Sticholysins I and II are potent cytolysins produced by the sea anemone *Stichodactyla helianthus*, characterized by forming oligomeric pores in natural and artificial membranes. Among its main applications are the construction of antitumoral or antiparasite immunotoxins and as a tool to deliver liposome associated molecules to cell cytosol. To optimize the efficiency of these preparations the mechanism of toxin action must be understood. In the present study, we evaluate the influence of the main lipid components on the functional activity of St II using lipid monolayers and liposomes. The amino terminal sequence of St II is a putative essential region for its lytic activity, here we characterize the functional activity of a synthetic peptide from this region using model membrane systems. The joint presence of sphingomyelin and cholesterol increases the association to the membrane and its permeabilizing capacity, which is probably related to the formation of lipid microdomains. The function of non-bilayer lipids is related to the formation of the lipid toroidal pore.

**Materials and methods**

**Purification of toxins**

Samples of the anemone *Stichodactyla helianthus* were collected in the shores of Havana. Protein purification was carried out according to Lanio et al [2].

**Interactions of Sticholysins with lipid monolayers**

The insertion of toxins into lipid monolayers was assessed with a m-Through equipment (Kibron, Finland). To prepare the monolayers, the corresponding lipid mix, previously prepared in chlorormethanol:buffer 10 mM, NaCl 200 mM, pH 7 (HBS), until the desired initial pressure was reached. Toxin (0.93 mM), was placed on the surface of Hepes buffer and surface pressure changes were monitored at 25 °C under constant stirring.

**Fluorescent Spectroscopy**

Titrations experiments with vesicles were conducted with a fixed amount of toxin, and adding increasing amounts of liposomes. The emission was cored at 334 nm, after excitation at 295 nm [5].

**References**

Production and characterization of liposomal vesicles

Lipid mixtures were prepared from chloroform: methanol solutions in the following lipids: egg yolk phosphatidylcholine (EYP), bovine brain sphingomyelin (BBS), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (PE) (Avanti Polar Lipids) and cholesterol (Sigma). The lipid mix was dried under vacuum in a rotovaporator, and the lipid film was hydrated with Hepes buffer 10 mmol.L⁻¹, NaCl 50 mmol.L⁻¹, ANTS 25 mmol.L⁻¹, DPX 90 mmol.L⁻¹, pH 7.5, through alternate cycles of exhaustive shaking in a vortex and ultrasonic bath, until reaching its complete suspension to obtain MLV. After five cycles of freezing/thawing liposomal suspensions were subjected to 10 extrusion cycles at 45 °C, using 100 nm polycarbonate filters (Nucleopore, USA).

Vesicles were separated from non-encapsulated material by size exclusion chromatography on a Sephadex G-75 column pre-equilibrated in HBS buffer. Phospholipid content in the liposome preparations was measured by the method described by Kates [13]. Cholesterol concentration was measured using a commercial kit (1.14830.001) from Merek (Germany).

Evaluation of the permeabilizing activity of the toxin in vesicles loaded with fluorophores.

The intensity the fluorophores released to the extravesicular media as a consequence of toxin activity was determined with a SLM AMINCO MC-200 spectrofluorometer (USA), according to the procedure described by Caeveiro et al [14]. The complete release of the probe was achieved with Triton X-100 at a final concentration of 0.1% (v/v).

Results and discussion

We used lipid monolayers and liposomal vesicles for a better understanding of the contribution of the main lipids in animal membranes in the interaction of St II with the membrane. Results are summarized in table 1.

The insertion of the toxin in lipid monolayers was characterized by the critical pressure (p_c), using the values of surface pressure on a biological membrane (35 mN.m⁻¹, 15), and the equilibrium pressure of the lipids in the monolayers (47 mN.m⁻¹, data not shown) as reference.

The lipid mixtures were classified in three groups: 1) mixtures lacking EM, where PC:Chol and FC:PE has p_c values < 35 mN.m⁻¹. 2) FC:EM and EM:FC:PE monolayers, with intermediate values for this parameter and 3) Monolayers containing both EM and Chol (EM:Chol:FC:PE and EM:FC:Chol) which displayed p_c values > 47 mN.m⁻¹.

The incorporation of the St II to the liposomal vesicles, constructed with the lipid mixtures under study, was assessed by monitoring the changes in fluorescence in Trp residues. Results were expressed as the relation between the fluorescence of the protein in the presence (F_b) or in the absence (F_f) of vesicles (Table 1). In every case (except for EYP:PE vesicles) the increase in fluorescence is proportional to the increase in the lipid concentration. This indicates a change toward a more hydrophobic environment of the average Trp residues of the toxin when this molecule is associated with vesicles. When mixed with BBS and Chol containing vesicles, the toxin showed an increase in fluorescence intensity that was higher than those observed with the EYP:BBS and BBS:PE treatments.

