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Cholesterol stimulates and ceramide inhibits Sticholysin II-induced pore formation in complex bilayer membranes

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ABSTRACT

The pore forming capacity of Sticholysin II (StnII; isolated from *Stichodactyla helianthus*) in bilayer membranes containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), palmitoylsphingomyelin (PSM) and either cholesterol or palmitoyl ceramide (PCer) has been examined. The aim of the study was to elucidate how the presence of differently ordered PSM domains affected StnII oligomerization and pore formation. Cholesterol is known to enhance pore formation by StnII, and our results confirmed this and provide kinetic information for the process. The effect of cholesterol on bilayer permeabilization kinetics was concentration-dependent. In the concentration regime used (2.5–10 nmol cholesterol in POPC:PSM 80:20 by nmol), cholesterol also increased the acyl chain order in the fluid PSM domain and thus decreased bilayer fluidity, suggesting that fluidity per se was not responsible for cholesterol's effect. Addition of PCer (2.5–10 nmol) to the POPC:PSM (80:20 by nmol) bilayers attenuated StnII-induced pore formation, again in a concentration-dependent fashion. This addition also led to the formation of a PCer-rich gel phase. Addition of cholesterol to PCer-containing membranes could partially reduce the inhibitory effect of PCer on StnII pore formation. We conclude that the physical state of PSM (as influenced by either cholesterol or PCer) affected StnII binding and pore formation under the conditions examined.

1. Introduction

Sticholysin II (StnII) is a member of the actinoporin family of pore forming toxins [1–3]. It is a single polypeptide toxin which in bilayer membranes oligomerizes to a pore structure [4]. StnII pore formation is efficient in sphingomyelin (SM) and cholesterol containing fluid membranes [5,6]. Cholesterol has been shown to enhance pore formation by both StnII and equinatoxin II (EqtII - another member of the actinoporin family of toxins) in a concentration dependent manner [5,7]. In the absence of cholesterol, StnII forms pores only in the presence of hydrogen bonding-competent SM [8]. However, when membranes are rich in cholesterol, pore formation by StnII or EqtII is also possible in bilayers lacking SM [5,9]. It is not fully understood how the bilayer lipid composition or the physical state of the membrane affect pore formation by StnII.

The effects of cholesterol on membrane properties are fairly well known. Cholesterol will abolish the gel phase (decrease acyl chain order or increase fluidity) in simple saturated phospholipid bilayers, but on the other hand it will increase acyl chain order (decrease fluidity) of already disordered phospholipid acyl chains [10,11]. At certain bilayer concentrations, which depend on the co-lipid properties, cholesterol can induce the formation of a liquid-ordered phase together with certain phospholipids [12]. Interestingly, cholesterol has been shown to associate with SM in bilayers [13], and this interaction is in part stabilized by hydrogen-bonding among SMs and between SM and cholesterol [14,15]. It has been speculated that domain formation (ordered cholesterol-enriched domains in an otherwise disordered bilayer) could help StnII or EqtII to form pores, even if the membrane affinity of the toxin is not very high (i.e., in the absence of SM) [5,9].

If membrane SM is partly degraded by a sphingomyelinase enzyme, the resulting ceramide is likely to interact with the remaining SM and form a gel-like ordered domain [16,17]. Cholesterol has been shown to be displaced from such domains [17–19]. It is unclear how StnII activity (i.e., pore formation) is affected in bilayers containing such ceramide-enriched domains. Therefore we have in this study performed detailed

Abbreviations: EqtII, equinatoxin II; LUV, large unilamellar vesicle; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PCer, palmitoyl ceramide; PSM, palmitoyl SM; SM, sphingomyelin; SPR, surface plasmon resonance; StnII, sticholysin II; tPA-SM, *trans* parinaroyl sphingomyelin

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studies on the effects of both cholesterol and ceramide, separately and together, on StnII-pore formation in SM containing POPC bilayers. We show that whereas the bilayer presence of cholesterol stimulated StnII pore formation, palmitoylceramide inhibited the process.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), palmitoyl ceramide (PCer) and egg SM were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Palmitoyl SM (PSM) was purified from egg SM using preparative HPLC on a reverse phase (C18) column, as described previously [20]. Cholesterol, calcein and Sephacryl S200HR were obtained from Sigma/Aldrich (St. Louis, MO, USA). *trans*-Parinaric acid was synthesized as described in [21]. *trans*-Parinaroyl-SM (tPa-SM) was synthesized from *trans*-parinaric acid and sphingosylphosphorylcholine as described previously [18,22]. StnII was produced in an *E.coli* expression system, and purified, as described previously [23].

