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Identification of PLA_2 and α-Neurotoxin Proteins in the Venom of *Pseudonaja affinis* (Dugite)

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The Western brown snake *Pseudonaja affinis* (dugite), common to the Perth area of Western Australia, possesses one of the most lethal venoms in the world. Little is known, however, about the toxic protein constituents of the venom, other than those causing coagulopathic and procoagulant effects. The current study was therefore undertaken in order to identify other protein constituents and activities present. Crude venom induced a contraction in rat tracheal preparations through phospholipase A_2 (PLA_2) activity, as shown by the complete and partial inhibition of contraction by PLA_2 inhibitors 4-bromophenacyl bromide and quinacrine. Further, a reduced degree of smooth muscle contraction in the presence of the leukotriene receptor antagonist SKF104353 suggested that this effect was mediated by leukotriene metabolites. The venom-induced contraction did not reoccur upon a second administration of the venom, despite the muscle retaining its contractile function and appearing histologically undamaged. Chromatographic separation of the protein constituents of the venom showed that PLA_2 activity was associated with all protein fractions. A low-molecular-weight component of the venom was further investigated through N-terminal sequencing and found to possess high identity to the short-chain α-neurotoxin family of toxins. Venom activity on cultured rat cardiac myocytes and cultured cortical neurons was also examined. The crude venom was found to temporarily inhibit the beating of the cardiac myocytes, after which the beating resumed erratically. Cortical neurons, however, were irreversibly affected, showing concentration-dependent cell death.

Key Words: Pseudonaja affinis **venom; PLA_2; short-chain α-neurotoxin; trachea; cardiac myocyte; cortical neuron.**

Snake venom components have been studied extensively for the past 40 years in an effort to better understand their toxic effects as well as to identify protein components with therapeutic potential. The venom toxins mediate a range of physiological effects such as hemorrhage (Bonta *et al.*, 1970), coagulation (Herzig *et al.*, 1970), paralysis (Campbell, 1975), cardiac arrest, respiratory failure (Sutherland *et al.*, 1981), as well as tissue necrosis (Smith and Figge, 1991). Some of the most lethal venoms are produced by the elapid snakes of Australia, with potent coagulopathic, phospholipase, and neurotoxic activities. Of these, only a subset of the protein components from the venom of the eastern brown snake, *Pseudonaja textilis*, has been investigated in any detail. Identified proteins include the prothrombin activator textarin (Stocker *et al.*, 1994), the long-chain (pseudonaja toxin b) and short-chain (Pt-sntx) α-neurotoxins (Tyler *et al.*, 1987; Gong *et al.*, 1999) and textilotoxin, the most potent neurotoxic PLA_2 yet discovered (Pearson *et al.*, 1993; Su *et al.*, 1983).

In contrast, very little is known about the protein constituents of the venom from the Western cousin of *P. textilis*, *Pseudonaja affinis* (also known as dugite). The characteristic feature of dugite envenomation in humans is hemorrhage due to a consumptive coagulopathy; hence, studies characterizing the venom components of the dugite have been restricted to the coagulopathic effects (Sprivulis *et al.*, 1996; Jelinek *et al.*, 1991). Consumptive coagulopathy is mainly attributed to an 80-kDa serine protease, found to be conserved over the genus *Pseudonaja* (Williams *et al.*, 1994). The additional clinical effects following severe dugite envenomation, such as cerebral hemorrhage, kidney damage, paralysis, cardiac arrhythmias, and even cardiac arrest, though less common, would suggest the presence of potent enzymes and toxins in the venom that have yet to be identified (Williams *et al.*, 1994; White, 1987; Jelinek *et al.*, 1991).

In an effort to further understand the properties of the venom, several biological assays have been employed in the current study, including the use of neurons and myocytes in culture, as well as nerve/muscle preparations in tension-recording experiments to identify the activity of toxins targeting these cell and tissue types. Identification of the toxins present in

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dugite venom may explain the occurrence of paralysis and cardiac arrest in victims of severe dugite envenomation.