The time period for pore formation by the action of ST II in artificial membranes was evaluated through permeabilization experiments of LUV with ANTS/DPX. Kinetic data, obtained under conditions in which half of the vesicles were permeated, were used for the qualitative evaluation of channel formation by St II in the different vesicles under study. The toxin concentration required for this effect (C_50) was different in each case (Table 1): EM and Chol containing vesicles required very little toxin (25 mM) for 50% permeabilization in less than a minute; compared to the EYP:BBS control which reached the same proportion with 48 mM of St II.

On the other hand, vesicles composed of EYP and Chol only reached the same state 4 minutes after the start of the assay and with a toxin concentration of 82 nM. The treatment consisting of a binary mix EYP:PE was not able to reach 50% permeation in these vesicles at the highest concentration tested (1.16 mM).

In summary, these results demonstrate that the presence of BBS in the lipid membrane is not an absolute requirement but it favored the speed of pore formation by St II. The simultaneous presence of both BBS and Chol in the membrane significantly favors the association and permeating activity of St II. The binding of the protein to the membrane is especially favored under these conditions. The high values of critical pressure (p_c) in the lipid monolayers system are an indication of the enhanced capacity of these monolayers to accommodate the protein. This phenomenon seems to be related to the presence of BBS and Chol orderly complexes [16], stabilized through hydrogen bond formation between the amide group in BBS and the hydroxyl group of Chol.

Moreover, the unique characteristics of BBS and Chol containing vesicles, confers a higher capacity for membrane association to St II, and the pore is rapidly structured in these membranes with a relatively small amount of toxin. If the presence of BBS and Chol in lipid microdomains is responsible for the increase in the regions with the preferential toxin binding, the high association of St II, which takes place in these

Table 1. Effect of lipid composition on St II action

<table>
<thead>
<tr>
<th>Lipid Composition</th>
<th>π_c(mN.m⁻¹)</th>
<th>F_b/F_f</th>
<th>C_50(nM)</th>
<th>t_50(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYP:BBS (50:50)</td>
<td>44.9</td>
<td>1.24±0.07</td>
<td>48</td>
<td>60</td>
</tr>
<tr>
<td>EYP:Col (70:30)</td>
<td>31.8</td>
<td>1.07±0.01</td>
<td>82</td>
<td>240</td>
</tr>
<tr>
<td>BBS:EM:Col (50:15:35)</td>
<td>51.8</td>
<td>1.57±0.09</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>EYP:PE (70:30)</td>
<td>32.2</td>
<td>1.00</td>
<td>&gt;1160</td>
<td>=</td>
</tr>
<tr>
<td>BBS:EYP:PE (50:35:15)</td>
<td>43.6</td>
<td>1.25±0.03</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>BBS:Col:PE (50:35:15)</td>
<td>51.9</td>
<td>1.53±0.03</td>
<td>25</td>
<td>20</td>
</tr>
</tbody>
</table>

1 Critical pressure calculated by extrapolating to the X axis in the regression equation of a Δπ graph, as a function of the initial pressure of the monolayer. In all cases r² values were higher than 0.76.
2 Relationship between fluorescence intensities of bound (F_b) and free toxins (F_f) for each lipid mixture, estimated by the adjustment of the Boltzmann function of the data of increases in fluorescence with lipid concentration. In all cases r² were lower than 1.72 x 10⁻⁴.
3 Toxic concentrations required to permeate half of the vesicles in the assay.
4 Time to achieve permeabilization of half of the number of vesicles in the assay.
regions, could lead to a high local concentration and therefore a closer proximity with protein molecules. This could facilitate the cooperative oligomerization of the toxin to form the functional aggregate.

Phosphatidylethanolamine (PE) is the typical lipid inductor of the non-laminar phase, characterized by the formation of the hexagonal phase when mixed with EYP. It is a zwitterionic lipid and therefore its use in this study makes it possible to generalize the effect of non-laminar phase inducing lipids in St II activity.

It was corroborated that the inclusion of PE in vesicles containing EYP and BBS simultaneously, did not substantially modify the final insertion of the protein in the membrane; however it caused an increase in the speed of pore formation. On the other hand, the presence of PE in lipid systems composed exclusively by EYP, did not ensure a substantial binding of the toxin to the membrane, assessed by the critical pressure or the exposure of Trp residues to the aqueous media in the presence of vesicles. The latter behavior is very similar to the events experienced by the protein in solution.

Pore formation under these conditions could be considered irrelevant, possibly caused by the low binding ability of the protein to the bilayer.

