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Isolation of a Postsynaptic Blocker from the Venom of the Green Mamba, *Dendroaspis Angusticeps*

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ABSTRACT

A blocker of postsynaptic acetylcholine receptors was isolated from venom of the Eastern green mamba *Dendroaspis angusticeps*, by gel filtration, ion-exchange and reverse phase high performance liquid chromatography. The isolated component blocks neuromuscular transmission and responses to exogenously applied acetylcholine in isolated chick biventer cervices preparations. These results suggest that postsynaptic blockers exist in venoms of all members of the *Dendroaspis* genus. *Iran. Biomed. J. 3 (1 & 2): 53-57, 1999*

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INTRODUCTION

Venoms of snakes belonging to the family of Elapidae, including the mambas, are extremely toxic and produce flaccid paralysis and respiratory failure in animals. These effects are caused by the α-neurotoxins contained in these venoms, which bind to nicotinic acetylcholine receptors at neuromuscular junctions [1]. Postsynaptic α-neurotoxins have been isolated from three of the four species of mambas: *Dendroaspis polylepis* (black mamba) [2], *Dendroaspis viridis* (West African green mamba) [3, 4], and *Dendroaspis jamesoni* (Jamesoni's mamba) [5,6]. However, the venom of *Dendroaspis angusticeps* (East African green mamba) does not appear to contain either long or short α-neurotoxins. From this venom, Viljoen and Botes [7, 8] purified and sequenced major polypeptides F7 and F8 of low toxicity. They are structurally homologous to the short α-neurotoxins and cytotoxin groups but have completely distinct immunochemical properties. Barrett and Harvey [9] observed an inhibition of responses to acetylcholine by using a high concentration of the whole venom from *D. angusticeps* and even a failure in muscle contractility with a higher concentration. Wangai *et al.* [10] also reported that one fraction of *D. angusticeps* venom blocked neuromuscular transmission. However, despite the amount of work done on this venom, there has been no report of the isolation and characterization of postsynaptic neurotoxins. In the present study, a postsynaptic blocker of acetylcholine receptors was isolated from the venom of *D. angusticeps*, and pharmacologically characterized.

METHODS AND MATERIALS

Toxin Isolation: Whole venom (*Dendroaspis angusticeps*, obtained from Sigma Co.) was initially introduced onto a 17 mm x 1000 mm glass column of Sephadex G50, equilibrated with 0.1 M ammonium acetate (pH 6.8). 500 mg of crude venom was dissolved in 2.5 ml of 0.1 M ammonium acetate buffer and eluted isocratically with the same buffer at a flow rate of 0.3 ml/min. Absorbance of eluate was monitored at 280 nm. Samples were collected in 3 ml aliquots. The gel filtration of the venom resulted in five major fractions (Figure 1A).

The fraction III (Figure 1A, shown by the horizontal bar), which contains the dendrotoxins [9] was pooled, freeze-dried and submitted to further fractionation, using a high pressure liquid chromatography (HPLC) ion exchanger. A preparative 21.5 mm x 150 mm column packed with TSK-GEL (SP 5PW) was used. Freeze-dried material from the third peak of gel filtration was dissolved in 1.5 ml of 0.1 M ammonium acetate buffer (pH 6.8) and eluted with

