In vitro neuromuscular activity of ‘colubrid’ venoms: clinical and evolutionary implications

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Abstract

In this study, venoms from species in the Colubrinae, Homalopsinae, Natricinae, Pseudoxyrhophiinae and Psammophiinae snake families were assayed for activity in the chick biventer cervicis skeletal nerve muscle preparation. Boiga dendrophila, Boiga cynodon, Boiga dendrophila gemincincta, Boiga drapiezii, Boiga irregularis, Boiga nigriceps and Telescopus dhara venoms (10 µg/ml) displayed postsynaptic neuromuscular activity as evidenced by inhibition of indirect (0.1 Hz, 0.2 ms, supramaximal V) twitches. Neostigmine (5 µM) reversed the inhibition caused by B. cynodon venom (10 µg/ml) while the inhibitory effects of Psammophis mossambicus venom (10 µg/ml) spontaneously reversed, indicating a reversible mode of action for both venoms. Trimorphodon biscutatus (10 µg/ml) displayed irreversible presynaptic neurotoxic activity. Detectable levels of phospholipase A₂ activity were found only in T. biscutatus, T. dhara and P. mossambicus venoms. The results demonstrate a hitherto unsuspected diversity of pharmacological actions in all lineages which may have implications ranging from clinical management of envenomings to venom evolution.

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1. Introduction

The evolution of the venomous function of snakes and the diversification of their toxins has been of tremendous research interest and considerable debate. The advanced snakes (superfamily Colubroidea) make up over 80% of the approximately 2900 species of snake currently described, and contain all the known venomous forms (Greene, 1997; Vidal, 2002). The origin and evolution of venom-secreting glands and venom toxins has been a subject of much speculation. At present, the evidence-based majority view is that venom-secreting glands evolved at the base of the colubroid radiation, with extensive subsequent ‘evolutionary tinkering’ (Vidal, 2002), including the multiple evolution of front-fanged venom delivery systems in the families Viperidae, Elapidae and Atractaspidae (Underwood, 1967; Underwood and Kochva, 1993; Vidal, 2002) and secondary loss in some other lineages. The remaining majority of the Colubroidea lack front fangs, but most lineages possess a venom gland (formerly termed Duvernoy’s gland; see Fry et al., 2003a, Fry and Wüster, 2004), and may or may not have differentiated posterior maxillary teeth to facilitate venom inoculation, including advanced, highly mobile and efficient rear fangs evolving at least once. These snakes have traditionally been lumped into the family Colubridae, but multiple studies of Colubroid phylogeny have shown this family to be paraphyletic, at least with respect to the Atractaspidae and Elapidae (Fig. 1 Underwood, 1967; Slowinski and Lawson, 2002; Vidal, 2002; Vidal and Hedges, 2002; Kelly et al., 2003). However, for

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convenience, we retain the term ‘colubrid’ as an informal designation for the colubroid snakes lacking front-fanged venom delivery systems.

Evidence for the origin of venom-secreting glands at the base of the colubroid radiation comes from comparative morphology and the demonstrated homology of venom-secreting glands of different colubroid lineages (Kochva, 1963, 1965, 1978; Underwood and Kochva, 1993; Underwood, 1967; Jackson, 2003) as well as the distribution of these glands across the full spectrum of colubrid lineages (Vidal, 2002). Other supporting evidence includes the isolation of a potent three finger neurotoxin from the colubrine Coelognathus radiatus (formerly Elaphe radiata) (Fry et al., 2003b), a toxin type previously thought to be unique to Elapidae venoms (Fry et al., 2003c). Extensive liquid chromatography/mass spectrometry analysis of a diverse array of venoms from across the Colubroidea revealed a tremendous diversity of toxins, including the apparent widespread distribution of three finger toxins (Fry et al., 2003a). Molecular phylogenetic analysis has confirmed the homology of toxin types found in elapid and viperid venoms as well as that in various colubrid lineages (Fry and Wüster, 2004).

