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Ladder-shaped polyether compound, desulfated yessotoxin, interacts with membrane-integral α-helix peptides

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Abstract—Ladder-shaped polyether compounds, represented by brevetoxins, ciguatoxins, maitotoxin, and prymnesins, are thought to possess the high affinity to transmembrane proteins. As a model compound of ladder-shaped polyethers, we adopted desulfated yessotoxin (**2**) and examined its interaction with glycopholin A, a membrane protein known to form a dimer or oligomer. Desulfated yessotoxin turned out to interact with the α-helix so as to induce the dissociation of glycopholin oligomers when examined by SDS and PFO gel electrophoresis. The results provided the first evidence that lapper-shaped polyethers interact with transmembrane helix domains.

1. Introduction

Ladder-shaped polyether (LSP) compounds, unique products of Dinophyceae and Haptophyceae, comprise continuous trans-fused cyclic ethers and are known to possess potent toxicity. Brevetoxin B, the first member of LSPs, had been extensively studied by Koji Nakanishi's group for a long time, leading to more than 10 publications, which include the first structure elucidation of LSP in collaboration with Clardy's group.[1] Some of the LSP toxins were discovered as a causative agent of seafood poisoning cases. Among those, ciguatera is the most serious illness, from which more than twenty thousand patients suffered annually. The main causative agents of the poisoning have been identified to be ciguatoxin, which have a LSP structure encompassing 13 rings.[2] Besides, maitotoxin,[3] prymnesins[4], and yessotoxins (YTXs)[5] were found to be in association with fish-killing red tides or toxic seafood. These LSPs are added up to more than 50 compounds that can be classified into nine skeletal structures; brevetoxin B,[1] brevetoxin A,[6] ciguatoxin[2] (Caribbean ciguatoxin[7]), gambieric acid,[8] gambierol,[9] gymnocin A[10] (gymnocin B[11]), maitotoxin,[3] prymnesin,[4] and yessotoxin (**2**)[5] (adriatoxin[12]). Although the number of LSP members has increased, the molecular mode-of-action studies lagged behind chiefly due to a short supply of materials and their non-specific affinity to biomembranes. Brevetoxins and ciguatoxins are rare examples where their molecular target has been identified; the toxins share a common binding site on voltage, sensitive sodium channels.[13] These LSPs have very high affinity for the channel, with the dissociation constants of nanomolar–subnanomolar concentrations. Although gambierol and other polyethers show a weak interaction with the sodium channels,[13e] LSPs are though to bind to different molecular targets upon exerting their toxicity. These observations suggest a notion that there is a general and weakly interacting motif in proteins for LSP and, when a certain LSP matches the binding part of a target protein, probably due to an arrangement of polyether oxygen atoms, its powerful activity is manifested. Thus, this general motif should provide a clue to solve their potent biological activity.

Keywords: Ladder-shaped polyether; Transmembrane peptides; Yessotoxin; Glycopholin.
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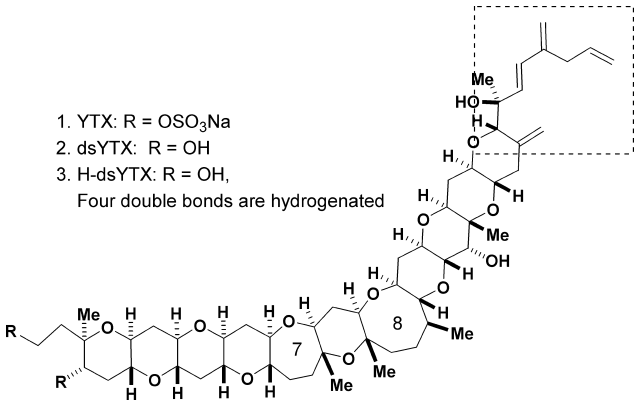

In the present study, we attempt to examine the above hypothesis using a simple method to assess the interactions between polycyclic ethers and membrane-integral α-helix peptides.

2. Materials and methods

2.1. Materials

Alamethicin I (crude) from *Trichoderma viride* and glycophorin A from blood type MM were purchased from Sigma. Purification of alamethicin I was carried out by HPLC on an ODS column (YMS-Pack ODS ϕ 10×150 mm), with a gradient elution using an aqueous acetonitrile system with 50 mM triethylammonium acetate. Melittin (synthetic, 97% purity by HPLC) was obtained from Sigma. Other chemicals were obtained from local venders.

