Steroid structural requirements for interaction of ostreolysin, a lipid-raft binding cytolysin, with lipid monolayers and bilayers

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Abstract

Ostreolysin, a cytolytic protein from the edible oyster mushroom (Pleurotus ostreatus), recognizes and binds specifically to membrane domains enriched in cholesterol and sphingomyelin (or saturated phosphatidylcholine). These events, leading to permeabilization of the membrane, suggest that a cholesterol-rich liquid-ordered membrane phase, which is characteristic of lipid rafts, could be its possible binding site. In this work, we present effects of ostreolysin on membranes containing various steroids. Binding and membrane permeabilizing activity of ostreolysin was studied using lipid mono- and bilayers composed of sphingomyelin combined, in a 1/1 molar ratio, with natural and synthetic steroids (cholesterol, ergosterol, β-sitosterol, stigmasterol, lanosterol, 7-dehydrocholesterol, cholesteryl acetate, and 5-cholesten-3-one). Binding to membranes and lytic activity of the protein are both shown to be dependent on the intact sterol β-OH group, and are decreased by introducing additional double bonds and methylation of the steroid skeleton or C17-isooctyl chain. The activity of ostreolysin mainly correlates with the ability of the steroids to promote formation of liquid-ordered membrane domains, and is the highest with cholesterol-containing membranes. Furthermore, increasing the cholesterol concentration enhanced ostreolysin binding in a highly cooperative manner, suggesting that the membrane lateral distribution and accessibility of the sterols are crucial for the activity of this new member of cholesterol-dependent cytolysins.

Keywords: Cholesterol; Cholesterol-dependent cytolysin; Liquid ordered phase; Pleurotus ostreatus; Pore forming protein; Sterol

1. Introduction

Sterols are essential components of eukaryotic membranes, which enable the cells to modulate and optimise certain physical properties of the membrane that relate to their biological function. This is connected especially with their ability to promote separation of lipid mixtures into co-existing liquid-disordered ($l_d$) and liquid-ordered ($l_o$) phases [1]. The $l_o$ membrane domains emerge as a result of a local enrichment in sterols, and are the basis for the formation of lipid rafts, transient membrane microdomains enriched in sterols and sphingolipids [2]. These membrane entities had been reported to be involved in a variety of biological functions, such as membrane traffic, signal transduction and pathogen entry [3–5]. Rafts have recently been detected in other eukaryotes as well as animals, e.g. yeast [6], and plants [7]. In these organisms, membrane cholesterol (Chol) is replaced by, or abundantly supplemented with, other membrane-active sterols such as ergosterol, campesterol, β-sitosterol and stigmasterol.

Lipid rafts serve as attachment sites for several cytolytic proteins, and recently some fluorescently labelled and chemically modified mutants of these cytolysins have been proposed as possible selective markers for rafts. These proteins appear not only to recognize the single lipid component that is enriched in rafts, but also to specifically sense its distribution in the membrane. The best
known examples are lysenin, whose truncated, non-toxic mutant specifically recognizes SM-enriched membrane domains\textsuperscript{[8,9]}, and a protease-nicked and biotinylated non-toxic derivative of perfringolysin O (θ-toxin), that selectively binds to Chol-rich lipid rafts\textsuperscript{[10,11]}. On the other hand, lipid rafts can enhance the membrane-permeabilizing activity of pore-forming agents by sequestering them into non-raft lipid domains. One example is a cytolytic peptide δ-lysin from \textit{Staphylococcus aureus}, whose exclusion from \textit{l}_{0}-domains results in concentration and aggregation of the lysin in \textit{l}_{d}-regions, leading to rapid and effective permeabilization of the membrane\textsuperscript{[12]}.