2.2. Calcein leakage assay

Calcein-entrapped large unilamellar vesicles (LUVs) were prepared from POPC and PSM (4:1 molar ratio) and contained indicated amounts of cholesterol or PCer. The LUVs were prepared by extrusion through 200 nm filters (Nucleopore, Whatman) at 60 °C. Briefly, the desired lipids were mixed and dried under a stream of nitrogen. The lipids were re-dissolved in chloroform and dried again before removal of any traces of remaining solvent in vacuum for 60 min. Prior to extrusion, the dry lipid films were hydrated for 30 min at 60 °C in Tris buffer (10 mM Tris, 140 mM NaCl, pH 7.4) containing calcein. The calcein concentration was 100 mM, and the total lipid concentration was 1.25 mM. LUVs were separated from non-entrapped calcein by gel filtration on Sephacryl S200HR. The LUVs were used for permeabilization studies within 24 h. The concentration of LUV phospholipids and StnII during calcein leakage experiments were about 70 μM and 80 nM, respectively. Phospholipid concentration was determined from Pi measurement [24] after dilution of vesicles during isolation, and protein concentration was determined from the absorbance at 280 nm (with knowledge of the extinction coefficient of StnII at 280 nm as given in [5]). Emission at 550 nm was followed at 23 °C as a function of time (Excitation at 480 nm). Fluorescence emission was measured with a PTI Quanta-Master spectrofluorimeter (Photon Technology International, Inc. NJ, USA). To ensure that no major spontaneous leakage occurred, the emission was measured for each sample during 5 min before addition of toxin. A steady signal level, indicating intact vesicles, was observed for all samples.

2.3. Surface plasmon resonance spectroscopy

The association of StnII with vesicle-coated gold chips was examined as follows: LUVs were prepared from POPC:PSM (4:1, molar ratio), and the indicated amounts of either cholesterol or PCer, in Tris buffer (10 mM Tris, 140 mM NaCl, pH 7.4) by extrusion through 100 nm polycarbonate filters (Nucleopore, Whatman) at 60 °C. StnII binding to the vesicles was studied at 23 °C with a BioNavis SPR Navi 200 instrument (BioNavis Ltd, Tampere, Finland). The sensor gold chip was coated with a carboxymethylated dextran layer which was treated with N-hydroxysuccinimide and N-ethyl-N′(dimethylaminopropyl) carbodiimide to activate the surface for capturing phospholipid membranes. All solutions used for SPR were filtered through 0.2 μm membrane filters and degassed by bath sonication before use. The running buffer was 10 mM Tris, 140 mM NaCl, pH 7.4 and the flow rate was 10 μl/min. First, the chip surface was cleaned (or regenerated after being coated with lipid) with two injections of 50 mM regeneration solution (NaOH:isopropanol, 2:3 by vol). Then extruded LUVs (0.5 mM lipid concentration) were applied on the surface (10 min injection) and unbound vesicles were removed by one (2 min) injection of 50 mM NaOH. Bovine serum albumin (0.1 mg/ml, 5 min injection) was used to verify that the chip did not have uncovered areas. The very limited binding of albumin to uncoated parts of the chips (data not shown) did not vary with liposome type, suggesting that coverage was similar within experimental error for all liposome types used in this study. Finally StnII (4 μM) was applied for 10 min. The chip was regenerated between different vesicle compositions with 50 mM regeneration solution (NaOH:isopropanol, 2:3 by vol).

2.4. Steady-state anisotropy measurements

Multilamellar vesicles were prepared by bath sonication, as described previously [25]. The final lipid concentration was 50 μM and tPa-SM was included at 1 mol%. The steady-state anisotropy measurements were performed with a T-format Quanta-Master spectrofluorimeter (Photon Technology International, Birmingham, NJ, USA) between 10 and 57 °C. The probe was excited at 305 nm and emission was detected at 410 nm. Steady state anisotropy values were calculated according to Lakowicz [26].

2.5. Binding of StnII to bilayer membranes measured with isothermal titration calorimetry

The interaction between StnII and LUVs prepared from POPC and SM (4:1 molar ratio, 100 nm diameter) with cholesterol or PCer was measured using a VP-ITC (MicroCal, Northampton, MA, USA), as described previously [27]. Briefly, protein solutions at 10 μM were titrated by injection of 20 μL aliquots of lipid suspensions (phospholipid concentration: 5 mM). Two separate injections for each compositon was performed. Binding isotherms were adjusted to a model were the protein binds the membrane involving *n* lipid molecules. ΔG and ΔS were calculated from the following relationship:

$$\Delta G = -RT\ln(K/0.8n) \quad \text{and} \quad \Delta G = \Delta H - T\Delta S$$

([27]).

