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Frontoxins, three-finger toxins from *Micrurus frontalis* venom, decrease miniature endplate potential amplitude at frog neuromuscular junction

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ABSTRACT

Neurotoxicity is a major symptom of envenomation caused by Brazilian coral snake *Micrurus frontalis*. Due to the small amount of material that can be collected, no neurotoxin has been fully sequenced from this venom. In this work we report six new three-finger like toxins isolated from the venom of the coral snake *M. frontalis* which we named Frontoxin (FTx) I–VI. Toxins were purified using multiple steps of RP-HPLC. Molecular masses were determined by MALDI-TOF and ESI ion-trap mass spectrometry. The complete amino acid sequence of FTx II, III, IV and V were determined by sequencing of overlapping proteolytic fragments by Edman degradation and by *de novo* sequencing. The amino acid sequences of FTx I, II, III and VI predict 4 conserved disulphide bonds and structural similarity to previously reported short-chain α-neurotoxins. FTx IV and V each contained 10 conserved cysteines and share high similarity with long-chain α-neurotoxins. At the frog neuromuscular junction FTx II, III and IV reduced miniature endplate potential amplitudes in a time-and concentration-dependent manner suggesting Frontoxins block nicotinic acetylcholine receptors.

1. Introduction

Snake venoms are a mixture of pharmacologically active proteins and polypeptides fundamentally important for the capture and digestion of prey, and for defense against predators (Hodgson and Wickramaratna, 2002). Elapid snake venoms are rich in three-finger scaffold neurotoxins. This toxin family adopts a flat, leaf-like shape formed by three adjacent loops that emerge from a small globular core, which is the location of four conserved disulphide bonds (Menez, 1998; Tsetlin, 1999). Three-finger toxins bind with high affinity and selectivity to nicotinic acetylcholine receptor (nAChR) affecting cholinergic synaptic transmission (Antil-Delbeke et al., 2000; Nirthanan et al., 2002). Neurophysiological alterations similar to those induced by α-neurotoxins are observed in coral snake venoms (genera *Micrurus*, *Leptomicrurus* and *Micruroides*) (Serafim et al., 2002).

The *Micrurus* genus is the major representative elapid in the Americas, with more than 70 species distributed in southwestern United States, Central and South America (Roze, 1996; Campbell and Lamar, 2004). These elapids are relatively small, non aggressive animals with aposematic coloration, that generally live underground. *Micrurus* venoms have been little studied because these animals are difficult to capture and to keep in captivity, have restricted and specialized diets, and produce limited amount of venom.

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Reports of snake bite by *Micrurus* are rare, but can be quite severe depending on the amount of venom injected and the age of the victims. In many cases, *Micrurus* bites can result in death due to respiratory arrest (Vital-Brazil, 1987), probably as a result of a progressive blockade at neuromuscular endplate. Experimental studies suggest that *Micrurus* venoms cause neurophysiological changes, cardiotoxic, haemolytic and myotoxic effects (Aird and Jorge da Silva, 1991; Gutiérrez et al., 1992; Francis et al., 1997; Cecchini et al., 2005).

Micrurus venom toxins block neuromuscular transmission and may be classified according to their mechanisms of action. *Micrurus corallinus* for example was classified as having both presynaptic and postsynaptic actions, because it causes reduction in the response to exogenously applied acetylcholine (postsynaptic action, presumably on muscle isoforms of the nicotinic receptors) as well as an increase of spontaneous release (a presynaptic action, possibly mediated by alpha-7 containing nicotinic receptors). On the other hand, *Micrurus frontalis* and *Micrurus lemniscatus* demonstrated only postsynaptic activity (Vital-Brazil and Fontana, 1983/1984). To date, individual toxins from *M. frontalis* venom have not been studied separately.

Although coral snakes have been studied as sources of neurotoxins, only a few complete toxin sequence data have been gathered from *M. corallinus* (Oliveira et al., 2000; Leão et al., 2009), *Micrurus nigrocinctus* (Rosso et al., 1996), *Micrurus surinamensis* (Olamendi-Portugal et al., 2008) and *Micrurus pyrrhocryptus* (Dokmetjian et al., 2009) venoms. Presently there are few studies with neurotoxins from Brazilian coral snake venoms. Three-finger neurotoxins from *M. corallinus* were recently identified by transcriptomic analysis, based on close similarities of the amino acid sequences deduced from complementary DNA cloning (Leão et al., 2009). N-terminal amino acid sequence of a postsynaptic action toxin from *M. frontalis* was partially sequenced by peptide purification (Francis et al., 1997). In this study we describe the isolation, molecular mass determination, complete and partial amino acid sequencing of short and long-chain three-finger toxins isolated from *M. frontalis* (Brazilian coral snake) venom and tested their ability to block miniature endplate potentials at the neuromuscular junction.

2. Material and methods

2.1. Venom

The venom of *M. frontalis* was obtained by manual gland compression using glass micropipettes, lyophilized and stored at −80 °C. The pool of *M. frontalis* venom was extracted from adult specimens ($n = 5$), which were kindly donated by Centro de Estudos e Pesquisas Biológicas at Universidade Católica de Goiás (Brazil).