Ostreolysin (Oly) is a 15 kDa acidic protein isolated from fruiting bodies of the edible mushroom \textit{Pleurotus ostreatus}\textsuperscript{[13]}. This protein, belonging to the aegerolysin family\textsuperscript{[14]}, is specifically expressed during the formation of primordia and young fruit bodies\textsuperscript{[13,15]}, and is lytic to a variety of erythrocytes and nucleated mammalian cells at sub-micromolar concentrations\textsuperscript{[16,17]}. Cell lysis is induced by a colloid-osmotic mechanism, and the estimated radius of the Oly-induced pore is \(\sim 2\) nm\textsuperscript{[16]}. Our previous study on its binding and pore-forming properties revealed its high affinity for the Chol-rich membrane phase, whose integrity could be disrupted by the addition of mono- and di-unsaturated glycerophospholipids and which was tentatively identified as a \textit{l}_{d}-phase. Further, after applying it to rodent cell lines and to SM/Chol (1/1) vesicles, Oly was found exclusively in detergent-resistant membranes (DRMs)\textsuperscript{[17]}. These membrane fractions have been proposed as to be lipid raft equivalents. However, recent experimental data revealed that DRMs should not be identified with the rafts, as they may be detergent-induced artifactual structures\textsuperscript{[2]}.

In contrast to lysenin or perfringolysin O, pure SM or Chol cannot inhibit the lytic activity of Oly. Its interaction with artificial lipid membranes requires a specific combination of these two lipids, or a combination of Chol with fully saturated phosphatidylcholine (PC). Its activity can additionally be inhibited by different lysophospholipids, however all our attempts to demonstrate direct binding of these molecules to Oly failed (([16], unpublished data). In SM/Chol vesicles, permeabilization appears only above 30 mol\% Chol, the concentration at which this sterol induces the formation of a \textit{l}_{d}-phase\textsuperscript{[18]}, and markedly increases with higher membrane Chol concentrations\textsuperscript{[17]}. Oly was found to be less active on vesicles in which Chol was replaced by ergosterol\textsuperscript{[17]}, although this sterol has been reported to induce the formation of ordered membrane domains even more efficiently than Chol\textsuperscript{[19]}.

In this work, we have studied the effect of the structure of membrane sterols and cholesterol derivatives on ostreolysin binding and permeabilization activity. It is known that both the sterol structure, and the structure of the adjacent phospholipids result in different effects on membrane ordering\textsuperscript{[19–22]}. Based on our previous results\textsuperscript{[17]}, one would expect that ostreolysin interaction with lipid membranes will correlate with the sterol \textit{l}_{d}-phase-forming ability. We have assayed binding of the protein to supported lipid monolayers and bilayers, and its lytic activity on bilayers composed of 1/1 molar mixtures of SM with different synthetic steroids (cholesteryl acetate, 5-cholestene-3-one), and natural sterols (cholesterol, ergosterol, β-sitosterol, stigmasterol, lanosterol, 7-dehydrocholesterol). We found that presence of the free sterol 3β-OH group is an essential requirement for the binding of Oly to supported lipid monolayers and lipid vesicles.

Fig. 1. Chemical structure of the natural sterols and cholesterol derivatives studied in this work.
The binding and vesicle permeabilizing activity is modulated by the number of double bonds, methylation of the steroid skeleton, and structure of the sterol alkyl chain. Although some of the tested sterols, e.g. ergosterol or 7-dehydrocholesterol, were found to induce higher ordering effects over cholesterol, maximal binding and permeabilization rates were always obtained with cholesterol-containing vesicles.

2. Materials and methods

2.1. Materials

Oly was purified from fresh young fruit bodies of *Pleurotus ostreatus* according to Berne et al. [13], and frozen in aliquots at -20 °C. Before use, the protein was diluted in 140 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 8.0 (vesicle buffer).

Porcine brain sphingomyelin (SM) and wool grease cholesterol (Chol) were from Avanti Polar Lipids (Alabaster, USA), β-sitosterol (Sito), lanosterol (Lano), stigmasterol (Stigma), ergosterol (Ergo), 7-dehydrocholesterol (7-DHC) were from Sigma (St. Louis, USA), and cholesteryl acetate (Ch-Ac) and 5-cholen-3-ene (Ch-one) were from Fluka (Buchs, Switzerland) and Aldrich (St. Louis, USA), respectively. The structure of the chosen sterols and Chol derivatives is shown in Fig. 1. All lipids were dissolved in chloroform or other organic solvents according to manufacturer’s instructions.

1,6-diphenyl-1,3,5-hexatriene (DPH) was from Aldrich Chem. Co. (Milwaukee, USA), and Triton X-100 and calcein were from Sigma. All chemicals were of the highest grade available.

