View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Toxicon 83 (2014) 15–21

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Short communication

Functional and structural study comparing the C-terminal amidated β-neurotoxin Ts1 with its isoform Ts1-G isolated from *Tityus serrulatus* venom

V.A. Coelho [a,1], C.M. Cremonez [a,1], F.A.P. Anjolette [a], J.F. Aguiar [b], W.A. Varanda [b], E.C. Arantes [a,*]

[a] *Department of Physics and Chemistry, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Av. do Café, s/n, 14040-903 Ribeirão Preto, SP, Brazil*

[b] *Department of Physiology, Ribeirão Preto Medical School, University of São Paulo, Av. Bandeirantes, 3900 14049-900 Ribeirão Preto, SP, Brazil*

ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form 10 February 2014
Accepted 12 February 2014
Available online 20 February 2014

Keywords:
Ts1 isoform
β-Neurotoxin
Tityus serrulatus venom
C-terminal amidation
Voltage gated sodium channels

ABSTRACT

Mature Ts1, the main neurotoxin from *Tityus serrulatus* venom, has its C-terminal Cys amidated, while the isolated isoform of Ts1, named Ts1-G, keeps the non-amidated Gly residue at the C-terminal region, allowing the study of the comparative functional importance of amidation at the C-terminal between these two native toxins. Voltage dependent sodium current measurements showed that the affinity of Ts1-G for sodium channels is smaller than that of the mature Ts1, confirming the important role played by the C-terminal amidation in determining Ts1 activity.

© 2014 Elsevier Ltd. All rights reserved.

Voltage-gated sodium channels (Na_vs) have received a great deal of attention both because they are widely expressed in both peripheral and central nervous system and because malfunctioning of its constitutive proteins is associated with several diseases (Saucedo et al., 2012). From a pharmacological point of view, several substances are able to alter Na_v function and, among them, there are different types of peptides extracted from scorpion venoms (Rodríguez de la Vega and Possani, 2005).

NaScTxs are 6500–8500 Da polypeptides composed of 58–76 amino acid residues folded and packed by 4 disulfide bridges. In addition, this group of toxins has a highly conserved common structure showing a βαββ topology (Quintero-Hernández et al., 2013; Saucedo et al., 2012; Schiavon et al., 2012; reviewed by Rodríguez de la Vega and Possani, 2007). They are currently classified into two categories based on their binding and physiological effects on Na_v gating: α-toxins (α-NaScTx) which bind to receptor site 3 of the extracellular portion of the channel and inhibit the inactivation process, making it slow and incomplete, and promoting the prolongation of the action potential (Marcussi et al., 2011; Kirsch et al., 1989; Possani et al., 1999; Catterall et al., 2007); and β-toxins (β-NaScTx) which bind to receptor site 4 and shift the threshold of the channel activation to more negative membrane potentials, triggering spontaneous and repetitious membrane depolarization (Cestèle and Catterall, 2000; Cologna et al., 2009; Quintero-Hernández et al., 2013; Rodríguez de la Vega and Possani, 2005).

Abbreviations: Na_v channel, voltage-dependent sodium channels; NaScTx, scorpion toxin affecting Na_v channels.

* Corresponding author. Tel.: +55 16 3602 4275; fax: +55 16 3602 4880.

E-mail address: ecabraga@fcfrp.usp.br (E.C. Arantes).

[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.toxicon.2014.02.010
0041-0101/© 2014 Elsevier Ltd. All rights reserved.

Toxins from the Brazilian scorpion *Tityus serrulatus* have been extensively studied and many of them have been isolated and characterized. The toxin named Ts1 is highlighted as the main β-neurotoxin present in the venom of *T. serrulatus* (Toledo and Neves, 1976; Barhanin et al., 1984; Arantes et al., 1989). It is the major constituent of the venom, representing approximately 15% of the soluble fraction (Cologna et al., 2009; Pessini et al., 2001). The amino acid sequence deduced from the cDNA nucleotide sequence indicated that Ts1 precursor undergoes a post-translational modification and contains a signal peptide of 20 residues, the mature toxin of 61 amino acid residues and an extra Gly-Lys-Lys tail before the termination codon. Thus, the generation of Ts1 requires, in addition to the removal of the signal peptide, a post-translational cleavage of the peptide bonds involving Lys residues by a carboxypeptidase, after which the remaining Gly residue has its NH_3^+ linked to the C-terminal residue Cys by the action of the α-amidating enzyme on its precursor (Martin-Eauclaire et al., 1992, 1994). In the end of this post-translational process, the mature Ts1 has its C-terminal Cys amidated.

