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Pharmacological characterisation of a neurotoxin from the venom of *Boiga dendrophila* (Mangrove catsnake)

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Abstract

In this study, we have pharmacologically characterised boigatoxin-A, a three finger toxin isolated from the venom of the colubrid, *Boiga dendrophila* (Mangrove catsnake). In the chick biventer cervicis nerve-muscle preparation boigatoxin-A (1 μM) displayed poorly reversible postsynaptic blockade as evidenced by the inhibition of indirect (0.1 Hz, 0.2 ms, supramaximal V) twitches and responses to exogenous acetylcholine (1 mM) and carbachol (20 μM). Boigatoxin-A (0.3–0.5 μM) caused a concentration-dependent depression of the maximum response of cumulative concentration response curves to CCh (0.6–80 μM). Boigatoxin-A (1 μM) induced readily reversible inhibition of electrically evoked (0.2 Hz, 0.3 ms, 70–100 V) twitches of the prostatic segment of the rat vas deferens. This inhibition was not significantly attenuated by 8-phenyltheophylline (20 μM) or idazoxan (1 μM). Boigatoxin-A (1 μM) did not affect α,β-mATP (10 μM) or noradrenaline (25 μM) responses in unstimulated epididymal segments of the rat vas deferens. Our data suggests that this toxin has weak postsynaptic neurotoxicity in skeletal muscle and also prejunctional neurotoxic activity in the smooth muscle of the rat vas deferens to inhibit the release of neurotransmitter(s), but not via prejunctional purinergic or adrenergic receptors. This is the first report of such activity for a toxin isolated from snake venom and reinforces the largely untapped potential of colubrid venoms.

Keywords: Opisthoglyph; Colubridae; Chick biventer cervicis; Rat vas deferens; Boiga dendrophila; Snake venom; Three finger toxin

1. Introduction

Within the higher snake phylogeny there is a large group defined by the presence of enlarged grooved posterior maxillary teeth. Many of these opisthoglyphs are commonly considered as belonging to the colubrid family and although this classification is known to be artificial, the extensive evolutionary history and diverse prey base of its members has led researchers to believe that their venoms may represent a vast source of novel toxins and biological activities (for a review see Mackessy, 2002). α-Neurotoxins, which target muscle nicotinic receptors, are a common component of proteoglyph (front-fanged) snake venoms (for a review see Chang, 1979; Hodgson and Wickramaratna, 2002). They have also been implicated in a number of colubrid venoms (Levinson et al., 1976; Broaders et al., 1999; Lumsden et al., 2004a). Only one neurotoxin, α-colubritoxin, has been isolated and fully sequenced from a colubrid venom (Fry et al., 2003a). This toxin displayed

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reversible and competitive antagonism at the skeletal muscle nicotinic receptor but poor amino acid sequence similarity with long chain (66–74 amino acids and five disulphide bridges) or short chain (60–62 amino acids and four disulphide bridges) α-neurotoxins (Endo and Tamiya, 1991). Although 11 and 12 kDa components were isolated from the venom of the colubrid, *B. dendrophila*, and shown to have AChR binding activity, these were pooled during the pharmacological analysis and remain unsequenced (Broaders et al., 1999). A biologically uncharacterised 8769 Da component (which we now name boigatoxin-A) was also isolated from this venom and shown to display sequence homology with α-colubritoxin (Fry et al., 2003b). The current study examines the activity of boigatoxin-A in the chick biventer cervicis preparation. To determine selectivity of boigatoxin-A, and in consideration of previous reports which show *B. dendrophila* venom to display inhibitory activity in the rat vas deferens (Lumsden et al., 2004b), we also examine the activity of boigatoxin-A in this preparation.

2. Materials and methods

2.1. Isolation and storage of boigatoxin-A

Boigatoxin-A was isolated from *B. dendrophila* venom as previously described (Fry et al., 2003b). It was later lyophilised and stored at −20 °C. Stock solutions were prepared in distilled water and stored at −20 °C until required.

