Pharmacological effects of two cytolysins isolated from the sea anemone *Stichodactyla helianthus*

T García[1,*], D Martinez[2], A Palmero[1], C Soto[2], M Tejuca[2], F Pazos[2], R Menéndez[1], C Alvarez[2] and A Garateix[1]

[1]*Centro de Bioproductos Marinos, Loma y 37, Plaza, CP 10600, La Habana, Cuba*

[2]*Centro de Estudios de Proteínas, Facultad de Biología, Universidad de La Habana, 25 y J, Plaza, La Habana, Cuba*

**Corresponding author (Email, cebimar@infomed.sld.cu)*

Sticholysins I and II (St I/II) are cytolysins purified from the sea anemone *Stichodactyla helianthus.* In this study, we show their pharmacological action on guinea-pig and snail models in native and pH-denatured conditions in order to correlate the pharmacological findings with the pore-forming activity of both isoforms. In guinea-pig erythrocytes (N = 3), St II possessed higher haemolytic activity in comparison with St I and this activity was lost at an alkaline pH. In molluscan central neurons (N = 30), they irreversibly decreased the amplitude of the cholinergic response; St I (EC_{50} 0.6 μmolL^{-1}) was more potent than St II (EC_{50} >6.6 μmolL^{-1}) and they both increased the duration of the action potential; these effects were absent at an alkaline pH. In guinea-pig isolated atrium (N = 25), both increased the amplitude of the contraction force, but St II was more potent than St I (EC_{50} 0.03 μmolL^{-1} and 0.3 μmolL^{-1}, respectively) and this effect persisted at an alkaline pH. In summary, both cytolysins have neuroactive and cardioactive properties. The main mechanism in molluscan neurons seems to be associated with the cytolytic activity of these molecules, whereas in guinea-pig atrium, the existence of an additional pharmacological mechanism might be contributing to the observed effect.

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1. Introduction

Sticholysins I and II (St I/II) are cytolysins produced by the sea anemone *Stichodactyla helianthus* with a high degree of sequence identity (93%), but St II exhibits higher haemolytic activity in human erythrocytes when compared with St I. N-terminal amino acids play an important role in the formation of functional pores and lytic activity of cytolysins. The differences at the polar face of the N-terminal amino acid sequences between them could be responsible for the observed differences in haemolytic activity between St I and St II (Huerta *et al*. 2001).

It has been demonstrated that red blood cell (RBC) permeabilization is pH- (Alvarez *et al.* 2001) and cell-type dependent (Lanio *et al.* 2001). Exposure of cytolysins to an alkaline pH produces permanent changes that lead to a significant loss of pore-forming activity in human erythrocytes (Alvarez *et al.* 2001; Martinez *et al.* 2001) and lipid vesicles (Tejuca *et al.* 1996).

In contrast with cytolysins from other sea anemones, the pharmacological properties of these compounds have rarely been studied and it is not known if the same region of the molecule is involved in haemolytic activity and could participate in the putative pharmacological action. Nevertheless, in a few studies carried out on different sea anemone cytolysins, it is not quite clear whether the pharmacological action observed is the result of their direct pore-forming activity (Ho *et al.* 1987; Khoo *et al.* 1995; Migues *et al.* 1999; Bunc *et al.* 1999). The cardioinhibitory effect of equinatoxin (EqTx) from *Actinia equina* on guinea-

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Abbreviations used: AA, arachidonic acid; Ach, acetylcholine; AP, action potential; EqTx, equinatoxin; GABA, gamma aminobutyric acid; HMg, heteractis magnificalysin; PBS, phosphate buffered saline; RBC, red blood cell; RMP, resting membrane potential; St I/II, sticholysins I and II

pig atrium is due mainly to the release of adenyl compounds while the positive inotropic action may result from the release of arachidonic acid and subsequent synthesis of prostaglandins (Ho *et al.* 1987). EqTx II and heteractis magnificalysins I and II (HMg I and HMg II) (from the sea anemones *Actinia equina* and *Heteractis magnifica,* respectively) are haemolytic and exert an inhibitory effect on synaptosomal gamma aminobutyric acid (GABA) and choline uptake, but the mechanisms of action of these two activities seem to be different (Khoo *et al.*1995). Bc2, a cytolytic fraction from the sea anemone *Bunodosoma caissarum*, induces massive liberation of glutamate [^{3}H] in rat brain synaptosomes without causing them to swell (Migues *et al.*1999). The observations of Bunc *et al.* (1999) with EqTx *in vivo* suggest that the release of vasoactive substances from leucocytes and thrombocytes may play a significant role in the cardioctive activity of these toxins. These studies demostrated that lytic activity besides other mechanisms is responsible for the effects of these compounds. The aim of this study was to describe the pharmacological actions of these compounds in guinea-pig and snail models in native and pH-denatured conditions, in order to correlate the pharmacological findings with the pore-forming activity of both isoforms.

