Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1715 (2005) 49 – 56
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

# *N*-acyl phosphatidylethanolamines affect the lateral distribution of cholesterol in membranes

Bohdana Térová[a], Gitte Petersen[b], Harald S. Hansen[b], J. Peter Slotte[a,*]

[a]*Department of Biochemistry and Pharmacy, Åbo Akademi, Turku, Finland*
[b]*Department of Pharmacology, the Danish University of Pharmaceutical Sciences, Copenhagen, Denmark*

Received 22 May 2005; received in revised form 6 July 2005; accepted 19 July 2005
Available online 28 July 2005

**Abstract**

*N*-Acyl phosphatidylethanolamines are negatively charged phospholipids, which are naturally occurring albeit at low abundance. In this study, we have examined how the amide-linked acyl chain affected the membrane behavior of the *N*-acyl-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (*N*-acyl-POPE) or *N*-acyl-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (*N*-acyl-DPPE), and how the molecules interacted with cholesterol. The gel→liquid crystalline transition temperature of sonicated *N*-acyl phosphatidylethanolamine vesicles in water correlated positively with the number of palmitic acyl chains in the molecules. Based on diphenylhexatriene steady state anisotropy measurements, the presence of 33 mol% cholesterol in the membranes removed the phase transition from *N*-oleoyl-POPE bilayers, but failed to completely remove it from *N*-palmitoyl-DPPE and *N*-palmitoyl-POPE bilayers, suggesting rather weak interaction of cholesterol with the *N*-saturated NAPEs. The rate of cholesterol desorption from mixed monolayers containing *N*-palmitoyl-DPPE and cholesterol (1:1 molar ratio) was much higher compared to cholesterol/DPPE binary monolayers, suggesting a weak cholesterol interaction with *N*-palmitoyl-DPPE also in monolayers. In bilayer membranes, both *N*-palmitoyl-POPE and *N*-palmitoyl-DPPE failed to form sterol-rich domains, and in fact appeared to displace sterol from sterol/*N*-palmitoyl-sphingomyelin domains. The present data provide new information about the effects of saturated NAPEs on the lateral distribution of cholesterol in NAPE-containing membranes. These findings may be of relevance to neural cells which accumulate NAPEs during stress and cell injury.


*Keywords:* Cholesterol; Cholesterol desorption; Cholestatrienol; Membrane domain; Quenching

## 1. Introduction

Phosphatidylethanolamines with an *N*-linked acyl chain (NAPE) are negatively charged phospholipids naturally occurring in many different species. Their abundance is not very high in most membranes of mammals, constituting less than 0.1% of total phospholipid [1]. The content of NAPEs, however, increases markedly in membranes of tissues and cells, which experience different types of stress and cell injury [for reviews, see [2,3]]. In mammals, the formation of NAPEs is catalyzed by a membrane-associated *N*-acyltransferase, which transfers the *sn*-1 acyl group of a phospholipid to the amino group of phosphatidylethanolamine [4]. In NAPEs, isolated from natural sources the *N*-acyl chain is often long (16 to 18 carbons) and saturated [5,6].

The current interest in NAPEs derives in part from the fact that NAPEs are precursors for *N*-acyl ethanolamines, which may function as endogenous ligands for cannabinoid receptor type 1 [7]. On the other hand, interest in NAPEs also comes from the findings that these lipids have been reported to be membrane-stabilizing compounds. It has for

*Abbreviations:* 7SLPC, 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; β-CyD, β-cyclodextrin; CTL, cholesta-5,7,9(11)-trien-3-beta-ol; DPH, 1,6-diphenyl-1,3,5-hexatriene; NAPE, *N*-acyl phosphatidylethanolamine; *N*-acyl-POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-acyl)ethanolamine; *N*-acyl-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho(*N*-acyl)ethanolamine; PSM, D-*erythro*-*N*-palmitoyl-sphingomyelin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; tParSM, D-*erythro*-*N*-*trans*-parinoyl-sphingomyelin

* Corresponding author. Tel.: +358405816521; fax: +35822154010.
*E-mail address:* jpslotte@abo.fi (J.P. Slotte).

0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.07.004

instance been shown that the presence of *N*-acyl egg phosphatidylethanolamine (PE) or *N*-palmitoyl dipalmitoyl PE in liposomes can reduce spontaneous or induced leakage across liposomal membranes [8]. The bilayer-stabilizing effects of NAPEs may relate to the finding that the *N*-linked acyl chain is firmly anchored within the bilayer if its length is at least 10 methylene units [9,10]. Whereas unsaturated phosphatidylethanolamines prefer non-bilayer phases under certain conditions [11], the *N*-acylation is known to convert the phosphatidylethanolamine to a lamellar phase preferring phospholipid [12,13]. NAPEs are known to interact with cholesterol in monolayer and bilayer membranes, since cholesterol has been demonstrated to exert an ordering effect on the acyl chains of NAPEs [14]. Cholesterol orders the *N*-linked acyl chain in a similar manner as is seen for the *sn*-2 acyl chain of the NAPEs [10]. The *N*-linked acyl chains in sphingomyelins are known to be crucial for stabilizing the intermolecular association between cholesterol and sphingomyelin [15,16]. In this study, we have explored the effects of the *N*-linked acyl chain in NAPEs on their membrane properties and interactions with cholesterol.

## 2. Material and methods

### 2.1. Material

D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids, Alabaster, AL, USA) by reverse-phase HPLC (Supelco Discovery C18-column, dimensions 250 × 21.2 mm, 5 ìm particle size, with 100% methanol as the mobile phase, flow 9 ml/min). The purity and identity of the product was verified on a Micromass Quattro II mass spectrometer (Manchester, UK). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, triethylamine, palmitic and oleic anhydride and dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) were from Sigma Chemicals (St. Louis, MO, USA). β-Cyclodextrin (β-CyD) was obtained from Fluka (Buchs, Switzerland) and was dried before use for 24 h at 50 °C in a vacuum drying oven. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes (Leiden, the Netherlands). All organic solvents were obtained from Merck (Darmstadt, Germany). Stock solutions of lipids were prepared in hexane/2-propanol (3/2, v/v), stored in the dark at −20 °C and warmed to ambient temperature before use. β-CyD solutions were prepared in pure water to a concentration of 40 mM. Millipore UF Plus produced water (resistivity 18.2 MΩcm) was used.

