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# The *in vitro* and *in vivo* pharmacological activity of *Boiga dendrophila* (mangrove catsnake) venom

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**Summary**

**1** The great taxonomic and prey base diversity of colubrids (non-front-fanged snakes) suggests that their venoms may represent a 'literal gold mine' for scientists eager to find novel pharmacological probes (Mackessy, 2002).

**2** While pharmacological characterization is lacking for most of these venoms, this is even more so with regard to activity of colubrid venoms on the mammalian autonomic nervous system. This study characterizes the activity of venom from the colubrid, *Boiga dendrophila* using *in vitro* smooth muscle preparations and the anaesthetized rat.

**3** In the prostatic segment of the rat vas deferens, cumulative additions of venom (1–150 μg $ml^{-1}$) induced concentration-dependent inhibition of electrically evoked (0.2 Hz, 0.3 ms, 70–100 V) twitches. The inhibitory effect of venom (100 μg $ml^{-1}$) was attenuated by 8-phenyltheophylline (8-PT) (20 μM) and 8-cyclopentyl-1, 3-dipropylxanthine (20 μM) but not idazoxan (1 μM), or a combination of ranitidine (0.2 μM) and thioperamide (10 μM). The inhibitory effect of venom (100 μg $ml^{-1}$) was augmented by dipyridamole (10 μM) but abolished by pretreatment with adenosine deaminase (7.5 units/100 μl) suggesting that it contains components with adenosine $A_1$ receptor activity, most likely adenosine.

**4** In isolated segments of guinea-pig ileum, venom (10–100 μg $ml^{-1}$) caused concentration-dependent contractions which were inhibited by the muscarinic receptor antagonist atropine (0.1 μM) but not by the histamine receptor antagonist mepyramine (0.5 μM).

**5** In the anaesthetized rat, venom (5–7.5 mg $kg^{-1}$, i.v.) caused a hypotensive effect.

**6** Our data suggest that the venom contains components with purinergic and muscarinic receptor activity.

**Keywords:** anaesthetized rat, colubrid, guinea-pig ileum, rat vas deferens, snake, venom

## Introduction

The colubrid assemblage (non-front-fanged snakes) includes a diverse array of families and genera within the Colubroidea superfamily (advanced snakes) (McDowell, 1987; Cadle, 1988; Knight & Mindell, 1994; Heise, Maxson, Dowling & Hedges, 1995; Kraus & Brown, 1998; Vidal, Kindl, Wong & Hedges, 2000; Vidal & Hedges, 2002). Due to a relatively inefficient venom delivery system, compared with the hypodermic-like fangs of the atractaspidid, elapid and viperid families, it has been assumed that most colubrids pose little risk to humans (Kardong & Lavin-Murcio, 1993) and therefore most of their venoms have remained uncharacterized. However, there is a growing interest in colubrid venoms as their extensive evolutionary history and prey base suggests that they may represent a vast source of novel toxins and biological activities (Mackessy, 2002). It is also envisaged that further research will help address unanswered questions regarding the biological role of colubrid venoms.

Most studies which have investigated colubrid venoms have focused upon toxicity determination, biochemical characterization (e.g. protease and phospholipase activities) and activities affecting haemostasis (for a review see Mackessy, 2002). Only two studies (Young, 1992, 1996) examining the action of colubrid venoms upon the autonomic nervous system, rather than the somatic division (Levinson, Evans & Groves,

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1976; Assakura, Salomao, Puorto & Mandelbaum, 1992; Prado-Franceschi *et al.*, 1998; Lumsden, Fry, Kini & Hodgson, 2004), are known to the authors.

In the present study, we investigate the *in vitro* pharmacological activity of venom from the colubrid, *Boiga dendrophila* in the rat vas deferens and guinea-pig ileum, preparations containing receptor types found throughout the autonomic nervous system. We also investigate the effects of intravenously injected venom on the blood pressure and heart rate of the anaesthetized rat to explore possible *in vivo* biological roles of the venom.

## Materials and methods

### *Snakes*

Snakes were collected from Bali and venom extracted as previously described (Fry *et al.*, 2003). Pooled samples from at least six adults were used to minimize the effects of individual variation (Chippaux, Williams & White, 1991).

### *Venom preparation and storage*

Upon collection, venom was filtered with a 0.22-μM membrane filter (Millipore, Bedford, MA, USA) and immediately frozen using liquid nitrogen. Venom was later lyophilized and stored at −20 °C. Stock solutions were prepared in distilled water and stored at −20 °C until required.

