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The pore forming capacity of Sticholysin I in dipalmitoyl phosphatidyl vesicles is tuned by osmotic stress

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ABSTRACT

The osmotic condition modulates the properties of liposomes, particularly those related to their stability and response to external agents such as membrane-active proteins or peptides. In a previous work, we have demonstrated that an osmotic shock can increase, *per se*, water influx/efflux and the exit of the fluorophore calcein entrapped in the aqueous pool of dipalmitoylphosphatidylcholine (DPPC) and DPPC: sphingomyelin (SM) large unilamellar vesicles (LUVs), suggesting a loss of integrity of the liposome bilayer. In the present work, we have extended our study in order to assess how an osmotic imbalance prior to or synchronous with the addition of a recombinant variant of the pore-forming toxin sticholysin I (rSt I) modifies its pore forming capacity in DPPC and DPPC:SM (1:1) LUVs. Our results conclusively show the capacity of hypotonic gradients to improve the pore forming capacity of rSt I molecules, even in pure DPPC liposomes, rendering pore-formation less dependent on the presence of sphyngomyelin. In fact, non-active toxins in DPPC liposomes become active by a hypotonic imbalance in a similar way to those containing SM as a second component.

1. Introduction

Actinoporins comprise a protein family of very potent cytolysins isolated from the venom of sea anemones (Kem and Dunn, 1988). In particular, the anemone *Stichodactyla helianthus*, widely distributed in the Caribbean Sea, produces a venom that contains at least two cytolysins (Kem and Dunn, 1988) named sticholysin I (St I) and sticholysin II (St II) exhibiting molecular masses around 20 kDa, basic properties and high affinity for sphingomyelin (SM)-containing membranes (Alvarez et al., 2009; Lanio et al., 2001). These toxins can generate pores of ca. 2 nm diameter both in cells (erythrocytes) and model membranes (Tejuca et al., 2001). The efficiency and rate of pore formation in large (LUVs) and small unilamellar vesicles (SUVs) by actinoporins increase when SM is included into the liposomes (Bernheimer and Avigad, 1976; Bonev et al., 2003; Caaveiro et al., 2001; Martinez et al., 2007; Schon et al., 2008; Tejuca et al., 1996) reaching the optimum activity at equimolar concentrations of phosphatydylcholine (PC) and SM (Tejuca et al., 1996). This positive effect of SM in toxins activities has been attributed to a negative curvature in the bilayer (Alvarez et al., 2009; Tanaka et al., 2013; Valcarcel et al., 2001) and to an increased adsorption of the toxin to the vesicles (Bakrač and Anderluh, 2010; Mui et al., 1994). Also, it has been proposed that the presence of lipidic microdomains enriched in cholesterol and/or sphyngomyelin could contribute to the pore forming capacity of actinoporins (Barlic et al., 2004; Martinez et al., 2007; Schon et al., 2008). In contrast, a high efficacy of binding and permeabilization by St I in sterol-containing membranes independently of their ability to form domains was observed and this was mainly explained by the molecular heterogeneity, fluidity, and negative curvature of the bilayer induced by the sterols in membranes of PC and SM (Valcarcel et al., 2001).

The pore forming capacity of actinoporins in model membranes and their haemolytic activity can be modified by a variety of factors such as ionic strength (Alvarez et al., 1998), temperature (Martinez et al., 2001), fluidity (Pedrera et al., 2014; Tomita et al., 1992), lipidic composition (Alvarez et al., 2003; Caaveiro et al., 2001; Tejuca et al., 1996; Valcarcel et al., 2001), pH (Alvarez et al., 2001), size of the liposomes (Tejuca et al., 1996), and surfactant addition (Lanio et al., 2002, 2007). On the other hand, a variety of studies have been performed in order to determine how the presence of an osmotic gradient modulates membrane properties in liposomes *per se* (Ahumada et al., 2015; Mui et al., 1993; Abuin et al., 1995) or in the presence of a variety of pore forming peptides or toxins such

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as plasma (Mui et al., 1994); imipramine (Ahyayauch et al., 2004); Class L amphipathic helical peptides (Polozov et al., 2001); Amphotericin B (Ruckwardt et al., 1998; Wolf and Hartsel, 1995); Melittin (Benachir and Lafleur, 1996); Sendai virus (Kundrot et al., 1983); and *Bacillus thuringiensis* toxins (Kirouac et al., 2006), but none of them with actinoporins. All these systems, in spite of their large differences, give similar results in the presence of osmotic unbalances: i) an increase in the rate and extent of pore formation; ii) the capacity to be active even in sterol-free liposomes; and iii) the largest effect takes place for *trans*-bilayer hypotonic gradients (Wolf and Hartsel, 1995).

