% CO₂/95% air at 37 °C. Cells were washed twice with PBS containing 2 mM EDTA and harvested by scraping into the same buffer with a Teflon policeman. The cells were centrifuged and aliquots of cell pellets were stored at -70 °C.

For the assay, a cell-free fibroblast extract was prepared just before use by sonication of thawed cell pellets in distilled water for five 10 s intervals on ice using a microtip on a probe sonicator. The probe particles were removed by centrifugation (13000 *g*, 30 s) and the supernatant was used to assay lipid degradation. Protein concentration was determined using BSA as standard [30].

Hydrolysis of pyrenyl lipids by fibroblast extracts

A lipid mixture in chloroform containing a pyrenyl phosphatidylcholine species, palmitoyl-oleyl-phosphatidylcholine and palmitoyl-oleyl-phosphatidic acid, in either a 1:9:1 or a 0.1:9:1 molar ratio with the monopyrenyl and dipyrenyl lipids respectively, was dried by evaporation under nitrogen and further dried under vacuum for 2 h. The dried lipids were then dispersed in a 50 mM sodium acetate/4 mM taurocholate/1 mM EDTA buffer, pH 3.9, by sonication in a water bath at 40 °C. This micellar substrate (2.2 or 10 nmol total lipid in the case of mono- and di-pyrenyl lipids respectively) was then incubated with cell-

free fibroblast extract at 37 °C for 3 h. Generally, 50 µg of the extract protein was used per assay, but in the case of di-Pyr_{*n*}PCs 300 µg was used to obtain adequate hydrolysis. Hydrolysis of Pyr_{*n*}PC and ¹⁴C-DPPC incorporated into reconstituted high-density lipoprotein particles (rHDL) was carried out similarly, but in the absence of taurocholate. The rHDL particles were prepared according to the method of Pittman et al. [31] as modified previously [32].

While it is generally useful to obtain true kinetic constants (*K_m* and *V_{max}*), this was not attempted here since the cell extract, used as the enzyme source, contains various phospholipase activities in unknown proportions, each of which could differ in its sensitivity to product inhibition or stimulation. Thus meaningful interpretation of the data in terms of individual kinetic parameters, which is complicated even in much simpler systems [33], would not be feasible. Therefore, the percentage of Pyr_{*n*}PC hydrolysed during a 3 h incubation is used as a (semiquantitative) measure of the rate of lipid hydrolysis. Pilot experiments with C₁₆Pyr₁₀PC showed that the reaction proceeded in a nearly linear fashion for at least 3 h.

The specific activity of different homogenates upon preparation varied somewhat and there was also some decrease in activity during storage at -70 °C. Since a constant amount of homogenate protein was used in the assays for practical reasons, the absolute degree of hydrolysis varied somewhat from one experiment to another. However, this variation did not affect significantly the relative degree of degradation of the different species and thus does not compromise the conclusions drawn below.

Extraction and analysis of lipids

The enzymic reactions were stopped by addition of chloroform and methanol (1:2, v/v), after which the lipids were extracted according to the method of Bligh and Dyer [34] in the presence of 0.5 M acetic acid to ensure quantitative partitioning of the released fatty acids to the chloroform phase. Extracted lipids were separated by HPLC on a Spherisorb 3 µm silica column (Phase Separations Ltd.) using initially a ternary solvent gradient capable of separating di- and mono-glycerides, fatty acids, PC and LPC [24,35]. However, since no di- or mono-glycerides were detected in the incubation mixtures with any pyrenyl lipid, an isocratic system employing chloroform/methanol/water (60:40:5, by vol.) as the solvent was used routinely to separate the degradation products, Pyr_{*n*}FA and Pyr_{*n*}LPC, from Pyr_{*n*}PC. This isocratic system is faster and produces a more reproducible fatty acid peak than the gradient system. For quantification, the eluent was passed through a Merck-Hitachi F-1150 fluorescence detector (with the excitation and emission monochromators set to 345 and 395 nm respectively) coupled to a Merck-Hitachi D-2000 integrator. Since the cell extracts contained an intrinsic fluorescent compound that co-eluted with Pyr_{*n*}FA, it was necessary to correct the Pyr_{*n*}FA peak area for the contribution of this (unidentified) compound. This was done by (1) co-running samples obtained from incubation mixtures that lacked the pyrenyl lipid and (2) subtracting the area corresponding to this unknown compound from the area of the Pyr_{*n*}FA peak. The data were also corrected for a minor amount of spontaneous hydrolysis.