### *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 at 32 °C under 0.75 g resting tension. Indirect twitches were evoked in the prostatic segment by electrical stimulation of the motor nerve (70–100 V, 0.3 ms, 0.2 Hz). Venom or agonists were left in contact with the prostatic preparation for 5 min before and after the addition of antagonists/inhibitors which were left in contact with the preparation for 30 min. Data were 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 antagonists/inhibitors. In the epididymal segment, contractile responses to ATP (10 μM) and noradrenaline (25 μM) were obtained before and after the addition of venom which was left in contact with the preparation for 1 h. For treatment with adenosine deaminase, venom (5 mg $ml^{-1}$), adenosine (0.2 mM) or deionized water were incubated for 2 h at room temperature with adenosine deaminase (7.5 units/100 μl sample). Control samples of venom and adenosine were kept incubated at room temperature for 2 h without exposure to adenosine deaminase.

### *Guinea-pig ileum*

Dunkin–Hartley guinea-pigs (1.0–1.4 kg) were killed by 80% $CO_2$ and cervical dislocation. The isolated ileum was dissected into segments (2 cm) which were then mounted on wire tissue holders in organ baths under 1 g resting tension at 34 °C. The activity of venom was measured as a relative percentage of the maximum response to histamine (30 μM). Responses to venom or agonists were measured before and after the addition of antagonists which were incubated with the preparation for 30 min.

### *Experimental conditions*

*In vitro* preparations were mounted in 5 ml isolated organ baths containing physiological salt 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 45 min before addition of drugs. Responses were measured via a Grass force displacement transducer (FTO3) and recorded on a MacLab system (ADInstruments, Castle Hill, NSW, Australia).

### *Anaesthetized rat preparation*

Sprague–Dawley rats (250–350 g) were anaesthetized with pentobarbitone sodium (60–100 mg $kg^{-1}$, i.p.). A midline incision was made in the cervical region, and tracheal and jugular cannulae inserted. The carotid artery was cannulated for the recording of arterial blood pressure which was recorded via a Gould P23 pressure transducer connected to a Powerlab system. The electrocardiogram was recorded via needle electrodes which were connected to an ADInstruments Bioamp (ML 136; ADInstruments). Drugs were administered via the jugular vein and flushed through with heparinized saline (0.2 ml). Pulse pressure was defined as the difference between systolic and diastolic blood pressure. Mean arterial pressure (MAP) was defined as diastolic blood pressure plus one-third of pulse pressure.

### *Drugs*

The following drugs were used: adenosine; adenosine deaminase; acetylcholine (ACh); atropine; 8-phenylltheophylline (8-PT), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX); dipyridimole; histamine; mepyramine; α,β-methyleneadenosine 5-triposphate (α,β-mATP); noradrenaline bitartate; ranitidine (Sigma Chemical Co., St Louis, MO,

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USA); clonidine (Boehringer Ingelheim, Artarmon, NSW, Australia); idazoxan (Reckitt & Coleman, Kingston upon Hull, UK); thioperamide maleate (ICN Pharmaceuticals, Plainview, NY, USA). Except where indicated, stock solutions were prepared in distilled water. Noradrenaline was prepared in catecholamine diluent (0.9% NaCl, 0.0156% $NaH_2PO_4.2H_2O$, 0.004% ascorbic acid, w/v). 8-phenyltheophylline (8-PT) was prepared in 80% MeOH, 20% 1 M NaOH. DPCPX and dipyridamole were prepared in 50% MeOH/50% distilled water and further diluted in 100% distilled water.

### *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.

## Results

### *Rat isolated vas deferens preparation: epididymal segment*

Venom (10–100 μg ml$^{-1}$) alone had no significant effect on the unstimulated epididymal segment of the rat vas deferens ($n = 3$–6; data not shown). In addition, venom (10–100 μg ml$^{-1}$) had no significant effect on contractile responses to α,β-mATP (10 μM) or noradrenaline (25 μM) in the epididymal segments ($n = 3$–4; data not shown).

