TOXICOLOGICAL SCIENCES **74,** 352–360 (2003)
DOI: 10.1093/toxsci/kfg144

Species-Dependent Variations in the *in Vitro* Myotoxicity of Death Adder (*Acanthophis*) Venoms

Janith C. Wickramaratna,* Bryan G. Fry,† and Wayne C. Hodgson*,[1]

*Monash Venom Group, Department of Pharmacology, Monash University, Victoria 3800, Australia; and †Australian Venom Research Unit, Department of Pharmacology, University of Melbourne, Victoria 3010, Australia

Received February 26, 2003; accepted April 30, 2003

Based on early studies on *Acanthophis antarcticus* (common death adder) venom, it has long been thought that death adder snake venoms are devoid of myotoxicity. However, a recent clinical study reported rhabdomyolysis in patients following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus*. Subsequently, a myotoxic phospholipase A_2 component was isolated from *A. rugosus* (Irian Jayan death adder) venom. The present study examined the venoms of *A. praelongus* (northern), *A. pyrrhus* (desert), *A. hawkei* (Barkly Tableland), *A. wellsi* (black head), *A. rugosus*, *A.* sp. Seram and the regional variants of *A. antarcticus* for *in vitro* myotoxicity. Venoms (10–50 μg/ml) were examined for myotoxicity using the chick directly (0.1 Hz, 2 ms, supramaximal V) stimulated biventer cervicis nerve-muscle preparation. A significant contracture of skeletal muscle and/or inhibition of direct twitches were considered signs of myotoxicity. This was confirmed by histological examination. All venoms displayed high phospholipase A_2 activity. The venoms (10–50 μg/ml) of *A.* sp. Seram, *A. praelongus, A. rugosus,* and *A. wellsi* caused a significant inhibition of direct twitches and an increase in baseline tension compared to the vehicle ($n = 4–6$; two-way ANOVA, $p < 0.05$). Furthermore, these venoms caused dose-dependent morphological changes in skeletal muscle. In contrast, the venoms (10–50 μg/ml; $n = 3–6$) of *A. hawkei*, *A. pyrrhus,* and regional variants of *A. antarcticus* were devoid of myotoxicity. Prior incubation (10 min) of CSL death adder antivenom (5 U/ml) prevented the myotoxicity caused by *A.* sp. Seram, *A. praelongus*, *A. rugosus,* and *A. wellsi* venoms (50 μg/ml; $n = 4–7$). In conclusion, clinicians may need to be mindful of possible myotoxicity following envenomations by *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* species.

***Key Words:* *Acanthophis*; *A. antarcticus*; antivenom; death adder; myotoxic; phospholipase A_2; *A. praelongus*; rhabdomyolysis; *A. rugosus*; venom.**

Death adders (genus *Acanthophis*) are unique among Australian snakes in both morphology and behavior. Although classified into the Elapidae family of snakes they are viper-like in appearance and habit (Campbell, 1966; Cogger, 2000). Death adders are the widest ranging of the Australian elapids being found not only in continental Australia, but north throughout the Torres Straight Islands, Papua New Guinea, Irian Jaya, and the Indonesian islands of Seram, Halmahera, Obi, and Tanimbar. Although up to 12 species and 3 subspecies of death adders have been described thus far (Hoser, 1998), considerable debate remains about species identification (Wuster *et al*., 1999). Of these, only the venom of the common (*A. antarcticus*) death adder has been studied in detail.

Acanthophis antarcticus venom has previously been examined for lethality, neurotoxicity, myotoxicity, and its effects on blood coagulation, both experimentally and clinically (Broad *et al*., 1979; Campbell, 1966; Kellaway, 1929a,b; Mebs and Samejima, 1980; Sutherland *et al*., 1981; Wickramaratna and Hodgson, 2001). In addition, five postsynaptic neurotoxins and four phospholipase A_2 (PLA_2) components have been isolated and sequenced from *A. antarcticus* venom (Chow *et al*., 1998; Kim and Tamiya, 1981a,b; Sheumack *et al*., 1979, 1990; Tyler *et al*., 1997; van der Weyden *et al*., 1997). However, no myotoxic components have been isolated from this venom.

Previously, using the chick isolated biventer cervicis nerve-muscle (CBCNM) preparation, we studied the venoms of the northern (*A. praelongus*), desert (*A. pyrrhus*), Barkly Tableland (*A. hawkei*), black head (*A. wellsi*), Irian Jayan (*A. rugosus*), and *A.* sp. Seram for *in vitro* neurotoxicity (Fry *et al*., 2001). All venoms (1–10 μg/ml) caused dose-dependent neurotoxicity, which was postsynaptic in nature. In the same study, CSL death adder antivenom (1 U/ml), which is raised against *A. antarcticus* venom, prevented the neurotoxic effects of *A. pyrrhus*, *A. praelongus*, and *A. hawkei* venoms. However, it was markedly less effective against the venoms of *A. rugosus*, *A. wellsi,* and *A.* sp. Seram (Fry *et al*., 2001). At a higher concentration, antivenom (5 U/ml) was effective against all venoms. In another study, the venoms of major species and regional variants of death adders were investigated by liquid chromatography/mass spectrometry (Fry *et al*., 2002). This study revealed a great diversity in venom composition.