2.2. Chick biventer cervicis nerve muscle preparation

Chicks (4–10 days) were killed by CO_2 and exsanguination and chick biventer cervicis nerve muscle preparations isolated and mounted in organ baths. Indirect twitches were evoked by electrical stimulation of the motor nerve (supramaximal voltage, 0.2 ms, 0.1 Hz) (Harvey et al., 1993). *d*-Tubocurarine (*d*TC; 10 μM) was then added with the subsequent abolition of twitches confirming selective stimulation of the motor nerve. The preparation was then washed thoroughly over a 30 min period to re-establish twitches and to ensure removal of *d*TC from the bathing medium. Contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s) and potassium chloride (KCl; 40 mM for 30 s) were obtained in the absence of stimulation. Electrical stimulation was then recommenced and the preparation equilibrated for a further 30 min before the addition of toxin or vehicle. Boigatoxin-A (1 μM) or vehicle were left in contact with the preparation for a maximum of 3 h and change in twitch height was expressed as a percentage of the original twitch height in the absence of toxin or vehicle. Contractile responses to ACh, CCh and KCl were then obtained as described above and expressed as a percentage of the initial response obtained in the absence of toxin or vehicle. To determine reversibility of the inhibitory activity of the toxin upon twitches, the contents of the organ bath were replaced at 10 min intervals over a 60 min period after the 3 h incubation period.

Concentration-response curves to CCh (0.6–80 μM) were obtained by adding cumulative additions of CCh to unstimulated preparations which had not previously been used for any other experiment. After the maximum contraction was confirmed (by adding higher concentrations of CCh and observing no further change in magnitude of contraction) the tissue was washed and allowed to recover for 30 min. Toxin or vehicle were then left in contact with the preparation for 3 h before the cumulative CCh concentration response curve was repeated as above. Responses to CCh were expressed as a percentage of the initial maximum CCh response obtained in the absence of toxin or vehicle.

2.3. Rat vas deferens preparation

Sprague Dawley rats (250–350 g) were killed by 80% CO_2 and decapitation. The vas deferens were isolated, bisected into epididymal and prostatic segments and mounted on wire tissue holders or electrodes, respectively, in organ baths. Indirect twitches were evoked in the prostatic segment by electrical stimulation of the motor nerve (70–100 V, 0.3 ms, 0.2 Hz). Toxin or agonists were left in contact with the prostatic segment for 5 min before and after the addition of antagonists/inhibitors which had been equilibrated for 30 min. Data was expressed as the maximum change of twitch height observed in the 5 min incubation as a percentage of the original twitch height obtained in the absence of toxin or vehicle. To determine the activity of nicotinic receptor blocking drugs in the electrically stimulated prostatic segments, *d*TC (2–20 μM) was added to untreated preparations for 5 min and data expressed as previously mentioned. In unstimulated epididymal segments, contractile responses to α,β-methyleneadenosine 5-triphosphate (α,β-mATP; 10 μM) and noradrenaline (NA; 25 μM) were obtained before and after the addition of toxin or vehicle which had been equilibrated with the preparation for 30 min. Final agonist responses were expressed as a percentage of the original agonist response obtained in the absence of toxin or vehicle.

2.4. Experimental conditions

Rat vas deferens or chick biventer cervicis nerve muscle preparations were mounted under 0.75 g or 1 g resting tension, respectively, in 5 ml isolated organ baths containing physiological solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; $KHPO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25 and glucose, 11.1. The solution was bubbled with carbogen (95% O_2 and 5% CO_2). Preparations were equilibrated for at least 30 min before commencement of the experiment and maintained at 32 °C

(rat vas deferens) or 34 °C (chick biventer cervicis). Responses were measured via a Grass Force-Displacement Transducer (FT03 C; Grass Instrument Co., Quincy, MA, USA) and recorded on a PowerLab 400 System (ADI instruments).

2.5. Drugs

The following drugs were used: ACh; adenosine; CCh; clonidine; α,β-mATP; noradrenaline; 8-phenyltheophylline (8-PT); *d*TC (Sigma Chemical Co., St Louis MO, USA); clonidine (Boehringer Ingelheim, Artarmon, NSW, Australia); idazoxan (Reckitt and Coleman, Kingston upon Hull, UK). Except where indicated, stock solutions were prepared in distilled water. NA was prepared in catecholamine diluent (0.9% NaCl, 0.0156% $NaH_2PO_4 \cdot 2H_20$, 0.004% ascorbic acid w/v). 8-PTwas prepared in 80% MeOH and 20% 1 M NaOH.