RESULTS

Effect of the length and the position of the pyrenylacyl chain

The effect of pyrenylacyl chain length on the degradation of Pyr_{*n*}PCs by lysosomal phospholipases was investigated first.

Figure 1 Chemical structures and degradation patterns of the Pyr_nPC species used

Top: $\text{Pyr}_n\text{C}_x\text{PC}$ species. The length of either the pyrenylacyl chain ($n = 4\text{--}14$ carbons) or the unlabelled chain ($x = 10\text{--}20$ carbons) is varied. Bottom: $\text{C}_n\text{Pyr}_n\text{PC}$ species. Again, either $n (= 4\text{--}14)$ or $x (= 10\text{--}20)$ is varied. The products obtained via degradation of the species by A_1 (PLA_1) or A_2 (PLA_2)-type phospholipases are also indicated.

Two sets of species were used (see Figure 1): one ($\text{C}_{16}\text{Pyr}_n\text{PC}$ species) that contained palmitic acid in the *sn*-1 position and a pyrenyl chain of variable length in the *sn*-2 position, and the other ($\text{Pyr}_n\text{C}_{16}\text{PC}$ species; n indicates the number of aliphatic carbons in the labelled acyl chain) that consisted of the positional isomers of the first set. The lipids were incorporated, together with an excess of unlabelled phospholipids, into taurocholate micelles which provide an environment favourable for the action of lysosomal phospholipases [36]. A crude fibroblast extract was used as the enzyme source, the reaction was run at pH 3.9 and the products were separated and quantified by HPLC with coupled fluorescence detection.

When the $\text{Pyr}_n\text{C}_{16}\text{PC}$ species were used as substrates, the hydrolytic activity varied considerably with the length of the pyrenyl chain (Figure 2A). $\text{Pyr}_{10}\text{C}_{16}\text{PC}$ species were hydrolysed most efficiently and markedly decreased hydrolysis was observed when the length of the pyrenyl chain was made either shorter or longer. With each species, Pyr_nFA was the major fluorescent product; only small amounts of Pyr_nLPC were detected (Figure

Figure 2 Hydrolysis versus the length of the pyrenyl chain in the *sn*-1 position

(A) Percentage hydrolysis of the $\text{Pyr}_n\text{C}_{16}\text{PC}$ species. (B) Relative amount Pyr_nFA ($\square$) and Pyr_nLPC ($\triangle$) product formed (% of total pyrenyl lipids). The pyrenyl lipids were incorporated, together with an excess of unlabelled phospholipids, into taurocholate micelles and the reaction was started by addition of cell-free fibroblast extract (50 μg of protein) and carried out at pH 3.9 for 3 h at 37 $^\circ\text{C}$. The extent of hydrolysis was determined by HPLC as outlined in the Experimental section. Averaged data from four independent experiments, normalized by using the Pyr_{10} species as the reference, is shown. The error bars indicate the standard deviation.

2B). This indicates that $\text{Pyr}_n\text{C}_{16}\text{PC}$ s are degraded either by a phospholipase A_1 (PLA_1 ; EC 3.1.1.32) and/or by PLA_2 together with a lysophospholipase. To study this issue further, *sn*-1- $\text{Pyr}_{10}\text{LPC}$ and *sn*-1- $\text{Pyr}_{12}\text{LPC}$ species were prepared and subjected to hydrolysis under the standard conditions. The hydrolysis of these lyso-derivatives were 13.6 (± 4.7) and 9.2 (± 0.12) % of that of the corresponding $\text{Pyr}_n\text{C}_{16}\text{PC}$ species studied in parallel respectively. This relatively inefficient hydrolysis of the lyso-derivatives strongly suggests that most of the Pyr_nFA released from $\text{Pyr}_n\text{C}_{16}\text{PC}$ s (Figure 2B) originates from a PLA_1 reaction, rather than being produced by the combined action of a PLA_2 and a lysophospholipase.

