Hypothesis

Differential interaction of the two cholesterol-dependent, membrane-damaging toxins, streptolysin O and *Vibrio cholerae* cytolysin, with enantiomeric cholesterol

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Abstract Membrane cholesterol is essential to the activity of at least two structurally unrelated families of bacterial pore-forming toxins, represented by streptolysin O (SLO) and *Vibrio cholerae* cytolysin (VCC), respectively. Here, we report that SLO and VCC differ sharply in their interaction with liposome membranes containing enantiomeric cholesterol (*ent*-cholesterol). VCC had very low activity with *ent*-cholesterol, which is in line with a stereospecific mode of interaction of this toxin with cholesterol. In contrast, SLO was only slightly less active with *ent*-cholesterol than with cholesterol, suggesting a rather limited degree of structural specificity in the toxin–cholesterol interaction.

Key words: Streptolysin O; Cholesterol-binding cytolysins; *Vibrio cholerae* cytolysin; Protein–cholesterol interaction; Enantiomeric cholesterol

1. Introduction

Bacterial cytolysins or pore-forming toxins must possess some degree of specificity for animal as opposed to bacterial cell membranes. With two distinct families of oligomerizing, pore-forming toxins, this specificity is mediated by membrane cholesterol. The sterol requirement of streptolysin O (SLO) has long been recognized, and SLO and the toxins homologous to it are frequently referred to as the 'cholesterol-binding cytolysins' [1]. The latter name is actually at odds with the following experimental observations:

1. With at least one member of this class of toxins (listeriolysin), binding (though not oligomerization) has been observed with liposomes not containing cholesterol [2].
2. Modification of the single cysteine residue shared by most toxins abrogates binding to membranes but not to cholesterol [3].

Binding to cholesterol and binding to the membrane, respectively, therefore do not seem to be equivalent. The crystal structure of one of the 'cholesterol-binding cytolysins' (perfringolysin O, secreted by *Clostridium perfringens*) has been determined [4]. It has since become clear that membrane binding of the monomer is mediated by the C-terminal domain 4 of the toxin molecule [5], apparently by a confined, tryptophan-rich motif around the single cysteine residue, which remains the only membrane-inserted part of that domain even after oligomerization and pore formation [6]. It is, however, not possible at present to assign individual amino acid residues that are directly involved in the interaction with the sterol.

More recently, the hemolytic cytolysin secreted by *Vibrio cholerae* El-Tor has been found to also require cholesterol for membrane permeabilization [7]. This toxin is not detectably related to the SLO family at the level of amino acid sequence, and known close homologues so far are restricted to the genus *Vibrio*. *V. cholerae* cytolysin (VCC) also differs considerably from SLO in pore size, which is ~1.5 nm in diameter [8] compared with up to 30 nm [9] for SLO. While a structural model of VCC has recently been reported [10], experimental data to assign residues involved in membrane interaction is still wanting.

In the present study, we have examined the specificity of SLO and of VCC for cholesterol using enantiomeric cholesterol (*ent*-cholesterol) as a probe. Cholesterol and *ent*-cholesterol have mirror image shapes, but identical physical properties; *ent*-cholesterol is therefore very suitable as a probe of stereospecificity in the interaction between proteins and cholesterol.

2. Materials and methods

SLO was recombinantly expressed in *Escherichia coli* and purified by successive affinity and hydroxylapatite chromatography [11]. VCC was purified from culture supernatants of *V. cholerae* by isoelectric focusing [12]. The synthesis of *ent*-cholesterol has been described elsewhere [13,14]. Egg yolk phosphatidylcholine and phosphatidylglycerol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Galactosylceramide (from bovine brain), cholesterol, 7α- and 19-hydroxycholesterol, cholesterol-3β-acetate, and 3β-thiocholesterol were obtained from Sigma. The commercially obtained lipids were used without further purification.

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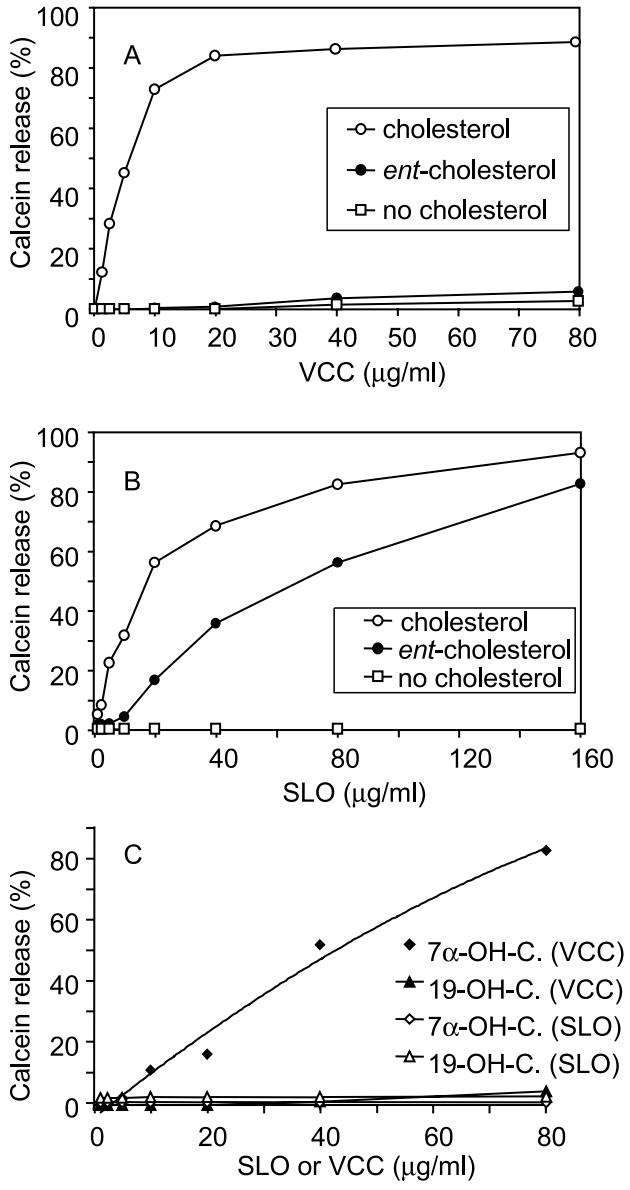

