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Toxin-induced pore formation is hindered by intermolecular hydrogen bonding in sphingomyelin bilayers

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ABSTRACT

Sticholysin I and II (StnI and StnII) are pore-forming toxins that use sphingomyelin (SM) for membrane binding. We examined how hydrogen bonding among membrane SMs affected the StnI- and StnII-induced pore formation process, resulting in bilayer permeabilization. We compared toxin-induced permeabilization in bilayers containing either SM or dihydro-SM (lacking the *trans* Δ^4 double bond of the long-chain base), since their hydrogen-bonding properties are known to differ greatly. We observed that whereas both StnI and StnII formed pores in unilamellar vesicles containing palmitoyl-SM or oleoyl-SM, the toxins failed to similarly form pores in vesicles prepared from dihydro-PSM or dihydro-OSM. In supported bilayers containing OSM, StnII bound efficiently, as determined by surface plasmon resonance. However, StnII binding to supported bilayers prepared from dihydro-OSM was very low under similar experimental conditions. The association of the positively charged StnII (at pH 7.0) with unilamellar vesicles prepared from OSM led to a concentration-dependent increase in vesicle charge, as determined from zeta-potential measurements. With dihydro-OSM vesicles, a similar response was not observed. Benzyl alcohol, which is a small hydrogen-bonding compound with affinity to lipid bilayer interfaces, strongly facilitated StnII-induced pore formation in dihydro-OSM bilayers, suggesting that hydrogen bonding in the interfacial region originally prevented StnII from membrane binding and pore formation. We conclude that interfacial hydrogen bonding was able to affect the membrane association of StnI- and StnII, and hence their pore forming capacity. Our results suggest that other types of protein interactions in bilayers may also be affected by hydrogen-bonding origination from SMs.

1. Introduction

Actinoporins are a group of soluble monomeric peptide toxins that have evolved to use sphingomyelin (SM) as their membrane recognition target [1–7]. These actinoporins include toxins such as sticholysin I and II (StnI and StnII, from *Stichodactyla helianthus* [8–10]), equinatoxin II (EqtII, from *Actina equina* [11]), and fragaceatoxin C (FraC, from *Actina fragacea* [12]). The water-soluble structure of the toxins is known in detail [13–16]. They have a β-sandwich motif consisting of 10 β-strands flanked by two α-helices, which interact with both sides of the β-sandwich. For the actinoporins, it is generally believed that monomeric peptides bind to the membrane interface (aided by SM) before oligomerization takes place, and a functional pore is finally formed [6,7].

The effects of membrane properties on toxin binding and pore formation by actinoporins have received a lot of attention over the years (for recent reviews, see [6,7,17]). Quite a lot is known about how membrane and SM properties affect pore formation by different actinoporins. In the absence of cholesterol, pore formation by StnII requires the hydrogen-bonding competent SM [18]. Indeed, all examined actinoporins appear to require the presence of SM in their target membranes [8,18–20]. However, under some conditions, both EqtII and StnII have been shown to increase bilayer permeability in the absence of SM [8,20]. Cholesterol has been shown to affect the pore-formation kinetics and efficiency of actinoporins [21,22]. The actual mechanism by which cholesterol affects rates and efficiency of pore formation is not fully understood, but cholesterol is known to abolish a gel phase in which SM could form in a unsaturated phospholipid bilayer (in the absence of cholesterol). In so doing, cholesterol increases diffusion in the SM-rich domain, but decreases diffusion in the unsaturated phospholipid domain [23,24]. Cholesterol may also affect hydrogen bonding in the membrane

Abbreviations: BA, benzyl alcohol; DPH, diphenyl hexatriene; EqtII, equinatoxin II; LUV, large unilamellar vesicle; FraC, fragaceatoxin C; OSM, *N*-oleoyl-D-*erythro*-sphingomyelin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, *N*-palmitoyl-D-*erythro*-sphingomyelin; SM, sphingomyelin; StnI, sticholysin I; StnII, sticholysin II.

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interface, since its polar hydroxyl can act as both hydrogen bond donor and acceptor. Palmitoyl ceramide, which interacts strongly with SM and induces gel phase formation [25,26], is known to abolish StnII-induced pore formation [21].

SM is a hydrogen-bonding molecule, and many of its bilayer properties (e.g., interaction with other co-lipids) are affected by hydrogen bonding [27,28]. Intermolecular hydrogen-bonding origination from the 2NH function of the sphingoid base is thought to result in SM clustering in the bilayer phase [29]. Intramolecular hydrogen bonding involving the 3OH of the sphingoid base and phosphate oxygens of the phosphocholine headgroup is very prevalent and is likely to regulate head group dynamics and orientation in SM [30,31]. The formation of StnII-pores was abolished when hydrogen bonding from both 2NH and 3OH was prevented by methylation [18]. This result was interpreted to be a consequence of decreased binding of toxin to methylated SM analogs. However, it is possible that intermolecular hydrogen bonding among SMs also affects the way in which pores are formed.

