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Original Article

Voltage-Gated Sodium Channels Modulation by Bothutous Schach Scorpion Venom

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Abstract

Buthotus schach is one of the dangers scorpions in Iran that belong to the Buthidae family. Toxins are existing in venom scorpion, modulate the ion channels by blocking or opening the pore of the channel or by altering the voltage gating and useful as pharmacological tools. In the present study, we investigated the effects of venom and its obtained fractions by gel filtration on electrophysiological properties of magnocellular supraoptic of hypothalamus by using whole cell patch clamp. Our results shown indicated scorpion crude venom and its fraction effect on voltage gated sodium channels. A significant decrease was revealed in amplitude firing, in venom various concentration and some of the venom fractions. Also, a significant increase was shown in half width and rise time 10% to 90% actions potential firing. Previous evidence revealed a change in electrophysiological properties such as amplitude and rise time 10% to 90%, related to voltage gated sodium channels. Sodium channels toxins existed on scorpion venom caused modulate on sodium channels. In order to access bioactive components, six fractions were collected by gel chromatography techniques. After bath application of fractions, F2 and F3 components show the same effects than venom on electrophysiological properties of magnocellular supraoptic.

Key words: *Buthotus schach,* scorpion toxin, Voltage gated sodium channels, whole-cell patch clamp.

1. Introduction

The scorpion venom is libraries of a variety of different biological activities such as neurotoxins, antimicrobial peptides, and proteases [1-3]. Neurotoxins are specifically bond on pharmacological targets, especially on ion channels and modify the pores of the ion channels, that have been useful in deciphering the structure and function of ion channels or useful as pharmacological tools and therapeutic

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agents [4, 5]. Scorpion neurotoxins are generally classified into two groups based on their molecular weights. The sodium ion channel toxins are a kind of toxin with 58-76 amino acids residues in length, tightly bound by four disulfide bonds [6, 7] that decrease neuronal excitation by modulating the function of voltage gated sodium channels [8, 9]. Further neurotoxins sodium channels due to their pharmacological effects were classified as α and β toxins. Voltage-gated sodium channels are integral membrane proteins. These kinds of ion channels have been caused initiation and propagation of action potentials therefore; play a central role in the electrical connection of excitable cells [10, 11]. Buthidae is known as the largest scorpion family and plays an important role in medicine [12-14]. *Buthotus Schach (BS) recently called hottentotta zagrosensis, a member of Buthidae family, can be distributed in Middle West region of Iran.* Specifically, BS scorpion can be found in the central and southern part of Iran and Iraq [15]. BS venom is a rich source of biologically active peptides with varied ion-channel specificities. That can cause various effects ranging from local pain, inflammation, convulsion, necrosis, respiratory depression, and cardiac arrest in human. In previous studies shown, BS venoms can cause paralytic effects on nerve-muscle preparations [16, 17]. Also, in other study, it was shown that the scorpion toxin OD1 was fraction of Odontobuthus Doriae venom (Iranian Scorpion) that is a potent modulator of Nav1.7, with a unique selectivity pattern [18, 19]. The Purpose of this research was to examine the effects of BS scorpion crude venom and its obtained fractions by use of gel chromatography on sodium channels of the magnocellular neurons (MCNs) of supraoptic nucleus (SON) in rat hypothalamus, by using whole cell patch-clamp techniques.

2. Materials and Methods

2.1. Animals

Young male rats weighting 70-100 g (Pasteur Institute, Tehran, Iran) were purchased for studying. The animals were housed in cages that were allowed free access to food and tap water under standardized housing conditions with a 12 h light–dark cycle and at room temperature of 25 ± 2 °C with a relative humidity of 40-60%. All procedures were carried out according to the guidelines of the National Institute of Health (NIH Publications No. 80-23, revised 1996) and were approved by Shahid Beheshti University of Medical Sciences of Ethics Committee.

2.2. Venom

BS crude venom was gifted by the Department of Poisonous Animals, Razi Vaccine and Serum Research Institute, Karaj, Iran. The venom was obtained by electrically stimulating of scorpions and then lyophilized [16].

2.3. Purification

Lyophilized crude venom *Buthotus schach* (300 mg) was dissolved in 5 mL of 0.1 mM ammonium acetate buffer (pH 8.2) and centrifuged at 14,000 rpm for 15 minutes at 4ºC. Fractioning of the clear supernatant of venom was applied with using a Sephadex G50 column (150 x 2 cm), previously equilibrated and eluted with a pH = 8.2, 0.1 M ammonium acetate buffer (16) Bradford method at 595 nm estimated for protein content by using spectrophotometrically. Finally, fractions were lyophilized and kept at room temperature.