One of the major remaining unanswered questions is the relative neurotoxicity (i.e., inhibition of neurotransmission at the skeletal neuromuscular junction) of the venoms from the non-front-fanged colubroid lineages. Neurotoxicity has been documented in vitro in a number of species belonging to

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Fig. 1. ‘Best guess’ phylogenetic tree for the species studied and representatives from other lineages. Phylogeny follows principally Vidal and Hedges, 2002; Slowinski and Lawson, 2002, with additional input from Scanlon and Lee, 2004 for Australasian elapids.
the Colubrinae (Levinson et al., 1976; Fontana et al., 1996; Broaders et al., 1999; Fry et al., 2003b) and Xenodontinae (Young, 1992; Prado-Franceschi et al., 1996). Only two colubrid species, *Boiga irregularis* (Colubrinae), and *Malpolon monspessulanus* (Psammophiinae), have been recorded in the literature as causing unequivocal, clinically significant neurotoxicity (González, 1979; Fritts et al., 1990), and there are less clear-cut reports for four others, the colubrines *Boiga blandingii*, *Coluber rhodorachis* and *Coluber viridiflavus* (Bedry et al., 1998; Fry et al., 2003a) and the xenodontine *Hydrodynastes gigas* (Manning et al., 1999). In this study, we examined the relative neurotoxicity of venoms of species from the Colubrinae, Homalopsinae, Natricinae, Pseudoxyrhophiinae and Psammophiinae snake families in order to study the greatest taxonomical breadth of the colubrid snakes. These results provide further information for use in understanding the evolution of venoms in the advanced snakes.

2. Materials and methods

2.1. Snakes

Species and localities for each venom are shown in Table 1. Snakes were milked as previously described by us (Fry et al., 2003a,b). Pooled samples from at least six unrelated adults were used for all species to minimize the effects of individual variation (Chippaux et al., 1991). All venoms underwent a 20-µm filtration to remove any potential mucousal contaminants. In all cases, 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.

### Table 1

<table>
<thead>
<tr>
<th>Species and localities of snake milked for this study</th>
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<tbody>
<tr>
<td><strong>Colubrinae</strong></td>
</tr>
<tr>
<td>Ahaetulla prasina</td>
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<tr>
<td>Boiga cynodon</td>
</tr>
<tr>
<td>Boiga dendrophila dendrophila</td>
</tr>
<tr>
<td>Boiga dendrophila gemmicincta</td>
</tr>
<tr>
<td>Boiga drapiezii</td>
</tr>
<tr>
<td>Boiga irregularis</td>
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<tr>
<td>Boiga nigriceps</td>
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<tr>
<td>Teleuscopus dhara</td>
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<tr>
<td>Trimorphodon biscutatus</td>
</tr>
<tr>
<td><strong>Homalopsinae</strong></td>
</tr>
<tr>
<td>Enhydris chinensis</td>
</tr>
<tr>
<td><strong>Natricinae</strong></td>
</tr>
<tr>
<td>Rhabdophis tigrinus</td>
</tr>
<tr>
<td><strong>Psammophiinae</strong></td>
</tr>
<tr>
<td>Psammophis mossambicus</td>
</tr>
<tr>
<td><strong>Pseudoxyrhophiinae</strong></td>
</tr>
<tr>
<td>Leioheterodon madagascariensis</td>
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solution, the plate shaken and then read every minute for 5 min at 405 nm using a CERES900C micro plate reader. The activity was expressed as micromoles of phosphatidylcholine hydrolysed per min per mg of enzyme.