MATERIALS AND METHODS

Materials

Lyophilized venom from *P. affinis* was purchased from Venom Supplies (Tanunda, South Australia). Venom (200 mg) was suspended in 10 ml of 0.1 M phosphate-buffered saline (PBS), centrifuged at 7500*g* for 10 min, and the supernatant stored at −20°C. All reagents, unless otherwise indicated, were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation and Purification of Active Components from Crude Venom

Venom components were separated by gel-filtration chromatography using a Superose-12 column (Pharmacia, NJ). Separated fractions, eluted with 0.1 M PBS (pH 7.4) at a flow rate of 0.5 ml/min and detected at 280 nm, were concentrated using centrifugal concentrators (1-kDa cutoff) for 4 h at 7500*g*. Fractions were electrophoresed on a 18% Tris-Tricine SDS polyacrylamide gel (1.8 mm) for 5 h at 80 V and visualized with Coomassie brilliant blue stain. Fraction 3 (containing the lowest molecular weight proteins) was further purified by reverse-phase HPLC on a Vydac C-18 column (5 μm, 4.6 × 250 mm; Hesperia, CA) equilibrated in 95% solution A (0.1% TFA and water) and 5% solution B (90% CH_3CN, 0.1% TFA in water) for 10 min. A gradient from 5 to 100% solution B over 29 min with a flow rate of 1 ml/min was used, and the venom components were monitored at 220 nm. The collected fractions were lyophilized overnight and stored at −20°C, with the main purified component submitted for amino-terminal sequencing (Protein Facility, Western Australia).

Peptide Sequencing and Analysis

N-terminal sequencing by automatic Edman degradation was conducted with 1–10 pmol of sample using a ABI476A protein sequencer (Applied Biosystems, Foster City, CA). The amino acid sequence of the first 40 residues of *P. affinis* protein was compared to other sequences using NCBI BLAST (Altschul *et al.,* 1990).

Tension-Recording Studies in Rat Isolated Trachea

(i) Crude venom. To study the effect of crude dugite venom on tracheal muscle preparations, 8- to 10-week-old male Wistar SPF rats (Animal Resource Centre, Western Australia) were anesthetized with sodium pentobarbital (200 mg/kg, intraperitoneal) and sacrificed, and the trachea was removed and sectioned into rings of 2 mm length. Tracheal sections were mounted onto stainless-steel hooks attached to force displacement transducers (Grass FT03C). A resting tension of 500 mg was applied. The tracheas were immersed in Sigmacoate-treated organ baths containing 2 mL Krebs bicarbonate solution (6.9 g/L NaCl, 2 g/L glucose, 2.1 g/L $NaHCO_3$, 5 mL of 80 g/L KCl, 2 mL of 70 g/L $MgSO_4$, 2 mL of 70 g/L KH_2PO_4, 2 mL of 40% $CaCl_2$) with indomethacin (3 μM bath concentration in 0.9% NaCI) bubbled with carbogen (95% O_2 and 5% CO_2) at 37°C. Changes in smooth muscle tension were recorded using a Grass Polygraph (Model 7B). After equilibration (1 h), tracheal contractile function was tested by the addition of a supramaximal, bolus dose of carbachol (30 μM). Following a 20-min rest and washout period, tissues were exposed to cumulatively added concentrations of carbachol (30 nM–30 μM), washed for a further 20 min, and then exposed to crude dugite venom (190 μg/mL of venom protein) for 15–30 min. The muscle was then washed and rested for a further 30 min, and a second dose-response curve to carbachol was completed.

(ii) Crude venom and phospholipase antagonists. In some experiments, preparations were incubated with the PLA_2 inhibitors 4-bromophenacyl bromide (4-BPB, 180 μM for 30 min) or quinacrine (10 μM, for 30 min), or the leukotriene receptor antagonist SKF104353 (10 μM, for 15 min) prior to venom addition (190 μg/mL). Venom-induced contractions were expressed as a percentage of the response observed to 30 μM carbachol obtained at the beginning of the experiment.

(iii) Venom fractions. Response of trachea to dugite venom fractions purified by gel filtration chromatography was determined with venom fraction protein concentrations of 500 μg/mL (bath concentration). The crude and purified venom protein content was determined by the BCA assay (Pierce Chemicals, Rockford, IL).

Statistical Analysis

All data are presented as the means ± SEM and group data compared using analysis of variance (one-way ANOVA), with a P value < 0.05 considered significant.

Light Microscopic Examination of Venom-Treated Trachea

Tracheal segments from organ bath experiments were processed in preparation for paraffin wax sectioning (Bancroft and Stevens; 1977). Sections of 5 μm thickness were mounted onto glass slides, stained with Harris's hematoxylin and eosin (H&E), and viewed at 200× magnification.

Electron Microscopic Examination of Venom-Treated Trachea

Following organ bath experiments, tracheal segments were removed and placed in 2% glutaraldehyde for 4 h, followed by cacodylate buffer. Tissues were postfixed with osmium tetroxide, and thin sections (50 nm) prepared and stained as described by Robertson *et al.* (2000). The transmission electron microscope used was Phillips 410LS at 4800× magnification.

