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Original Article

Effects of Black Scorpion *Androctonus crasicuda* Venom on Striated Muscle Preparation *in vitro*.

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Abstract

Effects of venom from black scorpion Androctonus crasicuda (AC) were determined on isolated chick biventer cervices nerve-muscle and mouse hemidiaphragm preparations using twitch tension method. The isolated nerves were stimulated by electrical stimulator and response to each stimulus was recorded. The venom mainly acted prejunctionally to facilitate neuromuscular activity due to an increase in acetylcholine release.

Keywords: Scorpion; Muscle; Neuromuscular; Transmission; Acetylcholine.

Introduction

Over 1500 species of scorpions have been described. They are commonly subdivided into two groups: Buthidae (40% of known species) and Chactoids. All human hazardous scorpions belong to the Buthidae (Buthoid) family. Envenomation by scorpion stings is common in tropical and subtropical regions (1). Many investigators have shown that mammalian scorpion toxins are low molecular weight proteins of about 7000 daltons, as well as a single polypeptide chain cross-linked by four disulfide bridges (2, 3) which are toxic to mammals (4). The toxin can cause a variety of symptoms from pain at the site of sting to death (5). The venom of Androctonus crasicuda is a potent autonomic stimulator. Severity of symptoms depends on the size of the victim, season, and time lapse between sting and hospitalization. Vomiting, profuse sweating, priapism, mild pain at the sting site, local urticaria, and cool extremities are early signs of autonomic stimulation due to scorpion sting. Fatality after scorpion envenoming may be the result of cardiovascular failure complicated by pulmonary oedema, as well as respiratory arrest (6). It has been proposed that the majority of the symptoms of envenoming are due to massive discharge of catecholamines during so-called autonomic storms (7). The underlying mechanism of transmitter release and the identity of the bioactive molecules in Androctonus crasicuda venom remain unknown. However, other scorpion venoms contain toxins that affect ion channels and synaptic transmission (8, 9). Scorpion venom toxicity to human has mainly been attributed to the pharmacological properties of toxic polypeptides active in mammals. Toxins that prolong the opening of Na^+ channels by slowing the inactivation process (the so called a-type scorpion toxins) lead to a prolongation of the action potential. This effect, which is dependent on membrane potential, has been observed with the toxins mainly isolated from old world scorpion venoms, including Androctonus australis Hector, leiurus quinquestriatus quinquestriatus, Buthus tamulus and Buthus epeus (10, 11). In contrast, there are toxins that cause Na^+ channel to open at membrane potentials at which they would normally be closed (the b-type scorpion toxins) and provoke spontaneous and repetitive firing of action potentials. This effect has been

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observed with venom and toxins from new world scorpion, such as Centruroides suffusus, Centruroides sculpturatus and Tityus serrulatus (12, 13), although in two of these venoms (i.e. Centruroides sculpturatus and Tityus serrulatus) toxins representative of the two classes have been identified (13). These toxins can alter neuronal action potentials and cause release of neurotransmitter from cholinergic and adrenergic neurones. A few venoms have, in addition, minor components, e.g. iberiotoxin from Buthus tamulus venom (14), that act selectively on voltage-gated or Ca^{2+}-activated K^+ channels (15-19).

In vitro models of cholinergic transmission (chick biventer cervicis, mouse hemidiaphragm nerve-muscle preparations) were used in order to study changes in twitch tension and in acetylcholine release. Almost 30 years ago, Del Pozo while working with Mexican scorpion, has reported the action of the venom from the genus Centruroides on peripheral nervous system and muscle preparations (20). The venom of the same species applied at the frog neuromuscular junction can induce an increase in the duration of the presynaptic nerve action potential as well as an increase in the mean number of quanta released by a nerve impulse (21). Vital has also shown that the venom of the South American Tityus serrulatus induces the release of an acetylcholine-like substance, which could account for the twitches observed and also for the decurarizing activity of scorpion venom (22). It has been suggested that this scorpion toxin has two sites of action presynaptically: it depolarizes the nerve terminal facilitating the spontaneouse release of transmitter (acetylcholine), and also acts on the membrane of the unmyelinated nerve terminal arborization prolonging the sodium current. It has been shown that scorpion Centruroides noxius Hoffman venom has a direct effect on K^+ channels. It reversibly blocks the delayed rectifier potassium current in the squid giant axon. Purified noxiustoxin blocks K^+ currents in the squid giant axon in a complex manner (23). At concentrations less than 1.5 μM, block by noxiustoxin is independent of membrane potential, while at higher concentrations toxin block is voltage-dependent, and the toxin probably dissociates when the preparation is repetitively depolarized. These effects are similar to the voltage-dependent properties of some Na^+ channel scorpion toxins.

