

# Ostreolysin, a pore-forming protein from the oyster mushroom, interacts specifically with membrane cholesterol-rich lipid domains

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**Abstract** Ostreolysin, a 15 kDa pore-forming protein from the edible oyster mushroom (*Pleurotus ostreatus*), is lytic to membranes containing both cholesterol and sphingomyelin. Its cytotoxicity to Chinese hamster ovary cells correlates with their cholesterol contents and with the occurrence of ostreolysin in the cells detergent resistant membranes. Moreover, ostreolysin binds to supported monolayers and efficiently permeabilizes sonicated lipid vesicles, only if cholesterol is combined with either sphingomyelin or dipalmitoylphosphatidylcholine. Addition of mono- or di-unsaturated phosphatidylcholine to the cholesterol/sphingomyelin vesicles dramatically reduces the ostreolysin's activity. It appears that the protein recognizes specifically a cholesterol-rich lipid phase, probably the liquid-ordered phase. © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

**Keywords:** Aegerolysin; Cholesterol; Lipid raft; Pore-forming protein; Sphingomyelin; *Pleurotus ostreatus*

## 1. Introduction

Ostreolysin (Oly) [1,2] is a representative of the aegerolysin protein family (Pfam Accession No. PF06355). So far, these proteins have been found in the bacteria *Clostridium bifermentans* [3] and *Pseudomonas aeruginosa* (TrEMBL Accession No. Q9I710), moulds *Aspergillus fumigatus* [4] and *Neurospora crassa* (TrEMBL Accession No. Q8WZT0), and mushrooms *Pleurotus ostreatus* and *Agrocybe aegerita* [1,5]. It has been suggested that they may have a role in processes such as

modulation of bacterial sporulation [3], virulence of *A. fumigatus* [4], and mushroom fruiting [6].

Oly interacts with lipids and forms pores in eukaryotic cell membranes and lipid vesicles prepared from total membrane lipids [2]. So far, searching for a specific lipid acceptor of the proteins belonging to the aegerolysin protein family has not been successful. Lysophospholipids have been reported to be inhibitors of Asp-hemolysin from *A. fumigatus* [7] and Oly, but no direct interaction with lysophospholipids could be proved for Oly [2]. Very recently, an Oly isoform from *P. ostreatus*, pleurotolysin A, was suggested to be an SM-inhibited hemolysin [5].

Here, we addressed the question of membrane lipid acceptor(s) for ostreolysin. We provide evidence that ostreolysin is partitioned in both cell and artificial lipid membrane detergent resistant membranes (DRMs). Moreover, its binding to lipid mono- and bilayers, and lipid vesicle permeabilization, is specifically dependent on the presence of both cholesterol (Chol) and sphingomyelin (SM). SM can be replaced by saturated dipalmitoylphosphatidylcholine (DPPC); however, this results in the decreased activity of Oly.

## 2. Materials and methods

### 2.1. Ostreolysin and lipids

Ostreolysin was isolated from immature mushroom fruit bodies and N-sequenced to check purity as described [1]. In addition, its homogeneity was confirmed by ESI-mass spectroscopy, which gave an  $M_r$  for ostreolysin of 14975.4. Dioleoylphosphatidylcholine (DOPC), DPPC, palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylethanolamine, wool grease Chol, and porcine brain SM were obtained from Avanti Polar Lipids (USA). Ergosterol was from Fluka (Switzerland) and ganglioside G<sub>M1</sub> from Serva (Germany).

### 2.2. Cytotoxicity assay

To determine the influence of cell membrane Chol on the Oly cytotoxicity, we used Chinese hamster ovary wild-type CHO-K1 and CHO-215 cells with deficient Chol synthesis (impaired 4-carboxysterol decarboxylase) [8], grown in a McCoy's 5A medium (Sigma, Germany) with 10% fetal calf serum (FCS) at 37 °C and 5% CO<sub>2</sub>. Cells were plated overnight in 96-well microtiter plates (Costar, USA) at a concentration of  $5 \times 10^3$  cells/well in the growth medium. After washing them with a Dulbecco's PBS buffer, fresh growth medium, with either 10% FCS or a lipoprotein poor serum (LPPS) with diminished Chol contents [8,9], was added. After 24 h, the cells were treated with 1 µg/ml Oly for 0–25 min. Cells were then washed three times with the