By choosing the right purification technique, it was possible to isolate an unique isoform of Ts1, just before it undergoes the last step of maturation, i.e., the action of the α-amidating enzyme on the Gly residue. The toxin named Ts1-G isolated by our group still has the Gly residue intact, and gave us the opportunity to study the implications of the C-terminal amidation in the function of two native toxins, the mature Ts1 and its isoform with a Gly residue tail (Ts1-G). We conduct the isolation and structural characterization of the precursor as well as a biological and electrophysiological study comparing Ts1 with its isoform Ts1-G.

Ts1 and Ts1-G were isolated by using a combination of cation exchange chromatography followed by reverse-phase liquid chromatography. The whole venom of *T. serrulatus* was fractionated using a CM-cellulose-52 column adapted to a *FPLC Äkta Purifier UPC-10* (Cerni et al., 2014) and this process afforded 18 fractions named I, II, III, IV, V, VIA, VIB, VII, VIIIA, VIIIB, IXA, IXB, X, XIA, XIB, XIIA, XIIB, XIII (data not shown). Fraction XIIB was submitted to a reversed-phase fast protein liquid chromatography (RP-FPLC) on an analytical C-18 column (4,6 × 250 mm, 5 μm, Shimadzu), previously equilibrated with Solution A (0.1% trifluoroacetic acid, v/v), and eluted at a flow rate of 1 mL/min, connected to a FPLC Äkta System equipment (GE, Uppsala, Sweden). The chromatographic profile of fraction XIIB (Fig. 1A) obtained from *T. serrulatus* venom showed more than five peaks, and Ts1-G was eluted on the major one. The peak containing Ts1-G was submitted to a third chromatographic step (Fig. 1B), under the same chromatographic conditions, to reassure the purity of the toxin for the subsequent assays. Ts1 was eluted on fraction XIII which was submitted to a RP-FPLC on C-18 column (4,6 × 250 mm, 5 μm, Vydac), providing highly purified mature Ts1 (Fig. 1C). The isolated toxins were characterized through amino acid sequencing, polyacrylamide gel electrophoresis for basic proteins (PAGE), performed as described by Arantes et al., 1989 (Fig. 1D and E), and mass spectroscopy (Fig. 1F). The amino acid sequencing was obtained by Edman's degradation method (Edman and Begg, 1967), using a PPSQ-33A equipment (Shimadzu Co., Kyoto, Japan), and the sequences were aligned (Fig. 1G) using the Mutalin interface (Corpet, 1988). The image was created using Expasy software (http://www.expasy.org/) and the mass spectroscopy was performed using Ultraflex II MALDI TOF/TOF (Bruker Daltonics, Alemanha).

Ts1 and Ts1-G were also functionally characterized (Fig. 2) by biochemical assays to evaluate plasma creatine kinase (CK-NAC KINETICS K010 kit -Bioclin®) and blood glucose levels (Optium® Mini Blood Glucose Monitoring System, Abbott) according to fabricant's guidance. Balb mice (20 g) were injected (i.p.) with 200 μL of 0.9% saline (control) and with Ts1 and Ts1-G at different doses (30, 60, 120 μg/kg). The animal's blood was collected 1 h after injections into heparinized tubes for plasma separation to be used in the assays.

Electrophysiological assays were performed using the *Patch Clamp* technique (Hamill et al., 1981) on neurons of dorsal root ganglion (DRGN), freshly isolated from male Wistar mice (30 days). Whole cell membrane currents, in response to depolarizing voltage steps were recorded using an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA, USA) interfaced to a computer via a Digidata 1200 running pClamp 10 (Axon Instruments). Sodium currents were filtered at 5 kHz and acquired at 10 kHz. Glass micropipettes were pulled from borosilicate glass capillaries and showed resistance between 2 and 4 MΩ. During measurements cells were bathed in a solution containing (in mM): 50 NaCl; 95 NMDG; 5.4 CsCl; 1.8 $CaCl_2$; 10 HEPES; 5 glucose and 100 μM $CdSO_4$. $CdSO_4$ was added to the external solution in order to block calcium currents and have a better resolution of the sodium currents. The pH 7.4 was adjusted with NaOH and the osmolality was 300 mOsm/(KgH_2O). The pipette was filled with (in mM): 100 CsF; 10 NaCl; 10 HEPES; 5 EGTA and 40 TEACl. The pH was adjusted to 7.4 with CsOH and the osmolality was 301 mOsm/(KgH_2O). Ts1 and Ts1-G (500 nM diluted in bath solution) were applied to DRG cells for up to seven minutes using a perfusion system controlled by solenoid valves (RSC-Bio-Logic Co., Claix, France). The cells were kept at a holding voltage of −70 mV, hyperpolarized to −100 mV for 150 ms and then stepped between −80 and +70 mV in 10 mV steps for 50 ms. The average cell capacitance was 30.3 ± 1.9 pF (mean ± SEM, $n = 33$ cells) indicating that the recorded cells have a diameter around 31 μm. This is consistent with medium sized neurons of the DGR.