The purpose of the present study is to investigate the effects of Androctonus crasicuda venom on the neuromuscular transmission. In vitro models of cholinergic transmission, including chick biventer cervicis and mouse hemidiaphragm nerve-muscle preparations, are used in order to study changes in muscle twitch tension, during the release of acetylcholine.

Experimental

Chick biventer cervicis nerve-muscle preparation and mouse phrenic nerve-hemidiaphragm preparation have been used for studying the effects of drugs and toxins on neuromuscular transmission. Both can be set up in small tissuse baths (5 ml or less), and both are robust and stable over several hours in vitro. These features make them suitable for studying the effects of toxins, which are often available in only small quantities, and which are often slow in action.

Chick biventer cervicis preparations

The chick biventer cervicis muscle contain both focally innervated twitch fibres and multiply innervated contracture-producing fibres. Thus, it can be stimulated by exogenously applied cholinomimetic agonists, as well as by stimulation of its motor nerve. This enables prejunctional effects to be distinguished from postjunctional effects. Biventer cervicis nerve-muscle preparations (24) were isolated from 3-14 day old chicks that had been killed by exposure to anaesthetics (CO_2 for 2-7 days old and halotane for 7-12 days old chicks) and mounted (in pairs) with a resting tension of 0.5-1g in 2-10 ml tissue baths containing Krebs-Henseleit physiological salt solution of the following composition (mM): NaCl, 118.5; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$ 2.5; $NaHCo_3$, 25.0; glucose, 11.1. The solution was maintained at 33°C and bubbled with 95% O_2 plus 5% CO_2 at pH 7.3.

For indirect stimulating, muscle contraction was induced by stimulation the motor nerve in the tendon, using ring electrodes every 10 sec with pulses of 0.2 ms duration and a voltage greater than that which produced a maximal twitch. In the absence of nerve stimulation, contractures to sub-maximal concentrations of

exogenously acetylcholine (1-3 mM), carbachol (20-40 uM), and KCl (40-50 mM) were obtained prior to the addition of the test compound and at the end of the experiment. Acetylcholine and potassium chloride were allowed to bathe the preparation for 30 sec and carbachol for 60 sec. Preparations were then washed by overflow for 15 sec at a rate of 5-10 ml/s. Preparations were finally allowed to stabilize for 20-40 min before the addition of test compound.

For direct muscle stimulation, neuromuscular transmission was blocked with tubocurarine (10-20 uM) and the stimulating electrodes were moved into contact with the belly of the muscle. Stimulation was performed directly at 0.1 Hz with pulses of 2 ms duration and a voltage greater than which was needed to induce maximum twitch height. Preparations were allowed to stabilize for approximately 30 min before the addition of test venoms or toxins.

Twitches and contractures were recorded isometrically on Narco-Trace polygraphs using F60 force displacement transducer.

Mouse hemidiaphragm nerve-muscle preparation

Mouse hemidiaphragms and attached phrenic nerve were isolated from 15-25 g male mice (Balb C strain) for the rat hemidiaphragm preparation. Preparations were mounted individually in 10 ml organ baths under the same conditions used for the biventer cervicis preparations, except that the temperature was maintained at 36.5°C.

For indirect stimulation, the phrenic nerve was stimulated (via a Burn and Rand electrode) at 0.1 Hz with pulses of 0.2 ms duration and voltage greater than that needed to produce maximal twitches.

