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Presynaptic neuromuscular activity of venom from the brown-headed snake (*Glyphodon tristis*)

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Abstract

The brown-headed snake (*Glyphodon tristis*) inhabits the forest regions of Papua New Guinea, Torres Strait Islands, and far northern Queensland, Australia. Although bites by *Glyphodon dunmalli* have been reported, *G. tristis* was regarded as innocuous until 1989 when a healthy 20 year old man was bitten (Sutherland, S.K., Tibballs, J., 2001. Australian Animal Toxins, the Creatures, their Toxins and Care of the Poisoned Patient. University Press, Oxford). Treatment of envenomation by this species is empirical with no specific antivenom available. While no published studies on the venom of *G. tristis* are available, unpublished studies suggest neurotoxicity as being the main symptom of envenomation. In this study, the in vitro effects of *G. tristis* venom were examined using the chick biventer cervicis nerve muscle (CBCNM) preparation. Venom (10 μg/ml) inhibited indirect (0.2 ms, 0.1 Hz, supramaximal V) twitches of the CBCNM. This inhibition appeared to be presynaptic in origin as evidenced by the lack of effect of venom on responses to exogenous acetylcholine (1 mM), carbachol (20 μM) and KCl (40 mM) in the non-stimulated CBCNM. Prior addition (10 min) of polyvalent snake antivenom (5 U/ml; CSL Ltd) attenuated twitch inhibition. The venom (10–50 μg/ml) also appears to be myotoxic as indicated by a slowly developing contracture and inhibition of direct (2 ms, 0.1 Hz, supramaximal V, in the presence of tubocurarine 10 μM) twitches. Myotoxicity was confirmed by subsequent histological examination of tissues. This myotoxicity was prevented by the prior addition of polyvalent snake antivenom (30 U/ml). The phospholipase A inhibitor 4-BPB (1.8 mM) significantly attenuated the inhibition of indirect and direct twitches of the CBCNM preparation, indicating the involvement of a PLA_2 component in the toxic action of the venom.
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1. Introduction

The brown-headed snake (*Glyphodon tristis*) inhabits the forest regions of Papua New Guinea, Torres Strait Islands, and far northern Queensland, Australia (Longmore, 1986). Being a nocturnal species it is rarely encountered by humans. Bites by *Glyphodon* species have been considered trivial although marked reactions to bites by *Glyphodon dunmalli* have been reported (Wilson and Knowles, 1988; Gow, 1989). In 1989 a healthy 20-year-old male was bitten by *G. tristis*, subsequently displaying respiratory distress, abdominal cramps, vomiting, hypotension and tachycardia. Polyvalent snake antivenom was administered although its contribution to the recovery of the patient, several days later, is unclear. A venom detection kit (VDK) failed to positively identify the species (Sutherland and Tibballs, 2001). No published studies on the venom of *G. tristis* have been located.

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An unpublished study (cited in Hobbins, 1994) showed transient hypotension and reduced cardiac output in an anaesthetised dog following administration of *G. tristis* venom. Another unpublished study (Hobbins, 1994), comparing *G. tristis* and tiger snake (*Notechis scutatus*) venoms, indicated that neurotoxicity was the main effect of *G. tristis* venom. Indeed, the murine LD_{50} of *G. tristis* (3.6 mg/kg, s.c.) is only 30 times more than that of *N. scutatus* (Hobbins, 1994). This study examined the neurotoxic and myotoxic effects of brown-headed snake venom in order to provide further insight into some of its components and their mechanism(s) of action.

2. Materials and methods

2.1. Venom preparation and storage

Specimens of *G. tristis* were collected from Weipa, Queensland and venom extracted by sliding pipette tips over the fang and wiggling to stimulate venom delivery. Pooled venom from at least six adults was used to minimize the effects of individual variation. The venom underwent a 20 μm filtration to remove any potential mucosal contaminants. Polyethylene materials (pipette tips, Eppendorf tubes, specimen bottles) were used to handle and contain the milkings due to the strong affinity some peptides possess for glass and polystyrene. Freeze dried venom and stock solutions of venom prepared in 0.1% bovine serum albumin in 0.9% saline (BSA) were stored at −20 °C until required.

