Characteristics of Pyrene Phospholipid/γ-Cyclodextrin Complex

Kimmo Tanhuanpää,* Kwan Hon Cheng,† Krista Anttonen,* Jorma A. Virtanen,‡ and Pentti Somerharju*
*Institute of Biomedicine, Department of Biochemistry, University of Helsinki, Finland; †Department of Physics, Texas Tech University, Lubbock, Texas 79409 USA; and ‡Institute of Chemistry, Department of Physical Chemistry, University of Helsinki, Finland

ABSTRACT Recently, it was demonstrated that γ-cyclodextrins (γ-CDs) greatly accelerates transfer of hydrophobic pyrene-labeled and other fluorescent phospholipid derivatives from vesicles to cells in culture (Tanhuanpää and Somerharju, 1999). To understand better the characteristics of this process, we studied the interaction of γ-CD with pyrene-labeled phosphatidylcholines (PyrPCs) using a variety of physical methods. Either one or both of the acyl chains of PC was labeled with a pyrene moiety (monoPyrPCs and diPyrPCs, respectively), and the length of the labeled chain(s) varied from 4 to 14 carbons. Fluorescent binding assays showed that the association constant decreases strongly with increasing acyl chain length. PyrPC/γ-CD stoichiometry was 1:2 for the shorter chain species, but changed to 1:3 when the acyl chain length exceeded 8 (diPyrPCs) or 10 (monoPyrPCs) carbons. The activation energy for the formation of $diPyr_{10}PC$/γ-CD complex was high, i.e., +92 kJ/mol, indicating that the phospholipid molecule has to fully emerge from the bilayer before complex formation can take place. The free energy, enthalpy, and entropy of transfer of monoPyrPC from bilayer to γ-CD complex were close to zero. The absorption, Fourier transform infrared, and fluorescence spectral measurements and lifetime analysis indicated that the pyrene moiety lies inside the CD cavity and is conformationally restricted, particularly when the labeled chain is short. The acyl chains of a PyrPC molecule seem to share a CD cavity rather than occupy different ones. The present data provide strong evidence that the ability of γ-CD to enhance intermembrane transfer of pyrene-labeled phospholipids is based on the formation of stoichiometric complexes in the aqueous phase. This information should help in designing CD derivatives that are more efficient lipid carriers then those available at present.

INTRODUCTION

Intracellular phospholipid trafficking is a phenomenon that is intimately involved in many crucial cellular phenomena, such as genesis of organelle membranes and signal transduction (Wirtz, 1997; van Meer, 1993; Rogers and Bankaitis, 2000). However, the mechanisms and regulation of phospholipid trafficking are poorly understood at present. The main reason for this is the lack of suitable tools for monitoring lipid-trafficking. Short-chain fluorescent phospholipids have been successfully used to resolve certain routes of trafficking (Pagano and Sleight, 1985; Pagano et al., 1999; Chen et al., 1997; van Helvoort et al., 1994; van Meer and Holthuis, 2000), but cannot be used to study transport between cytoplasmic membrane surfaces. This is because their high solubility in the aqueous phase allows them to bypass certain mechanisms (e.g., vesicular and protein-mediated transport) presumably responsible for the transfer of natural, far more hydrophobic species. Hydrophobic fluorescent derivatives have been used only rarely (van Meer and Simons, 1986) because there have been no simple and universally applicable methods available for their introduction to cells. Recently, we found that carboxyethyl-γ-cyclodextrin (carboxyethyl-γ-CD) greatly accelerates the transfer of long-chain fluorescent phospholipid derivatives from vesicles to cells in culture (Tanhuanpää and Somerharju, 1999). Thus it is possible to study also those trafficking routes that are not accessible when using truncated analogues. Unfortunately, however, the CD derivatives did not mediate efficient transfer of fluorescence phospholipids that are as hydrophobic as the most hydrophobic natural species. This is a significant drawback because the dependency of intermembrane transfer on lipid hydrophobicity is a very useful criterion when assessing the mechanism of transfer (Heikinheimo and Somerharju, 1998). Also, hydrophobic fluorescent derivatives could provide useful information on the properties and formation of membrane rafts, structures which probably consist mostly of hydrophobic (long-chain and/or saturated) phospho- and glycolipids, and seem to play a crucial role in lipid (and protein) trafficking as well as other cellular events (Simons and Ikonen, 2000).

To better understand the factors limiting the transfer of hydrophobic phospholipids by γ-CD and its derivatives, we have here studied the interaction of pyrene-labeled phospholipids with γ-CD by using a variety of physical methods and systematically constructed sets of labeled species. The particular advantage of using pyrene-labeled rather than other fluorescent species are that the physical and photophysical properties of these lipids are particularly well characterized (Pownall and Smith, 1989; Eklund et al., 1992; Sassaroli et al., 1995). We used unmodified γ-CD instead of the carboxyethyl derivative because interpretation of the data is not 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. The key result of this study is that the rate of efflux from the bilayer

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Address reprint requests to Pentti Somerharju, Institute of Biomedicine, P.O. Box 63, Haartmaninkatu 8, 00014 University of Helsinki, Finland. Tel.: 358-0-191-25410; Fax: 358-0-191-25444; E-mail: pentti.somerharju@helsinki.fi.

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is, most probably, the rate limiting step in γ-CD–mediated transfer of (phospho)lipids.

MATERIALS AND METHODS

Lipids and other reagents

All unlabeled lipids were obtained from Avanti Polar Lipids (Alabaster, AL). The pyrenylacyl phosphatidylcholines (PyrPCs) were synthesized as described previously (Somerharju and Wirtz, 1982; Somerharju et al., 1985) and were at least 99% pure as determined by high-performance liquid chromatography. γ-CD (> 99% pure) was obtained from Cyclolab (Budapest, Hungary). Solvents were obtained from Merck (West Point, PA) and other chemicals from Sigma (St. Louis, MO).