2.2. Preparation of sonicated vesicles (SV)

Lipid films were formed by removing the organic solvent from a lipid solution by rotary evaporation and vacuum drying. Lipids, at a final concentration 10 mg/mL, were swollen in vesicle buffer or in 80 mM calcein and vortexed vigorously to give multilamellar vesicles (MLV), that were further sonicated as described [16]. After a centrifugation (20 min, 16,000×g, 25 °C), the vesicles were incubated for 45 min at 45 °C. In the case of calcein-containing vesicles, extra-vesicular calcein was removed by a gel filtration on a Sephadex G-75 column. Concentration of DPH was 0.5 μM, and fluorescence anisotropy was measured at an excitation wavelength of 358 nm with the excitation polarizer oriented in the vertical position, while vertical and horizontal components of polarized emission light were recorded through a monochromator at 410 nm. The anisotropy was calculated using the instrument build-in software. The value of G-factor (the ratio of the sensitivities of the detection system for vertically and horizontally polarized light) was determined for each sample separately.

2.5. Inhibition of ostreolysin-induced hemolysis

Binding of Oly to SV with different lipid compositions (SM/sterol in a 1/1 molar ratio) was estimated by measuring the residual hemolytic activity of unbound Oly, using a kinetic microplate reader (MRX, Dynex Technologies, Denkendorf, Germany). Typically, 75 μL of SV at various lipid concentrations in vesicle buffer were pipetted onto a multilwell-plate. 25 μL of Oly (4 μg/mL) were then added and the plate incubated for 30 min at 25 °C to allow binding of Oly to SV. Hemolysis was then assayed by adding 100 μL of bovine erythrocyte suspension in vesicle buffer. The decrease in turbidity at 630 nm was recorded for 30 min to determine the time necessary for 50% hemolysis (t50%)

2.6. Surface plasmon resonance (SPR) measurements of ostreolysin binding to supported lipid monolayers

Binding of Oly to supported lipid monolayers was determined using a Biorec X SPR apparatus and an HPA Sensor Chip (Biacore AB, Uppsala, Sweden), pre-coated with long alkylthioether chains that form a flat, quasi-crystalline hydrophobic layer, and immobilize liposomes via surface interaction. In this way, the hybrid bilayer membrane, in which alkane thiols became part of the membrane bilayer, is formed. Such a system is a well-defined model for studying the surface adsorption step of pore-forming proteins [23,24]. All ligands and washing chemicals were prepared in the degassed vesicle buffer (140 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 8.0), which was also used as running buffer. The desired lipid monolayer was coupled to the chip using SV. The SV suspension with 0.5 mM lipid concentration, prepared from pure SM or from SM combined with a steroid, was injected over the chip at a flow rate of 2 μL/min and running buffer. The supported monolayer was treated with 100 μL of 0.1 mg/mL bovine serum albumin (20 μL/min) to minimize nonspecific binding of Oly to the microfluidic system. Oly, at 5, 10, 20, 40 or 55 μM, was injected over the prepared monolayer for 10 min, followed by injection of running buffer, to obtain dissociation kinetics. At a flow rate of 10 μL/min, sensorgrams were recorded at 25 °C. After the dissociation phase following the injection of each Oly concentration, the protein was removed from the monolayer surface with 20 μL of 100 mM NaOH (100 μL/min), and new Oly injection was applied. At the end of experiments, the chip surface was purged with 100 μL of 40 mM octylglucoside in water (20 μL/min), and a new lipid monolayer was applied following the above procedure. Bulk controls were run on monolayers with vesicle buffer alone. Sensorgrams were processed and evaluated using BIAevaluation Version 3.2 software (Biacore AB, Uppsala, Sweden).
2.7. Assay for vesicle aggregation

Possible aggregation of equimolar Chol/SM SV (14 μg/mL) after addition of Oly in the final concentration of 0, 0.1, 0.5, 1, and 2 μM was followed as an increase in light scattering at 25 °C with a Jasco FP-750 spectrofluorometer (Jasco Ltd., Essex, UK). The apparatus was equipped with a water thermostated cell holder using 1 cm path length, magnetically stirred (830 rpm) quartz cuvette. Slit widths with a nominal band-pass of 5 nm were used for both excitation and emission, which were both set to 400 nm.