2. Materials and methods

2.1 *Purification procedure*

St I and St II were purified from the sea anemone *Stichodactyla helianthus* according to the procedure described by Lanio *et al.* (2001). Specimens were collected along the coast of Havana City. A voucher sample (No. Ido 003-3-2-006) was deposited in the collection of the Cuban National Aquarium. Total extract was obtained by homogenizing the whole body of the anemone. Sticholysins were purified by combining gel filtration chromatography on Sephadex G-50 medium (Amersham Pharmacia Biotech, Uppsala, Sweden) and ionic exchange chromatography on CM-cellulose 52 (Whatman, Maidstone, UK). The fractions corresponding to St I and St II were concentrated and diafiltrated with distilled water using an Amicon ultrafiltration device equipped with a 1000 Da cut-off membrane.

2.2 *Assays of biological activity*

The effects of St I and St II were studied in different biological models: in guinea-pig RBC and isolated atrium as well as in molluscan neurons**.**

2.2.1 *Guinea-pig erythrocyte test:* To evaluate the haemolytic capacity of these proteins in guinea-pig erythrocytes, suspensions of RBCs from total blood, washed twice by 10 min centrifugation at 700 × *g* at room temperature, were prepared in buffer solution (phosphate buffered saline [PBS], in mmolL^{-1}): Na_2HPO_4 3.5, NaH_2PO_4 1.05, NaCl 145, pH 7.4. The haemolytic assay was carried out by preincubating the toxins for 30 min at room temperature. In each well, a fixed concentration of toxin was present at a final volume of 100 μl. The reaction was started by adding 100 μl of RBCs, with an initial absorbance of around 0.1 at 650 nm. The assay was performed under constant stirring and measurements were collected every 10 s for 10 min. Lyses of guinea-pig erythrocytes was assessed by measuring the decrease in turbidity of the RBC suspension as a function of time at room temperature.

2.2.2 *Snail neuron experiments:* A group of experiments was carried out on identified neurones of the Cuban land snail *Zachrysia guanensis* to study the effect of sticholysins on the action potential (AP) characteristics and on the amplitude of cholinergic responses. The suboesophageal ganglionic mass of the central nervous system of the snail was dissected according to the procedure described previously (Martínez-Soler *et al.* 1983). The preparation was kept in physiological solution for molluscans (in mmolL^{-1}): NaCl 80, KCl 4, $CaCl_2$ 7, $MgCl_2$ 4, Tris-HCl 10 and pH adjusted to 7.4. Intracellular recording was made using glass microelectrodes filled with 3 molL^{-1} KCl and resistances ranging from 10 to 20 MΩ as previously described (Garateix *et al.* 1996). Sticholysins were added to the external solution at the desired concentration and added to the bath fluid that surrounded the preparation for up to 30 min and, during this time, responses to acetylcholine (Ach) were tested every 3 min. At the end of the experiment, the preparation was repetitively washed with solution for molluscans to evaluate the recovery of the response. The amplitude of depolarization induced by the application of Ach was measured and compared with control responses. Experiments were carried out at room temperature (25°C). AP signals were digitalized at a sampling rate of 3 kHz using an analogue digital converter. Data analyses were performed automatically using the Analnew software (Version 6.1, Puebla University, Mexico). Resting membrane potential (RMP), amplitude and duration of AP were measured in 30 preparations.