In a previous work (Ahumada et al., 2015), we have shown that an osmotic shock can increase, *per se*, water influx/efflux and the exit of calcein entrapped in the aqueous pool of DPPC and DPPC:SM LUVs, suggesting a loss of integrity of the liposome bilayer. The response to a hypotonic imbalance was faster and more important than that associated to a hypertonic stress, being this difference particularly noticeable for calcein efflux. In the present work, we extend our study in order to assess how an osmotic unbalance prior to or synchronous with the addition of a recombinant St I (rSt I), modifies the pore forming capacity of this toxin in DPPC LUVs, lipid which under our current knowledge, no activity of rSt I has been reported whatsoever in order to render the pore formation less dependent on SM, particularly in the presence of hypotonic gradients.

2. Experimental

2.1. Chemicals and reagents

1,2-dipalmitoyl–sn–glycero–3–phosphatydylcholine (DPPC) and porcine brain sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 3,3-bis[*N,N*-bis(carboxymethyl)-aminomethyl] fluorescein (calcein), 1,6-diphenyl-1,3,5-hexatriene (DPH),N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien) phenylammonium *p*-toluenesulfonate (TMA-DPH) and Sephadex G-25 were acquired from Sigma-Aldrich (St. Louis, MO, USA). All these reagents were employed as received.

2.2. rSt I characteristics

Recombinant St I (rSt I) was obtained as previously described by Pazos (Pazos et al., 2006). It differs from the wild type (St I) in a single amino acid residue (E16Q). This pseudo-wild type is structurally similar to St I and shows an equivalent capacity to interact with and form pores in erythrocytes and model membranes (DPPC:SM LUVs).

2.3. Preparation of phospholipid vesicles

Large unilamellar vesicles (LUVs) were prepared by adding into a round-bottom flask a known amount of DPPC or DPPC:SM (1:1 molar ratio) dissolved in chloroform. The solvent was removed by slow evaporation at 40 °C in a water bath under gentle vacuum. Resuspension of the film was carried out in TRIS buffer (20 mM Tris-(hydroxymethyl) aminomethane, 140 mM NaCl, 1 mM EDTA, pH = 7.0) or TRIS buffer plus 80 mM calcein, prepared using ultrapure water obtained from a Modulab Type II equipment (Biostad, Québec, Canada). The suspensions were subjected to ten freeze-thaw cycles, transferred to a home-made extruder, and extruded 10 times through polycarbonate filters (400 nm nominal pore size, Nucleopore®polycarbonate, PCI Scientific, NJ, USA) at 52 °C, standard procedure that allow a fluid passage of the liposomes through the extruder membrane and maximize the formation of unilamellar liposomes (Cho et al., 2013; Hwang et al., 2012; Walde and Ichikawa, 2001), providing a liposomal ensemble with a narrow size distribution, with an average size of 200 nm. External calcein was removed by eluting the suspension through a column containing Sephadex G-25. The final lipid concentrations of liposomal stock suspensions were determined by phosphate titration, as reported by Stewart (Stewart, 1980). The values obtained were 1.0 and 0.64 mg/mL prior and after elution, respectively.

2.4. Osmotic shocks to phospholipid vesicles

Stock solutions of liposomes (0,64 mg/mL) with entrapped calcein were diluted till the desired analytical lipid concentrations. Osmotic gradients across the liposome bilayer were generated by employing different media as external solvent: TRIS buffer (isotonic), ultrapure water (hypotonic) and TRIS buffer containing 1.4 M NaCl (hypertonic). Note that pH in buffer conditions was 7.0, and in hypotonic media, ultrapure water had a pH value around 6.9. Furthermore, previous work have established that differences in the values near to neutral pH do not affect the binding and hemolytic activity of stycholysins (Alvarez et al., 2001). After a fast manual mixing, dye (calcein) efflux was estimated by the change in fluorescence intensity (Ahumada et al., 2015; Maherani et al., 2013). Once the fluorescence intensity reached a plateau, rStI was added to a 3 mL quartz cuvette. Kinetics profiles of calcein fluorescence intensity (excitation wavelength 485 nm; emission wavelength 538 nm) were performed at 25 °C in a Shimadzu RF-5301 PC spectrofluorometer (Kyoto, Japan). Assays were carried out in duplicate or triplicate and the fluorescence intensity values showed a variation of less than 10%. Association of the toxin to the bilayers was evaluated by following the change on the intrinsic rSt I fluorescence elicited by liposome addition (λ_{ex} = 295 nm and λ_{em} = 333 nm). A steady value of the protein fluorescence was achieved in few minutes. Fluorescence intensities were corrected to take into account dilution effects and the turbidity of the samples (Pazos et al., 2003).