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**Abbreviations:** CHO, Chinese hamster ovary; Chol, cholesterol; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DRMs, detergent resistant membranes; EDTA, ethylene-diaminetetraacetic acid; Erg, ergosterol; FCS, fetal calf serum; HEPES, 1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)-monosodium salt; L<sub>d</sub>, liquid-disordered; L<sub>o</sub>, liquid-ordered; LPPS, lipo-protein poor serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Oly, ostreolysin; PBS, phosphate-buffered saline; POPC, palmitoyloleoylphosphatidylcholine; SM, sphingomyelin; SPR, surface plasmon resonance; SV, sonicated vesicles

growth medium and cytotoxicity was determined with an MTT assay [2]. The treated cells were inspected under a phase-contrast microscope.

### 2.3. Isolation of DRMs and Oly Western immunoblotting

DRMs were isolated from the cells or sonicated vesicles (SV) by Triton X-100 (0.5% v/v) extraction as described in detail before [9,10]. In the growth media,  $2\text{--}5 \times 10^7$  cells were pre-treated with 2.5  $\mu\text{g}/\text{ml}$  Oly for 15 min at 23 °C before the detergent extraction. The pelleted cells, lysed for 30 min at 0 °C by 0.5% Triton X-100 in buffered saline (25 mM 1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)-monosodium salt (HEPES), 150 mM NaCl, 1 mM ethylene-diaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu\text{g}/\text{ml}$  aprotinin, pH 6.5), were mixed (1/1, v/v) with 85% sucrose (w/v) in the same buffer mixture. In a centrifuge tube, the resulting 42.5% sucrose mixture was overlaid successively with 5 ml of a 35 and 5% (w/v) sucrose gradient in the buffered saline supplemented with 1 mM EDTA and 1 mM  $\text{Na}_3\text{VO}_4$ . Following 18 h centrifugation at 4 °C (200 000 $\times$ g, SW41 rotor, Beckman L8-M preparative centrifuge), centrifuged sucrose fractions were collected and analyzed for Oly using Western immunoblotting. Proteins were stained with rabbit anti-Oly polyclonal and secondary goat anti-rabbit horseradish peroxidase-conjugated antibodies [1]. Chol contents/mg of cell DRMs protein were determined as reported [8,9]. Using the same procedure as for the cell DRMs, SV DRMs were isolated after treatment of 10 mg/ml SV with 100  $\mu\text{g}/\text{ml}$  Oly in 140 mM NaCl, 20 mM Tris-HCl, and 1 mM EDTA, pH 8.0, for 15 min at room temperature. In that case, only fractions containing visible Triton X-100-insoluble lipids were analyzed for Oly as described above.

### 2.4. Lipid vesicles and measurement of calcein release

Sonicated vesicles, either without or loaded with 80 mM calcein (Sigma), were prepared by sonication of 4–20 mg/ml lipids in 140 mM NaCl, 20 mM Tris-HCl, and 1 mM EDTA, pH 8.0. Non-encapsulated 80 mM calcein was removed on a Sephadex G-50 column. After sonication and centrifugation, the lipid concentration of SV suspensions (without calcein) was determined colorimetrically with Waco Free Cholesterol C and Wako test Phospholipids B (990-54009) kits (Waco Chemicals GmbH, Germany) in order to quantify total lipids in suspension and to check the cholesterol/phospholipid ratio. Before use, the sonicated vesicles were kept overnight at room temperature to avoid their instability due to fusion [11]. Size of SV was estimated by photon correlation spectroscopy on a Zeta Sizer instrument (Malvern Instruments, UK) as reported before [2].

Permeabilization of SV was measured as the increase in fluorescence intensity of calcein (exc./em. wave length, 485/535 nm), dequenched on release from SV, in a fluorescence microplate reader Tecan O2A (Tecan, Austria). The time-course of increasing fluorescence intensity,  $F_t$ , was fitted to a stretched exponential equation used in various chemical and physical rate processes in disordered media [12]:

$$F_t = F_{\min} + F_{\max} * (1 - \exp(-k \cdot t)^b) \quad (1)$$

where  $F_{\min}$  and  $F_{\max}$  are the starting and final fluorescence intensities,  $k$  is a rate constant, and  $b$  an exponential scaling factor. Cooperativity of the permeabilization process was estimated by determining the slope of the log–log dependence of the relative rate of permeabilization on concentration of Chol or Oly.