2.2. Purification

Aliquots from the pool of crude venom were initially fractionated by semi-preparative reverse phase chromatography using a C_4 column (204TP510 Grace Vydac, USA) on a Class VP chromatographer from Shimadzu Co. (Japan). All fractions eluted during the 120 min linear acetonitrile gradient (0.1% TFA to 95% acetonitrile containing 0.1% TFA) with 2.5 mL/min flow rate were submitted to further purification steps on analytical columns and Ultra Fast Liquid Chromatography on an UFLC Prominence system using Shim-pack XR-ODS (Shimadzu Co., Japan) from Shimadzu Co (Japan). The HPLC fractions containing neurotoxins were purified up to mass spectrometry grade using a C_{18} (218TP54 Grace Vydac, USA) and/or Source 5RPC (150 × 4.6 mm, GE Healthcare Bio-Sciences AB, Sweden) columns with optimized gradients of acetonitrile over 60 minutes for each molecule. All experiments were monitored at 216 and 280 nm and each fraction was collected manually, frozen in liquid nitrogen, lyophilized and stored at −20 °C.

2.3. Purity, molecular masses and cysteine content determinations

The purity and accurate molecular mass determination of each purified molecule were determined by ESI HCT-Ultra ETD II (Bruker Daltonics, Billerica, USA), MALDI Synapt HDMS (Waters Co. Manchester, UK) and on an UltraFlex III (Bruker Daltonics, Billerica, USA) according to the best possible signal/noise relation for each individual molecule.

Under electrospray ionization, the samples were reconstituted with 100–300 μL of solvent (50% acetonitrile containing 0.1% formic acid). The MS scans were performed in the range of m/z 300–3000 Da. For MALDI-TOF/MS experiments, the selected fractions were dissolved in Milli-Q water, mixed with a saturated matrix solution of a-cyano-4-hydroxycinnamic acid (1:3), spotted onto a MALDI-TOF plate and dried at room temperature. The MS spectra were generated in linear and reflector modes with external standard calibrations provided by Bruker Daltonics.

2.4. Toxins reduction and alkylation

Disulphide bond reduction and S-alkylation of each native polypeptide was achieved by incubation with 50-fold DTT (dithiothreitol, Sigma–Aldrich, St. Louis, MO) excess in 100 mM ammonium bicarbonate solution pH 8.0 buffer, for 1 h at 70 °C followed by the addition of 50 mM iodoacetamide (Sigma–Aldrich, St. Louis, MO) at 37 °C during 1 h, in the dark. The reduced and alkylated polypeptides were purified by RP-HPLC using an analytical C_{18} column (218TP54 Grace Vydac, USA) eluted with linear gradient (5–95% ACN). ESI-IonTrap/MS, ESI-Q-TOF/MS and MALDI-TOF/MS experiments were performed to determine average and accurate monoisotopic molecular masses of the reduced and S-alkylated samples.

2.5. Enzymatic cleavages

Five different proteolytic enzymes (sequencing-grade proteases Asp-N, chymotrypsin, endopeptidase Glu-C, endopeptidase Lys-C and trypsin) were used to obtain suitable peptide sequence overlaps for unambiguous

complete primary structure determination of each one of the *M. frontalis* neurotoxins.

Asp-N (Roche Applied Science, Germany) – the reduced and alkylated sample was dissolved in 50 mM sodium phosphate pH 8.0 and digested at an enzyme to substrate concentration of 1:100 (w/w) for 2 h at 37 °C.

Chymotrypsin (Roche Applied Science, Germany) – this enzymatic hydrolysis was performed dissolving each sample in 100 mM Tris–HCl, pH 7.8. Ten microliters of the enzyme were added and samples incubated with intermittent agitation overnight at 37 °C.

Glu-C (Roche Applied Science, Germany) – the samples were dissolved in 0.1 M ammonium bicarbonate pH 8.0 at a concentration of 10 mg/mL and added 2% (w/w) enzyme to substrate. The enzymatic reaction was incubated at 37 °C for 24 h.

Lys-C (Roche Applied Science, Germany) – in the treatment with endoprotease Lys-C the samples were dissolved in 0.1 M ammonium bicarbonate pH 8.0, to which 20 μL of enzyme were added and incubated with agitation for 24 h at 37 °C.

Trypsin (Pierce Chemical, USA) – the immobilized TPCK Trypsin was prepared for use by washing successively with 10 mM ammonium bicarbonate solution. Samples were dissolved in 0.1 M ammonium bicarbonate pH 8.0 and the enzyme solution was added to a final concentration of 2% (w/w). The reaction was incubated for 2 h at 37 °C with intermittent agitation.