Determination of PyrPC/γ-CD association and binding rate constants

To determine the binding of PyrPCs to γ-CDs, we made use of a previously described dequenching assay (Somerharju et al., 1987). Briefly, donor vesicles consisting of a pyrene-labeled PC, *sn*-1-palmitoyl, 2-oleoyl-phosphatidylcholine (POPC), *sn*-1-palmitoyl, 2-oleoyl-phosphatidic acid (POPA), and *N*-trinitrophenyl-phosphatidylethanolamine (TNP-PE) quencher (0.2:45:1:5 molar ratio) were prepared by probe sonication. An aliquot of γ-CD solution (100 mM in phosphate-buffered saline, pH 7.4) was added to achieve the desired concentration. The fluorescence intensity was then determined after an equilibration period that varied from 30–190 min, depending on the pyrene lipid acyl chain length. The long-chain species required a longer incubation time to reach the binding equilibrium (see Results). The association constant K was obtained by fitting the following equation to the fluorescence data:

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

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 CD concentration; and z is the γ-CD: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 then 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 used to obtain the rate constants:

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

F_0 is the initial fluorescence, t is the time, t_1 and t_2 are the halftimes of the two kinetic processes and A_1 and A_2 are fitting parameters. The pseudo first-order rate constants k_1' and k_2' are equal to $1/t_1$ and $1/t_2$, respectively.

Fluorescence spectrometry

Steady state fluorescence measurements were carried out with a PTI QuantaMaster spectrofluorometer (PTI, Lawrenceville, NJ) 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 multifrequency cross-correlation phase and modulation acquisition electronics. A He-Cd UV laser was used for excitation. An excitation polarizer with its transmission axis set at 35° with respect to the vertical was placed in the excitation beam to eliminate the contribution of the rotational diffusion effect of the sample to the measurements (Spencer and Weber, 1970). No polarizer was placed on the emission side. Phase delay and demodulation values of pyrene emission at 392 and 450 nm were collected and compared with that of a standard solution (*p-bis*[2-(5-phenyloxzaolyl]) benzene in pure ethanol, fluorescence lifetime = 1.53 ns) for frequencies ranging from 0.1 to 10 MHz. All fluorescence measurements were carried out at 23°C. The intramolecular excimer kinetic parameters of pyrene in $diPyr_4PC$ were obtained from the frequency-domain fluorescence data using a two-state kinetic model as described previously (Cheng and Somerharju, 1996). Sample concentrations and preparations were the same as those in the spectral measurements mentioned above.

Fourier transform infrared (FTIR) spectrometry

$DiPyr_4PC$ in chloroform was dried under vacuum and dissolved in ethanol. Dry powder of γ-CD was then added, the mixture was kept under vacuum for several hours and finally rehydrated by addition of 50 μl of phosphate-buffered saline, pH 7.4. The infrared spectra were recorded with a Magna-IR 560 FTIR spectrometer (Nicolet Inc., Madison, WI) equipped with a deuteriated triglycine sulfate detector operated at room temperature. All samples were applied on a single reflection horizontal ATR sample cell (Pike Technologies, Inc. Madison, WI) at 23°C. Typically, 50 interferograms were collected, averaged, Fourier transformed, and subtracted from the solvent background by standard procedure (Cheng et al., 1994) using the Omnic software provided by Nicolet. The overall spectral resolution of each spectrum was $>0.125\ cm^{-1}$.

RESULTS

Pyrene-labeled PCs

In this study, two sets of pyrene PyrPC species were used: 1) di-pyrenylacyl-phoshatidylcholines ($diPyr_nPCs$) in which both acyl chains contained a pyrene moiety linked to the terminal methylene unit of the acyl chain; and 2) monopyrenylacyl-phosphatidylcholines ($monoPyr_nPCs$) in which only the *sn*-2 chain is labeled with pyrene. $MonoPyr_nPCs$ usually contained a saturated 16-carbon acyl chain in the *sn*-1 position, but in some studies a species with 10 carbon chain was used. The length of the pyrenylacyl chain (n) varied from 4 to 14 carbons with two methylene unit intervals in both sets. Notably, variation of n in case of $diPyr_n$-PCs has a much larger relative effect on the lipid hydrophobicity than in the case of 16:0-Pyr_nPCs as the length of both acyl chains varies.

Binding of $diPyr_nPC$ and 16:0-Pyr_nPC species to γ-CD

To study the binding of pyrene PC species to γ-CD, quenched vesicles containing a dipyrenylacyl ($diPyr_nPC$) or a monopyrenylacyl PCs (16:0-Pyr_nPC) 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 of pyrene fluorescence intensity attributable to removal of the PyrPC molecules from the lipid bilayer containing the quencher molecules (TNP-PE). The fact that the increase of pyrene fluorescence upon addition of γ-CD strongly diminishes with PyrPC hydrophobicity (see below) shows that this increase is indeed attributable to removal of the pyrene lipid, rather than the