Effects of Crude Venom on Spontaneously Beating Cardiac Myocytes

Spontaneously beating ventricular cardiomyocytes were prepared from neonatal rat hearts as described in Bogoyevitch *et al.* (1995). The cells were treated with crude venom (200, 100, 20, 2, and 0.2 μg/mL protein concentration) 2 days after plating and incubation at 37°C. The beating pattern and morphology were monitored within a 24-h period at 200× magnification.

Effects of Crude Venom on Neurons

Rat fetal cortical neurons were isolated and cultured according to Namgung *et al.* (2000). Neurons were treated with varying amounts of crude venom (200, 100, 20, 2, and 0.2 μg/mL protein concentration) 2 days after plating following a change of culture media. Their morphology was monitored at 200× magnification in a 24-h period.

RESULTS

Crude Dugite Venom Induced Contraction in Rat Isolated Trachea

Crude dugite venom (190 μg/mL) induced a marked contractile response in rat isolated tracheal preparations (Fig. 1). The magnitude of the peak response was 75 ± 5% of the maximum carbachol-induced response. The peak was observed approximately 3 min after venom addition, and was maintained for at least 15–30 min (n = 10). Venom-induced contractions were reversed by washout, but did not reoccur upon administration of a second venom dose to the same tissue (n = 3). Venom did not impair tissue contractile responses to carbachol (EC_{50} before venom = 0.49 μM (95% confidence limits,

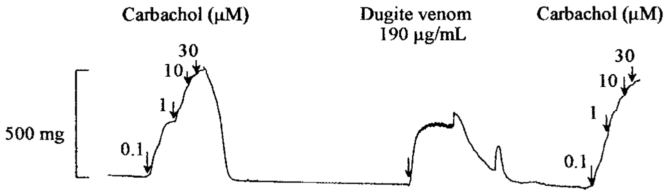


FIG. 1. Dugite venom causes a contraction of rat trachea. Venom (190 μg/mL protein concentration) was added to a tracheal segment (resting tension 500 mg), resulting in a significant contraction (75 ± 5% of that induced by 30 μM carbachol). Venom did not reduce tissue viability since carbachol-induced contractions obtained after venom administration were not attenuated.

0.42–0.60 μM, $n = 13$); EC_{50} after venom = 0.35 μM (95% confidence limits, 0.30–0.40 μM, $n = 13$)).

Contractile Response with PLA_2 Antagonists

Both quinacrine and 4-BPB significantly inhibited venom-induced contractions (Fig. 2; one-way ANOVA, $P < 0.05$). Quinacrine (10 μM) inhibited venom-induced responses by more than half (approximately 40% of the dugite-induced response), whereas 4-BPB (180 μM) caused complete abolition of the response ($n = 3$). These results indicate that PLA_2 proteins present in the venom were responsible for the contractions, and likely to act via downstream products of PLA_2 activity such as leukotrienes. To assess the possible role of leukotrienes in venom-induced contractions, tracheal preparations were incubated with a leukotriene receptor antagonist SKF104353 prior to venom addition ($n = 3$). SKF104353 reduced the venom-induced contractions to approximately 39% of the dugite-induced response, (one way ANOVA, $P < 0.05$), suggesting that leukotrienes are involved to some extent in the venom contractile response (Fig. 2).

Examination of Venom Fractions

In order to further characterize the proteins present in venom, crude dugite venom (1 mg/mL) was fractionated using gel-filtration chromatography, and fractions were examined by gel electrophoresis and tested for their ability to cause rat tracheal contractions. Six main fractions were collected (Fig. 3A). The gel electrophoretogram (Fig. 3B) shows that the venom fractions contain a wide range of protein components, ranging in size from 200 to 6 kDa, with the high-molecular-weight proteins confined to the earliest eluting fractions 1a and 1b. Fractions 2a, 2b, and 3 contained protein components of between 22 and 6 kDa, with species of approximately 20, 13, and 6 kDa predominating. Fraction 4 did not give rise to any protein bands, and is likely to represent a low-molecular-weight non protein component in the venom. Since fractions 1a and 1b contained common components, as did fractions 2a and 2b, these were pooled for measurement of rat tracheal contractility and are referred to in these studies as fractions 1 and 2, respectively.

Venom fractions 1, 2, and 3 (500 μg/mL) all induced a tracheal muscle contractile response. These were 31 ± 4, 41 ± 5, and 49 ± 10% of the carbachol-induced response, respectively (one-way ANOVA, $P < 0.05$), representing a significant response compared to the crude venom response of 75 ± 5% (Fig. 4; $n = 3$).