3. Results

3.1. Cholesterol enhances StnII pore formation kinetics

The pore-forming capability of actinoporins is often detected from their membrane permeabilization capacity [28]. Using POPC and PSM as the main bilayer components, at a 4:1 molar ratio, we measured how addition of cholesterol to the unilamellar bilayers affected the kinetics of StnII-induced calcein release from the vesicle-entrapped aqueous compartment. The ratio of vesicles to StnII was adjusted so that the POPC:PSM (80:20 nmol) system gave "intermediate" kinetics for calcein release (Fig. 1A). Addition of cholesterol to the bilayers (up to 10 nmol) increased the kinetics of calcein release in a concentration-dependent manner (Fig. 1B shows the concentration-dependence at time 200 s after StnII addition from several experiments). Triton X-100 addition to the LUVs at the end of the experiment (at time ~1300 sec) resulted in signals equivalent to the normalized value of 1 (one Triton X-100 addition is shown for curve 1). This suggest that the LUVs were not completely unilamellar. Since we did not measure calcein release to the end-point, and because of some apparent multilamellarity, comparison of the maximal extent of calcein-release as a function of cholesterol was not made. It is also evident from Fig. 1A that the calcein signal (after the plateau was reached) decreased slightly, most probably due to light-induced quenching.

Measuring StnII binding to bilayers on a solid support (using surface plasmon resonance), we observed that addition of increasing amounts of cholesterol to the POPC:PSM (80:20 nmol) bilayer led to increased

