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Isolation of a Neurotoxin (α-colubritoxin) from a Nonvenomous Colubrid: Evidence for Early Origin of Venom in Snakes

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Abstract. The evolution of venom in advanced snakes has been a focus of long-standing interest. Here we provide the first complete amino acid sequence of a colubrid toxin, which we have called α-colubritoxin, isolated from the Asian ratsnake *Coelognathus radiatus* (formerly known as *Elaphe radiata*), an archetypal nonvenomous snake as sold in pet stores. This potent postsynaptic neurotoxin displays readily reversible, competitive antagonism at the nicotinic receptor. The toxin is homologous with, and phylogenetically rooted within, the three-finger toxins, previously thought unique to elapids, suggesting that this toxin family was recruited into the chemical arsenal of advanced snakes early in their evolutionary history. LC-MS analysis of venoms from most other advanced snake lineages revealed the widespread presence of components of the same molecular weight class, suggesting the ubiquity of three-finger toxins across advanced snakes, with the exclusion of Viperidae. These results support the role of venom as a key evolutionary innovation in the early diversification of advanced snakes and provide evidence that forces a fundamental rethink of the very concept of nonvenomous snake.

Key words: Neurotoxin — Venom — Evolution — Multi-gene — Three finger — Snake

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Introduction

The advanced snakes (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 evolution of the venomous function and its role on the diversification of this clade has been a central focus of interest in these animals. Only approximately 20% of colubroids (families Elapidae, Viperidae, and Atractaspididae) have front-fanged venom delivery systems and are dangerous to humans. However, at least some species of most other families/subfamilies have toxin-secreting, serous Duvernoy's glands, often in association with differentiated posterior maxillary teeth (Hill and Mackessy 2000; Mackessy 2002; Vidal 2002). Although the phylogenetic relationships among the major colubroid groups remain inadequately resolved (Kraus and Brown 1998; Slowinski and Lawson 2002; Vidal and Hedges 2002) (Fig. 1), the presence of front-fanged venom delivery systems in several unrelated lineages and the wide distribution of Duvernoy's glands and differentiated maxillary dentitions across colubroid lineages suggest that toxic secretions are a synapomorphy of the Colubroidea, subsequently lost in some lineages (Underwood and Kochva 1993; Vidal 2002).

Whereas the venoms of front-fanged snakes have been studied extensively, those of the paraphyletic Colubridae, which make up the bulk of the advanced

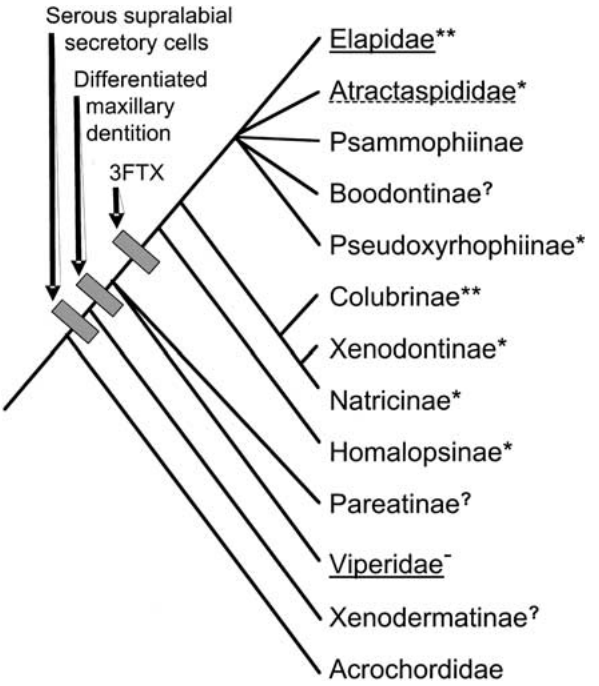

Fig. 1. Phylogeny of the Colubroidea (modified from Vidal and Hedges 2002, taking into account the data of Slowinski and Lawson 2002). Underlined taxa consist entirely of front-fanged forms, taxa with stippled underlining include some front-fanged forms. **3FTXs demonstrated by sequencing. *3FTXs shown by LC-MS. *Not studied; presence of 3FTXs likely in Boodontinae, unlikely in Pareatinae and Xenodermatinae. The relative rooting of the Viperinae and Pareatinae is presently not robustly resolved.

snakes, have been largely neglected. The few published studies focused upon the medically important species such as the *Dispholidus typus* (Kamiguti et al. 2000) but have not examined the evolutionary development of venom or the relationship between colubrid venoms and elapid venoms. This applies especially to the neurotoxic activities of colubrid venoms, almost entirely ignored until recently (Broaders et al. 1999). Here we describe our studies on a polypeptide toxin isolated from the Duvernoy's secretion of *Coelognathus radiatus*, an Asiatic member of the family Colubrinae and an archetypal nonvenomous snake. This snake was until recently known as *Elaphe radiata* (Helfenberger 2001).