Light and Electron Microscopic Analysis of Venom-Treated Tracheal Segments

Following treatment with crude dugite venom, the tracheal segments were analyzed for evidence of tissue damage. Light microscopic analysis revealed that tracheal segments treated with crude venom (190 μg/mL venom concentration for 30 min at 37°C) exhibited complete degradation of the epithelial layer (E). This was in contrast to control tracheal segments from organ baths that were not exposed to the dugite venom (Fig. 5A untreated trachea, Fig. 5B venom-treated trachea). Muscle (M) and collagen (C) appeared to be unaffected by the addition of venom. Closer inspection by transmission electron microscopy verified the intact state of smooth muscle cells and collagen. However, a spatial separation between muscle and collagen fibers was observed, suggesting an edematous response to venom exposure (Fig. 5C untreated trachea, Fig 5D venom-treated trachea).

Venom Toxicity Assay with Cultured Cardiac Myocytes

Crude dugite venom was applied to cultures of spontaneously beating cardiac myocytes in order to observe the venom effects. Application of the venom was found to alter the beating pattern of normal healthy cardiac myocytes, which beat strongly in a uniform manner ($n = 6$). Venom (200 and 100

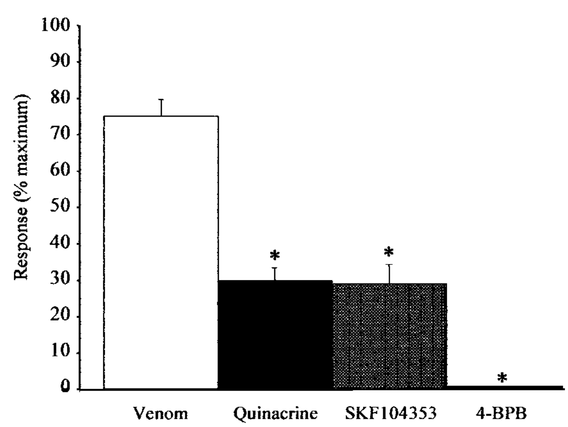


FIG. 2. Inhibition of dugite-induced contraction of rat trachea by eicosanoid antagonists. The PLA_2 inhibitor quinacrine (10 μM) partially abolished venom-induced contractility (30 ± 2% of carbachol-induced contraction, $P < 0.05$) when compared to the control response (75 ± 5% of carbachol-induced-contraction, $P < 0.05$), whereas the PLA_2 inhibitor 4-BPB (180 mM) completely abolished the contraction. SKF104353 (100 μM) also partially abolished venom contraction (29 ± 6% of carbachol induced contraction; *$P < 0.05$).

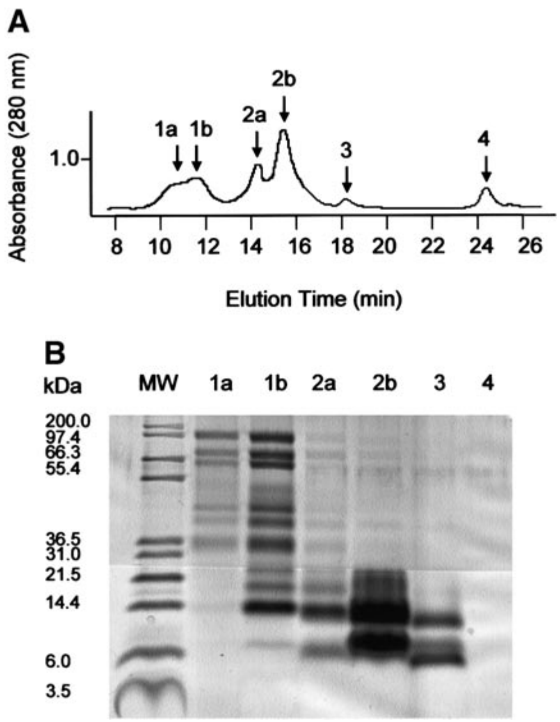


FIG. 3. (A) Size-exclusion chromatography profile of dugite venom. A 0.1 M PBS (pH 7.4) was used to elute 6 venom fractions detected at 280 nm with a flow rate of 0.5 mL/min. Fraction 1a eluted at 10 min, fraction 1b at 11 min, fraction 2a at 14 min, fraction 2b at 15 min, fraction 3 at 18 min, and fraction 4 at 23 min. (B) Tris-Tricine electrophoretogram of dugite venom fractions. The electrophoresis of six fractions shows the venom to contain a wide range of protein species ranging from 200 to 6 kDa in size, with the high-molecular-weight proteins found in the earliest eluting fractions, and the low-molecular-weight proteins found in the later eluting fractions. Fraction 4 did not contain any protein species.