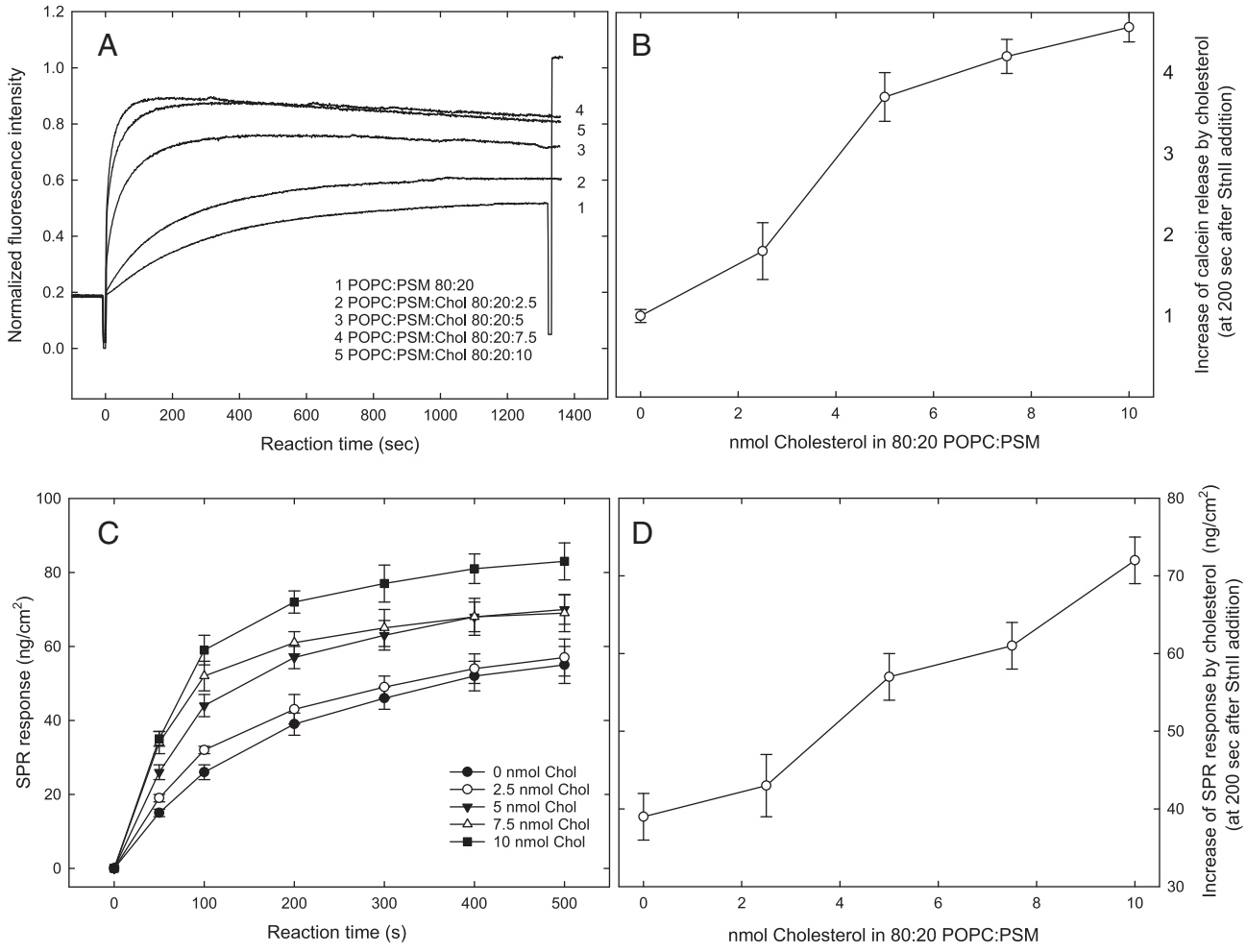

Fig. 1. Effect of cholesterol on StnII interaction with bilayer membranes. Calcein-entrapped vesicles were prepared with extrusion, and contained POPC and PSM (80:20 nmol) and increasing amounts of cholesterol (0 to 10 nmol). Calcein release was measured at 23 °C (panel A). All intensities were normalized. Towards the end of the measurement, Triton X-100 was added to dissolve the LUVs and release all calcein. In panel B, the cholesterol-induced calcein increase (fold increase relative to 0 cholesterol) is shown for the situation 200 s after StnII addition (average ± SEM). Panel C gives the SPR sensogram showing the binding of StnII to immobilized vesicles of different lipid composition. The vesicles consisted of POPC and PSM (80:20 nmol) and the indicated amounts of cholesterol (0 to 10 nmol). Each value is the average ± SEM (n = 3) from measurements at 23 °C. Panel D shows the cholesterol concentration dependence of StnII binding at 200 s after StnII addition.

SPR response (Fig. 1C). The concentration dependence of the SPR signal at 200 s is also shown (Fig. 1D). Taken together, both calcein release kinetics and the SPR response suggest that cholesterol addition to the POPC:PSM (80:20 nmol) system facilitated the StnII/bilayer interaction and the subsequent formation of StnII pores.

ITC analysis of StnII binding to POPC:PSM (80:20 by mol) bilayers is shown in Fig. 2 and Table 1. The binding constant of StnII for POPC:PSM bilayers was $2.1 \pm 0.3 \times 10^{-6}$ M^{-1}, whereas inclusion of cholesterol (POPC:PSM:Chol 80:20:10 by mol) increased the K to $6.1 \pm 0.1 \times 10^{-6}$ M^{-1}. The binding of StnII to cholesterol-containing bilayers was thermodynamically slightly more favorable (ΔG −6.8 ± 0.2 kcal/mol) compared to the cholesterol-free bilayers (ΔG −6.4 ± 0.8 kcal/mol).

3.2. Palmitoylceramide attenuates the formation of StnII pores in POPC:PSM 4:1 bilayers

To measure the effects of PCer on StnII pore formation kinetics, we measured both calcein release-kinetics and SPR response as a function of added PCer to POPC:PSM LUVs. As shown in Fig. 3, addition of up to 10 nmol PCer to POPC:PSM (80:20 nmol) bilayers led to decreased calcein release (Fig. 3A), again in a concentration-dependent manner (Fig. 3B, shows concentration-dependence at 200 s after addition of StnII from several experiments). The SPR response was similarly attenuated when the bilayers contained increasing amounts of PCer (Fig. 3C). In Fig. 3D, the concentration-dependence of the SPR signal to PCer addition is shown at time 200 s post StnII addition.

Binding of StnII to POPC:PSM bilayers containing PCer (POPC:PSM:PCer 80:20:10 by mol) was reduced, as determined by ITC analysis (Fig. 2). The K-value was 0.4 ± 0.1 x10^{-6} M^{-1}, down from the control value of $2.1 \pm 0.3 \times 10^{-1}$ M^{-1} (Table 1). Binding of StnII to PCer-containing membranes was again accompanied by a slightly less favorable free energy (ΔG 5.2 ± 0.2 kcal/mol), when compared to control bilayers (ΔG −6.4 ± 0.8 kcal/mol; Table 1).

