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Title: Characterization of functional effects of two new active fractions isolated from scorpion (Buthotus schach) venom on neuronal Ca^{2+} spikes: A possible action on Ca^{2+}-dependent K^+ channels

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Abstract

Background: It is a long time that natural toxin research is being carried out aims to unlock the medical potential of toxins. Although venoms-toxins cause pathophysiological conditions, they may turn out to be effective for therapy of many several diseases. Since toxins, including scorpion toxins target voltage-gated ion channels, they may have profound effects on excitable cells. Therefore, elucidating the cellular and electrophysiological impacts of toxins, particularly scorpion toxins would be helpful in future drug development opportunities.

Methods: Intracellular recording was made from F1 cells of *Helix aspersa* in the presence of calcium Ringer solution in which Na^+ and K^+ channels were blocked. Then, the modulation of channel function in the presence of extracellular application of F4 and F6 toxins and Kaliotoxin (50nM & 1µM) was examined by assessing the electrophysiological characteristics of calcium spikes.

Results and Conclusion: The two active toxin fractions, similar to Kaliotoxin, a known Ca^{2+}-activated K^+ channel blocker, reduced the amplitude of AHP, enhanced the firing frequency of calcium spikes and broadened the duration of Ca^{2+} spikes. Therefore it might be inferred that these two new fractions induce neuronal hyperexcitability possibly, in part, by blocking calcium-activated potassium channel current. However, this supposition requires further investigation using voltage clamping technique.

Keywords: Scorpion toxin; Intracellular recording; Calcium Spike; *Buthotus schach*

Introduction

Venoms are composed of a large number of bioactive substances, which may have specific effects on the biological systems (Biswas et al., 2012). Although venoms/toxins mainly result in pathophysiological consequences on human, there are several studies that support the potential medicinal properties of natural animal and insect venom neurotoxins, including scorpion toxins (Hwang et al., 2015; Fang et al., 2016). Same target molecules can be affected by many natural toxins in order to control and/or treat several diseases (Mouhat et al., 2004; Mouhat et al., 2008; Fang et al., 2016). In this context, ion channels could be common biological targets affected by both diseases and venomous neurotoxins. Functional alterations of many neuronal ion channels in diseases and/or following exposure to venoms have been extensively reported (Mouhat et al., 2004; Possani et al., 1999; Catterall et al., 2007; Han et al., 2010; Quintero-Hernández et al., 2013). Ion channels have different fundamental regulatory roles in neuronal excitability; therefore they could be considered as potential therapeutic and /or preventive targets. Heterogeneity in the expression of ion channels proteins shapes action potential characteristics and discharge firing pattern (Bean, 2003; Palacio et al., 2010); therefore, analysis of natural toxins on the shape of action potential or cell excitability would be beneficial in the early stages of drug development (Mohan et al., 2006; Akanda et al., 2009). Among them, voltage-gated Na^+, Ca^{2+} and K^+ channels are important therapeutic candidates which can be modulated by various neurotoxins, including scorpion toxins (Batista et al., 2002; Zuo and Ji 2004; Quintero-Hernández et al., 2013; Fang et al., 2016; He et al., 2016). Voltage-gated K^+ channels are crucial for regulating the neuronal excitability, through contribution to the repolarization following an action potential. Their blockade results in neuronal hyperexcitability by reducing the membrane hyperpolarization potential. Several types of potassium channels, including Ca^{2+}-activated

K^+ channels have been reported to be exist in different neuronal cell types (Humphries & Dart, 2015).Therefore, characterizing the functional effects of new scorpion toxin fractions that may affect the potassium channels function, particularly K_{Ca}^{2+} is important and could be a promising candidate as a K_{Ca}^{2+} channel blocker to treat diseases (Devaux, 2010; Bittner & Meuth, 2013; Ehling et al., 2011; Martin et al., 2017). Calcium-activated K^+ channels contribute to the regulation of vesicular release of neurotransmitter (Lee and Cui, 2010).

Kaliotoxin (KTX), an *Androctonus mauretanicus mauretanicus* peptidyl neurotoxin, has been reported to block neuronal maxi Ca^{2+}-activated K^+ channels in snail neurons (Crest et al., 1992). KTX has been widely used to treat experimental autoimmune encephalomyelitis (Beeton et al., 2001) and inflammatory lesions of periodontal disease (Valverde et al., 2004). It was also used to facilitate cognitive processes as learning (Kourrich et al., 2001), therefore it was suggested that KTX-sensitive potassium channels contribute to the repolarization of the presynaptic action potential of hippocampal inhibitory neurons and thereby induced facilitation of synaptic transmission (Martin-Eauclaire et al., 2012).