### *Rat isolated vas deferens: prostatic segment*

Venom (1–150 μg ml$^{-1}$) caused concentration-dependent inhibition of electrically stimulated contractions of the prostatic segment of the rat vas deferens (Fig. 1a,b; $n = 4$). Prior addition of idazoxan (1 μM) prevented the inhibitory activity of clonidine (3 nM) but not venom (100 μg ml$^{-1}$) or histamine (2 μM) (Fig. 2a; $n = 3$–4). Prior addition of a combination of ranitidine (0.2 μM) and thioperamide (10 μM) prevented the inhibitory activity of histamine (2 μM) but not venom (100 μg ml$^{-1}$) or adenosine (2 μM) (Fig. 2b; $n = 4$–5). Prior addition of 8-PT (20 μM) prevented the inhibitory activity of venom (100 μg ml$^{-1}$) or adenosine (2 μM) but not histamine (2 μM) (Fig. 2c; $n = 4$–5). Prior addition of DPCPX (20 μM) prevented the inhibitory activity of venom (100 μg ml$^{-1}$) or adenosine (2 μM) but not histamine (2 μM) (Fig. 2d; $n = 4$–5). Prior addition of dipyridamole (10 μM) augmented the inhibitory activity of venom (100 μg ml$^{-1}$) and adenosine (2 μM) but not histamine (2 μM) (Fig. 2e; $n = 4$–7). Prior treatment of venom (100 μg ml$^{-1}$) and adenosine (2 μM) with adenosine deaminase (7.5 units/100 μl venom) resulted in loss of inhibitory activity but had no significant effect on clonidine (3 nM) (Fig. 2f; $n = 4$–5).

### *Guinea-pig ileum preparation*

Venom (10–100 μg ml$^{-1}$) caused concentration-dependent contractile responses in the guinea-pig ileum (Fig. 3a,b; $n = 3$–9). Subsequent additions (three) of venom (50 μg ml$^{-1}$) with 10-min intervals between each addition did not result in a significant change of the contractile response (Fig. 3c; $n = 4$). Prior addition of atropine (0.1 μM) prevented the contractile activity of ACh (2 μM) and venom (100 μg ml$^{-1}$) but not histamine (2 μM) (Fig. 3d; $n = 3$–4). Prior addition of mepyramine (0.5 μM) prevented the contractile activity of histamine (2 μM) but not venom (100 μg ml$^{-1}$) or ACh (2 μM) (Fig. 3e; $n = 3$–4).

### *Anaesthetised rat preparation*

Venom produced a significant ($P < 0.05$) 17 ± 8 and 30 ± 5 mmHg decrease in MAP (5 and 7.5 mg kg$^{-1}$, respectively, $n = 4$ each; Fig. 4) without significant effect upon heart rate (19 ± 15 and 4 ± 7 bpm, respectively, $n = 4$ each). An equivalent volume of vehicle (i.e. distilled $H_2O$,

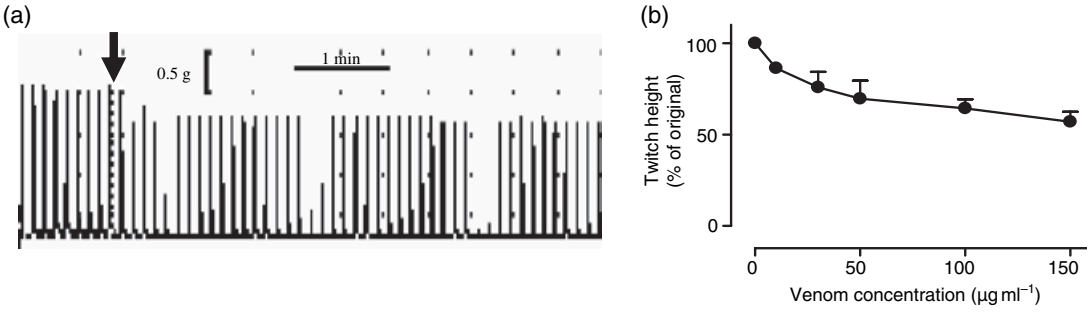


**Figure 1** (a) Trace showing the effect of venom (100 μg ml$^{-1}$) on the prostatic segment of the stimulated (70–100 V, 0.3 ms, 0.2 Hz) rat vas deferens segment. Arrow indicates addition of venom. (b) Concentration–response curve for the inhibitory effect of venom (1–150 μg ml$^{-1}$; $n = 4$) on stimulated rat vas deferens prostatic segment. Error bars are the SEM.