3.3. How does cholesterol or PCer addition to POPC:PSM bilayers affect bilayer properties?

Both cholesterol and PCer are able to interact with PSM in the bilayers. With cholesterol, PSM becomes more liquid-ordered [20], and with PCer a gel phase is likely to arise [18,29]. To test for these possibilities, the steady-state anisotropy of tPa-SM was measured. This SM probe partitions into the PSM-rich domains [18], since it can hydrogen-bond to PSM as well as native SM, and since the *all trans* parinaroyl chain is extended and also prefers ordered domains [30,31]. As shown in Fig. 4, the POPC:PSM 80:20 (nmol) bilayers did not show domain

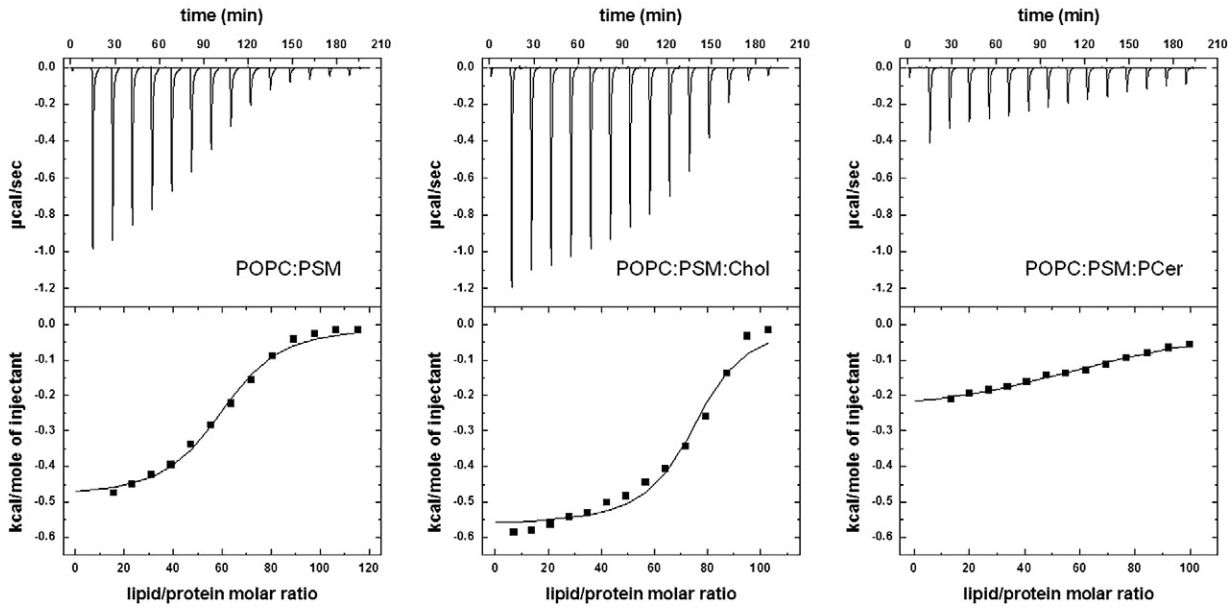

Fig. 2. Interaction of StnII with lipid vesicles as determined with isothermal titration calorimetry. To a StnII solution at 10 μM was added a 20 μL aliquot of vesicles of indicated composition (by molar ratio: 80:20 POPC:PSM or 80:20:10 POPC:PSM:cholesterol or PCer, or PSM alone) about every 15 min, until binding enthalpy approached zero (for a total of 14 injections). Binding isotherms were adjusted to a model were the protein binds the membrane involving *n* lipid molecules.

melting at temperatures above 20 °C, suggesting that PSM in the POPC matrix was disordered by the POPC and was not present as a gel phase. This interpretation is fully consistent with phase diagrams of POPC and PSM at ambient temperature [32]. Addition of cholesterol to the POPC:PSM bilayer (POPC:PSM:Chol 80:20:10 nmol) increased the tPa-SM anisotropy value slightly, suggesting an ordering effect of cholesterol on the acyl chains of PSM (and tPa-SM; Fig. 4). However, when PCer was added to the POPC:PSM fluid bilayer (POPC:PSM:PCer 80:20:10 nmol), a highly ordered (gel) phase was clearly seen at temperatures below 35 °C (Fig. 4). At temperatures above this, the apparent gel phase melted (complete melting around 42–43 °C), but even in the melted state the acyl chains of PSM and tPa-SM were more ordered by the presence of PCer.