2.5. Steady-state anisotropy measurements

Fluorescence anisotropy measurements were performed in a phase shift and modulation I.S.S. K2 spectrofluorometer interfaced to personal computers employing I.S.S. software. All measurements were carried out in 1 cm path-length quartz cuvettes whose temperature was controlled by an external Cole Palmer bath circulator. The actual temperature was measured in the sample cell before and after each determination using an Omega digital thermometer. Polarization measurements were done in the "L" configuration using Glan-Thompson prism polarizers in both excitation and emission beams. Excitation wavelength was set at 360 nm. The emission was filtered through a WG-420 Schott high pass filter of negligible fluorescence.

3. Results and discussion

3.1. Adsorption of rSt I to LUVs

Evaluation of rSt I adsorption to LUVs was carried out at 25 °C by following the changes in toxin intrinsic fluorescence elicited by LUVs addition to a toxin solution, keeping constant the pertinent osmotic unbalance and the rSt I concentration. Generally, toxins that lack a pore forming capacity towards liposomes comprising a single phospholipid (such as DPPC) become active when a particular second component, such as a sterol or SM, is included in the liposomal preparation (Dalla Serra et al., 1999; Tejuca et al., 1996; Valcarcel et al., 2001). This activity could be promoted by an increase in the amount of toxin associated to the liposomes. In fact, if the presence of SM enhances the association of sticholysins to PC

LUVs, it could favour the competent organization of the toxin (Martinez et al., 2007; Tejuca et al., 1996). Fig. 1, shows titration curves obtained employing DPPC and DPPC:SM LUVs in iso-, hypo- and hypertonic conditions.

It is observed that SM presence into the liposomes drastically reduces the lipid concentration that must be added to reach a plateau in the intrinsic rSt I fluorescence intensity vs analytical lipid concentration plot. Furthermore, at low lipid concentrations the plot of F°/F vs lipid concentration is almost linear up to the plateau, suggesting a total incorporation of the toxin and, hence, a high affinity for the liposomes (saturation behaviour). The slope of the line provides an estimation of the number of lipid molecules needed to sustain a toxin (Alvarez et al., 2001; Lanio et al., 2002). On the other hand, a different behaviour is observed when DPPC liposomes under hypotonic conditions are employed (Fig. 1). In this system, the Langmuir-like behaviour observed in the absence of SM implies a smaller affinity of the toxin towards the liposomes and allows an evaluation of the lipid concentration $[M]_{1/2}$ required to bind half of the added toxin and, hence, the equilibrium binding constant

$$K_b = 1/[M]_{1/2} \quad (1)$$

The data collected in Table 1 show that the hypotonic shock, as well as the incorporation of SM to the LUVs, increases rSt I binding to liposomes, as it has been reported in closely related systems employing EqT II (Maček et al., 1994). Parallel to this increase in affinity, osmotic shocks reduce F°/F values measured under conditions of almost total adsorption (See Fig. 1 and Table 1). Furthermore, adsorption of the toxin by LUVs renders a small but measurable shift of the protein fluorescence towards the blue, indicating a moderate change in Trp groups localization when the toxin migrates from the aqueous solution to the liposomes.

The data of this Table allow concluding that:

(i) The presence of SM and osmotic unbalances cooperatively favours the association of r St I to the liposomal ensemble. However, SM addition under isotonic conditions produces only a minor change in rSt I binding that can not explain the noticeable effect of SM on rSt I pore forming capacity.
(ii) In all the systems, the association of St I to the liposomes produces at most a modest shift of its Trp intrinsic fluorescence towards the blue. This suggests that the association to the liposomes does not change significantly the surroundings of emitting Trp groups.

Taking together these data would indicate that the incorporation of SM and osmotic imbalances are factors that maximize the incorporation of the toxin to the liposomes and could explain, at least partially, the high efficiency of rSt I pore formation in these systems.