Based on early studies on *A. antarcticus* venom it was thought that death adder venoms were devoid of myotoxic

[1] To whom correspondence should be addressed. Fax: +61-3-99055851. E-mail: wayne.hodgson@med.monash.edu.au.

activity (Sutherland *et al*., 1981). *A. antarcticus* venom displayed no myotoxic activity *in vivo* in Rhesus monkeys (*Macaca fascicularis*; Sutherland *et al*., 1981). In another study, Mebs and Samejima (1980) fractionated *A. antarcticus* venom by ion-exchange chromatography. None of the isolated fractions caused myoglobinuria in mice after sc injection. However, a clinical study reported myotoxic activity following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus* (Lalloo *et al*., 1996). In this study one patient developed renal failure following delayed presentation and two-thirds of envenomed patients had significantly elevated creatine kinase levels (Lalloo *et al*., 1996). This is suggestive of rhabdomyolysis and the possible presence of myotoxic activity in the venom (Sutherland *et al*., 1981). Recently, we have shown that venom of the Irian Jayan death adder (*A. rugosus*) causes dose-dependent *in vitro* myotoxicity, and subsequently isolated the first myotoxic PLA_2 from a death adder venom (Wickramaratna *et al*., 2003). However, no studies have been performed to determine the effectiveness of CSL death adder antivenom, which has been raised against *A. antarcticus* venom, in neutralizing the myotoxic activity of *A. rugosus* venom.

The first aim of this study was to examine the venoms of *A. praelongus*, *A. pyrrhus*, *A. hawkei*, *A. wellsi*, *A.* sp. Seram, and the regional variants of *A. antarcticus* for *in vitro* myotoxic activity. The second was to determine the effectiveness of CSL death adder antivenom in neutralizing the myotoxic activity of death adder venoms.

MATERIALS AND METHODS

Venom preparation and storage. *A. antarcticus* venoms were obtained from populations in New South Wales (NSW), Queensland (Qld), South Australia (SA), and Western Australia (WA). *A. praelongus* venom was from populations in Cairns, Queensland; *A. pyrrhus* venom from Alice Springs, Northern Territory; *A. wellsi* venom from the Pilbarra region of Western Australia; *A. hawkei* venom from the Barkly Tableland region of Northern Territory; *A. rugosus* venom from Irian Jaya (West Papua), and *A.* sp. Seram from the island of Seram, Indonesia. Venoms were either purchased from Venom Supplies Pty. Ltd., South Australia, or milked from specimens caught by Dr. Bryan Fry. For each geographic variant or species, venoms were collected and pooled to minimize the effects of individual variations (Chippaux *et al*., 1991). Freeze-dried venoms and stock solutions of venoms prepared in 0.1% bovine serum albumin (BSA) in 0.9% saline were stored at –20°C until required.

Determination of phospholipase A_2 activity. The PLA_2 activity of death adder venoms was determined using a secretory PLA_2 colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). The assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine as a substrate. Free thiols generated upon hydrolysis of the thio ester bond at the *sn*-2 position by PLA_2 are detected using DTNB (5,5′-dithiobis(2-nitrobenzoic acid)). Color changes were monitored by the CERES900C microplate reader (Bio-Tek Instruments, Winooski, VT) at 405 nm, sampling every min for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolysed per min per mg of enzyme.

Inactivation of PLA_2 activity with 4-bromophenacyl bromide. The PLA_2 activity of *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms were inhibited by alkylation with 4-bromophenacyl bromide (4-BPB). *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (10,000 μg/ml) were made up in sodium cacodylate-HCl buffer (25 μl, 0.1 M, pH 6.0), and 4-BPB made up in acetone was added to give a final concentration of 1.8 mM (Abe *et al*., 1977; Bell *et al*., 1998; Crachi *et al*., 1999b). Each vial containing the above solution was then incubated at 30°C for 16 h. As a positive control, *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (10,000 μg/ml) made up in sodium cacodylate-HCl buffer were incubated with acetone. As a negative control, sodium cacodylate-HCl buffer was incubated with 1.8 mM 4-BPB in acetone.

Chick isolated biventer cervicis nerve-muscle preparation. Male White leg horn chicks aged between 9 and 11 days were killed with CO_2 and both biventer cervicis nerve-muscle preparations were removed. These were mounted under 1 g resting tension in organ baths (5 ml) containing Krebs 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 Krebs solution was bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 34°C. Direct twitches were evoked by stimulating the muscle directly every 10 s with pulses of 2 ms duration at a supramaximal voltage (Harvey *et al*., 1994) using a Grass S88 stimulator. After a 30-min equilibration period, to ensure selective stimulation of muscle, d-tubocurarine (10 μM) was added and left in the organ bath for the duration of the experiment. Death adder venoms (10–50 μg/ml), 4-BPB modified *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) or relevant controls were left in contact with the preparations for a 3 h period. A significant contracture of skeletal muscle (i.e., a rise in baseline) and/or inhibition of direct twitches were considered signs of myotoxicity (Harvey *et al*., 1994). Where indicated, CSL death adder antivenom (5 U/ml) was added 10 min prior (Barfaraz and Harvey, 1994; Crachi *et al*., 1999a; Fry *et al*., 2001; Wickramaratna and Hodgson, 2001) to the addition of death adder venoms (50 μg/ml).

Morphological studies. After the conclusion of the functional myotoxic experiments, the tissues were quickly placed in Tissue Tek and frozen with liquid nitrogen. The tissues were stored at –80°C until required. Using a Leica CM1800 cryostat, tissues were cut into transverse sections (14 μm) and placed onto gelatin-coated slides. Tissue sections were post fixed for 15 min in a solution containing 4% paraformaldehyde in phosphate buffered saline (PBS; [mol/l] NaCl, 0.137; KH_2PO_4, 0.002; and Na_2HPO_4, 0.008). Tissue sections were routinely stained with haematoxylin and eosin, and examined under a light microscope (Olympus BX 51, Olympus Optical Co., Japan). Areas exhibiting typical pathological changes were photographed using an Olympus C-4040ZOOM (Olympus Optical Co., Japan) digital camera.