(7-Doxyl)-stearic acid was obtained from TCI (Zwijndrecht, Belgium) and was used for the synthesis of 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC), done by a slight modification of the method describe in [17]. Cholesta-5,7,9(11)-trien-3-beta-ol (cholestatrienol, CTL) was synthesized from 7-dehydrocholesterol using the method published in ref. [18]. D-*erythro*-*N*-*trans*-parinoyl-sphingomyelin (tParSM) was synthesized from *trans*-parinaric acid (Molecular Probes, Eugene, OR, USA) and D-*erythro*-sphingosylphosphorylcholine (Matreya LLC, Pleasant Gap, PA, USA) according to [19]. The fluorescent probes were purified by reverse-phase HPLC on an RP-18 column with methanol/acetonitrile (70:30, by vol) as eluent for CTL, or with 100% methanol as eluent for tParSM. All compounds were identified by mass spectrometry. Probes were stored dry under argon in the dark at −87 °C until solubilized in argon-purged ethanol (CTL) or methanol (tParSM). Stock solutions of fluorescent lipids were stored in the dark at −20 °C and used within a week.

### 2.2. Synthesis of NAPEs

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-palmitoyl)ethanolamine (*N*-palmitoyl-POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)ethanolamine (*N*-oleoyl-POPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho(*N*-palmitoyl)ethanolamine (*N*-palmitoyl-DPPE) were prepared basically as described in ref. [20]. Briefly, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine or 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine were dissolved in dichloromethane. A two-fold molar excess of either oleic or palmitic anhydride and a ten-fold excess of triethylamine were added to the solution. The mixture was stirred for 30 min at room temperature, then evaporated to dryness and dissolved in chloroform. The formed NAPEs were purified by Bond Elut LRC SI 500 mg column (Varian, Palo Alto, CA, USA) using step-wise solvent gradients consisting of chloroform and methanol (100:0, 98:2, 95:5, 90:10 and 80:20 by vol, respectively). The NAPEs were almost completely eluted after the 90:10 chloroform/methanol step. To verify the purity and identity of the compounds, mass spectrometric analysis was done. For the accurate determination of the concentration of the NAPE stock solutions, GC-MS for fatty acid analysis with internal standards was performed.

### 2.3. Cholesterol desorption kinetics

To measure the relative association of cholesterol with co-lipids in monolayer membranes, we determined cholesterol desorption rates from mixed phospholipids/cholesterol monolayers to β-CyD in the subphase. Mixed monolayers containing phospholipids and 50 mol% cholesterol were prepared at the air/water interface at 22 °C. The trough used was of zero-order type, with a reaction chamber (23.9 ml volume, 28.3 $cm^2$ area) separated by a glass bridge from a lipid reservoir. The monolayers were compressed to 20 mN/m with a KSV surface barostat. After a stable baseline had been obtained, β-CyD (in a volume not exceeding 1 ml) was injected into the stirred reaction chamber without penetrating

the monolayer. The final concentration of β-CyD in the subphase was 1.7 mM. The removal of cholesterol from the monolayer to the subphase was determined from the area decrease of the monolayer at constant surface pressure, as described by [21].

To determine whether the association between cholesterol and PSM was disturbed by *N*-palmitoyl-DPPE, cholesterol desorption rates were determined from mixed monolayers containing equimolar amounts of cholesterol and PSM with increasing amounts of *N*-palmitoyl-DPPE. The other experimental conditions were as described above.

### 2.4. Steady-state fluorescence anisotropy

To characterize packing properties of NAPE bilayer membrane in both pure and cholesterol containing vesicles, the steady-state anisotropy of DPH as a function of temperature was determined. The small vesicles were prepared from pure phospholipids, or from a mixture of 33 mol% cholesterol and 66 mol% phospholipids and 1 mol% DPH as a reporter molecule. The lipids were dried under nitrogen, resuspended in water, heated above the transition temperature of the particular lipid. The warm samples were vortexed briefly and sonicated for 2 min (20% duty cycle, power output 15 W) using a Branson W-250 probe sonifier (Branson Ultrasonics, MA, USA). The samples with the fluorescent probe were protected from light during all steps. Excitation was carried out at 355 nm and the emission was recorded at 425 nm as the temperature was scanned from 5 to 80 °C using PTI QuantaMaster 1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ, USA). The steady-state anisotropy was determined as described in [22].

### 2.5. Quenching of steady state cholestatrienol fluorescence

To follow the formation of sterol-rich domains in bilayer membranes, a CTL quenching assay was used, which is described in more detail in [23]. Briefly, vesicles with a total lipid concentration of 50 μM with 1 mol% CTL were used. Fluorescence emission intensity was measured in sample *F* (quenched) consisting of POPC/7SLPC/phospholipids/cholesterol/CTL (30:30:30:9:1, or 45:30:15:9:1 molar ratio), and compared to $F_o$ samples (nonquenched), in which 7SLPC (30 mol%) was replaced by POPC (totaling 60 or 75 mol%). The small vesicles were prepared by probe sonication as described above. The solvent water was saturated with argon before being used to minimize the risk of oxidation and the samples containing fluorescent probe were protected from light during all steps. The phospholipids used were PSM, *N*-palmitoyl-POPE and *N*-palmitoyl-DPPE and the fluorescence emission intensity of cholestatrienol was measured with excitation and emission wavelengths of 324 nm and 374 nm, respectively, as a function of temperature.