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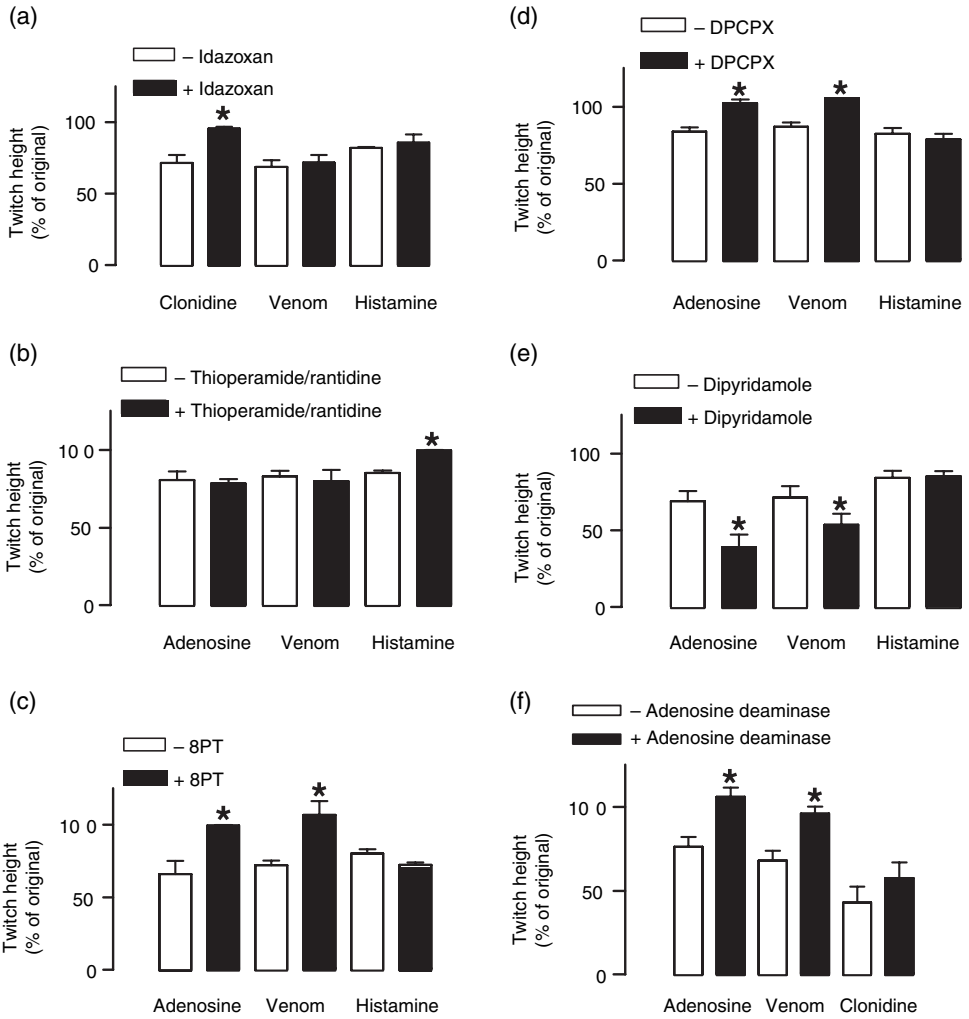


**Figure 2** Responses of the electrically stimulated (70–100 V, 0.3 ms, 02 Hz) rat vas deferens prostatic segment. (a) The effect of idazoxan (1 μM) on venom- (100 μg ml$^{-1}$; $n = 4$), clonidine- (3 nM; $n = 3$) or histamine- (2 μM; $n = 4$) treated tissue. (b) The effect of thioperamide (10 μM) and ranitidine (0.2 μM) on venom- (100 μg ml$^{-1}$; $n = 5$), adenosine- (2 μM; $n = 4$) or histamine- (2 μM; $n = 5$) treated tissue. (c) The effect of 8-PT (20 μM) on venom- (100 μg ml$^{-1}$; $n = 5$), adenosine- (2 μM; $n = 4$) or histamine- (2 μM; $n = 4$) treated tissue. (d) The effect of DPCPX (20 μM) on venom- (100 μg ml$^{-1}$; $n = 5$), adenosine- (2 μM; $n = 4$) or histamine- (2 μM; $n = 4$) treated tissue. (e) The effect of dipyridamole (10 μM) on venom- (100 μg ml$^{-1}$; $n = 7$), adenosine- (2 μM; $n = 5$) or histamine- (3 nM; $n = 4$) treated tissue. (f) The response of venom- (100 μg ml$^{-1}$; $n = 5$), adenosine- (2 μM; $n = 4$) or clonidine- (2 μM; $n = 5$) treated tissue after incubation with adenosine deaminase (7.5 units/100 μl sample). *$P < 0.05$, significantly different to original response by Student's paired $t$-test.

i.v.) did not have any significant effect on blood pressure (3 ± 1 mmHg change in MAP, $n = 4$). Basal MAP of anaesthetized rats before administration of venom (5 and 7.5 mg kg$^{-1}$; 87 ± 8 and 79 ± 4 mmHg respectively; $n = 4$) was not significantly different from the basal MAP of anaesthetized rats before the administration of vehicle (82 ± 9 mmHg; $n = 4$).

