Oligomerization of Vibrio cholerae Cytolysin Yields a Pentameric Pore and Has a Dual Specificity for Cholesterol and Sphingolipids in the Target Membrane*

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Vibrio cholerae cytolysin permeabilizes animal cell membranes. Upon binding to the target lipid bilayer, the protein assembles into homo-oligomeric pores of an as yet unknown stoichiometry. Pore formation has been observed with model liposomes consisting of phosphatidylcholine and cholesterol, but the latter were much less susceptible to the cytolysin than were erythrocytes or intestinal epithelial cells. We here show that liposome permeabilization is strongly promoted if cholesterol is combined with sphingolipids, whereby the most pronounced effects are observed with monohexosylceramides and free ceramide. These two lipid species are prevalent in mammalian intestinal brush border membranes. We therefore propose that, on its natural target membranes, the cytolysin has a dual specificity for both cholesterol and ceramides. To assess the stoichiometry of the pore, we generated hybrid oligomers of two naturally occurring variants of the toxin that differ in molecular weight. On SDS-polyacrylamide gel electrophoresis, the mixed oligomers formed a pattern of six distinct bands. Ordered by decreasing electrophoretic mobility, the six oligomer species must comprise 0 to 5 subunits of the larger form; the pore thus is a pentamer. Due to both lipid specificity and pore stoichiometry, Vibrio cholerae cytolysin represents a novel prototype in the class of bacterial pore-forming toxins.

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inferred. The results of this study are relevant to both molecular and pathogenetic aspects of *V. cholerae* cytolsin.

**MATERIALS AND METHODS**

**Bacterial Strains**—*V. cholerae* O1 El Tor strain 8731, which was used for isolation of the VCC 65-kDa form, was a generous gift of Dr. R. Hall, Washington D. C. The 50-kDa form was isolated from *V. cholerae* O1 El Tor strain KM 169 (4).

**Purification of V. cholerae Cytolsin**—Both forms of VCC were isolated according to published procedures. Briefly, the 65-kDa form was precipitated from culture supernatants of strain 8731 with ethanol and purified by sequential isoelectric focusing in sucrose density gradients and hydroxyapatite chromatography (15). For the isolation of the 50-kDa form, *V. cholerae* strain KM 169 was cultured in liquid minimal medium. The cytolsin was purified by ammonium sulfate fractionation and sequential chromatographic on DE52 cellulose (Whatman), Ultragel AcA-44 (Amer sham Pharmacia Biotech), and a Mono Q column (Amer sham Pharmacia Biotech) (7).

**Preparation of Liposomes**—Cholesterol, phosphatidylethanolamine (PE), and PC from egg yolk, ceramide, t-sphingosine, ganglioside *G* *α* 1, and bovine brain extract (composed of phosphatidylserine (PS) 10–15%, PC 15–20%, PE 20–25%, sphingomyelin (SPM) 10–15%, glycerolamides 30–40%, and cholesterol 1–2%) were purchased from Fluka AG, Buchs, Switzerland. The enzymatic constituents of the brain extract were also purchased singly in purified form (purity ≥ 98%) from either Sigma or Fluka; glucosylceramide from human spleen was obtained from Sigma. The lipids were dissolved in chloroform with or without 33% methanol and admixed at the desired molar ratios (see “Results”) in a 250-ml round bottomed flask. The solvent was evaporated under a stream of nitrogen, and the lipid film was dried for 50 min under vacuum. Following resuspension of the lipids in Hesper/NaCl to 5 mg/ml, large unilamellar vesicles (LUV) were formed by repeatedly extruding the suspension through polycarbonate membranes (Nuclepore, CA; 100-nm pore size) (17), whereby the extrusion apparatus (Lipex Bi omembranes, Vancouver, Canada) was thermostatted at 40 or 50 °C if required. The lipid concentration in the final sample was determined using the amipic acid method (Boehringer Mannheim). If the liposomes did not contain cholesterol, phospholipids were quantitated by phosphorus analysis (18).

**Calcein Release Assay**—Large unilamellar vesicles (LUV) were produced as above, whereas the lipids after drying were resuspended in Hepes/NaCl containing 50 mM calcium (2, 7-bis-[N,N-bis(carboxymethyl)aminomethyl]fluorescein). Following extrusion, the liposome suspension was passed over a column of Sephadex G-50 (Amer sham Pharmacia Biotech) equilibrated with Hepes/NaCl to remove nonentrapped calcein. The void volume fractions were pooled, and the lipid concentration was determined. Small unilamellar vesicles (SUV) were prepared in an analogous manner, except that sonication (Branson probe sonifier 250) for 6 min was substituted for membrane extrusion. Liposomes of total lipid were incubated for 10 min at 37 °C, and the fraction of calcein released was quantitated fluorimetrically. As shown in Fig. 1A, the LUV proved even less susceptible toward the cytolsin than SUV. Since LUV are generally considered a more appropriate model of natural membranes than SUV, this finding even accentuates the gap in susceptibility between these artificial target membranes and natural ones.