The statistical significance of results was evaluated using analysis of variance (one-way ANOVA) followed by Tukey test, considering p values <0.05 as significant. All experiments followed the ethical standards for animal experiments in toxinological research recommended by the International Society of Toxinology and was approved by the Committee for Ethics in Animal Utilization of Ribeirão Preto – Universidade de São Paulo (protocol number: 08.1.1352.53.8).

The isoform Ts1-G (Fig. 1B) and the mature toxin Ts1 (Fig. 1C) were isolated in 3 and 2 chromatographic steps, respectively. The differences in retention time on reverse phase chromatography between Ts1 and Ts1-G is explained by the glycine positioned at the C-terminal region of Ts1-G, This additional non-amidated residue gives to the Ts1-G

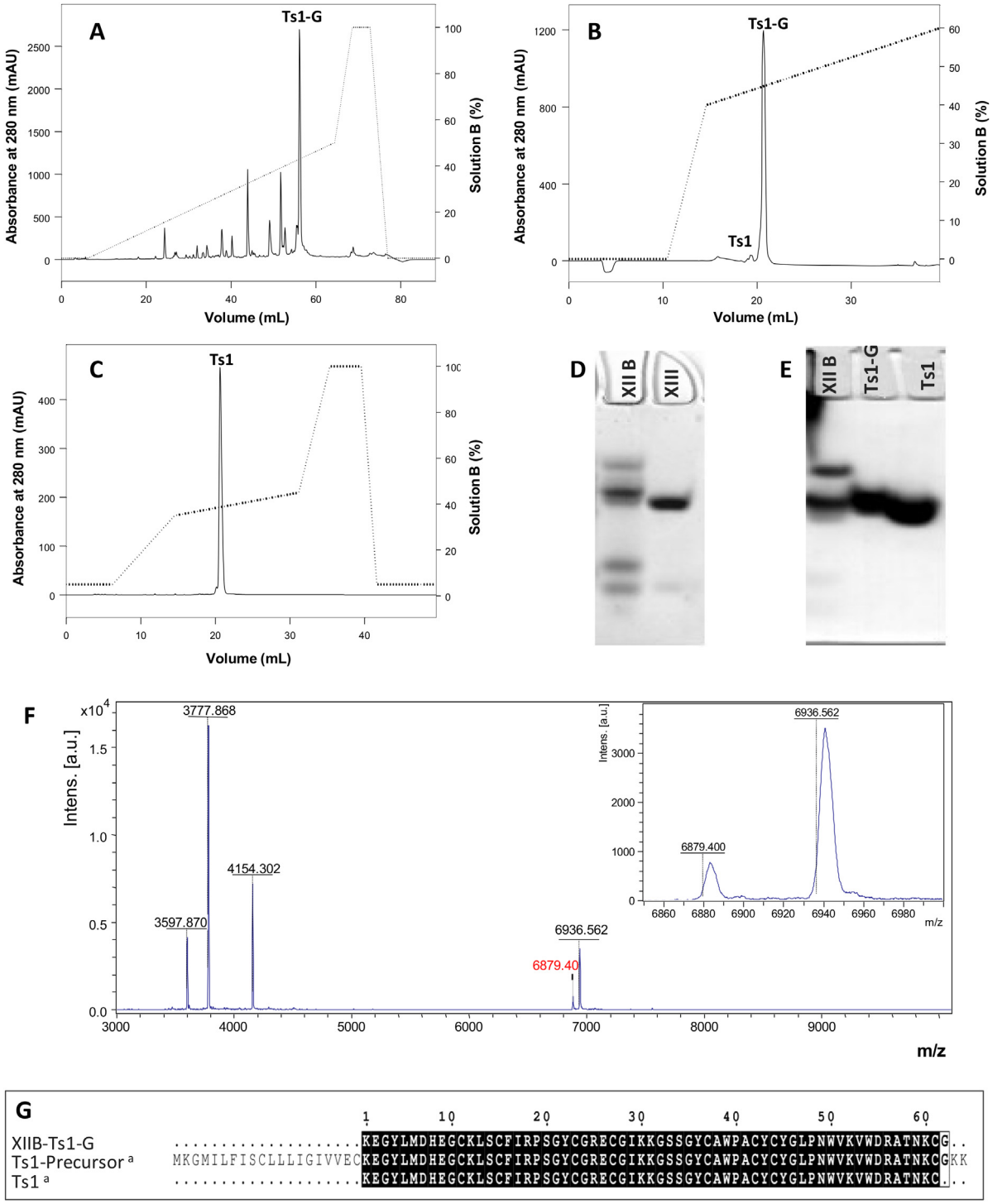

Fig. 1. **(A)** Chromatographic profile of fraction XIIB (0.5 mg) obtained from *Tityus serrulatus* venom by C-18 RP-FPLC. The column was previously equilibrated with Solution A (0.1% trifluoroacetic acid, v/v) and Fraction XIIB was eluted (1 mL/min) with a segmented concentration gradient (0–100%) of Solution B (80% acetonitrile in 0.1% trifluoroacetic acid, v/v), represented by the dotted line. **(B)** Chromatographic profile of Ts1-G by C-18 RP-FPLC. **(C)** Chromatographic profile of fraction XIII by C-18 RP-FPLC. **(D)** PAGE comparing Fraction XIIB (Lane 1) with Fraction XIII (Lane2) from *T. serrulatus* venom. **(E)** PAGE comparing Fraction XIIB (Lane 1), pure Ts1-G (Lane 2) and pure Ts1 (Lane3). **(F)** MALDI-TOF/TOF mass spectrum of fraction XIIB, analyzed using DHB matrix and linear mode. The insert shows the presence of Ts1 with a mass of 6879.400 Da, and Ts1-G with a mass of 6936.562 Da. **(G)** Alignment of the sequences of pure Ts1-G purified from fraction XIIB, the complete Ts1 Precursor sequence with the signal peptide and C-terminus, and mature Ts1 ([a]Cologna et al., 2009).