## Discussion

This study presents, for the first time, the *in vitro* activity of *B. dendrophila* venom on smooth muscle function. The primary activity of *B. dendrophila* venom in the rat vas deferens is likely to be due to interaction with the presynaptic adenosine $A_1$ receptor site as antagonists of this receptor (i.e. 8-PT and DPCPX) abolished the inhibitory activity of the venom. The lack of effect on α,β-mATP- (adenosine P2X receptor agonist) or noradrenaline- (α-adrenoceptor agonist)induced contractions in the epididymal segment implies lack of activity at the postsynaptic membrane. It is likely that the activity of the venom involves adenosine as the inhibitory effect of the venom was abolished after pretreatment with adenosine deaminase. This is further indicated when the adenosine uptake inhibitor, dipyridamole,

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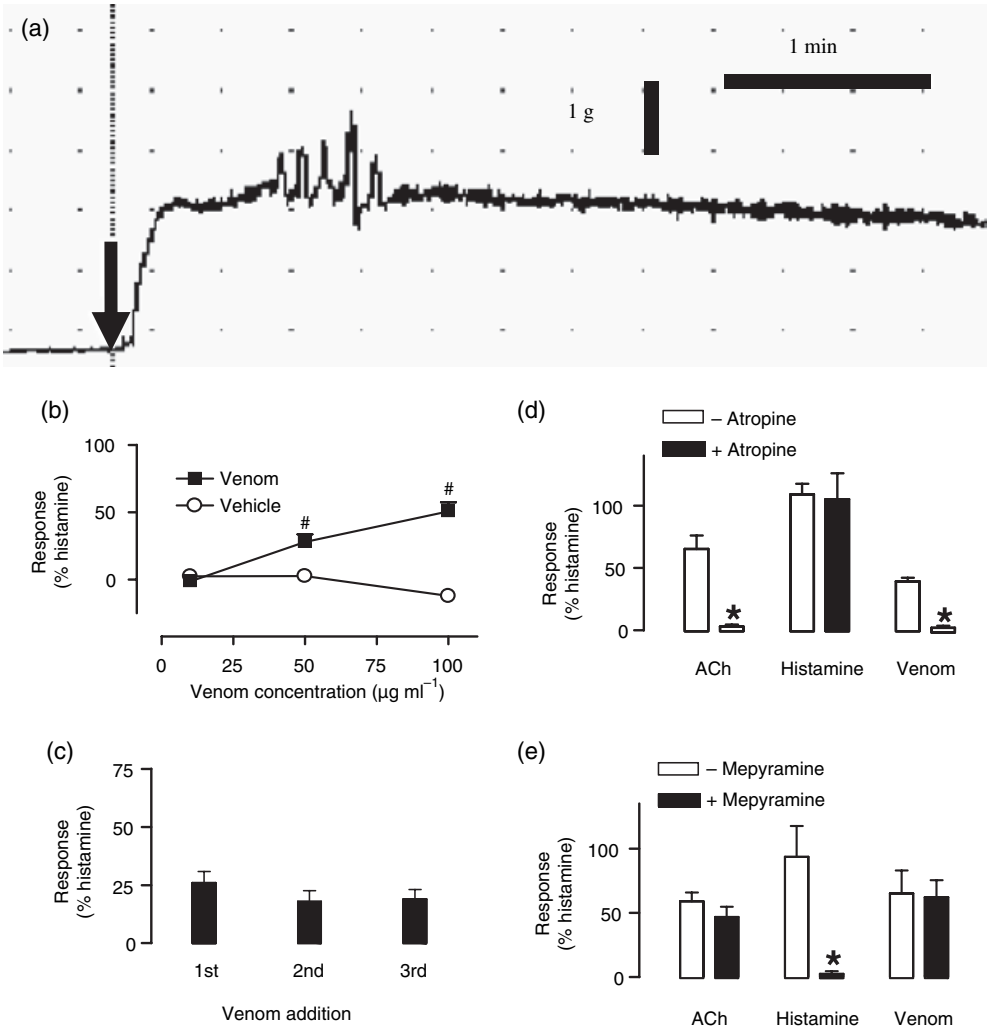


