

Tityus serrulatus venom and its toxins Ts1 and Ts5 increase cytosolic Ca^{2+} concentration in isolated vascular smooth muscle cells*

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ABSTRACT

Voltage-gated Na^+ channel (Na_v channel) scorpion toxins are classified as α - and β -neurotoxins. Ts5 (α -neurotoxin) and Ts1 (β -neurotoxin) from *Tityus serrulatus* venom (TsV) interact with Na_v channels, increasing Na^+ influx and activating voltage-dependent Ca^{2+} channels. This study aimed to investigate the effect of TsV, Ts1 and Ts5 on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in rat aortic smooth muscle cells. Toxins were isolated by ion exchange chromatography (Ts1) followed by RP-HPLC (Ts5). The rat aortic smooth muscle cells were isolated in Hanks buffer pH 7.4 and loaded with 5 μ mol/L of Fura-2AM (45 minutes at 37°C), in order to measure $[Ca^{2+}]_c$ by fluorescence of Fura-2/AM (ratio 340/380nm). The fluorescence was measured in one single cell (excitation: 340 and 380 nm; emission: 510 nm). TsV (100 and 500 μ g/mL) and its toxins Ts1 and Ts5 (50 and 100 μ g/mL each) led to a concentration-dependent increase in $[Ca^{2+}]_c$. Tetrodotoxin (1 μ mol/L), a Na_v channel blocker, and verapamil (1 μ mol/L), a voltage-operated Ca^{2+} channel blocker, inhibited the increase in $[Ca^{2+}]_c$ induced by TsV (500 μ g/mL). In conclusion, TsV and its toxins induce a concentration-dependent increase in $[Ca^{2+}]_c$ that probably occurs through interaction with Na_v channels, thus inducing depolarization and consequent Ca^{2+} influx. This assumption is based on the fact that this effect is inhibited by tetrodotoxin and verapamil, showing a direct action of TsV toxins on aorta smooth muscle cells.

*Conflict of Interest: There are no conflicts of interest with regards to this manuscript.

Keywords: Ts1; Ts5; Na^+ Channel; Ca^{2+} Channel; *Tityus serrulatus*

1. INTRODUCTION

Tityus serrulatus is the most dangerous scorpion species in Brazil, yielding a mortality rate of 1.04% among children [1]. Most of the symptoms and signs observed in the scorpion envenomation, such as fever, psychomotor agitation, salivation, lachrymation, increased gastrointestinal tract mobility, cardiac and respiratory arrhythmias, arterial hypertension followed by hypotension, cardiac failure, pulmonary edema and shock, is a consequence of widespread peripheral nerve stimulation, culminating in a massive release of neurotransmitters [2-9].

Scorpion venoms are rich sources of different classes of peptides that represent useful tools for biological research. These peptides disrupt the normal function of excitable tissues found in muscles and nerves by interaction with voltage-gated Na^+ , K^+ , Ca^{2+} or Cl^- channel [10-13]. Voltage-gated Na^+ (Na_v) channel neurotoxins are the most important components of the scorpion venom, and they are the main agents responsible for the toxic effects of scorpion envenoming. Two types of toxins (α and β) that are active on the Na_v channels have been described, and they are classified according to their specificity toward two different sites on the channel [12-14]. The α -neurotoxins bind to receptor site 3 retarding Na_v channel inactivation and inducing a prolongation of the action potential depolarization phase. The β -neurotoxins bind to receptor site 4 and shift the voltage dependence of Na_v channel activation to more negative potentials, giving rise to an increased tendency of the cell to fire spontaneously and repeatedly, independent of the membrane potential [13,14].

Scorpion neurotoxins (α and β) act on the Na_v channel

of nerve terminals, resulting in increased Na^+ influx, which in turn causes depolarization of the cell membrane. Depolarization induces the opening of the voltage-activated calcium channel, allowing the entrance of Ca^{2+} . This represents a key step in the regulation of a variety of cellular processes, such as the neurotransmitter release [7,15,16].