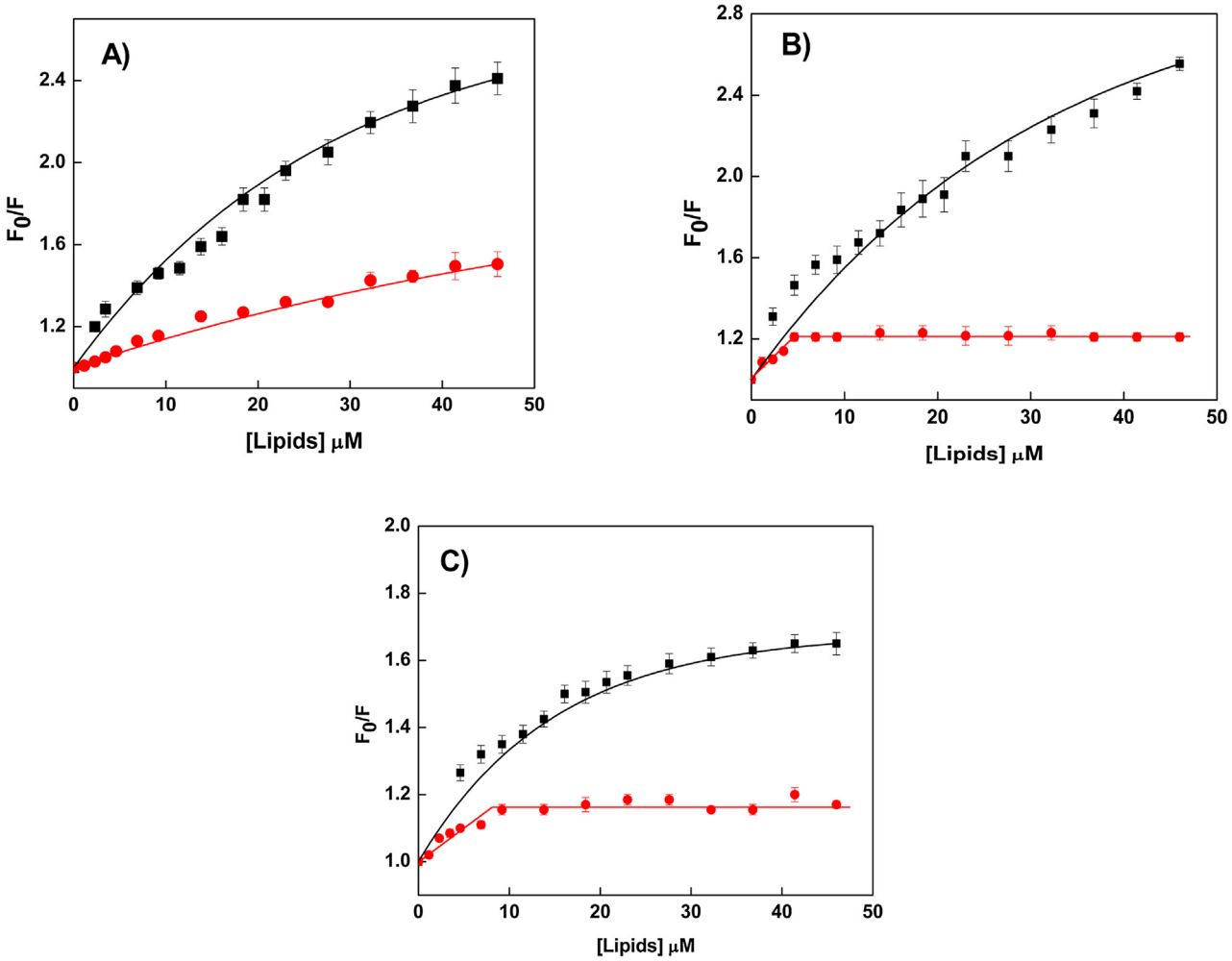

Fig. 1. Change in rStI (500 nM) intrinsic fluorescence intensity as a function of the analytical lipid concentration under osmotic stress conditions. LUVs of DPPC (■) and DPPC: SM 1:1 (●). A) isotonic; B) hypotonic; C) hypertonic. F_0 corresponds to the toxin fluorescence in absence of lipids and F is the fluorescence at the given lipid concentration. All measurements were carried out at 25° C in triplicate.

Table 1
Adsorption of rSt I (500 nM) to DPPC and DPPC:SM LUVs, under differents osmotic gradients (25° C).

Property	DPPC Iso	DPPC/SM Iso	DPPC hypo	DPPC/SM hypo	DPPC hyper	DPPC/SM hyper
***n* at saturation**[a]	–	–	–	8	–	20
K_b[b]	≈ 0.025	0.05	0.067	–	0.10	–
F°/F_∞[c]	2.4	1.5	2.6	1.2	1.6	1.2
$\Delta\lambda$[d]	1.5	1.0	0	1.5	0.5	0.5
Behaviour	Langmuir	Langmuir	Langmuir	Saturation	Langmuir	Saturation

*Data obtained from experiments carried out in triplicate (Fig. 1) with a variation lower than 10%.
[a] *n*: Minimum number of lipids required to sustain a toxin molecule.
[b] K_b: Equilibrium constant for the association of rSt I to LUVs (in M^{-1}).
[c] F°/F_∞:Quotient between the fluorescence of the free toxin and that totally bound to the liposomes.
[d] $\Delta\lambda$: Shift in the wavelength of maximum fluorescence intensity (measured in nm) when the toxin location changes from the external solvent to the liposome.

Table 2
DPH and TMA-DPH fluorescence anisotropy in LUVs submitted to different osmotic gradients.