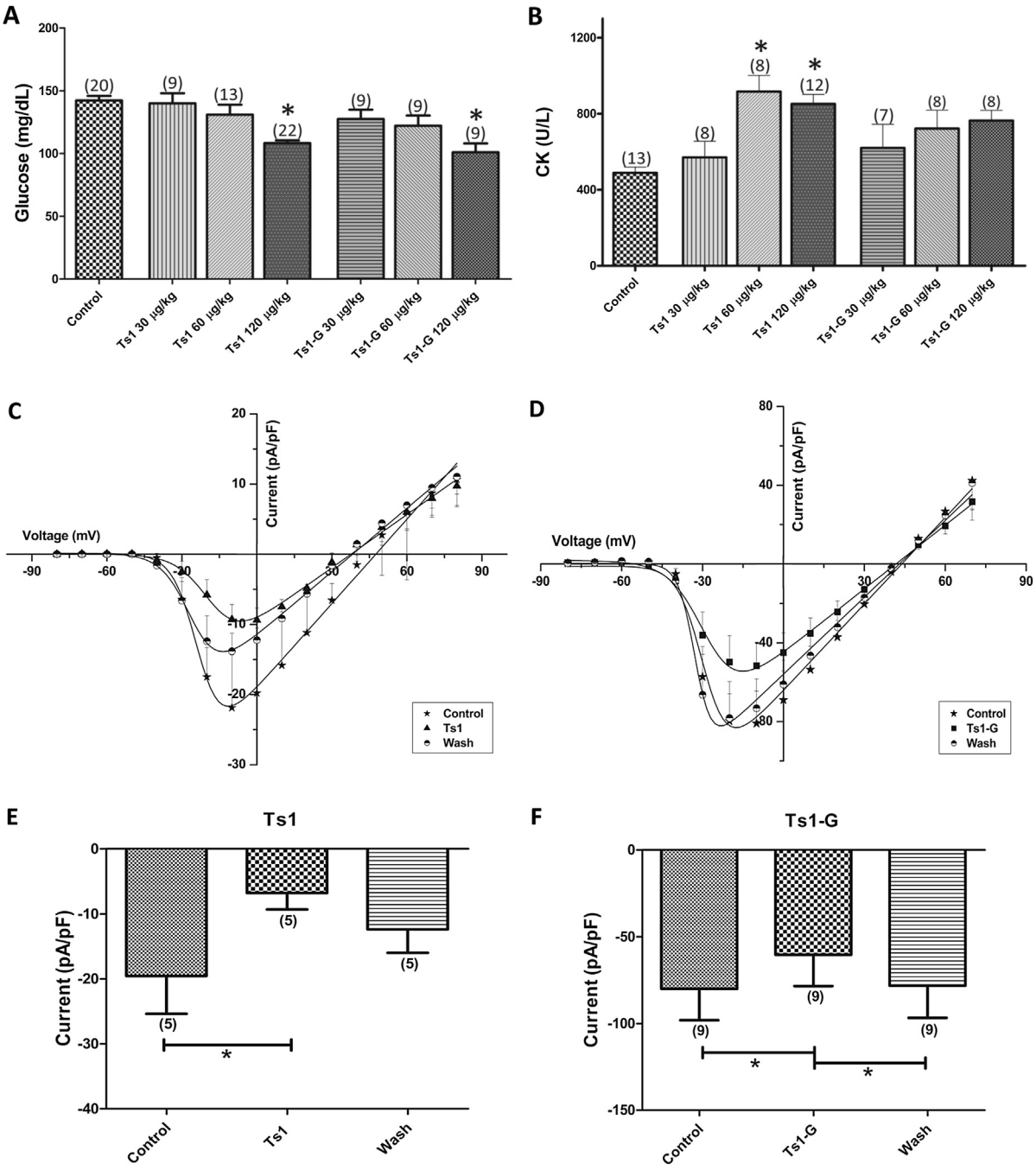

Fig. 2. **(A)** Blood glucose and **(B)** plasma creatine kinase concentrations of mice injected with 0.9% saline (control), Ts1 and Ts1-G at different doses (30, 60, 120 µg/kg). $^*p < 0.05$, values significantly different from the control group. The number of animals used in each group is above the bars. **(C)** and **(D)**, Sodium currents measured in DRGNs in control conditions and in the presence of Ts1 (500 nM) and Ts1-G (500 nM), respectively. I × V graphs were constructed by plotting the peak sodium current, normalized by the cells capacitance, against the respective clamp voltage. Points correspond to mean ± SEM of observations made in 5 cells for Ts1 and 9 cells for Ts1-G. Total sodium currents were measured in medium sized neurons of the DGR cells. **(E)** and **(F)** Statistical analysis of the blockage of sodium currents in the presence of Ts1 (500 nM) and Ts1-G (500 nM), respectively. $^*p < 0.05$, values significantly different from the control group or between the two test groups.

less positive charges and higher apolar characteristics and because of it, presents more interaction with the reverse-phase column.

Polyacrylamide gel electrophoresis of Fractions XIIB and XIII (Fig. 1D) shows that the major component of Fraction XIIB (Ts1-G) presents electrophoretic mobility smaller than Ts1 (Fraction XIII), which confirms that Ts1-G is less positively charged than Ts1. The same electrophoretic behavior can be observed for Ts1-G and purified Ts1 (Fig. 1E). PAGE confirms the purity of the toxins and makes possible to

observe the presence of an electrophoretic band of low intensity with the same mobility as Ts1, indicating the presence of small amounts of this toxin in fraction XIIB.

Mass spectrum of fraction XIIB (Fig. 1F) shows a peak with mass (m/z) of 6936.562 Da corresponding to Ts1-G and the peak of 6879.400 Da corresponding to Ts1, confirming the presence of a small amount of Ts1 in Fraction XIIB (Fig. 1F insert). The mass difference between these two peaks is 57.162, confirming the presence of an additional glycine residue in the molecule of Ts1-G.

Alignment of the sequences of Ts1-G (this work), the complete Ts1-Precursor sequence with the signal peptide and C-terminus GKK, and mature Ts1 (for review see Cologna et al., 2009) is shown in Fig. 1G. The N-terminal sequencing of the Ts1-G confirmed that it is identical to Ts1 up to residue 61, but presents Gly as C-terminal residue.