Chemicals and drugs. The following drugs and chemicals were used: 4-bromophenacyl bromide (4-BPB), BSA, cacodylic acid (sodium cacodylate), d-tubocurarine chloride, eosin, Mayer's Hematoxylin solution (Sigma Chemical Co., St. Louis, MO). Except where indicated, stock solutions were made up in distilled water. 4-BPB was made up in acetone. Death adder antivenom, which is raised against *A. antarcticus* venom in horses, was obtained from CSL Ltd. (Melbourne, Australia).

Analysis of results and statistics. For isolated tissue experiments, responses were measured via a Grass force displacement transducer (FT03) and recorded on a MacLab System. The twitch height was expressed as a percentage of the initial twitch height (i.e., prior to the addition of venom or vehicle). Full data (i.e., response curves over a 3 h period) are shown for twitch height and baseline tension at 50 μg/ml venoms. However, for brevity full data are not shown at venom concentrations of 10 μg/ml and 30 μg/ml. Instead, data for all venom concentrations are summarized in Figures 2a and 2b using only the twitch height and baseline tension values at the 180-min time point. Statistical difference was determined by a two-way ANOVA on the twitch heights, at the 180-min time point, at different concentrations of venoms. Likewise, a two-way ANOVA was performed on the contractile responses induced by the venoms at different concentrations at the 180-min time point (i.e., only the data at the 180-min time point have been statistically analyzed). For 4-BPB modified venom studies, statistical difference was determined by a two-way ANOVA on the data at the 180-min time point. Statistical difference between the PLA_2 activity of 4-BPB treated and untreated venom was determined by a Student's unpaired *t*-test. All ANOVAs were followed by a

Bonferroni-corrected multiple *t*-test. Statistical significance was indicated when $p < 0.05$. All statistical tests were carried out using the SigmaStat (ver. 1.0) software package.

RESULTS

Phospholipase A_2 Activity

High PLA_2 activity was detected in all death adder venoms (Table 1). While there was a large variation in the PLA_2 activity of death adder venoms, *A. pyrrhus* venom had the highest specific activity, 476.4 ± 12.4 μmol/min/mg ($n = 12$). The positive control, bee venom PLA_2, had a specific activity of 287.5 ± 17.5 μmol/min/mg ($n = 4$). 4-BPB treated *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms had significantly reduced PLA_2 activities of 2.0 ± 0.9, 1.7 ± 1.1, and 1.8 ± 1.0 μmol/min/mg ($n = 8$) compared to their untreated venoms with specific activities of 140.2 ± 10.4, 420.4 ± 10.8, and 255.0 ± 8.6 μmol/min/mg, respectively ($n = 6–8$; Student's unpaired *t*-test, $p < 0.05$).

Chick Isolated Directly-Stimulated Biventer Cervicis Nerve-Muscle Preparation

Myotoxic studies. The venoms (10–50 μg/ml) of *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei*, *A. pyrrhus,* and *A. wellsi* had no significant inhibitory effect on the direct twitches compared to the vehicle ($n = 3–6$; two-way ANOVA, $p < 0.0001$; Figs. 1a and 2a). In contrast, *A.* sp. Seram venom (10–50 μg/ml) caused a significant inhibition of direct twitches compared to the vehicle ($n = 4–6$; Figs. 1a and 2a). However, this effect was not concentration-dependent as there was no significant difference in the twitch inhibition caused by *A.* sp. Seram venom at 10 μg/ml and 50 μg/ml ($n = 4$; Fig. 2a). Both *A. praelongus* venom (30–50 μg/ml) and *A. rugosus* venom (30–50 μg/ml) caused a significant inhibition of direct twitches compared to the vehicle ($n = 4–6$; Figs. 1a and 2a). This effect was concentration-dependent with *A. praelongus* (50 μg/ml) and *A. rugosus* (50 μg/ml) venoms causing a significantly greater inhibition of direct twitches compared to *A. praelongus* and *A. rugosus* venom at 10 μg/ml, respectively ($n = 5–6$; Fig. 2a). When taking all concentrations into consideration *A.* sp. Seram venom was significantly more potent in causing direct twitch inhibition than either *A. praelongus* venom or *A. rugosus* venom ($n = 4–6$). In contrast, there was no significant difference between *A. praelongus* venom and *A. rugosus* venom ($n = 5–6$; Fig. 2a).

The venoms (10–50 μg/ml) of *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei,* and *A. pyrrhus* had no significant effect on the baseline tension compared to the vehicle ($n = 3–6$; two-way ANOVA, $p < 0.0001$; Figs. 1b and 2b). While *A. wellsi* venom (10–30 μg/ml) had no significant effect on the baseline tension, *A. wellsi* venom (50 μg/ml) induced a significant increase in baseline tension compared to the vehicle ($n = 4–6$). The venoms (10–50 μg/ml) of *A.* sp. Seram, *A. praelon-*

