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Sticholysin I–membrane interaction: An interplay between the presence of sphingomyelin and membrane fluidity

Lohans Pedrera [a], Maria Laura Fanani [b,*], Uris Ros [a], María E. Lanio [a], Bruno Maggio [b], Carlos Álvarez [a,**]

[a] Centro de Estudio de Proteínas, Facultad de Biología, Universidad de la Habana, CP 10400 La Habana, Cuba
[b] Departamento de Química Biológica, Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC), Facultad de Ciencias Químicas-CONICET, Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina

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ABSTRACT

Sticholysin I (St I) is a pore-forming toxin (PFT) produced by the Caribbean Sea anemone *Stichodactyla helianthus* belonging to the actinoporin protein family, a unique class of eukaryotic PFT exclusively found in sea anemones. As for actinoporins, it has been proposed that the presence of sphingomyelin (SM) and the coexistence of lipid phases increase binding to the target membrane. However, little is known about the role of membrane structure and dynamics (phase state, fluidity, presence of lipid domains) on actinoporins' activity or which regions of the membrane are the most favorable platforms for protein insertion. To gain insight into the role of SM on the interaction of St I to lipid membranes we studied their binding to monolayers of phosphatidylcholine (PC) and SM in different proportions. Additionally, the effect of acyl chain length and unsaturation, two features related to membrane fluidity, was evaluated on St I binding to monolayers. This study revealed that St I binds and penetrates preferentially and with a faster kinetic to liquid-expanded films with high lateral mobility and moderately enriched in SM. A high content of SM induces a lower lateral diffusion and/or liquid-condensed phases, which hinder St I binding and penetration to the lipid monolayer. Furthermore, the presence of lipid domain borders does not appear as an important factor for St I binding to the lipid monolayer.

1. Introduction

Pore forming toxins (PFTs) play an active role in the defense systems of different kingdoms of life [1,2]. Besides its biological relevance, pore formation in lipid membrane by PFT has received special attention as model systems to understand basic molecular mechanism of protein insertion into membranes. Actinoporins are highly basic proteins, of a single polypeptide chain, with molecular weight around 20 kDa exclusively produced by sea anemones whose putative receptor is SM [2,3]. They are classified as α-PFT because their mechanism of pore formation involves the insertion of the N-terminal α-helix in the membrane [4,5]. St I is an actinoporin of 176 amino acid residues purified from the Caribbean Sea anemone *Stichodactyla helianthus* which displays a hemolytic activity at rather low concentrations [6].

The mechanism of pore formation proposed for actinoporins is based on an initial binding step followed by oligomerization and membrane insertion leading to pore formation [7]. For actinoporins, it has been proposed that binding to membranes is favored both by the presence of SM [3,8–13] and/or the coexistence of lipid phases [14–17]. However, the role of membrane structure and dynamics on actinoporins' initial binding or which regions of membrane are the most favorable platforms for protein recognition are not well understood. In fact, based on indirect evidence it was proposed that lipid packing defects arising at the interface between coexisting lipid phases may function as preferential binding sites for equinatoxin II (Eqt II), an actinoporin from *Actinia equina* [14]. However, there is no robust experimental data regarding the preferential membrane localization of sticholysins or in a more general sense it has not been described how the lipid molecular packing or acyl chain alignment affects the initial binding of actinoporins to membranes. In particular, the influence of phospholipid acyl chain length and unsaturation, two features related to membrane fluidity, have not been systematically studied in the effect of the actinoporin family.

Abbreviations: St I, sticholysin I; St II, sticholysin II; PFT, pore-forming toxin; SM, sphingomyelin; PC, phosphatidylcholine; Eqt II, equinatoxin II; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; eSM, egg sphingomyelin; SM 18:0, N-stearoyl sphingomyelin; SM 18:1, N-oleoyl sphingomyelin; BAM, Brewster angle microscopy; FM, fluorescence microscopy; FRAP, fluorescence recovery after photobleaching; DMPC, 1,2-dimyristoyl phosphatidylcholine; DPPC, 1,2-dipalmitoyl phosphatidylcholine; DSPC, 1,2-distearoyl phosphatidylcholine; DAPC, 1,2-diaraquidoyl phosphatidylcholine; DOPC, 1,2-dioleoyl phosphatidylcholine; Rho-PE, L-α-phosphatidylethanolamine-N-(lissaminerhodamine B sulphonyl) ammonium salt egg transphosphatidylated; NBD-DPPE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine-triethyl ammonium salt; π, surface pressure; π_0, initial surface pressure; Δπ, increment in surface pressure; V_0, initial maximal rate; Cs^{-1}, compressibility modulus; MMA, mean molecular area; LUV, large unilamellar vesicles; Le, liquid expanded; Lc, liquid condensed; TBS, Tris-buffered saline