### 2.5. Hemolysis assay and inhibition of ostreolysin by lipids

Binding of Oly to SV (without encapsulated calcein) was estimated by measuring the hemolytic activity of the free Oly fraction after pre-incubation of 0.2  $\mu\text{g}/\text{ml}$  Oly with varied concentration of SV lipids for 30 min at 37 °C. The lipid concentration necessary for reducing the rate of hemolysis of bovine erythrocytes by 50%,  $\text{IC}_{50}$ , was determined in a MRX microplate reader (Dynex Technologies, Germany) as described [2].

### 2.6. EPR spectroscopy

EPR spectroscopy was used to evaluate potential effect of ostreolysin on the domain structure of lipid vesicles and their fluidity characteristics [13]. SV (20 mg/ml lipids, without calcein) were spin-labeled with MeFASL (10.3) (*N*-oxyl-2-undecyl-2-(3'-methoxycarbonyl)-propyl-4,4-dimethylloxazolidine, provided by Prof. S. Pečar, University of Ljubljana) using a molar ratio of 200:1. Spectra were taken on a X-band EPR spectrometer ESP 300 (Bruker, Germany) at temperatures

ranging between 20 and 40 °C as described [14]. EPR spectra were analyzed with an EPRSIM Ver 4.9 software package for simulation of the spectra of nitroxide spin labels [13]. The simulation method takes into account the fact that the spectra are composed of several components that belong to the spin labels in different types of coexisting membrane regions with different ordering and dynamics of the alkyl chains. From the best fit to the experimental spectra, a number of the coexisting domains, their order parameter, rotational correlation time, and the corresponding portion of each region in the membrane of SV were derived. The effect of Oly on the parameters  $p$  and  $S$  was studied at a lipid/protein molar ratio of 100:1.

### 2.7. SPR determination of binding of ostreolysin to lipid monolayers

Oly binding to lipid monolayers was determined in a Biacore X surface plasmon resonance (SPR) apparatus (Biacore AB, Sweden). Monolayers were deposited on a HPA Chip (Biacore AB) using SV (~0.5 mM lipids in 140 mM NaCl, 20 mM Tris-HCl, and 1 mM EDTA, pH 8.0) as recommended by the producer. Dissolved in the same buffer, Oly (0.081, 0.162, 0.325, 0.75, and 1.2 mg/ml) was injected at a flow rate of 40  $\mu\text{l}/\text{min}$  and sensorgrams were recorded at 25 °C. Bulk controls were run without protein.

## 3. Results and discussion

In membranes, dynamic complexion of cholesterol with sphingomyelin and saturated glycerophosphatides results in liquid-ordered,  $L_o$ , and disordered,  $L_d$ , lipid phases [15]. This is the basis for the formation of separate lipid domains, lipid rafts (operationally DRMs). In cell membranes, rafts are enriched in cholesterol, sphingolipids, and specific proteins. They have roles in membrane trafficking and signaling [16–18], and are attachment/entry sites for cell pathogens, toxins and other ligands [19,20].

Our preliminary screening for lipid acceptor(s) had demonstrated that only vesicles reconstituted from erythrocyte neutral lipids (mainly Chol) and phospholipids (SM and glycerophosphatides) could be permeabilized by Oly. This observation has suggested a specific cholesterol-induced lipid microdomain, such as  $L_o$  phase, which is characteristic of lipid rafts, to be a possible binding site. To check this hypothesis, we first employed wild-type CHO-K1 and mutant CHO-215 cells, with impaired Chol synthesis, to assess dependence of Oly cytotoxicity on the cell membrane contents of Chol and occurrence of Oly in DRMs. Fig. 1 demonstrates that (i) Oly is more cytotoxic to cells, both wild-type and mutant, grown in the presence of serum Chol and (ii) the higher the cellular Chol level, the more abundant is Oly in the cells' DRMs. Despite the lower content of Chol, the mutant cells appear more sensitive to Oly, which could be the consequence of the lower viability of the cells with deficient Chol-synthesis as observed before [9,10]. Microscopy revealed that the extent of cytotoxicity correlated well with changes of cell shape, cell membrane blebbing, and cell lysis (not shown), similar to that observed for some tumor cells exposed to Oly [2].