2.2.3 *Experiments in guinea-pig isolated atrium:* Guinea-pig isolated atrium was dissected according to the procedure described by Sainz *et al.* (1972). Briefly, the heart was rapidly removed from the animal and bathed in modified Krebs solution (in mmolL^{-1}): NaCl 136.88; KCl 4; $NaHCO_3$ 12; glucose 5; $MgCl_2$ 0.5; NaH_2PO_4 0.9; $CaCl_2$ 2.0; pH 7.4–7.6; aerated with a mixture of 97% O_2 and 3% CO_2. The left atrium was cut off from the heart, suspended in a 5 ml organ bath maintained at 37 ± 0.5°C and electrically stimulated with square pulses of 5 ms duration, 1.2 Hz frequency and constant voltage at twice the threshold intensity. Contractile

force was recorded with a force-displacement transducer attached to a multipurpose polygraph by conventional techniques. Taking into account that an alkaline pH induces changes in the toxins' capacity to form pores (pH 11.5), a group of experiments was carried out to study the effects of pH-denatured toxins on the pharmacological activity of St I and St II in order to distinguish between the contribution of the pore-forming activity and pharmacological activity.

2.3 *Effect of alkaline pH on the lytic activity of St I and St II on guinea-pig erythrocytes*

The haemolytic assay was performed by pre-incubating the toxins at pH 11.5 for 30 min with NaOH (1molL^{-1}) at room temperature. To ensure the same pH in the assay, the pH of PBS was adjusted to pH 11.5 by addition of NaOH (1 molL^{-1}). Control experiments, in which the RBCs