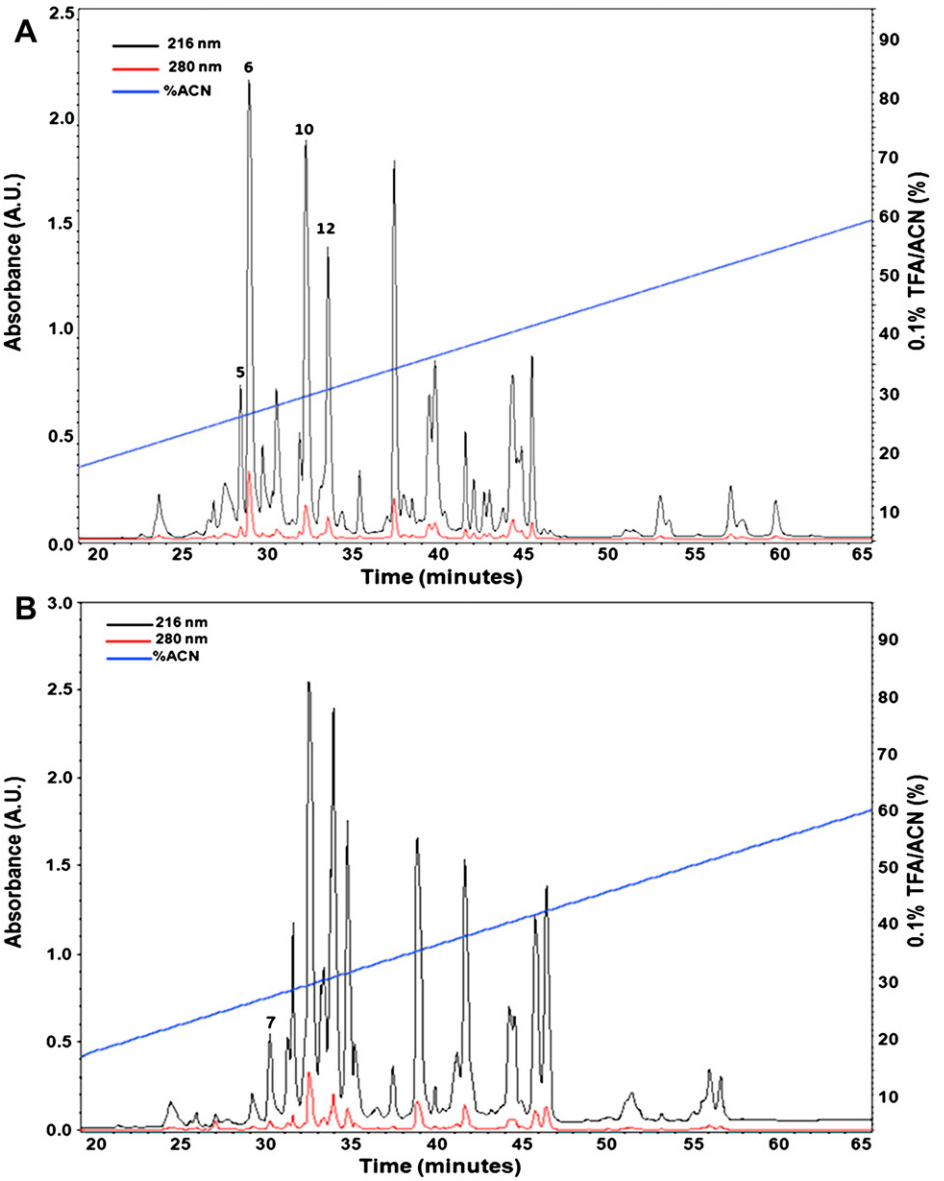

Fig. 1. Chromatographic profile of *M. frontalis* crude venom in a semipreparative C_4 (A) and analytical C_2C_{18} (B) columns. The venom was eluted with trifluoroacetic acid (TFA) (0.1%) (Solvent A) and acetonitrile (95% + 0.1% TFA) (solvent B), under a linear gradient of solvent B for 100 min. The absorbance was monitored at 216 and 280 nm in arbitrary units (A.U.). Fractions 5, 6, 10, 12 (A) and 7 (B) were further purified for amino acid sequencing.

In all enzymatic treated toxins the resulting peptides were separated by RP-HPLC on a Vydac 218TP54 C_{18} analytical column using a linear gradient (5–95% ACN) and their grades of purity, molecular masses and parent ion fragmentations were obtained by a number of specific mass spectrometry experiments.

N-terminal amino acid sequencing of the intact and the S-alkylated neurotoxins were performed by the automatic Edman Degradation in a PPSQ-23 Sequencer (Shimadzu Co. Japan). Sequence similarity searches and alignments were performed using the MPsrch (http://www.ebi.ac.uk/MPsrch/) and Clustal W multiple alignment tool available on Bioedit v. 7.0.4.1 software.

2.6. Electrophysiological assay

All experiments were carried out using protocols approved by the institutional animal care and use committee of Universidade Federal de Minas Gerais (CETEA – UFMG). The experiments were performed at room temperature using cutaneous *pectoralis* muscle from *Rana catesbeiana*. The animals were sacrificed by decapitation and the muscle was pinned onto a bed of Sylgard (Dow Corning, USA) in a 4 mL chamber containing Ringer solution with the following composition (in mM): NaCl 115; KCl 2.5; $CaCl_2$ 1.8; and N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) 5, pH 7.4. Miniature endplate potentials (MEPPs) were recorded by intracellular impalement of the endplate region of muscle fibers with 5–15 MΩ microelectrodes filled with 3 M KCl. Details of our methods are described in Oliveira et al. (2007). The toxins were added directly to the bath. For recovery conditions, the preparation was washed with toxin free Ringer solution (5 mL/min) for at least 30 min. Quantal size was measured and corrected to a standard membrane potential of −90 mV using the method of Katz and Thesleff (1957). We measured MEPP amplitude as the mean of 100 MEPPS in control conditions and at least 30 MEPPS at the various times studied after toxin application. To evaluate the statistical significance of changes in MEPP size and frequency, each