**Brain Lipids Strongly Enhance Liposome Permeabilization by VCC**—For an initial test of the hypothesis that lipids other than cholesterol are important in the interaction of *V. cholerae* cytolsin with target membranes, we employed a crude mixture of phospholipids and glycolipids extracted from bovine brain. These lipids were supplemented with cholesterol to the same molar content as above (30%) and used for the preparation of LUV. Fig. 1B shows that calcein was released from the cholesterol-enriched brain lipid liposomes at very much lower cytolsin dosages than those containing PC (cf. Fig. 1A). Obviously, the brain extract does contain one or more lipid species that greatly augment VCC pore formation.

We next examined the contribution of cholesterol to the sensitivity of the brain lipid liposomes. Since the crude brain extract has a residual cholesterol content of 1–2%, we employed a blend of purified bovine brain lipids instead of the crude extract for the preparation of LUV without cholesterol. PC, PE, PS, galactosylceramide, and SPM were admixed at their respective molar fractions also prevalent in the crude extract (see “Materials and Methods”). As seen in Fig. 1B, the cholesterol-free liposomes required a fairly high dosage of cytolsin to yield any detectable calcein release at all, and only very few cytolsin oligomers were detected on the liposome membranes by SDS-PAGE. This observation reinforces the previously established important role of cholesterol (14). On the other hand, it may be stated that the blended brain lipid liposomes are still similarly sensitive to VCC as are those composed of egg yolk PC and cholesterol (cf. Fig. 1A). Thus, it appears that both cholesterol and the incremental brain lipid species impart a low level sensitivity to membranes when present alone, but they have a strong cooperative effect when employed in combination.

In membrane permeabilization by VCC, monomer binding can be distinguished from oligomerization and pore formation, and the promoting effect of particular membrane constituents might be related to either of these steps. It has previously been shown that VCC efficiently binds to membranes consisting of egg yolk PC alone (15). PC was a major constituent of all liposome species that were employed here. Accordingly, with all of these liposome preparations, the extent of toxin binding ranged from 50 to 90% as assayed by hemolytic titration. Therefore, the binding step could only account for a 2-fold

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Liposomes with entrapped calcein were added to a dilution series of V. cholerae cytolysin (final concentration of lipid, 150 μg/ml). After 10 min at 37 °C, the calcein released was quantitated fluorimetrically; 100% release corresponds to the fluorescence of a detergent-lysed sample. A, SUV and LUV consisting of phosphatidylcholine (molar content, 70%) and cholesterol (30%). B, LUV prepared from crude bovine brain lipids, supplemented with cholesterol to a molar content of 30% or from a corresponding mixture of purified brain lipids lacking cholesterol. Inset, SDS-PAGE of VCC incubated with liposomes. The upper bands correspond to the oligomer. Left, brain lipid LUV with cholesterol; right, brain lipid LUV without cholesterol.

Variation in membrane susceptibility, which means that the much larger differences that were observed experimentally must mainly be due to the oligomerization step.

Galactosylceramide Is the Major Constituent Responsible for the Sensitivity of Brain Lipid Liposomes to VCC—We then sought to identify the individual lipid constituents of the crude brain extract involved in promoting the oligomerization of VCC. To this end, the major lipid species were obtained in pure form and incorporated into liposomes at their respective molar fraction in the crude extract (see “Materials and Methods”); the residual lipids were cholesterol (molar fraction, 30%) and egg yolk PC. As an exception, bovine brain PC was employed at 70% with no egg yolk PC. The liposomes were added to serial dilutions of VCC, Fig. 2 displays the fraction of calcein released from the various lipid species. The most pronounced sensitization was evidently induced by galactosylceramide (GalCer), which was employed at 40% by mole and approached the crude brain lipid mixture in efficacy. A slight enhancement of sensitivity was also observed with SPM (molar content in the membrane, 10%). In contrast, very little sensitization was evident with any of the glycerophospholipids, with the sole exception of bovine brain PE (membrane content, 20%) which was similar in efficacy to SPM and clearly superior to bovine brain PC. A similar relationship was observed with PE and PC derived from egg yolk (not shown). The main difference between the two lipid species should consist in their respective choline and the ethanolamine head groups; the choline head group therefore appears to have an inhibitory effect upon VCC pore formation.