2.4. Slice preparation

*R*ats were anaesthetized with isoflorane and then decapitated. The brains were immediately removed, blocked in the Coronal plane and chilled in ice-cold oxygenated slicing solution containing (in mM): 206 sucrose, 2.8 KCl, 1 $MgCl_2$, 2 $MgSO_4$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 D-glucose. The brain Coronal slices containing SON area were prepared using a microtome (Campden Instruments, UK). The slices were then incubated in ACSF chamber containing (in mM): 124 NaCl, 2.8 KCl, 2 CaCl2, 2 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, and 10 D-glucose at pH 7.4, (Osmolarity = 290 mOsm) bubbled with a mixture of O2 (95%) and CO2 (5%) at temperature of 32- 34 ºC for 1h and kept at room temperature until being transferred to the recording chamber.

2.5. Whole cell patch-clamp recordings

The slices were transferred to a submerged recording chamber and were continuously perfused with ACSF (1–2 mL/min) at room temperature, set on the stage of an upright microscope (Olympus BX51W1, Japan). Patch clamp recording was made from magnocellular neurons of supraoptic nucleus, which were identified based on method described by Hirasawa et al [20]. The slices were perfused by artificial cerebrospinal fluid (aCSF) solution bubbled with a mixture of O2 (95%) and CO2 (5%). Somatic whole-cell recordings were obtained under visual control using infrared difference interference contrast (IR-DIC) optics (Hamamatsu, Japan) with a 40x water immersion objective. Whole cell recordings were made using Multiclamp 700B amplifier (Axon Instruments, USA) equipped with Digidata 1320 A/D converter (Axon Instruments, USA). Recordings were made using borosilicate glass capillary (1.2 mm O.D., 0.9 mm I.D.) pulled with a two-stage vertical puller (PC10, Narishige, Japan). The pipettes were with 4–7MΩ resistance when filled with intracellular solution containing (in mM): 90 potassium gluconate, 30 KCl, 2 $MgCl_2$, 2

EGTA, 5 NaCl, 10 HEPES (pH = 7.25, Osmolarity = 290 mOsm). Recordings were accepted for further analysis, if the series resistance was less than 25MΩ and less than 20% change during the experiment.

After establishing the whole-cell recording configuration in current-clamp condition, the cell was permitted to stabilize for 1–2 min to allow equilibration between the cell interior and the micropipette solution. Then, Resting membrane potential was calculated. Crude venom in various doses 0.3, 1, 3 and 10 µg/ml and each fraction dose was 1 µg/mL, which were included in bath solution for recording (10). Samples are recorded at 10 kHz, filtered at 5 kHz and stored for offline analysis. In order to investigate the electrophysiological properties of MNCs after application of venom and its fractions in current clamp mode, depolarizing currents (50 pA; 10 ms) were elicited and an action potential was shown, while the cell was hold at -60 mV. Action potential properties were determined based on the single Action potential elicited by the depolarizing current of 50 pA [21]. Electrophysiological parameters including the Amplitude of APs, AP duration at half-width and Rise time were measured. Action potential amplitude was measured as the voltage difference between the baseline and peak amplitude of the spike. The half width of spike at one half of its maximum amplitude was determined as Action potential half-width; the rise time of APs was measured as the maximum time of rising phase of the spike. After SON reached an equivalent baseline level of firing, we measured the effect of venom on neuron excitability. Electrophysiological parameters were measured before and after 10 min bath application of BS venom to ensure that maximum effects were obtained.

2.6. Statistical Analysis

All of the data were analyzed by using the pClamp software, version 10.5 (Molecular device Inc.). Also, the statistical analysis was performed by using GraphPad Prism version 6 (Graph Pad Software Inc.). Repeated measures two-way analysis of variance ANOVA followed by Bonferroni post-test multiple comparison post–hoc test was compared to analyze differences between groups. A value of 0.05 or less was considered statistically significant. All data are expressed as mean ± standard error of mean (SEM).