### 2.6. Quenching of steady state trans-parinoyl sphingomyelin fluorescence

To follow the formation of sphingolipid-rich domains, an analogous quenching assay was used as described for the formation of sterol-rich domains, but instead of CTL, the quenching of tParSM by 7SLPC was measured. Similarly as above, the fluorescence emission intensity was measured in sample *F* (quenched) consisting of POPC/7SLPC/phospholipid/cholesterol/PSM/tParSM (30:30:15:10:14:1, molar ratio), and compared to $F_o$ samples (nonquenched), in which 7SLPC was replaced by POPC. The small vesicles were prepared by probe sonication as described above. The phospholipids used were PSM, *N*-palmitoyl-POPE and *N*-palmitoyl-DPPE and the fluorescence emission intensity of tParSM was measured with excitation and emission wavelengths of 305 nm and 410 nm, respectively, as a function of temperature.

### 2.7. Differential scanning calorimetry

The transition temperature of pure NAPE vesicles and the miscibility of *N*-palmitoyl-DPPE with PSM were determined by differential scanning calorimetry using a Nano II high-sensitivity scanning calorimeter (Calorimeter Science Corporation, Provo, UT, USA). Samples containing pure *N*-palmitoyl-DPPE, or *N*-palmitoyl-DPPE and 50 mol% PSM were dried under nitrogen and excess solvent was removed by vacuum drying at a room temperature for at least 4 h. The small vesicles were prepared by probe sonication as described above. The final concentration of lipids in the solution was 1 mM. Two consecutive heating and cooling scans from 0 °C to 100 °C and a scan rate of 0.5 °C /min were performed.

## 3. Results

### 3.1. Steady-state anisotropy of DPH in NAPE bilayers with and without cholesterol

To obtain information about packing properties in the hydrophobic core of NAPEs bilayer membranes consisting of NAPEs in both the presence and absence of cholesterol, we measured the anisotropy of DPH as a function of temperature. In Fig. 1A, the steady-state anisotropy of DPH in pure NAPE bilayers is plotted against temperature. The gel to liquid disordered phase transition was clearly reported as a steep decrease in the anisotropy of DPH. The mid-temperature of this transition was 14 °C for *N*-oleoyl-POPE, 41 °C for *N*-palmitoyl-POPE and 71 °C for *N*-palmitoyl-DPPE. Inclusion of 33 mol% cholesterol removed the phase transition in *N*-oleoyl-POPE containing membranes, but only partially abolished the phase transition in *N*-palmitoyl-POPE and *N*-palmitoyl-DPPE bilayers (Fig. 1B). Cholesterol at a similar concentration

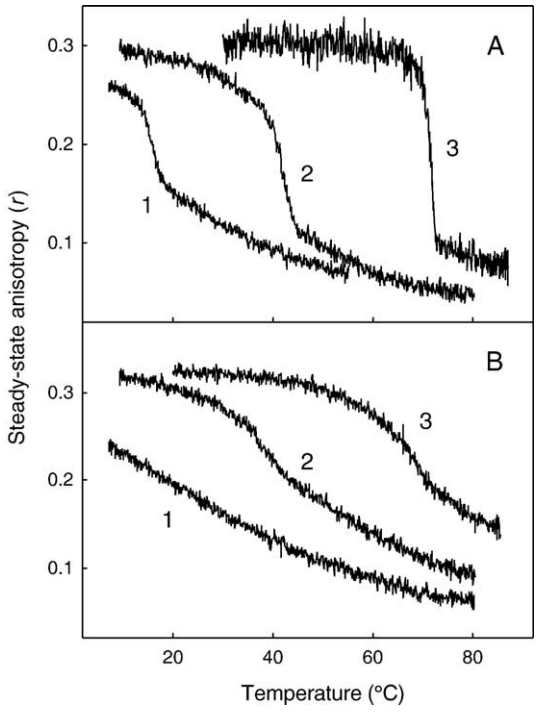


Fig. 1. Steady state anisotropy ($r_{ss}$) of DPH in NAPE or cholesterol/NAPE bilayers as a function of temperature. The steady state anisotropy of pure NAPEs bilayers containing 1 mol% DPH is shown in panel A. Panel B shows results obtained from vesicles containing 66 mol% NAPE and 33 mol% cholesterol with 1 mol% DPH. The curves shown are representative traces from at least two separate experiments and correspond to (1) *N*-oleoyl-POPE, (2) *N*-palmitoyl-POPE, (3) *N*-palmitoyl-DPPE with or without cholesterol.

also failed to abolish the phase transition in *N*-palmitoyl-DPPE bilayers as determined by DSC (data not shown). The failure of cholesterol to abolish the gel-liquid transition in *N*-palmitoyl-DPPE bilayers suggests a rather weak effect of cholesterol on the cooperative chain-melting of the saturated NAPEs, which may relate to poor interaction or miscibility of cholesterol with the fully saturated NAPE. Interestingly, when the gel to liquid disordered phase transition of pure NAPE vesicles was determined using differential scanning calorimetry, the measured $T^m$:*s* were lower by a few degrees from the $T^m$:*s* measured with DPH (11.6, 39.3 and 65 °C, for *N*-oleoyl-POPE, *N*-palmitoyl-POPE, and *N*-palmitoyl-DPPE, respectively). The fact that DPH anisotropy reported a 6-degree higher $T^m$ for *N*-palmitoyl-DPPE than the DSC method suggests that DPH had some miscibility problems in the fully saturated *N*-palmitoyl-DPPE vesicles.