In this study, the electrophysiological consequences of two new fractions (F4 and F6) isolated from *Buthotus schach* scorpion venom were investigated on the properties of neuronal Ca^{2+} spikes. The scorpion *Buthotus schach*, which belongs to the Buthidae family, is widely found in the western and tropical area of Iran. In the previous report, the effect of these two new fractions was investigated on the release of Ach in neuromuscular junctions (Vatanpour et al., 2012), where these two fractions transiently increased the amplitude of muscle twitch associated with a huge contracture and then followed by muscle paralysis in chick and mice (Vatanpour et al., 2012). We showed also that application of both fractions affected the Na^+ action potential waveform of F1 neurons of *Helix aspersa* (Tamadon et al., 2014). Here, it was attempted to demonstrate the functional effects of the

two fractions on the electrophysiological properties of Ca^{2+} spikes in F1 neurons of snail neurons. Findings of the present account extend the findings presented in our previous work (Tamaddon et al., 2013), by providing additional details regarding the effects of the two active toxin fractions on Ca^{2+}-dependent neuronal excitability.

Materials and Methods

All experiments were approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences.

All recordings were performed on the soma membrane of F1 neuronal cells, located on the right parietal of sub-oesophageal ganglia of *Helix aspersa* (Iranian garden snail). The ganglionic mass was dissected out and then pinned on the bottom of the recording chamber covered by Sylgard 184(Dow Corning Midland, MI, USA). Thereafter, the superficial connective tissue overlying the ganglia was gently removed using fine forceps. F1 neurons were then visualized under stereomicroscope (Nikon, Japan) by their location and size within the right parietal ganglion. Intracellular recordings were done in the presence of calcium Ringer solution in which Na^+ was replaced by TEA chloride and voltage-gated K^+ channel current was blocked by bath application of 4-aminopyridine (4-AP) and tetraethyl ammonium (TEA). The Ca^{2+} bathing solution contained (in mM): 80 TEA, 4 KCl, 10 $CaCl_2$, 5 $MgCl_2$, 10 glucose, 5 HEPES. In order to examine the electrophysiological consequences of neuronal exposure to the two toxin fractions or Kaliotoxin, as a standard scorpion toxin, on Ca^{2+} excitability, two doses (50nM and 1µM) of the toxin solution were applied on the basis of respective literature and prior works in our laboratory (Tamadon et al., 2014). The six toxin fractions were isolated and purified (Vatanpour et al., 2012; Aboutorabi et al., 2016) and the two fractions that had action mostly

prejunctionally on Ach release from the neuromuscular junctions of chicks and mice (Vatanpour et al., 2012).

Intracellular recording technique was made under current-clamp condition using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA). An Ag/AgCl electrode within an agar bridge (4% agar in snail Ringer) was used as a reference or ground electrode. Spontaneous Ca^{2+} spikes were recorded in the presence or absence of either toxins fractions (F4 or F6) or KTX. Voltage signals were filtered at 10 kHz and digitized at 20 kHz using a 16bit A/D converter (ADInstrument Pty Ltd., Sydney, Australia) and stored on a computer for further offline analysis using Lab Chart pro7 and Excel softwares.

2.1 Statistical analysis: Results were reported as mean ±S.E.M, with '*n*' being the number of cells on which the recording was performed. Data were subjected to statistical analysis with GraphPad Prism 6 software, using unpaired Student's t-test or one-way ANOVA followed by Tukey's test as the post hoc analysis. $P \leq 0.05$ was considered to be significant.

3. Results

Calcium channel modulators can regulate membrane excitability in many neurons in part by changing the AHP amplitude. Thus, the Ca^{2+} spikes were recorded from the soma after blockade of the inward Na^{+} channel and outward voltage-gated K^{+} channels (Fig. 1). Under this condition, the mean of neuronal resting membrane potential (RMP) was -37.43±0.8mV (Fig. 2A), the spike firing frequency was 0.95±0.02Hz (Fig. 2B), the amplitude of after hyperpolarization potential (AHP) was -2.55±0.09mV and the half-width of Ca^{2+} spike was 42.52±1.84ms (Figs. 3A-C).

When F1 neurons were exposed to Ca^{2+} Ringer containing F4 fraction at a concentration of 50nM a slight depolarization in the membrane voltage was occurred (-36.38±0.31mV), but at 1µM a shift in

the membrane potential towards more hyperpolarized voltages occurred (-40.14±0.46mV, P≤0.01; Fig .2A). The Ca^{2+} spike frequency was significantly increased in response to an exposure to both concentrations of F4 toxin fraction (1.21±0.02Hz, P≤0.001 and 1.52±0.03Hz, P≤0.001; Fig. 2B). Moreover, application of Ca^{2+} Ringer solution containing F4 fraction led to a significant reduction in the amplitude of AHP (-1.73±0.08mV, P<0.001 and -1.91±0.07mV, P<0.001; Fig. 3A). The recorded Ca^{2+} spikes were significantly broadened, when cells were exposed to both concentrations of F4 toxin fraction (54.63±1.67ms, P≤0.001 and 48.95±1.49ms, P≤0.01; Fig. 3B).