2.6. Analysis of results and statistics

Students' paired *t*-test was used to compare before and after responses in the same tissue. Multiple comparisons were made using a one-way analysis of variance (ANOVA) followed by a Bonferroni test. Values of $p<0.05$ were considered significant. Data are expressed as mean ± SEM.

3. Results

3.1. Chick biventer cervicis nerve muscle preparation

Boigatoxin-A (1 μM) caused time-dependant inhibition of indirect twitches of the chick biventer cervicis nerve muscle preparation (Fig. 1a; $n=5$). After 3 h incubation with the toxin, washing (at 10 min intervals for 60 min) did not result in any significant recovery of the twitch height (Fig. 1a; $n=4$; arrow indicates commencement of washing). In addition, boigatoxin-A (1 μM) significantly inhibited contractile responses to the exogenous nicotinic agonists (i.e. ACh and CCh; $p<0.05$) but not KCl (Fig. 1b; $n=5$). Boigatoxin-A (0.3 μM; $n=4$, 0.5 μM; $n=3$) caused a significant ($p<0.05$) depression of the maximum response obtained in the cumulative concentration response curve to CCh when compared to vehicle ($n=5$) (Fig. 1c and d).

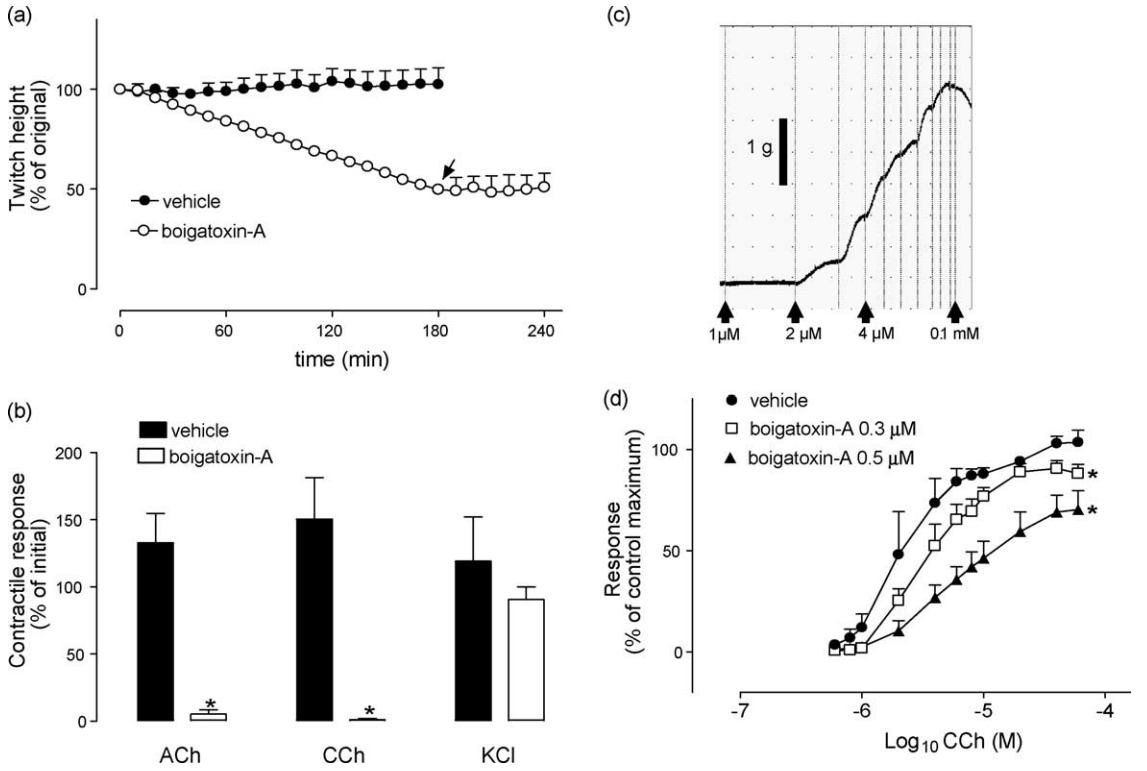

Fig. 1. (a) The effect of boigatoxin-A (1 μM; $n=5$) or vehicle ($n=8$) on twitch height of the chick indirectly stimulated biventer cervicis muscle and the effect of washing on the inhibition produced by the toxin (arrow indicates commencement of washing). Note: error bars are smaller than symbols. (b) The effect of boigatoxin-A (1 μM; $n=5$) or vehicle ($n=8$) on ACh, CCh and KCl responses in the unstimulated chick biventer cervicis nerve-muscle preparation. (c) Trace showing cumulative concentration response curve to CCh in the presence of boigatoxin-A (0.5 μM; vertical dotted lines indicate each addition of CCh). (d) The effect of boigatoxin-A (0.3–0.5 μM; $n=3$–4) or vehicle ($n=5$) on cumulative concentration response curves of the chick biventer cervicis nerve muscle preparation to CCh. *$P<0.05$; response was significantly different to that of vehicle control.

3.2. Rat isolated vas deferens: prostatic segment

Boigatoxin-A (1 μM), but not *d*TC (2–20 μM) or vehicle, produced inhibition of electrically induced contractions of the prostatic segment (Fig. 2a and b; $n=4$ each; arrow indicates addition of toxin in Fig. 2a) which was reversed by washing ($n=4$; data not shown). Prior addition of 8-PT (20 μM) prevented the inhibitory activity of adenosine (2 μM; $n=5$) but not boigatoxin-A (1 μM; $n=4$) (Fig. 2c). Prior addition of idazoxan (1 μM) prevented the inhibitory activity of clonidine (3 nM; $n=3$) but not boigatoxin-A (1 μM; $n=4$) (Fig. 2d).