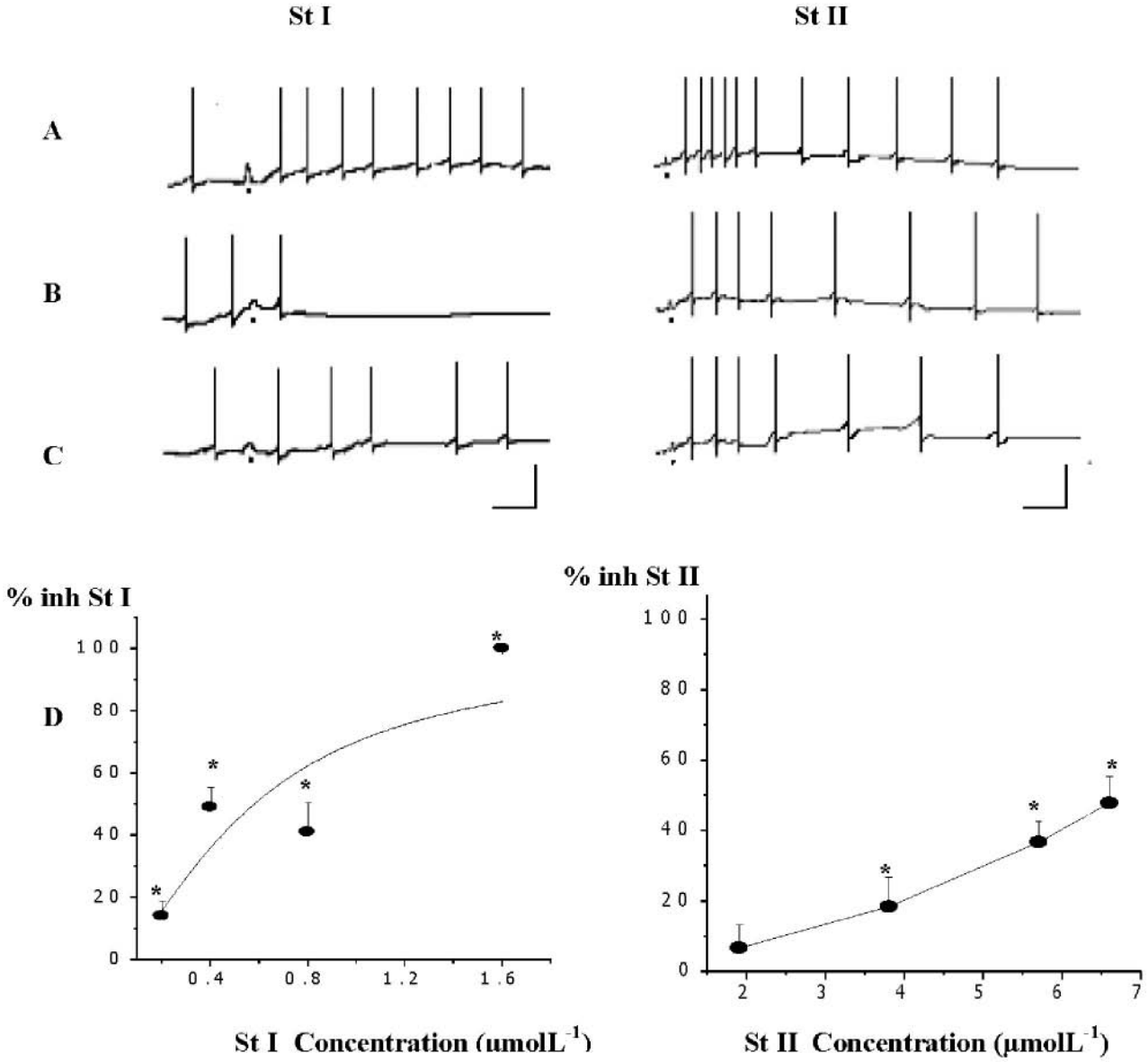

Figure 1. Effects produced 3 min after St I and St II perfusion in central neurons of the snail *Zachrysia guanensis*. The point indicates the application of Ach. Calibration: horizontal=20 ms; vertical= 40 mV. **(A)** Control responses to Ach (30 V, 800 ms). (**B**) Partial blockade of cholinergic responses produced by perfusion of St I (0.8 μmolL^{-1}) and St II (6.6 μmolL^{-1}).(**C**) Absence of recovery of Ach responses, 15 min after washing. (**D**) Concentration–effect relationship (fitting by Hill equation) of St I (EC_{50} 0.6 μmolL-1) and St II (EC_{50} not determined) action on the amplitude of the cholinergic responses evoked by the iontophoretical application of Ach. Each point represents the means of at least three tests of a given concentration ± SEM. * indicates significant difference compared to control, P<0.05.

were preincubated with the alkaline buffer, were also performed.

2.4 *Effects of pH-inactivated sticholysins on snail neurons and guinea-pig isolated atrium*

In these experiments, pH-denatured toxins were assayed at concentrations higher than EC_{50} and determined for both sticholysins in each model. St I and St II were pH-denatured as described in section 2.3.

2.5 *Statistical analyses*

The parametric Student *t*-test and non-parametric Kruskal–Wallis and Mann–Whitney U tests were used to evaluate statistical significance. The software used was STADGRAPH and differences between the treated and control groups were considered statistically significant when $P \leq 0.05$. The concentration–response relationship for cytolysins in both the models was expressed as a mean percentage of change with respect to control conditions ± SEM and they were adjusted to the Hill equation ($Y = 100/[1+(x/CI_{50})^N]$); where Y is the inhibition percentage, x is the concentration of the compound, N is the Hill coefficient and CI_{50} is the concentration that induces 50% inhibition.

3. Results

3.1 *Effects of St I and St II on molluscan neurons*

3.1.1 *Effects on cholinergic responses:* The amplitude of the depolarization evoked by the microiontophoretical application of Ach was reduced by the perfusion of St I (0.2–1.8 μmolL^{-1}) and St II (0.2–6.6 μmolL^{-1}) in a concentration-dependent manner. Figure 1 shows a typical experiment in a molluscan central neuron 3 min after application of the toxins, a time at which the peak effect occurred. Figure 1A depicts the excitatory response evoked by the iontophoretical application of Ach, which is characterized by an increase in the discharge of the APs and membrane depolarization. As shown in figure 1B, 3 min after the application of these compounds, a total blockade of the cholinergic response was produced for St I at 1.6 μmolL^{-1}. In contrast, a reduction of only about 59.7% was achieved for St II when added at concentrations as high as 6.6 μmolL^{-1}. No recovery of cholinergic responses was observed after washing the preparation of both cytolysins (figure 1C).

The concentration–response relationship adjusted by the Hill equation is reflected in figure 1D. As depicted, the potency of the inhibitory action of St II on cholinergic responses in molluscan neurons is considerably lower than that for St I (EC_{50}=0.6 μmolL^{-1}). The EC_{50} for St II was not determined because of the low potency of this toxin.

3.1.2 *Effects on AP:* Table 1 summarizes the effects of perfusion of the sticholysins on the RMP, and duration and amplitude of the AP.

St I and St II (1.8 μmolL^{-1}) produced an increase in duration of the AP. This effect became significant 3 min after the perfusion of St I, while the effects of St II were not significant until 7 min. These effects were not reversible after repeated washing of the preparation. Besides, St I and St II did not affect either the AP amplitude or the magnitude of the RMP. St I increased the duration of the AP even at lower concentrations (0.8 μmolL^{-1}). However, St II was ineffective at similar doses, which highlights the differences in potency between both isoforms (data not shown).