Fig. 1. Activity of VCC and SLO on membranes containing cholesterol, *ent*-cholesterol, and 7α- or 19-hydroxycholesterol. Liposomes containing the sterol in question (33% by mol; see Section 2 for residual lipid composition) and filled with calcein were incubated with VCC or SLO at the concentrations indicated, and the extent of calcein release was determined by an increase in fluorescence. A: Activity of VCC with cholesterol and *ent*-cholesterol. B: Activity of SLO with cholesterol and *ent*-cholesterol. C: Activity of VCC and SLO with 7α- and 19-hydroxycholesterol.

2.1. Liposome permeabilization experiments

Liposomes containing trapped calcein were prepared from egg yolk phosphatidylcholine (45% by mol), phosphatidylglycerol (2%), galactosylceramide (20%), and the sterol in question (33%) by extrusion through Nuclepore polycarbonate membranes [15,16]. For controls without any sterols, the content of phosphatidylcholine was increased to 58%, whereas the fractional content of the other components remained unchanged. Calcein release assays were performed using twofold serial dilutions of VCC or SLO, to which constant amounts of the liposomes in question were added (final concentration of total lipid: 0.1 mg/ml). The percentage of calcein released was determined fluorometrically after incubation at 37°C for 10 min. Additional details can be found elsewhere [16].

3. Activity of VCC and SLO on membranes containing cholesterol and *ent*-cholesterol

Fig. 1A,B shows the permeabilization of liposomal model membranes containing cholesterol or *ent*-cholesterol (33% by mol) in a background of PC and galactosylceramide. Both VCC and SLO efficiently permeabilize membranes with cholesterol. If *ent*-cholesterol replaces cholesterol, VCC shows very limited permeabilization, only marginally exceeding that observed with control vesicles not containing any sterol at all (Fig. 1A). This finding is in accordance with the concept of a specific toxin–sterol interaction. SLO, too, is inactive with vesicles lacking cholesterol; however, in contrast to VCC, it displays a very considerable activity with *ent*-cholesterol (Fig. 1B).

4. Interaction of SLO and VCC with other cholesterol derivatives

Fig. 1C shows the permeabilization by VCC and SLO of liposome membranes containing two cholesterol derivatives, 7α- and 19-hydroxycholesterol. VCC shows considerable activity with 7α-hydroxycholesterol but is virtually inactive with the 19-hydroxysterol, as is SLO with either derivative. These findings are compatible with the assumption that both toxins interact with cholesterol in a structurally specific way, whereby the 7α-hydroxyl group would be in an obtrusive position with SLO but not VCC.

SLO did not induce any detectable permeabilization of membranes containing either 3β-thiocholesterol or cholesterol acetate. VCC was also inactive with thiocholesterol but displayed a low degree of activity with cholesterol acetate (data not shown).

5. Conclusion

The collective results are compatible with a stereospecific mutual interaction of cholesterol and VCC. In contrast, there seems to be a discrepancy in the case of SLO: The inactivity observed with most cholesterol analogs suggests a high degree of specificity; however, the opposite is favored by the fact that SLO is active with *ent*-cholesterol. The latter finding imposes a rather surprising twist to the customary concept of a mutually specific toxin–cholesterol interaction [17,18]. If discrimination between enantiomers is taken as a measure of structural specificity, then SLO appears to have a strikingly low degree of specificity for the sterol. On the other hand, any modification of the 3β-hydroxy group completely abrogates the activity of SLO or its homologous toxin perfringolysin O [19]. This suggests that the toxins' specificity is largely confined to the most surface-exposed part of the sterol molecule. The latter conclusion is in line with recent structural data on perfringolysin O, which indicate that, even after completion of toxin oligomerization and pore formation, only a rather limited part of the domain that is responsible for the initial, sterol-dependent membrane binding actually inserts into the lipid bilayer [6].

The question then arises why both 7α- and 19-hydroxycholesterol fail to support SLO activity to any detectable extent. If these sterols were to assume the same orientation in the membrane as cholesterol, then their respective accessory hydroxyl groups should be located remote from the membrane

surface, thereby precluding their interference with the toxin–sterol interaction. This suggests a deviant orientation of these two sterols in the membrane. With 7α-cholesterol, a previous study indeed suggests that both the 3β–hydroxyl group and the 7α-hydroxyl group are in contact with the aqueous phase, and the body of the molecule is accordingly tilted [20].

In summary, our study supports a stereospecific mode of cholesterol interaction with VCC, but not with SLO, the 'classic' cholesterol-binding toxin. The findings illustrate the usefulness of *ent*-cholesterol as a probe of structural specificity in protein–cholesterol interaction.

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