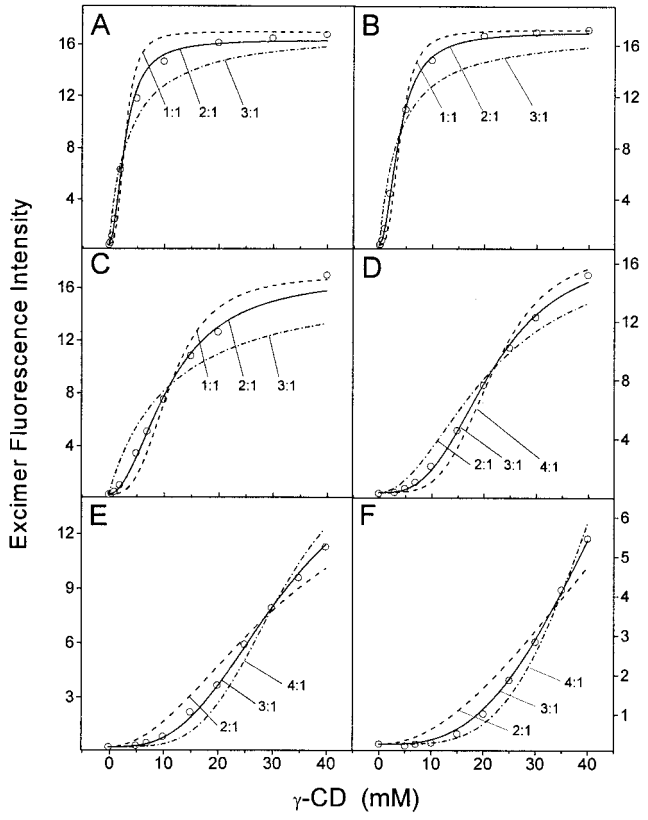

FIGURE 1 Binding of diPyr$_n$PC species to γ-CD. Vesicles consisting of diPyr$_n$PC, POPC, POPA, and TNP-PE (0.2/45/1/5 nmol) were mixed with buffer containing varying concentrations of γ-CD and the excimer intensity was recorded after an equilibration time of 30–180 min, depending to the acyl chain length (see Materials and Methods). Eq. 1 was then fitted to the binding data to determine complex stoichiometry and association constant. The assumed z values, i.e., cyclodextrin/PyrPC ratio in the complex, are indicated in the figure. n = 4 (*A*), 6 (*B*), 8 (*C*), 10 (*D*), 12 (*E*), or 14 (*F*). Note the different y scales.

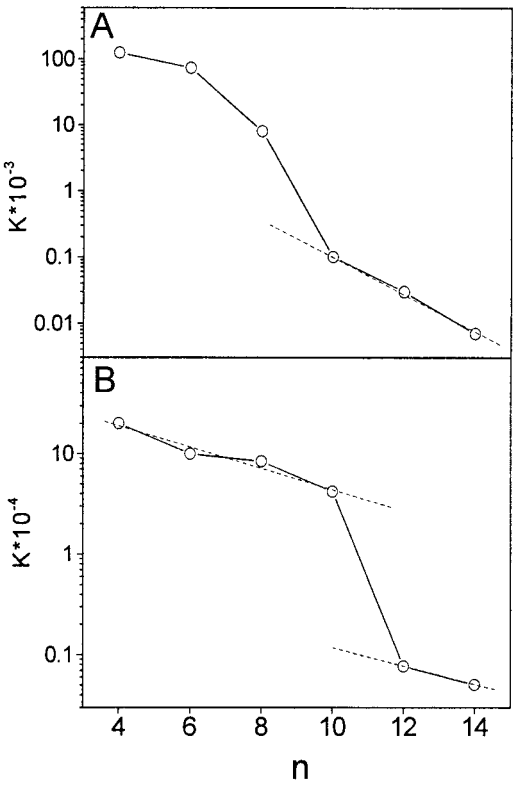

FIGURE 2 PyrPC/γ-CD association constant versus the length pyrenylacyl chain. Association constants for diPyr$_n$PC (*A*) and 16:0-Pyr$_n$PC (*B*) γ-CD complexes were obtained by fitting Eq. 1 to the data shown in Fig. 1 for diPyr$_n$PC. Original binding data for 16:0-Pyr$_n$PC were of similar quality (not shown). The *dashed lines* are drawn to guide the eye.

quencher, from the bilayer (Somerharju et al., 1987; Tanhuanpää and Somerharju, 1999).

Eq. 1 was fitted to the fluorescence titration data to obtain the association constants. As shown in Fig. 1, for diPyr$_4$PC, diPyr$_6$PC, and diPyr$_8$PC, best fits were obtained when assuming the stoichiometry of two γ-CD molecules for each PC, whereas for diPyr$_{10}$PC, diPyr$_{12}$PC, and diPyr$_{14}$PC, best fits were obtained when assuming a 3:1 stoichiometry. Thus, a change in stoichiometry occurs when the length of the pyrene-labeled chain of diPyr$_n$PC exceeds eight carbon units.

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 (data not shown). The association constant versus chain length plots (Fig. 2) support the proposition that a change in complex stoichiometry from 1:2 to 1:3 occurs when the length of the pyrene labeled chain exceeds 8 with diPyr$_n$PCs (Fig. 2 *A*) or 10 carbon units with 16:0-Pyr$_n$PCs (Fig. 2 *B*).

Temperature dependency of PyrPC/γ-CD association constant

The temperature dependency of the association constant K for the 10:0-Pyr$_{10}$PC/γ-CD complex was determined next to obtain the thermodynamic parameters ΔG, ΔH, and ΔS for the complex formation (Fig. 3). The following values were obtained: ΔG = 0.34 kJ/mol; ΔH = 0.475 kJ/mol; $T_{298} \cdot \Delta S$ = 0.13 kJ/mol (ΔS = 0.43 J/mol · K). These numbers indicate that the transfer of 10:0-Pyr$_{10}$PC form the bilayer to the γ-CD cavity is a nearly energy-neutral process.

Effect of pyrenylacyl chain length on the rate of complex formation

To study the effect of the pyrenylacyl chain length on the rate of complex formation, γ-CD was mixed with quenched donor vesicles containing a diPyr$_n$PC or a monoPyr$_n$PC species and the fluorescence intensity was then recorded versus time. The data for diPyr$_n$PC species are shown in Fig. 4 *A*. As expected, the rate of association was very fast with the species with a short pyrenylacyl chain (the rate for diPy$_4$PC could not be reliably measured) but diminished progressively when the chain length increased. Notably, the