So far, screening of dispersions of pure Chol, SM, DPPC, and some other phospholipids, or binary mixtures of phospholipids for inhibition of hemolysis produced by Oly has failed to reveal specific acceptor lipid(s) [1,2]. Following this fact and the results in Fig. 1, we used artificial lipid membranes to study Oly preference for lipid mixtures typical of DRMs. Only SV composed of Chol combined with either SM or DPPC exerted inhibition of hemolysis, as indicated by  $\text{IC}_{50}$  values in Table 1. None of the other combinations tested (up to 20 mg/ml lipids), such as those including POPC, DOPC or egg phosphatidylethanolamine, was inhibitory. The striking find-

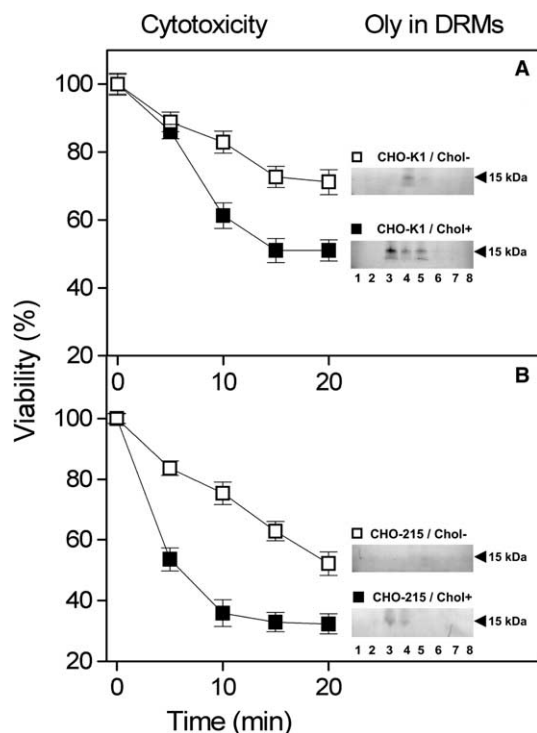


Fig. 1. Cytotoxicity of Oly and its occurrence in DRMs. Wild-type CHO-K1 (A) and CHO-215 cells with deficient Chol synthesis (B) grown in the presence of 10% FCS (normal cholesterol level) (closed squares, Chol+) or a lipoprotein poor serum with decreased Chol (open squares, Chol-) were treated with 1  $\mu$ g/ml Oly for the indicated time, and viability was assayed with an MTT test. Viability (%) was expressed as the ratio between formazan absorbance at 570 nm of treated cells at different time intervals and control cells at the beginning of the experiment (0 min). Means and S.E. values of 5–8 replicates are shown. DRMs were isolated as described in Section 2 and fractions 1–8 immunoblotted for Oly detection (insets). For CHO-K1 cells grown in the presence of FCS or LPPS, the concentration of Chol in DRMs was approx. 22 or 15  $\mu$ g/mg protein, while for CHO-215 12 or 6  $\mu$ g/mg. Visible cholesterol-rich fractions were 3–5.

Table 1  
Lipid composition of sonicated vesicles, dimensions, and inhibition of ostreolysin-induced hemolysis

Composition (molar ratio)	Diameter (nm)	IC <sub>50</sub> (mg/ml)
Chol:SM 1:9	104 ± 1	~17
Chol:SM 2:8	123 ± 2	~15
Chol:SM 3:7	88 ± 1	~11
Chol:SM 4:6	94 ± 1	6.6
Chol:SM 5:5	91 ± 1	1.0
Chol:SM 6:4	97 ± 3	0.1
Chol:DOPC 1:1	119 ± 2	≥20
Chol:POPC 1:1	71 ± 6	≥20
Chol:DPPE 1:1	98 ± 3	~3

Mean diameter  $D \pm$  S.D. (standard deviation) was estimated by photon correlation spectroscopy. IC<sub>50</sub> is the concentration of lipids that decreases the rate of hemolysis of bovine erythrocytes by 50% [2].

ing that, while inhibition is essentially dependent on Chol, this is only in combination with either SM or the saturated phosphatidylcholine species, strongly suggested that the protein might recognize a specific Chol-induced lipid phase.