3.4. Can cholesterol overcome the inhibition caused by PCer?

To test the pore-forming properties of StnII in POPC:PSM (80:20 nmol) bilayers which simultaneously contain both PCer and cholesterol, we measured calcein-release kinetics from different bilayer systems (Fig. 5). Addition of 20 nmol PCer to POPC:PSM (80:20 nmol) bilayers decreased the calcein release signal by about 90% (Fig. 5). Addition of 2.5 nmol cholesterol to this bilayer increased StnII-induced calcein release slightly, and at 5 nmol cholesterol (in POPC:PSM:PCer 80:20:20 nmol) increased calcein-release significantly despite the presence of PCer (Fig. 5). This increase was not due to surface dilution of PCer (bars 3 vs 2), but to the presence of cholesterol. However, PCer still inhibited calcein release by StnII, since the addition of 5 nmol cholesterol to POPC:PSM (without PCer) enhanced calcein release significantly (Fig. 1A and Fig. 5). These results show that cholesterol and PCer modulate the StnII pore formation process, and that they appear to compete with each other reversibly.

4. Discussion

Although SM is not obligate for StnII pore formation in bilayers [5], it greatly facilitates bilayer binding of StnII and pore formation [27]. It is likely that the hydrogen-bonding groups of SM (2-NH and 3-OH) stabilize the interaction between the SM head group and the POC binding site of StnII [8] and EqtII [7]. If this interaction is crucial also for pore formation, alterations of the SM head group orientation or dynamics may have consequences for StnII pore formation.

Cholesterol is known to affect membrane fluidity via its effects on phospholipid acyl chain order [20]. With our bilayer model (POPC:PSM 4:1) cholesterol will increase mostly PSM acyl chain order (Fig. 4), but will likely also influence POPC acyl chain order to some extent. Cholesterol will also interfere with SM-SM interlipid hydrogen bonding [33], which is likely to slightly affect SM properties in the SM-rich domains [34,35]. Phospholipid head group mobility and tilt is known to be different in gel and fluid states [36]. Further, cholesterol appears to affect the SM phosphocholine head group orientation and dynamics similarly as seen with glycerophospholipids [33,37,38]. In this study, cholesterol at about 10 mol% increased pore formation kinetics significantly (Fig. 1), while the tPa-SM order parameter increased only moderately (Fig. 4). Since the phosphocholine head group is an important recognition site for StnII, we find it likely that that cholesterol's effect on SM head group tilt and dynamics affected StnII binding and/or oligomerization more than cholesterol's effect on bilayer fluidity. In the POPC:PSM:Chol 80:20:10 (by mol) system, a gel phase-restricted mobility of SM is not a likely explanation of cholesterol's effect on

Table 1
Interaction of StnII with LUVs prepared to the indicated compositions. To a buffer solution containing 10 μM of StnII, 20 μl injections of a 5 mM solution of LUVs were performed (see Fig. 2). Values are average ± deviation from two separate measurements.

Vesicles	n	$K \times 10^{-6}$ (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
POPC:PSM	54 ± 5	2.1 ± 0.3	−6.4 ± 0.8	−29.0 ± 0.2	−76 ± 1
POPC:PSM:Chol	75 ± 2	6.1 ± 0.1	−6.8 ± 0.2	−40.5 ± 0.8	−113 ± 3
POPC:PSM:PCer	66 ± 8	0.4 ± 0.1	−5.2 ± 0.2	−16.2 ± 2.6	−36 ± 8