LUVs	DPH	TMA-DPH
DPPC:SM isotonic	0.305 ± 0.005	0.335 ± 0.004
DPPC isotonic	0.325 ± 0.004	0.330 ± 0.003
DPPC:SM hypotonic	0.375 ± 0.005	0.335 ± 0.004
DPPC hypotonic	0.340 ± 0.003	0.360 ± 0.005
DPPC:SM hypertonic	0.340 ± 0.004	0.315 ± 0.004
DPPC hypertonic	0.340 ± 0.005	0.350 ± 0.004

Values given are averages of three independent determinations carried out at 25 °C.

Another possible explanation for the lack of rSt I activity in isotonic conditions and in the absence of SM could be a large difference in the bilayer microviscosity, as proposed for other toxins (Shimanouchi et al., 2009; Tomita et al., 1992). In order to test this possibility, we measured DPH and TMA-DPH anisotropy in the DPPC and DPPC:SM LUVs in isotonic and hypotonic conditions. The data are collected in Table 2.

The data of this table show only small differences in the anisotropy value of the probes. These differences are not enough to explain the large difference in pore forming activities elicited by SM incorporation to isotonic DPPC liposomes.

3.2. Activity of rSt I in DPPC:SM liposomes under hypo and hyperosmotic conditions

In order to assess the pore forming capacity of rSt I in the presence of osmotic gradients the toxin was added to LUVs ensembles comprising DPPC or DPPC:SM that have been previously submitted to osmotic unbalances. Calcein release experiments were monitored at 25 °C to facilitate the evaluation of the dye release elicited by the osmotic shocks and/or the rSt I addition, minimizing the leakage induced by the enhanced membrane fluidity, usually observed at temperatures near or above the Tm of the selected lipids (~42 °C) (Shimanouchi et al., 2009). Typical results are shown in Fig. 2.

In agreement with previous results the osmotic unbalance *per se* promotes the partial release of entrapped calcein, most likely through transient pores (Ahumada et al., 2015). As expected, addition of rSt I (200 nM) increases the liberation of calcein in the liposomes containing SM in any conditions, but this response is faster and more important for LUVs exposed to a negative osmolarity (external minus internal, hypo-osmotic conditions) (Fig. 2B). In these conditions, almost total release of calcein is achieved in few seconds, and the rate and extent of the process are dependent upon the osmolarity gradient and toxin concentration. The value of X% (percentage of entrapped calcein liberated by the toxin after a long incubation time, ca. 50 min) as a function of the toxin concentration is shown in Fig. 3, and t_{50} values (time required to release 50% of the entrapped calcein) are collected in Table 3, in both cases under hypo-osmotic conditions. Regarding the pre-exposure of liposomes to a hypertonic shock, there is a small enhancement of rSt I promoted leakage of calcein only in DPPC:SM liposomes (Fig. 2B).

In order to assess the efficiency of rSt I acting on hypo- and hyper-osmotically stressed LUVs ensembles, X and t_{50} values were measured as a function of the external NaCl concentration at a fixed toxin concentration (200 nM). The results are shown in Fig. 4, where t_{50} values corresponds to the time required to permeabilize

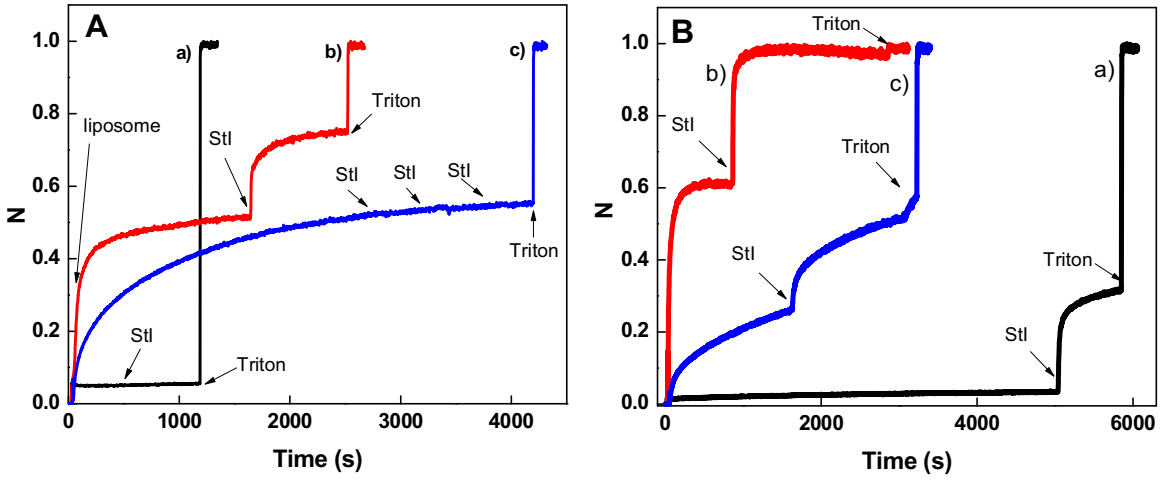

Fig. 2. Fraction of calcein released from the LUVs (N) plotted as a function of time. Arrows indicate the times at which the liposomes or rSt 1 (200 nM) are added. A corresponds to DPPC vesicles. B corresponds to DPPC:SM vesicles. Data include results obtained in isotonic (a); hypotonic (b) and hypertonic (c) conditions.