### 3.2. Cholesterol desorption from monolayer membranes

One way to measure the relative association between cholesterol and a co-lipid in a monolayer membrane is to determine the rate of cholesterol desorption from the mixed monolayer to β-CyD in the subphase [16]. If the interaction between cholesterol and a co-lipid is strong or of high affinity, desorption rates will be slower than for systems where the interaction is weaker. We measured cholesterol desorption from monolayers of different compositions (50 mol% cholesterol and a co-lipid), which were kept at constant lateral pressure (20 mN/m and 22 °C), to 1.7 mM β-CyD in the subphase. As detailed in Table 1, the cholesterol desorption rate was highest from pure cholesterol monolayers, as previously shown by [16]. The interaction between PSM and cholesterol resulted in a very low desorption rate for cholesterol, as seen in many previous studies, and in part, this slow desorption depends on the *N*-linked acyl chain of sphingomyelin [17,24]. When DPPE and DPPC were compared, it was observed that cholesterol had a much weaker association with DPPE as compared with DPPC because the desorption rate was significantly higher from DPPE than from DPPC monolayers. Apparently, the difference in headgroup size and hydrogen bonding properties of these lipids may explain the difference in cholesterol desorption rate. The rate of cholesterol desorption was also higher from POPE than from DPPE mixed monolayers, as can be expected for monounsaturated two-chain phospholipid membranes [25,26]. The rate of cholesterol desorption was very high from monolayers containing the fully saturated *N*-palmitoyl-DPPE but was lower from *N*-palmitoyl-POPE monolayers. These result may imply that cholesterol was not fully miscible (giving a high desorption rate) with *N*-palmitoyl-DPPE (because of its high $T^m$), and was better soluble (giving lower desorption rate) in the partially unsaturated *N*-palmitoyl-POPE monolayer. Further, the cholesterol desorption rate was lower in *N*-palmitoyl-POPE monolayers than it was in POPE monolayers, implying some effect of the *N*-palmitoyl residue for cholesterol interaction in the membrane. These results clearly show the effects of the head group size, the hydrogen bonding properties and the acyl chain number and degree of unsaturation on cholesterol interaction.

Table 1
Cholesterol desorption from mixed monolayers to β-CyD in the subphase

| Monolayer composition | Desorption rate (pmol/cm$^{-2}$, min) | % of rate of pure cholesterol |
|---|---|---|
| *N*-palmitoyl-SM/chol 1:1 | 0.6±0.2[a] | 1.5 |
| DPPC/chol 1:1 | 1.8±0.1[a] | 4.6 |
| DPPE/chol 1:1 | 7.1±0.7 | 18.1 |
| POPE/chol 1:1 | 23.1±1.3[b] | 58.9 |
| *N*-palmitoyl-DPPE/chol 1:1 | 17.0±0.2 | 43.3 |
| *N*-palmitoyl-POPE/chol 1:1 | 9.2±1.2 | 23.4 |
| *N*-oleoyl-POPE/chol 1:1 | 10.7±0.9 | 27.3 |
| Cholesterol | 39.2±0.6 | 100 |

Mixed monolayers containing a phospholipid and 50 mol% cholesterol were prepared at the air/water interface. The monolayers were compressed to 20 mN/m and maintained at a constant surface pressure (at 22 °C). The desorption values are given as the average desorption rate±SD from at least three different monolayer experiments.

[a] Desorption data taken from [16].

[b] Desorption data taken from [17].

### 3.3. Formation of the sterol-rich domains

In model membranes, cholesterol promotes phase separation with sphingomyelins, commonly containing long saturated acyl chains, and with saturated phosphatidylcholines [27–29]. We used fluorescence quenching to examine whether cholesterol can form liquid ordered domains also with NAPEs containing two (*N*-palmitoyl-POPE) or three (*N*-palmitoyl-DPPE) saturated acyl chains. The fluorescent cholesterol analog used was cholestatrienol, which is known to behave similarly as cholesterol with respect to orientation in the membrane and condensation of phospholipid membranes [30] 7SLPC, which is located mainly outside the ordered domains [29] because of the bulky doxyl group at carbon 7 of the *sn*-2 acyl chain, was used as a quencher of cholestatrienol emission intensity. The fluorescence emission intensity was measured in sample $F$ (quenched) consisting of POPC/7SLPC/phospholipid/cholesterol/CTL, and compared to $F_0$ samples (nonquenched), in which 7SLPC was replaced by POPC. The ratio $F/F_0$ was plotted against temperature. The melting of PSM and sterol-rich domains was clearly detected as a decrease in $F/F_0$ ratio in the temperature range 10–30 °C (Fig. 2). No such domain

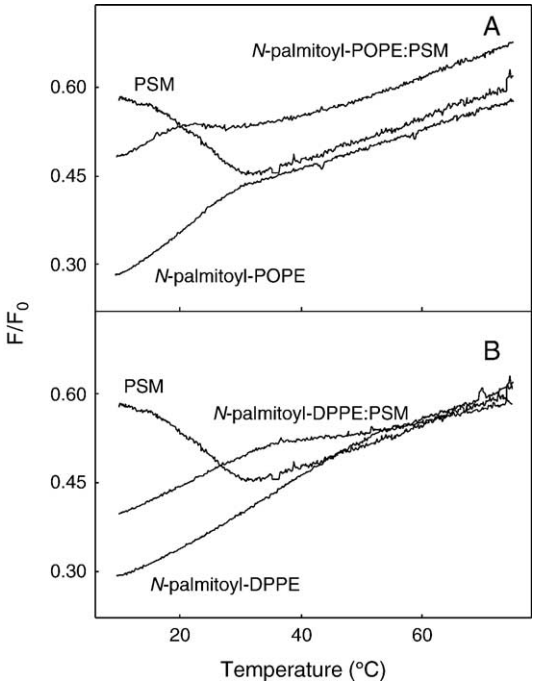


Fig. 2. Quenching of CTL emission by 7SLPC in phospholipids bilayers as a function of temperature. Sample $F$ (quenched) consisted of POPC/7SLPC/PSM/cholesterol/CTL (45:30:15:9:1 molar ratio), 7SLPC was replaced by POPC in sample $F_o$ (unquenched). In NAPE containing bilayers, the composition was POPC/7SLPC/*X*/cholesterol/CTL (30:30:30:9:1 molar ratio), where *X* was NAPE alone (30 mol%) or mixed with PSM (15 mol% each). Panel A shows the $F/F_o$ ratio plotted against temperature for bilayers containing PSM (15 mol%), *N*-palmitoyl-POPE (30 mol%) or PSM/*N*-palmitoyl-POPE (15:15 mol%), panel B for bilayers containing PSM (15 mol%), *N*-palmitoyl-DPPE (30 mol%) or PSM/*N*-palmitoyl-DPPE (15:15 mol%). The temperature was increased by 5 °C/min and the $F/F_o$ ratio was calculated.