2.2. Culture of dinoflagellate and isolation of yessotoxin

The dinoflagellate *Protoceratium reticulatum* was isolated in the Mutsu Bay, Japan.[14] The algae were cultured in 2L GSe medium in a 2-LFernbach flask for 28–35 days with 16-light/8-dark photocycles. The harvested cells were extracted with methanol and then acetone. The extracts obtained after removal of solvents were dissolved in aqueous 80% methanol and defatted with hexane. The methanolic layer was evaporated and subjected to a 1-butanol–water partition. The crude toxin in the butanol layer was purified by HPLC; column, YMC-Pack ODS-AM ϕ 4.6×150 mm; mobile phase, 35 mM phosphate buffer (pH 6.5) in aqueous 70% methanol, flow rate, 0.5 mL/min. YTX (**1**) was eluted at 19 min when monitored at 230 nm. From the culture media, YTX was trapped by a column of HP-20 and eluted with methanol. The crude YTX was subjected to HPLC purification, as described above. In average, 0.8 mg of YTX was obtained from 1 L of the culture.

2.3. Preparations of yessotoxin analogues

Desulfated YTX (dsYTX, **2**) was prepared by an acid hydrolysis of YTX. *p*-Toluenesulfonic acid (5.3 μmol) and YTX (0.84 μmol) were dissolved in 250 μL dioxane and left to stand for 20 min at room temperature. After addition of a saturated $NaHCO_3$ solution, the product was extracted with ethyl acetate and purified by HPLC on an ODS column (YMS-Pack ODS, ϕ 4.6×150 mm), with a mobile phase of aqueous 15% methanol at a flow rate of 0.5 mL. The structure of dsYTX was confirmed by ^{1}H NMR, that was identical to reported data.[5c] A hydrogenated product of dsYTX (**3**) was prepared by catalytic hydrogenation. A methanolic solution of **2** (1 μmol, 200 μL) in the presence of PtO_2 was stirred under H_2 for 50 h. After removal of the catalysis, the product that gave a single spot on TLC was subjected to NMR (no olefinic signals between 4 and 6 ppm) and MS (ESI MS *m*/*z* 1014.3 corresponding to $M+Na^+$). The purity of the product (**3**) was judged to be sufficient for further experiments.

2.4. Biological assays for yessotoxin and its analogues

Antifungal assays were carried out using *Aspergillus nigar*. The fungus was inoculated to a 1.5% agar plate consisting of 2% glucose, 0.2% malts extracts, 0.05% $MgSO_4$, 0.5% polypeptone, and 0.1% KH_2PO_4. A sample was added to a paper disk (8 mm diameter) and placed on the agar plate, which was incubated for two days at room temperature. Antifungal activities were evaluated by the diameter of an inhibitory zone of the fungus. The hemolytic activity of YTX analogues was measured, as described previously.[15] Briefly, 20% erythrocyte suspension was prepared from human blood, which was washed with 10 mM Tris–HCl buffer three time and diluted to 20% with the same buffer. The blood suspension was diluted further to 20-fold before use. A sample in 20 μL DMSO was added to a 1% suspension and incubated at 37 °C for 3 h. After centrifugation of the suspension, the resultant supernatant was subjected to a colorimetric determination of hemoglobin at 490 nm. Those upon addition of distilled water to the 20% suspension were taken as 100% hemolysis test. The hemolytic activity of YTX and the analogues, was expressed with the dose causing 50% hemolysis as EC_{50}. The effects of dsYTX on the hemolytic activity of amphiphilic peptides were examined by the same method, as described above, except for the addition of 14 μM alamethicin I or 1.4 μM melittin to DMSO solutions of dsYTX.

2.5. Gel electrophoresis

The dissociation of glycopholin A (GpA) was reportedly measured by SDS gel electrophoresis.[16] We followed the method by Bormann et al. Briefly, GpA (1.2 μg) was dissolved in 170 μL of 0.2% SDS buffer (50 mM Tris–HCl, 30% glycerol, and 1 ppm bromophenol blue at pH 6.8). A sample in 2 μL methanol or DMSO was added to 14 μL of the SDS solution, left for 1 h, and loaded on SDS–PAGE. The gel (10–20% gradient precast gel) was developed with a buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS at 10–20 mA for 90 min, which was then subjected to periodate oxidation (0.2% for 15 min at 4 °C) to enhance the band of GpA before using Coomassie brilliant blue (CBB) or silver staining (Silver Staining Kit Protein®, Amersham). As an alternative method, particular for the transmembrane

peptide of GpA (GpA-TM), PFO–PAGE using perfluorooctanoic acid (caution: hazardous chemical) was adopted for facilitating the formation of oligomers. PFO-PAGE was carried out with essentially the same methods as those in SDS–PAGE experiments, except for using, a 1.3% PFO buffer (80 mM Tris, 24% glycerol, and 1 ppm bromophenol blue at pH 8.5) for incubation and 0.5% PFO for electrophoresis. GpA-TM consisting of 29 amino acid residues, EPEITLIIFGVMAGVIGT ILLISYGIRRL, was synthesized and purified as previously reported.[17]