Most SM species have a *trans* Δ^4 double bond in their long-chain base. However, dihydro-SM, which lacks the unsaturation in the long-chain base, is a fairly common SM present in most cells [32,33]. Dihydro-SM is the predominant SM present in the eye-lens membrane [34]. SM and dihydro-SM are known to display very different intra- and intermolecular hydrogen-bonding properties [30,31,35]. Hydrogen bonding among dihydro-SM molecules was recently shown to contribute to the lateral segregation and stabilization of a fluid dihydro-SM domain in disordered PC bilayers at high temperature [36]. A similar segregation of a fluid SM domain was not observed. The much stronger hydrogen bonding in dihydro-SM bilayers, as compared to acyl-chain matched SM bilayers, was also shown to have a fairly large effect on cholesterol and ceramide interactions with SMs [37].

Now, we have examined how pore formation by two different toxins, with highly similar structures but quite different hemolytic activities [19,38], is affected by the differing hydrogen-bonding properties of SM and dihydro-SM bilayers. We used *N*-palmitoyl-SM (PSM) or dihydro-PSM in mixed bilayers with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (1:4 molar ratio) and *N*-oleoyl-SM (OSM) or dihydro-OSM in pure SM bilayers (the latter was possible because of the low gel–liquid crystalline-phase transition temperature of OSM/dihydro-OSM [39]). Our results show that StnII binding to bilayers and the subsequent pore formation were dramatically attenuated in bilayers containing dihydro-SM bilayers as compared to SM-containing bilayers. The addition of benzyl alcohol rescued the pore formation process in dihydro-SM bilayers, possibly by rearranging interfacial hydrogen bonding in a way that favored pore formation also in dihydro-OSM bilayers. We speculate that also other protein-driven processes in membranes may be affected by strong interfacial hydrogen bonding among dihydro-SM molecules.

2. Materials and methods

2.1. Materials

POPC, egg SM, sphingosyl-phosphorylcholine (lyso SM), sphinganin-phosphorylcholine (lyso dihydro-SM), and OSM were obtained from Avanti Polar Lipids (Alabaster, AL, USA). PSM was purified from egg SM as described previously [40]. Dihydro-PSM was prepared from PSM by hydrogenation using H_2-gas and palladium (10%) on charcoal (Sigma-Aldrich, St. Louis, MO, USA) as a catalyst, as described previously [39]. Dihydro-OSM was prepared from oleic acid anhydride (Sigma-Aldrich) and sphinganin–phosphorylcholine, as described previously [41]. Benzyl alcohol and calcein were obtained from Sigma-Aldrich. DPH was obtained from Molecular Probes (Eugene OR, USA). StnI and StnII were produced in an *Escherichia coli* expression system and purified, again, as previously [38].

2.2. Calcein release from LUVs

Calcein-entrapped large unilamellar vesicles (LUVs) were prepared from POPC/PSM and POPC/dihydro-PSM (4:1 molar ratio) and from pure OSM and dihydro-OSM by extrusion through 200-nm filters at 60 °C [18]. Briefly, the desired lipids were mixed and dried under a stream of nitrogen. 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 final 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 12 h. The concentrations of LUV and StnII during calcein leakage experiments were 2.5 μM and 20 nM, respectively. Emission at 550 nm was followed at 23 °C as a function of time (Ex 480 nm). Fluorescence emission was measured in a PTI Quanta-Master spectrofluorimeter (Photon Technology International, Inc. NJ, USA). The released fraction of calcein was determined based on the maximum calcein release induced by Triton X-100. To ensure that no spontaneous leakage occurred, the emission was measured for each sample for 5 min before addition of toxin. A steady signal level, indicating intact vesicles, was observed for all samples. When benzoyl alcohol was used, it was added to preformed LUVs 30 min prior to initiation of experiments.

2.3. Surface plasmon resonance measurements

The association of StnII with lipid bilayers on coated gold chips was performed as follows. LUVs were prepared from OSM and dihydro-OSM in Tris buffer by extrusion through 100 nm polycarbonate filters at 60 °C. StnII binding to the coated bilayers 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 treated with N-hydroxysuccinimide and N-ethyl-N′(dimethylaminopropyl) carbodiimide to activate the surface for capture of phospholipid membranes [18]. 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 5 μl/min.

First, the chip surface was cleaned with two injections of 10 mM CHAPS. 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, 2 min injection) was used to verify that the chip did not have uncovered areas. Finally StnII (1.0 μM) was applied for 10 min, after which buffer alone was injected for 2 min to study toxin dissociation. The chip was regenerated with CHAPS, as in the beginning of the experiment.

2.4. ζ-Potential measurements

To measure toxin/LUV bilayer interactions, vesicles were prepared from OSM and dihydro-OSM in Tris buffer (pH 7.4, 140 mM NaCl). They were exposed to increasing concentrations of StnII for 20 min prior to measuring the ζ-potential of the aggregates. A Malvern Zetasizer ZS (Worcestershire, UK) was used for the measurements. Values given are averages ± SD of $n = 3$.

2.5. Interaction of StnII with OSM or dihydro-OSM monolayers

To measure the penetration of StnII into OSM and dihydro-OSM monolayers, these were spread to an initial surface pressure of 9 mN/m. Following injection of StnII into the buffer subphase of the monolayers (0.8 μM final concentration), which were kept at constant surface area, the penetration of toxin into the monolayer led to a time-dependent increase in lateral surface pressure [42]. The experiments were repeated three times, and a set of representative penetration curves are shown.