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Acyl chain length affects ceramide action on sterol/sphingomyelin-rich domains

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Abstract

The effects of ceramides with varying saturated *N*-linked acyl chains (C2–C14) on cholesterol displacement from sphingomyelin-rich domains and on the stability of ordered domains were studied. The bilayers examined were made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM), D-*erythro*-*N*-*acyl*-sphingosine, and cholesterol (60:15:15:10 mol%, respectively). Cholestatrienol (CTL) or D-*erythro*-*N*-*trans*-parinoyl-sphingomyelin (tParSM) were used as reporter molecules (at 1 mol%) for the ordered domains, and 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) as a fluorescence quencher (30 mol%, replacing POPC) in the liquid-disordered phase. The results indicate that the ceramide had to have an *N*-linked acyl chain with at least 8 methylene units in order for it to displace cholesterol from the sphingomyelin-rich domains at the concentration used. The melting of the sphingomyelin-rich domain shifted to higher temperatures (compared to the ceramide-free control) with C2, C12 and longer chain ceramides, whereas C4–C10 ceramides led to domain melting at lower temperatures than control. This study shows that short-chain ceramides do not have the same effects on sterol- and sphingomyelin-rich domains as naturally occurring longer-chain ceramides have.

Keywords: Sterol partitioning; Cholestatrienol; Trans-parinaric acid; Quenching; 7-doxyl phosphatidylcholine

1. Introduction

Sphingomyelins are major solubilizers of cholesterol in the plasma membrane compartment in most cell types [1,2]. Sphingomyelins are well suited to interact with cholesterol, since their acyl chains are long and mostly saturated [3,4], and since they have more possibilities for hydrogen bonding as compared with phosphatidylcholines [5,6]. It has been argued that most of the cellular cholesterol is confined to plasma membranes [7,8], where a substantial fraction of it is found in sphingomyelin-rich domains [9,10]. However, conditions exist where cells appear to enrich plasma membranes with cholesterol even if their sphingomyelin-level is markedly reduced [11]. When plasma membrane sphingomyelin is enzymatically degraded, the cellular homeostasis of cholesterol is dramatically changed and cholesterol is translocated from the cell surface to intracellular membranes [12]. Ceramide is a much less effective solubilizer of cholesterol than sphingomyelin, in part because ceramide has no protecting head group to shield the exposed hydrophobic portions of the molecules [13]. The miscibility of ceramide with sphingomyelin is, on the other hand, good based on, e.g., DSC analysis of equimolar mixtures of PSM and C16-ceramide [14]. On a macroscopic scale, ceramide has also been shown to partition favorably into sphingomyelin-rich domains [5,12,15]. Very recent data from both model membrane systems [14,16], lipoproteins [17], and caveolin-rich lipid rafts [18] show that ceramide is able to displace cholesterol from sphingomyelin-rich [14,17,18] or saturated phosphatidylcholine-rich [16] domains. It was also shown that the partitioning of ceramide into the sphingomyelin-rich domain (from which cholesterol was displaced) resulted in a marked stabilization of the ceramide/sphingomyelin domain against temperature-induced melting [14].

Abbreviations: 7-SLPC, 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; CTL, Cholestatrienol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, D-*erythro*-*N*-palmitoyl-sphingomyelin; tParSM, D-*erythro*-*N*-*trans* parinoyl-sphingosylphosphorylcholine

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Since ceramides can have such dramatic effects on the sterol content of sphingomyelin-rich domains, it would be of interest to evaluate the effect of ceramide structure on the displacement process. This would be important in light of the common use of short-chain and medium-chain ceramides as tools to induce or inhibit apoptosis in cells [19,20]. It is possible that some of the effects of ceramides reported in the literature stems from their effects on cholesterol/sphingomyelin domains rather than from direct effects on target proteins [21,22]. In this study, we have used cholestatrienol (CTL) and *N-trans* parinoyl-sphingomyelin (tParSM) as fluorescent probes which partition into sterol- and sphingomyelin-rich domains, respectively, and 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) as a quencher lipid mainly located in the liquid-disordered phase. We have determined the effect of the ceramide *N*-linked chain length (C2–C14) on sterol displacement from and the temperature-dependent stability of sphingomyelin-rich domains in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles containing 10 mol% sterol.