Materials and Methods

Snakes and Milking

We obtained adult *Coelognathus radiatus* from Bali, Indonesia, as well as adults from a number of other colubrids representing some of the major Colubroid radiations, including the Colubrinae (*Ahaetulla prasina*, Bali; *Boiga drapiezii*, Bali), Homalopsinae (*Cerberus rynchops*, Singapore), Pseudoxyrhophiinae (*Leioheterodon madagascarensis*, Madagascar), Psammophiinae (*Psammophis mossambica*, Tanzania), and Xenodontinae (*Heterodon nasicus*, Texas, USA). We also obtained samples of *Python reticulatus* saliva (Singapore) as a negative control, *Ophiophagus hannah* venom (Singapore) as a positive control, and *Atheris squamigera* venom (Uganda) for comparison purposes. Colubrid and python specimens were milked using a variation of recently developed colubrid-specific method (Hill and Mackessy 1997). Rather than ketamine, snakes were anesthetized with Zoletile 100 (Tiletamine 50 mg and Zolazepam 50 mg) at a starting dose of 3 mg/kg. Rather than intramuscular injections on the body, pilocarpine was injected subcutaneously periglandular to the duvernoy's gland. As an additional step, atropine was injected subsequent to milkings at a concentration of 0.04 to 0.08 mg/kg in order to counteract the excessive salivation produced by the pilocarpine. Pooled samples from at least six unrelated adults were used for all species to minimize the effects of individual variation (Chippaux et al. 1991). 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.

Liquid Chromatography–Mass Spectrometry, Protein Isolation and Characterization

On-line liquid chromatography–mass spectronomy (LC-MS) of venom samples dissolved in 0.1% TFA (Trifluroacetic acid) to a concentration of ~3 mg/mL was performed on a Phenomenex Jupiter C_{18} column (1 × 150 mm, 5 μ particle size, 300 Å) with Solvent A (0.05% TFA) and Solvent B (90% acetonitrile in 0.045% TFA) at a flow rate of 50 μL/min. The solvent delivery and gradient formation of a 1% gradient from 0 to 60% acetonitrile/0.05% TFA over 60 min was achieved using a Shimadzu LC 10AD solvent delivery system. Electrospray mass spectra are acquired on a PE-SCIEX API 300 system with a turbo ionspray atmospheric pressure ionization source. Samples (25 μL) were injected manually into the LC-MS system and analysed in positive ion mode. Full scan data was acquired with the electrospray sprayer voltage at 4600 V, orifice 45 V, and ring 350 V over the ion range 600–3000 m/z with a step size of 0.2 amu. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Canada). Preparative, reverse-phase, high-performance liquid chromatography and Edman degradation sequencing of a prominent component of the secretions of *Coelognathus radiatus* were as previously described by us (Nirthanan et al. 2002). Removal of pyroglutamic acid was accomplished using pyroglutumase (Takara Biomedicals, catalog number 7334) following the standard protocol.

Phylogenetic Analysis

The sequenced toxin from *C. radiatus* displayed suspected sequence homology with elapid three-finger toxins (3FTX). We therefore aligned the sequence of this *C. radiatus* toxin with a selection of elapid 3FTX sequences chosen to reflect the major clades of elapid toxins (Fry et al. 2003). The alignment was executed using the program CLUSTAL-X (Thompson et al. 1997), followed by visual inspection for errors. The endogenous vertebrate neuropeptide Lynxl (Miwa et al. 1999) was selected as an outgroup to root the trees. The final alignment consisted of 110 amino acid sites, 63 being parsimony-informative. A copy can be obtained by emailing Dr. B. G. Fry.