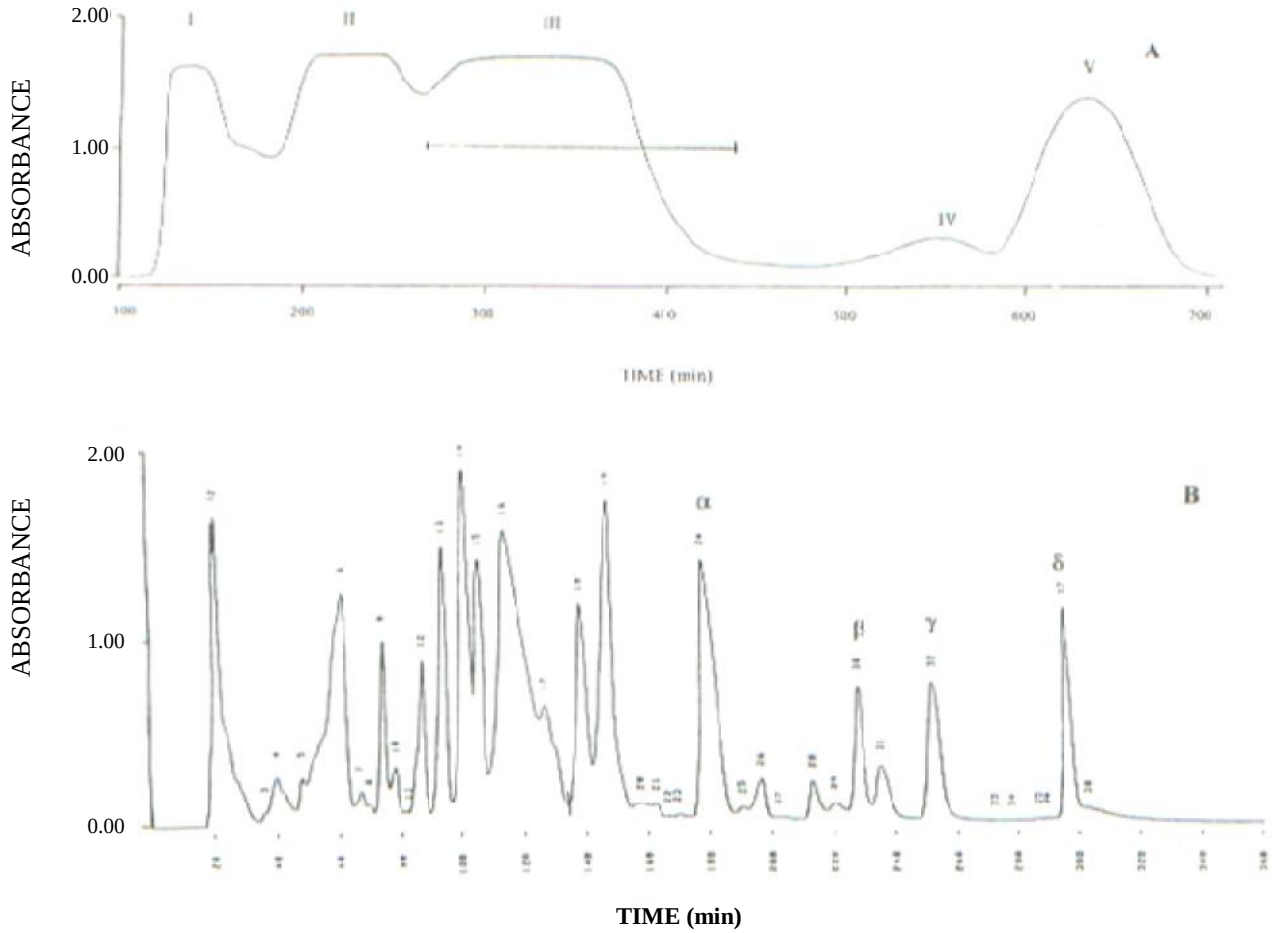

Fig. 1. Isolation of the α-neurotoxin from green mamba venom. (A): Gel filtration of whole venom of Eastern green mamba (*Dendroaspis angusticeps*). (B): Ion-exchange chromatography of fraction III from gel filtration of the venom. Peak corresponding to α-dendrotoxin is shown. Absorbance of eluate was monitored at 280 nm.

a two step linear gradient of ammonium acetate buffer (0.1 to 1.6 M, pH 6.8) with a flow rate of 2 ml/min.

Samples were collected in 8 ml aliquots. The ion exchange chromatography of fraction III gave more than 30 peaks (Figure 1B). Peaks relevant to α-dendrotoxin were identified by comparison with the chromatographic profiles of Harvey and Karlsson [11] and Benishin *et al.* [12]. This fraction was pooled and freeze-dried and its effects on chick biventer cervices (CBC) preparation were examined. The α-dendrotoxin fraction (10 μg/ml), unexpectedly, after a slight increase, greatly reduced the twitch height (Figure 3A).