The venom of the Brazilian scorpion *Tityus serrulatus* Lutz & Mello (Buthidae family) contains both types of neurotoxins, represented by Ts5 (α -neurotoxin) and Ts1 (β -neurotoxin). Ts5 was first isolated and characterized by Arantes *et al.* (1994) [17] as a basic ($\text{pI} = 8.0$) toxic polypeptide ($\text{LD}_{50} = 94 \pm 7 \mu\text{g}/\text{kg}$, i.v. in mice) that induces a prolongation of Na_v channel inactivation. The amino acid sequence of the toxin Ts5 has also been determined. It has 64 amino acid residues and a calculated M_r of 7230 [18].

The major toxic component from *T. serrulatus* venom (TsV) is a long-chain β -neurotoxin Ts1 [19,20], also called TsTX-I, toxin VII or toxin- γ (updated nomenclature by Cologna *et al.*, 2009 [21]), and it has an intravenous LD_{50} of $76 \pm 9 \mu\text{g}/\text{kg}$, compared to $375 \pm 45 \mu\text{g}/\text{kg}$ for TsV [22]. Ts1 plays a major role in *T. serrulatus* envenoming, accounting for approximately 16% of the soluble venom, while Ts5 represents only 2% of TsV. In rats, they induce intense catecholamine release, with concomitant increase in the mean arterial pressure [7].

Despite the fact that catecholamines and acetylcholine mediate most of the toxicological actions of scorpion venoms, other neurotransmitters are also involved in these actions [23-30]. Vasconcelos *et al.* (2005) [7] reported that the toxicity of Ts5 is not only related to its ability to release catecholamines, but modulation of the release of other neurotransmitters may be involved. The Ts5 causes a reduction in ^3H -GABA and ^3H -DA uptake in a Ca^{2+} -dependent manner, not directly affecting GABA transporters, but, it involves Na_v channels as a consequence of depolarization [15].

Thus, both α - and β -neurotoxins may modulate the neurotransmitter release via a Ca^{2+} -dependent mechanism, although these toxins act on the Na_v channel, allowing increased Na^+ influx. This in turn causes membrane depolarization, leading to the activation of voltage-dependent Ca^{2+} channels.

Most of the actions of the scorpion toxins are indirect and due to neurotransmitter release, but Teixeira Jr. *et al.* (2001) [31] have reported a possible direct action of TsV on the cardiac muscle. The venom-induced positive inotropic effects are not modified by pretreatment with metoprolol, or chemical sympathetic denervation with 6-OH-dopamine. These results demonstrate that the inotropic effects of the venom on the rat heart are not dependent on neurotransmitter release. The increase in the contractile force appears to be a direct effect of the

venom on the cardiomyocyte [31]. Cardiac voltage-gated Na^+ channels are also targets of scorpion toxins [32]. It has been demonstrated in earlier studies that vascular smooth muscle contraction is induced by Na^+ channel activators (like Na_v channel scorpions neurotoxins), veratridine and batrachotoxin [33].

Bearing in this background in mind and considering that the direct action of neurotoxins from scorpion venoms on vascular muscle cells are not yet fully understood, the objective of this study was to investigate the effect of TsV and its purified toxins Ts5 and Ts1 on the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) in isolated rat aorta smooth muscle cells, as well as the mechanism involved in this effect.

2. MATERIAL AND METHODS

2.1. Animals

Male Wistar rats (180 - 200 g) were obtained from the animal facility of the Campus of Ribeirão Preto, University of São Paulo (USP), and maintained under standard laboratory conditions of 12 h dark/light cycle, at a temperature of 21°C . The rats were housed in polystyrene cages (4 rats/cage, $50 \times 35 \times 15$ cm) containing stainless steel coverlids and wooden shavings as bedding. The animals were anesthetized and euthanized by decapitation on the day of the experiment and a segment of the thoracic aorta (15 mm) was isolated and maintained in Hanks solution.

All the experimental procedures were conducted according to the Institutional Ethics Committee of the University of São Paulo-Brazil (Protocol Nr. 03. 1.1069.53.0).

2.2. Ca^{2+} Measurements

The aortic smooth muscle cells were isolated on the day of the experiment and loaded with $5 \mu\text{mol}/\text{L}$ Fura-2AM for 45 minutes, in order to measure $[\text{Ca}^{2+}]_c$ by fluorescence of Fura-2AM (ratio 340/380 nm). Experimental protocols were developed using the venom (TsV) and its toxins (Ts1 and Ts5) in the presence or in the absence of a sodium channel blocker (tetrodotoxin) and a calcium channel blocker (Verapamil) added 10 minutes prior to stimulation with the venom or toxins. Data are expressed as the difference between the basal and final fluorescence intensities ($\Delta\%IF$), which reflects the increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) obtained for each protocol.

