CARDIOTOXIC ACTIONS OF STHICHOLYSINS I AND II, TWO CYTOLYSINS ISOLATED FROM THE SEA ANEMONE *Stichodactyla helianthus*

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Summary

The sthicholysins I y II, are cytolytic proteins from the sea anemone *Stichodactyla helianthus* that interact with biologic membranes and form an oligomeric pore and thus produce the cellular lysis. In general the cytolysins are very lethal in mammals. The cardiotoxic activity of the cytolysins seems to be the most important effect that lead animals to death, so our purpose was to study the actions of sthicholysins I and II on action potential and contraction of isolated perfusing rat heart (Langendorff) and rat ventricular papillary muscle and on the sodium and calcium currents of isolated rat ventricular cardiomyocytes using the patch-clamp technique in whole cell variant. Also we characterized a possible pharmacologic action of theses cytolysins, additional and independent of the forming pore activity, analysing all the experiments mentioned here, but incubating the cytolysins in a basic pH (pH=11.5), because at this pH value these proteins lost the cytolytic action. Both cytolysins produced a powerful cardiotoxic action, characterized by bradycardia and depressed contraction. Also they produced arrhythmias and death of the preparations. Both compounds depressed the sodium current and without effect on calcium current and they produced the cellular death. All effects were irreversibles. When the cytolysins were preincubated to pH = 11.5, also they produced bradycardia and depressed the contraction of perfusing isolated rat hearts (Langendorff), but less powerful and the preparations did not die. However, the cytolysins had not effects on sodium and calcium currents and they did not produced cellular death. These results suggest that the additional pharmacologic action seen in Langendorff experiments is not linked with these ionic channels.

Key Words: sthicholysins, *Stichodactyla helianthus*, cardiotoxicity, cytolysins.

The sthicholysins I (St I) and II (St II), are the two most potent cytolysins purified from the Caribbean sea anemone *Stichodactyla helianthus* (order Actiniaria) (1). *Stichodactyla helianthus* is a sea anemone relatively abundant along Cuban sea coasts. Both St I and St II exert their cytolytic action by forming oligomeric pores in the cell membranes (1-3), and therefore belong to the large protein superfamily of pore-forming toxins. Much work has been done to purify and characterize the molecular entities of this anemone (1-4). The cardiotoxic activity of the cytolysins seems to be the most important effect that lead animals to death, as have been reported for some of them (5). The cardiotoxic characterization of St I and St II have not been studied yet, so our purpose was to study the cardiovascular actions of St I and St II. Also we characterized a possible pharmacologic action of these cytolysins, additional and independent of the forming pore activity, incubating the cytolysins in a basic pH (pH=11.5), because at this pH value these proteins lost the cytolytic action (2).

Methods

Specimens of the anemone *Stichodactyla helianthus* were collected along the coast of Havana city. St I and St II were purified as described (1-4) by combining gel filtration chromatography on Sephadex G-50 medium and ionic exchange chromatography on CM-cellulose 52. In some experiments St I and St II were preincubated to pH = 11.5 by 10 min.

Langendorff perfused rat hearts. Hearts were dissected out from pentobarbital anaesthetized rats and mounted in a Langendorff column. They were perfused at constant flow (10 ml/min) with a normal Tyrode solution gassed with O_2 at 35°C. The apex was attached to a force transducer and the hearts were left to contract, spontaneously, under a constant load of 1 g. The aortic cannula was used as a reference electrode and the active electrode was fixed by a needle to the left ventricular surface. The surface electrogram was recorded with a biophysical preamplifier.

Ventricular papillary muscles. Ventricular papillary muscles were dissected out from adult rat hearts (Wistar). See details in Galán et al. 2001 (6).

Isolation of adult ventricular cardiomyocytes and patch-clamp recording**.** Single rat ventricular cells were dispersed by a collagenase/trypsin enzymatic method similar to that previously described (6). The "whole cell" variant of the patch-clamp method was used (7). See more details in Galán et al. 2001 (6).