**Figure 3** (a) Trace showing the effect of venom (100 μg ml$^{-1}$) in guinea-pig ileum. Arrow indicates addition of venom. (b) Concentration–response curve for the contractile response to venom (10–100 μg ml$^{-1}$; $n = 3$–9) in guinea-pig ileum. (c) The contractile activity of three additions of venom (each 50 μg ml$^{-1}$; $n = 4$) in the same tissue. (d) The effect of atropine (0.1 μM) on contractile activity of venom (100 μg ml$^{-1}$; $n = 3$), ACh (0.3 μM; $n = 4$) and histamine (2 μM; $n = 4$). (e) The effect of mepyramine (1 μM) on contractile activity of venom (100 μg ml$^{-1}$; $n = 3$), ACh (0.3 μM; $n = 4$) and histamine (2 μM; $n = 4$). #$P < 0.05$, significantly different to vehicle response by one-way ANOVA followed by Bonferroni's *t*-test. *$P < 0.05$, significantly different to original response by Student's paired *t*-test.


**Figure 4** Trace showing the effect of venom (7.5 mg kg$^{-1}$, i.v.) on blood pressure in the anaesthetized rat. Arrow indicates addition of venom.

potentiated the inhibitory activity of the venom (and adenosine). While purinergic activity has previously been indicated for venoms from elapid and viper venoms (for a review see Aird, 2002), this is the first such report for a colubrid venom. Previous studies have shown *B. dendrophila* venom

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to display phosphodiesterase (PDE) activity (Broaders & Ryan, 1997) but this is unlikely to be responsible for the observed inhibitory effects of the venom as PDE activity in smooth muscle is generally associated with increased contractility while inhibitors of PDE demonstrate relaxant effects (Karsten, Derouet, Ziegler & Eckert, 2003; Mehats *et al.*, 2003; Rybalkin, Yan, Bornfeldt & Beavo, 2003; Oger *et al.*, 2004). It is therefore more probable that adenosine is endogenous to the venom.

In the guinea-pig ileum the concentration-dependent contractile activity of *B. dendrophila* venom appears to be mediated by muscarinic receptor activation as this could be blocked by atropine. Phospholipase $A_2$ activity is unlikely to be responsible for the observed effects, as was previously observed for *Oxyuranus microlepidotus* venom in this preparation (Bell, Sutherland & Hodgson, 1998) as repetitive doses of the *B. dendrophila* venom did not result in tachyphylaxis. Previous reports have shown venom from the colubrids, *Dispholidus typus* and *Heterodon platirhinos*, display contractile activity in smooth muscle, although the exact mechanism of action was unclear (Young, 1992, 1996). Future studies would be useful in determining whether *B. dendrophila* venom directly interacts with the muscarinic receptor, e.g. due to the presence of ACh or an analogue or whether it acts indirectly by mediating the release of ACh from the nerve terminal.

The biological role(s) of Duvernoy's gland secretions are still the centre of considerable debate. Suggested roles include immobilization of prey, lubrication for passage of prey, tooth hygiene, and/or neutralization of toxins secreted by the prey (Kardong, 1980, 1996; Jansen, 1983; Weinstein & Kardong, 1994). It is possible that the purinergic and muscarinic receptor activity observed in the present study, along with the *in vitro* neurotoxicity (most likely due to activity at the skeletal muscle ACh receptor), mild coagulopathy, PDE, acetylcholinesterase and protease activities previously reported (Sakai, Honma & Sawai, 1984; Broaders & Ryan, 1997; Hill & Mackessy, 2000; Lumsden *et al.*, 2004), may act synergistically to reduce prey struggle during capture and ingestion. Indeed, both hypotensive and purinergic activities have been suggested to play a role in envenomation strategies such as prey immobilization (Aird, 2002).

In conclusion, this study has provided further evidence of colubrid venoms displaying activity upon components of the autonomic nervous system in addition to the somatic nervous system. The similar protein banding patterns shared between *B. dendrophila* venom and venoms from other congeneric species such as *B. cyanea* (Hill & Mackessy, 2000) suggest the presence of purinergic and muscarinic activity in other *Boiga* venoms. Isolation and characterization of the active components may give further insight into the potential development of novel pharmacological probes.

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