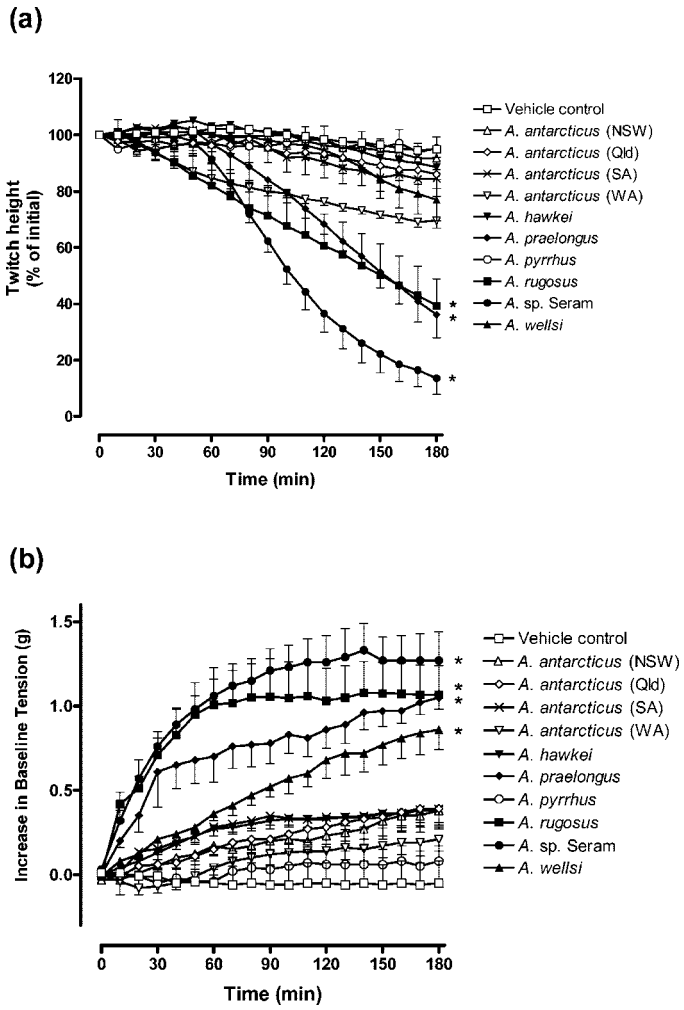

FIG. 1. The effect of *Acanthophis* venoms (10–50 μg/ml; $n = 3–6$) or vehicle ($n = 6$) on (a) direct twitches or (b) baseline tension of the CBCNM preparation at the 180-min time point. *$p < 0.05$, significantly different from vehicle, two-way ANOVA.

TABLE 1
Phospholipase A_2 Activity of Death Adder Venoms

Venoms	PLA_2 activity (μmol/min/mg)[a]
A. antarcticus (NSW)	145.8 ± 8.4
A. antarcticus (Qld)	142.1 ± 1.6
A. antarcticus (SA)	125.0 ± 6.5
A. antarcticus (WA)	344.8 ± 14.0
A. hawkei	318.0 ± 6.3
A. praelongus	255.0 ± 8.6
A. pyrrhus	476.4 ± 12.4
A. rugosus	140.2 ± 10.4[b]
A. sp. Seram	420.4 ± 10.8
A. wellsi	119.8 ± 6.2

[a]Data represent the mean ± SEM ($n = 6–12$).
[b]From Wickramaratna *et al.* (2003).

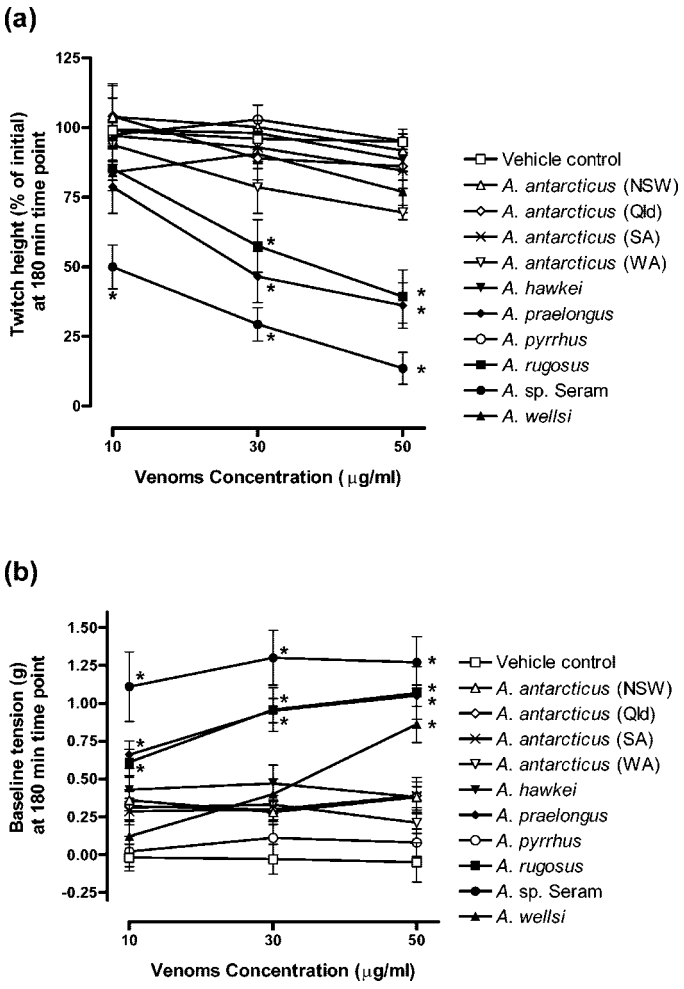

FIG. 2. The effect of *Acanthophis* venoms (50 μg/ml; $n = 3–6$) or vehicle ($n = 6$) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. *$p < 0.05$, significantly different from vehicle, two-way ANOVA.

gus, and *A. rugosus* induced a significant increase in baseline tension compared to the vehicle ($n = 4–6$). However, there was no significant difference in the baseline contraction caused by *A.* sp. Seram venom at 10 μg/ml and 50 μg/ml ($n = 4$; Fig. 2b). This was also the case with *A. praelongus* and *A. rugosus* venoms.