As shown in Fig. 2, we studied in more detail the dependence of SV permeabilization on specific Chol binary or ternary lipid mixtures allowing formation of the  $L_o$  phase and lipid rafts

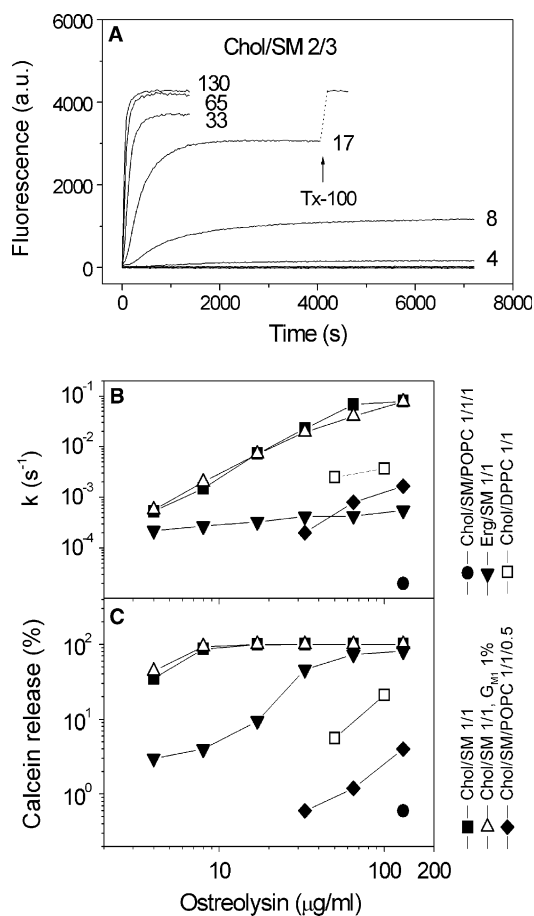


Fig. 2. Permeabilization of sonicated lipid vesicles by ostreolysin. (A) Representative time course of changes of dequenched fluorescence intensity of calcein released from Chol/SM 2:3 SV upon Oly treatment at the indicated  $\mu$ g/ml concentrations. 1 mM Triton X-100 (Tx-100) was used for 100% release of encapsulated fluorophore as designated for 17  $\mu$ g/ml Oly by the arrow. Permeabilization of 20  $\mu$ g/ml SV with indicated molar ratio of lipids was characterized by rate constant  $k$  (B) and percentage of calcein release (C) derived by fitting a time course to a stretched exponential function (see Section 2).

[21–23]. Time courses of calcein release, quantifying permeabilization activity as exemplified in Fig. 2A, were used to derive a rate constant  $k$  and the percentage of maximal calcein release at different final Oly concentrations (Fig. 2B and C). The highest rate and extent of calcein release is pertinent to the Chol/SM SV, while those obtained with Chol/DPPE are approx. 20 times lower. POPC, 20 mol%, in SV containing Chol/SM (1:1) reduces both  $k$  and the percentage of release by approx. 60-fold, while at 33%, it results in a decrease of  $k$  by ~1000-fold. Inclusion of 1% ganglioside  $G_{M1}$ , well-known to be preferentially associated into the  $L_o$  lipid phase [24], has no effect on Oly permeabilizing activity. Replacement of Chol by the fungal sterol ergosterol in SV has a similar effect, but at a very much lower efficiency (Fig. 2B, C). In this respect, it is known that sterols other than Chol, typical for fungi or plants, and Chol precursors, have a similar function in lipid raft maintenance [25,26].

Detergent extraction of cell and artificial lipid membranes may produce artifact DRMs [27,28] and, therefore, inclusion of Oly in the cell and lipid DRMs might be an artifact. However, this seems unlikely due to the SPR results in Fig. 3,