Phylogenetic trees were reconstructed using Bayesian inference, implemented on MrBayes, version 3.0b4 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The method uses Markov-chain Monte Carlo methods to generate posterior probabilities for each clade represented in the tree. The analysis was performed by running 2×10^6 generations in four chains, and saving every hundredth tree. The log-likelihood score of each saved tree was plotted against the number of generations to establish the point at which the log likelihood scores of the analysis reached their

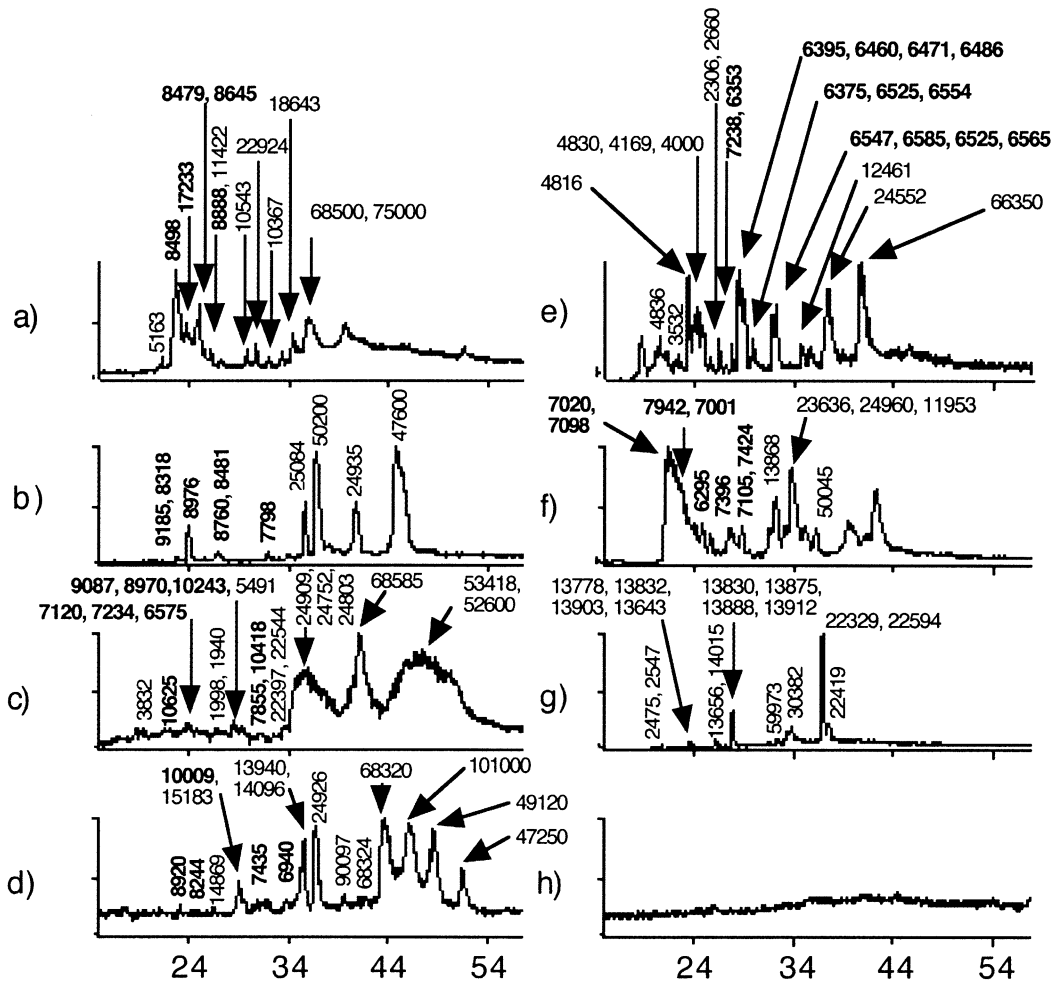

Fig. 2. LC-MS profiles of representatives from major colubroid radiations (**a**) *Coelognathus radiatus* (Colubrinae), (**b**) *Cerberus rhynchops* (Homalopsinae), (**c**) *Heterodon nasicus* (Xendontinae), (**d**) *Leioheterodon madagascariensis* (Pseudoxyrhophiinae), (**e**) *Psammophis mossambica* (Psammophiinae), (**f**) *Ophiophagus hannah* (Elapidae), (**g**) *Atheris squamiger* (Viperidae) venoms, and (**h**) *Python reticulatus* (Pythonidae) saliva. Reconstructed masses are given above each peak. X-axis is the percentage of acetonitrile at the time of elution and the y-axis is the relative intensity (0–100%).

asymptote, and the posterior probabilities for clades established by constructing a majority rule consensus tree for all trees generated after the completion of the burn-in phase.