Antivenom studies. Prior incubation (10 min) of CSL death adder antivenom (5 U/ml) prevented the inhibition of direct twitches and the increase in baseline tension caused by *A.* sp. Seram, *A. praelongus*, *A. rugosus,* and *A. wellsi* venoms (50 μg/ml; $n = 4–7$; Figs. 3a,b). *A.* sp. Seram, *A. praelongus*, *A. rugosus,* and *A. wellsi* venoms (50 μg/ml) in the presence of antivenom (5 U/ml) had no significant inhibitory effect on the direct twitches compared to the antivenom control ($n = 4–7$; Fig. 3a; one-way ANOVA, $p = 0.49$). Furthermore, *A.* sp. Seram, *A. praelongus*, *A. rugosus,* and *A. wellsi* venoms (50 μg/ml) in the presence of antivenom (5 U/ml) had no significant effect on the baseline tension compared to the antivenom control ($n = 4–7$; Fig. 3b; one-way ANOVA, $p = 0.40$).

4-BPB modified venom studies. *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) incubated with 4-BPB had no significant inhibitory effect on direct twitches compared to 4-BPB plus vehicle ($n = 4–6$; two-way ANOVA, $p < 0.0001$; Fig. 4a). However, *A. rugosus* *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) incubated with vehicle (ace-

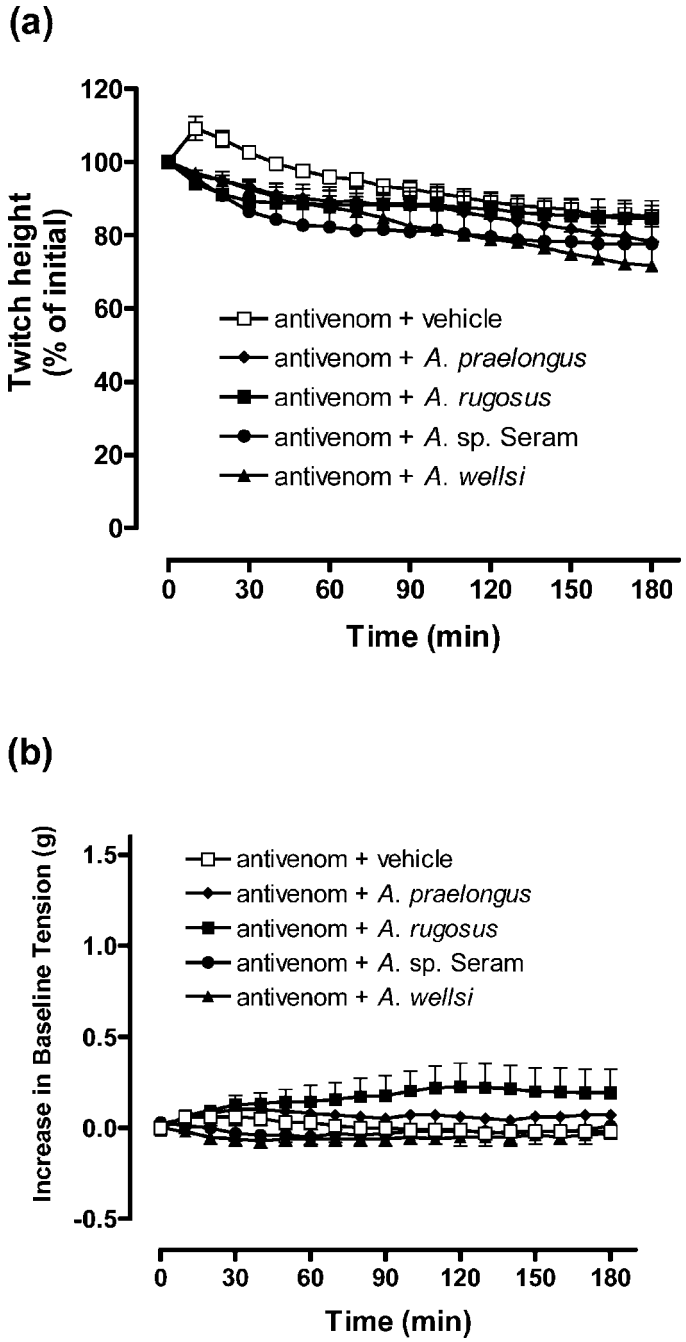

FIG. 3. The effect of *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* venoms (50 μg/ml; $n = 4–7$) or vehicle (BSA; $n = 4$) in the presence of antivenom (5 U/ml) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. *$p < 0.05$, significantly different from antivenom control, one-way ANOVA.