2.5. Chick biventer cervicis nerve muscle preparation

Chick biventer cervicis nerve muscle preparations were isolated from chicks (4–10 days) killed by CO2 and exsanguination. Preparations were mounted in 5 ml isolated organ baths containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; MgSO4, 1.2; KH2PO4, 1.2; CaCl2, 2.5; NaHCO3, 25 and glucose, 11.1. The Krebs solution was bubbled with carbogen (95% O2 and 5% CO2) and maintained at 34 °C. Indirect twitches were evoked by electrical stimulation of the motor nerve (supramaximal voltage, 0.2 ms, 0.1 Hz; Harvey et al., 1994). d-Tubocurarine (10 μM) was then added, with the subsequent abolition of twitches confirming selective stimulation of the motor nerve. The preparation was then washed thoroughly to re-establish twitches. Contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 1 min) and potassium chloride (KCl; 40 mM, for 30 s) were obtained in the absence of stimulation. Electrical stimulation was then recommenced and the preparation was equilibrated for 30 min before the addition of venom. Venom (3–10 μg/ml) was left in contact with the preparation for a maximum of 3 h or until twitch height was abolished. Contractile responses to ACh, CCh and KCl were then obtained as described above. Reversibility of the effects of the venoms was examined by adding neostigmine (5 μM) after venoms had produced a 50% inhibition of nerve-mediated twitches (i.e. at t50).

2.6. Drugs

The following drugs were used: ACh chloride (Sigma); bovine serum albumin (Sigma); carbamylmethyl chloride (CCh; Sigma); neostigmine (Sigma); d-tubocurarine chloride (Calbiochem). Except where indicated, stock solutions were prepared in distilled water.

2.7. Analysis of results and statistics

All responses were measured via a Grass force displacement transducer (FT03) and recorded on a MacLab system. In the skeletal muscle preparations t50 and, where necessary, t50 values were calculated to compare the neurotoxicity of venoms. Contractile responses to ACh, CCh and KCl were expressed as a percentage of the respective initial response and analysed by a one-way ANOVA. Data was expressed as mean ± SEM.

3. Results

3.1. Protein content and PLA2 activity

All venoms demonstrated moderate to high levels of protein (Table 2). Detectable levels of PLA2 activity were only found in T. biscutatus, T. dhara and P. mossambicus venoms (Table 2).

3.2. Chick isolated biventer cervicis nerve muscle preparation

Venoms (10 μg/ml) of Boiga cynodon, Boiga dendrophila, Boiga dendrophila gemincincta, Boiga drapiezii, B. irregularis, Boiga nigriceps and Telescopus dhara venoms caused time-dependant inhibition of indirect twitches of the chick isolated biventer cervicis nerve muscle preparation (Fig. 2a). All of these venoms (10 μg/ml) significantly inhibited contractile responses to the exogenous nicotinic agonists (i.e. ACh and CCh) but not KCl (Fig. 2b). T. biscutatus (10 μg/ml) also caused time-dependant inhibition of indirect twitches in this preparation (Fig. 3a), but had no effect on the contractile responses (Fig. 3b). P. mossambicus (10 μg/ml) demonstrated time-dependant inhibition of indirect twitches of the chick isolated (Fig. 4a) which spontaneously reversed after approximately 30 min incubation, consequently, little effect was demonstrated on the contractile responses (Fig. 4b). Venoms (10 μg/ml) of A. prasina, E. chinensis or L. madagascarenisis lacked inhibitory effects on indirect twitches (Fig. 5a) or contractile responses (Fig. 5b). The addition of neostigmine (5 μM) to tissues treated with B. dendrophila, B. d. gemincincta, B. drapiezii, B. irregularis, T. dhara (3 μg/ml; Fig. 6a,c–f , respectively) or T. biscutatus (10 μg/ml; Fig. 6b) venoms was unable to restore twitch height. In tissues treated with B. cynodon venom (10 μg/ml; Fig. 6g) or dTC (8 μM; Fig. 6h),