3. Results and discussion

3.1. Biological activities of yessotoxin analogues and their effects on hemolysis of amphipathic peptides

Among LSPs of natural origins, we focused on YTX (**1**) that could be obtained by dinoflagellate culture.[14] However, YTX is a hydrophilic compound unlike other LSPs, most of which are insoluble in water. To prepare a model compound, the hydrophilicity of YTX should be reduced. Thus, we prepared desulfated YTX (dsYTX) as a LSP model by hydrolyzing the ester under acidic conditions. Hydrogenated dsYTX (H-dsYTX) was also prepared to know the effect of a polyene side chain on the biological activities.

Hemolytic and antifungal activities were measured for YTX, desulfated-YTX (**2**), and hydrogenated-dsYTX (**3**) in comparison with previously reported toxicity (Table 1).[5e,13e] The two analogues **2** and **3** showed comparable potency in antifungal tests. A similar lethal activity between **1** and **2**, despite a marked difference in hydrophilicity, also implies that the biological activities are chiefly attributable to an LSP structure of YTX.

Next, we attempted to evaluate the interaction of dsYTX with amphipathic peptides, alamethicin I and melittin. Both of them are known to form an α-helix in lipidic environments[18] and induce membrane permeabilization,[19] which can be monitored by hemolysis tests. If dsYTX interacts with the peptides, their hemolytic activity should be somewhat affected. As shown in Figure 1a, hemolysis elicited by alamethicin I was markedly enhanced by dsYTX in the concentration range of 10–33 μM. Similarly, hemolysis by melittin was augmented by dsYTX in a concentration-dependent manner (Fig. 1b). To examine changes in the α-helix structure of alamethicin I, CD spectra[18a,b] were determined for the membrane-bound peptide in the presence of increasing

Table 1. Biological activities of YTX **1** and its analogues **2** and **3**

Compound	Antifungal (μg/disk)[a]	Hemolytic (μM)[b]	Mouse lethal (μg/kg)[c]
YTX	10	90	286
dsYTX	5	>300	301
H-dsYTX	10	—	—

[a] Minimum effective dose against *Aspergillus niger*.
[b] EC_{50} value for human erythrocytes.
[c] Mouse lethal potency by intraperitoneal injection.[5e,13e]

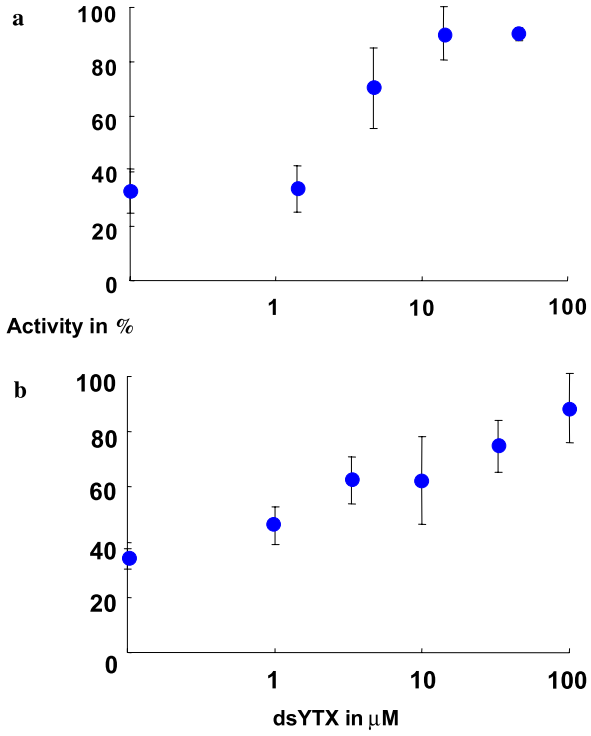

Figure 1. Effects of desulfated yessotoxin (dsYTX) on the hemolytic activities of alamethicin I (a) and melittin (b). DsYTX enhanced the hemolytic activity (*y*-axis in percent) of alamethicin I at 14 μM and melittin at 1.4 μM in a dose-dependent manner.

concentrations of dsYTX. The CD curves of alamethicin I (16 μM in EggPC liposomes, data not shown) derived from an α-helix slightly changed to a less polarized direction; the molar ellipticity (θ) per amino acid residue at 222 nm was shifted in a concentration-dependent manner from −11.7° in the absence of dsYTX to −9.7° in the presence of 33 μM dsYTX. This small change may suggest that the binding of dsYTX does not significantly alter the conformation of the peptide or the affinity of dsYTX is not high enough to change the peptide conformation.