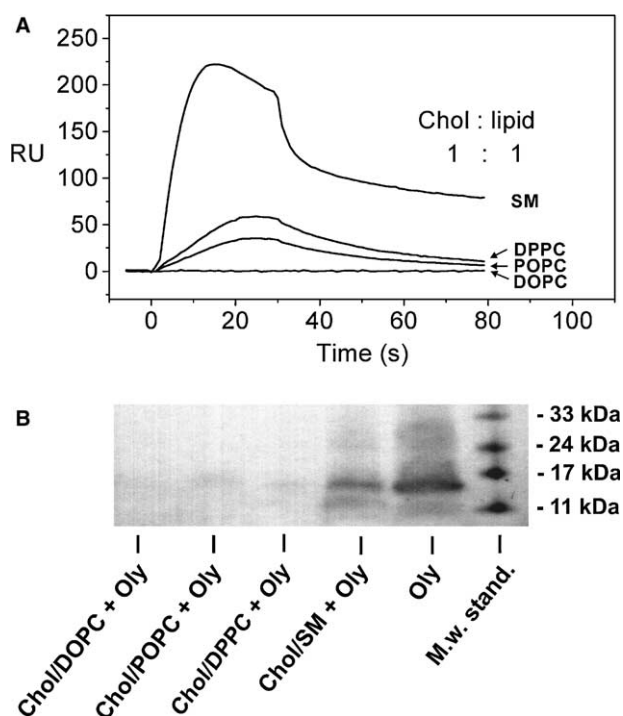


Fig. 3. Binding of ostreolysin to lipid mono- and bilayers. (A) 1.2 mg/ml Oly in 140 mM NaCl, 20 mM Tris–HCl, and 1 mM EDTA, pH 8.0, was injected at a flow rate of 40  $\mu$ l/min over indicated Chol/lipid 1/1 monolayers. The sensorgrams were corrected for bulk effects; RU, response units. Decrease of the signal at the end of injection is either an artifact due to a rather low injected volume or results from Oly's induced lipid desorption. (B) Binding of Oly to Chol/lipid 1/1 SV. In 140 mM NaCl and 20 mM Tris–HCl, pH 8.0, 0.5 ml of SV (10 mg/ml) was pre-incubated with 50  $\mu$ l of Oly (1 mg/ml) for 15 min at room temperature and visible DRMs were isolated and immunoblotted to detect DRM-bound Oly, as compared to Oly control without lipids and kaledoscopic molecular weight markers.

where direct binding, without intervention of the detergent, is shown. Further, incubation of spin labeled Chol/SM (1: 1) SV with Oly had no effect on their EPR spectra (not shown). This evidence thus supports the conclusion that the permeabilizing effect of Oly results from its direct recognition of an existing specific lipid complex, rather than any effect on recruiting and ordering of lipids.

Formation of a transmembrane pore by pore-forming proteins is a multi-step process that includes binding to the membrane and in-plane protein oligomerization [29,30]. Certain of such proteins may exploit cholesterol or SM for binding or protein insertion. Chol is crucial for permeabilization of bacterial cholesterol-dependent cytolysins but not for binding and pre-pore formation on the membrane [30]. An exception is perfringolysin O and its C-terminal domain, of which binding step is cholesterol-specific [31]. Another cytolysin, lysenin, uses SM as a binding acceptor [32], as also suggested for pleurotolysin A [5]. To discriminate between the possible effects of the specific Chol/lipid composition on either binding or pore-formation by Oly, we used SPR to detect directly its binding to lipid monolayers [29,33]. In Fig. 3, only sensorgrams obtained at the highest Oly concentration of 1.2 mg/ml are shown because no Oly binding to Chol/POPC could be observed below this concentration. On the Chol/SM monolayer, saturation with ostreolysin was observed by increasing the protein con-

centration (not shown); a half-saturation concentration was  $\sim$ 90  $\mu$ g/ml. The efficiency of Oly binding, in the order Chol/SM  $\gg$  Chol/DPPC > Chol/POPC and no binding to the Chol/DOPC monolayers (Fig. 3A), strongly suggests that the binding step is essentially dependent on the specific Chol/lipid composition. This is also supported by partition of Oly into SV DRMs (Fig. 3B). There is evidence that the coexistence of  $L_o$ ,  $L_d$ , and some other lipid domains, together with their contents, is dependent on the Chol/lipid(s) ratio [23,34,35]. We, therefore, analyzed in more detail the effect of the Chol/SM ratio on the permeabilizing activity of Oly in SV (Fig. 4). The rate constants and extent of calcein release coincide with increased Chol. The concentrations of Chol (above 30 mol%) that produce detectable permeabilization are similar to those inducing the  $L_o$  lipid phase in mono- and bilayers [23,34]. If the marked