Briefly, the aortas were dissected and longitudinally opened, and smooth muscle cells were isolated by enzyme digestion with collagenase in Hanks solution. The resulting cell solution was centrifuged at $200 \times g$ and suspended in Hanks solution. The cells were placed on glass coverslips and kept in a humidified 37°C incubator

gassed with 5% CO₂ for 4 h. Then, the viability of the cells at this stage was determined by exclusion of 0.4% trypan blue dye, being invariably larger than 95%. The cells were then incubated with 5 μmol/L Fura-2AM in 1 mg/mL bovine soroalbumin (BSA) for 45 minutes, at 37°C, and then washed for 20 minutes. Dishes containing Fura-2AM loaded cells were placed in a temperature regulated (37°C) chamber mounted on the Nikon inverted microscope. Each fluorescence measurement was performed in one isolated cell, in the window of a dual wavelength spectrofluorometer (Deltaram, Photon Technology Intl), at excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm. Fura-2 fluorescent signals originated from the cells were collected and stored using a software package from Photon Technology International (Felix). Results represent the Δ%IF of the F/F₀ ratio and are expressed in percentage. The F values represent the final fluorescence, after pharmacological stimulation, and F₀ represents the basal fluorescence [34].

2.3. Venom Extraction and Ts1 and Ts5 Isolation

2.3.1. Soluble Venom (TsV)

Lyophilized TsV was purchased from Phoneutria Biotechnology and Services (Belo Horizonte-MG, Brazil) and stored at -20°C. The venom was dissolved in 0.9% (w/v) NaCl and centrifuged at 11,270 × g at 27 °C, for 5 minutes. TsV quantification was based on total proteins.

2.3.2. Protein Determination

Determination of total proteins was carried out according to the method described by Bradford [35], using commercially acquired kits (Bio-Rad Protein assay, Bio-agency®), and confirmed by the method of absorbance at 205/280 nm [36]. BSA was used as standard.

2.3.3. Ion Exchange Chromatography

TsV was fractionated at 5°C by ion exchange chromatography as previously described [22]. The soluble extract from the crude venom (400 mg) was chromatographed on a 2.5 × 63.0 cm carboxymethyl cellulose-52 column (Whatman), which had been equilibrated and initially eluted with 0.01 mol/L ammonium bicarbonate buffer pH 7.8 up to 468 mL effluent, when a convex concentration gradient was started from 0.01 mol/L to 1.00 mol/L buffer. The flow rate was 26.7 mL/h. The resulting pools designated I-XIII were then lyophilized until salt-free, followed by determination of the buffer concentration of each fraction by measurement of the corresponding conductivity. Pure Ts1 (pool X-III) was obtained in this single chromatographic step. The recovery was calculated in terms of the absorbance at 280 nm.

2.3.4. Reverse-Phase Liquid Chromatography

RP-HPLC of the lyophilized pool XI was performed in a Shimadzu HPLC system, using a C-18 reverse-phase analytical column Shim Pack CLC-ODS (M) 5 μm, 0.46 × 25.0 cm (Shimadzu Instruments Corp., Tokyo, Japan) at room temperature. The column had been previously equilibrated with buffer A (5% acetonitrile + 0.1% trifluoroacetic acid). Elution was performed with a linear gradient of Buffer B (60% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 1.0 mL/min. Absorbance was monitored at 214 nm. The resulting highly purified toxin was previously named TsTX-V [17]. The RP-HPLC of Ts1 was carried out under the same conditions.

2.4. Drugs and Solutions

Albumin bovine fraction V, fatty acid-free, Albumin bovine fraction V, Dimethyl sulfoxide (Grade I) DM-SO, Poly-L-lysine solution, tetrodotoxin and verapamil were obtained from Sigma Chem. Co. (St Louis, MO-USA). Fura-2AM was purchased from Molecular Probes (Eugene, Oregon, USA).