3.2 *Effects of St I and St II on guinea-pig erythrocytes*

The haemolytic activity displayed by both proteins on guinea-pig RBC was time- and concentration-dependent. Upon addition of St I and St II (0.6 nmolL^{-1}), total lysis of guinea-pig erythrocytes took place in 10 min (figure 2A). The rate of haemolysis induced by St II measured as the time required to lyse half of the RBC ensemble (t_{50}), was nearly 4-fold higher than that of St I.

Table 1. Time-dependent effect of St I and St II (both at 1.8 μmolL^{-1}) on RMP, and amplitude and duration of AP

Time	RMP (mv)		Amplitude (mv)		Duration (ms)	
	St I (*N*=4)	St II (*N*=3)	St I (*N*=5)	St II (*N*=3)	St I (*N*=5)	St II (*N*=3)
Control	56.3±1.6	55.3±1.3	82.4±1.2	82.9±2.2	9.9±0.5	9.6±0.6
1 min	54.1±1.2	53.8±1.4	83.3±1.2	84.0±2.3	9.7±0.5	9.4±3.2
3 min	53.0±0.7	52.0±1.2	83.7±0.5	83.0±2.3	13.6±1.2*	9.9±4.0
5 min	54.5 ±1.3	54.3±1.3	78.6±2.3	80.5±1.0	16.3±3.3*	12.9±2.3
7 min	53.3±8.3	56.7±1.6	81.5.±5.4	80.87±1.0	20.5±3.2*	16.9±0.7*
Washing	55.8±3.2	54.3±2.2	80.5±5.2	83.0±0.7	18.4±3.3*	14.6±0.3*

Number of experiments are indicated in the table. Results are presented as means ± SEM. * $P<0.05$, compared with the control group (Student *t*-test and Mann–Whitney *U* test).

3.2.1 *Effects of alkaline pH on the lytic activity of sticholysins on guinea-pig erythrocytes:* In order to assay the activity of sticholysins denatured in an alkaline pH, the assay was carried out at a concentration of 0.2 μmolL^{-1}, which was high enough to elicit total lysis of the RBC ensemble. Therefore, this concentration was used as a reference point in both molluscan neuron and guinea-pig atrium experiments.

When St I was preincubated at pH 11.5 for 30 min, the cytolytic activity was lost while no decrease in turbidity of the RBC suspension was observed upon addition of the toxin (0.2 μmolL^{-1}). In contrast, at pH 7.4, the toxins produced total lysis as expected at this concentration (figure 2B). Similar results were obtained for St II (data not shown).

3.3 *Effects of St I and St II on isolated guinea-pig atrium*

Perfusion of both sticholysins on guinea-pig atrium qualitatively produced a transient fall in the amplitude of the contraction force followed by a more sustained rise (figure 3A) with respect to control conditions. However, the brief negative inotropic effect was significant only 1 min after St I perfusion at 0.1 μmolL^{-1}, while St II had no significant effect at any time and/or concentration assayed. Thereafter, this effect was followed by a positive inotropic action (the maximum effect was reached in about 2–3 min); this was observed for both cytolysins at different times and concentrations. The concentration–response relationship