The comparative functional characterization between Ts1 and Ts1-G is shown in Fig. 2. The action of NaScTx on voltage-dependent sodium channels induces massive release of mediators such as adrenaline and noradrenaline from adrenals and noradrenergic nerve terminal, giving rise to several pathophysiological effects (Vasconcelos et al., 2005). Blood glucose levels and plasma CK levels were chosen to evaluate the systemic effects of Ts1 and Ts1-G, because they undergo important changes in *T. serrulatus* envenomation. These biochemical parameters were previously analyzed by Corrêa et al. (1997), Vasconcelos et al. (2004) and Cusinato et al. (2010) using *T. serrulatus* venom and/or Ts1. They showed that both, toxin and whole venom, induced fast hyperglycemia and increase of creatine kinase in rats, attributed to the intense discharge of catecholamines. These mediators induce hepatic glycogenolysis, which causes an initial increase in blood glucose, which in turn induces insulin release. Insulin peak was observed 1 h after *T. serrulatus* venom injection (Vasconcelos et al., 2004).

In our work, the blood glucose level of animals 1 h after injection of Ts1 and Ts1-G at different doses (Fig. 2A) showed a dose-dependent reduction. Glucose levels of animals injected with the toxins (120 μg/kg) were significantly lower when compared to control animals, but did not present significant differences among each other. However the levels observed with Ts1 were always slightly lower than those obtained with Ts1-G. The reduction of blood glucose induced by the toxins can be explained by increased insulin release and liver glycogen depletion (Vasconcelos et al., 2004).

Gly residue at the C-terminal region of the Ts1-G sequence was not sufficient to induce significant differences in the blood glucose levels of mice when compared to Ts1. On the other hand, the creatine kinase (CK) levels (Fig. 2B) were significantly higher in the animals injected with 60 and 120 μg/kg of Ts1 ($p < 0.005$), when compared with the control group, whereas the animals injected with Ts1-G showed a dose-dependent increase in CK levels, but without statistical significance. These results showed that Ts1-G still has some activity, but not as high as mature Ts1. There were no significant differences between the CK levels observed with both toxins. Patients stung by *T. serrulatus* scorpion that presented cardiac insufficiency and pulmonary edema showed increased levels of CK and its isozyme CK-MB (Hering et al., 1993). Significant increases of CK levels were also observed in rats injected with *T. serrulatus* venom and Ts1 (Corrêa et al., 1997; Cusinato et al., 2010). The cardiocirculatory failure observed in severe scorpion envenomations has been attributed to the massive release of catecholamines and/or to a direct toxic effect of the venom on cardiac fibers (Hering et al., 1993).