2.2. Chick biventer cervicis nerve–muscle preparation

Chickens (4–10 day old male) were killed with CO_2 and both biventer cervicis nerve–muscle preparations were dissected. These were mounted under 1 g resting tension in 5 ml organ baths containing physiological salt solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25 and glucose, 11.1. The solution was maintained at 34 °C bubbled with carbogen (95% O_2 and 5% CO_2). Motor nerves were stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator (Harvey et al., 1994). d-Tubocurarine (dTC) was added (10 μM) and the subsequent abolition of twitches confirmed the selective stimulation of nerves. Responses to nerve stimulation were re-established by thorough washing. 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 (Harvey et al., 1994). The preparations were then equilibrated for at least 30 min with continuous nerve stimulation before addition of venom. In all experiments venom was left in contact with the preparations until responses to nerve stimulation were abolished, or for a maximum of 3 h if total twitch blockade did not occur. At the conclusion of the experiment, responses to ACh, CCh and KCl were obtained as previously described. Time taken to reduce the amplitude of the indirect twitches by 90% (t_{90}) was calculated in order to provide a quantitative measure of neurotoxicity (Crachi et al., 1999).

Where indicated snake polyvalent antivenom (5–30 U/ml) was added 10 min prior (Barfaraz and Harvey, 1994) to the addition of venom (10 μg/ml). Reversibility of the inhibitory effects of venom was examined in additional experiments in which antivenom (5 U/ml) was added at t_{90}. Twitch height was then monitored for a further 1.5 h.

In experiments examining the myotoxic effects of venom, the biventer cervicis muscle was directly stimulated every 10 s with pulses of 2 ms duration at supramaximal voltage. In these experiments the electrodes were placed around the belly of the muscle and d-tubocurarine (10 μM) remained in the organ bath for the duration of the experiment. Venom was left in contact with the preparation until twitch blockade occurred, or for a 3 h period (as above). Venom was considered to be myotoxic if it inhibited directly mediated twitches or caused a contracture of the skeletal muscle (Harvey et al., 1994).

2.3. Phospholipase A_2 (PLA_2) inhibition with 4-bromophenacyl bromide (4-BPB)

PLA_2 activity of venom was inhibited by alkylation with 4-BPB. Venom (3–50 μg/ml), made up in sodium cacodylate–HCl buffer (50 μl, 0.1 M, pH 6.0), and 4-BPB made up in acetone was added to produce a final concentration of 1.8 mM (Abe et al., 1977; Bell et al., 1998; Crachi et al., 1999). Vials containing the above mixture were incubated for 16 h at 30 °C. Where indicated venom made up in sodium cacodylate–HCl buffer incubated with acetone, and cacodylate–HCl buffer incubated with 1.8 mM 4-BPB were used as vehicle controls for 4-BPB and venom, respectively (Wickramaratna et al., 2003).

2.4. Determination of PLA_2 activity

PLA_2 activity of the venom was determined using a secretory colourimetric assay kit (Cayman Chemical, USA). This assay uses the 1,2-dithio analog of diheptanoyl phosphatidylcholine which serves as a substrate for PLA_2 enzymes. Free thiols generated following the hydrolysis of the thio ester bond at the sn-2 position by PLA_2 are detected using DTNB (5,5′-dithio-*bis*-(2-nitrobenzoic acid)). Colour changes were monitored using a CERES900C micro-plate reader (Bio-Tek Instruments, USA) at 405 nm, sampling every minute for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolysed per min per milligram of enzyme.

2.5. Analysis of results and statistics

For isolated tissue experiments, responses were measured via a Grass force displacement transducer

(FTO3) and recorded on a MacLab System. In both neurotoxicity and myotoxicity studies, twitch height was expressed as a percentage of the twitch height prior to the addition of venom or antivenom. Paired Student's *t*-test was used to compare agonist responses before and after treatment in the same preparation. A one-way ANOVA was performed for multiple comparisons at the time point indicated. Data are expressed as mean ± SE with statistical significance whenever $P<0.05$.

2.6. Morphological studies

At the conclusion of the in vitro myotoxicity studies, tissues were removed from the organ bath, immediately placed in Tissue Tek, frozen in liquid nitrogen, and stored at −80 °C until required. Tissues were cut into transverse sections (14 μm) using a Leica CM1800 cryostat and placed onto gelatin-coated slides. Tissue sections were post fixed for 15 min in 4% formaldehyde, stained with haematoxylin and eosin and examined under a light microscope (Olympus BH-2, Olympus Optical Co., Japan). Areas exhibiting pathological changes typical of myotoxicity were photographed using Olympus C-35AD camera (Olympus Optical Co., Japan).