2.8. Permeabilization of SV

Vesicle permeabilization was determined using a fluorescence microplate reader (Tecan Genios, Grödig/Salzburg, Austria) with excitation and emission at 485 nm and 535 nm. 100 μL of Oly at various concentrations in vesicle buffer were dispensed in a multiwell microplate, followed by an appropriate amount of calcein-loaded SV or MLV composed of SM and steroids in a 1/1 molar ratio. Release of calcein was then recorded for 1 h at 25 °C. Maximal (100%) calcein release was obtained by solubilization of SV with Triton X-100, 1 mM final concentration, and the percentage of calcein release was calculated for each well [25]. The maximal reaction rate was defined as the maximal slope of the permeabilization kinetics.

3. Results

3.1. Characterization of the phase state of SV by DSC and anisotropy measurements

By applying DSC measurements, we followed the lipid thermotropic phase behaviour of SM and SM/steroid (1/1 molar ratio) vesicles. Porcine brain SM undergoes clear phase transition at 37 °C from the solid state to the liquid-disordered state. 1/1 (mol/mol) mixtures of SM with tested steroids do not show any phase transitions. In the temperature range from 0 to 70 °C, all the lipid mixtures, even those containing Ch-on or Ch-Ac, showed very broadened thermograms, suggesting coexistence of several lipid phases as expected for the high content of sterols (data not shown). Therefore, the steady-state DPH anisotropy measurements were additionally performed to compare the level of order in some of the tested lipid mixtures. Anisotropy values are the highest in the gel state, lowest in the liquid-disordered state, and intermediate in the liquid-ordered state [22]. Results of anisotropy measurements of DPH added to equimolar mixtures of SM with different steroids at 25 °C are listed in Table 1. Anisotropy values of DPH in pure SM before (at 25 °C) and after (50 °C) gel-liquid phase transition are shown for comparison. The highest value of DPH anisotropy was observed for SM in the gel state while the lowest for the SM in liquid state. The anisotropy values of DPH in different equimolar SM/sterol mixtures are decreasing in the order: Ergo ≥ Stigma > 7-DHC > Sito > Chol >> Ch-one.

3.2. Ostreolysin binding to steroid/SM (1/1) SV

Inhibition of Oly-induced hemolysis [16] was measured as an indirect assessment of the binding affinity of Oly to steroid-containing lipid vesicles. Chol-containing vesicles were the most effective in inhibiting hemolysis (IC50 = 1 mg/mL), followed by other sterol/SM mixtures in the order: Sito > 7-DHC > Ergo > Stigma > Lano (Table 2). Up to a lipid concentration of 10 mg/mL, vesicles containing Ch-one and Ch-Ac showed no inhibition of Oly-induced hemolysis.

<table>
<thead>
<tr>
<th>Composition (1/1 molar ratio)</th>
<th>Diameter (nm)</th>
<th>IC50 (mg/mL)</th>
</tr>
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<tbody>
<tr>
<td>SM/Chol</td>
<td>91±1</td>
<td>1.0</td>
</tr>
<tr>
<td>SM/7-DHC</td>
<td>138±12</td>
<td>1.3</td>
</tr>
<tr>
<td>SM/Ergo</td>
<td>110±2</td>
<td>1.6</td>
</tr>
<tr>
<td>SM/Sito</td>
<td>137±15</td>
<td>1.2</td>
</tr>
<tr>
<td>SM/Stigma</td>
<td>125</td>
<td>1.7</td>
</tr>
<tr>
<td>SM/Lano</td>
<td>99</td>
<td>2.3</td>
</tr>
<tr>
<td>SM/Ch-Ac</td>
<td>110</td>
<td>&gt;&gt;10</td>
</tr>
<tr>
<td>SM/Ch-one</td>
<td>141</td>
<td>&gt;&gt;10</td>
</tr>
</tbody>
</table>

IC50 denotes the concentration of SV at which the rate of hemolysis is decreased by 50% (see Materials and methods).