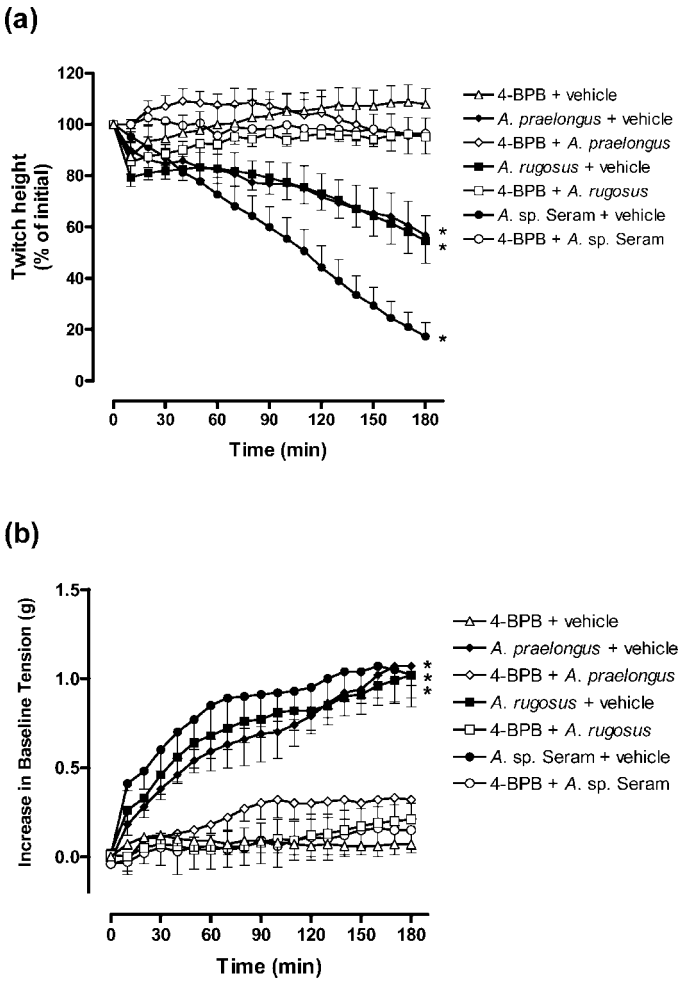

FIG. 4. The effect of *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml; $n = 4-6$) or vehicle (sodium cacodylate; $n = 6$) incubated with 4-BPB (1.8 mM) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. Positive control was *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml; $n = 5$) incubated in vehicle (acetone). $*p < 0.05$, significantly different from 4-BPB plus vehicle, two-way ANOVA.

tone) significantly inhibited direct twitches compared to 4-BPB plus vehicle (sodium cacodylate; $n = 4-6$; Fig. 4a). *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) incubated with 4-BPB had no significant effect on the baseline tension compared to 4-BPB plus vehicle ($n = 4-6$; two-way ANOVA, $p < 0.0001$; Fig. 4b). However, *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) incubated with vehicle induced a significant increase in baseline tension compared to 4-BPB plus vehicle ($n = 4-6$; Fig. 4b).

Morphological studies. Light microscopy studies of tissues exposed to *A.* sp. Seram, *A. praelongus*, *A. rugosus,* and *A. wellsi* venoms (10–50 μg/ml) showed dose-dependent morphological changes in skeletal muscle compared to the vehicle control tissues (Figs. 5a–e; data not shown for other venoms). These changes included the appearance of necrotic cells, vacuoles, edema, and cellular infiltrate. In contrast, tissues exposed to *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei,* and *A. pyrrhus* venoms (10–50 μg/ml) were similar in morphology to the vehicle control tissues (Fig. 5f; data not shown for other venoms). Prior incubation of CSL death adder antivenom (5 U/ml) prevented most of the morphological changes from occurring due to *A.* sp. Seram, *A. praelongus*, *A. rugosus,* and *A. wellsi* venoms. In the case of *A. rugosus* venom (50 μg/ml) a few vacuoles were evident in some tissues even in the presence of antivenom (5 U/ml; Fig. 5g). There were no detectable morphological changes in tissues equilibrated with antivenom alone (data not shown). *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) incubated with vehicle (i.e., acetone) induced morphological changes similar to the corresponding venom (50 μg/ml). However, no detectable morphological changes were seen in tissues exposed to *A. rugosus* (Fig. 5h), *A.* sp. Seram and *A. praelongus* venoms (50 μg/ml) incubated with 4-BPB or vehicle incubated with 4-BPB (data not shown).

DISCUSSION

Based on earlier studies on *A. antarcticus* venom it was thought that death adder venoms were devoid of myotoxic activity (Mebs and Samejima, 1980; Sutherland *et al.,* 1981). However, a recent clinical study reported evidence of rhabdomyolysis in patients following death adder envenomations, in Papua New Guinea, by a species different to *A. antarcticus* (Lalloo *et al.,* 1996). More recently, a myotoxic PLA_2 from *A. rugosus* venom was isolated (Wickramaratna *et al.,* 2003). Consequently, the present study examined the venoms of *A. praelongus*, *A. pyrrhus*, *A. hawkei*, *A. wellsi*, *A.* sp. Seram, and the regional variants of *A. antarcticus* for *in vitro* myotoxic activity. In addition, this study examined the effectiveness of CSL death adder antivenom in neutralizing the myotoxic activity of death adder venoms.

Death adder venoms were examined for *in vitro* myotoxicity using the directly stimulated CBCNM preparation. *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei,* and *A. pyrrhus* venoms did not cause a significant inhibition of the direct twitch height or an increase in the baseline tension. Furthermore, light microscopy studies indicated that tissues treated with these venoms had morphology similar to vehicle control tissues. Thus, these studies have shown that *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei,* and *A. pyrrhus* venoms are devoid of *in vitro* myotoxic activity. While several previous studies have shown that *A. antarcticus* venom is devoid of myotoxic activity none have examined the regional variations of this venom (Mebs and Samejima, 1980; Sutherland *et al.,* 1981; Wickramaratna and Hodgson, 2001). Liquid chromatography–mass spectrometry studies have shown variations in venom composition among the venoms of *A. antarcticus* regional variants (Fry *et al.,* 2001, 2002). Furthermore, functional studies have shown variations in neurotoxicity among the venoms of *A. antarcticus* regional variants (Fry *et al.,* 2001).