melting can be seen in POPC/sterol bilayers [23]. Cholestatrienol did not report the melting of sterol-enriched domains in a bilayer system containing 30 mol% of *N*-palmitoyl-POPE (Fig. 2A) or *N*-palmitoyl-DPPE (Fig. 2B). When 15 mol% of *N*-palmitoyl-POPE (Fig. 2A) or *N*-palmitoyl-DPPE (Fig. 2B) were included in the bilayer membranes together with 15 mol% PSM and sterol, the PSM/sterol domain properties were clearly affected in a way suggesting that the NAPEs displaced some or most of the sterol in the PSM domains. To further verify this, we tested the quenching susceptibility of tParSM (which is miscible with PSM) by 7SLPC in analogous membrane systems. In the absence of NAPEs, PSM and the sterol formed domains which appeared to melt in the temperature range 10–30 °C, as reported by the increased quenching of tParSM (Fig. 3). This temperature interval is similar irrespective of the probe (cholestatrienol or tParSM), since both probes very likely occupy the same domain when the bilayer contains PSM and sterol in a matrix of POPC. The inclusion of 15 mol% of *N*-palmitoyl-DPPE into the bilayer system markedly stabilized PSM-enriched domains against temperature-induced melting, while the presence of 15 mol% *N*-palmitoyl-POPE did not markedly affect the apparent stability of the PSM-enriched domains.

To further verify the indication that NAPEs displace sterol from PSM-enriched domains, we measured the effects of *N*-palmitoyl-DPPE on cholesterol desorption from PSM monolayers. As seen in Fig. 4, the incremental addition of *N*-palmitoyl-DPPE to a monolayer containing an equimolar mixture of cholesterol and PSM led to an increased rate of cholesterol desorption. This clearly implies that *N*-palmitoyl-DPPE mixed with PSM and displaced cholesterol which eventually became available for rapid desorption.

### 3.4. Miscibility of N-palmitoyl-DPPE with PSM

To further verify that *N*-palmitoyl-DPPE indeed was miscible with PSM, we measured the miscibility of *N*-palmitoyl-DPPE with PSM in an equimolar bilayer mixture using DSC (Fig. 5). The miscibility of PSM with *N*-palmitoyl-DPPE appeared to be fairly good, since the thermogram revealed no melting from pure PSM (which would occur at 41 °C [31]), and since the melting temperature of *N*-palmitoyl-DPPE was decreased only by about 3.5 °C. The cooperativity of melting of the binary system was markedly decreased, as can be expected for non-ideal mixing.

## 4. Discussion

The membrane behavior of acyl-chain defined NAPEs was recently thoroughly characterized by electron spin resonance spectroscopy [10]. In that study, it was demonstrated that an *N*-linked myristoyl moiety inserted directly into the hydrophobic core of the membrane, and that the

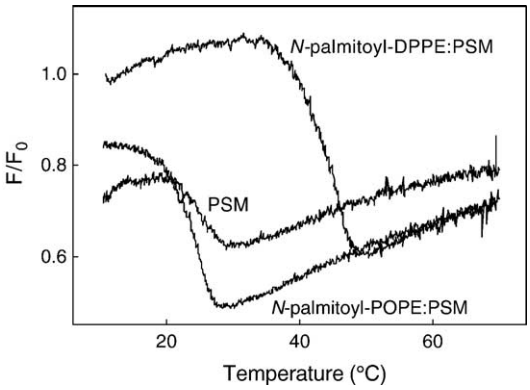


Fig. 3. Quenching of tParSM emission by 7SLPC in phospholipids bilayers as a function of temperature. Sample $F$ (quenched) consisted of POPC/7SLPC/PSM/cholesterol/tParSM (45:30:14:10:1 molar ratio), 7SLPC was replaced by POPC in sample $F_o$ (unquenched). In NAPE containing bilayers, the composition was POPC/7SLPC/$X$/cholesterol/tParSM (30:30:29:10:1 molar ratio), where $X$ was NAPE alone (29 mol%) or mixed with PSM (15 mol% NAPE and 14 mol% PSM). The $F/F_o$ is plotted against temperature, which was increased by 5 °C/min.

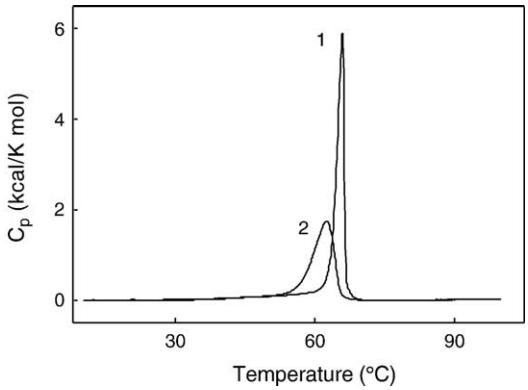


Fig. 5. Miscibility of *N*-palmitoyl-DPPE with PSM. The representative DSC heating thermograms of (1) pure *N*-palmitoyl-DPPE bilayer, or (2) *N*-palmitoyl-DPPE bilayer containing 50 mol% PSM are shown. The samples were heated at a rate of 0.5 °C/min. The measured $T^m$ for *N*-palmitoyl-DPPE was 65.9 °C and that for the 1:1 mixture of *N*-palmitoyl-DPPE and PSM was 62.4 °C.

vertical location of the *N*-linked acyl chain was comparable to the location of the *sn*-2 chain of the NAPE. It was further demonstrated that cholesterol affected the mobility and order of the *N*-linked acyl chain similarly as it affected *O*-linked acyl chains in glycerophospholipids [10]. The recent study [14] characterized the monolayer properties of *N*-myristoyl DMPE and the effects of cholesterol on molecular packing in the monolayer, showing that cholesterol indeed was able to condense the lateral packing of *N*-myristoyl DMPE. The focus of the present study was to study how the *N*-linked acyl chain of POPE and DPPE affected their membrane properties, and how the *N*-linked acyl chain affected interactions with cholesterol in model membranes.