3. Results and Discussion

One of the ion channels are Voltage-gated sodium channels (VGSCs), which regulate generation and propagation of the action potentials in neurons and other excitable cells [22, 23]. Sodium channel toxins (NaTX) are the major group of toxins recognized in Buthidae family[24]. *Buthotus schach* is a dangerous scorpion in Iran and the effects of BS venom on ion channels is not reported yet. Also to seek the active biomolecules, venom were isolated by gel filtration and their effects on electrophysiological properties of action potential of SON neurons found out. The main

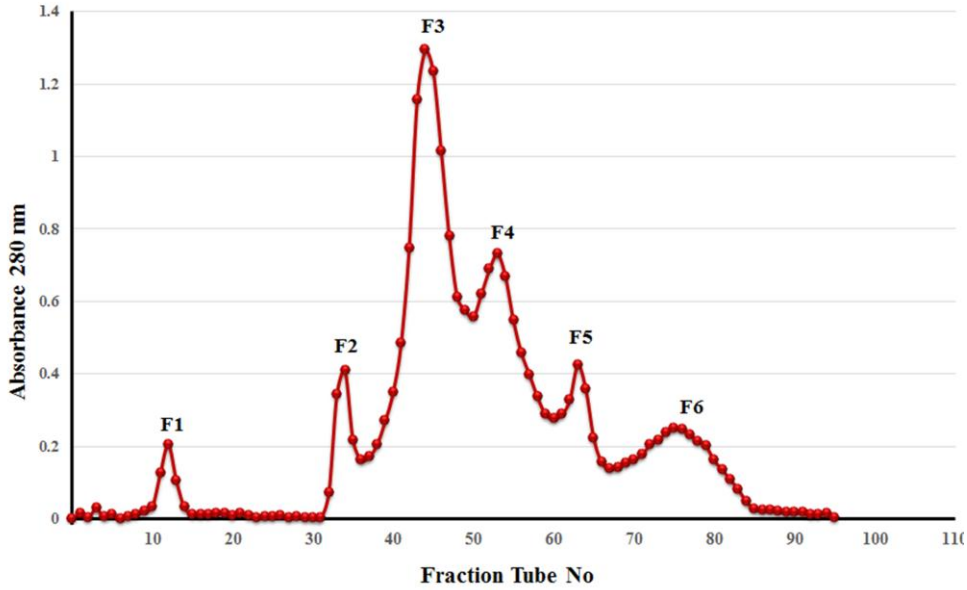

Figure 1. *Buthotus schach* scorpion venom (300 mg) was dissolved in Ammonium acetate buffer and loaded on Sephadex G50 column with 1 mL/min flow rate. Six fractions were obtained.

purpose of this study was to investigate the effect of BS venom and its fractions of gel chromatography on sodium channels via changes in the firing properties of SON. Current clamp recording for investigating the effect of BS venom and its fractions on membrane properties were performed and the changes in the action potential properties of SON neuron were evaluated. As shown in figure 1, in order to achieve the bioactive molecules responsible for pharmacological effects of the BS scorpion venom, it was fractionated by Gel filtration technique using Sephadex G-50 column and ammonium acetate buffer with 1 mL/min flow rate. Subsequently, to follow the pharmacological experiments for crude venom and venom fractions, attempt were made for six collected fractions to see if there is any affect similar to venom. Each fraction was used at 1 µg/mL concentrations. In this experiment, a significantly decrease in the action potential amplitude after venom application, a depolarization of the resting membrane potential restoring the amplitude and shape of evoked action potentials were revealed. Also after fractions application, asignificant change in action potential amplitude was showed. Tree active fractions (F2, F3 and F5) decreased significantly action potential amplitude in neurons. The reduced of amplitude of APs by various concentrations and some fractions of venom might result from the Na^{+} and Ca^{2+} channel inactivation due to membrane depolarization[25]. Previous study has been shown that resting membrane potential shifted to more depolarized values may cause the

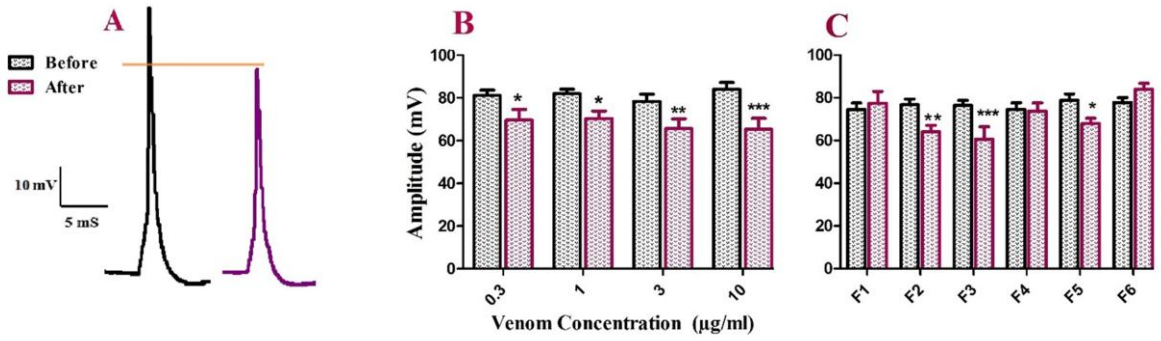

Figure 2. Effects of bath application of BS scorpion venom and fractions on peak amplitude. Representative traces of the spike firing by 50-pA depolarizing current injection in SON neurons before and after application of venom. The dashed line shows the amplitude of spike after application of venom was changed (A) .Effects of bath application of BS scorpion venom on peak amplitude (B) and BS scorpion fractions venom [F1, F2, F3, F4, F5 and F6] on peak amplitude (C) of SON neurons. Data are shown as mean ± SEM. *P < 0.05, **p < 0.01 and ***p < 0.001 significant difference compared with the values before venom application.