To isolate the postsynaptic blocking contaminant from the α-dendrotoxin obtained from ion exchange chromatography, the freeze-dried material (12 mg) was loaded to a semi-preparative reverse phase HPLC column (10 mm x 250 mm) packed with Spherisorb ODS-II equilibrated with 0.1% trifluoroacetic acid (TFA) in HPLC grade water and eluted at room temperature with a three step linear gradient of 5-100% acetonitrile and 0.1% TFA at a flow rate of 2 ml/min. Absorbance was monitored at 280 nm and fractions of 4 ml were collected.

In order to find the postsynaptic blocker, the major peak (Figure 2A) was divided into five fractions. Eluates relating to each fraction were pooled and freeze-dried, and were used for bioassay experiments. The postsynaptic contaminant was related to the small peak which eluted just before the α-dendrotoxin peak and consisted of only 0.03% of the material. To separate the contaminant from α-dendrotoxin, fraction containing postsynaptic blocker was loaded onto an analytical reverse phase HPLC column (Spherisorb ODS-II, 4.6 mm x 250 mm), equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted at room temperature with a three

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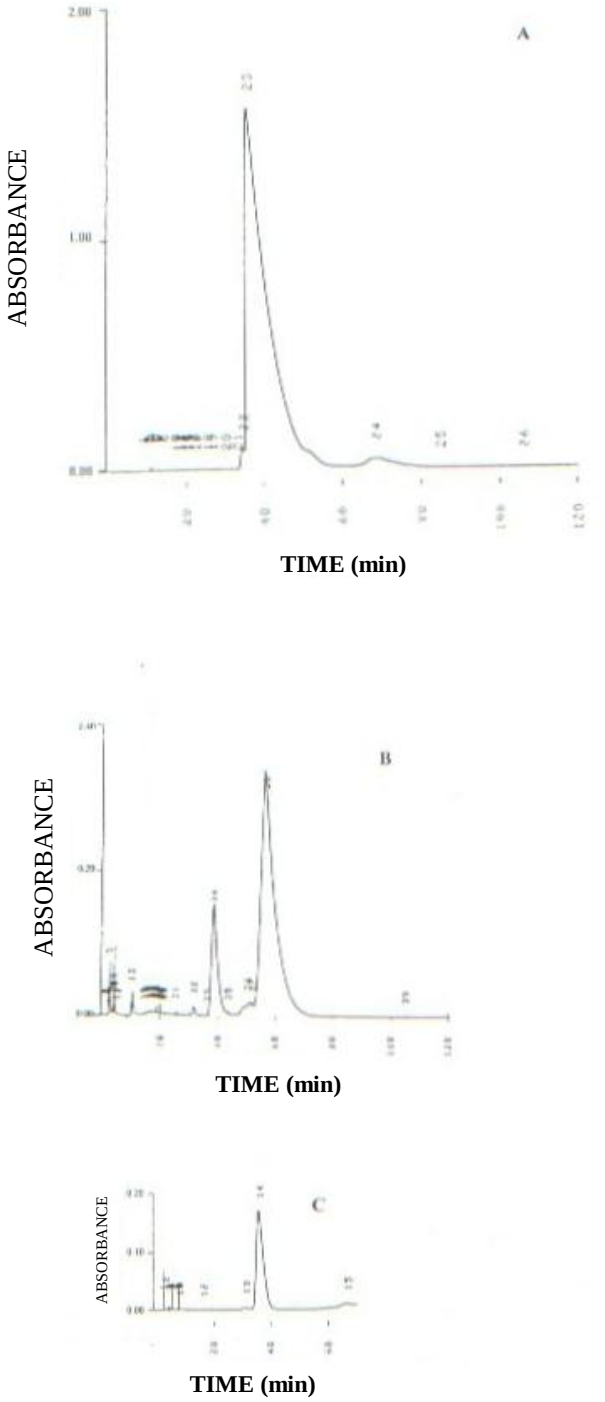

Fig. 2. (A): Reverse phase high pressure liquid chromatography of the α-dendrotoxin peak from ion-exchange chromatography, using a preparative column. The small peak (number 22) just before the main peak was responsible for the postsynaptic blocking effect. (B): Reverse phase high pressure liquid chromatography of the fraction containing the postsynaptic blocking toxin from the previous chromatography, using an analytical column. The two major peaks correspond to postsynaptic blocking toxin and α-dendrotoxin, respectively. (C): Further purification of the postsynaptic blocking toxin by reverse phase HPLC. Absorbance of eluate was monitored at 280 nm.

step linear gradient of 5-100% acetonitrile (Figure 3 B) at a flow rate of 1 ml/min. This separation resulted in two separate peaks, the postsynaptic blocker and α-dendrotoxin.