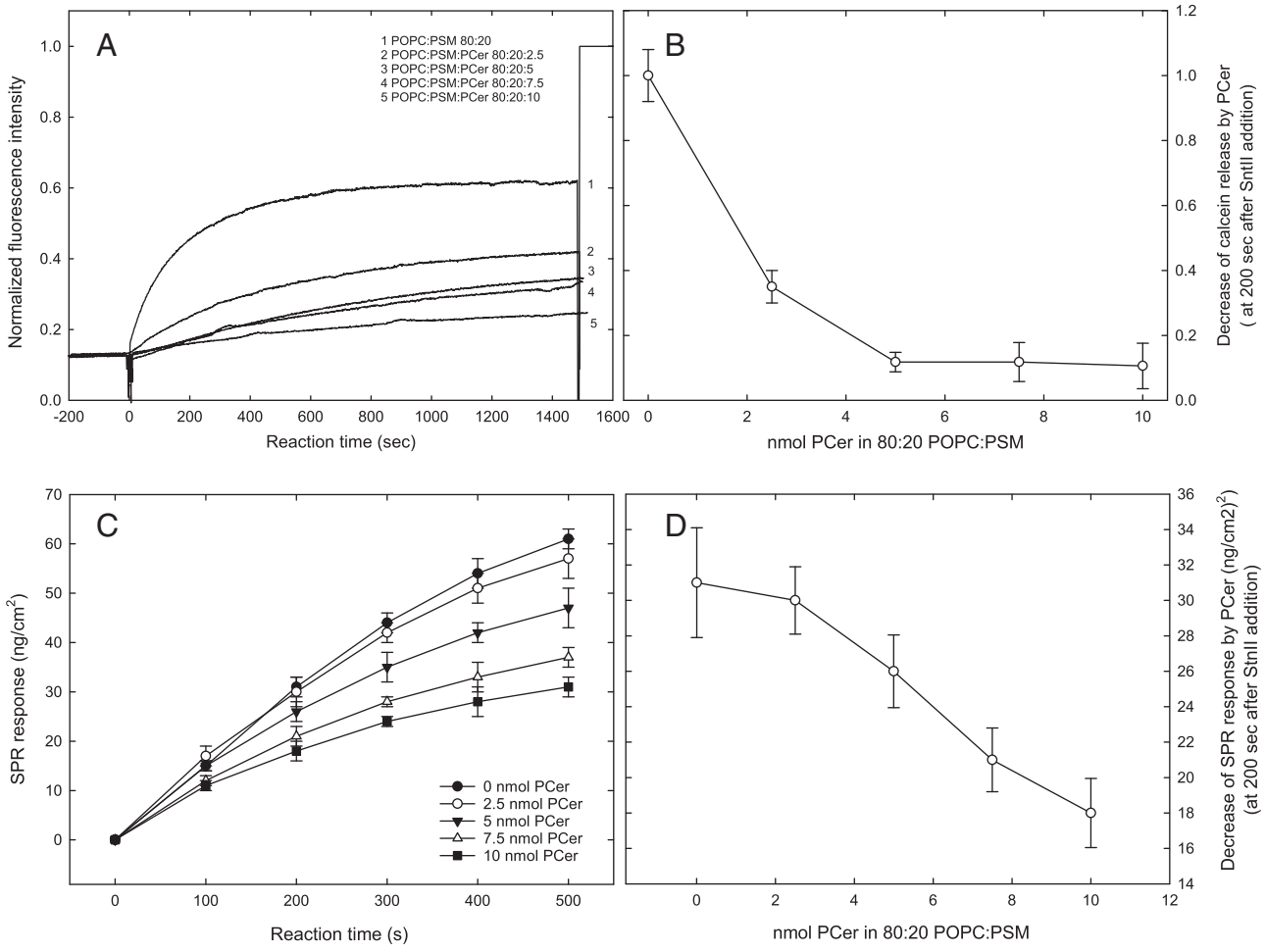

Fig. 3. Effect of PCer on StnII interaction with bilayer membranes. Calcein-entrapped vesicles were prepared with extrusion, and contained POPC and PSM (80:20 nmol) and increasing amounts of PCer (0 to 10 nmol). Calcein release was measured at 23 °C (panel A). All intensities were normalized. Towards the end of the measurement, Triton X-100 was added to dissolve the LUVs and release all calcein. In panel B, the effect of PCer addition on calcein release is given, relative to the PCer-free system, for the situation 200 s after StnII addition. Panel C gives the SPR sensogram showing the binding of StnII to immobilized vesicles of different lipid composition. The vesicles consisted of POPC and PSM (80:20 nmol) and the indicated amounts of PCer (0 to 10 nmol). Each value is the average + SEM (n = 3) from measurements at 23 °C. Panel D shows the PCer concentration dependence of StnII binding at 200 s after StnII addition.

StnII pore formation (Fig. 4). ITC data further suggest that StnII binding and pore formation led to an ordering effect in the bilayer (large negative ΔS, Table 1), possibly reflecting in part ordering of the SM head group by StnII binding. Our data and molecular interpretation is compatible with the suggestion that SM-binding toxins (e.g., EqtII) preferentially bind to molecules, carrying a phosphocholine head group, at

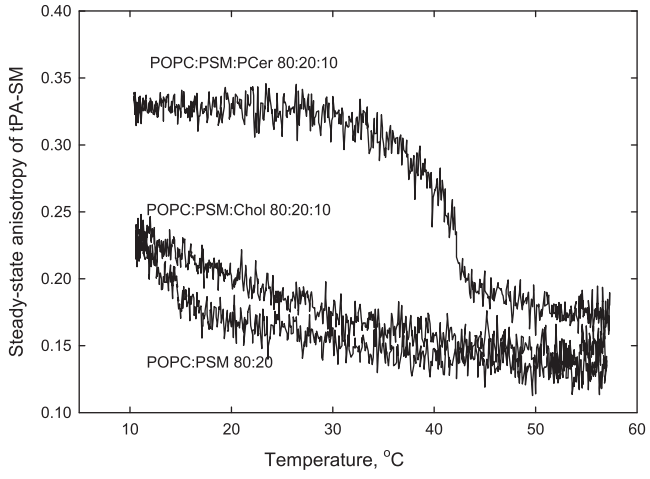

Fig. 4. Determination of steady-state anisotropy of tPa-SM in multilamellar vesicles. Vesicles were prepared to contain POPC and PSM (80 and 20 nmol) and either 10 nmol of cholesterol or PCer. The temperature gradient was 5 °C/min, and each curve is representative of at least three separate measurements.