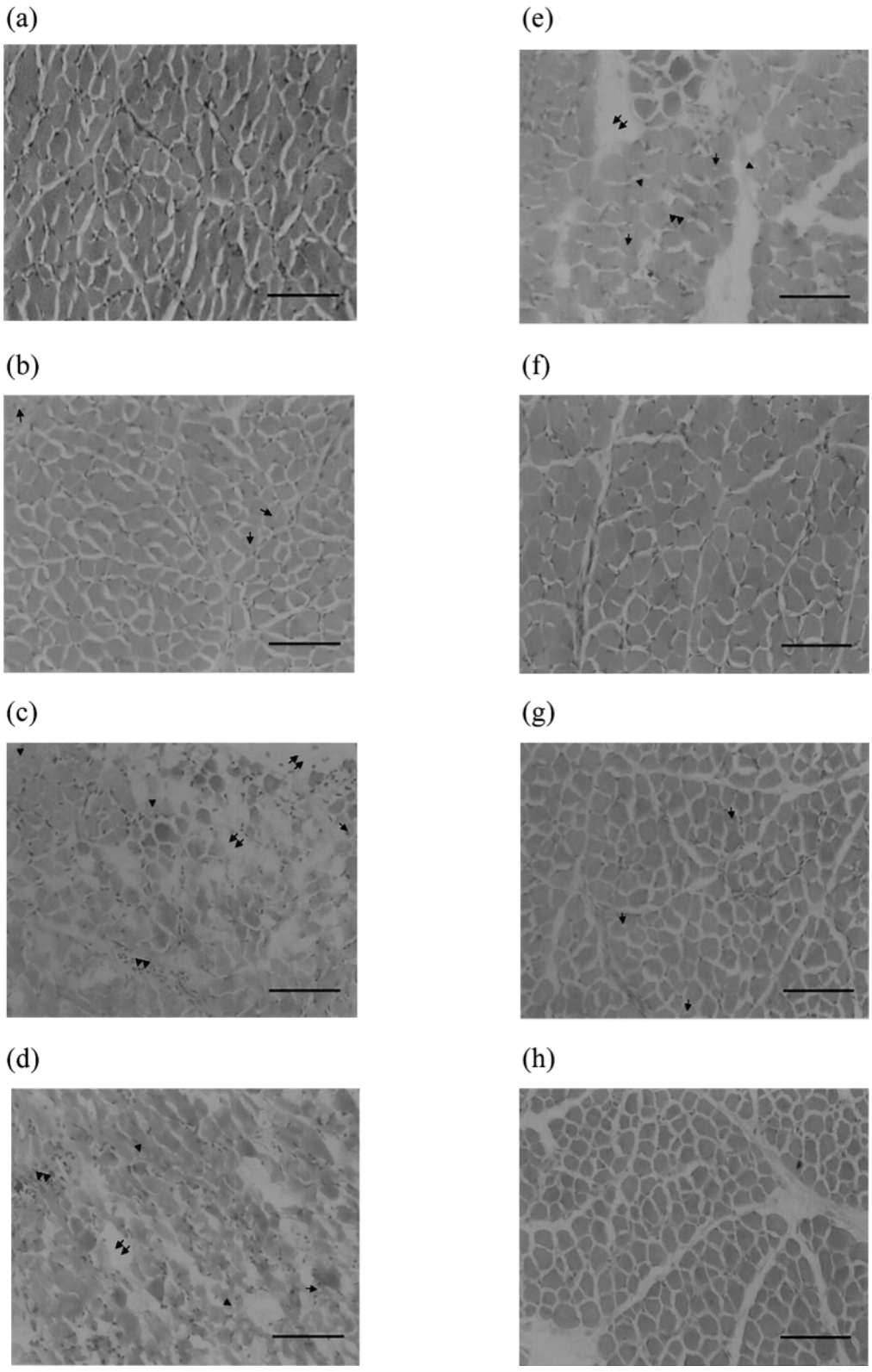

FIG. 5. Transverse sections of CBCNM preparations exposed to (a) vehicle (BSA); (b) *A. rugosus* venom (10 μg/ml); (c) *A. rugosus* venom (50 μg/ml); (d) *A.* sp. Seram venom (50 μg/ml); (e) *A. wellsi* venom (50 μg/ml); (f) *A. antarcticus* venom (WA; 50 μg/ml); (g) *A. rugosus* venom (50 μg/ml) in the presence of antivenom (5 U/ml); (h) *A. rugosus* venom (50 μg/ml) incubated with 4-BPB (1.8 mM). Scale bars, 100 μm in all micrographs. Arrows indicate prominent vacuoles; arrowheads indicate necrotic cells; double arrows indicate edema; double arrowheads indicate cellular infiltrate.

Although *A. wellsi* venom had no effect on the direct twitch height it induced a dose-dependent increase in baseline tension. At the higher concentration, *A. wellsi* venom also caused morphological changes in skeletal muscle. Thus, suggesting that at higher concentrations this venom causes *in vitro* myotoxic activity. Both *A. praelongus* and *A. rugosus* venoms caused concentration-dependent inhibition of direct twitches, and an increase in baseline tension. Inhibition of direct twitches and a rise in baseline tension have been postulated to be indicative of myotoxic activity (Harvey *et al.,* 1994). Light microscopy studies showed that tissues exposed to *A. praelongus* and *A. rugosus* venoms caused dose-dependent morphological changes. Although we have previously shown that *A. rugosus* venom causes *in vitro* myotoxic activity (Wickramaratna *et al.,* 2003), this venom was included in the present study to allow for a comparison between venoms. In contrast to this study, a previous study showed that *A. praelongus* venom at 30 μg/ml did not cause a significant inhibition of direct twitches compared to the vehicle control (Wickramaratna and Hodgson, 2001). However, in that study the venom did cause a significant increase in baseline tension (Wickramaratna and Hodgson, 2001). This previous study neither examined a higher concentration of *A. praelongus* venom nor the morphology of exposed tissues. The use of younger chicks in the previous study may have contributed to this variability between the two studies (Harris, 1991).