μg/mL protein concentration) caused myocyte beating to cease for approximately 2 min immediately after addition. Beating resumed in an erratic fashion characterized by patches of hypercontracting cells and patches of cells that were beating very slowly. A strong, uniform beating pattern was observed to resume approximately 24 h after venom administration. Lower venom concentrations (20, 2, and 0.2 μg/mL), however, did not mediate any perturbations of the myocyte beating pattern. Morphologically, the myocytes exposed to varying concentrations of venom retained their shape, with no evidence of necrosis or cellular damage.

Venom Toxicity Assay with Cultured Cortical Neurons

Toxicity assays were carried out to observe the *in vitro* effect of dugite venom on cortical neurons. The effect of crude venom 30 min, 60 min, and 24 h after treatment is illustrated in Fig. 6. Venom administered at 200 μg/mL resulted in partial degradation of the nerve cell body and axons after 30 min. After 60 min, degradation was severe and complete, with the appearance of predominantly particulate matter. This did not further increase after 24 h. A dose dependency of this effect was observed, with lower doses (100, 20, 2, and 0.2 μg/mL) being slower to induce degradation, and not showing as severe damage after 24 h.

Amino-Terminal Sequencing

It was of interest to determine the identity of the low-molecular-weight protein(s) present in the venom. For this reason, fraction 3, already partially purified by gel-filtration chromatography (Fig. 3), underwent further purification by reverse-phase HPLC. One main fraction was eluted, and this was subjected to amino-terminal sequencing. A total of 40 residues were successfully sequenced, and this partial sequence data were compared to other snake amino acid sequences using BLAST (Altschul *et al.*, 1990; Fig. 7). The dugite venom sequence was found to possess considerable sequence identity to elapid short chain α-neurotoxins, particularly to the *P. textilis* neurotoxins. The *P. textilis* short-chain α-neurotoxin isoforms (Pt-sntx) were found to be 77% (isoform 1), 80% (isoform 2), 82% (isoform 3), 74% (isoform 5), 72% (isoform 6), and 80% (isoform 7) identical to the *P. affinis* 40 amino acid sequence, respectively. Other short chain α-neurotoxins showed lower identity to the dugite sequence: *Pseudechis australis* (Pa a) 30%, *Acanthopis antarcticus* (Aa c) 27%, *Naja nigricollis* (Toxin α) 35%, *Naja mossambica mossambica* (Nm III) 35%, *Laticauda semifasciata* (Ls Ec) 30%, and *Dendroaspis polylepis* (Dp a) 30%.

DISCUSSION

The current investigation was undertaken in order to provide a more comprehensive study of the nature of toxin components in dugite venom. The results presented here have identified for the first time the presence of PLA_2 and a short-chain α-neu-

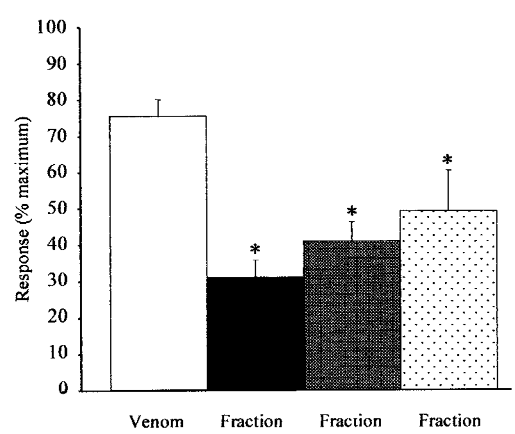
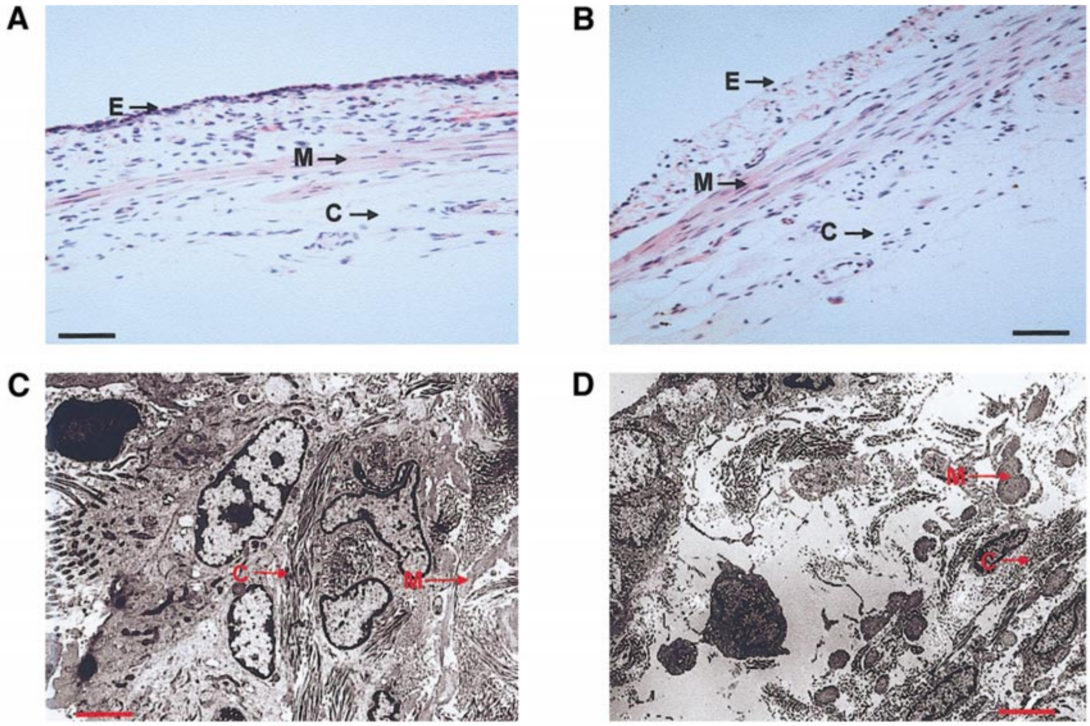