For direct muscle stimulation, the preparation was impaled (just below the rib) upon two silver electrode. Pulses of 2 ms were applied at a frequency of 0.1 Hz with sufficient strength to produce a maximal twitch. Tubocurarine (10-20 uM) was used to prevent neuromuscular transmission. Alternatively preparations can also be stimulated, indirectly via the phrenic nerve and directly via the hook electrodes at a frequency of 0.05 Hz. Under these conditions, tubucurarine was always added prior to the experiment to ensure that

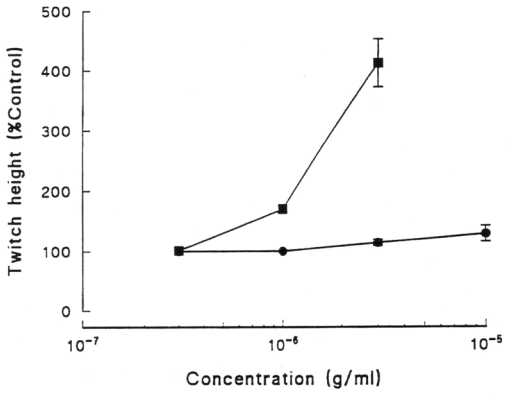

Figure 1. Effects of Androctonus crasicuda venom on response of chick biventer cervicis preparation to direct (■) and indirect (□) stimulation. Each point represents the maximum response for that concentration (vertical bars are sem, n=4).

activation of nerve endings by the field stimulation do not contribute to the overall tension recorded in direct stimulation. In cases with more than a 10% decrease in response to direct stimulation in the presence of tubucurarine, the hook electrodes were repositioned to avoid this artifact. After washout of the tubocurarine, preparations was allowed 30-60 min to stabilize prior to the addition of toxin.

Results

Androctonus crasicuda venom (AC) was tested on directly and indirectly stimulated preparations at 0.3, 1, 3 and 10 µg/ml

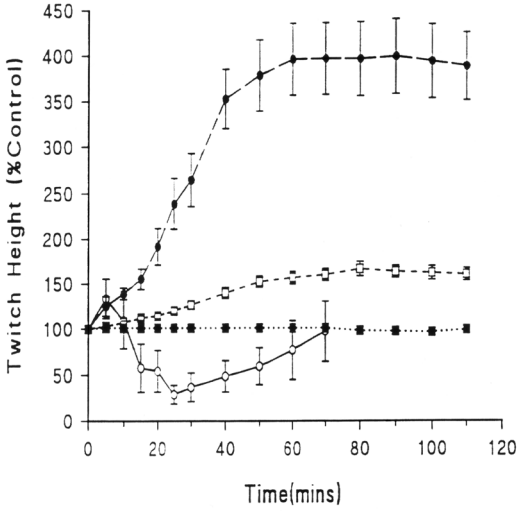

Figure 2. Effects of Androctonus crasicuda venom on responses of indirectly stimulated chick biventer cervicis nerve-muscle preparation (■) 0.3 µg/ml, (□) 1 µg/ml, (●) 3 mg/ml and (■) 10 µg/ml. Each point represents the mean ± sem (n=4).

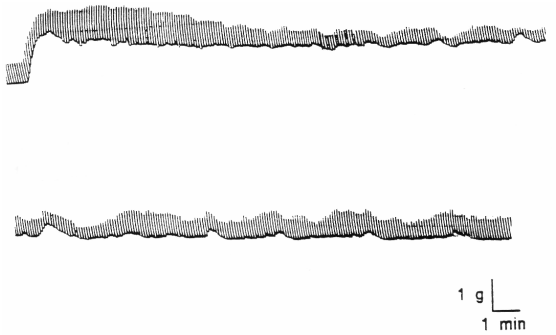

Figure 3. Androctonus crasicuda venom (10 µg/ml) induces a marked contracture followed by periodic oscillations of the resting tension.s