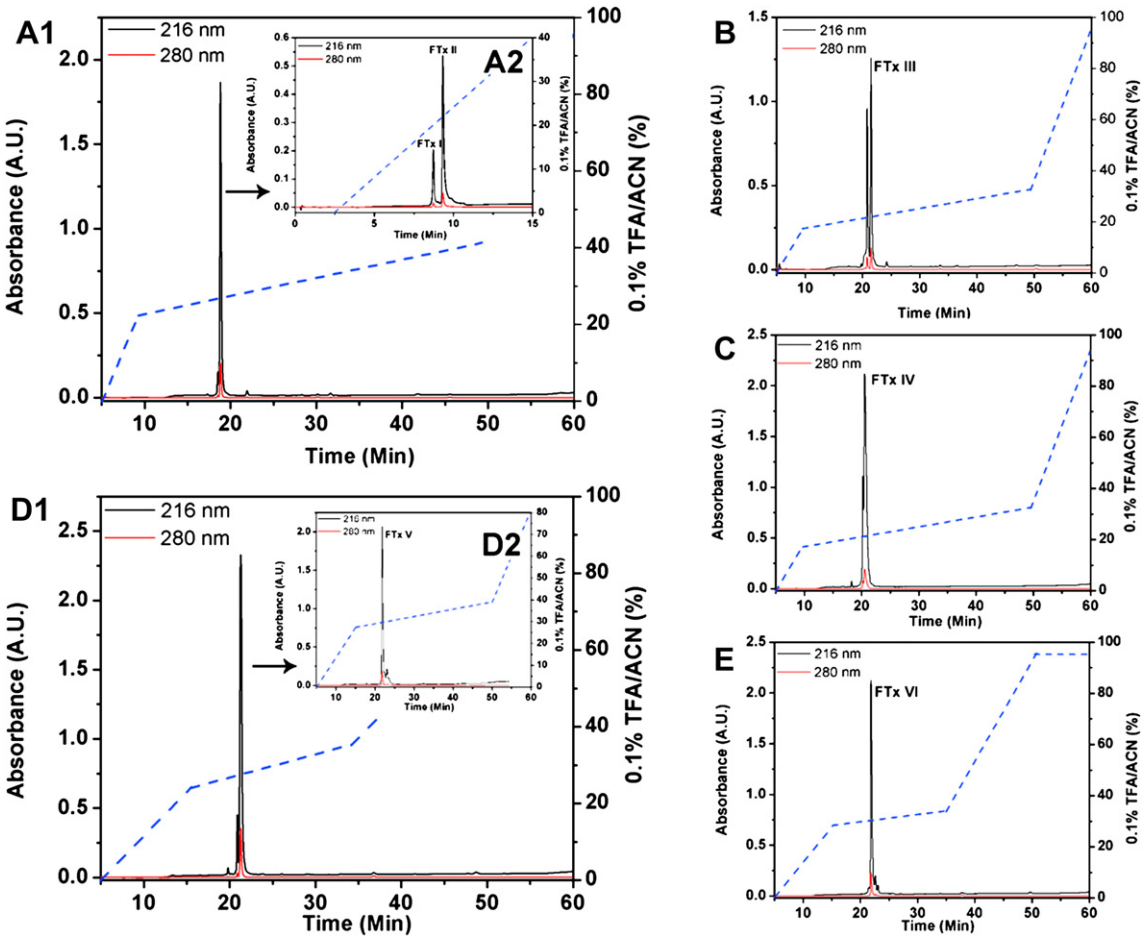

Fig. 2. Isolation of FTxs, neurotoxins from *M. frontalis* venom. (A) FTx I and II were purified after a C_{18} reverse-phase chromatography (A1) followed by Ultra Fast Liquid Chromatography using a Shimpack-XR-ODS column under a linear gradient of acetonitrile at a flow rate of 0.4 mL/min (A2). FTx III (B), IV (C) and VI (E) were purified after previous chromatographic steps using a shallow gradient of acetonitrile (25–35% over 20–60 min) (blue line) on a C_{18} reverse-phase column. FTx V (D) was purified by reverse-phase HPLC on C_{18} column under a shallower gradient of acetonitrile (20–35% over 60 min) (D1) and a final reversed-phase chromatography using a Source 5RTP column under the same conditions used on C_{18} reverse-phase (D2). In all purifications the absorbance was measured at 216 (black line) and 280 nm (red line).

Table 1
Molecular mass and number of cysteine residues of purified components of *M. frontalis* venom.

FTx	MM (native) (Da)	MM (Red_Alk) (Da)	Number Cys residues
I	6642.8	7098.8	8
II	6548.9	7013.2	8
III	7032.8	7496.2	8
IV	7411.3	7996.6	10
V	7166.1	7746.4	10
VI	7327.4	7790.8	8

neuromuscular junction served as its own control. For each experiment, we calculated the mean and 95% confidence limits for these ratios based on Student's distribution. When the range defined by mean ± 95% confidence limit did not contain the value 1.0, we considered the effect statistically significant.