Pharmacological Analysis

Chick biventer cervicis nerve muscle preparations were isolated from chicks (4–10 days old) killed by CO_2 and exsanguination. Preparations were mounted under 1 g resting tension in 5 mL organ baths containing Krebs solution (NaCl, 118.4 m*M*; KCl, 4.7 m*M*; $MgSO_4$, 1.2 m*M* KH_2PO_4, 1.2 m*M*; $CaCl_2$, 2.5 m*M*; $NaHCO_3$, 25 m*M* and glucose, 11.1 m*M*), maintained at 34°C, and bubbled with 95% O_2 and 5% CO_2.

Indirect twitches were evoked by electrical stimulation of the motor nerve (supramaximal voltage, 0.2 ms, 0.1 Hz; Harvey et al. 1994) using a Grass S88 stimulator. 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 reestablish twitches. Contractile responses to acetylcholine (ACh; 1 m*M* for 30 s), carbachol (CCh; 20 μM for 1 min), and potassium chloride (KCl; 40 m*M*, 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 or toxin. Venom (3–10 μg/mL) or toxin (1 μM) 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 previously described. In some experiments, reversibility of the toxin was determined by washing the preparation at 10 min intervals for 90 min.

A cumulative concentration response curve to carbachol (0.6–80 μM) was obtained in the chick unstimulated biventer cervicis nerve-muscle preparation. After completion of the curve the tissue was thoroughly washed over 30 min, the toxin (0.1–1 μM) added and allowed to equilibrate for 1 h. The cumulative concentration response curve to carbachol was then obtained in the presence of toxin.

The following drugs were used: bovine serum albumin (Sigma, St. Louis, Missouri); d-tubocurarine chloride (Sigma); acetylcholine chloride (Sigma); carbamylcholine chloride (carbachol; Sigma). Stock solutions were prepared in distilled water.

Contractile responses were measured on a MacLab system (PowerLab/400) via a Grass force displacement transducer (FT03). Twitch height was expressed as a percentage of the twitch height before the addition of venom or toxin. Contractile responses to exogenous ACh, CCh, and KCl were expressed as a percentage of the respective initial response and analyzed using one way ANOVA followed by a Bonferroni post hoc test. Statistical significance was indicated when $P < 0.05$. Responses to cumulative additions of CCh in the presence of toxin were expressed as a percentage of the

maximum response obtained in the absence of toxin. Schild plot analysis was used to calculate the pA_2.

Results

LC-MS, Protein Isolation, and Characterization

LC-MS analysis of *Coelognathus radiatus* revealed that the most abundant component in the Duvernoy's gland secretion was a peptide of 8498 Daltons (Fig. 2), which was the protein selected for further detailed analyses. Isolation and sequence characterization of the toxin from *C. radiatus* included the removal of an N-terminal pyroglutamic acid through the use of pyroglutamate aminopeptidase. It was subsequently revealed that this peptide has a high degree of homology to the elapid three-finger toxins (Fig. 3).

Numerous other peptides in this molecular weight were also present in the secretion of *C. radiatus*. Our LC-MS of the variety of other colubrid venoms in this study revealed the ubiquity of components in the 6–10 kDa range (representatives of major families/subfamilies shown in Fig. 2). These components have retention times similar to the 3FTX of *C. radiatus*. Further, we have partially sequenced purified toxins from *Boiga dendrophila* and confirmed them also to be 3FTXs.

LG-MS analysis of the negative control, the basal snake *Python reticulatus*, did not reveal any components of this molecular weight, whereas LC-MS analysis of the positive control, venom from the elapid *Ophiophagus hannah*, showed that the colubrid 3FTX retention times and mass patterns were highly similar to those of elapids. The LC-MS of the African bush viper *Atheris squamigera* demonstrated that no components with molecular weights in the 6–10 kDa molecular weight range are present in viperine venoms.