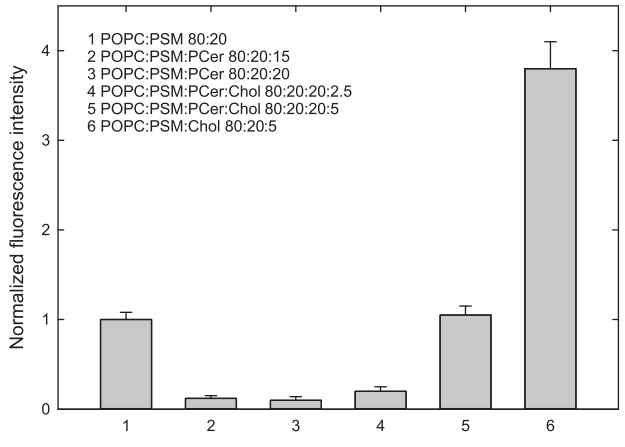

Fig. 5. Effect of the simultaneous presence of cholesterol and PCer (at different ratios) on StnII pore formation in POPC:PSM bilayers. Calcein release was determined from LUVs containing POPC:PSM at 80 and 20 nmol, to which indicated amounts of cholesterol and/or PCer were added. Calcein release at 200 s after StnII addition is shown, normalized to the POPC:PSM system. Values are averages ± SEM for two or three separate experiments for each composition.

the disordered/ordered domain interfaces [39]. Toxin (EqtII) oligomerization to pores apparently occurs in the liquid disordered phase [40].

Addition of PCer to SM-containing bilayers is known to lead to the formation of SM and PCer-rich domains [29,41], mainly because of their mutual affinity for each other and because of entropically favored mixing. In this study, PCer addition to POPC:PSM bilayers markedly inhibited StnII bilayer binding (Fig. 2) and pore formation (Fig. 3). PCer prefers to interact with PSM over POPC [42–44], and the resulting interaction leads to the formation of a highly ordered PCer- and PSM-rich domain [45,46], as also demonstrated by the high tPA-SM anisotropy at ambient temperature (Fig. 4). The shift of PSM from a fluid to a gel-like state (by PCer) will affect SM phosphocholine head group orientation and dynamics [47], in addition to increasing acyl chain order and lateral packing. The gel-phase formation most likely also sequesters SM (together with PCer [29]) and in that way may affect SM availability to StnII for binding. Our results do not allow us to distinguish the mechanism for how PCer addition to SM-domains hinder StnII-pore formation so markedly.

Both cholesterol and ceramide compete for interacting with PSM under certain conditions [18,19]. Usually cholesterol gets displaced from PSM domains by PCer, probably because PCer has higher affinity for PSM compared to cholesterol [18], but the ratios of cholesterol to ceramide, or cholesterol/ceramide to PSM also affect the final outcome of interaction [48]. In our present study, cholesterol was able to (partially) reverse the inhibitory effect of PCer on StnII pore formation, as determined from calcein release kinetics (Fig. 5). While cholesterol at the concentration used (about 4 mol%) was not likely to affect the over-all gel nature of the PSM/PCer domains (16 mol% each), it is possible that cholesterol affected properties of PSM in the POPC-rich phase, or of PSM at the gel phase boundary in a way that facilitated StnII/SM interaction and pore formation.

Considering the biological implications of our results, we may first speculate that since StnII is most likely targeted to SM-rich membranes, the protein was apparently evolutionarily optimized to function best in cholesterol-containing membranes, since cholesterol and SM coexist in membranes [49,50]. If that is the case, is it not surprising that pore formation is attenuated in SM-containing membranes in the absence of cholesterol. Secondly, we may conclude that the inhibitory effects of SM degradation (by sphingomyelinase) on red blood cell hemolysis by StnII [6] is probably not caused by SM depletion from membranes per se, but rather from ceramide generation. We conclude that StnII/membrane interaction, leading to pore formation, may critically respond to changes in PSM head group properties, which can be efficiently modulated by cholesterol or ceramide.

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