Fig. 2C and D shows the current × voltage relationships of control and treated DRGNs. As can be seen, both Ts1 (500 nM, 5 cells) and Ts1-G (500 nM, 9 cells) induced a reduction in the peak amplitude of the sodium currents. Nevertheless, Ts1 had a stronger effect when compared with its precursor. Ts1 reduced the maximal observed current by 57.1% (Fig. 2E) and the Ts1-G by 38.5% (Fig. 2F). Recovery after washing out the toxin was almost complete for the Ts1-G and no more than 63% of the current was recovered in the case of Ts1. It's also observed a negative shift of Erev induced by Ts1, but not by Ts1-G. Jonas et al. (1986) observed the same negative shift of Erev induced by Ts1, probably caused by the Na^+ accumulation during permanent Na current flow at rest.

Studies with post-translational modifications began in 1989, when Bougis and colleagues came to the conclusion that the structures of the precursors of toxins passed through a process of post-translational modifications analyzing precursors and mature toxins of the scorpion *Androctonus australis*. Auguste et al. (1990) confirm the importance of C-terminal amidation in the toxin Leiurotoxin I (*scyllatoxin*), a 31 amino acids polypeptide from the scorpion *Leiurus quinquestriatus hebraeu*. They compared binding inhibition of ^{125}I-apamin of a synthetic analogue of C-terminal amidated *scyllatoxin* (-$CONH_2$*scyllatoxin*) with another analogue without this amidation (-COOH*scyllatoxin*) in the preparation of rat brain membranes, which resulted in lower affinity for the non-amitated toxin analogue (-COOH*scyllatoxin*).

Benkhadir et al. (2004) expressed recombinant analogues with carboxylate C-terminal (rBotIII-OH and rAahII-OH) of two native toxins (BotIII and AahII) isolated from the venom of the scorpion *Buthus occitanus tunetanus* and *A. australis*, respectively, both with amidated C-terminal. In this study it was shown that LD_{50} values are 4–10 times smaller for recombinant toxins compared to native ones, confirming the loss of activity of the toxins by the absence of C-terminal amidation. In the study performed by Estrada et al. (2007) the relevance of C-terminal amidation to the toxin activity was also confirmed, since native CssII from the scorpion *Centruroides suffusus suffusus* has the C-terminal amidation and affinity for Na_v channels 15 times higher when compared to the recombinant C-terminal carboxylated toxin.

Legros et al. (2005) reported no differences between the native toxin AaHII which has its His64 C-terminal amidated, and other two mutants, one with suppression of H64 amidation and other with additional Gly (G65). The mutants exhibited exactly the same affinity for sodium channels as the native toxin. The same result was found by Saucedo et al., 2012: both native (C-terminal amidated) and recombinant CssII (non-amidated C-terminal) showed almost identical affinity for Na_vs heterologous expressed in CHO cells (Chinese hamster ovary cells).

It has been shown that C-terminal amidation in scorpion toxins usually plays a significant role in toxicity and/or stability against exopeptidases. The additional charge conferred by amidation appears to be required for maximum biological activity of the toxins, but there are inconsistent data in the literature (Rodríguez de la Vega and Possani, 2005; Legros et al., 2005; Benkhadir et al., 2004) and lack of standardized methodology. Although C-terminal amidation displays an important role in activity for some neurotoxins, for others it is not relevant (Merkler, 1994; Estrada et al., 2011).

In conclusion, this work is very relevant since it shows that the affinity of Ts1-G for the voltage-dependent sodium channels is reduced compared to the mature Ts1 toxin. The data obtained in this study revealed the important role of the C-terminal amidation for the biological activity of Ts1. Additionally, as long as we know, it is the first study comparing a mature amidated NaScTx with its non-amidated precursor, both isolated from the same venom. This ensures that Ts1, as well as its precursor, were perfectly folded and that the only difference between them is the non-amidated Gly at the C-terminal region of the precursor and the C-terminus amidation of mature Ts1. Other studies presented in the literature were performed with precursors obtained by heterologous expression that can generate toxins not perfectly folded. Our results indicate that the C-terminal amidation is important to Ts1 activity on Na_v channels, supporting the suspicion that incorporation of basic charged amino acid residues at the C-terminal tail of some scorpion toxins was favored by natural selection. Further studies are required to fully characterize the molecular interactions involved in the Ts1 and Ts1-G actions on specific subtypes of Na_vs.