2.7. Drugs

The following drugs were used: acetylcholine chloride; bovine serum albumin, carbamylcholine chloride, d-tubocurarine, 4-bromophenacyl bromide, sodium cacodylate, eosin, Mayer's Haematoxylin (Sigma), Polyvalent Snake Antivenom (CSL Ltd, Melbourne, Australia). Except where indicated all stock solutions were made up in distilled water.

3. Results

3.1. Chick isolated biventer cervicis muscle

Venom (10 μg/ml) abolished indirect twitches of the chick biventer muscle preparation (Fig. 1(a); $t_{90}=99.7\pm7.7$ min). Reduction of indirect twitches by *G. tristis* venom appeared to be triphasic showing a small initial decrease followed by a transient increase and finally complete inhibition of twitches. Venom (10 μg/ml) had no significant inhibitory effect on the responses to ACh (1 mM), CCh (20 μM) or KCl (40 mM) (Fig. 1(b)). Vehicle (i.e. BSA) had no significant effect on indirect twitches nor response to exogenous agonists (data not shown, $n=4$).

The phospholipase A_2 inhibitor 4-BPB (1.8 mM) significantly attenuated the inhibition of indirect twitches by venom (10 μg/ml) (Fig. 1(a); $n=5$; $P<0.05$ one-way ANOVA). Venom and vehicle caused an inhibition of indirect twitches, which was not significantly different to venom alone.

Prior addition (10 min) of polyvalent snake antivenom (5 U/ml) significantly attenuated twitch inhibition produced

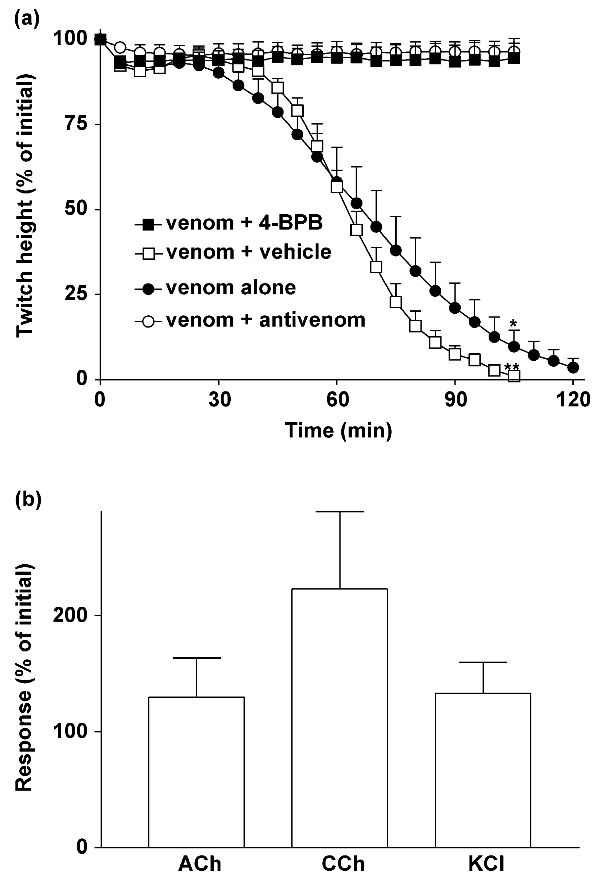

Fig. 1. (a) The effect of venom (10 μg/ml; $n=6$) alone, venom incubated with 4-BPB (1.8 mM; $n=5$), venom incubated with acetone ($n=5$) and venom in the presence of antivenom (5 U/ml; $n=4$) on indirect twitches of the chick biventer muscle. *$P<0.05$ significantly different from venom in the presence of antivenom, one-way ANOVA. **$P<0.05$ significantly different from venom in the presence of 4-BPB. (b) The effect of venom (10 μg/ml) on responses of the chick biventer cervicis muscle preparation to acetylcholine (ACh, 1 mM), carbachol (CCh, 20 μM) or potassium chloride (KCl, 40 mM) ($n=6$).

by venom (10 μg/ml, Fig. 1(a)). Vehicle (i.e. 0.1% BSA), in the presence of antivenom, had no effect on twitch height (data not shown, $n=4$). The addition of antivenom (5 U/ml) at t_{90} failed to reverse the inhibition of indirect twitches by venom (10 μg/ml, Fig. 2). Antivenom alone had no significant effect on twitch height (data not shown, $n=4$).