3. Results and discussion

3.1. Isolation and purification of M. frontalis venom components

Fractionation of *M. frontalis* venom by RP-HPLC on a semipreparative C_4 and an analytical C_2C_{18} column yielded more than 30 different components (Fig. 1). The major neurotoxin-rich fractions could be separated at the early stages of the acetonitrile gradient as four intense UV absorbance peaks revealing a number of polypeptides with molecular masses ranging from 6 to 8 kDa. The five most abundant toxins eluted between 30% and 35% acetonitrile were submitted to further purification steps and later named Frontoxins (FTx). Fraction 7 from analytical C_2C_{18} chromatography (Fig. 1B) was also rechromatographed although it was not an intense UV absorbance fraction.

Less abundant fractions FTx together with PLA2-like proteins, L-amino acid oxidase and whey acid protein (WAP) broadly identified and characterized during this work do not belong to the objectives of the present manuscript but considering the lack of information on this venom, particularly considering anti-venom manufacturing, it is worth mentioning in advance the presence of these molecules that will soon be reported elsewhere. Moreover, it is of great importance to point out that each semi-preparative fraction of *M. frontalis* venom was highly populated with a consistent number of distinct isoformes of the major polypeptides purified which imposed a skillful and laborious effort in the optimization of the protein separation techniques available not only to reach the levels of purity required but also to obtain a suitable amount of material required for every FTx isolated.

The polypeptide content of fraction 6 (Fig. 1A) was submitted to two chromatographic steps. The first step was performed using a C_{18} analytical RP-HPLC and the resulting fractions were subsequently applied to a high performance Shim-Pack XR-ODS column on an Ultra Fast Liquid Chromatographer (UFLC) with a flow rate of 0.4 mL/min at 50 °C. Frontoxin I (FTx I) and Frontoxin II (FTx II) showing molecular mass values of $[M+H]^+ = 6642.8$ and 6548.9 Da, respectively (Fig. 2A1 and A2) were isolated as major components among at least four other less abundant isoforms. Similar purification strategies were used with fractions 5, 10 (Fig. 1A) and 7 (Fig. 1B) yielding Frontoxin III (FTx III), Frontoxin IV (FTx IV) and Frontoxin VI (FTx VI) (Fig. 2B, C and E) with corresponding molecular mass values of $[M+H]^+ = 7032.8$, 7411.3 and 7327.4 Da. An additional step of purification, using a Source 5RTP column, was performed to isolate fraction 10, which resulted on Frontoxin V (FTx V), showing a molecular mass of $[M+H]^+ = 7166.1$ Da. Mass spectrometry data of all native Frontoxins are shown in Fig. S1(supplementary material).

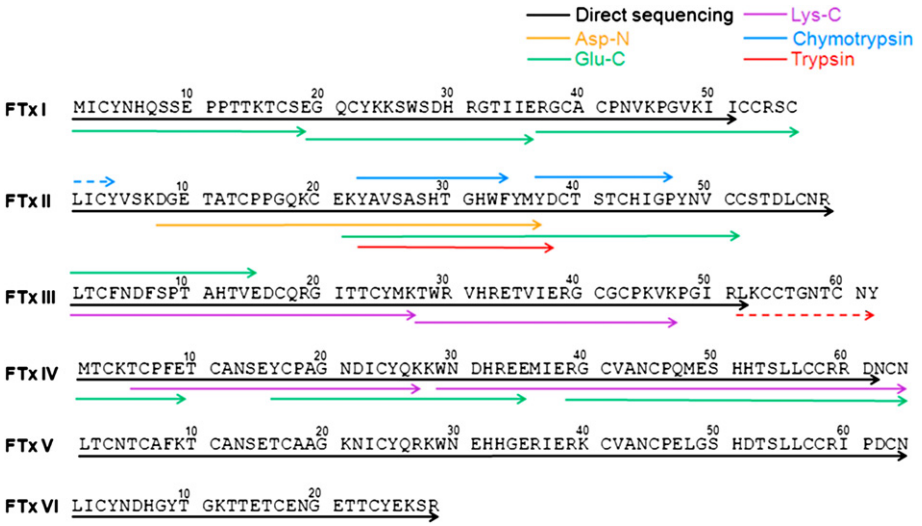

Fig. 3. Determination of the complete primary sequence of FTx II, III, IV and V and parcial sequence of FTx I and VI. The sequences were determined after reduction, alkylation and in some cases after enzimatic cleavage with Asp-N, Lys-C, Glu-C, trypsin and chymotrypsin. The fragments of peptides were purified by HPLC and identified by Edman degradation (solid lines) and MS/MS (dashed lines).

Table 2
Molecular mass and localization in the sequence of peptides obtained from the enzimatic treatment of Frontoxins with different endoproteases. Molecular masses were determined by MALDI-TOF/MS, using an UltraFlex III instrument.