The Ceramide Moiety Is Essential in Enhancement of VCC Oligomerization by Sphingolipids—In the above experiments, the sphingolipids SPM and GalCer had performed quite differently in sensitizing the respective liposomes to VCC. However, GalCer had been employed to a four times higher amount, which might conceivably account for its superior effect. For a more precise comparison of their interaction with the cytolysin, liposomes were produced with matched contents of SPM and GalCer. In this series of experiments, further lipid species were also included to learn more about the importance of various structural features of the sphingolipid molecule to the oligomerization of VCC.

Fig. 3 displays the cytolsin dosages required for calcein release of ≥50% from liposomes containing 10, 20, or 40% of the respective sphingolipids (missing values for 20 or 40% indicate that homogeneous and stable lipidome preparations could not be obtained under our experimental conditions.) The essential findings can be stated as follows: SPM is clearly inferior to GalCer also if both are employed at equivalent amounts. The ganglioside GM1 (which possesses a tetrasaccharide head group) is also included to learn more about the importance of various structural features of the sphingolipid molecule to the oligomerization of VCC.

The 50-kDa Form of V. cholerae Cytolysin Lacks the C Terminus of the 65-kDa Form—In a previous report, we showed that a naturally occurring 50-kDa form of VCC shares the N terminus of the 65-kDa form, implying that it must have been
proteolytically cleaved close to its C terminus (4). In those experiments, the 50-kDa form was only characterized by SDS-PAGE, so the possibility was not ruled out that the proteolytic fragments remain associated under non-denaturing conditions. With the homologous V. vulnificus cytolysin, a proteolytically nicked form has been described, the fragments of which are held together by disulfide bonds (20). Fig. 4A shows that this is not the case with the 50-kDa form of VCC, since identical migration was observed under reducing and non-reducing conditions. If electrophoresis was performed in the presence of the non-denaturing detergent deoxycholate, the 50-kDa form again migrated ahead of the 65-kDa form (Fig. 4B). This confirms that the C terminus is indeed lost upon proteolytic cleavage and is not required for cytolytic activity.

The V. cholerae Cytolysin Pore Is a Pentamer—The 65-kDa form assembles into SDS-resistant oligomeric pores on suitable membranes (14). This also holds for the 50-kDa form, whereby the two oligomer species differ in electrophoretic mobility in good correlation with the difference in molecular mass of the respective monomers (Fig. 5, lanes 1 and 5). We reasoned that a mixture of the two cytolysin forms should yield hybrid oligomers. The composition of these hybrids should be randomly distributed, and it should be reflected by their respective electrophoretic mobility. Fig. 5 (lanes 2–4) shows that this is indeed the case. The mixed samples formed patterns of six evenly spaced bands, which included those representing the homogeneous oligomers. The oligomers migrating in the lowest of these six bands thus consisted of 50-kDa subunits only. With each of the five subsequent bands, the number of 65-kDa subunits increased by one, reaching a maximum of five with the topmost band. The total number of subunits thus is always five; there-fore the VCC pore is a pentamer. When the oligomers were dissociated by boiling in SDS, their subunits were recovered with unaltered electrophoretic mobility, which ruled out any artifacts due to changes in covalent structure (e.g. by protease contamination; Fig. 5B).

**DISCUSSION**

The cytolysin of V. cholerae belongs to a homologous family of toxins that are widespread among Vibrio and Aeromonas species (21). Several of these are capable of eliciting diarrheal disease, including enterotoxin-negative strains of V. cholerae O1 El Tor (22, 23) and non-O1 serotypes (24, 25). In the latter instance, VCC most probably is pathogenetically significant, since experimentally the purified protein imparts marked damage to the small intestine epithelium of rabbits and mice (6, 7, 26).

The susceptibility of the epithelial cells to these cytolysins probably reflects the adaptation of V. cholerae and related species to the intestinal environment, but it has not yet been explained in molecular terms. The mammalian intestinal brush border membrane is distinguished by its high content of glycolipids, which may contribute to the tubular shape of the microvilli (27, 28) and, like phospholipids and cholesterol, amount to one-third of the total lipids. Monohexosylceramides or ceramide represent the major glycolipids in rats (29, 30) and humans (31). This composition of lipids very much resembles the most susceptible synthetic liposomes characterized in the present study. We therefore propose that its high content of glycolipids, in conjunction with cholesterol, accounts for the sensitivity of the brush border membrane toward V. cholerae cytolysin.

One of the most striking findings about the effect of lipids upon the oligomerization of VCC consists in the pronounced cooperativity between sphingolipids and cholesterol. Various sphingolipids have been reported to associate with cholesterol in mixed membranes, which raises the possibility that the oligomerization of VCC is mediated by sphingolipid-cholesterol complexes rather than by the individual lipid molecules. The interaction with cholesterol has been most thoroughly studied with sphingomyelin. Model monolayers of sphingomyelin were significantly condensed upon addition of cholesterol, and the
cholesterol liposomes at 37 °C. In the mixtures, oligomers were generated by incubation with brain lipid/cytolysin forms of 65 and 50 kDa were admixed at varying ratios. From lysin resist dissociation by SDS at room temperature (15). The two oligomers by SDS-PAGE (silver staining).