Venom (10–50 μg/ml) caused a dose-dependent inhibition of direct twitches (Fig. 3(a)). This inhibition (i.e. at 50 μg/ml) was statistically significant compared to the vehicle alone ($n=3$–5; one-way ANOVA, $P<0.05$). Venom (10–50 μg/ml) also induced a significant increase in baseline tension (1.3 ± 0.2 g) compared to vehicle (0 ± 0.2 g; $n=3$–5; one-way ANOVA).

The phospholipase A_2 inhibitor 4-BPB (1.8 mM) attenuated the inhibition of direct twitches and the increase

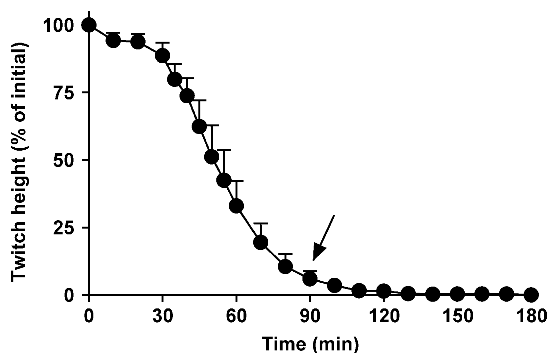

Fig. 2. Effect of antivenom (5 U/ml; administration indicated by arrow) added at t_{90} on inhibition of indirect twitches of the chick biventer cervicis nerve muscle preparation caused by venom (10 μg/ml, $n=4$).

in baseline tension (0.2±0.2 g; one-way ANOVA, $n=4$) produced by venom (50 μg/ml). In contrast, venom and vehicle (i.e. acetone) still caused a significant decrease in twitch height (Fig. 3(b); $n=3$–4 one-way ANOVA, $P<0.05$).

Prior addition (10 min) of polyvalent snake antivenom (30 U/ml) significantly attenuated twitch inhibition but not the increase in baseline (0.7±0.2 g, $n=4$) produced by venom (50 μg/ml) (Fig. 3(a); $n=4$, one-way ANOVA, $P<0.05$).

3.2. Morphological studies

Light microscopy studies of tissues exposed to venom (10–50 μg/ml) showed dose-dependent morphological changes (Fig. 4(b) and (c)). These changes included edema, myofibre damage and vacuolation. No detectable morphological changes were seen in the tissues exposed to the vehicle (i.e. BSA, Fig. 4(a)). Prior incubation of 4-BPB with venom (Fig. 4(d)) resulted in only slight edema while swollen cells were detected when venom was added in the presence of antivenom (Fig. 4(e); 30 U/ml). No detectable morphological changes were observed in tissues exposed to antivenom alone or to the combination of vehicle (sodium cacodylate) and 4-BPB (data not shown).

3.3. Phosoholipase A_2 (PLA_2) activity

PLA_2 activity of 141.5±2.9 μmol/min mg^{-1} ($n=6$) was detected in venom. This activity was significantly reduced by prior incubation of the venom with 4-BPB (2.5±0.3, $n=6$).

4. Discussion

Previous unpublished in vitro pharmacological studies have shown that *G. tristis* venom has no effect on