The membrane properties of the NAPEs with different *N*-linked acyl chains were markedly different depending on the

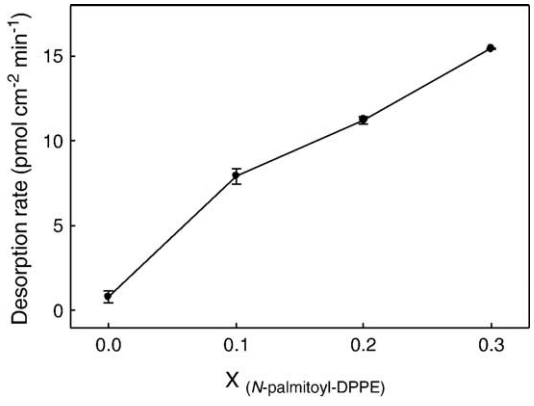


Fig. 4. Desorption of cholesterol from PSM/cholesterol/*N*-palmitoyl-DPPE monolayers to β-CyD in the subphase. The mixed monolayers were prepared at air/water interface, compressed to 20 mN/m and maintained at a constant surface pressure (at 22 °C). The ratio PSM/cholesterol in the monolayer was kept at 1:1 while *N*-palmitoyl-DPPE content increased from 0 to 30 mol%. The desorption values are given as the average desorption rate±SD from at least three different monolayer experiments and plotted against *N*-palmitoyl-DPPE molar ratio.

*N*-linked chain length and degree of unsaturation. In bilayer membranes, it was observed that *N*-palmitoyl-DPPE was the most ordered lipid since its gel-to-liquid transition temperature was highest of all the palmitoylated NAPEs. The $T^m$ was 71 °C based on changes in DPH steady-state anisotropy (Fig. 1), and 65 °C based on DSC analysis (Fig. 5). The discrepancy in reported $T^m$ is likely due to constraints in DPH miscibility in the gel-phase *N*-palmitoyl-DPPE. In another DSC study, the melting temperature of *N*-palmitoyl-DPPE was determined to 64.2 °C [32], which is in good agreement with our DSC measurements. There was a much better correlation in the measured $T^m$ values for *N*-palmitoyl-POPE (41 °C versus 39.3 °C) and *N*-oleoyl-POPE (14 °C versus 11.6 °C) with both DPH anisotropy and DSC measurements. However, the discrepancy in the reported $T^m$:*s* clearly weakens the value of DPH anisotropy measurements for accurate $T^m$ determination. Whereas cholesterol at 33 mol% was able to eliminate the transition from *N*-oleoyl-POPE, it failed to do so with the *N*-palmitoyl NAPEs. Although 33 mol% cholesterol is not enough to completely remove a gel–liquid transition from e.g. saturated phosphatidylcholines [33], the gel–liquid transition is not usually reported by DPH steady state anisotropy measurements at cholesterol concentrations above 30 mol% in at least *N*-palmitoyl ceramide phosphoethanolamine [34] or *N*-palmitoyl sphingomyelin bilayers [24]. Consequently, one can argue that a cholesterol concentration of 33 mol% is not enough to disorder the palmitoyl acyl chains of the NAPEs examined, and this may relate to packing constraints and/or miscibility problems of cholesterol in the three-chain (partly or completely saturated) phospholipid matrix.

It was of interest to study how the *N*-linked acyl chain in NAPEs affected their association with cholesterol. Cholesterol desorption from PSM monolayers is known to be very slow compared to desorption from DPPC monolayers, as seen previously [21]. Decreasing the head-group size (going from DPPC to DPPE) of the matrix phospholipid increased

desorption rates markedly, which may suggest that cholesterol was more easily squeezed out from the DPPE monolayer as the phospholipid molecules could pack closer and interact through hydrogen bonding. The bigger head group size of DPPC would prevent such close interaction and possibly allows for better solubility of cholesterol in the phospholipid matrix. In fact, the maximum solubility of cholesterol in bilayers of phosphatidylcholines and phosphatidylethanolamines are different, with phosphatidylcholine being a higher capacity solubilizer of cholesterol [35–37]. When cholesterol desorption from *N*-palmitoyl-DPPE was determined, a very high desorption rate was seen, possibly suggesting that cholesterol was not completely miscible with *N*-palmitoyl-DPPE and hence was more readily available for desorption. A much slower desorption rate was seen from *N*-palmitoyl-POPE monolayers, which may relate to a better miscibility of cholesterol in the partially unsaturated phospholipid monolayer and/or some interaction of cholesterol with the *N*-linked palmitoyl residue in combination with the monounsaturated nature of the *N*-palmitoyl-POPE. Although the phase state in monolayers containing 50 mol% cholesterol and a phospholipid was not directly measured in this study, it is clear that DPPE and *N*-palmitoyl-DPPE monolayers were the most condensed ones (based on surface pressure-molecular area isotherms). It is therefore likely that some of the differences seen in cholesterol desorption rates may be influenced by differences in phase states in the monolayer systems examined.