(Figure 1). AC induced a large increase in the response of indirectly stimulated nerve-muscle preparation, but not directly stimulated preparation. The threshold concentration was around 1 µg/ml. Maximal twitch augmentation with indirect stimulation occurred after about 60 min and was 69 ± 7% and 300 ± 40% of control twitch height at 1 and 3 µg/ml, respectively (Figure 2). At 10 µg/ml, there was a transient augmentation (34 ± 22%) of twtich height followed by a contracture of 148 ± 20% of control twitch height. Thereafter, there was a series of large periodic (approximately one every 5 min) increase in resting tension, coinciding with the reduction of twitch height (Figure 3). The effect of venom was reversed by washing intermittenly for about 45 min. AC (1-3 µg/ml) caused little change in post-junctional sensitivity, as assessed by contracture to exogenously applied acetylcholine, carbachol and KCl (Figure 4). In preparations without tubocurarine pretreatment, AC induced a 64 ± 10% increase in the contracture to exogenously applied KCl at 10 µg/ml, but no significant changes in contractures to acetylcholine and carbachol. However, contractures to KCl did not change when AC (10 µg/ml) was tested in preparations exposed to tubocurarine, indicating that the enhanced contracture to KCl may be due to an increase in the amount of acetylcholine released from nerve terminals by K^+ induced depolarization. In preparations stimulated

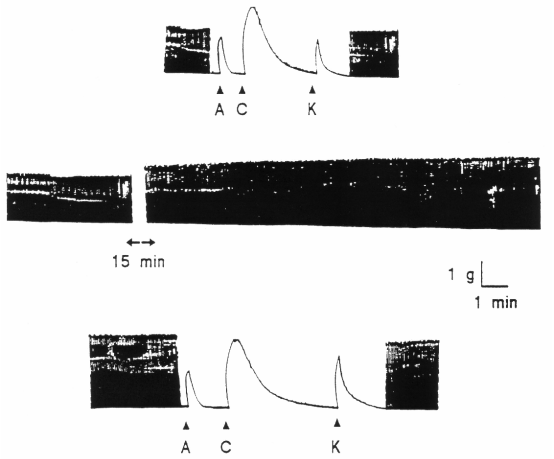

Figure 4. Effects pf Androctonus crasicuda venom (1 µg/ml) on chick biventer cervicis preparation at 33°C. The muscle was stimulated indirectly once every ten seconds and, in the absence of twitches, by exogenous acetylcholine (1 mM), carbachol (40 µM) and potassium chloride (40 mM).

directly in the presence of tubocurarine, 10 ug/ml AC caused a small increase in twitch responses (29 ± 13%) and a small contrature (21 ± 5% of control twitch height). Additionally, in the absence of stimulation as well as in indirectly stimulated preparations, AC (10 µg/ml) caused contracture of around 15% of control twitch height. The contractures was abolished by higher concentrations of tubocurarine (10-100 µM), and was not seen in preparations pretreated with tubocurarine (Figure 5). In order to investigate whether the facilitatory effect was due to AC acting presynaptically or postsynaptically, AC (3 and 10 µg/ml) was tested on partially paralysed preparations (50-80%) with tubocurarine, $MgCl_2$ or procaine. In the presence of tubocurarine or Mg^{2+}, AC at 3 µg/ml increased twitch height to about 120% greater than control after about 60 min; AC reversed the block induced by procaine by only 70%. At 10 µg/ml, AC reversed the reduction in twitch height induced by tubucurartine and procaine more rapidly, following by augmentation in the twitch height. The partial block induced by Mg^{2+} was incompletely reversed by AC (Figure 6).

AC at 3 and 10 µg/ml increased twitch height of indirectly stimulated preparations by 53 ± 11% and 307 ± 90% of control twitch height, respectively. This increase did not cause contracture on mouse hemidiaphragm frenic nerve muscle preparations (Figure 7). Maximum augmentation was reached after 60 min. At 10 µg/ml, AC augmented responses of directly stimulated preparations by 34 ± 9% of the control twitch height.