The Hanks solution was as follows (in mmol/L): 145.0 NaCl, 1.6 CaCl₂, 5.0 KCl, 1.0 MgCl₂, 0.5 Na-H₂PO₄, 10.0 dextrose and 10.0 HEPES (pH 7.4).

2.5. Statistical Analysis

The effect on the cytosolic Ca²⁺ concentration ([Ca²⁺]_c) elicited by TsV or its toxins (Ts1 and Ts5) was represented by the percentage of the Δ of fluorescence intensity of each protocol in relation to the positive control (100%) KCl 60 mmol/L. Analysis of the data and plotting of the figures were performed with the aid of the software GraphPad PRISM™, Version 3.02 (2000) (San Diego, CA, USA). Data are expressed as mean ± SEM. In each set of experiments, *n* indicates the number of cells studied. Differences between mean values were assessed by one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test, and values of *P* < 0.05 were considered significant.

3. RESULTS

The effects of TsV, Ts1 and Ts5 in increasing [Ca²⁺]_c in isolated rat aorta smooth muscle cells were evaluated and compared to the depolarizing effect of KCl 60 mmol/L (positive control: 100%).

TsV (100 and 500 μg/mL) increased the [Ca²⁺]_c in 49.6% ± 2.6% and 103.7% ± 5.2%, respectively, when compared with the positive control (100%), showing a concentration-dependent effect (**Figures 1 and 2**). The lower TsV concentration induced an increase in [Ca²⁺]_c that was different from those induced by the control (*P* < 0.001; *n* =

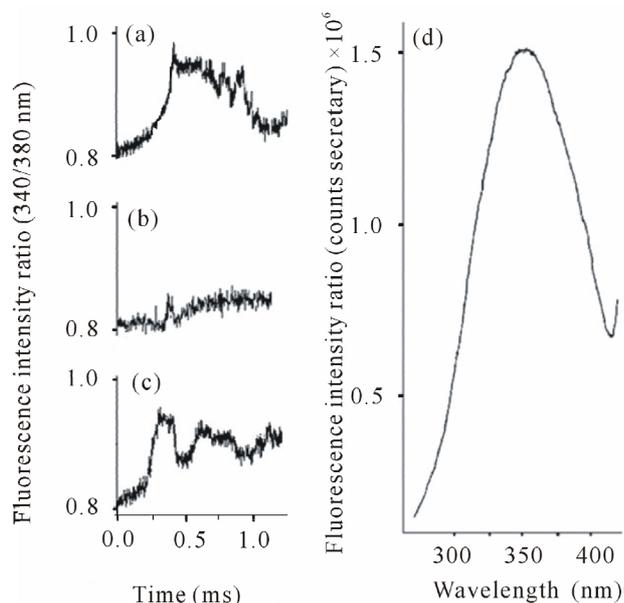


Figure 1. Representative records of assays performed with vascular smooth muscle cells of rat aorta. Ratio of fluorescence intensity (340/380 nm) after perfusion of the cells with: (a) KCl 60 mmol/L; (b) TsV 100 µg/mL; and (c) TsV 500 µg/mL. (d) Representative fluorescence profile of cell loaded with the probe Fura-2AM (counts/sec).

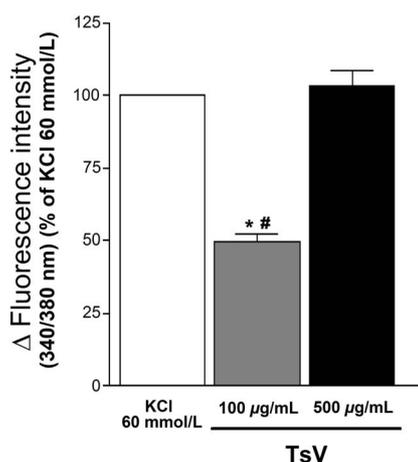


Figure 2. Effect of the TsV on Ca²⁺ cytosolic concentration ([Ca²⁺]_c) in rat aortic smooth muscle cells. Bars represent [Ca²⁺]_c stimulated with TsV 100 and 500 µg/mL. # P < 0.001: TsV 100 µg/mL vs KCl 60 mmol/L (n = 3, ANOVA); *P < 0.001: TsV 100 µg/mL vs TsV 500 µg/mL (n = 3, ANOVA).

3) and the higher TsV concentration (P < 0.001; n = 3).