* Corresponding author. Tel.: +54 351 4334168; fax: +54 351 4334074.
** Corresponding author. Tel.: +54 537 8324830; fax: +54 537 8321321.
E-mail addresses: lfanani@fcq.unc.edu.ar (M.L. Fanani), calvarez@fbio.uh.cu (C. Álvarez).

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In the present study, we used lipid monolayers as a model membrane to study the first step of St I binding to membrane. The combination of monolayer compression isotherms, Brewster angle microscopy (BAM) and fluorescence recovery after photobleaching (FRAP) experiments provided detailed information on the structural and rheological properties of the monolayers and allow correlating them with the affinity of St I for lipid films. Additionally, fluorescence microscopy (FM) imaging revealed the preferential localization of St I in different lipid phases. These results provided further insight into the initial binding and recognition of St I to lipid membrane, and reveal that the toxin association to model membranes is a result of a subtle interplay between the presence of SM and membrane fluidity.

2. Materials and methods

2.1. Chemicals and reagents

St I was purified from the sea anemone *S. helianthus* by combining gel filtration chromatography on Sephadex G-50 medium (Pharmacia-LKB, Sweden) and cationic exchange chromatography on CM-cellulose 52 (Whatman, Maidstone, UK) as previously described [6]. Lipids were from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The lipophilic fluorescent probes Rho-PE and NBD-DPPE, and the amine-reactive probe Alexa Fluor 488 carboxylic acid succinimidyl ester were purchased from Invitrogen (Eugene, OR). Solvents and chemicals were of the highest commercial purity available. Water was purified by a Milli-Q system (Millipore, Billerica, MA), to yield a product with a resistivity of ~18.5 $M\Omega \cdot cm^{-1}$. The absence of surface-active impurities was routinely checked as described elsewhere [18].

2.2. Binding of St I to lipid monolayers

Surface pressure (π) measurements were carried out with a μTrough-S system (Kibron, Helsinki, Finland) at 23 ± 2 °C under constant stirring. The subphase consisted of 300 μL of Tris-buffered saline (TBS: 145 mM NaCl, 10 mM Tris–HCl, pH 7.4). Lipid monolayers were formed by spreading a lipid chloroformic solution over the surface to attain an initial surface pressure (π_0) of ~20 $mN \cdot m^{-1}$. After 5 min for allowing solvent evaporation, St I was injected into the subphase to achieve 0.8 μM protein final concentration. Similar to St II [17], at this subphase concentration St I has no effect on surface tension of the air–buffer interface. The increment in surface pressure (Δπ) was recorded as a function of time until a stable signal was obtained. The initial maximal rate (V_0) was evaluated within the first 10 s of toxin penetration experiments, where a linear relationship of π with time was observed.

2.3. Monolayer compression isotherms

Compression isotherms were obtained for pure or mixed lipid monolayers by spreading 25 μL of a chloroformic solution onto a Teflon through filled with 145 mM NaCl, pH 5.6. The film was relaxed for 5 min at 0 $mN \cdot m^{-1}$ and subsequently compressed at a rate of 1 $Å^2 \cdot molecule^{-1} \cdot min^{-1}$ to the collapse pressure. π and the film area were continuously measured and recorded with a KSV Minitrough equipment (KSV, Helsinki, Finland). The phase transition points were estimated by the third derivate method [19]. In order to compare the phase state and the rheology properties of the film, the compressibility modulus (Cs^{-1}) was calculated at 20 $mN \cdot m^{-1}$ from the isotherm data as [19]:

$$Cs^{-1} = -MMA\left(\frac{\partial \pi}{\partial MMA}\right)_T \quad (1)$$

where *MMA* is the mean molecular area of the lipid monolayer at 20 $mN \cdot m^{-1}$ at a constant temperature (*T*).