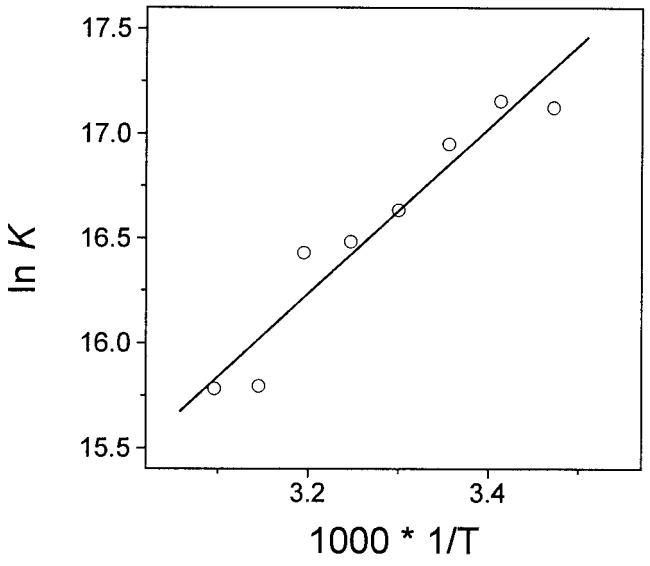

FIGURE 3 Effect of temperature on the association of 10:0-Pyr$_{10}$PC for γ-CD. The association constant was determined at the indicated temperatures as described in the legend to Fig. 1.

binding was biphasic with each species, i.e., there was a rapid phase followed by a much slower one (see below). The rate constants for these two processes are plotted in Fig. 4, *B* and *C*. The value of k_2' chain length plot shows a major deviation at $n = 8$ (Fig. 4 *C*), probably because a change in the binding stoichiometry occurs at this chain length.

The corresponding kinetic data for monoPyr$_n$PC species are shown in Fig. 5 *A*. As with the diPyr$_n$PC species, the rate of complex formation was strongly dependent on the length of the pyrenylacyl chain and the binding process was best modeled by assuming two kinetic processes. The rate constant for these processes are given in Fig. 5, *B* and *C*. Notably, the k_2' versus chain length plot shows a clear deviation when $n = 10$, again probably because the complex stoichiometry changes from 1:2 to 1:3 at this chain length.

Whereas the fast, kinetic phase in Figs. 4 and 5 obviously represents binding of the PyrPC molecules derived from the outer leaflet of the donor vesicles to γ-CD, the nature of the slower process is not clear at present. One possibility is that it represents some kind of rearrangement of the PyrPC/γ-CD complexes leading to a higher quantum yield of pyrene. Alternatively, it could represent binding of PyrPC molecules moving (flipping) from the inner to the outer leaflet of the vesicle bilayer. This latter possibility, however, seems less likely because the process is several orders of magnitudes faster than what has been reported previously for the transbilayer movement of PyrPC (Homan and Pownall, 1988).

Activation energy of complex formation

To obtain information on the rate-limiting step of binding of PyrPCs with γ-CD, the rate constant for diPyr$_{10}$PC binding

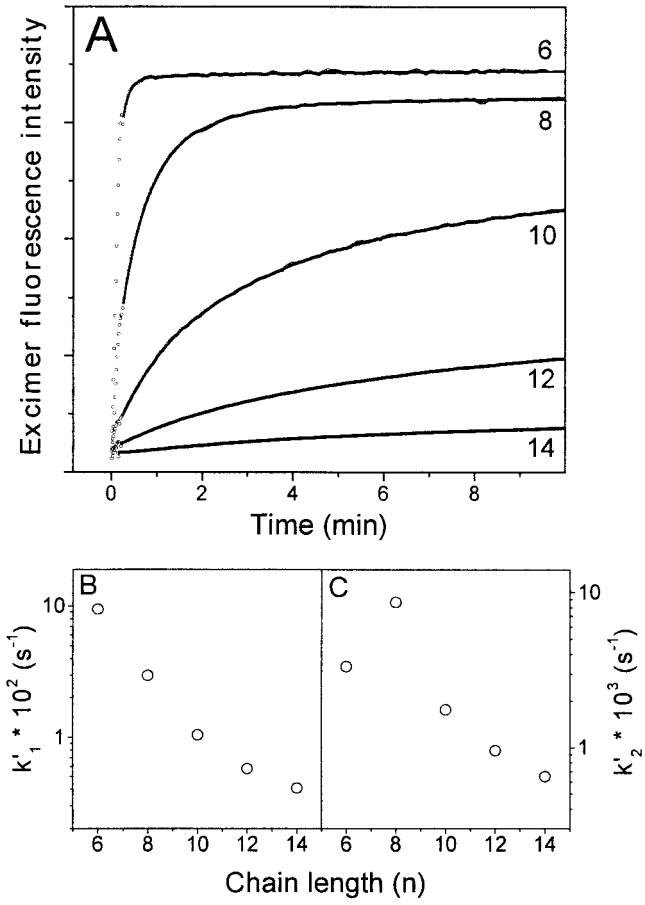

FIGURE 4 Kinetics of diPyr$_n$PC binding to γ-CD (*A*). At time 0, γ-CD (30 mM final concentration) was added to a cuvette containing quenched vesicles consisting of diPyr$_n$PC, POPC, POPA, and TNP-PE (0.2/45/1/5 nmol) and the excimer emission intensity at 475 nm was recorded with time. The binding process was biphasic and the pseudo-first order rate constants k_1' (*B*) and k_2' (*C*) were obtained by fitting Eq. 2 to the data. Early datapoints were excluded from fitting to avoid bias because of a delay in instrument response.

to γ-CD was determined at different temperatures (Fig. 6). From these data, the activation energy was calculated to be +92 kJ/mol. This value is very similar to that obtained previously for the efflux of phospholipid molecules from a bilayer to an aqueous phase (see Discussion).