Acknowledgments

ECA was supported by the following grants: São Paulo State Research Foundation (FAPESP # 2008/10761-0 and Scientific Initiation scholarship to VAC 2008/07373-9); The National Council for Scientific and Technological Development (CNPq, PhD scholarship for CMC 143472/2011-9), Coordination of the Advancement of Higher Education (CAPES, PhD scholarship for FAPA) and NAP-TOXAN-USP. WAV was supported by a grant from São Paulo State Research Foundation (FAPESP # 2010/16600-9).

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Arantes, E.C., Prado, W.A., Sampaio, S.V., Giglio, J.R., 1989. A simplified procedure for the fractionation of *Tityus serrulatus* venom: isolation and partial characterization of TsTX-IV, a new neurotoxin. Toxicon 27, 907–916.

Auguste, P., Hugues, M., Gravé, B., Gesquière, J.C., Maes, P., Tartar, A., Romey, G., Schweitz, H., Lazdunski, M., 1990. Leiurotoxin I (Scyllatoxin), a peptide ligand for Ca^{2+}-activated K^+ - Channels. J. Biol. Chem. 265, 4753–4759.

Barhanin, J., Ildefonse, M., Rougier, O., Sampaio, S.V., Giglio, J.R., Lazdunski, M., 1984. *Tityus gamma* toxin, a high affinity effector of the Na^+ channel in muscle, with a selectivity for channels in the surface membrane. Pflüg. Arch. 400, 22–27.

Benkhadir, K., Kharrat, R., Cestèle, S., Mosbah, A., Rochat, H., El Ayeb, M., Karoui, H., 2004. Molecular cloning and functional expression of the alpha-scorpion toxin BotIII: pivotal role of the C-terminal region for its interaction with voltage-dependent sodium channels. Peptides 25, 151–161.

Bougis, P.E., Rochat, H., Smith, L.A., 1989. Precursors of *Androctonus australis* scorpion neurotoxins: structures of precursors, processing outcomes, and expression of a functional recombinant toxin II. J. Biol. Chem. 264, 19259–19265.

Catterall, W.A., Cestele, S., Yarov-Yarovoy, V., Yu, F.H., Konoki, K., Scheuer, T., 2007. Voltage-gated ions channels and gating modifier toxins. Toxicon 49, 124–141.

Cerni, F.A., Pucca, M.B., Peigneur, S., Cremonez, C.M., Bordon, K.C.F., Tytgat, J., Arantes, E.C., 2014. Electrophysiological characterization of Ts6 and Ts7, K+ channel toxins isolated through an improved Tityus serrulatus venom purification procedure. Toxins 6 (3), 892–913.

Cestèle, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 82, 883–892.

Cologna, C.T., Marcussi, S., Giglio, J.R., Soares, A.M., Arantes, E.C., 2009. *Tityus serrulatus* scorpion venom and toxins: an overview. Protein Pept. Lett. 16, 920–932.

Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16, 10881–10890.

Corrêa, M.M., Sampaio, S.V., Lopes, R.A., Mancuso, L.C., Cunha, O.A.B., Franco, J.J., Giglio, J.R., 1997. Biochemical and histopathological alterations induced in rats by *Tityus serrulatus* scorpion venom and its major neurotoxin tityustoxin-I. Toxicon 35, 1053–1067.

Cusinato, D.A., Souza, A.M., Vasconcelos, F., Guimarães, L.F., Leite, F.P., Gregório, Z.M., Giglio, J.R., Arantes, E.C., 2010. Assessment of biochemical and hematological parameters in rats injected with *Tityus serrulatus* scorpion venom. Toxicon 56, 1477–1486.

Edman, P., Begg, G., 1967. A protein sequenator. Eur. J. Biochem. FEBS 1, 91.

Estrada, G., Garcia, B.I., Schiavon, E., Ortiz, E., Cestele, S., Wanke, E., Possani, L.D., Corzo, G., 2007. Four disulfide-bridged scorpion beta neurotoxin CssII: heterologous expression and proper folding in vitro. Biochim. Biophys. Acta 1770, 1161–1168.

Estrada, G., Restano-Cassulini, R., Ortiz, E., Possani, L.D., Corzo, G., 2011. Addition of positive charges at the C-terminal peptide region of CssII, a mammalian scorpion peptide toxin, improves its affinity for sodium channels Nav1.6. Peptides 32, 75–79.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflug. Arch. 391, 85–100.

Hering, S.E., Jurca, M., Vichi, F.L., Azevedo-Marques, M.M., Cupo, P., 1993. 'Reversible cardiomyopathy' in patients with severe scorpion envenoming by *Tityus serrulatus*: evolution of enzymatic, electrocardiographic and echocardiographic alterations. Ann. Trop. Paediatr. 13, 173–182.