Phylogenetic Analysis

In the Bayesian analysis, the log-likelihood scores of trees asymptote after approximately 8000 generations. We discarded the first 100 retained trees (representing 10,000 generations), and constructed a majority-rule consensus tree from the remaining trees (Fig. 3B). The results show that the elapid three-finger toxins are paraphyletic with respect to the peptide isolated from *Coelognathus radiatus*. (Fig. 3B). The rooting of the *C. radiatus* toxin within the Elapid three-finger toxins is supported through two strongly supported nodes.

Pharmacological Analysis

C. radiatus venom (3–10 μg/mL) and α-colubritoxin (1 μM) caused time-dependent inhibition of indirect

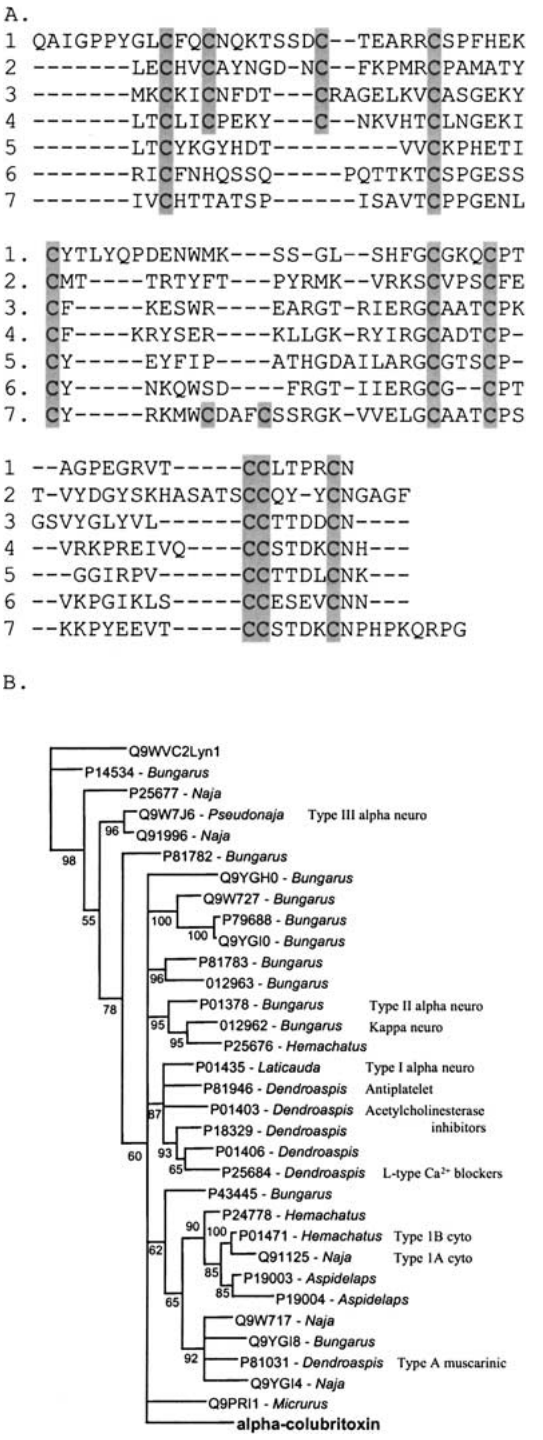

Fig. 3. (**A**) Alignment of (**1**) α-colubritoxin with (**2**) the human brain neuropeptide Lynx-1 (Q9WVC2) and the following postsynaptic nicotinic acetylcholine receptor antagonistic snake venom toxins: (**3**) *Bungarus* reversible neurotoxin (P81783); (**4**) *Naja* weak neurotoxin (P29180); (**5**) *Pseudonaja* Type I neurotoxin (Q9W7J9); (**6**) erabutoxin (*Laticauda* Type I neurotoxin P01435); (**7**) α-bungarotoxin (*Bungarus* Type II neurotoxin, P01378). Swiss-Prot accession numbers are in parenthesis after each component. Cysteines are highlighted in grey. The N-terminal Q is pyroglutamic acid. (**B**) Bayesian tree of aligned sequences of 31 elapid three-finger toxins, α-colubritoxin and the Lynx-1 neuropeptide. Numbers on branches indiate percentage posterior clade probability.

twitches of the chick isolated biventer cervicis muscle preparation (Fig. 4a). After washing of the tissue exposed to α-colubritoxin, twitch height was restored to approximately 60% of the initial height (Fig. 4a). In addition, *C. radiatus* venom (3–10 μg/mL) and α-colubritoxin (1 μM) significantly inhibited contractile responses to the exogenous nicotinic agonists (ie. ACh and CCh) but not KCl, indicating a postsynaptic action (Fig. 4b).