Table 2

Lipid composition of sonicated vesicles, dimensions, and inhibition of ostreolysin-induced hemolysis
3.3. Kinetics of interaction of ostreolysin with supported monolayers

Sensorgrams, without corrections for bulk effects (primary sensorgrams), display the association–dissociation kinetics of Oly interaction with supported monolayers made of either pure SM, Chol/SM at various ratios, or SM/steroid (1/1) mixtures (Figs. 2A, 3A, 4A). In all kinetic evaluations, primary sensorgrams were used for fitting to theoretical curves. Injection of the buffer control revealed that, at the flow rate of 10 μL/min, there was a delay in maximal RU response of 4.5 s following both sample and buffer injection. In evaluating sensorgrams, we therefore assumed that rapid changes in RU at the very beginning of association and dissociation should be ascribed to changes of refractive index, due to bulk effects, and not to protein adsorption to or dissociation from the supported monolayer. Using numerical integration analysis [26], primary sensorgrams were fitted to a two-state reaction model, the second step being a conformational change without an increase in RU. The choice of this model is supported by the experimental evidence that a compact native-like conformation of Oly, and a subtle local conformational change are both characteristic of the protein binding to the lipid membrane[14].

In the model, we assume that the binding, observed as an increase in RU, reflects both protein adsorption and possible insertion in the supported monolayer. This model, which has been successively applied to describe the interaction of some other proteins with lipid bilayers [27,28], is depicted by the scheme:

$$Oly + L \overset{k_{a,1}}{\underset{k_{d,1}}{\rightleftharpoons}} OlyL \overset{k_{a,2}}{\underset{k_{d,2}}{\rightleftharpoons}} ^*OlyL,$$

where Oly is ostreolysin, L is lipid, the asterisk designates Oly with changed conformation, and $k_{a,1}$, $k_{a,2}$, $k_{d,1}$, and $k_{d,2}$ are association and dissociation rate constants. When fitting single sensorgrams, goodness of the fits was characterized by values of the $\chi^2$ test ranging from 0.2 to 3; however, global fitting gave values over 10. It is considered that global fits with $\chi^2$ values over 10 are not satisfactory [29], and therefore, we do not report the calculated kinetic and equilibrium constants.

The association–dissociation kinetics of Oly interaction with supported monolayers made of either pure SM or Chol/SM with increasing Chol contents, are shown by sensorgrams (Fig. 2A). Derived sensorgrams (after subtraction of the calculated bulk response) and best fits of the association–dissociation component are shown in Fig. 2B. Spikes at the start and end of the sample injection result from mismatches between the real-time changes of the bulk response, which exhibit a 4 to 5 s delay, and the calculated theoretical bulk response, which is a rectangular pulse. We also observed that, despite the use of rather high protein concentrations, no binding equilibria could be obtained. In good agreement with previous permeabilization results [17], SPR experiments confirmed that (i) Oly does not bind to pure SM and that inclusion of Chol above 30 mol% is a prerequisite, and (ii) the theoretical maximum number of the lipid binding sites increases in a highly cooperative manner (Fig. 2B, inset). In contrast, a concentration-dependent linear relationship is observed for the binding of Oly (up to 55 μM) to a SM/Chol (1/1) supported monolayer (Fig. 3). Sensorgrams showing the interaction of 20 μM Oly with supported monolayers made of steroid/SM (1/1) showed again that Chol-containing mixtures were the most susceptible for binding (Fig. 4). Dissociation of Oly from the Chol-containing monolayer was negligible, reflecting close to irreversible binding. Binding to monolayers containing other natural sterols was considerably lower. A rather high extent of binding was observed with monolayers containing Stigma, however, almost 50% of the signal was lost during the dissociation step, suggesting a loose interaction with Oly. In contrast, most of the signal persisted after the dissociation step with Ergo-, Sito and 7-DHC-containing monolayers. Binding to monolayers containing Lano or one of the synthetic Chol derivatives was poor and comparable to binding to a pure SM-monolayer.

![Image of sensorgrams showing the interaction of ostreolysin with cholesterol/sphingomyelin (1/1 molar ratio) supported monolayers. Primary sensorgrams (A) and sensorgrams after subtraction of the calculated component corresponding to the bulk response (B) are shown. Ostreolysin concentration (μM) is indicated by numbers. Solid line, experimental curves; dotted line, best fits. All other conditions are the same as in Fig. 2. Inset, relationship between calculated response without the bulk response at $t=1400$ s, RU$_{1400s}$, and concentration of Oly. The SPR experiments were run in duplicate or triplicate.](image-url)
3.4. Permeabilization of steroid-containing SV by ostreolysin