Jonas, P., Vogel, W., Arantes, E.C., Giglio, J.R., 1986. Toxin gamma of the scorpion *Tityus serrulatus* modifies both activation and inactivation of sodium permeability of nerve membrane. Pflug. Arch. 407, 92–99.

Kirsch, G.E., Skattebol, A., Possani, L.D., Rown, A.M., 1989. Modification of Na^+ channel gating by a scorpion toxin from *Tityus serrulatus*. J. Gen. Physiol. 93, 67–83.

Legros, C., Ceard, B., Vacher, H., Marchot, P., Bougis, P.E., Martin-Eauclaire, M.F., 2005. Expression of the standard scorpion alpha-toxin AaH II and AaH II mutants leading to the identification of some key bioactive elements. Biochim. Biophys. Acta Gen. Subj. 1723, 91–99.

Marcussi, S., Arantes, E.C., Soares, A.M., Giglio, J.R., Mazzi, M.V., 2011. Escorpiões: Biologia, envenenamento e mecanismos de ação de suas toxinas, first ed. Funpec, Ribeirão Preto.

Martin- Eauclaire, M.F., Ceard, B., Ribeiro, A.M., Diniz, C.R., Rochat, H., Bougis, P.E., 1994. Biochemical, pharmacological and genomic characterization of ts-iv, an alpha-toxin from the venom of the South-American scorpion *Tityus serrulatus*. Febs Lett. 342, 181–184.

Martin-Eauclaire, M.F., Ceard, B., Ribeiro, A.M., Diniz, C.R., Rochat, H., Bougis, P.E., 1992. Molecular-cloning and nucleotide-sequence analysis of a cdna-encoding the main beta-neurotoxin from the venom of the South-American scorpion *Tityus serrulatus*. Febs Lett. 302, 220–222.

Merkler, D.J., 1994. C-terminal amidated peptides – production by the in-vitro enzymatic amidation of glycine-extended peptides and the importance of the amide to bioactivity. Enzym. Microb. Technol. 16, 450–456.

Pessini, A.C., Takao, T.T., Cavalheiro, E.C., Vichnewski, W., Sampaio, S.V., Giglio, J.R., Arantes, E.C., 2001. A hyaluronidase from *Tityus serrulatus* scorpion venom: isolation, characterization and inhibition by flavonoids. Toxicon 39, 1495–1504.

Possani, L.D., Becerril, B., Delepierre, M., Tytgat, J., 1999. Scorpion toxins specific for Na^+-channels. Eur. J. Biochem. FEBS 264, 287–300.

Quintero-Hernández, V., Jiménez-Vargas, J.M., Gurrola, G.B., Valdivia, H.H., Possani, L.D., 2013. Scorpion venom components that affect ion-channels function. Toxicon 76, 328–342.

Rodríguez de la Vega, R.C., Possani, L.D., 2005. Overview of scorpion toxins specific for Na^+ channels and related peptides: biodiversity, structure-function relationships and evolution. Toxicon 46, 831–844.

Rodríguez de la Vega, R.C.R., Possani, L.D., 2007. Novel paradigms on scorpion toxins that affects the activating mechanism of sodium channels. Toxicon 49, 171–180.

Schiavon, E., Pedraza-Escalona, M., Gurrola, G.B., Olamendi-Portugal, T., Corzo, G., Wanke, E., Possani, L.D., 2012. Negative-shift activation, current reduction and resurgent currents induced by β-toxins from Centruroides scorpions in sodium channels. Toxicon 59, 283–293.

Saucedo, A.L., Rio-Portilla, F., Picco, C., Estrada, G., Prestipino, G., Possani, L.D., Delepierre, M., Corzo, G., 2012. Solution structure of native and recombinant expressed toxin CssII from the venom of the scorpion *Centruroides suffusus suffusus*, and their effects on Nav1.5 sodium channels. Biochim. Biophys. Acta 1824, 478–487.

Toledo, D.V., Neves, A.G.A., 1976. Purification and partial characterization of a second toxin from the scorpion *Tityus serrulatus*. Comp. Biochem. Physiol. 55, 249–253.

Vasconcelos, F., Sampaio, S.V., Garófalo, M.A., Guimarães, L.F., Giglio, J.R., Arantes, E.C., 2004. Insulin-like effects of *Bauhinia forficata* aqueous extract upon *Tityus serrulatus* scorpion envenoming. J. Ethnopharmacol. 95, 385–392.

Vasconcelos, F., Lanchote, V., Bendhack, L.M., Giglio, J.R., Sampaio, S.V., Arantes, E.C., 2005. Effects of voltage-gated Na^+ channel toxins from *Tityus serrulatus* venom on rat arterial blood pressure and plasma catecholamines. Comp. Biochem. Physiol. Toxicol. Pharmacol. CBP 141, 85–92.