The oligomers of the cytolysin were used: PAGE without prior heat treatment. The following ratios (65:50 kDa) were used: lane 1, 100:0; lane 2, 90:10; lane 3, 70:30; lane 4, 40:60; lane 5, 0:100. B, the samples correspond to those in A but were dissociated at 95 °C prior to electrophoresis.

sterol was also more firmly retained by these monolayers than by ones consisting of PC, respectively (32, 33). Similar findings have been reported for dihexosylceramides, whereas monohexosylceramides did not appreciably associate with cholesterol (34). To our knowledge, evidence of stable complexes consisting of cholesterol and free ceramide is lacking as well. Since monohexosylceramides and free ceramide are clearly superior to SPM with respect to the enhancement of VCC oligomerization, it appears that association of sphingolipids and cholesterol is not essential for their interaction with the cytolysin.

Another interesting example of a protein simultaneously requiring ceramides and cholesterol in its target lipid membrane is provided by the E1 envelope protein of Semliki Forest virus (35, 36), which triggers fusion of the viral envelope to the endosome membrane. Fusion, like pore formation, requires physical separation of laterally interacting lipid molecules, which might constitute the common rationale behind the unusual combined specificity for two lipid molecules, both of which are largely buried within the apolar core of the bilayer. In line with this interpretation, both ceramide and cholesterol are not required in binding of the cytolysin monomer but essentially contribute to the subsequent event of oligomerization and pore formation.

Apart from the apolar moieties of the membrane lipids, their polar head groups also appear to play a role in the oligomerization of VCC. Of note, a choline head group is present in both PC and sphingomyelin, and the efficiency of oligomerization in the presence of either molecule was clearly inferior to that observed with homologous lipid species (PE and ceramide, respectively). The inhibitory effect of the choline head group apparent from these results may be shared by the complex polar tetrasaccharide moiety of the ganglioside G_{M1}. In this context, it should be noted that a reduction in sensitivity of rabbit erythrocytes to VCC has been obtained by neuraminidase treatment (37). Removal of sialic acid from the membrane glycolipids exposed terminal galactose moieties, and susceptibility of the cells could be restored by treating the cells with galactose oxidase. It thus appears that VCC may interact with terminal galactosyl residues (which also occur in the ganglioside G_{M1}) in a non-functional manner. On the other hand, galactosylceramide was very similar in its capability to sensitize membranes to VCC as were glucosylceramide and free ceramide. Possibly, the distance separating galactose from the ceramide moiety determines whether or not oligomerization of VCC proceeds following its binding to the sugar residue.

Sensitive model liposomes containing both cholesterol and glycosphingolipids were utilized to assess the stoichiometry of the V. cholerae cytolysin pore. The electrophoretic analysis of heteromers applied here to elucidate the oligomer stoichiometry was inspired by previous work on the heptamer of Staphylococcus aureus α-hemolysin (38). In the work cited, the two toxin variants required were obtained by chemical modification of single cysteine mutants. Where chemical modification or limited proteolysis are inappropriate, extending or truncating the termini of a protein molecule at the DNA level might be used to produce the variant species for heteromer analysis. The latter method should thus be more generally useful to determine the stoichiometries of toxin pores and, if combined with non-denaturing analytical separation, other homo-oligomeric proteins.

Among the bacterial pore-formers, α-hemolysin provides the only example of an oligomer structure determined at high resolution (39). Its transmembrane portion consists of a β-barrel with 7-fold rotational symmetry. Heptameric stoichiometry and β-barrel structure has also been confirmed with the protective antigen component of anthrax toxin (40, 41). As a pentamer, VCC so far is unique within this particular class of toxins, but there are several previous examples of pentameric transmembrane channels in general. One of those is provided by the B subunit oligomer of V. cholerae enterotoxin. The hollow center of the latter is lined by five α-helices (42). Helical structure has also been suggested for the intramembranous portion of the nicotinic acetylcholine receptor (43) and for the cardiac calcium channel phospholamban (44). It would be most interesting to determine which of the two above structural paradigms applies to the transmembrane part of the VCC pore.

In sum, the present study shows that V. cholerae cytolysin has a dual specificity for both cholesterol and ceramides, which reflects the composition of its natural target membranes and that the pore is a pentamer. Both these properties qualify VCC as a novel prototype within the class of bacterial pore-forming toxins, and they probably apply to a series of homologous toxins of other Vibrionaceae as well.

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