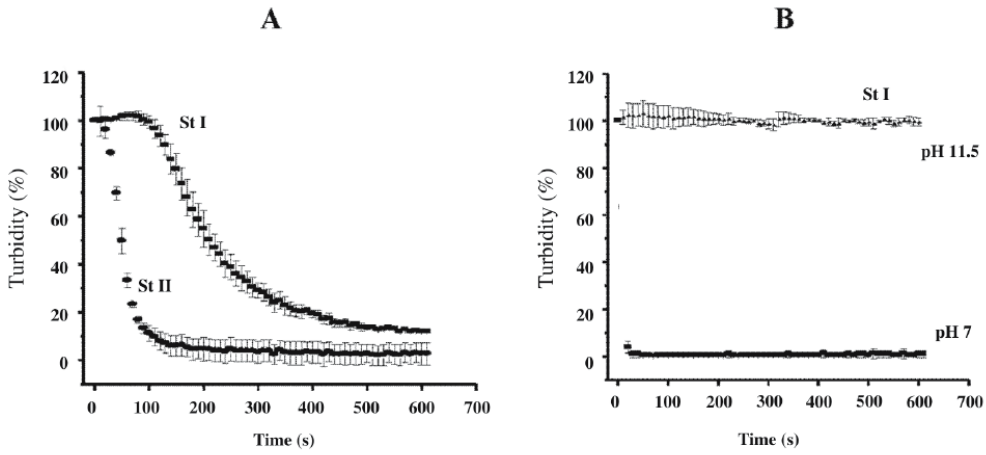

Figure 2. Guinea pig erythrocyte lyses induced by sticholysin I and II. (**A**) Decrease in the sample turbidity, measured by absorbance at 650 nm, following the addition of sticholysin I and II at 0.6 nmolL^{-1}. (**B**) Effect of preincubation of St I (0.2 μmolL^{-1}) at pH 11.5 for 30 minutes to show its haemolytic activity. St II produced a similar action (data not shown).

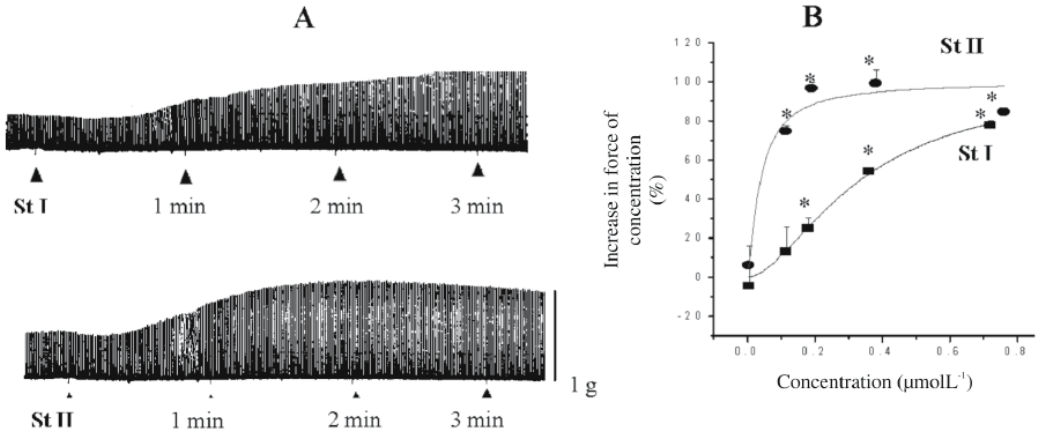

Figure 3. (**A**) Effect produced by the perfusion of sticholysin St I and St II (0.8 μmolL^{-1}) on guinea pig isolated atrium. (**B**) Concentration–effect relationship of St I (■) (EC_{50} = 0.3 μmolL^{-1}) and St II (●) (EC_{50}=0.03 μmolL^{-1}) action on the force of contraction. Each point represents means ± SEM of at least three tests' concentration at 3 min. *P<0.05 (Student t-test).

adjusted by the Hill equation showed (figure 3B) that the increase in amplitude of the contraction force was time- and concentration-dependent for both proteins. However, the potency of St II (EC_{50} 0.03 μmolL^{-1}) was markedly higher than that of St I (EC_{50} 0.3 μmolL^{-1}). Continuous washing of the preparation for 20 min led to recovery of the amplitude of the concentration force.

3.4 *Effects of pH-inactivated St I and St II on molluscan neurons and isolated guinea-pig atrium*

Preincubation at an alkaline pH of both sticholysins induced changes in their pharmacological action at the concentration tested. Figure 4A compares the temporal course of the cholinergic response in molluscan neurons under St I and St II perfusion in their native and pH-inactivated states. A decrease in the Ach response caused by both sticholysins in their native state was observed; in contrast, the denatured sticholysins practically did not affect this response.

In isolated guinea-pig atrium, perfusion of both sticholysins produced an increase in the amplitude of the contraction force compared with control conditions (figure 4B). This positive inotropic effect was also time-dependent and reversible after the preparation was washed. Interestingly, this action was statistically significant even