Discussion

Envenoming by the Iranian scorpion Androctnus crasicuda can cause massive discharge of catecholamine. The relationship of these effects to the clinical situation is unclear. Results from the present study show that venom from Iranian scorpion Androctonus crasicuda has some effects on neuromuscular transmission. Present results revealed some of the underlying mechanisms: AC venom can increases the release of acetylcholine at the neuromuscular junction by both increasing quantal content as well as the release after single shock stimulation. There is, however, no change in postsynaptic sensitivity. AC, at high concentration, causes very large periodic increases in resting tension on both indirectly stimulated and unstimulated chick biventer cervicis preparations. As these contractures are abolished by tubocurarine, they are probably caused by the release of acetylcholine induced by AC. Although toxins responsible for this action are still to be identified, but the initial steps toward their isolation have been undertaken-in this study.

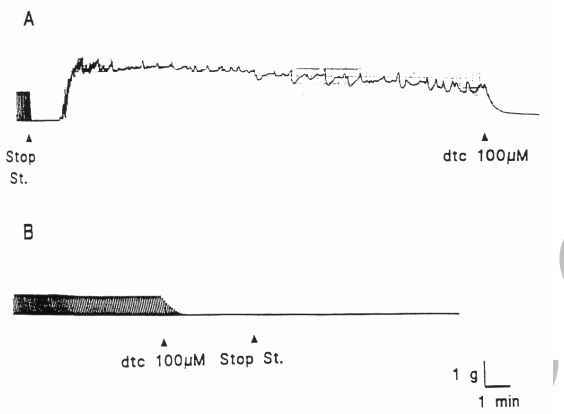

Figure 5. Effects of Androctonus crasicuda venom (10 µg/ml) on chick biventer cervicis preparation, A: It caused a large contracture in the absence of stimulation, B: tubocurarine pretreatment prevented the effect of venom in the lack of stimulation.

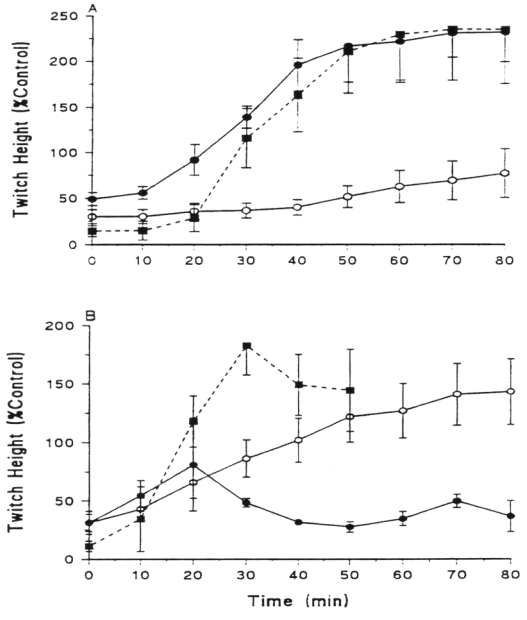

Figure 6. Reversal of tubocurarine (●), Mg^{2+} (■), procaine (o) blockade by Androctonus crasicuda venom on responses of stimulated chick biventer cervicis nerve-muscle preparation. (A) Experiments in the presence of 3 µg/ml venom. (B) Experiments in the presence of 10 µg/ml venom. (mean ± sem, n=4).

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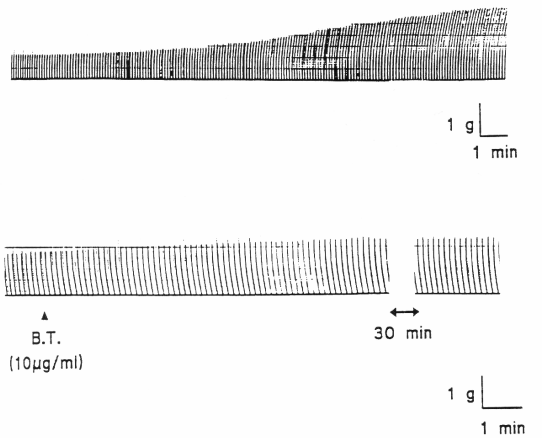

Figure 7. Androctonus crasicuda venom (10 µg/ml) markedly increased twitch height evoked by indirectly (upper panel), but not direct (lower panel) stimulation of mouse hemidiaphragm nerve-muscle preparations.

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