α-colubritoxin (0.1–1 μM) produced a rightward shift of cumulative concentration response curves to CCh in the chick isolated biventer cervicis muscle (Fig. 4c). Schild plot analysis demonstrated a pA_2 value of 7.36 and slope of 1.005 ± 0.21.

Discussion

The three-finger toxins (3FTXs) form a broad superfamily of nonenzymatic polypeptides, hitherto thought to be restricted to elapids (e.g. sea snakes and cobras) (Kini 2002). They encompass a large variety of toxins with diverse functional activities, making them interesting from a molecular evolutionary perspective as well in biochemical and biomedical research (Ménez 1998). The members of this multigene family contain 60–74 amino acid residues and are rich in disulphide bonds, with four such bonds being conserved in all family members (Endo and Tamiya 1987). The consequent pattern of protein folding consists of three loops extending from a central core containing the four conserved disulphide bridges (Ménez 1998). The endogenous peptides of vertebrates with three-finger fold play a significant role in cell-cell adhesion complement system (CD59), brain (Lynx-1), and lymphocytes (Ly-6). Such endogenous three-finger proteins may be the ancestors of the 3FTXs (Fleming et al. 1993; Gumley et al. 1995).

The three-finger toxin from *Coelognathus radiatus* has the same ancestral ten cysteine pattern as the endogenous vertebrate peptides such as the neuropeptide Lynx-1 that modulates neuronal nicotinic acetylcholine receptors (Fry et al. 2003). Intriguingly, this same pattern is found in elapid three-finger nicotinic acetylcholine receptor antagonistic peptides, such as the so-called weak α-neurotoxins (Utkin et al. 2001), as well as a reversible α-neurotoxin from *Bungarus candidus* (Nirthanan et al. 2002). It is notable that typically the second and third ancestral cysteine are lost in the more potent elapid α-neurotoxins such as α-bungarotoxin (Fry et al. 2003).

With 79 amino acid residues, the toxin from *Coelognathus radiatus* is slightly larger than the average elapid 3FTX, but this is accounted for by a seven-residue extension at the N-terminus. Other colubrid 3FTXs also appear to have a slightly larger upper range (10 kDa) than elapid 3FTXs, which typically do not exceed 8.5 kDa but have been recorded above