Thereafter, the effect of F6 fraction was examined on the electrophysiological properties of Ca^{2+} spikes in a separate set of experiments.

Extracellular application of the F6 fraction at concentrations of either 50nM or 1µM significantly shifted the RMP to the hyperpolarized potential (-45.01±0.46mV, P≤0.001 and -40.74±0.27mV, P≤0.001, Fig.2A, respectively). In addition, the amplitude of AHP was significantly decreased when cells were exposed to both doses of F6 toxin fraction (-1.81±0.06mV, P<0.001 and -1.23±0.03mV, P<0.001, Fig. 3A, respectively) and this led to a significant increase in the spike firing frequency (1.61±0.02Hz, P≤0.001 and 1.81±0.02Hz, P≤0.001 in the presence of 50nM and 1µM, Fig. 2B, respectively). Application of F6 fraction was also associated with a slight insignificant prolongation of Ca^{2+} spike duration both at a lower dose (43.27±1.01ms) and higher dose (43.13±1.33ms, Fig. 3B).

3.1 The electrophysiological consequences of Kaliotoxin exposure on the Ca^{2+} spikes

Following application of Kaliotoxin, a known scorpion neurotoxin to block KCa current, the RMP shifted towards hyperpolarization potential either in the presence of 50nM (-49.41±0.39mV, P≤0.001) or 1µM concentration of neurotoxin (-43.43±0.26mV, P≤0.001, Fig. 2A). In addition, exposure to Kaliotoxin significantly dampened the AHP amplitude and an increase in the firing rate (1.02±0.01Hz,

P≤0.05 and 1.03±0.02Hz, P≤0.05, Fig. 3A, respectively). However, application of Kaliotoxin had dose dependently opposite effects on the duration of Ca^{2+} spike. At low concentration, Kaliotoxin exposure led to a significant spike prolongation (37.91±0.99ms, P≤0.05), but at high concentration resulted in shortening of the spike (57.75±0.92ms, P≤0.001; Fig.3B).

3.2 Comparison the effects of Kaliotoxin and *Buthotus schach* scorpion toxin fractions of F4 & F6 on Ca^{2+} spikes

Comparing the action potential electrophysiological parameters measured in the presence of Kaliotoxin and the two active fractions demonstrated that all neurotoxin treatments had the same effects on the measured variables. So that the RMP became more hyperpolarized. In addition, although all applied neurotoxins caused a decrease in AHP amplitude, exerted an increasing effect on the spike frequency.

4. Discussion

Present study attempted to determine the electrophysiological consequences of exposure to the two active toxin fraction isolated from *Buthotus Schach* venom on the Ca^{2+}-dependent neuronal excitability. To this end, the ionic conditions were manipulated by replacing tetraethyl ammonium hydrochloride for sodium chloride and by adding 4-AP (5mM) to block IA channel current. Under this condition, F1 neurones generated overshooting Ca^{2+}-dependent spikes. Then, the Ca^{2+} spike parameters, including RMP, AHP amplitude, spike duration and firing frequency were measured and compared in the presence of the two active fractions with those obtained in the presence of Kaliotoxin, as a known scorpion neurotoxin particularly acting on KCa channel.

Natural toxins have been widely used as tools to identify the new biomedical molecules and pathways and also as experimental probes for membrane structures comprising their targets ().In addition, the natural toxins can be turned and evolved into life-saving drugs and powerful medications. Therefore,

identifying and characterising the impact of the new toxin fractions at the cellular level may be helpful for treating the diseases and for the new drug development. Here, it was investigated whether exposure to the two new scorpion toxin fractions may affect the Ca^{2+}-based excitability in the F1 neurone in *Helix aspersa*.