The formation of liquid-ordered domains in membranes is mainly due to lipid–lipid interactions, which are largely dependent on the structure and biophysical properties of the lipid components. Formation of ordered domains in mixed lipid bilayers is favored by the presence of long-chain saturated sphingolipids and by the presence of cholesterol [38–40]. Cholesterol influences the physical properties of the surrounding phospholipids in these membranes and causes phase-separation into liquid-disordered ($L_d$) cholesterol-poor and liquid-ordered ($L_o$) cholesterol-rich domains [41,42]. We measured the formation of sterol-rich domain using the cholestatrienol quenching assay, as described in more detail in [23]. Whereas cholesterol and PSM formed ordered domains which became more disordered at higher temperatures (Fig. 2), cholesterol failed to form such ordered domains with *N*-palmitoyl-POPE or *N*-palmitoyl-DPPE. This finding is in good agreement with both the DPH steady-state anisotropy results, as well as with the cholesterol desorption data, which all suggest that cholesterol does not interact well with the NAPEs examined in this study, although *N*-acylation of the natural occurring POPE seems to increase its interaction with cholesterol (Table 1). Instead, it appeared that the NAPEs showed so good miscibility in PSM-rich domains that the NAPEs displaced (at least in part) the sterol from the PSM-domains. The good miscibility of NAPEs with PSM was verified both by DSC analysis and by analysis of the thermal stability of PSM domains, as reported by the tParSM-probe. In fact, the good miscibility of *N*-palmitoyl-DPPE in PSM domains markedly stabilized these domains, as seen from the increased melting temperature (Fig. 3). It is possible that the *N*-linked acyl chain and its hydrogen-bonding properties enable the palmitoylated NAPEs to mix favorably with PSM and displace cholesterol from the PSM-rich domains. A similar displacement of cholesterol from PSM-rich domains is also induced by ceramides [23,43], which also have *N*-linked acyl chains and show good miscibility with sphingomyelins. Additionally, our monolayer desorption data (Fig. 4) clearly showed a dramatic effect of *N*-palmitoyl-DPPE on cholesterol/PSM interaction. The increased desorption of cholesterol from PSM monolayers containing increasing amounts of *N*-palmitoyl-DPPE strongly suggests that also in the monolayer system did *N*-palmitoyl-DPPE displace cholesterol from interacting with PSM.

Taken together, our present results using a variety of biophysical techniques to study the membrane properties of *N*-acylated phosphatidylethanolamines suggest that their properties are markedly influenced by the type of acyl chain linked to the head group. In comparison with PSM, the NAPEs do not interact well with cholesterol, and in fact appear to affect the lateral distribution of cholesterol in membranes containing both unsaturated phospholipid species and saturated sphingolipids. Since at least 50% of NAPEs in rat brain cells are located in plasma membranes [44], our novel finding that saturated or mono-unsaturated NAPEs may in fact displace cholesterol from raft-like structures may have dramatic implications for neural cell membrane function during stress and injury.

## Acknowledgements

This study was supported by generous grants from the Academy of Finland, the Sigrid Juselius Foundation, and the Danish Medical Research Council.

## References

[1] H.S. Hansen, B. Moesgaard, H.H. Hansen, A. Schousboe, G. Petersen, Formation of *N*-acyl-phosphatidylethanolamine and *N*-acylethanolamine (including anandamide) during glutamate-induced neurotoxicity, Lipids 34 (1999) S327–S330.

[2] H.H.O. Schmid, P.C. Schmid, V. Natarajan, *N*-acylated phospholipids and their derivatives, Prog. Lipid Res. 29 (1990) 1–43.

[3] H.S. Hansen, L. Lauritzen, B. Moesgaard, A.M. Strand, H.H. Hansen, Formation of *N*-acyl-phosphatidylethanolamines and N-acyl-ethanolamines—Proposed role in cytotoxicity, Biochem. Pharmacol. 55 (1998) 719–725.

[4] V. Natarajan, P.V. Reddy, P.C. Schmid, H.O. Schmid, On the biosynthesis and metabolism of *N*-acylethanolamine phospholipids in infarcted dog heart, Biochim. Biophys. Acta 664 (1981) 445–448.

[5] D.E. Epps, V. Natarajan, P.C. Schmid, H.H.O. Schmid, Accumulation of *N*-acylethanolamine glycerophospholipids in infarcted myocardium, Biochim. Biophys. Acta 618 (1980) 420–430.

