REPORT

# *Membrane lipids act as modulators of the permeabilizing activity of Sticholysin II, a pore-forming protein with biomedical applications*

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***ABSTRACT***

**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 membrane 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 sphyngomyelin 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.**

## *I*ntroduction

Sticholysins are pore-forming cytolysins produced by the sea anemone *Stichodactyla helianthus*, widely distributed throughout Cuban shores. These proteins belong to the actinoporin family and display a high degree of sequence conservation, approximately 20kDa of molecular mass, a basic isolectric point, a predominant b-sheet secondary structure and the ability to form oligomeric pores in natural and model membranes [1]. Sticholysins exhibit a potent lytic activity in the picomolar range against erythrocytes from different mammalian species. Moreover these toxins present mild type A2 phospholipase activity and exert a potent cytotoxic effect against cells of the *Giardia duodenalis* parasite [2, 3]. Pore-forming toxins are unique molecules due to their capacity to spontaneously insert themselves into the membrane, forming hydrophilic channels.

Besides being considered important virulence factors or defensive systems developed by many organisms, Sticholysins are model systems when studying the processes of insertion and self assembly of proteins into membranes.

In recent years an extensive study on Sticholysins has been carried out, which includs their purification and molecular characterization [2], primary [4] and secondary structure determination [5, 6]; X ray resolution of their three-dimensional structure [7]; and the mechanism used for pore formation in the membranes [8, 9]. Other studies have demonstrated the effect of different environmental factors, such as ionic strength [10] and pH [11], on the conformation and lytic activity of these proteins.

The mechanism of action of actinoporins includes two main steps, which are mediated by different regions of the molecule. The initial attachment to the membrane is conducted through a patch of aromatic amino acids, exposed on the surface of the molecule. Afterwards the amphiphilic helix, located in the amino terminal segment, is transferred to the lipid interphase and enters into the membrane. Finally, four monomeric helices form the transmembranic pore [12].

Actinoporins are very potent toxins, affecting almost all eukaryotic cells tested so far. The reason for this apparent lack of specificity is that they use sphyngomyelin, a very ubiquitous lipid in animal cells, as a low affinity acceptor. In this study the influence of the main lipid components of membranes on the functional activity of a protein from the actinoporin family is assessed, using different membrane model systems, such as lipid monolayers and liposomes.

## *M*aterials 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 chloroform: methanol (2:1, v:v), was placed on the surface of Hepes buffer 10mM, NaCl 200mM, pH 7 (HBS), until the desired initial pressure was reached. Toxin (0.93 $mmol.L^{-1}$) was added at the subphase, and surface pressure changes were monitored at 25 °C under constant stirring.

### Fluorescent Spectroscopy

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

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### Production and characterization of liposomal vesicles

Lipid mixtures were prepared from chloroform: methanol solutions in the following lipids: egg yolk phosphatidylcholine (EYP), bovine brain sphyngomyelin (BBS), 1,2-dioleil-sn-glycero-3-phosphatidilethanolamine (PE) (Avanti Polar Lipids) and cholesterol (Sigma). The lipid mix was dried under vacuum in a rotoevaporator, 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 Merck (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 Caaveiro et al [14]. The complete release of the probe was achieved with Triton X-100 at a final concentration of 0.1% (p/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 FC:Chol and FC:FE has $p_c$ values < 35 mN.$m^{-1}$, 2) FC:EM and EM:FC:FE monolayers, with intermediate values for this parameter and 3) Monolayers containing both EM and Chol (EM:Chol:FE 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 ($F_L$) or in the absence ($F_o$) 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 hydrophilic 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:EYP:PE treatments.

Table 1. Effect of lipid composition on St II action

| Lipid Composition | $\pi_c$[1] (mN.$m^{-1}$) | $F_L/F_O$[2] | $C_{50}$[3] (nM) | $t_{50}$[4] (s) |
|---|---|---|---|---|
| EYP:BBS (50:50) | 44.9 | 1.24 ± 0.07 | 48 | 60 |
| EYP:Col (70:30) | 31.8 | 1.07 ± 0.01 | 82 | 240 |
| BBS:EYP:Col (50:15:35) | 51.8 | 1.57 ± 0.09 | 25 | 20 |
| EYP:PE (70:30) | 32.2 | 1.00 | > 1160 | ∞ |
| BBS:EYP:PE (50:35:15) | 43.6 | 1.25 ± 0.03 | 50 | 50 |
| BBS:Col:PE (50:35:15) | 51.9 | 1.53 ± 0.03 | 25 | 20 |

[1] Critical pressure calculated by extrapolating to the X axis in the regression equation of a $\Delta\pi$ graph, as a function of the initial pressure of the monolayer. In all cases $r^2$ values were higher than 0.76.

[2] Relationship between fluorescence intensities of bound ($F_L$) and free toxins ($F_0$) for each lipid mixture, estimated by the adjustment of the Boltzman function of the data of increases in fluorescence with lipid concentration. In all cases $\chi^2$ were lower than 1.72 x $10^{-4}$.

[3] Toxin 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.

In contrast, the toxin showed very few changes in the environment of Trp residues when mixed with EYP:Chol containing vesicles.

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 nM) for 50% permeabilization in less than a minute; compared to the EYP:BBS control which reached the same proportion with 48 nM 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 ($\pi_c$) in the lipid monolayers system are an indication of the enhanced capacity of these monolayers to accommodate the proteins. 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

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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 $p_c$ 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 $Å^2$ 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 helixes to the inner surface of the pore is of only 1 600 $Å^2$. From here we can infer that the pore can not be made exclusively by four amphiphilic St II helixes. 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 helixes 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 helixes 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

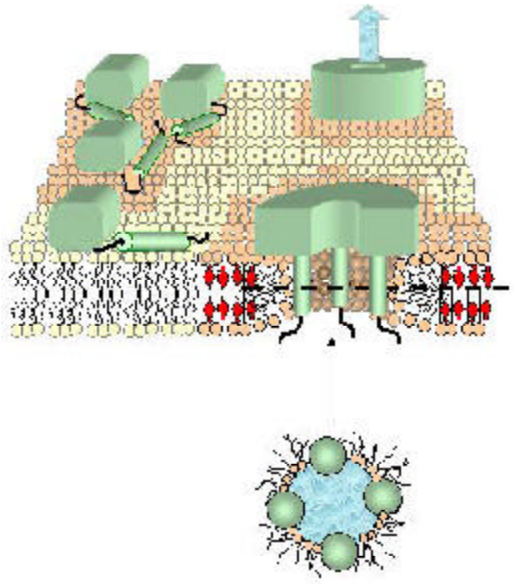

Figure 1. Schematics of the toroidal pore generated by Sticholysins. The toxin is represented by an amphiphilic helix resembling a cylinder. Spheres represent phospholipid polar heads, and the lines are the acyclic chains. Cholesterol molecules are represented in black. The toxins are preferentially associated to the membrane by EM and Chol microdomains, oligomerizing and forming the tetrameric pore. During these processes, the lipid membrane is reorganized originating the toroidal lipid pore, where the lipid inductors of the non laminar phase are distributed between amphiphilic helixes, forming the walls of the pore (lower view). Helixes are arranged with hydrophilic faces toward the pore lumen and the hydrophobic faces in contact with hydrocarbonate lipid chains.

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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 immunoconjugates, 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 B*acillus 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.

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