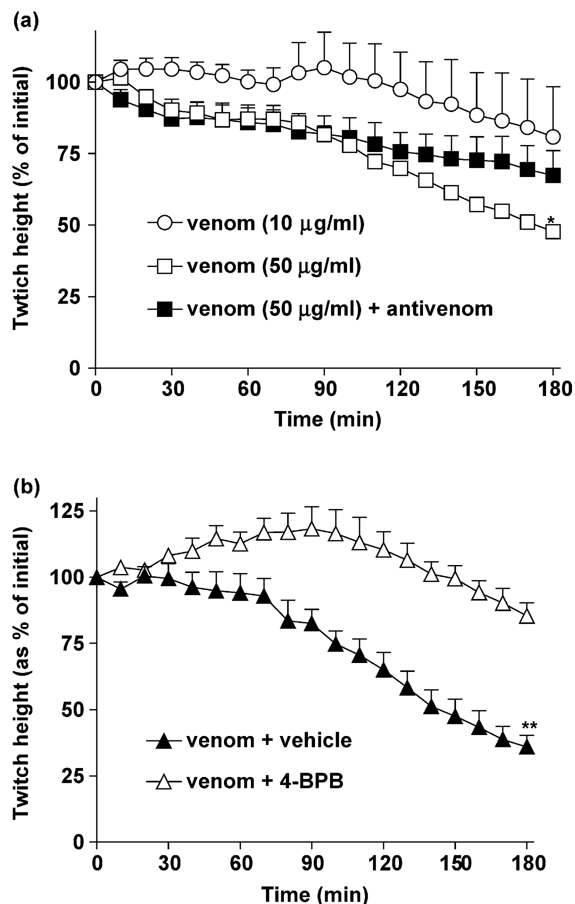

Fig. 3. (a) The effect of venom (10–50 μg/ml) or venom in the presence of antivenom (30 U/ml) on direct twitches of the chick biventer cervicis nerve muscle preparation ($n=3$–5). *$P<0.05$, significantly different from vehicle control and venom (50 μg/ml) in the presence of antivenom one-way ANOVA. (b) The effect of venom (50 μg/ml) incubated with 4-BPB (1.8 mM) or venom (50 μg/ml) incubated with acetone, on direct twitches of the chick biventer muscle ($n=3$–4). **$P<0.05$ significantly different from venom with 4-BPB, one-way ANOVA.

the contractions of the rat vas deferens or vascular smooth muscle. Although the venom causes a slight increase in heart rate in vivo, neurotoxicity appears to be the primary life threatening symptom of envenomation by this species (Hobbins, 1994). The myotoxic effects of this venom are unknown. Therefore the present study examined the myotoxic effects, nature of neurotoxicity, and their neutralisation with a commercially available polyvalent antivenom.

The in vitro neurotoxicity of *G. tristis* venom was investigated using the CBCNM. Venom caused inhibition of indirect twitches but failed to inhibit the contractile responses to exogenous ACh, CCh or KCl. This appears to indicate the presence of a presynaptic neurotoxin(s) in the venom. Reduction of indirect twitches by *G. tristis* venom was triphasic showing a small initial decrease followed by

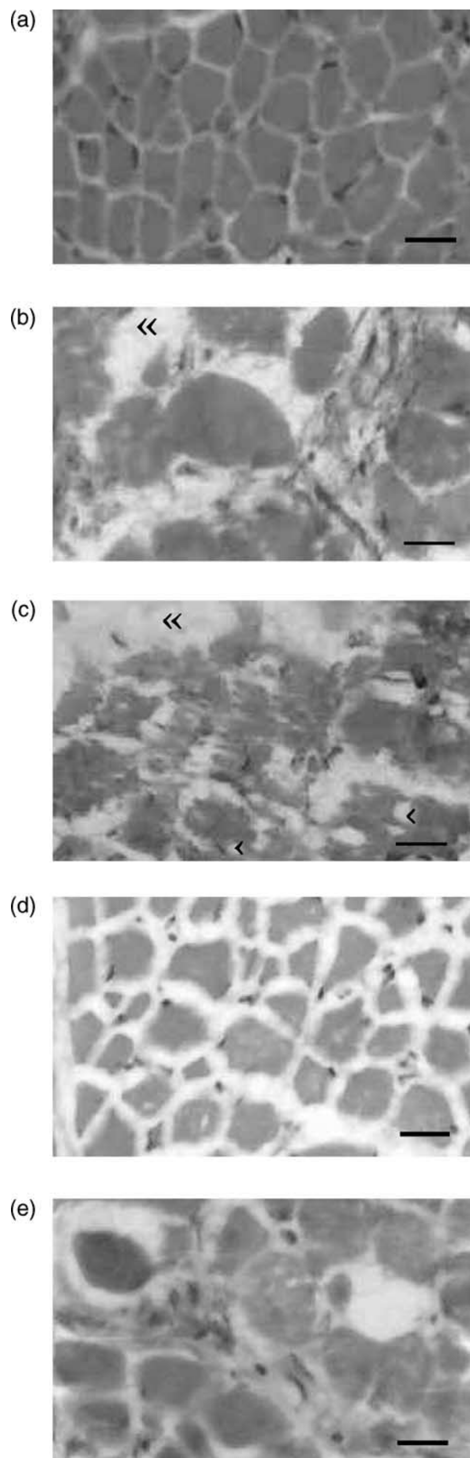

Fig. 4. Transverse sections of chick biventer muscle exposed to (a) vehicle (i.e. BSA); (b) venom (10 μg/ml); (c) venom (50 μg/ml); (d) venom (50 μg/ml) in the presence of 4-BPB; (e) venom (50 μg/ml) in the presence of antivenom (30 U/ml). Scale 50 μm in all micrographs. Single arrowheads indicate prominent vacuoles and double arrowheads indicate edema.

a brief transient increase and finally complete inhibition of twitches. This triphasic effect on neurotransmission is similar to that caused by the snake presynaptic neurotoxins taipoxin, notexin and β-bungarotoxin (Harris, 1991). These changes are particularly evident when the safety factor of transmission is lowered by reducing the Ca^{2+} or increasing the Mg^{2+} content of the bathing medium (Chang et al., 1977).