<table>
<thead>
<tr>
<th>Venom</th>
<th>Protein content (μg/ml)</th>
<th>PLA2 activity (μmol/min/mg)</th>
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<tbody>
<tr>
<td>A. prasina</td>
<td>813.9 ± 29.4</td>
<td>nd</td>
</tr>
<tr>
<td>B. cynodon</td>
<td>977.6 ± 49.8</td>
<td>nd</td>
</tr>
<tr>
<td>B. dendrophila</td>
<td>854.2 ± 485.2</td>
<td>nd</td>
</tr>
<tr>
<td>B. d. gemincincta</td>
<td>857.1 ± 87.9</td>
<td>nd</td>
</tr>
<tr>
<td>B. drapiezii</td>
<td>911.1 ± 18.7</td>
<td>nd</td>
</tr>
<tr>
<td>B. irregularis</td>
<td>913.6 ± 20.8</td>
<td>nd</td>
</tr>
<tr>
<td>B. nigriceps</td>
<td>668.8 ± 20.2</td>
<td>nd</td>
</tr>
<tr>
<td>E. chinensis</td>
<td>805.4 ± 32.6</td>
<td>nd</td>
</tr>
<tr>
<td>L. madagascarenisis</td>
<td>334.9 ± 10.4</td>
<td>nd</td>
</tr>
<tr>
<td>P. mossambicus</td>
<td>459.2 ± 20.4</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>T. biscutatus</td>
<td>847.0 ± 79.8</td>
<td>10.2 ± 0.4</td>
</tr>
<tr>
<td>T. dhara</td>
<td>814.3 ± 31.7</td>
<td>26.9 ± 0.7</td>
</tr>
</tbody>
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nd, Not detected.
neostigmine (5 μM) restored twitch height to approximately 80% of the initial height.

4. Discussion

4.1. Phylogenetic–bioactivity relationships

A conspicuous finding of this study was the phylogenetic patterns of postsynaptic neurotoxic action of the venoms. The colubrine venoms were the most potently neurotoxic, the psammophiine and pseudoxyrhophiine venoms so less and the homalopsine and natricine venoms virtually devoid of neurotoxicity. These results are consistent with the relative abundance of molecular weights consistent with three finger toxins in the different venoms (Fry et al., 2003a).

Comparison of the $t_{90}$ values obtained in this study (Table 3) indicate that B. dendrophila, B. d. gemincincta, B. irregularis, B. nigriceps and T. dhara venoms demonstrated neurotoxic activity which was just as potent as that demonstrated by elapid venoms such as Oxyuranus microlepidotus ($t_{90} = 29 \pm 3$; Crachi et al., 1999), Notechis scutatus ($t_{90} = 21.7 \pm 1.6$; Hodgson et al., 2003) and Austrelaps superbus ($t_{90} = 25.6 \pm 2.5$; Hodgson et al., 2003). This is most likely a reflection of the presence of α-neurotoxins with similar potency. However, it should be noted that $t_{90}$ values are highly influenced by the presence of rapidly acting α-neurotoxins rather than the slower acting potent β-neurotoxins present in many highly venomous elapids. In contrast, a different mode of activity was implied for P. mossambicus venom since after approximately 30 min exposure to the venom, the inhibition of indirect twitches spontaneously reversed. Since, only B. cynodon was reversed by neostigmine, a unique reversible mode of action is indicated here compared to the other venoms screened in this study.

The relative PLA2 activity was also variable (Table 2). However, this is not necessarily indicative of the relative
abundance of this toxin class in the venoms (Fry et al., 2003a) and numerous studies of elapid PLA2 toxins have shown that the non-toxic esterase activity is distinct from the toxic activity (e.g. Mollier et al., 1989; Yang and Chang, 1991). Thus, detectable levels of PLA2 activity merely confirms the presence of PLA2 toxins that retain the esterase activity but the absence of PLA2 esterase activity is not evidence of the absence of PLA2 toxic activity. Nevertheless, our studies clearly demonstrate the presence of PLA2 toxins in colubrine and psammophine venoms.

The *T. biscutatus* venom was intriguing in having neurotoxic activity at the skeletal neuromuscular junction as shown by time-dependant inhibition of indirect twitches of the chick isolated biventer cervicis nerve muscle preparation. The effect of *P. mossambicus* venom (10 μg/ml; *n* = 4) on responses of chick isolated biventer cervicis muscle to ACh (1 mM), CCh (20 μM) or KCl (40 mM).