FIG. 4. Effect of partially purified venom fractions on tracheal contractility. Fractions 1–3 (500 μg/mL protein concentration) induced contraction of rat trachea that were 31 ± 4, 41 ± 5, and 49 ± 10% of the carbachol-induced response, compared with a crude venom response of 73 ± 0.6% (*$P < 0.05$).



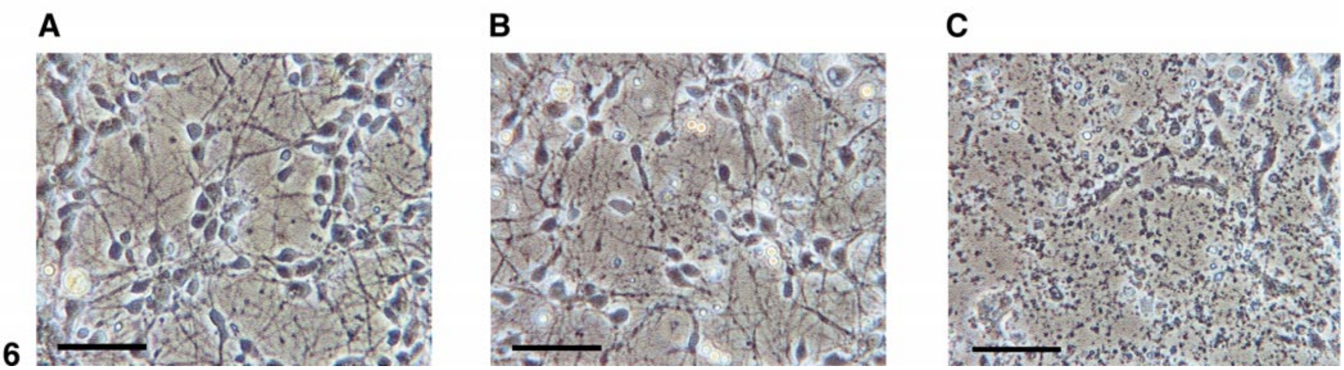


FIG. 5. (A and B) H&E-stained sections of rat trachea following organ bath experiments. Panel A shows trachea that have not been exposed to dugite venom. No disruption to muscle (M), collagen (C), nor epithelium (E) is seen. Panel B illustrates trachea following exposure of 30 min to dugite venom. Both muscle (M) and collagen (C) remain intact; however, the epithelium (E) is notably disrupted and absent in parts. Bar = 50 μm. (C and D) Electron micrograph of rat trachea following organ bath experiments. Untreated trachea (C) contains muscle (M) and collagen (C) with a minimal amount of intercellular space. In contrast, venom-treated trachea (panel D) shows separation of muscle (M) and collagen (C), characterizing edema. Bar = 2 μm.

FIG. 6. Effect of dugite venom on cultured cortical neurons. Panel A depicts healthy, untreated neurons that possess the characteristic features of elongated cell body and axons, at 30 min after venom addition (200 μg/mL protein concentration), loss of neuronal axons became apparent (B). At 60 min after venom addition, neuronal cell bodies and axons were completely degraded (C). Bar = 50 μm.

rotoxin in dugite venom. Furthermore, the activity of venom components mediating neuron death and perturbations of myocyte beating has been reported.