The postsynaptic blocker and dendrotoxin peaks were collected. The fractions relevant to the blocker were directly loaded onto the same column but eluted with a modified three step linear gradient (Figure 3C). The resulting material from this final purification was used for bioassay experiments.

The chick biventer cervices nerve-muscle preparation: Biventer cervices muscles and associated nerves [13] were dissected from 4-12 day old chicks killed by exposure to a lethal dose of halothane or CO_2. Two preparations were mounted in 10 ml tissue baths containing modified Krebs-Henseleit solution maintained at 32°C, pH 7.3-7.4 and bubbled with 95% O_2 + 5% CO_2. The modified Krebs-Henseleit solution was composed of: NaCl, 118.4 mM; KH_2PO_4, 1.2 mM; glucose, 11.1 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.4 mM and KCl, 4.7mM.

In twitch tension recording, twitches were evoked by stimulating the motor nerve at 0.1 Hz with pulses 0.2 msec duration and a voltage greater than that required to produce the maximum response.

To detect any changes in postsynaptic sensitivity, responses to submaximal concentrations of acetylcholine (1-2 mM), carbachol (20-40 μM), and KCl (20-40 mM), in the absence of nerve stimulation prior to the addition of toxin and at the end of the experiment were recorded. The preparations were washed free of these drugs and allowed 20-30 min to stabilize before the application of toxins. In the absence of toxin, twitch height or responses to exogenously applied acetylcholine, carbachol or KCl did not change in control experiments (up to at least 2 hours).

Twitches and contractures were recorded isometrically using Washington, Grass model 79 and Grass model 79B polygraphs, and SRI or Grass FT03 force transducers.

RESULTS

Postsynaptic blocking effect of ~-dendrotoxin fraction: Materials from the α-dendrotoxin fraction of ion exchange chromatography, as it was identified by comparison with the chromatographic profiles of Harvey and Karlsson [11] and Benishin *et*

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al. [12], was assessed by functional assays using chick biventer cervices (CBC) preparations.

The α-dendrotoxin fraction (10 μg/ml), unlike purified α-dendrotoxin, after a slight increase, greatly reduced the twitch height (Figure 3A). This reduction was not reversed by washing out the toxin fractions.

The α-dendrotoxin fraction abolished the responses to acetylcholine and carbachol, while it had no significant effect on the KCl response (Figure 3B). The postsynaptic effects of the toxin fractions were not affected by washing out, indicating the irreversibility of the effects. The effects observed with α-dendrotoxin fraction were similar to those with postjunctional α-neurotoxins (e.g. α-bungarotoxin).

Dose-response relationship of the isolated postsynaptic blocker: The effects of purified postsynaptic blocking contaminant on neuromuscular transmission were characterized, using chick biventer cervices preparations. The isolated contaminant, dose-dependently, reduced the twitch height (without any initial augmentation) followed by a complete postsynaptic block (Figure 3A). It also abolished the postsynaptic responses to acetylcholine and carbachol. However, it had no significant effect on the postsynaptic response to KCl (Figure 3B). These results suggested that the contaminant was a postjunctional blocking α-neurotoxin.