Absorbance and excitation spectra for PyrPCs in γ-CD-complex versus bilayer

To study the structure of PyrPC/γ-CD complexes, we obtained the absorption spectrum for of DiPy$_4$PC, diPyr$_6$PC, and diPyr$_8$PC in the complex as well as in a bilayer. In each case pyrene absorption peaks were strongly broadened in the complex as compared with the bilayer (data not shown). Such peak broadening is a clear indication of ground state pyrene/pyrene association (Winnik, 1993). Obviously, the pyrenes linked to the two acyl chains are forced to lie close

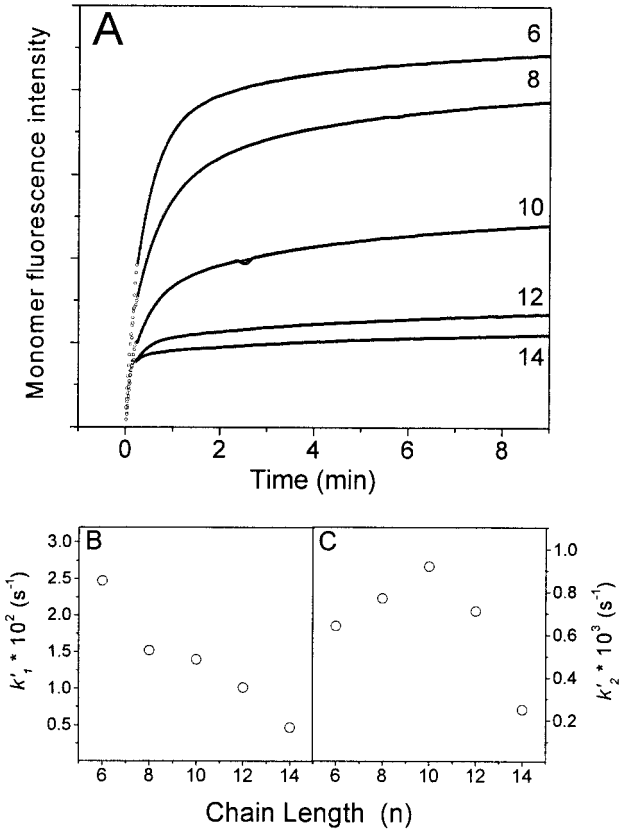

FIGURE 5 Kinetics of 16:0-Pyr$_n$PC binding to γ-CD (*A*). At time 0, γ-CD (30 mM final concentration) was added to a cuvette containing 16:0-Pyr$_n$PC, POPC, POPA, and TNP-PE (0.2/45/1/5 nmol) vesicles and the monomer fluorescence intensity at 378 nm was recorded. The numbers next to curves indicate the length of the labeled chain (*n*). The binding process was biphasic and the individual rate constants k_1 (*B*) and k_2 (*C*) were obtained by fitting Eq. 2 to the data. Early datapoints were excluded from fitting to avoid bias because of a delay in instrument response.

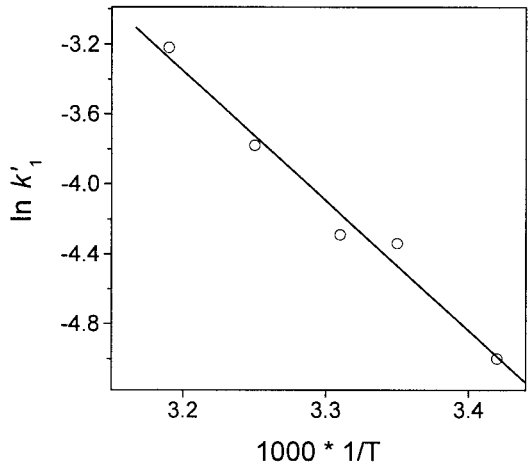

FIGURE 6 Effect of temperature on rate of diPyr$_{10}$PC binding to γ-CD. The pseudo first order rate constant (k'_1) for complex formation was determined at indicated temperatures as outlined in the legend to Fig. 4. The activation energy E_a calculated from the slope of the plot was 92 kJ/mol.

and parallel to one another in the complex. This conclusion is supported by the excimer excitation spectra obtained for diPyr$_4$PC and diPyr$_{10}$PC/γ-CD complexes: pronounced broadening (and shift) of the peaks was observed for the complex as compared with the bilayer (Fig. 7, *A* and *B*). Notably, the broadening was somewhat more extensive for diPy$_4$PC than for diPyr$_{10}$PC, suggesting stronger ground state association of the pyrenes when linked to a shorter acyl chain, which is logical considering the more restricted conformational freedom of the shorter chain. The excitation spectra for the monomer emission of diPyr$_{10}$PC or diPyr$_4$PC do not show such broadening (Fig. 7, *A* and *B*), which is to be expected because the pyrenes emitting monomer fluorescence are typically not associated with another pyrene at the time of excitation. However, the fraction of such molecules is rather small as indicated by prevalence of the excimer emission (see below).

Although it seemed likely that the two acyl chains of a diPyr$_n$PC molecule share a cavity (consisting of 2–3 γ-CD molecules, see above), the chains could, in principle, also occupy different cavities together with a chain from a different molecule, i.e., the excimer formation could be, at least in part, an intermolecular process. To study this possibility, we determined the excitation spectra for two monopyrenylacyl species, i.e., 16:0-Pyr$_4$PC and 10:0-Pyr$_{10}$PC. As shown by Fig. 7, *C* and *D*, no broadening of the excitation peaks was observed for the complexes, indicating that there is only a single pyrenylacyl chain per cavity. This finding suggests that there is no significant tendency for a pyrenylacyl chain (of a monoPyrPC) to share a cavity with another such chain. Therefore, the prominent ground-state pyrene-pyrene interaction observed for diPyr$_n$PC/γ-CD complexes (Fig. 7) most probably occurs between pyrenes of a single molecule located in the same cavity, rather than between pyrenes belonging to different diPyr$_n$PC molecules.

Interestingly, the monomer excitation peaks of 16:0-Pyr$_4$PC showed a significant red shift in the γ-CD complex, whereas hardly any shift was observed for 10:0-Pyr$_{10}$PC (Fig. 7, *C* and *D*). The reason for this difference is not clear, but it could result from chain length-dependent positioning of the pyrene moiety within the γ-CD cavity. A pyrene attached to a short acyl chain might not be able to penetrate the cavity as deeply as one attached to a longer chain and would thus be more exposed to water, i.e., would lie in a more polar environment.