4.2. Clinical and evolutionary implications

In this study, five of the venoms studied displayed neurotoxic potency similar to venoms collected from elapids known to cause serious envenomation, and thus may be capable of significant human envenomation. In particular, the potent *T. dhara* venom combined with the very high venom yield (Fry et al., 2003a) indicates that this species (and perhaps the entire genus) may be capable of clinically significant envenomation. By way of comparison, while the *Psammophis mossambicus* venom was relatively weakly neurotoxic, the highly complex nature of the venom (Fry et al., 2003a) indicates that it may possess other, more potent activities. This, combined with the high venom yield (Fry et al., 2003a), means that this genus may also be capable of clinically significant envenomation. Similarly, the lack of neurotoxic activity demonstrated by *A. prasina*, *E. chinensis* or *L. madagascarensis* venoms does not preclude the venoms from having potent effects elsewhere. Reports have
shown that envenomation by *E. chinensis* can result in systemic haemorrhage (Sakai et al., 1983; Toriba and Sawai, 1990), therefore, further study of these other two venoms upon the haemostatic system may be revealing.

Although, reports have shown the yield and delivery of venom in colubrids is relatively poor compared to that demonstrated by highly dangerous elapids and vipers, if given enough contact time the potential may be there to
cause significant envenomation. Given the taxonomical distances separating the lineages (Fig. 1), it is unlikely that any of the commercially available elapid antivenoms will be clinically useful against any neurotoxicity produced by colubrine venoms. Previous work has shown that there is cross-reactivity between some boigine venoms and elapid antiserum (e.g. Weinstein and Smith, 1993). While an antivenom is produced against the colubrine Dispholidus typus, 3FTx are relatively scarce in this venom in comparison to the large quantity of the prothrombin-activating enzyme (Fry et al., 2003a). Thus, this antivenom would be expected to be poorly effective against the neurotoxicity produced by other colubrines and even less so against more divergent lineages. As only B. cynodon venom neurotoxicity was reversed by neostigmine, anticholinesterase therapy is likely to have limited clinical effectiveness.

Some colubrids have a long history of interaction with humans due to their popularity in herpetoculture, and one can thus be reasonably confident that the full envelope of possible reactions to bites is known. For instance, Thamnophis spp. (garter snakes) have been kept safely by many herpetologists, with a very low incidence of mild local symptoms (Vest, 1981; Hayes and Hayes, 1985; Nichols, 1986), so that the possibility of life-threatening garter snake bites can be discounted. However, as different genera that were previously rarely kept become available, it is possible that some of these may eventually turn out to be more dangerous than previously suspected. This has already happened: Rhabdophis species were very popular in the pet trade in the 1970s and 1980s, until they caused several life-threatening envenomings (Mather et al., 1978; Cable et al., 1984). The potent neurotoxicity of the T. dhara venom revealed in this study may be cause for concern, particularly when coupled to the large venom yield of this species. C. rhodorachis similarly has become recently available and a neurotoxic envenomation resulting in paralysis has already been reported (Fry et al., 2003a). Therefore, studies such as the present one are useful in providing information in regards to potential clinical effects.

The potent neurotoxicity of the boigine venoms confirms previous reports of ACh receptor binding activity for some species in this genus (Broaders et al., 1999; Weinstein and Smith, 1993) but is in contrast to the relatively high LD₅₀ values previously reported (Vest et al., 1991; Weinstein et al., 1991). However, the LD₅₀ calculations were based on a murine model while the current study examined the neurotoxic effects upon avian tissue, a model much more reflective of the natural prey items for these species. The neurotoxic mode of action of the colubrine venom is most consistent with the role of killing or incapacitating prey, rather than digestion or other functions (Fry et al., 2003a,b). This is in agreement with previous work regarding the importance of the evolution of a venomous function as a foraging adaptation early in the diversification of the Colubroidea, followed by secondary loss in some lineages (Greene, 1997; Vidal, 2002).

In conclusion, this study demonstrates the potent bioactive effects that different Colubroidea snake venoms may have. These results not only have clinical and evolutionary implications, but they also underscore the largely untapped nature of colubrid venoms and their potential for providing novel ligands or leads for drug design and development. These venoms provide a rich opportunity for research in these areas.

Acknowledgements

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