3.2. SDS–PAGE and PFO–PAGE of glycophorin-A and its transmembrane peptide in the presence of desulfated yessotoxin

Glycophorin A (GpA), a heavily glycosylated membrane protein occurring in erythrocyte membrane, is known to form a dimer[16,20] in membrane environments by interaction mainly between two α-helices at glycin-valine residues. In addition, GpA tends to aggregate in water, even in the presence of detergents. Bormann et al.[16] have reported that GpA migrated on SDS–PAGE as a dimer or oligomer, which can be dissociated into a monomer by peptides corresponding to the transmembrane domain. One of these peptides (GpA-TM, see Section 2.5 for the amino acid sequence) was reported to take on an α helix structure in SDS micelles.[21] The dissociation can, thus, be accounted for by directly binding of GpA-TM to the transmembrane part of GpA. We attempted to utilize this method to know whether dsYTX bound to a membrane-integral α-helix peptide.

As shown in Figure 2, addition of dsYTX markedly reduced the oligomers and increased the dimers and monomers. Appearance of a monomer band in the presence of dsYTX, as is the case with GpA-TM, is attributable to the binding of dsYTX to an α-helical part of GpA. This dsYTX binding is further supported by PFO–PAGE, as shown in Figure 3; PFO is known to stabilize oligomers of hydrophobic peptides.[22] In the presence of dsYTX, the oligomers of GpA-TM were mostly dissociated into dimers and, at higher PFO concentrations, monomers were partly formed (data not shown). These observations again imply that dsYTX binds to a hydrophobic α-helix and prevents aggregations of GpA-TMs.

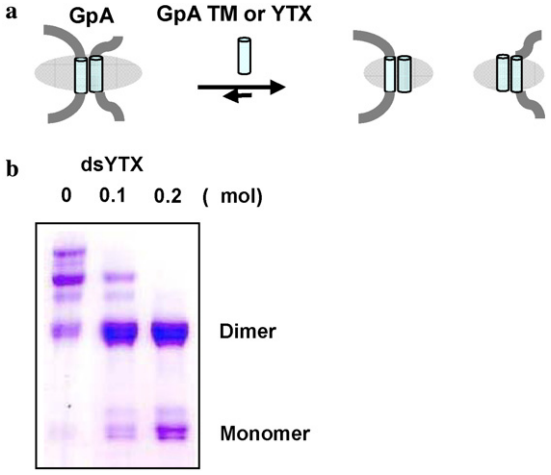

Figure 2. SDS–PAGE of glycopholin A in the presence of desulfated yessotoxin (dsYTX). (a) Chematic illustration of dissociation of glycopholin dimers in SDS micelles by its transmembrane peptide (GpA-TM) or by dsYTX. (b) SDS–PAGE of glycopholin-A (530 pmol, 20 μg) in the presence of 0, 0.1, and 0.2 μmol dsYTX stained by CBB. Large amounts of GpA and dsYTX compared with those by silver staining (Figs. 4 and 5) were used to make the monomer band clearer by CBB staining. Similar dissociation of the dimers into monomers elicited by a transmembrane peptide of glycopholin A has previously been described by Bormann et al.[16]

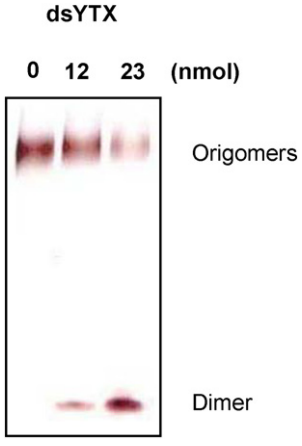

Figure 3. PFO-PAGE of transmembrane peptide of Glycophorin-A (GpA-TM) in the presence of desulfated yessotoxin (dsYTX). Perfluorooctanoic acid (caution. hazardous chemical) was used instead of SDS. Oligomers of GpA-TM (0.25 nmol, 0.8 μg) were mostly formed under these conditions, which was effectively dissociated into dimers by 23 nmol dsYTX.