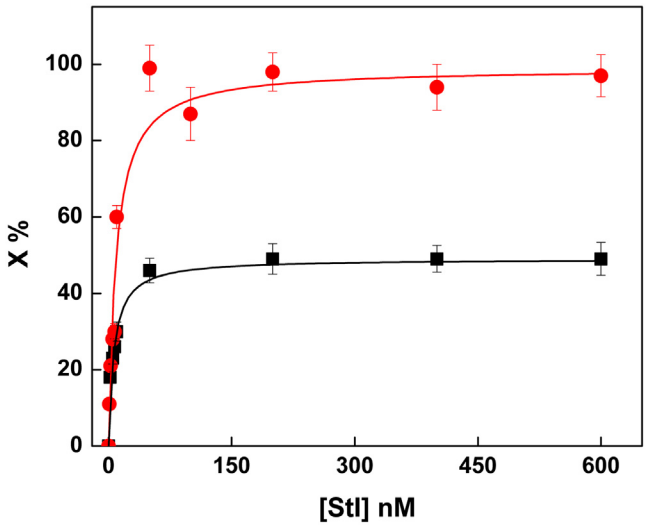

Fig. 3. Effect of rSt I concentration on calcein exit from the liposomes previously exposed to a hypotonic osmotic shock. LUVs of DPPC (■) and DPPC:SM 1:1 (●). Data values given are averages of three independent determinations carried out at 25 °C. X corresponds to the percentage of entrapped calcein liberated by the toxin after a long incubation time, ca. 50 min.

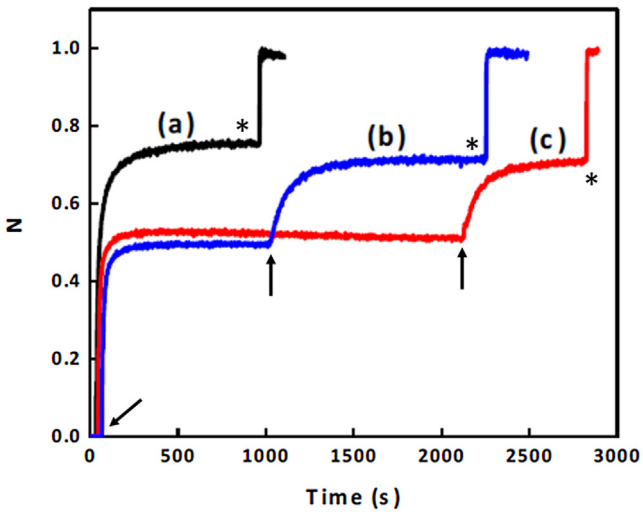

Fig. 5. Effect of rSt I (7 nM) addition to hypotonically stressed DPPC:SM liposomes. Plots obtained for calcein exit in assays carried out after different incubation times. (a) Simultaneous addition (t = 0) of rSt I and the hypotonic shock; (b) addition of rSt I 1000 s after hypotonic shock; (c) addition of rSt I 2100 s after hypotonic shock. Asterisks indicate the time at which Triton X-100 was added to the liposomal suspensions and arrows indicate the time of rSt I addition for each experiment.

Table 3
Effect of rSt I on the rate of calcein exit from liposomes previously exposed to a hypotonic osmotic shock (25 °C).

	t_{50} (s)	
[StI] (nM)	DPPC	DPPC:SM
200	62 ± 4.3	3 ± 0.3
100	63 ± 4.7	5 ± 0.3
50	66 ± 5.1	5 ± 0. 2
10	83 ± 6.4	<3 ± 0.1
7.5	145 ± 9.0	<3 ± 0.3
5	242 ± 18.2	<3 ±0.2
2.5	257 ± 13.8	4 ± 0.4
1	–	8 ± 0.4

Values given are averages of three independent determinations.

50% of the intact liposomes remaining at the moment of rSt I addition.

rSt I pore forming activity increases when the liposomes are stressed by a hypotonic shock (Fig. 4). This is evidenced both in smaller t_{50} times and larger X% values. Conversely, the hypertonic shock gives X% values similar to those of the isotonic liposomes, irrespective of the magnitude of the osmotic imbalance. In fact, in the more drastic conditions employed in the present work, the osmotic imbalances amounts to 2800 and −383 mosM for the hyper- and hypo-osmotic shocks respectively. In spite of these differences, the liposomal suspension is much more vulnerable to rSt I addition in hypo-osmotic conditions. For the shrunk vesicles, it takes longer times to achieve the competent organization of the toxin in the bilayers but the final state is little dependent of the magnitude of the hyper-osmotic imbalance.