When the isomeric $\text{C}_{16}\text{Pyr}_n\text{PC}$ s were subjected to hydrolysis, the species containing a Pyr_{10}FA was again the optimal substrate, and the hydrolysis diminished considerably with decreasing length of the pyrenyl chain (Figure 3A). With this set of lipids the main product was Pyr_nLPC (Figure 3B), as expected if the pyrenyl lipids were mainly degraded by PLA_1 -type enzymes, as suggested by the data given above. However, since considerable amounts of Pyr_nFA were also detected, PLA_2 enzyme(s) could also have acted on these lipids. Based on the facts that (1), on average, the amount of fatty acid is less than half of that of the lysolipid (Figure 3B) and (2) some of the lysolipid has probably been degraded to Pyr_nFA by lysophospholipases (see above), we estimate that at least 70–80 % of the diacyl pyrenylphosphatidylcholines is hydrolysed by PLA_1 -type enzymes.

Effect of the length and position of the unlabelled acyl chain

In principle, the low activity of the lysosomal phospholipases toward the PC species with short pyrenyl chains could be due to:

Figure 3 Hydrolysis versus the length of the pyrenyl chain in the *sn*-2 position

(A) Percentage hydrolysis of C₁₆Pyr_xPC. (B) Relative amounts of Pyr_nFA (□) and Pyr_nLPC (△) formed (% of total pyrenyl lipids). Other details as given in the legend to Figure 2. Owing to lack of material, the data for C₁₆Pyr₁₄PC (●) is from a single experiment only.

(1) problems of incorporation of the bulky pyrenyl group into the active site of the enzyme(s); or (2) lower total hydrophobicity of the molecule (or the labelled acyl chain). To resolve this issue,

Figure 4 Hydrolysis versus the length of the unlabelled acyl chain in the *sn*-1 or *sn*-2 position

Percentage hydrolysis of Pyr₁₀C_xPC (A) and C_xPyr₁₀PC (B) species is plotted versus the length of the unlabelled chain ($x = 10$ –20 carbons). Other details are as given in the legend to Figure 2. The data represent averages from three independent experiments.

Figure 5 Hydrolysis of the diPyr_xPC species versus the length of the pyrenyl chains

Percentage hydrolysis is plotted versus the length of the two identical pyrenyl chains. $x = 4$ –14. For other details see the Experimental section. The data are averaged from four independent experiments.

we studied the effect of the length of the unlabelled chain on the rate of hydrolysis. Two isomeric sets of PC species were used, both containing Pyr₁₀FA as the labelled fatty acid and a saturated acyl chain of 10–20 carbon units either in the *sn*-1 position (C_xPyr₁₀PCs) or in the *sn*-2 position (Pyr₁₀C_xPCs). With the Pyr₁₀C_xPC species, the extent of hydrolysis decreased steadily with the length of the unlabelled acyl chain (Figure 4A). Also with the isomeric set, i.e. C_xPyr₁₀PCs, hydrolysis decreased with increasing length of the unlabelled acyl chain (Figure 4B), albeit not quite as smoothly.

The fact that lipids with a short unlabelled acyl chain are well hydrolysed (Figures 4A and 4B), while those with a short pyrenyl chain are not (Figures 2 and 3), strongly suggests the impaired hydrolysis of the latter results from steric interference due to the bulky pyrenyl moiety rather than diminished hydrophobicity of the acyl chain or the whole molecule (see the Discussion section). It is relevant to note here that the removal of a methylene unit from the unlabelled chain or from the pyrene-labelled chain changes the hydrophobicity of the molecule in a nearly identical manner as determined by reverse-phase chromatography (results not shown).

Degradation of dipyrenylphosphatidylcholines

The hydrolysis of dipyrenylphosphatidylcholines (diPyr_nPCs), which contain two pyrenylacyl chains of equal length was studied next. As shown in Figure 5, hydrolysis of such lipids was also markedly dependent on the length of the pyrene chain. In general, the effect of chain length is similar to those obtained with the monopyrenyl derivatives (Figures 2A and 3A). However, hydrolysis of the diPyr₄ species was more extensive than would be expected from the trends observed for the monopyrenyl derivatives. Because of its short acyl chains, this species is relatively soluble in aqueous medium [20] and could thus serve as a substrate for non-specific esterases that cannot act on the other, more hydrophobic lipids embedded in the micelles. We also note that one should not compare directly the degradation efficiencies of the diPyr_nPC species with those of the monopyrenyl species because considerably higher amounts of both the lysosomal extract and the carrier lipids had to be used with the former (see the Experimental section) to obtain significant hydrolysis. As expected, both Pyr_nFA and Pyr_nLPC, in a somewhat species-dependent ratio, were the fluorescent degradation products detected (results not shown).