In order to investigate the Na⁺-dependence of the Ca²⁺ influx elicited by TsV, the rat aortic smooth muscle cells were pre-incubated with tetrodotoxin (TTX), a Na_v channel blocker, or verapamil, a voltage-operated Ca²⁺ channel blocker. After incubation for 10 minutes, the cells were stimulated with 500 µg/mL of TsV, and the increase in [Ca²⁺]_c was determined and compared with

the control (100%). TsV at 500 µg/mL was selected because at this concentration its effect on the [Ca²⁺]_c was similar to that of the control, more precisely 103.7% ± 5.2%. When the cells were incubated with TTX (1 µmol/L), the increase in [Ca²⁺]_c elicited by TsV (500 µg/mL) was reduced to 15.7% ± 3.1% (P < 0.001). However, when the cells were incubated with verapamil (1 µmol/L), the increase in [Ca²⁺]_c elicited by TsV (500 µg/mL) was reduced to 47% ± 5.8% (P < 0.01) (**Figure 3**). Cells pre-incubation with TTX was more effective in reducing the effect of TsV on the [Ca²⁺]_c than pre-incubation with verapamil (P < 0.05).

The effects of Ts1 and Ts5 (50 and 100 µg/mL of each) on the increase in [Ca²⁺]_c was investigated and compared with the control (100%) (**Figure 4**). Ts1, 50 µg/mL and 100 µg/mL, increased the [Ca²⁺]_c in 43.9% ± 3.1% and 121.8% ± 8.9%, respectively (**Figure 5**). The effect of the higher Ts1 concentration was different from that of lower concentration (P < 0.001; n = 3), as observed with the TsV. Ts5 exhibited a similar concentration-dependent effect. The concentrations of 50 µg/mL and 100 µg/mL increased the [Ca²⁺]_c in 52.6% ± 8.3% and 79.5% ± 6.1%, respectively (**Figure 5**). However, the effect of Ts1 (100 µg/mL) was greater than that of Ts5 at the same concentration.

4. DISCUSSION

Scorpion venoms are extremely rich on bioactive peptides (toxins) that often carry diverse functions and are presumably needed to achieve synergistic effects for rapid prey immobilizing and consequent defense. The

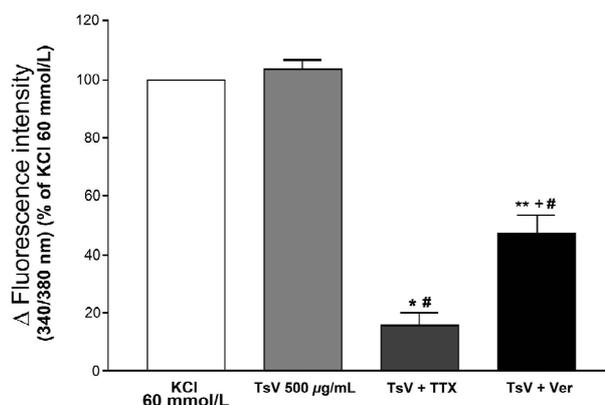


Figure 3. Effects of tetrodotoxin (TTX) and verapamil (Ver) on the effect elicited with TsV on Ca²⁺ cytosolic concentration ([Ca²⁺]_c) in rat aortic smooth muscle cells. Bars represent [Ca²⁺]_c stimulated with TsV 500 µg/mL, TsV (500 µg/mL) pre-incubated with TTX (1 µmol/L), and TsV (500 µg/mL) pre-incubated with verapamil (1 µmol/L). #P < 0.001: each group vs KCl 60 mmol/L (n = 3, ANOVA); *P < 0.001: TsV 500 µg/mL vs TsV + TTX (n = 3, ANOVA); **P < 0.01: TsV 500 µg/mL vs TsV + Ver (n = 3, ANOVA); +P < 0.05: TsV + TTX vs TsV + Ver (n = 3, ANOVA).