As shown in Figure 4, YTX (**1**) and hydrogenated-dsYTX (**3**) showed similar action and efficacy as dsYTX. Although YTX bearing two sulfate groups is a water-soluble and a more hydrophilic compound than dsYTX, both the LSPs revealed a similar potency. Brevetoxin-B, generously gifted by Professor Nakanishi, revealed essentially same activity and potency as those of dsYTX (Fig. 4). The SDS–PAGE results may infer that the LSP structure can generally interact with an α-helix motif of GpA and induce the dissociation of the oligomers into dimers and monomers.

3.3. SDS–PAGE of glycopholin-A in the presence of detergents and miscellaneous compounds

To know what types of structural moieties other than LSPs dissociate oligomers of GpA, more than 20 compounds were subjected to SDS–PAGE experiments. Among the polyether compounds tested, polyethylene glycol (average molecular weight around 1000), or 18-crown-6, did not induce any dissociation of oligomeric GpA. On the other hand, amphipathic polyether compounds, such as dicyclohexyl-18-crown-6 and Triton X-100, elicited the dissociation at 24 nmol (approximately one-tenth the potency of dsYTX), as shown in Figure 5; all the chemicals in Figure 5 did not induce any significant dissociation at 2.4 nmol. Ionic detergents, such as additional SDS, CHAPS, and cholic acid,

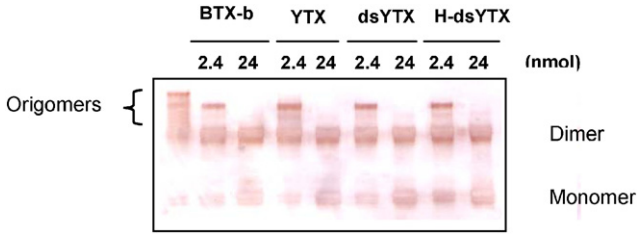

Figure 4. SDS–PAGE of glycopholin A in the presence of ladder-shaped poly ether compounds. SDS–PAGE of glycopholin-A (2.6 pmol, 0.1 μg) in the presence of 2.4 and 24 nmol of brevetoxin-B (BTX-b), YTX, dsYTX, and H-dsYTX visualized by silver staining.

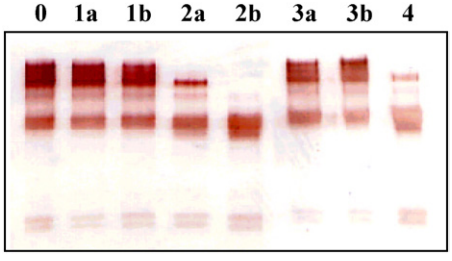

Figure 5. SDS–PAGE of glycopholin A in the presence of synthetic polyether compounds. Line 0, GpA alone; lines 1, 25 nmol (a) and 250 nmol (b) of 18-crown-6; lines 2, 25 nmol (a) and 250 nmol (b) of dicyclohexyl-18-crown-6; lines 3, 25 nmol (a) and 130 nmol (b) of polyethyleneglycol (average molecular weight 1000); and Line 4, 25 nmol Triton X-100. Glycopholin-A (2.6 pmol, 0.1 μg) was visualized by silver staining.

showed weak activity at 250 nmol (data not shown) and partly reduced high oligomers of GpA. Non-ionic detergents, such as sucrose monocaprate, sucrose monocholate and glucose octanoate caused the dissociation of oligomers into dimers but not into monomers at 250 nmol (data not shown), which were of much higher doses than those necessary for LSPs.

These results strongly suggest that polyether functionality with moderate hydrophobicity possesses affinity to a transmembrane α-helix motif. In particular, LSPs revealed a 10-fold higher activity than artificial polyethers in GpA dissociation assays (Figs. 4 and 5). On the other hand, efficacy was similar among the LSPs tested, which implied that the bilateral arrangement of ether oxygen atoms enhanced interaction with a transmembrane α-helix part of glycophorin A. Detailed examinations of the structure of the complex between dsYTX and GpA-TM are currently underway.

4. Conclusion

As a model of ladder-shaped polyether compounds, we adopted yessotoxin, which could be obtained from dinoflagellate culture, and examined its interaction with α-helix peptides. Desulfated yessotoxin (**2**) was shown to interact with the transmembrane part of glycopholin A to induce dissociation of oligomers of the protein by SDS–PAGE. The results provided evidence that ladder-shaped polyether compounds interact with membrane-integral helix domains of membrane proteins, which may partly account for the powerful biological activities.

Acknowledgments

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