Table 1 Comparison of the hydrolysis of pyrene-labelled and natural phosphatidylcholine species

The hydrolysis of the lipids co-incorporated into taurocholate micelles or rHDL was determined as detailed in the Experimental section.

	C_{16} Pyr $_{10}$ PC		14 C-DPPC	
	Relative rate*	FA/LPC†	Relative rate	FA/LPC
Micelles	1.00	0.89 ± 0.22	0.37 ± 0.06	1.38 ± 0.44
rHDL	1.00	2.18 ± 0.51	0.40 ± 0.17	0.59 ± 0.12

* The rates of hydrolysis are expressed as relative (normalized) values obtained by setting the rate of C_{16} Pyr $_{10}$ PC hydrolysis equal to 1.0. The S.D.s are also indicated ($n = 4$).

† The ratio of labelled fatty acid to labelled lysophosphatidylcholine.

The reason for the poor hydrolysis of the diPyr $_6$ and diPyr $_8$ species (Figure 5) is not clear, but preliminary investigations suggest that these two lipids, unlike the other dipyrenyl derivatives, may not be well dispersed in the taurocholate micelles, in spite of the presence of a 100-fold excess of unlabelled phospholipids. It is feasible that such undispersed (aggregated) lipids may not be readily available to the phospholipases.

We also studied the hydrolysis of all di- and mono-pyrenyl phosphatidylcholine species at pH 7.4 in the presence and absence of 1 mM $CaCl_2$. No significant hydrolysis of any of the pyrenyl lipids was detected (results not shown), thus demonstrating that non-lysosomal phospholipases contribute negligibly to degradation of pyrenyl lipids when studied at pH 3.9. While these results might also suggest that the pyrenyl derivatives are stable against degradation by non-lysosomal phospholipases, such a conclusion cannot be drawn at present, since neither was there any hydrolysis of 14 C-DPPC at pH 7.4. The reason for the lack of hydrolysis of the pyrene- and radio-labelled phosphatidylcholines at this pH is not known, but could be due to: (1) low levels of neutral phospholipases in human fibroblast; (2) their high substrate specificity; or (3) their inhibition by taurocholate present in the assay mixture.

Hydrolysis of pyrenyl phosphatidylcholines versus natural phosphatidylcholines

To compare of hydrolysis of pyrenyl phospholipids by lysosomal phospholipases with that of natural lipids, C_{16} Pyr $_{10}$ PC and 1-palmitoyl-2-[1- 14 C]-palmitoyl phosphatidylcholine (14 C-DPPC) were incorporated into taurocholate micelles or rHDL and then incubated in the presence of lysosomal extracts at pH 3.9. As shown in Table 1, C_{16} Pyr $_{10}$ PC was hydrolysed more efficiently than 14 C-DPPC species both in micelles and in rHDL particles. Thus, it appears that many of the pyrenyl phospholipid species studied here are as good substrates for the lysosomal phospholipases as their natural counterparts.

DISCUSSION

Lysosomes of mammalian cells contain various lipolytic enzymes that are capable of degrading complex lipids originating either from the outside of the cell [37,38] or from the endogenous membranes [38]. The principal enzymes of intralysosomal phospholipid catabolism are A $_1$ - and A $_2$ -type phospholipases [40–43]. In addition, phospholipase C [39,44] and lysophospholipase [45,46] activities have been detected in lysosomes. In the present study, Pyr $_n$ FA and Pyr $_n$ LPC were the only fluorescent degradation products obtained from the various pyrenylphos-

phatidylcholines studied. This strongly suggested that these derivatives are degraded mainly by A-type phospholipases. Collectively, the ratio of Pyr $_n$ FA to Pyr $_n$ LPC observed with the isomeric sets (Figures 2B and 3B) strongly suggest that the pyrenyl derivatives are degraded mainly by PLA $_1$ (see the Results section). However, PLA $_2$ -type enzymes also act on these lipids and appear to be responsible for 10–30 % of the A-type degradation.

While the absence of fluorescent di- or mono-glycerides indicates that phospholipase C and lysophospholipase C [36,44] are not involved in the degradation of Pyr $_n$ PCs, we cannot completely exclude a (minor) contribution by C-type phospho- or lysophospho-lipases [36,44], since it is possible that the products of these enzymes are degraded so rapidly that they escape detection. Since the pH optimum of phospholipase C is close to 5 [44], the low pH (3.9) used here may also have inhibited this enzyme.