Emission spectra of mono- and diPyr$_n$PCs in γ-CD complex versus bilayer

To study further the properties of PyrPC/γ-CD complexes, emission spectra were obtained for mono- and dipyrenyla-

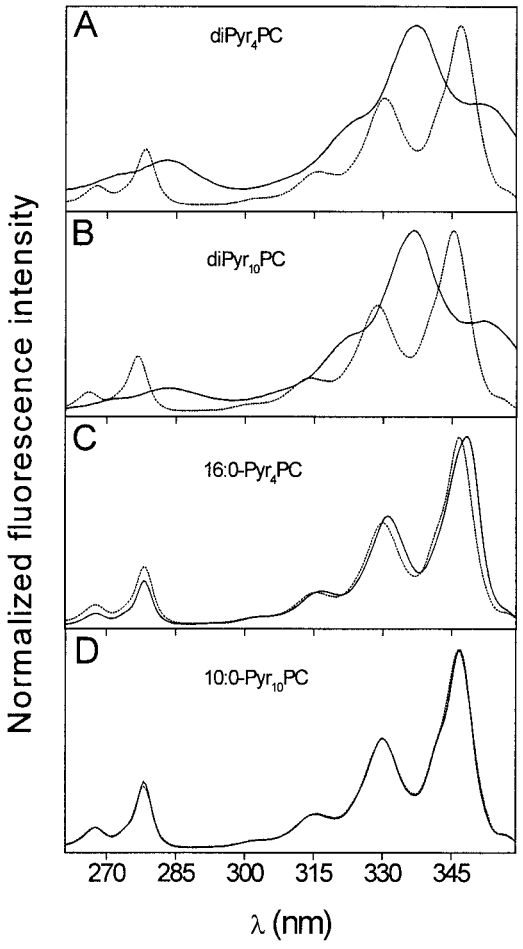

FIGURE 7 Excitation spectra for diPyr$_n$PCs and monoPyr$_n$PCs in γ-CD-complex versus bilayer. To obtain the spectrum for PyrPC/γ-CD-complex, γ-CD (10 mM final concentration) was added on quenched vesicles consisting of PyrPC, POPC, POPA, and TNP-PE (1/18/0.8/2 nmol). The bilayer spectrum was obtained with vesicles consisting of PyrPC, POPC, and POPA (0.2/19/0.8 nmol). *Continuous line*: excimer excitation spectrum for PyrPC/CD-complex. *Dashed line*: excimer excitation spectrum for PyrPC in bilayer. The monomer excitation spectra for PyrPC in the bilayer as well in the complex are not shown but coincide fully with one another as well as with the excimer spectrum in the bilayer. (*A*) diPyr$_4$PC; (*B*) diPyr$_{10}$PC; (*C*) 16:0-Pyr$_4$PC; (*D*) 10:0-Pyr$_{10}$PC.

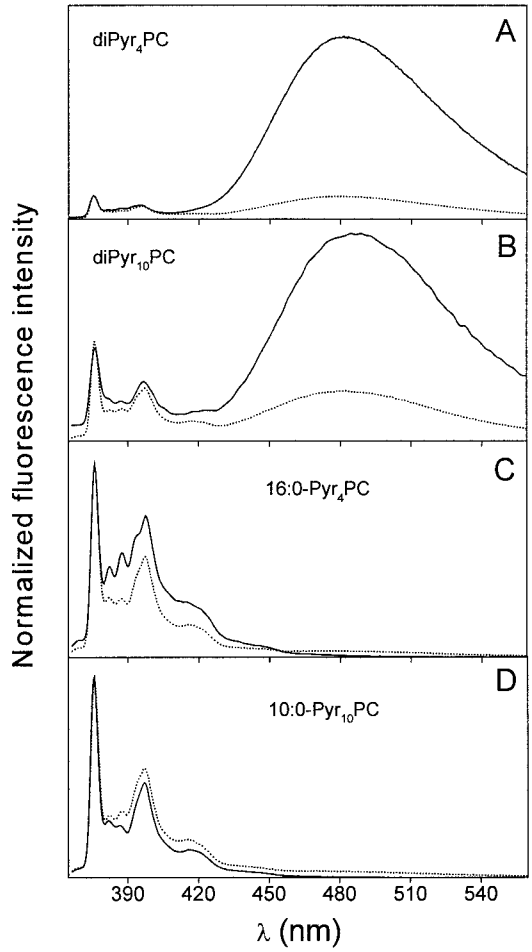

FIGURE 8 Emission spectra for diPyr$_n$PCs and monoPyr$_n$PCs in γ-CD complex versus bilayer. To obtain the spectrum for the complex, γ-CD (10 mM final concentration) was added on quenched vesicles consisting of PyrPC, POPC, POPA, and TNP-PE (1/18/0.8/2 nmol). The bilayer spectrum was obtained with vesicles consisting of a PyrPC, POPC, and POPA (0.2/19/0.8 nmol). Excitation was at 345 nm with 1-nm slit. *Continuous line*: PyrPC in complex. *Dashed line*: PyrPC in bilayer. (*A*) diPyr$_4$PC; (*B*) diPyr$_{10}$PC; (*C*) 16:0-Pyr$_4$PC; (*D*) 10:0-Pyr$_{10}$PC.

cyl lipids in γ-CD complexes and phospholipid bilayers. For diPyr$_4$PC the excimer to monomer ratio (E/M) is dramatically higher (10×) in the complex as compared with bilayer (Fig. 8 *A*), obviously because the pyrenes are forced to lie closely parallel to one another within the γ-CD cavity. A similar effect was observed for diPyr$_{10}$PC (Fig. 8 *B*). However, the E/M for the diPyr$_{10}$PC in γ-CD complex is not as high as that observed for diPyr$_4$PC. Presumably, the longer chains allow more conformational freedom for the linked pyrenes, thus allowing the two pyrenes to be separated within the complex. Previous studies have indicated that the centroids of two acyl-linked pyrenes have to coincide better than 0.3 Å for the excimer formation to take place (Eklund et al., 1992).