Statistical evaluation**.** When it was possible, results were analysed by the students' "t"-test and are expressed as means and standard deviations. The criterion for significance was $p < 0.05$.

Results

<u>Langendorff perfused rat hearts.</u> St I (n = 5) and St II (n = 6) decreased the spontaneous sinus rate of perfused rat hearts, in a concentration-dependent manner. Concentrations as low as 0.1 ng/ml of St I and St II significantly ($p < 0.05$) increased the R-R interval, thus both cytolysins produced bradycardia. Figure 1 shows an example of the effects of St II on the surface electrogram and on the contraction of one perfused rat heart. At 0.3 μg/ml, R-R interval was increased by more than 180 ms by both StI and St II, regarding to control value, these prove a potent bradycardia. In all concentrations studied (0.1 ng/ml a 1.3 μg/ml) arrhythmias were observed. In this experimental serie, the ventricular force of contraction (spontaneous rate) was decreased by both cytolysins in time and concentration-dependent manner. These demostrate the potent cardiotoxic action of these toxins. All these effects were irreversibles by both cytolisins and they produced asystolia between 10-15 min of the application, even stimulated, the hearts did not have electrical and contractil activities.

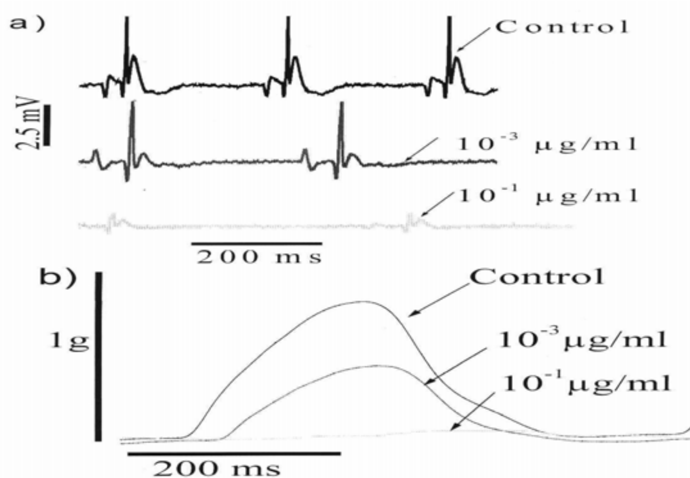

Figure 1: St II effects on Langendorff preparations. a) Surface electrocardiogram and b) Force of contraction; both with growing concentration of St II.

<u>Ventricular papillary muscles.</u> Both St I and St II depressed the force of contraction of papillary muscle in concentration and time-dependent manner. The action potential showed biphasic changes. At the highest concentration proved (48 μg/ml) St I inhibited the force of contraction by 60% and St II by 75 %. In general St I and St II at the lowest concentrations (4 – 16 μg/ml), reduced the action potential duration at 0 and –60 mV and these become accentuated to higher concentrations. At highest concentrations (32 – 48 μg/ml) those effects were inverse, the action potential duration at 0 and –60 mV were increased markedly. Usually, around 15 min of application of two cytolysins, all preparations died.

<u>Patch-clamp recordings.</u> I_{CaL} did not change by the application of St I and St II, even at the hihgest concentration proved (2 µg/ml). At 2 µg/ml St I decreased I_{Na} by 26% and St II by 30 %.
But without changes in the inactivation time constants (τ_1 and τ_2) of I_{Na} in all cases. Around 10 min of application of two cytolysins, all cells were swolled and showed contracture, which mean that cells died. Figure 2 shows the actions of St I and St II on calcium (I_{CaL}) and sodium (I_{Na}) currents.