2.4. Brewster angle microscopy of lipid monolayers

The monolayers were observed while compressed using BAM. A KSV Minitrough equipment (KSV, Helsinki, Finland) was placed on the stage of an autonulling Nanofilm EP3 Imaging Elipsometer (Accurion, Gottingen, Germany) used in the BAM mode. Zero reflection was set with a polarized laser (λ = 532 nm) incident on the bare aqueous surface at the experimentally calibrated Brewster angle (≈53.1°). After monolayer formation and compression, the reflected light was collected with a 20× objective.

2.5. Fluorescence recovery after photobleaching assay

FRAP experiments were performed using a confocal Olympus FV1000 microscope equipped with a CCD camera. The lipid mixture containing the fluorescent probe NBD-DPPE (1 mol%) was spread over a surface of TBS, until reaching a π_0 of ~20 $mN \cdot m^{-1}$. Afterwards, the subphase level was reduced to ~1 mm thickness to minimize convection. Before bleaching, a stack of five images was scanned to record the pre-bleach fluorescence. The bleaching spot was set by a circular region of 20 μm diameter illuminated at maximal potential for 15 s using a 20× objective. The fluorescence recovery was recorded in a series of images with a temporal spacing of 1.2 s. In order to characterize the lateral diffusion of the lipid in the monolayers we calculated the $t_{3/4}$ parameter which is the time at 75% recovery, by fitting the normalized fluorescence ($f_{(t)}/f_{(0)}$) *vs* time (*t*) to the single exponential function:

$$\left(f_{(t)}/f_{(0)}\right) = a - bc^t \quad (2)$$

where *a*, *b*, and *c* are the fitting coefficients of the exponential.

2.6. Conjugation of St I with Alexa dye

Conjugation of St I with the dye was performed in 100 mM $NaHCO_3$ (pH 8.3) buffer. St I was incubated with the amine-reactive dye Alexa Fluor 488 carboxylic acid succinimidyl ester, which was dissolved in dimethylsulfoxide and immediately added to the protein solution. The final dimethylsulfoxide dilution in the examined sample was 1:10. The reaction mixture was incubated for 1 h in the dark at room temperature under constant stirring. The conjugated protein was separated from the unreacted dye by filtering through Amicom Ultra 0.5 10 K filter (Millipore Corp., USA). The obtained conjugate had an average ratio of 2:1 Alexa/St I (calculated from absorbance measurements). The Alexa-St I conjugate was 1:5 diluted with unlabeled toxin and stored in the dark at 4 °C until use. The activity of the toxin mixture was similar to the unlabeled toxin as demonstrated by a hemolytic assay described elsewhere [20].

2.7. Visualization of St I-Alexa conjugate bound to supported monolayers

A combined Langmuir–Blodgett/Langmuir–Schaefer technique was used to visualize Alexa-St I insertion to lipid monolayers [21,22]. A first Langmuir–Blodgett transference of DPPC monolayer (at 30 $mN \cdot m^{-1}$) to clean glass coverslips was performed in a KSV Minitrough equipment (KSV, Helsinki, Finland) with the aim of obtaining an hydrophobic surface: after DPPC monolayer formation the film was transferred to the glass coverslips by moving it vertically upwards at a rate of 3 $mm \cdot min^{-1}$, and keeping automatically a constant π. Then, a second lipid monolayer was added by the Langmuir Schaefer method as previously described [22]. Briefly, after forming the lipid-Rho-PE (1 mol%) film at a π_0 of 20 $mN \cdot m^{-1}$ in a 1 mL circular compartment of a μTrough-S equipment (Kibron, Helsinki, Finland) the 1:5 mixture of Alexa-St I/unlabeled St I was injected into the subphase to reach a final concentration of 0.8 μM. When a stable π signal was obtained, the hydrophobic coverslip was lowered horizontally until touching the film. The