Sample	Enzyme cleavage	Peptide position	Molecular mass		
			Calculated[a]		Experimental
			Native	Red-Alk	
FTx I	Glu-C	1–19	2155.9	2269.9	2270.6
	Glu-C	20–36	2137.0	2194.0	2194.3
	Glu-C	37–56	2107.0	2392.1	2392.3
FTx II	Chymotrypsin	1–4	511.2	568.2	568.3
	Asp-N	8–37	3364.4	3478.4	3478.2
	Glu-C	22–51	3441.4	3612.5	3612.4
	Chymotrypsin	23–34	1362.6	1362.6	1362.9
	Trypsin	23–38	1934.3	1934.3	1934.5
	Chymotrypsin	38–47	1512.5	1683.6	1684.7
FTx III	Glu-C	1–15	1681.7	1738.7	1738.1
	Lys-C	1–27	3081.3	3252.4	3252.4
	Lys-C	28–47	2127.0	2241.1	2240.9
	Trypsin	52–62	1219.4	1390.5	1390.7
FTx IV	Glu-C	1–9	1059.4	1173.4	1173.4
	Lys-C	5–27	2557.0	2785.0	2785.1
	Glu-C	16–35	2600.1	2714.1	2714.1
	Lys-C	29–64	4414.8	4700.0	4700.9
	Glu-C	39–49	1207.5	1321.5	1321.5
	Glu-C	50–64	1758.7	1929.8	1929.8

[a] Calculated molecular mass values were determined by PeptideMass program available on Expasy (http://www.expasy.org/tools/peptide-mass.html).

3.2. Frontoxins primary structure determinations

The half-cystine content of the isolated Frontoxins was evaluated after reduction and S-alkylation. The number of S-alkylated cysteine residues was obtained by comparisons of the molecular mass values of the iodoacetamide treated DTT reduced neurotoxins and their respective native mass forms, divided by the mass increased by alkylation of one thiol group (57.1 Da). The obtained mass value differences indicated the presence of eight half-cystine residues in FTx I, II, III and VI and ten in FTx IV and V (Table 1). Mass spectrometry data of S-alkylated FTx are shown in Fig. S2 (supplementary material).

Native and S-alkylated Frontoxins (I–VI) were submitted to N-terminal sequencing by automatic Edman Degradation. Five different proteases (Asp-N, chymotrypsin, Glu-C, Lys-C and trypsin) were used to confirm the complete primary structure of five neurotoxins isolated from *M. frontalis* and the first 29 N-terminal residues of FTx VI (Fig. 3). After proteolysis of each Frontoxin, the resulting peptides were separated by RP-HPLC, mass analyzed and sequenced by CID fragmentation experiments and Edman Degradation (Table 2). The results were compared with other protein sequences at the National Center for Biotechnology Information (NCBI) database using the MPsrch (http://www.ebi.ac.uk/MPsrch/) and BLAST (Fig. 4). Sequence alignments based on the conserved cysteine residues for all Frontoxins and neurotoxins from available *Micrurus* species (Fig. 3) suggested that these proteins belong to the alpha-neurotoxins, which are non-enzymatic polypeptides rich in disulphide bonds (4 or 5) folded in a three-stranded loops extending from a central core containing four conserved disulphide bonds (Menez, 1998; Tsetlin, 1999). Frontoxins I, II and III belong to the short-chain neurotoxin family (Fig. 4A). Correspondingly, Frontoxins IV and V displayed high similarity scores with the long-chain neurotoxins (Fig. 4B). Long-chain toxins have five disulphide bonds, which bind to both muscle type and neuronal α-7 nicotinic receptor. Generally the additional disulphide bond is located in loop II and it appears to be essential for the acetylcholine receptors binding (Servent et al., 1997). By similarity it is feasible to propose that FTx IV and FTx V might possess a fifth disulfide bond located in the loop I (Cys-6–Cys-11). Proteins with this structural characteristics are classified as weak toxins or long neurotoxin homologs (Utkin et al., 2001a, b) which are capable of interacting with muscle and neural nicotinic acetylcholine receptors like long-chain neurotoxins with varying degree of affinity (Ogay et al., 2005). Only Trp-29 was conserved in *M. frontalis* long-chain toxins reinforcing