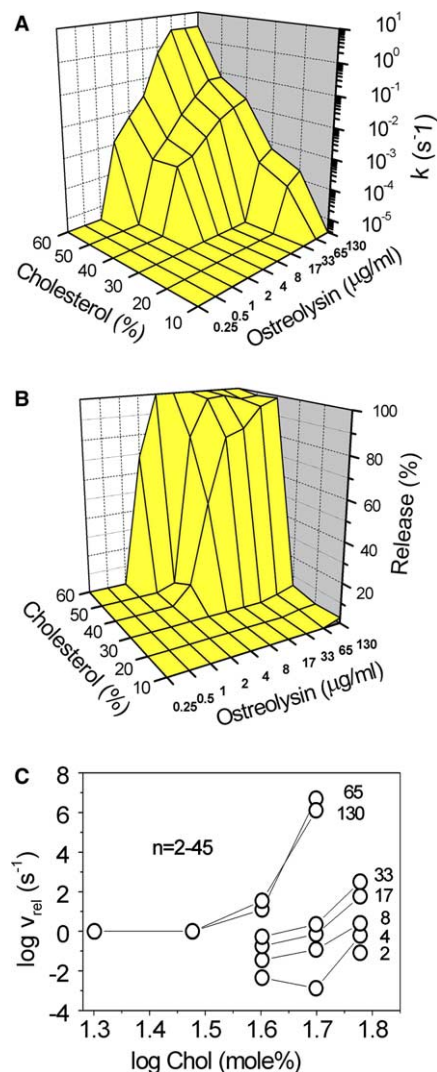


Fig. 4. Effect of cholesterol to sphingomyelin ratio on permeabilization of SV by Oly. Calcein-loaded Chol/SM SV containing indicated mole% of Chol were treated with 0.25–130  $\mu$ g/ml Oly to obtain the time course of calcein release, then rate constant  $k$  (A) and percentage of release (B) were derived (see Fig. 2 and Section 2). (C) Relative rate of release, normalized to 100% release (see (B)), was calculated as  $v_{rel} = k^*$  (maximal percentage of release) and used to estimate slope of the curve  $n$  from a standard double-log plot. Coefficient  $n$  (2–45) implies degree of cooperativity of the permeabilization process with respect to Chol. In (C), numbers show the respective  $\mu$ g/ml concentration of Oly.

activity is dependent on a Chol-rich phase, then one should expect that Oly interacts with certain Chol complexes. We used the method of initial rate estimates of the permeabilization curves to derive the coefficient  $n$  with respect to Chol concentration (Fig. 4C). The slope reveals unusual ever-increasing values for  $n$ , from  $\sim 2$  to about 45. In contrast,  $n$  determined for Oly from the plot of  $\log v_{\text{rel}}$  vs.  $\log \text{conc. of Oly}$  was uniformly equal to  $\sim 2.3$  over its whole concentration range (not shown), in agreement with the previously reported Oly oligomerization on vesicles made of erythrocyte lipids [2]. The increasing Chol cooperativity suggests that Oly interacts with specific Chol-containing complexes, which may grow with increasing Chol concentration. On the other hand, the opposing effect of POPC (see Fig. 2) implies that mono- and di-unsaturated lipids promote dissociation of productive Chol-rich complexes and thus prevent interaction with Oly.

#### 4. Conclusions and perspectives

So far, the majority of studies of lipid domains were performed on monolayers or giant unilamellar vesicles. Our study suggests, however, that the specific cholesterol-rich phases could be studied just as well in vesicles of a smaller diameter (see Table 1). It is evident that Oly does not interact with either pure Chol or SM but that it does react, specifically, with a Chol-rich lipid phase, the integrity of which is highly sensitive to unsaturated glycerophosphatides. The underlying mechanism could be dissociation of Chol-complexes by unsaturated phospholipids and, in turn, increase of chemical activity of Chol [24]. According to recent data, we suggest that the Chol-induced phase may be the  $L_o$  phase, however, some other unprecedented Chol-rich lipid phase, dependent on the presence of saturated phosphatidylcholines in the membrane, cannot be excluded. In fact, the diversity of Chol-rich lipid phases in membranes in terms of the stoichiometry of Chol/Chol and/or Chol/other lipid complexes is not obvious [15,24]. The properties of Oly described here thus make Oly and its engineered mutants – for example, fused with fluorescent proteins or labeled with fluorescent, as reported for perfringolysin O [36], or spin probes – a new tool in further studies of Chol-rich lipid phases and Chol-complexes in natural and artificial lipid membranes.

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