Since we did not observe any increase in light scattering in vesicle aggregation assay (not shown) we concluded that calcine release from SV is a reliable assay for membrane permeabilization by Oly. The characteristics of permeabilization of steroid/SM vesicles (Fig. 5) by Oly correlated well with the data on its binding to supported monolayers (Fig. 4) and SV (Table 2). Examples of time courses of calcine release from vesicles supplemented with various steroids are presented in Fig. 5A. The relationship between maximum calcine release from the various vesicles and the concentration of Oly (Fig. 5B) shows that Chol-containing membranes are the most susceptible to Oly-induced lysis, followed by those enriched with Sito and 7-DHC. With these three kinds of vesicles, 50% calcine release occurred between 0.5 and 5 μM Oly. Additionally, 2.7 μM Oly caused 75% permeabilization of SM/Chol (1/1) SV, while it was able to release only 47% of calcine from MLV with the same composition, added in the same protein/lipid molar ratio (not shown). This indicates that the mechanism of permeabilization does not arise from detergent effect, by likely from binding of the protein to the outer lipid leaflet of MLV and subsequent formation of the pore, suggesting that the pore-forming mechanism is responsible for both permeabilization of cells [16] and lipid vesicles. Ergo-, Stigma- and Lano-containing vesicles were considerably less sensitive, and those containing either Ch-one or Ch-Ac could not be permeabilized by Oly at all. The overall susceptibility of steroid/SM (1/1) vesicles to Oly-induced lysis was as follows: Chol > Sito > 7-DHC > Ergo > Stigma > Lano > Ch-one = Ch-Ac. Although the maximal permeabilization of Sito-containing vesicles approaches that of vesicles containing Chol, the shape of the kinetic curves at the beginning of the reaction (Fig. 5A), as well as the maximum permeabilization rates (Fig. 5C), show that calcine release is considerably faster in the presence of Chol.

![Figure 4](image-url)  
**Fig. 4.** Sensorgrams of ostreolysin (20 μM) interaction with supported monolayers of various steroids combined with SM in a 1/1 molar ratio. (A) Primary sensorgrams (solid line) and best fits (dotted line). (B) Corrected sensorgrams after subtraction of calculated component corresponding to bulk response. The experimental conditions are as described in Fig. 2. The SPR experiments were run in duplicate or triplicate.

![Figure 5](image-url)  
**Fig. 5.** Permeabilization by Oly of sonicated sphingomyelin/steroid (1/1 molar ratio) lipid vesicles. (A) Time course of permeabilization of calcine-loaded SM/steroid SV (20 μg/mL) treated with 6.7 μM Oly. (B) Percentage of calcine release and (C) the maximal rate of permeabilization of SM/steroid (1/1) SV treated with 0.067–6.7 μM Oly. (D) Relationship between RU_{1400s}, calculated response without the bulk response at t=1400 s (see Fig. 4), and maximal rate of calcine release from SM/steroid SV. The vesicles were composed of a 1/1 (mol/mol) ratio of SM and Chol (●), Sito (□), 7-DHC (▼), Stigma (▲), Ergo (■), Lano (○), Ch-Ac (♦), and Ch-one (◇).
4. Discussion

4.1. Cholesterol-dependence of ostreolysin resembles that of bacterial cholesterol-dependent cytolysins

Recent results suggest that the global membrane structure, in particular its domain organization, has considerable implications for the susceptibility to different cytolytic proteins, especially those whose membrane activity depends basically on the presence of Chol. For more than 20 years, this sterol was believed to be the receptor for a large group of highly similar toxins isolated from different Gram-positive bacteria and known as “cholesterol-dependent cytolysins” (CDCs). Recently, this paradigm has been extensively revised. For example, only membrane-bound Chol can induce oligomerization of listeriolysin [30], while another CDC, intermedialysin, interacts exclusively with human cells [31], making it hard to believe that Chol could be its exclusive acceptor. Although most CDCs can bind to and oligomerize with crystalline Chol, recent analyses suggest that the involvement of Chol in their cytolytic activity is more complex and even more important during the pore formation than during the binding step [32,33]. Binding of certain CDCs sharply increases above a threshold Chol concentration in lipid vesicles [34], which is also true for Oly ([17], this work), suggesting furthermore that the distribution of Chol molecules on the membrane, and the specific patterns that are subsequently formed, could be of crucial importance for the activity of this group of cytolysins. Another example of a Chol-binding pore-forming toxin is Vibrio cholerae cytolsin (VCC), whose crystal structure strongly resembles those of CDCs [35]. VCC was shown to bind pure Chol and oligomerize with it in solution, although it preferred association with sphingolipid/Chol complexes rather than with individual lipid molecules [36]. Compared to the bacterial toxins, (i) Oly cannot be bound to pure Chol [13,16] but only to Chol or other sterol combined with SM or saturated PC ([17], this work), and (ii) it appears that the role of sterols is essential in the Oly binding step. There is conclusive evidence that Oly, and probably other members of the aegerolysins, could be functionally classified as CDCs, and that bacteria are thus not exclusive producers of this class of proteins.