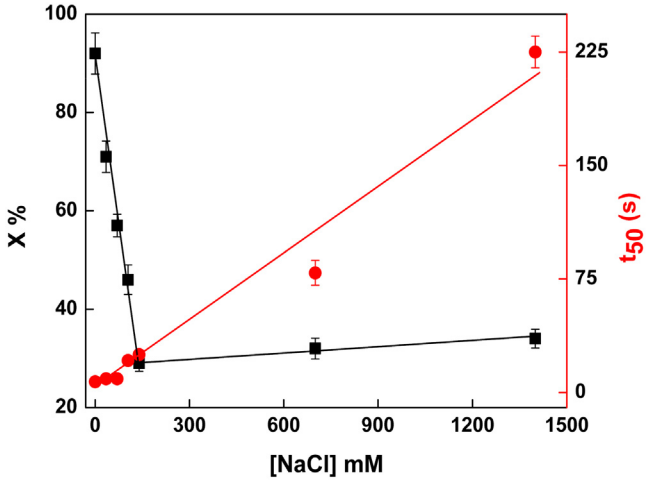

Fig. 4. X% (■) and t_{50} (●) values of DPPC:SM LUVs as a function of NaCl concentration at a fixed (200 nM) analytical toxin concentration. Total lipids: 13.6 μM. Value were obtained 50 min after toxin addition.

The stability of the hypotonically stressed ensembles was tested by adding rSt I (7 nM) at different times after the shock. The data, given in Fig. 5, show only a small decrease in efficiency from time zero (St I added simultaneously to the shock) to that observed when the toxin is added 2100 s after the shock. This implies that most of the stressed ensemble remains, in the absence of toxin, unchanged after long time periods. Further, previously we established that in DPPC and DPPC:SM LUVs, water outflux/influx takes place faster than the release of calcein in both hypo- and hypertonic condition, based on t_{50} values (Ahumada et al., 2015). However, the differences in t_{50} for water and calcein transport were significantly smaller in hypotonic conditions, suggesting that under an osmotic regime, water transport takes place in a similar timeframe than the calcein release. When the addition of toxin is simultaneous with the osmotic shock (Fig. 5), the pore forming process has a t_{50} nearly two times larger than that observed for the water transport, suggesting that the toxins exert its influence over vesicles when they are already subjected to a pressure induced stress, interacting with the bilayer during the process of reorganize itself, thus, we conclude that the effect of the toxin is dependent on the rearrangement resulting from an osmotic shock.

3.3. rSt I pore forming capacity in DPPC liposomes pre-exposed to an hypotonic stress

In isotonic conditions, St I does not show a measurable pore forming activity in the absence of SM (Fig. 2A). This result is in agreement with those previously reported (Tejuca et al., 1996). The

same can be concluded for the toxin action tested after a hyperosmotic shock. On the other hand, after one hyposmotic shock the addition of rSt I (200 nM) allows the exit of ca. 50% of the calcein remaining in the interior of the liposomes. The amount of calcein released depends on the rSt I concentration. However, calcein exit becomes independent of this factor at large rSt I concentrations (ca 200 nM) but rapidly decreases when toxin concentration is smaller than 10 nM (Fig. 3). This data show large differences in the rSt I capacity to form pores in osmotically stressed LUVs. In particular it is interesting to note that the presence of SM has a dramatic effect on the rate of calcein exit over all the rSt I concentration range. Conversely, with regard to the total extent of calcein release, the presence of SM promotes it at high toxin concentrations, with only minor differences at low rSt I concentrations.

Another noticeable difference is that in the absence of SM, only half of the calcein is release from the liposomes, even at very high concentrations of rSt I (600 nM) while, in the DPPC:SM liposomes, almost 100% of calcein leaves the liposome internal pool when StI $\geq$ 50 nM. The independent value of X% in DPPC LUVs at high rSt I concentrations can be interpreted in terms of saturation of the bilayer at these toxin concentrations (Fig. 3). Similar results have been reported in the release of calcein promoted by EqT-II in SM:PC 1:1 SUVs (Belmonte et al., 1993). However, the remaining non-permeated liposomes observed at high rSt in DPPC vesicles could also reflect a certain degree of inhomogeneity of the liposomal ensemble, for example the presence of a fraction of multilamellar vesicles insensitive to the influence of the toxin or up to a lesser extent, due to different response of the liposomes based on their size, where smaller liposomes might be less sensitive than larger liposomes, even with narrow size distributions.