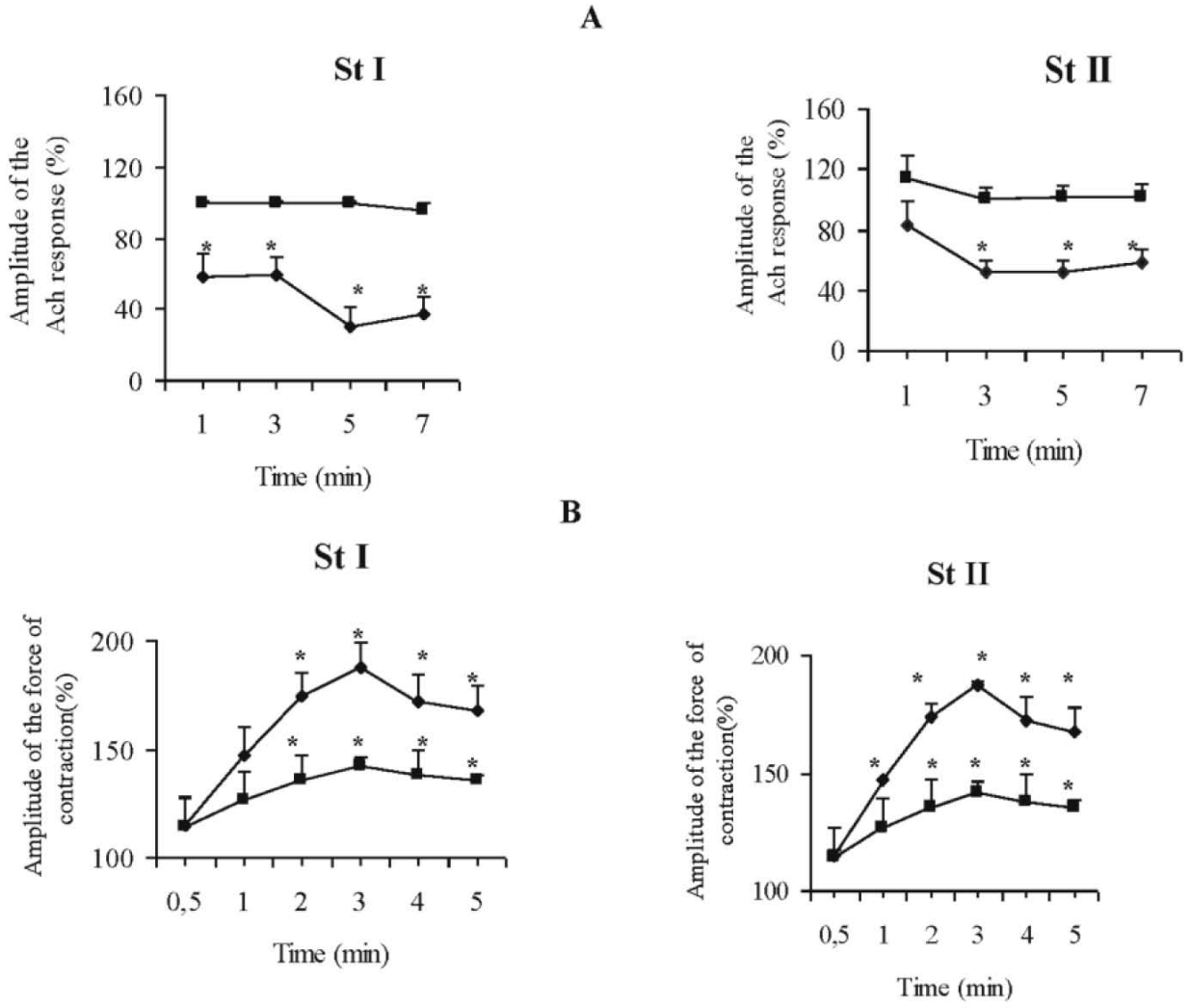

Figure 4. Influence of alkaline pH (11.5) pretreatment on pharmacological activity of St I and St II. Each point represents the means ± SEM of at least three tests of a given concentration, * P<0.05 (Student t-test) as compared with control (100%). (**A**) Temporal course of the inhibition of the acetylcholine response in a molluscan neuron when perfusing St I (0.8 μmolL^{-1}) and St II (6.6 μmolL^{-1}) in its native (♦) and inactive (■) state. (**B**) Effects on the force of contraction on guinea pig atrium by the perfusion of both cytolysins (0.8 μmolL^{-1}) in the native (♦) and inactive states (■).

with the inactive proteins, albeit at a smaller magnitude, when compared with native sticholysins.