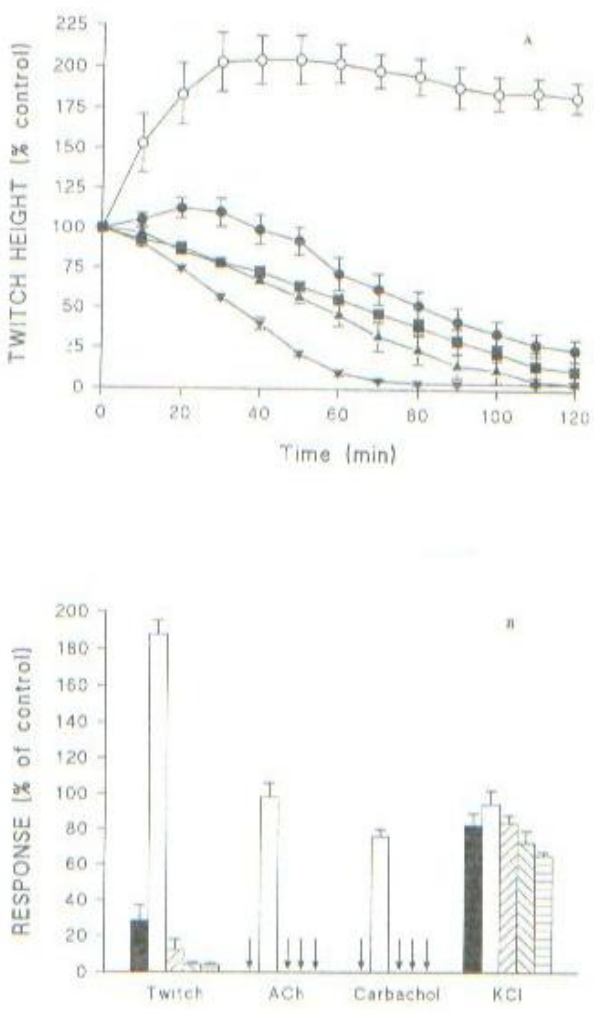

Figure 3. Effects on chick biventer cervices preparation. (A): Effects of purified α-dendrotoxin (10 μg/ml) (❍), α-dendrotoxin peak (10 μg/ml) from ion exchange chromatography (●) and different concentrations of the isolated postsynaptic blocking toxin (■, 0.2; ◆, 0.3; ◆, 0.6 μg/ml) on the twitch responses of chick biventer cervices. Note that the α-dendrotoxin peak, unlike purified α-dendrotoxin, progressively blocked the responses to indirect stimulation. Points are means of 4 experiments and s.e.ms are indicated by the bars. (B) Effects of purified α-dendrotoxin (10 μg/ml, □), α-dendrotoxin peak (10 μg/ml, ■) from ion exchange chromatography and different concentration of the postsynaptic blocking toxin (0.2, □; 0.3, □; and 0.6 μg/ml, □) isolated from the α-dendrotoxin peak on the responses of muscle responses to exogenously applied agonists. Columns show the responses to indirect stimulation (Twitch), acetylcholine (ACh, 2 mM), carbachol (40 μM) and potassium chloride (KCl, 80 mM). Note that muscles treated with α-dendrotoxin peak did not respond to acetylcholine and carbachol, shown by arrows. S.e.ms are indicated by the bars, n = 4.

DISCUSSION

Purification of venom components together with sensitive functional assessment of the compounds remains a critical criterion for further study of the properties of the compounds. Availability of highly efficient techniques and equipment (such as reverse phase high performance liquid chromatography) has provided significant progress in purification of toxins. The toxic peptides have also been shown to retain their biological properties after passing through different purification stages. The results of twitch tension recording experiments revealed that contamination (with a postsynaptic blocker) as low as 0.03% W/W can result in a substantial change in response to the contaminated fraction. Although functional experiments can help to detect such impurities, it may not always be the case. Firstly, if it is the first time that a compound is being tested, it is not possible to distinguish between the effect of the compound and any accompanying contaminant(s).

Secondly, in some bioassays such small contaminations may not be detected. For example, in the present case of a postsynaptic blocker, the contami-

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nant would not be detected by a dendrotoxins competitive binding assay. Therefore, secure purification of novel compounds is of great importance.

So far, such postsynaptic toxins have not been isolated from the venom of Eastern green mamba (*D. angusticeps*), although Barrett and Harvey [9] showed evidence of a postsynaptic blocking effect by the whole venom. The data presented in this study reveal that venom of the forth member of the *Dendroaspis* genus (*D. angusticeps)* also contains, at least one postsynaptic blocking toxin. The physical characteristics of this toxin remains to be studied.

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