At all concentrations tested, *A.* sp. Seram venom caused a significant inhibition of direct twitches and an increase in baseline tension. However, the twitch inhibition and the increase in baseline tension were not dose-dependent. Perhaps, had lower concentrations been tested, a dose-dependent effect may have been observed. Morphological studies however, showed dose-dependent skeletal muscle changes in tissues exposed to *A.* sp. Seram venom. Clearly, of all death adder venoms tested, *A.* sp. Seram venom was the most myotoxic.

While death adder envenomations have been uncommon in Australia in recent times due to habitat destruction and consequent decimation of populations, they are still significant health problem in Papua New Guinea and Irian Jaya (Currie, 2000; Currie *et al*., 1991; Lalloo *et al*., 1995, 1996; Sutherland, 1992). CSL death adder antivenom is the principal therapy for envenomation by any death adder species (AMH, 2003; White, 1998). Since *A. antarcticus* venom lacks myotoxic activity, and given that death adder antivenom has been raised against *A. antarcticus* venom, it was of clinical relevance to examine the efficacy of death adder antivenom against the *in vitro* myotoxicity of *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* venoms. Prior incubation of antivenom totally prevented the inhibition of direct twitches and the increase in baseline tension caused by *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* venoms. In addition, antivenom prevented most of the morphological changes from occurring due to these venoms. Therefore, CSL death adder antivenom is effective in neutralizing the *in vitro* myotoxic activity of death adder venoms. Previously, we have shown that death adder antivenom was effective in neutralizing the *in vitro* myotoxic activity of acanmyotoxin-1 (Wickramaratna *et al*., 2003).

Since the most important clinical symptoms of death adder envenomations are due to postsynaptic neurotoxicity, anticholinesterase therapy has been suggested to supplement death adder antivenom (Currie *et al*., 1988). Indeed, several clinicians have used anticholinesterases successfully to reduce the amount of antivenom administered (Currie *et al*., 1988; Lalloo *et al*., 1996; Little and Pereira, 2000). Anticholinesterase therapy has proven especially useful in Papua New Guinea and Irian Jaya to reduce the high costs associated with the use of death adder antivenom (Currie, 2000). However, given the results of the present study, clinicians may need to be mindful of possible myotoxicity following envenomations from *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* species. With concomitant anticholinesterase therapy the neurotoxicity of death adder envenomations may resolve, however, unchecked myotoxicity could cause myoglobinuria and then renal failure.

Previously it was shown that acanmyotoxin-1, a myotoxic component from *A. rugosus* venom, contained high PLA_2 activity (Wickramaratna *et al*., 2003). Studies have also shown that myotoxic fractions from other Australian elapid venoms contain PLA_2 activity (Harris and MacDonell, 1981; Mebs and Samejima, 1980). Liquid chromatography–mass spectrometry studies have shown that death adder venoms contain numerous components with molecular weights representative of PLA_2s (Fry *et al*., 2002). Therefore, death adder venoms were examined for PLA_2 activity. While high PLA_2 activity was detected in all death adder venoms, *A. pyrrhus* venom had the highest specific activity. In order to examine whether the PLA_2 activity of *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms is necessary for the myotoxic action, these venoms were subjected to 4-BPB modification. Although a myotoxic PLA_2 component has previously been isolated from *A. rugosus* venom this venom was subjected to 4-BPB modification to determine the presence of other components that may cause myotoxicity but are not mediated by PLA_2 activity. Studies have shown that PLA_2 activity can be inhibited by acylation using 4-BPB (Abe *et al*., 1977; Volwerk *et al*., 1974). 4-BPB treated *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms had significantly reduced PLA_2 activity and no myotoxic activity. Thus, suggesting that PLA_2 activity is necessary for the myotoxic activity of these death adder venoms. However, no direct relationship was found between the degree of PLA_2 activity and the myotoxic activity of death adder venoms. For example, while *A. pyrrhus* venom had the highest PLA_2 activity it was devoid of myotoxic activity. This suggests the presence of other non-myotoxic PLA_2 components in those non-myotoxic death adder venoms. In fact, several PLA_2 components with antiplatelet activity have been isolated from *A. antarcticus* and *A. praelongus* venoms (Chow *et al*., 1998; Sim, 1998). Similarly, it is possible that other non-myotoxic

PLA_2 components may also contribute to the PLA_2 activity of myotoxic death adder venoms.

In conclusion, *A*. sp. Seram, *A*. *praelongus*, *A*. *rugosus,* and *A*. *wellsi* venoms caused *in vitro* myotoxicity in the CBCNM preparation. In contrast, *A*. *antarcticus* (NSW, Qld, SA, WA), *A*. *hawkei,* and *A*. *pyrrhus* venoms were devoid of myotoxic activity. Although CSL death adder antivenom has been raised against *A*. *antarcticus* venom it is effective in neutralizing the myotoxic activity of *A*. *praelongus*, *A*. *rugosus*, *A*. sp. Seram, and *A*. *wellsi* venoms. Given the results of this study clinicians need to be mindful of possible myotoxicity following envenomations by *A*. *praelongus*, *A*. *rugosus*, *A*. sp. Seram, and *A*. *wellsi* death adder species.

ACKNOWLEDGMENTS

We thank Dr. Geoff Isbister (Clinical Toxicologist and Emergency Physician, Newcastle Mater Misericordiae Hospital, Australia) for his useful comments. We are grateful to CSL Ltd. for the generous gift of death adder antivenom. This research was funded by a Monash University Small Grant and from financial assistance by the Australia and Pacific Science Foundation. We would also like to thank the relevant departments in Queensland, the Northern Territory, Victoria, and Western Australia for the granting of scientific permits necessary to undertake collection of death adder specimens.

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