[6] H.S. Hansen, B. Moesgaard, H.H. Hansen, G. Petersen, *N*-acylethanolamines and precursor phospholipids—Relation to cell injury, Chem. Phys. Lipids 108 (2000) 135–150.
[7] W.A. Devane, L. Hanus, A. Breuer, R.G. Pertwee, L.A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, R. Mechoulam, Isolation and structure of a brain constituent that binds to the cannabinoid receptor, Science 258 (1992) 1946–1949.
[8] J.C. Domingo, M. Mora, M. Africa de Madariaga, Incorporation of *N*-acylethanolamine phospholipids into egg phosphatidylcholine vesicles: characterization and permeability properties of the binary systems, Biochim. Biophys. Acta 1148 (1993) 308–316.
[9] D. Marsh, M. Swamy, Derivatised lipids in membranes. Physicochemical aspects of *N*-biotinyl phosphatidylethanolamines, *N*-acyl phosphatidylethanolamines and *N*-acylethanolamines, Chem. Phys. Lipids 105 (2000) 43–69.
[10] M.J. Swamy, M. Ramakrishnan, B. Angerstein, D. Marsh, Spin-labeled electron spin resonance studies on the mode of anchoring and vertical location of the *N*-linked chain in *N*-acylphosphatidylethanolamines, Biochemistry 39 (2000) 12476–12484.
[11] P.R. Cullis, B. de Kruijff, $^{31}$P NMR studies of unsonicated aqueous dispersions of neutral and acidic phospholipids. Effects of phase transitions, pH and divalent cations on the motion in the phosphate region of the polar headgroup, Biochim. Biophys. Acta 436 (1976) 523–540.
[12] J.L. Newman, D.L. Stiers, W.H. Anderson, H.H.O. Schmid, Phase behavior of synthetic *N*-acylethanolamine phospholipids, Chem. Phys. Lipids 42 (1986) 249–260.
[13] Y. Ogawa, R.M. Epand, Effect of *N*-acyl-phosphatidylethanolamine on the membrane fusion between Sendai virus and liposome, Biosci. Rep. 17 (1997) 401–408.
[14] X.-M. Li, M. Ramakrishnan, H.L. Brockman, R.E. Brown, M.J. Swamy, *N*-myristoylated phosphatidylethanolamine: interfacial behaviour and interaction with cholesterol, Langmuir 18 (2002) 231–238.
[15] R. Bittman, C.R. Kasireddy, P. Mattjus, J.P. Slotte, Interaction of cholesterol with sphingomyelin in monolayers and vesicles, Biochemistry 33 (1994) 11776–11781.
[16] H. Ohvo-Rekilä, B. Ramstedt, P. Leppimäki, J.P. Slotte, Cholesterol interactions with phospholipid in membranes, Prog. Lipid Res. 41 (2002) 66–97.
[17] B. Ramstedt, J.P. Slotte, Interaction of cholesterol with sphingomyelins and acyl-chain matched phosphatidylcholines—A comparative study of the effects of the chain length, Biophys. J. 76 (1999) 908–915.
[18] R.T. Fischer, F.A. Stephenson, A. Shafiee, F. Schroeder, Delta 5,7,9(11)Cholestatrien-3 beta-ol: a fluorescent cholesterol analogue, Chem. Phys. Lipids 36 (1984) 1–14.
[19] R. Cohen, Y. Barenholz, S. Gatt, A. Dagan, Preparation and characterization of well defined D-erythro sphingomyelins, Chem. Phys. Lipids 35 (1984) 371–384.
[20] T. Shangguan, C.C. Pak, S. Ali, A.S. Janoff, P. Meers, Cation-dependent fusogenicity of an *N*-acyl phosphatidylethanolamine, Biochim. Biophys. Acta 1368 (1998) 171–183.
[21] H. Ohvo, J.P. Slotte, Cyclodextrin-mediated removal of sterols from monolayers: effect of sterol structure and phospholipids on desorption rate, Biochemistry 35 (1996) 8018–8024.
[22] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Kluvert Academic/Plenum Publishers, New York, 1999.
[23] Y.J. Björkqvist, T.K.M. Nyholm, J.P. Slotte, B. Ramstedt, Domain formation and stability in complex lipid bilayers as reported by cholestatrienol, Biophys. J. 88 (2005) 4054–4063.
[24] M. Kuikka, B. Ramstedt, H. Ohvo-Rekilä, J. Tuuf, J.P. Slotte, Membrane properties of D-erythro-*N*-acyl sphingomyelins and their corresponding dihydro species, Biophys. J. 80 (2001) 2327–2337.
[25] L. Fugler, S. Clejan, R. Bittman, Movement of cholesterol between vesicles prepared with different phospholipids or sizes, J. Biol. Chem. 260 (1985) 4098–4102.
[26] S. Lund-Katz, H.M. Laboda, L.R. McLean, M.C. Phillips, Influence of molecular packing and phospholipid type on rates of cholesterol exchange, Biochemistry 27 (9) (1988) 3416–3423.
[27] M.B. Sankaram, E. Thompson, Interaction of cholesterol with various glycerophospholipids and sphingomyelin, Biochemistry 29 (1990) 10670–10675.
[28] J.R. Silvius, D. del Giudice, M. Lafleur, Cholesterol at different bilayer concentrations can promote or antagonize lateral segregation of phospholipids of differing acyl chain length, Biochemistry 35 (1996) 15198–15208.
[29] S.N. Ahmed, D.A. Brown, E. London, On the origin of sphingolipid/cholesterol-rich detergent soluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent insoluble, liquid ordered lipid phase in model membranes, Biochemistry 36 (1997) 10944–10953.
[30] H.A. Scheidt, P. Muller, A. Herrmann, D. Huster, The potential of fluorescent and spin-labeled steroid analogs to mimic natural cholesterol, J. Biol. Chem. 278 (2003) 45563–45569.
[31] T.K.H. Nyholm, M. Nylund, J.P. Slotte, A calorimetric study of binary mixtures of dihydrosphingomyelin and sterols, sphingomyelin, or phosphatidylcholine, Biophys. J. 84 (2003) 3138–3146.
[32] M.J. Swamy, D. Marsh, M. Ramakrishnan, Differential scanning calorimetry of chain-melting phase transitions of *N*-acylphosphatidylethanolamines, Biophys. J. 73 (1997) 2556–2564.
[33] T.P.W. McMullen, R.N. McElhaney, New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry, Biochim. Biophys. Acta 1234 (1995) 90–98.
[34] B. Térová, R. Heczko, J.P. Slotte, On the importance of the phosphocholine methyl groups for sphingomyelin/cholesterol interactions in membranes: a study with ceramide phosphoethanolamine, Biophys. J. 88 (2005) 2661–2669.
[35] J.J. Collins, M.C. Phillips, The stability and structure of cholesterol-rich codispersions of cholesterol and phosphatidylcholine, J. Lipid Res. 23 (1982) 291–298.
[36] J. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta 1417 (1999) 89–100.
[37] D. Bach, E. Wachtel, Phospholipid/cholesterol model membranes: formation of cholesterol crystallites, Biochim. Biophys. Acta 1610 (2003) 187–197.
[38] R.E. Brown, Sphingolipid organization in biomembranes: what physical studies of model membranes reveal, J. Cell Sci. 111 (1998) 1–9.
[39] A. Rietveld, K. Simons, The differential miscibility of lipids as the basis for the formation of functional membrane rafts, Biochim. Biophys. Acta 1376 (1998) 467–479.
[40] E. London, D.A. Brown, Insolubility of lipids in Triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts), Biochim. Biophys. Acta 1508 (2000) 182–195.
[41] J.H. Ipsen, G. Karlstrom, O.G. Mouritsen, H. Wennerstrom, M.J. Zuckermann, Phase equilibria in the phosphatidylcholine-cholesterol system, Biochim. Biophys. Acta 905 (1987) 162–172.
[42] M.R. Vist, J.H. Davis, Phase equilibria of cholesterol/dipalmitoyl phosphatidylcholine mixtures: 2H nuclear magnetic resonance and differential scanning calorimetry, Biochemistry 29 (1990) 451–464.
[43] Megha, E. London, Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function, J. Biol. Chem. 279 (2004) 9997–10004.
[44] H. Cadas, S. Schinelli, D. Piomelli, Membrane localization of N-acylphosphatidylethanolamine in central neurons: studies with exogenous phospholipases, J. Lipid Mediators Cell Signal. 14 (1996) 63–70.