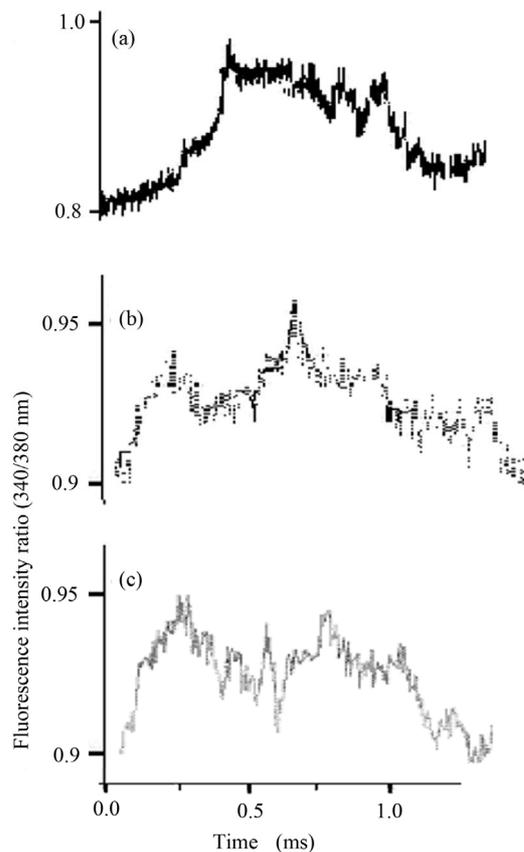


Figure 4. Representative records of assays performed with vascular smooth muscle cells of rat aorta. Ratio of fluorescence intensity (340/380 nm) after perfusion of the cells with: (a) KCl 60 mmol/L; (b) Ts1 50 µg/mL; and (c) Ts5 50 µg/mL.

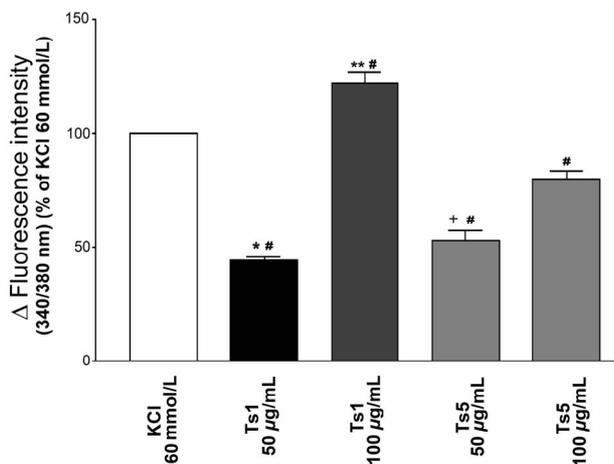


Figure 5. Effect of the toxins Ts1 and Ts5 on Ca^{2+} cytosolic concentration ($[Ca^{2+}]_c$) in rat aortic smooth muscle cells. Bars represent $[Ca^{2+}]_c$ stimulated with Ts1 50 and 100 µg/mL, and Ts5 50 and 100 µg/mL. # $P < 0.05$: group vs KCl 60 mmol/L ($n = 3$, ANOVA); * $P < 0.001$: Ts1 50 µg/mL vs Ts1 100 µg/mL ($n = 3$, ANOVA); ** $P < 0.01$: Ts1 100 µg/mL vs Ts5 100 µg/mL ($n = 3$, ANOVA); + $P < 0.01$: Ts5 50 µg/mL vs Ts5 100 µg/mL ($n = 3$, ANOVA).

major cause of death observed in victims stung by scorpions of the Buthidae family has been mainly attributed to cardiovascular toxicity resulting from the massive release of catecholamines from adrenal and noradrenergic nerve terminals, together with complications arising from the onset of pulmonary edema and respiratory arrest [2-4,7-9,16]. In addition to the role of catecholamines and other neurotransmitters, a direct action of scorpion toxins on the cardiovascular system has been repeatedly reported [29,37,38].

The synergistic actions of α - and β -toxins as well as the K^+ channel toxins are responsible for the lethality of the *Tityus serrulatus* venom (TsV). The α - and β -toxins are capable of exerting two distinct effects on the gating mechanism of Na^+ currents in nerves and muscles: α toxins block voltage-dependent inactivation of Na^+ channels and β toxins shift the voltage dependent activation of these channels to a more negative membrane potential. The α -toxins induce a prolongation of the repolarization phase of the action potential while β -toxins promote a repetitive firing after a unique stimulation [13, 14].