The emission spectrum obtained for 16:0-Pyr$_4$PC and 10:0-Pyr$_{10}$PC in the complex and the bilayer are shown in Fig. 8, *C* and *D*. In the bilayer, the monomer spectra of the species are quite similar, indicating that the pyrene is in similar environment in both cases. This is not unexpected, because a pyrene linked to an acyl chain of a phospholipid is located in the hydrocarbon region of the bilayer, independent of the length of the labeled chain (Eklund et al., 1992; Sassaroli et al., 1995). The spectra for 16:0-Pyr$_4$PC and 10:0-Pyr$_{10}$PC γ-CD complexes differ significantly from the bilayer spectra as well as from one another. With 16:0-Pyr$_4$PC, the long wave-length vibrational peaks are enhanced relative to the 378 nm peak, whereas the opposite is true for 10:0-Pyr$_{10}$PC. These findings indicate that the environment of the pyrene moiety in the complex is significantly different from that in the bilayer and, secondly, is

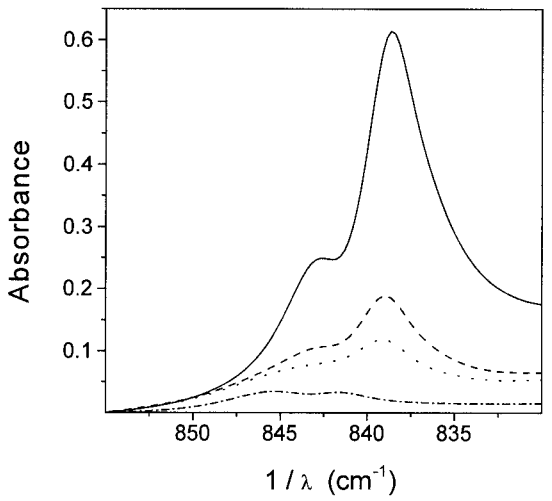

FIGURE 9 Effect of γ-CD addition on the FTIR spectrum of diPyr$_4$PC. Increasing amounts of γ-CD was mixed with neat diPyr$_4$PC (see Materials and Methods for details) and the FTIR spectrum was recorded. *Solid line*: diPyr$_4$PC only. *Dashed line*: γ-CD/diPyr$_4$PC 1:1. *Dotted line*: γ-CD/diPyr$_4$PC 3:1. *Dash-dotted line*: γ-CD/diPyr$_4$PC 10:1. The baseline has been set to zero at 855 cm^{-1} for all the spectra. The spectrum of neat γ-CD has been subtracted from the spectra of γ-CD-containing samples.

dependent on the length of the pyrene labeled chain (see Discussion). Notably, no detectable excimer fluorescence is observed for either PyrPC/γ-CD complex (Fig. 8, *C* and *D*), supporting the above notion that there is no significant tendency for two pyrene chains (of different molecules) to share a γ-CD cavity.

Time-resolved fluorescence studies

Time-resolved fluorescence measurements were carried out for diPyr$_{10}$PC in a phospholipid bilayer or in the γ-CD complex. Using a two-state excimer kinetic model (Cheng and Somerharju, 1996), k_{dm}, the association rate constant of pyrene dimer formation was determined from the decay of the monomer and excimer fluorescence. For diPyr$_{10}$PC in the bilayer or the complex, the rate constant was $0.68 \cdot 10^8$ or $3{,}4 \cdot 10^8\ s^{-1}$, respectively. Thus, the rate of excimer formation increased 5-fold upon transfer from the bilayer to γ-CD complex. This, in agreement with the steady state fluorescence data discussed above, indicates that the pyrenes are forced to lie close together in the complex.

FTIR studies

Pyrene and pyrene-labeled phospholipids display a characteristic peak at 839 cm^{-1} (Califano and Abbondanza, 1963; Lotta et al., 1988). This peak probably relates to a pyrene ring deformation mode (Califano and Abbondanza, 1963). Fig. 9 shows how mixing of γ-CD with diPyr$_4$PC affects this peak. As the γ-CD to diPyr$_4$PC molar ratio increases, the intensity of this pyrene-specific peak decreases progressively and its maximum shifts from 839 to 842 cm^{-1}. Such a strong decrease in intensity as well as the wavelength shift indicates that pyrene ring deformational vibration is markedly suppressed in the complex, probably because the pyrenes are forced to pack tightly together in the cavity of γ-CD. Thus the high-frequency shoulder of this peak, whose relative intensity increased with increasing γ-CD concentration, could perhaps derive from the pyrene dimer. In conclusion, these FTIR studies support the notion that pyrenes of diPyr$_n$PCs are closely apposed in the complex.

DISCUSSION

CDs are cyclic polymers consisting of 6 (α-CD), 7 (β-CD), 8 (γ-CD), or more glucose units. These compounds are capable of solubilizing hydrophobic molecules in aqueous media because of the hydrophobic character of their internal cavity. The hydrophobicity of the cavity results from its being lined with skeletal carbons and ethereal oxygens of the glucose residues (Szejtli, 1988). CDs have been of interest to pharmacologists because of their ability to enhance drug solubility and delivery (Szejtli, 1988; Loftsson and Brewster, 1996). However, during recent years, CDs have become popular tools among cell biologist as well. Most notably, 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). There are also some indications that α-CD could extract phospholipid molecules from cell membranes (Ohtani et al., 1989). However, formation of soluble phospholipid/CD-complexes was not unequivocally demonstrated. Recently, we found that γ-CDs can greatly enhance the transfer of fluorescent phospholipid derivatives between lipid vesicles or from vesicles to living cells (Tanhuanpää and Somerharju, 1999). The purpose of this study was to characterize this process in detail by using several different physical methods as well as systematically constructed sets of pyrene-labeled phospholipids. The key findings and their implications are discussed below.

Mechanism of complex formation

There are two principal, alternative models for the formation of a soluble PyrPC/γ-CD complex. One model assumes that the PyrPC molecule first fully effluxes from the bilayer and then associates with 2–3 γ-CD molecules in the aqueous phase, whereas the other assumes that the γ-CD molecules become sequentially complexed with lipid molecule while the latter is effluxing from the bilayer. The present data strongly support the former model. First, the activation energy of the complex formation (+92 kJ/mol) is very high and similar to that obtained previously for the efflux of a

pyrene-labeled or 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). One would expect the activation energy to be much lower for route B as the lipid acyl chain would not need to interact with water upon formation of the complex. Second, the rate of complex formation decreases exponentially with the increasing acyl chain length of PyrPC (Fig. 4). Analogous behavior has been observed for spontaneous interparticle transfer of PyrPCs (Massey et al., 1982a) 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.