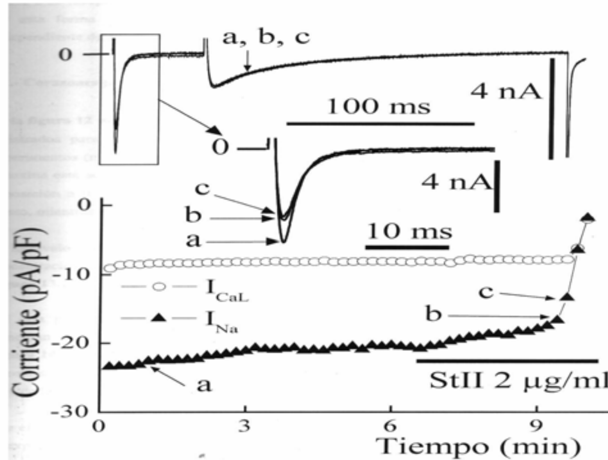

Figure 2: Effects of St II (2µg/ml) on the sodium current (I_{Na}) and on the L-type calcium current (I_{CaL}) of isolated rat ventricular cardiomyocytes. a) Control, b) After 4 min of to add St II, c) 7 min of to add St II.

<u>St I and St II preincubated to pH = 11.5</u>. The preincubation of St I and St II at high pH (pH ≥ 10), reduces irreversibly the capacity of these toxins to form pores without a significant decrease in its binding capacity and these reduces their hemolytic activity too (2). Thus, we can study if St I and St II has additional pharmacologic action.

<u>Langendorff perfused rat hearts.</u> St I (n = 4) and St II (n = 5) decreased the spontaneous sinus rate (bradycardia) and the force of contraction of perfused rat hearts, in a concentration-dependent manner, like St I and St II actives, but in less degree.
<u>Patch-clamp recordings</u>
I_{CaL} and I_{Na} did not change by the application of St I and St II in all concentration studied (0.03- 0.6 µg/ml). The cells did not die in all time of exposition to toxins.

Discussion

The present results show that the two citolysyns St I and St II provoke differents effects on electrical and contractil activities of cardiac tissues. St I and St II depressed the cardiac contraction progressively, while the electrical activity (action potential) manifested biphasic changes, at lowest concentrations of St I and St II reduced the action potential duration and at higher concentration of both citolysyns the action potential duration was increased. The R-R interval of the electrograme increased at growing concentration of St I and St II, showing a potent bradycardia.

In patch-clamp recording experiments on isolated cardiomyocytes, was observed a decreasing of sodium current (I_{Na}) and the L-type calcium current (I_{CaL}) was not varied by St I and St II. These results evidence a potent cardiotoxic action of St I and St II. In a brief time (~ 10 min) both toxins provoked cellular and tissular death.
When St I and St II binding to receptor in cellular membranes, sphingomyelin, form a oligomeric pore of 2 nm. Thus the permeability barrier of cells is broken and beginning the flux of ions (Na^+, Ca^{2+}, K^+ and Cl^-) according with their electrochemical gradient, at consequence enter water to the cell and the swelling happen. The damage that produce these citolysins in membranes is so big that the mechanisms of volume cellular regulation are not enough for contrarrest the cellular swelling and the consequent cellular lysis. Before this mechanism occurs, the cytoskeleton is broken (8). Because of the cytoskeleton rupture provoke changes in ionic channels of cardiomyocytes (9), the biphasic effects on action potential duration by both toxins can be the result of variations on cardiomyocyte ionic current. The decressing in I_{Na} by St I and St II observed by us, can be the responsable of the reducing of action potential duration. The inhibition of I_{Na} can be consequence to the cytoeskeleton disruption because of cellular swelling (10). At higher concentration of two toxins, we observed and increasing in the action potential duration, these can be produced by the lesions at cellular membranes that produce a desbalance in ionic currents.
It is possible that St I and St II affect the I_f current, that fact can explique the bradycardia produced. The bradycardia conduces to the decrease in force of contraction (11), and our result agree with these.
When the toxins were incubated to pH= 11.5 the effects on Langendorff preparations were similars to the result with the toxins actives but less powerfull. The bradycardia and the force of contraction inhibited by toxins can be a colinergic effect. Besides Lanio et al. (3) reported a phospholipasic actions for St II, which can produce too the bradycardia and the decreassig on force of contraction.
In conclusion, our results indicate for the first time a potent cardiotoxic actions of St I and St II and a possible additional independent actions of their cytolytic activity.

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