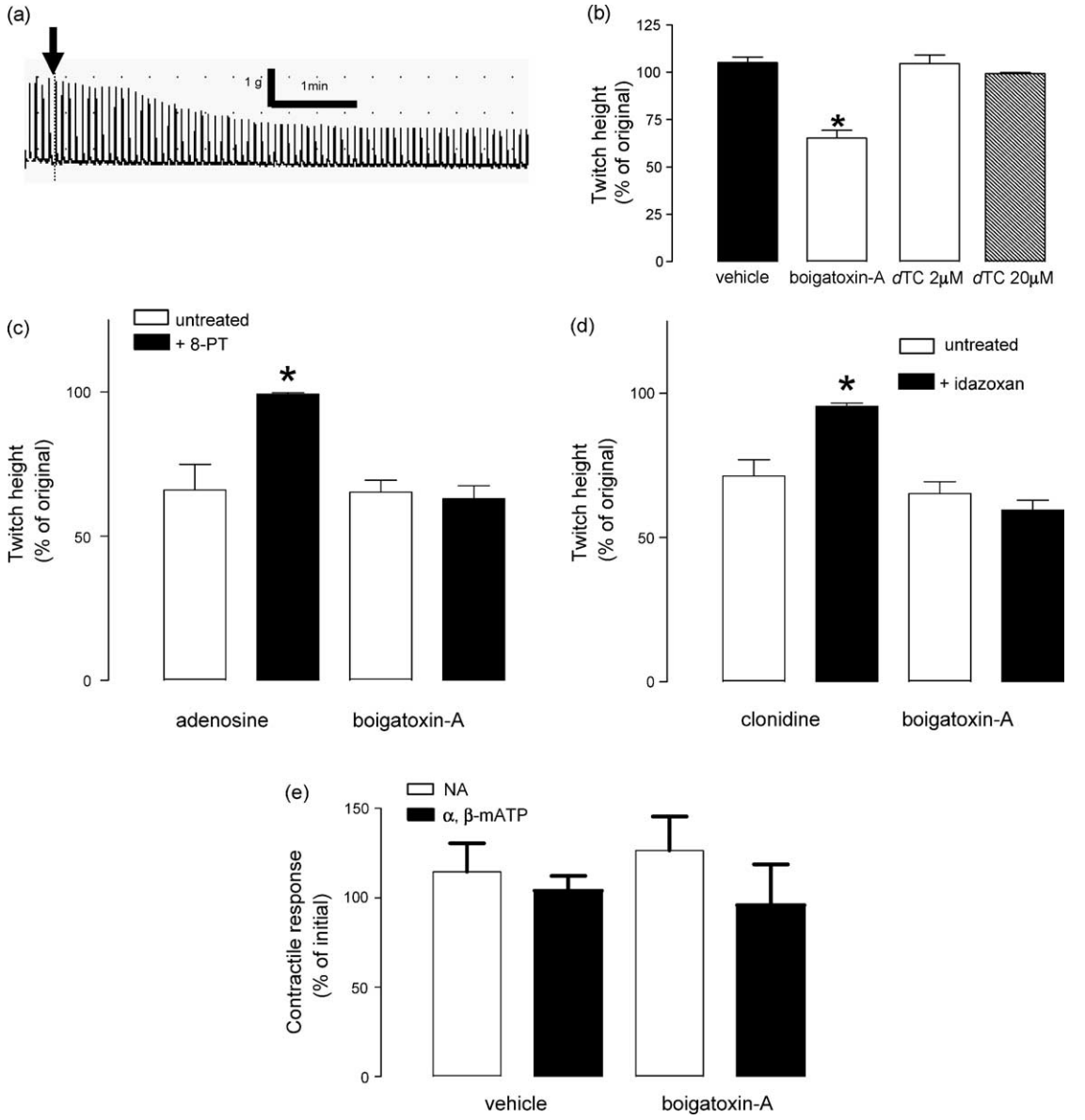

Fig. 2. (a) Trace showing the effect of boigatoxin-A (1 μM) on electrically stimulated rat vas deferens prostatic segment (arrow indicates addition of toxin). (b) The effect of boigatoxin-A (1 μM; $n=4$), *d*TC (2–20 μM; $n=4$) or vehicle ($n=4$) on twitch height of the prostatic segment of the electrically stimulated (70–100 V, 0.3 ms, 0.2 Hz) rat vas deferens segment. (c) The effect of 8-PT (20 μM) on electrically stimulated (70-100 V, 0.3 ms, 0.2 Hz) rat vas deferens prostatic segment treated with boigatoxin-A (1 μM; $n=5$) or adenosine (2 μM; $n=4$). (d) The effect of idazoxan (1 μM) on electrically stimulated (70–100 V, 0.3 ms, 0.2 Hz) rat vas deferens prostatic segment treated with boigatoxin-A (1 μM; $n=4$) or clonidine (3 nM; $n=4$). (e) The effect of boigatoxin-A (1 μM; $n=4$–5) or vehicle ($n=4$) on contractile responses to α, β-maTp (10 μM) or NA (25 μM) in the unstimulated epididymal segment. *$P<0.05$, response was significantly different to that of vehicle control or to the response obtained in the absence of antagonists.

3.3. Rat isolated vas deferens preparation: epididymal segment

Boigatoxin-A (1 μM) alone had no significant effect on the unstimulated epididymal segment of the rat vas deferens ($n=5$; data not shown). In addition, boigatoxin-A (1 μM) had no significant effect on contractile responses to α, β-maTP (10 μM) or noradrenaline (25 μM) (Fig. 2e; $n=5$ each).

4. Discussion