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 virtually energy-neutral. The very small value of ΔG is not surprising as the PyrPC molecule is transferred from one amphipathic environment to another, i.e., from a lipid bilayer to the CD. Neither the acyl chains nor the head group are expected to experience a significant change in their environment.

The lack of an entropy effect may seem unexpected considering that the motional freedom of the PyrPC acyl chains is likely to be diminished in the complex as compared with the bilayer, as also indicated by the close proximity of the pyrenes of diPyr$_n$PCs (see Results). However, this is a complicated issue because there are many additional factors involved. First, the rotational entropy of the PyrPC molecule may increase upon complex formation because the PyrPC molecule can tumble isotropically when in the complex, but this is not possible in the anisotropic bilayer environment. Second, the removal of a PyrPC molecule from the bilayer is likely to increase the entropy of the latter because of increased conformational freedom of the matrix lipid acyl chains that were proximal to the fairly bulky and rigid pyrene moiety. Third, the cavity on the CD molecule contains water molecules that have lower entropy than the bulk water molecules (Tabushi et al., 1978). The release of these constrained/immobilized water molecules upon formation of the PyrPC/γ-CD complex is thus an entropically favorable process. Fourth, the entropy of the γ-CD molecules participating in the complexes should decrease because of diminished rotational and translational degrees of freedom as compared with uncomplexed molecules.

That ΔH of transfer of a PyrPC from the bilayer to the γ-CD complex is very small and, again, probably a result of many compensating factors. The van der Waals interactions of the phospholipid acyl chains are probably better satisfied in the bilayer because of tight packing and flexibility of the acyl chains. In the complex, these interactions must be somewhat compromised because the combined cross-sectional area of the acyl chains (excluding pyrene) is significantly smaller (~50%, according to tests with space-filling models) than that of the γ-CD cavity. Although this cross-sectional disparity is obviously somewhat reduced because of kinking of the acyl chains (see below), it is likely that the van der Waals interactions cannot equal those in the bilayer. The water molecules located inside the hydrophobic γ-CD cavity cannot fully satisfy their hydrogen-bonding potentials; therefore they are of higher enthalpy than the bulk water molecules. The release of these water molecules upon binding of the phospholipid molecule should thus be enthalpically favorable.

Characteristics of the PyrPC/γ-CD-complex

DiPyr$_n$PCs with short acyl chains form a 1:2 complex with γ-CD, although those with longer chains seem to form a 1:3 complex (Fig. 1). The shift from a 1:2 to a 1:3 complex occurs when the length of the pyrene labeled chains is ≥10 carbons. Tests with space-filling models (not shown) indicate that the labeled chains can be nearly 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. With 16:0-Pyr$_n$PC species, the shift from a 1:2 to 1:3 complex seems to occur when the length of the labeled chain (n) is ≥12 carbons, rather than 10 carbons of the diPyr$_n$PCs. The reason for this difference is not obvious. One possibility is that, because the overall cross-sectional area of monoPyrPCs (in its all-*trans* conformation) is less 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 that of diPyr$_n$PCs.

Several lines of evidence indicate that the two acyl chains of a Pyr$_n$PC molecule share the same γ-CD cavity, consisting of 2–3 γ-CD molecules. First, the absorption as well as the excimer excitation spectra obtained for DiPyr$_n$PC/γ-CD complexes are strongly broadened (Fig. 7). This is a clear sign of ground-state dimer formation (Winnik, 1993). Second, 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 with bilayer-associated lipids (Fig. 8). This indicates that the pyrenes are constrained to lie close to one another in the complex. Time-resolved studies, showing a greatly enhanced excimer formation rate, strongly support this view. Third, FTIR-studies indicate marked suppression of certain pyrene ring deformation modes in the complex of diPyr$_4$PC (Fig. 9). This is compatible with the idea that pyrenes are forced to lie close together in the complex; the pyrene ring is a quite rigid and thus close apposition of two pyrenes is expected to limit ring deformations. Fourth, no broadening of the excitation peaks or no excimer formation was observed for mono-PyrPCs (Figs. 7 and 8).

Implications on the use of CDs as (phospho)lipid carriers

One of the main goals of this study was to clarify the factor(s) that determine the rate of CD-mediated (phospho)-lipid transfer. Such information could help to develop more efficient lipid carriers. The high activation energy of complex formation, as well as the strong logarithmic dependency of the association and binding rate constants versus hydrophobicity strongly suggest that the phospholipid molecule must first efflux from the bilayer to the aqueous phase before the complex formation can take place. Thus, it is the rate of lipid efflux to the aqueous phase, which is determined by its hydrophobicity (Massey et al., 1982b, 1985; McLean and Phillips, 1984; Wimley and Thompson, 1990; Reinl and Bayerl, 1994) that probably sets the ultimate limit for CD-mediated interbilayer lipid transfer. Assuming that the efflux is indeed the rate limiting step, one can speculate that the rate of CD-mediated lipid transfer could be enhanced by using smaller donor particles. It has been shown that the rate of lipid efflux is inversely correlated with the radius of the donor particles (Massey et al., 1984). Also the addition of bilayer-perturbing molecules, such as bile salts (Nichols, 1986), hydrophobic peptides, or solvents could further enhance the rate of efflux.

Although γ-CD and its derivatives seem to enhance the transport of pyrene-labeled phospholipids more efficiently than β- or α-CDs (Tanhuanpää and Somerharju, 1999), 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 whereas the cavity of α-CD accommodates 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). However, formation of soluble phospholipid/CD complexes was not unequivocally demonstrated. Based on the present finding that hydrophobicity-dependent efflux from the bilayer is the rate limiting step in CD-mediated phospholipid transfer, it seems unlikely that even the optimal CD-derivatives would mediate efficient transfer of typical natural phospho- or glycolipid species unless donors providing very high efflux rates can be devised.

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