In the BBS, Chol and PE containing mixtures, the effects of the three lipids were combined to reach a maximum toxin binding and membrane permeation. In a similar way BBS and Chol containing mixtures (BBS:Chol and BBS:EYP:Chol), conditioned a favorable St II accommodation, evidenced by the high p value in the monolayer system and the considerable change in the environment of Trp residues of the toxin molecule associated to the vesicular system. Those results were complemented with a good capacity for pore formation displayed by St II in BBS:Chol:PE vesicles.

From previous observations it can be inferred that the enhancing effect of EFE on St II activity in BBS containing lipid systems, is more related to the pore formation stage than to bilayer binding. The effect of these lipids on the toxin permeating capacity might be associated to a reorganization of lipids in the membrane, induced by the oligomerization of the toxin.

The structural region having a greater potential to span the lipid bilayer in an alpha helix conformation is located at the Nt segment and has a high homology with melitin and other cytolytic peptides. The diameter of the pore formed by St II, experimentally determined in the presence of osmotic protectors, is of approximately 2 nm [9], therefore its internal area will be of 2512 Å² in a 40 Å thick membrane.

Considering that the average diameter of a helix forming peptide is of approximately 1 nm, and Sticholysins-induced pore is formed by 3 or 4 monomers [8], it can be assumed that the contribution of the four helices to the inner surface of the pore is of only 1 600 Å². From here we can infer that the pore can not be made exclusively by four amphiphilic St II helices. Hence, it can be assumed that the inner surface of the St II pore is formed by the combination of the hydrophilic faces of amphiphilic helices of the toxin and the polar head of the membrane lipids. The channel opening induced by peptides could be linked to the formation of a toroidal lipid pore surrounding the protein structure [17]. Therefore, the lipid monolayer curves in a continuous way from outside to inside through a toroidal hole, so that the amphiphilic helices get stacked between the lipid polar heads and are perpendicularly oriented in relation to the bilayer plane (Figure 1).

A number of experimental evidences support the hypothesis of toroidal pore formation for Sticholysins. One of them is related to the demonstration of the transbilayer movement of lipids, once the toxin has been inserted in the membrane [18]. With the use of spin markers, located at different depths in the bilayer, it was demonstrated that the probe is not perturbed when located at position 16 of fatty acids, towards the centre of the bilayer. This was interpreted, under the light of the toroidal pore hypothesis [19], as a consequence of lipid reorganization in the presence of the protein, placing position 16 far from the contact with the polypeptide chain.

The toroidal pore hypothesis, applied to a protein toxin for the first time in our laboratory, has been confirmed in different ways by other researchers for other pore forming proteins from this [20] and other protein families. [21-24].

Among the possible applications of Sticholysins are the construction of immunotoxins against unwanted cells and their use as tools for the cytosol delivery of liposome encapsulated molecules. The conjugation of these proteins to monoclonal antibodies
specific for tumor associated antigens is the most promising alternative use of these toxins. Such is the case of conjugates obtained from monoclonal antibodies IorC5 and St I, with cytotoxic activity against SW948 human colon cancer [25]. Sticholysins have been cloned and expressed in Escherichia coli. Recently a recombinant form of St I was purified and characterized, as well as mutant St I W111C, with the aim of constructing unspecific immunotoxins. To efficiently form these immunotoxins, it is essential to understand the structure-function relationship of these proteins, its mode of action, particularly the molecular events involved in pore formation.

The contribution of the N-terminal region to the lytic mechanism pore formation by St II, the functional characteristics of the P1-30 peptide, reproducing the first thirty residues of the protein, were also studied. This peptide exhibits a 10^4 time higher hemolytic activity than St II, which seems to be related to the presence of a toxin in the phosphatidylcholine binding site described by Mancheño et al [7]. The radius of the pore generated in erythrocytes by P1-30 (0.95 ± 0.01 nm), was very similar to the one generated by the toxin (0.99 ± 0.01 nm) (26).

Interestingly, this peptide displayed antibacterial activity against Bacillus subtilis strains (Gram-positive), Pseudomonas aeruginosa and Escherichia coli (Gram-negative). This is the first report of a 3 kDa model peptide that can reproduce the pore forming activity of an actinoporin (20 kDa) and it inaugurates a new approach to the study of these proteins, through the use of small molecules mimicking their function. This type of compound could become a useful biotechnological tool.

 Acknowledgements

This research was financed by a MUTIS scholarship (Spanish Association for International Cooperation), a CITMA-CONCYIT project (Cuba-Chile), CNPq-MES project (Brasil), CNR, and Trentino Institute of Culture (Italy) and the IFS project No F/3773-1.