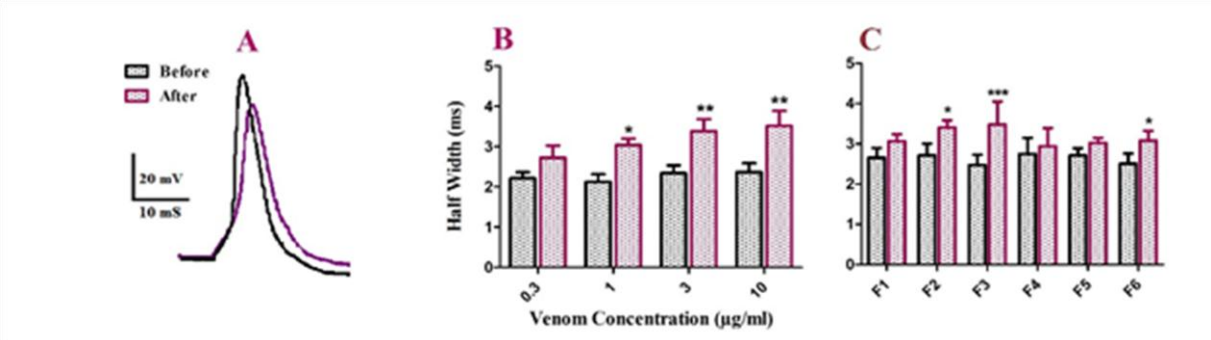

Figure 3. Effects of bath application of BS scorpion venom and fractions on half width action potential. Representative traces of the spike firing by 50-pA depolarizing current injection in SON neurons before and after application of venom (A). Effects of bath application of BS scorpion venom on Half- width action potential (B) and BS scorpion fractions venom [F1, F2, F3, F4, F5 and F6] on half width action potential (C) of SON neurons. Data are shown as mean ± SEM. *P < 0.05, **p < 0.01 and ***p < 0.001 significant difference compared with the values before venom application.

inactivation of voltage-dependent sodium channels. So, significant decrease in amplitude action potential may imply the fewer availability of Na+ channels to be opened [26-28]. Also previous reports have demonstrated effects of riluzole on amplitude of action potential. It has been shown that application of riluzole significantly reduced amplitude of action potential. It could indicate the probable inhibitory effect on voltage-gated Na+ channels on muscle [29]. Previous study suggested that this parameter is affected by activity of voltage-dependent sodium channels