Due to sequence homology with α-colubritoxin and the previously reported in vitro neurotoxic activity of venom and pooled venom components from *B. dendrophila* (Broaders et al., 1999; Fry et al., 2003b; Lumsden et al., 2004a), boigatoxin-A was examined for neurotoxic activity in the chick biventer cervicis nerve muscle preparation. Boigatoxin-A inhibited indirect twitches and exogenous agonist responses indicating a postsynaptic site of action which appears to be pharmacologically similar to that demonstrated by elapid venom α-neurotoxins. The activity of boigatoxin-A appears to be pseudo-irreversible (i.e. the toxin slowly dissociates from site of action), like that reported for α-Bungarotoxin and Acantoxin IVa (Wickramaratna et al., 2004), since the toxins inhibitory activity on twitches did not readily reverse upon washing and a depression of the maximum obtained in the CCh concentration response curve was also observed. A distinguishing feature of boigatoxin-A, is the slow inhibition of twitches (i.e. approximately 50% inhibition of twitches in 3 h) in comparison to that reported for previously studied snake venom neurotoxins including α-colubritoxin, which, at the same concentration as used in our study, typically abolish 90% of the original twitch height of the indirectly stimulated chick biventer cervicis within 1 h (Nirthanan et al., 2003; Fry et al., 2003a; Wickramaratna et al., 2004). Presently, both α-colubritoxin, and boigatoxin-A share some amino acid sequence homology with the poorly studied weak-type (also known as miscellaneous-type) neurotoxins, in that they display a similar cysteine residue pattern at the N-terminal (Fry et al., 2003a,b)