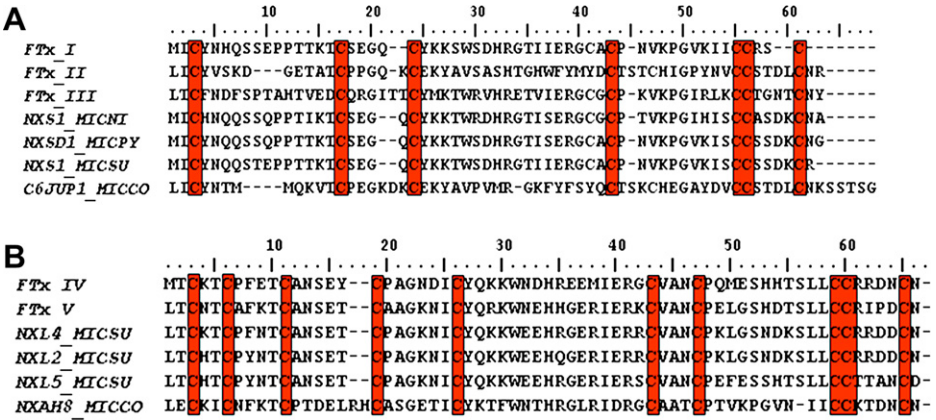

Fig. 4. Amino acid sequence comparison of Frontoxins with neurotoxins from *Micrurus* venom. (A) FTx I, II and III were compared with short-chain neurotoxins. (B) FTx IV and V were compared with long-chain neurotoxins. Gaps (-) have been inserted to maximize similarity. Conserved cysteine residues are shaded. The sequence data were obtained from National Center for Biotechnology Information (NCBI) protein database. MICNI, *Micrurus nigrocinctus*; MICSU, *Micrurus surinamensis*; MICPY, *Micrurus pyrrhocryptus*; MICCO, *Micrurus corallinus*.

the point that each type of toxin utilizes specific residues for receptor recognition. A conserved residue, therefore, even if it is functionally important in one toxin member, may not necessarily be critical in all members of the toxin family (Antil et al., 1999).

The first 35 amino acid residues of FTx I were 100% identical with the partial amino acid sequence of the short-chain neurotoxin of *M. frontalis* published by Francis et al. (1997), suggesting that the two results may refer to the same polypeptide. Due to insufficient amounts of protein in the studied venom, Frontoxin VI was partially N-terminal sequenced (Fig. 3) and disulphide content determination indicated the presence of 8 half-cystines in the sequence (Table 1). These results strongly suggest that FTx VI could be considered the fourth short-chain neurotoxin present in the *Micrurus frontalis* venom.

The protein sequence data reported in this work were deposited in the UniProt Knowledgebase under the accession number(s) P86420, P86421, P86422, P86423, P86424 and P86425.

3.3. Inhibition of miniature endplate potentials

FTxs showed high sequence similarity to α-neurotoxins, which block the nAChRs. We therefore tested their effect on miniature endplate potential. Our results show that after 45 min application, 1 μM FTx II, III and IV decreased MEPP amplitudes by approximately 50% (Figs. 5 and 6). In addition, there was a reduction of approximately 48 ± 29% (mean in ±95% confidence limits) in MEPP frequency for the three toxins tested. The effect on the frequency was probably due to disappearance of the smaller MEPPs into the baseline noise. The same concentration of d-tubocurarine inhibited MEPP amplitude by 55 ± 25% (Figs. 5 and 6), and reduced MEPP frequency by 78 ± 35%. At 2 μM, the short-chain neurotoxins FTx II and III completely abolished MEPPs whereas at this dose FTx IV inhibited MEPPs by only 61 ± 40% (mean ± 95% confidence limits) (Fig. 6). MEPP amplitudes did not recover even after extensive washing (>20 min) with toxin-free Ringer solution (data not shown).

Although the short-chain toxins FTx II and FTx III exhibit similar potency on frog muscle preparation (Fig. 6) they possess very different primary structures, having in common just the conservative Cys residues. Although FTx III contains some of the amino acid residues postulated to be important on nAChR binding, FTx II does not. Clearly, our knowledge of the structural requirements for nicotinic receptor blockade is incomplete.

FTx IV belongs to the superfamily of three-fingered toxins with five disulphide bonds located in the first loop, that can bind to muscle (α1) and to neuronal (α-7) nAChRs. These toxins, also called weak toxins, typically have lower toxicity than α-neurotoxins and are at least partially