4.2. Steroid structure and Oly activity

In our study, both binding and permeabilizing activity of Oly on SM/sterol (1/1) bilayers, as well as binding to supported lipid monolayers, proceeded in the same order, showing maximal activities with Chol-containing vesicles, followed by those containing Sito, 7-DHC, Ergo, Stigma, and Lano (Figs. 2–5, Table 2). In all these experiments, Oly was found to be ineffective on mono- and bilayers containing Ch-Ac or Ch-one, and on those made of pure SM. Our results indicate that the ability of a steroid to form the \( l_n \)-lipid phase [19–22] mainly correlates with increased susceptibility of the membrane for Oly binding. In terms of domain formation, the low susceptibility of Lano-containing vesicles to Oly binding and permeabilization is not surprising, as there is strong evidence that this evolutionary precursor of Chol has very low packing abilities with other phospholipids [22,37–39], exhibits the existence of the \( l_n \) phase only at quite low temperatures and/or high Lano concentration [40], and shows no coexisting liquid imiscible phases over a wide range of temperature and composition [41]. Binding of Oly and its permeabilization activity was decreased by the presence of sterols with an additional double bond in the alkyl chain (Ergo, Stigma, Lano, see Fig. 1). Finally, the absence of Oly activity on bilayers containing Ch-Ac or Ch-one correlates well with the reduced ability of these steroids to induce domain formation [42–44] (see also Table 1).

4.3. Cholesterol is preferred by CDCs

Our results on preference of Oly for cholesterol resemble those obtained with CDCs, whose membrane activity is impaired on interaction with either pure or membrane-bound steroids with modification of the \( 3\beta \)-OH group and/or C17 isocholesteryl chain [30]. Pneumolysin, for example, showed just 50% hemolysis inhibition with Ergo, and no inhibition at all with Lano [45], approaching the values obtained here with Oly. Similarly, binding of VCC to several crystalline sterols and steroids showed that this cytolsin needs both a free \( 3\beta \)-OH group and an unmodified C17 isocholesteryl chain for optimal oligomerization [46]. Interestingly, interaction of VCC with lipid vesicles containing enantiomeric Chol was negligible, suggesting a stereospecific mode of interaction [47].

The observed preferential permeabilization ([17], Fig. 5), and binding (Figs. 2, 4) of Oly to Chol-containing membranes are highly cooperative with respect to Chol membrane concentration, reflecting the importance of membrane lateral distribution and accessibility of Chol molecules for protein binding. Indeed, the maximal rates of vesicle permeabilization correlate very well with maximal binding of Oly to supported lipid monolayers (Fig. 5D), suggesting that the surface lipid organization of an SM/Chol lipid mixture is the most favourable one for the interaction with Oly, and furthermore, that the role of steroids is more important in the binding step rather than in later events leading to pore-formation.

It has been argued that increasing the Chol concentration in binary and ternary lipid mixtures leads to an increased chemical activity coefficient of Chol, and yet its tendency to leave the membrane [48]. Actually, there are pieces of experimental evidence that such cholesterol clusters may exist in cholesterol-enriched membranes [49,50]. It is thus suggestive that small Chol clusters, arranged specifically in the plane of the membrane, can serve as docking sites for Oly. Another possibility is that such Chol clusters include SM molecules. In fact, binary mixtures of Chol and phospholipids [51,52], or Chol and SM [53] are thought to form complexes with more than one stoichiometry at different membrane compositions. Such small Chol-rich patches may be identical with lipid nano-domains forming the \( l_n \)-lipid phase, as discussed recently by London [54].

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