The selectivity of boigatoxin-A was investigated using the rat vas deferens, a preparation containing several receptor types (e.g. histamine, muscarinic, adrenergic, opioid) and which has been previously used to demonstrate purinergic receptor activity of the adenosine A_1 subtype by *B. dendrophila* venom (Lumsden et al., 2004b). The inhibition of electrically stimulated twitches in the prostatic segment by the toxin was similar to that observed by the whole venom and an interesting finding, since snake venom α-neurotoxins, including α-Bungarotoxin (Gotti et al., 1985), have not been previously reported to display such inhibitory activity in this preparation.

Similar to *B. dendrophila* venom, boigatoxin-A appears to be acting presynaptically in the rat vas deferens since it did not block responses to the exogenous agonists αβ-mATP or NA which act on the postjunctionally located P2X purinoceptors and α_1-adrenoceptors, respectively (Leedham and Pennefather, 1982; Sneddon and Westfall, 1984). However, the toxin does not appear to be acting at the presynaptically located purinoceptors (including the adenosine A_1 subtype) and α_2-adrenoceptors, since their antagonists (i.e. 8-PT and idazoxan; Wooten et al., 1973; Rand et al., 1975) did not block the toxins activity. Therefore, boigatoxin-A appears to display a different mechanism of action to the whole venom from which it was isolated to produce the inhibitory effects. Neither does boigatoxin-A appear to be acting like Vipoxin (from *Daboia russelli russelli*), an unclassified snake venom toxin which has been shown to exert α_2, and also α_1, -adrenoceptor agonist effects in the rat vas deferens (Chappinelli, 1991). Since electrically evoked twitches of the rat vas deferens prostatic segment are predominantly mediated by the release of ATP (MacDonald and McGrath, 1980; Brown et al., 1983), it is apparent that boigatoxin-A is acting in some way to inhibit the release of neurotransmitter. However, it is inconclusive as to whether the toxin specifically blocks release of ATP or NA.

Considering snake venom toxins like κ-Bungarotoxin (isolated from *Bungarus multicinctus* venom) demonstrate affinity for both ganglionic and muscle nicotinic receptors (Chappinelli, 1991) it may be possible that boigatoxin-A is acting at ganglionic nicotinic receptors of the rat vas deferens. However, we were unable to find evidence in the literature or from our own experiments with *d*TC (which has similar affinity for both receptors), that drugs which target these receptor types have an inhibitory effect upon twitches in the rat vas deferens prostatic segment.

Alternatively, the activity of boigatoxin-A may be due to ion channel interaction like that observed for lignocaine, a sodium channel blocker which reduces twitch height in the electrically stimulated rat vas deferens preparation (Fatani et al., 2000) and at higher doses reduces the amplitude of indirectly stimulated twitches and ACh contractures in the chick biventer cervicis preparation (Wali, 1986). However, with previous reports indicating that *B. dendrophila* venom contains AChR binding components (Broaders et al., 1999), we believe it is highly likely that the postsynaptic blockade observed by boigatoxin-A in the chick biventer cervicis is due to direct interaction with the muscle nicotinic receptor. Future studies using electrophysiological techniques and/or radio ligand binding studies could be useful in confirming the exact site(s) of action of boigatoxin-A.

In conclusion, we present for the first time the pharmacological activity of a novel colubrid snake venom toxin. Boigatoxin-A appears to have poorly reversible postsynaptic blocking activity in the chick biventer cervicis preparation and readily reversible inhibitory activity at a presynaptic site in the rat vas deferens prostatic segment

most likely to prevent the release of neurotransmitter(s). This study reinforces the largely untapped pharmacological potential of colubrid snake venoms.

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