PLA_2 in snake venoms function to hydrolyze membrane phospholipids, liberating fatty acids and lysophosphoglycerides. While these enzymes have an important digestive function, displaying considerable homology to mammalian pancreatic PLA_2, they have also evolved to possess a wide range of pharmacological functions including neurotoxicity (Chang and Lee, 1963), myotoxicity (Mebs and Ownby, 1990), induction or inhibition of platelet aggregation (Marsh, 1994), and cardiotoxicity (Huang *et al.*, 1997), and thus also play a role in prey capture. In the current study, the presence of PLA_2 in dugite venom was shown to induce contractions of tracheal smooth muscle. The venom-induced muscle contraction was completely inhibited in the presence of the PLA_2 inhibitor 4-BPB, and partially abolished in the presence of quinacrine. Similar PLA_2 activity has also been identified in the venom of the taipans *O. microlepidotus* (Bell *et al.*, 1998) and *O. scutellatus* (Crachi *et al.*, 1999), causing contractions of guinea pig ileum smooth muscle. Muscle contraction did not occur upon a second administration of dugite venom, and this has

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P.affinis       LTCNKSYYD----TVVCKPHETICYRYHVPATHGNVITXRGCGT
Pt-sntx1        LTCYKGYHD----TVVCKPHETICYEYFIPATHGNAILARGCGTSCP---GGIRPVCCRT
Pt-sntx2        LTCYKGYHD----TVVCKPHETICYRYLIPATHGNAIPARGCGTSCP---GGNHPVCCST
Pt-sntx3        LTCYKGYHD----TVVCKPHETICYRYLVPATHGNAIPARGCGTSCP---GGNHPVCCST
Pt-sntx5        LTCYKGYHD----TVVCKPHETICYEYFIPATH-DAILARGCGTSCP---GGIRPVCCRT
Pt-sntx6        LTCYKSLSG----TVVCKPQETICYRRLIPATHGNAIIDRGCSTSCP---GGNRPVCCST
Pt-sntx7        LTCYKRYFD----TVVCKPHETICYEYIIPATHGNAITYRGCSTSCP---SGIRLVCCST
Pa a            MTCCNQQSSQPKTTTICAGGESSCYKKTWSDHRG-SRTERGCG--CPHVKPGIKLTCCKT
Aa c            MQCCNQQSSQPKTTTTCPGGVSSCYKKTWRDHRG-TIIERGCG--CPRVKPGIRLICCKT
Toxin α         LECHNQQSSQPPTTKTCPG-ETNCYKKVWRDHRG-TIIERGCG--CPTVKPGIKLNCCTT
NmIII           LNCHNQMSAQPPTTTRCSRWETNCYKKRWRDHRG-YKTERGCG--CPTVKKGIQLHCCTS
Ls Ec           RICFNHQSSQPQTTKTCSPGESSCYHKQWSDFRG-TIIERGCG--CPTVKPGINLSCCES
Dp a            RICYNHQSTTRATTKSCE--ENSCYKKYWRDHRG-TIIERGCG--CPKVKPGVGIHCCQS
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FIG. 7. Alignment of dugite toxin sequence (N-terminal 40 residues) with sequences of other snake short-chain α-neurotoxins. Pt-Sntx (1–3, 5–7), *Pseudonaja textilis*; Pa a, *Pseudechis australis*; Aa c, *Acanthopis antarcticus*; Toxin α, *Naja nigricollis*; NmIII, *Naja mossambica mossambica*; Ls Ec; *Laticauda semifasciata*; Dp a, *Dendroaspis polylepis*. Amino acid residues are represented by single letter code. (−) Indicates a gap due to insertion or deletion. (*) Indicates conserved amino acids.

also been previously observed in other snake species (Bell *et al.*, 1998; Ohara *et al.*, 1995). In the case of administration of *Vipera ammodytes* venom, a reduced secondary contraction of the ileum was reported (Sket and Gubensek, 1976).

Despite the absence of a second muscle contraction, the muscle itself was not damaged, as demonstrated by the consistency of the carbachol response before and after venom application. Furthermore, histopathological examination by light microscopy of the venom-treated tracheal segment verified the intact state of the tracheal smooth muscle. Thus, tracheal myonecrosis mediated by PLA_2 does not appear to be a feature of dugite envenomation. Complete eradication of the epithelial layer, however, was observed, a finding consistent with the epithelial damage observed with *B. asper* envenomation (Rucavado *et al.*, 1998).

Muscle contraction due to snake venom PLA_2 is thought to occur as a direct result of lipase activity. PLA_2 activity results in arachidonic acid release from membrane phospholipids (Kini and Evans, 1989). Arachidonic acid itself mediates the activation of the eicosanoid pathway and the subsequent generation of cyclooxygenase metabolites, such as thromboxanes and prostaglandins, and 5-lipoxygenase metabolites, such as leukotrienes (Kaiser *et al.*, 1990). In the current contractile studies, the cyclooxygenase inhibitor indomethacin was present; therefore, the observed contraction was not due to cyclooxygenase metabolites. Application of the leukotriene receptor antagonist SKF104353 resulted in a reduced tracheal contraction, suggesting leukotrienes were involved in tracheal contractility.