Implications on the structure of the acyl binding sites of PLAs

Systematic variation of the length and position of the pyrene-labelled and unlabelled acyl chains revealed that the pyrene moiety close to the carbonyl moiety severely interferes with binding to the active site of PLAs. This interference was independent of whether the pyrenyl moiety was in the *sn*-1 or *sn*-2 position of the glycerol moiety. Thus the phospholipase(s) involved appear to recognize both acyl chains of the lipid substrate, and secondly, the protein sites (or cavities) interacting with the carboxy-terminal parts of lipid acyl chains appear to be narrow. It is of interest to note also that other A-type phospholipases or related enzymes have been shown previously to interact poorly with lipids having bulky groups in the acyl chains close to the carbonyl moiety [47,48]. Furthermore, we have found that lysosomal cholesterol ester hydrolase (acid lipase) degrades poorly cholesterol esters containing a short pyrenyl chain, as compared with those having a pyrenyl chain of medium length (S. Lusa and P. Somerharju, unpublished work). Yet, similar rejection of lipids with short pyrenyl chains has been observed previously both for the phosphatidylcholine transfer protein [28] and the phosphatidylinositol/phosphatidylcholine transfer protein [49]. Thus, it appears that lipases and other proteins interacting with monomeric lipids may generally have narrow hydrophobic cavities for binding the acyl residue(s) of the lipid substrate. This would not be unexpected, since such narrow cavities should provide an optimal fit with the (non-bulky) acyl chains present in most natural lipids.

Poor degradation of short-chain pyrenyl lipids by lysosomal phospholipases implies also that the hydrolysis of other phospholipids having altered acyl chains, for example peroxidized or polymerized, could be impaired as compared with native lipids. If so, this could be a major reason for the accumulation of lipofuscin in lysosomes [27]. The poorly degraded pyrenyl species could be useful when modelling lipid accumulation in lysosomes.

Interestingly, the hydrolysis of pyrenyl lipids having a very long labelled or unlabelled chain is often less than optimal (Figures 4 and 5). This suggests that the lysosomal A-type phospholipases, which appear to be the major species hydrolysing the pyrenyl lipids, are not able to penetrate the micellar (or lipid) surface significantly, but the lipid molecule has to move up toward the aqueous phase to be incorporated into the active site. The excessive hydrophobicity of the long-chain derivatives is expected to make such a movement more difficult, thus resulting in impaired hydrolysis. Previously, excessive hydrophobicity has been suggested to be a reason for the impaired incorporation of

long-chain phospholipids into the lipid-binding site of phospholipid transfer proteins [28,49,50].

Pyrene phospholipids as reporters of natural lipid hydrolysis

Comparison of the extent of hydrolysis of C₁₆Pyr₁₀PC with that of a natural phosphatidylcholine species (Table 1) indicates that many of the Pyr_nPC species studied here are equally good substrates for the lysosomal lipases as their natural counterparts. Thus, the pyrenyl derivatives could be used as reliable reporters of phospholipids hydrolysis *in vitro* or in living cells. For instance, pyrenyl phospholipids could be introduced into intact lipoprotein particles by the use of phospholipid transfer proteins [51], and degradation of the lipids of such labelled lipoproteins could be followed either by using liquid chromatography with coupled fluorescence detection, as here, or by making use of quantitative fluorescence microscopy. For the latter approach, dipyrenyl derivatives may be particularly suitable, since their excimer-to-monomer fluorescence intensity ratio decreases linearly as a function of the degree of hydrolysis [52], thus allowing for accurate determination of A-type phospholipases at the sub-cellular level as exemplified by recent experiments [53].

Concluding remarks

The present data demonstrate that many pyrene-labelled phosphatidylcholine species are degraded efficiently by lysosomal phospholipases of human fibroblast. Therefore, these derivatives should be useful to study lysosomal phospholipid degradation by using either biochemical or microscopic methods of detection. Under the conditions used, mainly A₁-type phospholipases acted on the pyrenyl derivatives, as deduced from the relative abundance of the labelled products obtained with the different sets of lipids. Poor degradation of the species having a short pyrene-labelled chain indicates that these phospholipases have a narrow binding site for both the *sn*-1 and *sn*-2 acyl chains of the phospholipid. Finally, the impaired degradation of the most hydrophobic species suggests that upward movement of the lipid substrate is required for the hydrolysis to take place.

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