Postsynaptic neurotoxins on the other hand bind to nicotinic acetylcholine receptors with a high affinity and competitively antagonise the actions of acetylcholine (Stroud et al., 1990) and carbachol. Normally the presence of postsynaptic neurotoxins, which have a much quicker onset of action than presynaptic neurotoxins, mask the effects of presynaptic neurotoxins making it difficult to confirm their presence in whole venoms. As *G. tristis* whole venom displays presynaptic neurotoxic activity, this appears to indicate a lack of postsynaptic neurotoxic activity.

PLA_2 enzymes are found in the venom and oral secretions of snakes of all families (Harris, 1991) but are the result of two independent toxin recruitment events (Fry and Wuster, 2004). Two major properties of toxic snake venom PLA_2 enzymes are neurotoxicity and myotoxicity. The importance of PLA_2 activity to the toxic functions of enzymes has been the subject of much debate. In order to test if PLA_2 activity of the whole venom is necessary for the observed neurotoxic action, the venom was subjected to chemical modification by 4-BPB. Many studies have shown that PLA_2 activity can be inhibited by acylation of the His-248 residue using 4-BPB (Volwerk et al., 1974; Abe et al., 1977). Inhibition of indirect twitches of the chick preparation was attenuated when venom was incubated with 4-BPB. This suggests that PLA_2 activity of *G. tristis* venom is essential for its neurotoxic activity and is in agreement with previous studies which have shown that chemical inactivation of the PLA_2 active site blocks neurotoxic activity (Yang, 1997).

The in vitro myotoxic effects of *G. tristis* venom were also investigated. Venom caused a dose-dependent inhibition of direct twitches in the chick preparation, as well as an increase in baseline tension, indicative of myotoxicity (Harvey et al., 1994). In addition to these results, light microscopy studies showed obvious morphological changes in the biventer muscle exposed to venom, compared to that exposed to vehicle only. PLA_2 activity is essential for myotoxicity although no quantitative relationship between the two has been established (Gopalakrishnakone et al., 1997). It has also been suggested that the parts of the molecule responsible for toxicity are not the same as those responsible for hydrolytic activity. Chemical modification by 4-BPB appears to significantly reduce the myotoxic effects of *G. tristis* venom indicating that PLA_2 activity is important for the myotoxic effects.

Envenomation by the broad-headed snake is rare given the remoteness of the area it inhabits (Sutherland and Tibballs, 2001) and decimation of populations due to the introduction of the cane toad into Australia. However, an increase in the number of visitors to these areas makes it

important to determine the efficacy of currently available antivenoms in reversing the neurotoxic effects of this venom. Due to the lack of a specific antivenom against *G. tristis* venom, and due to its previous clinical use in treating a case of envenomation by *G. tristis*, CSL Ltd polyvalent snake antivenom was chosen for this study. Prior addition (10 min) of antivenom (5 U/ml) attenuated the venom-mediated inhibition of indirect twitches. In order to further study the efficacy of the antivenom, and to better approximate a clinical situation, antivenom was added at the t_{90} time point. However, antivenom failed to reverse the twitch height inhibition. This supports the suggestion that *G. tristis* venom contains a presynaptic neurotoxin as these are unresponsive to antivenom therapy once bound and internalised. It is also of clinical relevance to determine the efficacy of polyvalent snake antivenom in preventing the myotoxic effects of *G. tristis* venom. Prior incubation with antivenom significantly attenuated the inhibition of direct twitches of the CBCNM. Furthermore, the observed morphological changes were attenuated in the presence of antivenom.

In conclusion, brown-headed snake venom causes neurotoxicity and produced presynaptic inhibition of twitches in the CBCNM. PLA_2 activity appears to be necessary for the neurotoxic and myotoxic effects of the venom, which are effectively blocked by CSL Ltd polyvalent snake antivenom. While this study presents a pharmacological profile of the brown-headed snake venom, there is still scope for further investigation. Fractionating the venom would help identification and isolation of the components responsible for the above mentioned effects. In the light of these findings other *Glyphodon* venoms should be examined for similar activity.

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