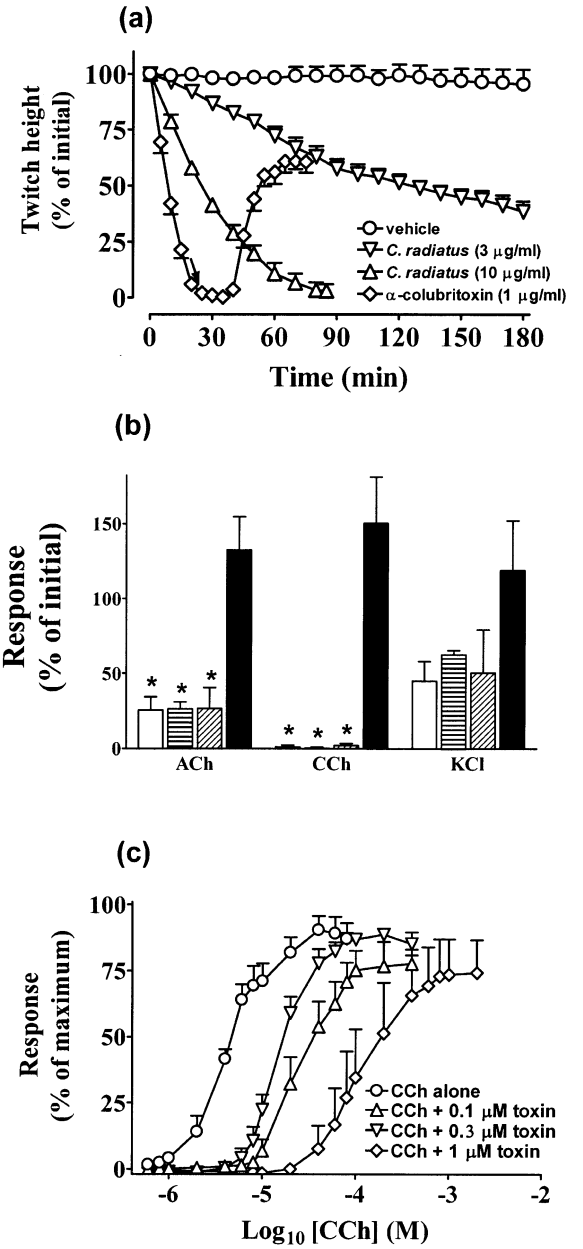

Fig. 4. (**a**) The effect of *C. radiatus* venom (3–10 μg/mL) or α-colubritoxin (1 μM) on indirectly-evoked (supramaximal voltage, 0.2 ms, 0.1 Hz) twitches of the chick isolated biventer cervicis muscle (arrow indicating commencement of washing; $n = 4$–8). (**b**) The effect of *C. radiatus* venom (3 μg/mL, open column; 10 μg/mL, horizontal hatching) or α-colubritoxin (1 μM, diagonal hatching) on responses of chick isolated biventer cervicis muscle to Ach (1 m*M*), CCh (20 μM), or KCl (40 m*M*) ($n = 4$–16). Vehicle (filled bars) had no significant inhibitory effect on responses. $^*p < 0.05$, significantly different from vehicle, one-way ANOVA followed by a Bonferroni post hoc test. (**c**) The effect of α-colubritoxin (0.1–1 μM) on responses to cumulative additions of CCh in chick isolated biventer cervicis muscle ($n = 4$–8).

9 kDa (e.g. Swiss-Prot accession numbers Q9YGHO and Q9PW19).

The reversibility of α-colubritoxin contrasts with the widely used pharmacological tool α-bungarotoxin (Chang 1999), an α-neurotoxin valued for its potency

and specificity but greatly limited by its irreversibility as an investigational ligand and also as a scaffold for drug design and development. Thus, α-colubritoxin may represent an excellent tool for neuropharmacology research and may even be a significant lead in drug design and development. This underscores the largely untapped potential of colubrid venoms for biomedical research, as investigational ligands or even as scaffolds for drug design. We have isolated a number of other three finger toxins from colubrid venoms and are in the process of fully characterizing the pharmacology and NMR structures. Further, the three finger toxin class as a whole have been largely untapped relative to the phylogenetic diversity present (Fry et al. 2003).

Our phylogenetic analysis of representative 3FTX sequences demonstrated with considerable statistical support that α-colubritoxin is rooted within the gene tree of the elapid 3FTXs (Fig. 3). Together with its structural and functional homology to elapid 3FTXs, this suggests that the 3FTX gene family was recruited into the chemical arsenal of the Colubroidea, and started to diversify, before the lineages leading to the present-day Elapidae and Colubrinae diverged. Moreover, our LC-MS results indicate the near-ubiquitous presence of toxins with similar molecular weights among multiple colubrid families/subfamilies. No other families of peptidic toxins in this molecular weight range have been identified in elapid venoms, which suggests that the corresponding molecular masses in other colubrid venoms are indeed three-finger toxins. This ubiquity of 3FTXs across practically all colubroid lineages indicates that the 3FTX gene family was recruited into the chemical arsenal of the Colubroidea early in their evolution, and began to diversify before the split between most of the major colubroid clades (Fig. 1). The absence of 3FTXs in the well-studied viper venoms (Kini 2002) suggests that this recruitment postdates the split between vipers and other colubroids, supporting the basal phylogenetic position of vipers among colubroids (Vidal 2002).

The biological role of toxicity in *Coelognathus radiatus* is unknown, as is the case in most colubrids (Mackessy 2002). However, neurotoxic activity in venoms is most consistent with the role of killing or incapacitating prey, rather than digestion or other functions. Since *C. radiatus* venom, like those of the colubrines *Boiga drapiezii* (Fig. 3) and *B. blandingi* (Levinson et al. 1976), as well as elapid 3FTXs, show α-neurotoxic activity, this suggests that a true venomous function may have been a part of the ancestral biological role of Duvernoy's gland. This forces a fundamental reconsideration of the very concept of nonvenomous snake. Our data thus strongly support 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). Our results thus demonstrate the potential of toxin gene sequences from the hitherto neglected colubrid lineages to shed new light on the origin of venom in snakes, and its functional context.

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