The above results imply that incompetent toxins in DPPC liposomes become competent by a hypotonic imbalance in a similar manner to the presence of the second component as SM. These results are comparable to those reported employing Amphotericin B (AmB). This polyene antibiotic readily forms pores in the presence of sterols and/or osmotically stressed liposomes (Ruckwardt et al., 1998; Wolf and Hartsel, 1995) at relatively low concentrations (ca. 34 AmB molecules per POPC LUV).

Regarding the time scale of the process, DPPC:SM liposomes in hyposmotic conditions liberate their calcein in few seconds, independently of the rSt I concentration, while $\mathbf{t_{50}}$ values in the absence of SM increase when the toxin concentration decreases, reaching values as large as 260 s (Table 3).

Further open questions are the reversibility of the pore-forming process and how a given liposome losses its entrapped calcein (Rapaport et al., 1996). Regarding the first point, we consider that reaching a plateau in the fluorescence intensity of calcein would suggest that, at least some step(s) must be reversible and/or the toxin bearing liposomes do not behaves as a homogeneous ensemble (Ahumada et al., 2015). However, this is a relevant point that has not been yet settled in this type of systems. In fact, F°/F can be used to determine the extent of dye release at equilibrium (Ahyayauch et al., 2004). Conversely, Rapaport et al. (1996) developed a model that considers that all steps but the last one (pore assembling) are reversible in Pardaxin promoted leakage from egg PC liposomes (Rapaport et al., 1996).

Under isotonic conditions, formation of pore(s) can lead to calcein exit by a progressive process, involving the entire liposome ensemble in which the rate of the process is proportional to the average intra-pool concentration or due to an *all-or-none* process in which total loss of the entrapped calcein takes place in any liposome bearing at least one active pore (Martinez et al., 2001; Valcarcel et al., 2001). Furthermore, pore formation under hyposmotic conditions can lead to calcein exit from the liposome pools through the pores and/or associated to a catastrophic collapse of liposomes resulting from the increase in internal pressure due to the (faster) solvent incorporation into the vesicles (Ahumada et al., 2015). Experiments carried out employing larger dyes unable to permeate through the pores could provide information about the relative contribution of both mechanisms.

The present results conclusively show the capacity of hypotonic gradients to improve the pore forming capacity of rSt I molecules, even in the pure DPPC liposomes. rSt I can be then considered as a molecular harpoon, an expression coined to describe substances that can selectively target osmotically stressed or highly curved membranes (Naka et al., 1992; Wolf and Hartsel, 1995). Modulation of peptide activity by osmotic gradients using liposomes is a rather common issue taking place with high density lipoproteins (Mui et al., 1994), melittin (Benachir and Lafleur, 1996), L class amphipathic helical peptides such as mastoparan and K18 L, and other membrane active peptides such as the class A model peptide Ac-18A-NH_2 (Polozov et al., 2001). This general behaviour can be related to the membrane expansion associated to a hypotonic stress, expansion that should favour proteins incorporation to the bilayer and the reversible pore formation. This change could increase the number or/and the size of pores in the liposome. Interestingly, by dark-field microscopy it has been observed that pores opened in liposomal membranes by talin became larger with increasing talin concentration (Saitoh et al., 1998).

In spite of the large number of studies regarding osmotic imbalance and pore formation in liposomes, further studies are needed to be able to answer a series of question regarding the mechanism of calcein exit and the main factors that condition the rate and extent of the process:

(i) Why is necessary SM in isotonic conditions to make rSt I active?
(ii) Why, even in excess of toxin, some systems are unable to liberate the total amount of calcein incorporated to the liposomes?
(iii) Why hypotonic conditions favours the formation of pores by rSt I both in the presence and in the absence of SM, while only DPPC:SM LUVs are responsive to hypertonic shocks?

We have already mentioned that an increased activity of rSt I resulting only from an increase in toxin-liposomes interactions elicited by SM addition is unlike due to the similarity in the binding isotherms, particularly in the absence of osmotic gradients. The best explanation for the positive effect of SM is then multifactorial, comprising an enhanced association of the toxin to the liposomes and the presence of microdomains and increased number of packing defects in the mixed system (Pedrera et al., 2014; Polozov et al., 2001; Schon et al., 2008).

The answer to point ii) could be the saturation of the liposomes with toxin (Caaveiro et al., 2001). At the working lipid concentration (0.6 mg/mL) saturation takes place when St I $\approx$ 10 nM. This implies that, if total capture of the toxin is assumed, approximately 136 lipids per toxin are involved in the formation of competent aggregates. Regarding point iii), expansion of the bilayer and melting microdomains could increase the tendency of rSt I to achieve its competent organization. Similar conclusions have been reached regarding the closely related actinoporin EqT II (Schon et al., 2008).

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