Induction of edema through PLA_2 activity, observed for *Bothrops* (Soares *et al.*, 2000; 2001; de Faria *et al.*, 2001) and *Naja naja atra* (Zhang and Gopalakrishnakone, 1999) venoms, is thought to be the result of eicosanoid generation of leukotrienes and prostaglandins, leading to inflammation and subsequent increased vascular permeability, and thus edema (Lloret and Moreno, 1993; Chiu *et al.*, 1989). In the case of dugite venom, generation of leukotrienes from PLA_2 hydrolysis is thought to underlie the observed edema.

The presence of a range of PLA_2 species is consistent with the studies of dugite venom fractions. Fractionation of the venom by size-exclusion chromatography and gel electrophoresis showed that, while a number of protein species are present, a significant proportion were of approximately 13 kDa, the expected size for PLA_2 (Grieg-Fry, 1999). In addition, all protein-containing fractions induced muscle contraction, suggesting the presence of PLA_2 in all three fractions, although this was not confirmed using PLA_2 inhibitors.

In a novel assay, the dugite venom was found to temporarily halt the beating of cardiac myocytes in culture, after which beating resumed irregularly. Cardiac arrhythmias are a phenomenon that has been observed in victims of envenomation by the cobra (Wang *et al.*, 1997) and taipan (Fantini *et al.*, 1996), as well as bee (Okamoto *et al.*, 1995) and spider (Pascarel *et al.*, 1997) venoms, but have not previously been reported for elapid venom. While further research is required to determine the basis of this effect, it may involve the modulation of Ca^{2+} flux in the cells. Cobra toxin was found to increase cellular contraction, and taipan toxin decrease contraction by elevating calcium influx into the cells through specific or nonspecific Ca^{2+} channels or $Na^{+}-Ca^{2+}$ exchangers, as well as impaired sequestration of Ca^{2+} in the sarcoplasmic reticulum (Fantini *et al.*, 1996; Wang *et al.*, 1997). It is possible that such Ca^{2+} flux is effected through PLA_2 activity. PLA_2 metabolites have been shown to mediate arrhythmias by the release of free fatty acids through the modulation of sodium and calcium currents (Kang and Leaf, 2000; Pi and Walker, 2000; Fantini *et al.*, 1996).

While the cardiomyocytes were only temporarily affected by the venom, cortical neurons in culture underwent a concentration-dependent cell death. The rapid destruction of the cell body is consistent with PLA_2 activity. Studies of bee venom PLA_2 (Kolko *et al.*, 1996; Clapp *et al.*, 1995) and venom from the snake *Crotalus durissus terrificus* (Mello and Cavalheiro, 1989) have been shown to promote neuronal injury by the destruction of neuronal axons and shrunken cell bodies. Traumatic and ischemic injury resulting in neuronal damage has long been known to be mediated by phospholipase metabolites (Bazan, 1970; Farooqui and Horrocks, 1994). Cellular damage may have occurred as a result of arachidonic acid release as well as elevation of Ca^{2+} levels (Kolko *et al.,* 1996; Bazan *et al.,* 1995).

In addition to the identification of PLA_2 in dugite venom, a protein species with considerable sequence identity to the *P. textilis* short-chain α-neurotoxin is reported. These toxins function by binding postsynaptically to the nicotinic acetylcholine receptor, thus inhibiting propagation of nerve transmission (Tu *et al.,* 1998). Relative to the archetype neurotoxins such as α-bungarotoxin, the *P. textilis* neurotoxin has a lower potency (Gong *et al.,* 1999). The activity of the dugite neurotoxin may also exhibit a similar low level of potency.

In conclusion, this study is the first to identify the activity of PLA_2, and the presence of a short chain α-neurotoxin in the venom of the Australian elapid, *P. affinis*. PLA_2 was found to affect tracheal smooth muscle, and is also thought to underlie the observed cortical neuron death and perturbations of cardiac myocyte beating. The successful use of cardiac myocyte and neuron cell cultures for venom research has been shown and appears to be an attractive alternative to animal experimentation for the detection of venom activities. While previous studies of dugite venom have reported the procoagulant activities responsible for incoagulable blood in human envenomation, this study has taken the first step toward identifying dugite toxins that are likely to underlie the more rare envenomation effects of cardiac arrest and paralysis. It is anticipated that a better understanding of the full spectrum of toxin activities in dugite venom will result in more advanced treatment for such snakebites.

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