The main goal of this study was to show for the first time the effect of *Tityus serrulatus* scorpion venom and its toxins Ts1 and Ts5 on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in isolated rat aorta smooth muscle cells. The scorpion venom and its toxins increased the fluorescence intensity related to the increase in $[Ca^{2+}]_c$. Consequently, the contractile effect on the vascular smooth muscle cells was increased in a concentration-dependent manner.

The TsV (100 and 500 µg/mL) and toxins (50 and 100 µg/mL) concentrations were chosen on the basis of their respective effects. The higher TsV and Ts1 concentrations (500 and 100 µg/mL, respectively) induced effects similar to those observed with the positive control (KCl 60 mmol/L), which produced the maximum contractile effect on aorta smooth muscle cells. The lower concentrations were important to demonstrate that the venom and toxin effects are concentration-dependent and allowed a comparative analysis of the effectiveness of TsV, Ts1 and Ts5.

Our results show that TsV and its toxins Ts1 and Ts5 cause an increase in $[Ca^{2+}]_c$ in rat aortic smooth muscle cells. We suggest that the contractile effect induced by the scorpion venom and its toxins are due to the Na^+ influx through voltage-gated Na^+ channels (Na_v), which induces membrane depolarization and results in activation of voltage-dependent Ca^{2+} channels. This is supported by the fact that the change in fluorescence intensity induced TsV was powerfully reduced by tetrodotoxin (TTX), a Na^+ channel blocker. Since both neurotoxins have been obtained from the venom, the effect elicited by TsV is, at least in part, dependent on the presence of Ts1 and Ts5. The contribution of Ts1 to the venom effect is probably greater than the one of Ts5, because it is present in a lar-

ger proportion in the venom and causes a larger increase in the $[Ca^{2+}]_c$ at the concentration of 100 $\mu\text{g/mL}$.

To confirm the hypothesis of extracellular Ca^{2+} influx via voltage-dependent Ca^{2+} channels, we incubated the cells with verapamil, a selective Ca^{2+} -L channel blocker. Since the increase in $[Ca^{2+}]_c$ induced by TsV is partially inhibited by verapamil, we suggest that voltage-operated Ca^{2+} channels sensitive to verapamil are indirectly activated by the scorpion venom. However, it is possible that activation of voltage-operated Ca^{2+} channel is not the only mechanism involved in the $[Ca^{2+}]_c$ increase caused by TsV, since the block observed in the presence of verapamil was only partial. Based on these experimental evidences, the increase in $[Ca^{2+}]_c$ that is not inhibited by verapamil could be explained by activation of mechanisms that release Ca^{2+} from intracellular organelles such as the sarcoplasmic reticulum, CIRC (Ca^{2+} -induced release of Ca^{2+}), and Ca^{2+} released via IP_3 and ryanodine receptors [39].

Grolleau *et al.* [40] reported that other targets such as high voltage activated calcium channels and non capacitive calcium entry (NCCE) may be involved in the neurotoxicity of toxin VII, a β -neurotoxin from TsV. These findings have generated many interesting questions relating envenoming physiopathology relationships. NCCE is thought to be an important pathway for calcium influx into many cellular types including excitable as well as nonexcitable cells such as fibroblasts, vascular smooth muscle cells and neurosecretory neurons [40-42].

Most of the effects of scorpion toxins are indirect and due to the release of adrenergic and cholinergic neurotransmitters. However, the results obtained in this work show that TsV and the toxins Ts1 and Ts5 are also able to interact directly with Na_v channel in vascular smooth muscle cells (VSMC) and increase $[Ca^{2+}]_c$, which causes contraction of the blood vessel. The presence and function of Na_v channel in VSMC has been shown [43-45].

Clinically, these results could be related with many symptoms promoted by scorpion venom, such as respiratory paralysis, altered consciousness, convulsions [46] and cardiovascular abnormalities related to arterial hypertension, in human envenomation [2].

5. CONCLUSION

In conclusion, TsV and its toxins Ts1 and Ts5 produce a direct effect on rat aorta smooth muscle cells, inducing a concentration-dependent increase in $[Ca^{2+}]_c$ probably by activation of Na_v channels sensitive to tetrodotoxin. Consequently, there is particular extracellular Ca^{2+} influx, at least in part via Ca^{2+} channels sensitive to verapamil.

6. ACKNOWLEDGEMENTS

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