4. Discussion

Our study stresses that the biological activity displayed by the pore-forming proteins St I and St II depends on the biological model studied, St II being more potent in guinea-pig atrium, and St I in snail neurons. To gain further insight into the pharmacological activities of both cytolisins, we used native and pH-denatured sticholysins. In snail neurons, the activities of both cytolysins can be correlated with their pore-forming activities, whereas in guinea-pig atrium, an additional pharmacological mechanism might be involved. In guinea-pig RBC, St II exhibited higher haemolytic activity than that of St I, as previously reported for human erythrocytes (Martinez *et al.* 2001). Also, incubation of the toxins at an alkaline pH induces a loss of the haemolytic activity due to less competent conformation of the toxins (Alvarez *et al.* 2001).

The decrease produced by both sticholysins on the amplitude of the cholinergic response in molluscan neurones resembles the effect reported by Cline (1997) for the cytolysin Up I (isolated from *Urticinia piscivora*) on cholinergic receptors of isolated ileum. On the other hand, St I and St II significantly increased the AP duration of molluscan central neurons without variation either in the AP amplitude or in the RMP. Previous studies have shown that pacemaker AP in snail neurons is calcium dependent (Kostyuk 1976). Hence, the observed enhancement of AP duration in this study may be due to the influx of calcium ions through the pores formed by St I and St II. In this regard, cation-selective pores formed by St I and St II have been demonstrated in earlier studies (Tejuca *et al.* 1996; Celedon *et al.* 2005).

The pharmacological actions of sticholysins on guinea-pig atrium agree with the dual effect previously described by Ho *et al.* (1987) for EqTx. The initial brief decrease in amplitude of the contraction force was significant only for St I during the first minute after perfusion. The positive inotropic effect observed by the addition of these molecules was significant for both toxins, St II being more potent than St I. This last effect could be related to an increase in the influx of calcium ions as a consequence of pore formation by sticholysins. Therefore, the putative entrance of Ca^{2+} induced by sticholysins into the cytoplasm could trigger molecular mechanisms (Katz 1996) leading to contractility of the cardiac muscle. Thus, the effects induced by St I and St II in guinea-pig atrium suggest that their actions resemble those previously described for EqTx (Lee *et al.* 1987) and EqTx II (Bunc *et al.* 1999; Frangez *et al.* 2000).

Incubation of sitcholysins in an alkaline pH elicited a loss of their haemolytic activity in guinea-pig erythrocytes as previously described in human erythrocytes (Alvarez *et al.* 2001; Martinez *et al.* 2001). Therefore, we next investigated the effects of pH-inactivated cytolysins on guinea-pig atrium and molluscan neurons. pH-inactivated sticholysins failed to modify the cholinergic responses and AP characteristics in molluscan neurons. Thus, the lack of effect in these experiments supports our hypothesis that pore formation is the main mechanism underlying the effects of St I and St II in this model. Besides, the fact that the action of native sticholysins on molluscan neurons was irreversible may also help to explain our hypothesis, bearing in mind that pore formation is an irreversible event (Alvarez-Valcárcel *et al.* 2001).

On the other hand, when the pH-inactivated cytolysins were assayed in guinea-pig atrium, a significant increase in amplitude of the contraction force was observed, albeit at a lesser magnitude than with the native molecules. This suggests that mechanisms other than pore-forming activity could account for the increase in the contraction force. Preincubation at an alkaline pH irreversibly reduces the capacity of St II to form pores without a significant decrease in its binding ability (Alvarez *et al.* 2001). Therefore, the observed effect of the inactive sticholysins on guinea-pig atrium as well as the reversibility of the pharmacological response with the native toxins suggest that sticholysins, once bound to membrane, can trigger other mechanisms that could explain this pharmacological effect.

The positive inotropic effects of tenebrosin-C (Galletis and Norton 1990) and EqTx II (Hong *et al.* 2002) seem to involve a direct stimulation of the arachidonic acid (AA) pathway. In this regard, even though phospholipase A_2 activity (10^{-3} U/mg protein) has been described for St I and St II (Lanio *et al.* 2001), it seems to be too weak to account for such effects. However, the possibility of activation of the AA or any other pathway by St I and St II after binding to membrane cannot be ruled out.

In summary, our findings indicate that the haemolytic activity displayed by these proteins on guinea-pig erythrocytes resembles their action on human erythrocytes: St II exhibits higher haemolytic activity and this activity is pH-dependent and inactivated at an alkaline pH. Our experiments also reveal that St I and St II display different activities on molluscan nervous system and mammalian cardiac tissues. Till date, the molecular basis of these differences remain unknown; however, it should be considered that differences in tissue and membrane characteristics as well as the pharmacological pathways involved in both models may be causing a different response to both toxins.

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