2. Material and methods

2.1. Materials

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 μm particle size) using 100% methanol as eluent. The purity and identity of the products were verified on a Micromass Quattro II mass spectrometer (Manchester, UK). POPC was obtained from Avanti Polar Lipids. (7-Doxyl)-stearic acid was obtained from TCI (TCI Europe N.V., Belgium) and was used for the synthesis of 7SLPC [23]. D-*erythro*-*N*-*acyl*-sphingosines were obtained from Larodan Fine Chemicals (Malmö, Sweden) or synthesized from D-*erythro*-sphingosine and fatty acid [24]. All products were characterized by mass spectrometry. Stock solutions of lipids were prepared in hexane/2-propanol (3:2 by vol), stored in the dark at −20 °C, and warmed to ambient temperature before use CTL (cholesta-5,7,9(11)-trien-3-beta-ol) was synthesized and purified using the method published by Fisher and coworkers [25]. tParSM was synthesized from *trans*-parinaric acid (Molecular Probes, Eugene, OR, USA) and D-*erythro*-sphingosylphosphorylcholine (Matreya LLC, Pleasant Gap, PA, USA) according to Cohen and co-workers [24]. The fluorescent probes were purified by reverse-phase HPLC on a RP-18 column with methanol/acetonitrile (70:30, by vol) as eluent for CTL and 100% methanol for tParSM. All compounds were positively identified by mass spectrometry. CTL and tParSM 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. Fluorescence quenching method

The F samples contained quencher (7SLPC) and a complex lipid mixture as described above, while POPC replaced 7SLPC in F_o samples. The fluorescence intensity in the F samples was compared to the fluorescence intensity in F_o samples giving the fraction of quenched fluorescence. In lipid vesicles in which ordered and disordered domains co-exist, POPC together with the nitroxide labeled quencher (7SLPC) formed the disordered phase in the vesicles [26]. Fluorophores residing in the quencher-rich disordered domains give a weaker fluorescence than fluorophores in the ordered domains, which are quencher-poor. CTL is a fluorescent cholesterol analogue that has been shown to mimic the membrane behavior of cholesterol quite well [25,27–30]. In complex lipid bilayer vesicles, in which lateral domain formation is expected, CTL can be used as a fluorescent sterol analogue that associates with sterol-rich domains [14]. The amount of CTL exposed to quenching by 7SLPC gives a measure of CTL distribution between ordered and disordered membrane domains. The stability of the ordered domains containing CTL is measured by looking at the quenching susceptibility as a function of temperature. This quenching method can be used with other fluorescent reporter molecules as well. In this study, we used CTL and tParSM as fluorescent probes which partition predominantly into domains enriched in sterol and PSM, respectively.

2.3. Preparation of vesicles

Vesicles used in steady-state fluorescence measurements were prepared at a lipid concentration of 50 μM. The lipid mixtures were dried under nitrogen, dispersed in argon-purged water and heated above the gel- to liquid-crystalline phase-transition temperature. The samples were vortexed and then sonicated for 2 min (20% duty cycle, power output 15 W) with a Branson probe sonifier W-250 (Branson Ultrasonics, CT, USA). This procedure gives liposomes with a rather large size distribution with a mean diameter of 200 nm (determined using a Malvern 4700 multiangle laser spectrometer at an angle of 90°). The water used in the experiments was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system, to yield a product with a resistivity of 18.2 MΩ cm.

In fluorescence quenching studies, F samples consisted of POPC: 7SLPC: PSM: ceramide: cholesterol (30:30:15:15:10 molar ratio) and in F_o samples POPC replaced 7SLPC. The samples were studied with CTL or tParSM as the fluorescent probe, which replaced cholesterol or PSM, respectively, to give a final probe concentration of 1 mol%. The fluorescent probes were protected from light during all steps. Solvents were saturated with argon before use in order to minimize the risk of oxidation. All experiments were performed in duplicate or triplicate and curves shown are representative of reproducible experiments.

2.4. Steady-state fluorescence measurements

Fluorescence measurements were performed on a PTI QuantaMaster-1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ, USA). The excitation and emission slits were set to 1–4 nm. The temperature was controlled by a Peltier element, with a temperature probe immersed in the sample solution. The samples were heated from 8 °C to 90 °C at a rate of 5 °C/min during the measurements. The measurements were performed in quartz cuvettes and the sample solutions were kept at constant stirring (260 rpm) throughout the measurements. Fluorescence intensity of CTL was detected with excitation and emission wavelengths at 324 nm and 374 nm, respectively. Fluorescence emission of tParSM was detected at 410 nm, while excitation occurred at 305 nm.

3. Results

The basic membrane system examined in this study contained POPC, PSM and cholesterol (75:15:10). According to the phase diagram of de Almeida and coworkers [31] the only phases present in such membranes at room temperature are the liquid disordered phase (L_d) containing mostly POPC, and the liquid ordered phase (L_o) containing PSM and most of the cholesterol. The "melting" of the L_o domains with increasing temperature can be detected using DSC [32] or CTL quenching [32] (see also Fig. 1). The amount of CTL exposed to quenching by 7SLPC gives a measure of CTL distribution between ordered and disordered membrane domains. The bottom trace in Fig. 1 shows the change in CTL quenching susceptibility as the temperature of the sample is increased. The $\Delta(F/F_o)$ change is not very