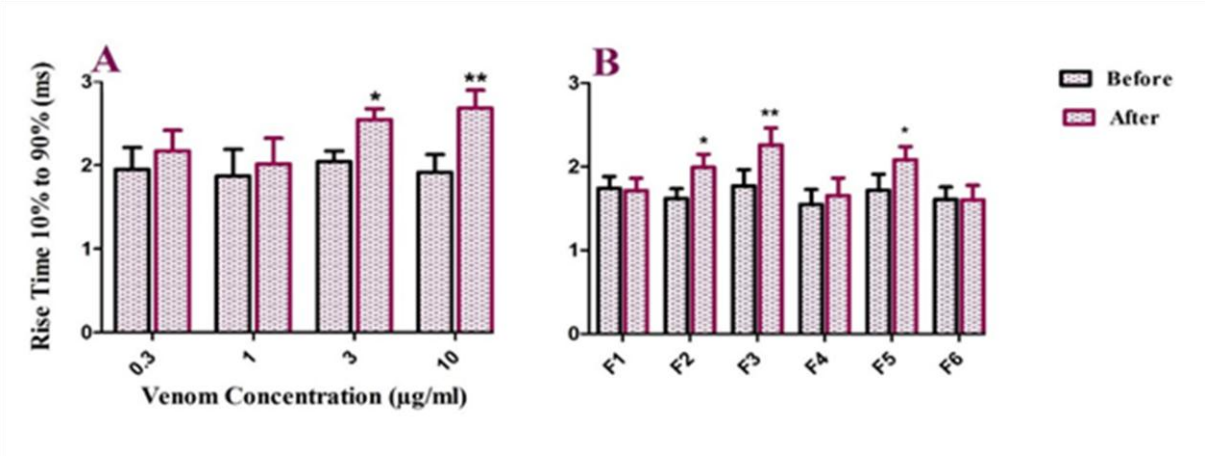

Figure 4. Effects of bath application of BS scorpion venom and fractions on Rise time 10% to 90% action potential. Effects of bath application of BS scorpion venom Rise time 10% to 90% action potential (A) and BS scorpion fractions venom [F1, F2, F3, F4, F5 and F6] on Rise time 10% to 90% action potential (B) of SON neurons. Data are shown as mean ± SEM. *P < 0.05, **p < 0.01 and ***p < 0.001 significant difference compared with the values before venom application.

Current clamp condition in whole cell patch clamp method was used for measuring the electrophysiological properties of SON neurons. Characteristics of AP were determined on the actions potential elicited in the cells by applying depolarizing current injection during 10 ms in 50 pA at holding potential of -55 mV. In order to investigate the effects of BS crude venom and its fractions on the electrophysiological properties of SON neurons, venom application made considerable changes in action potential properties of SON neurons.

A significant change was observed in the peak amplitude firing after bath application of crude venom [F (1, 32) = 50.50, p<0.0001]. Moreover, after application of venom fractions a significant change was revealed in peak amplitude firing [F (1, 36) = 11.88, p<0.01] in SON neurons (Fig 2 A). Analyses revealed significant decrease in peak amplitude of APs after bath application of venom at 0.3 (p<0.05), 1 (p<0.05), 3 (p<0.01) and 10 µg/ml (p<0.001) (Fig 2 B). Also after bath application of venom fractions, a significant decrease at F2 (p<0.01), F3 (p<0.001), and F5 (p<0.05) was observed (Fig. 3 C).

Half-width of action potential revealed significantly change after bath application of venom [F (1, 32) = 32.08, p<0.0001]. Also after application of venom fractions, Half widths of action potential were significantly changed [F (1, 30) = 40.52, p<0.0001] (Fig. 3A). Further analyses have shown significant increase in Half-width of action potential after venom application at 1 µg/ml (p<0.05), 3, and 10 µg/ml (p<0.01) compared to the values before venom application (Fig. 3B). In addition, after bath application of BS venom fractions, Half width of action potential were significantly increased at

F2 ($p<0.05$), F3 ($p<0.001$) and F6 ($p<0.05$) (Fig. 3C) compared with the values before venom application. The observed increase in half-width was in part due to an increase in Rise time 10% to 90% of action potential, as shown in Fig. 2B. Bath application of BS venom caused a significant change in Rise time 10% to 90% [$(1, 25) = 20.34$, $p < 0.001$]. Moreover, venom fractions application showed significant change in rise time 10% to 90% [$(1, 30) = 19.47$, $p < 0.001$]. Analyses revealed significant increase in Rise time 10% to 90% after bath application of venom at 3 µg/ml ($p<0.05$) and 10 µg/ml ($p<0.01$) compared with the values before venom application (Fig. 4A). Further bath application of venom fractions were significantly increased at F2 ($p<0.05$), F3 ($p<0.01$), and F5 ($p<0.05$) compared with the values before venom application (Fig. 4B). Our data showed that the half width and rise time (10% to 90%) of evoked single action potential were significantly increased after application of crude venom and its fractions. In the previous investigation, it was demonstrated that the increase in rise time of action potential may be related to these changes in sodium channels activity [26, 27, 30]. In particular, initiation and propagation of AP of neurons in most excitable cells in CNS are related to voltage- gated sodium channels [31]. Also the rising phase of the action potential is mediated by voltage-gated sodium channels (VGSCs)[32]. Consequently, crude venom and its fraction applications may decrease in available sodium channels and perhaps stop the alteration in sodium channel kinetics; however, this needs further experiments using voltage-clamp technique.

4. Conclusion

Study on venom of scorpions provides information regarding the use of these venoms as a powerful molecular tool for studying ion channels physiology and biophysics. In this study, our results show that Buthotus Schach scorpion crude venom and fractions could change electrophysiological properties of SON neuron probably by affecting on voltage gated sodium channels. Our data suggest that BS venom could be as a pharmacologic tool that allows better understanding of the excitation mechanism of neurons. In addition, more research are needed to study the effect of different components of the venom on ion channels subtypes. Moreover, the effects of BS toxins should be investigated on various subtypes of sodium channels by using single channel recording. Furthermore, these findings may act as good approaches for the use of BS venom, or its specific components, with modulating the kinetics of sodium channels, as an alternative therapeutic tool.

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