Several studies have proposed that transient and delayed rectifier K+ outward currents (Thompson, 1977; Solntseva, 1995; Bal et al., 2000, 2001; Sakakibara et al., 2005; Janahmadi et al., 2008), and Ca^{2+} activated K^+ channels (Hermann and Erxleben, 1987; Gola et al., 1990; Crest and Gola, 1993) are responsible for generating AHP following action potential in snail neurons. In our previous work, we demonstrated that functional blockade of Ca^{2+} activated potassium channels increased the frequency of Ca^{2+} spikes by eliminating the AHP, which follows action potential (Vatanparast et al., 2006; Janahmadi et al., 2008). Therefore, increasing effect of the two new active fractions and Kaliotoxin on the firing frequency could be possible due to the inhibition of KCa channels. There are several evidences reporting the effect of Kaliotoxin (KTX) on either voltage-gated or calcium-activated potassium channels including Kv1.3 and BK channels, respectively (Lange et al., Crest et al., Zachariae et al., Aiyar et al., 1996). The blocking effect of Kaliotoxin on calcium-activated potassium channels has been reported by Crest et al. reported (1992). The function of these channels has been demonstrated to be a key link between the rise in intracellular free Ca^{2+} and neuronal excitability by affecting the amplitude of AHP and firing frequency (MacDonald et al., 2006; Lin et al., 2010). There are also other reports indicating that exposure to scorpion venom peptides causes the enhancement of neuronal excitability by suppressing the AHP (Ishii et al., 1997; Juhng et al., 1999; Pedarzani et al., 2002). In many neurons, Ca^{2+} entry through activation of Ca^{2+} leads to opening of Ca^{2+} dependent potassium channels and thereby regulates cell excitability (Lancaster et al., 1986; Sah and Faber., 2002; Janahmadi et al., 2008; Duménieu et al., 2015). In the present account, in common with Kaliotoxin both two new active scorpion toxin fractions enhanced firing frequency by reducing the amplitude of AHP (Haghdoost et al., 2008). However, the further voltage-clamp analysis is needed to address this issue.

Another finding of the present work was hyperpolarization of the membrane potential following scorpion toxins treatment. Although, there are several reports in the literature showing that involvement of Ca^{2+}-activated K^+ channels in the generation of AHP, which thereby contribute to the repolarization phase and the duration of action potential (Storm, 1987; Liu et al., 2014), but not

resting membrane potential, blockade of these channels by scorpion toxins caused membrane hyperpolarization. It is hard to provide a decisive causative mechanism for this effect, but it can be hypothesized that blockade of KCa channels by scorpion toxins causes less K^+ efflux and thereby led to the accumulation of more positive ions inside the cell. This, in turn, may increase the Na^+-K^+ pump activity leading to membrane hyperpolarization.

Neuronal exposure to either two active fractions isolated from *Buthotus schach* or Kaliotoxin resulted in spike broadening. Since in the present study voltage-gated sodium and potassium channels were blocked, one possible explanation for the alteration in the spike duration could be changes in the balance between inward Ca^{2+} current and outward KCa current. Particularly, possible inhibitory effect of applied neurotoxin could be more effective on the membrane repolarization and the duration of action potential (Ma and Koester, 1996; Faber and Sah, 2003; Battonyai et al., 2014). It has been very well documented that Helix *aspersa* neurons possess many types of ion channels including voltage and Ca^2 dependent K^+ channels (Azanza et al., 2008). Ca^{2+}-activated K^+ channels are divided into three types based on the conductance: big conductance, intermediated and small conductance KCa channels. The first group consisted of two subtypes (Azanza, 2008), including one sensitive to intracellular Ca^{2+} concentration (BKCa) and one sensitive to the scorpion toxin, charybdotoxin (HLK3 channels). The second type includes two kinds of SK channels: SK2 and SK3. BKCa channels activation is involved in action potential repolarization, while SKCa channels contribute to underlie the AHP (Sah, 1996).

In conclusion, findings of the present investigation suggest that the two new scorpion toxin fractions isolated from *Buthotus Schach* venom, similar to the known scorpion neurotoxin, Kaliotoxin, caused hyperexcitability, possibly by blocking calcium-activated potassium channel current, although further voltage-clamp investigation needs to be done to explore the properties of ion channels affected by examined venom.

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Figures Legend

Figure 1: Effects of two different concentrations of F4 and F6 fractions isolated from *Buthotus schach* scorpion toxin and Kaliotoxin on the spontaneous calcium spike firing. Extracellular application of either F4 and F6 or Kaliotoxin resulted in the neuronal hyperexcitability.

Figure 2: Effect of two neurotoxins and Kaliotoxin on the AHP amplitude and the half-width of Ca^{2+} spike

(A) Application of either two active fractions, F4 and F6, or Kaliotoxin caused a significant reduction in the AHP amplitude. (B) Neuronal exposure to all applied neurotoxins led to a significant spike broadening, except Kaliotoxin 50nM, which reduced the duration of calcium spike.(C) Superimposed Ca^{2+} spikes in control condition and after application of two active toxin fractions and Kaliotoxin. * indicates significant difference between control group and all neurotoxins treated groups (***p<0.001, **p<0.01). # shows significant difference between Kaliotoxin and the two active toxin fractions (#p<0.05, ##p<0.01, ###p<0.001).

Figure 3: Effect of F4, F6 and Kaliotoxin on the F1 cell electrophysiological properties.

The impact of toxins on the resting membrane potential (A), action potential duration (B) and spike frequency (C).* indicates significant difference between control group and all neurotoxins treated groups (*p<0.05, **p<0.01, ***p<0.001). # shows significant difference between Kaliotoxin and the two active toxin fractions (#p<0.05, ##p<0.01, ###p<0.001).