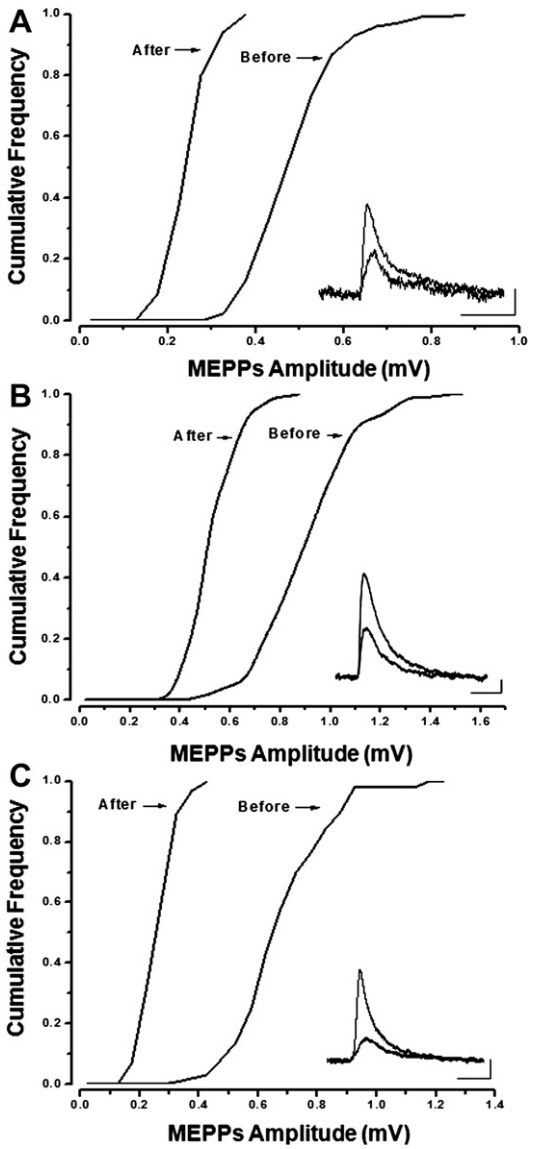

Fig. 5. Frontoxins decrease miniature endplate potentials (MEPPs) at frog neuromuscular junction. Shown are the distributions in MEPP amplitude before and 45 min after application of 1 μM FTx II (A), 1 μM FTx IV (B), or 1 μM d-tubocurarine (C). Insets: average MEPP waveforms before and after treatment. Calibration bars: 10 ms (horizontal), 0.1 mV (vertical).

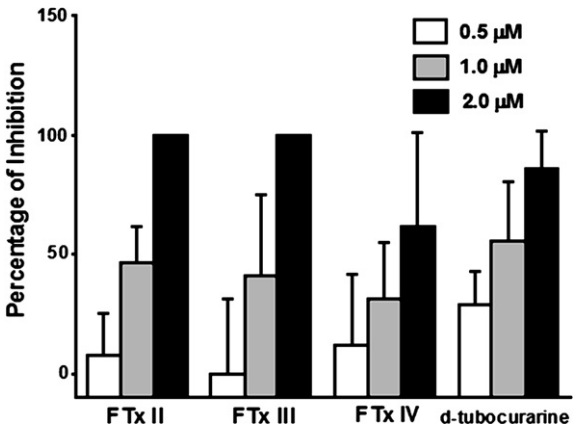

Fig. 6. Dose-dependent inhibition of MEPP size by Frontoxins at the frog neuromuscular junction. Each point represents the mean of the percentage of inhibition obtained after 45 min incubation with FTx II, III, IV or d-tubocurarine. Error bars are the 95% confidence limits of each mean ($N = 3–6$).

reversible (Kini, 2002). However, weak toxins do not appear to be functionally homogeneous class of toxins since both reversible and irreversible inhibition of muscle and neural AChRs has been reported (Servent et al., 2000). It has been postulated that the absence of Asp-31 may be associated with easy reversibility of neuromuscular blockade (Nirthanan et al., 2003). Our data corroborate this hypothesis because FTx IV assayed in this study contains an Asp-31 in primary structure and irreversible inhibited MEPP amplitudes and frequency.

The great variability observed in the primary sequence of neurotoxins within a single species has been attributed to the diversity, availability and susceptibility of prey (Daltry et al., 1996). In Australian elapids may have arisen as response to the variation in AChR targets presented by a wide range of target prey organisms including mammals, amphibians and other reptiles (St Pierre et al., 2007). Coral snakes often have restricted and specialized diets, feeding almost exclusively on other small snakes and/or amphisbaenians. Previous studies of coral snake venoms have suggested a strong predator–prey selective regime as an important determinant of venom composition (Jorge da Silva and Aird, 2001). However, *M. frontalis* venom shows high diversity of three-finger toxins as observed in *M. surinamensis* proteome (Olamendi-Portugal et al., 2008), *M. corallinus* venom gland transcriptome (Leão et al., 2009) and *M. pyrrhocryptus*.(Dokmetjian et al., 2009).

4. Conclusion

This study reports the isolation and characterization of Frontoxins, the first three-finger-like neurotoxins complete primary structures identified in Brazilian coral snake *Micrurus frontalis* venom. Frontoxins I, II, III and VI are short chain toxins with 4 disulphide bonds while FTx IV and V displayed long-chain neurotoxin. Both produces neuromuscular blockage by interaction with postsynaptic nicotinic acetylcholine receptors, probably playing a role in the immobilization of prey. Due to the insufficient amount of purified FTx I, V and VI, these toxins were not submitted to the biological assay.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.toxicon.2010.02.030.

Conflict of interest

The authors do not recognize any conflict of interest regarding any information and/or data presented in this work.

References

Aird, S.D., Jorge da Silva Jr., N., 1991. Comparative enzymatic composition of Brazilian coral snake (*Micrurus*) venoms. Comp. Biochem. Physiol. 99B, 287–294.

Antil, S., Servent, D., Menez, A., 1999. Variability among the sites by which curaremimetic toxins bind to torpedo acetylcholine receptor, as revealed by identification of the functional residues of alpha-cobratoxin. J. Biol. Chem. 274 (49), 34,851–34,858.

Antil-Delbeke, S., Gaillard, C., Tamiya, T., Corringer, P.J., Changeux, J.P., Servent, D., Ménez, A., 2000. Molecular determinants by which a long chain toxin from snake venom interacts with the neuronal alpha 7-nicotinic acetylcholine receptor. J. Biol. Chem. 275 (38), 29549–29601.

Campbell, J.A., Lamar, W.W., 2004. The Venomous Reptiles of Western Hemisphere: coral snakes and sea snakes. Elapidae Family. Cornell University Press, Ithaca, NY.

Cecchini, A.L., Marcussi, S., Silveira, L.B., Borja-Oliveira, C.R., Rodrigues-Simioni, L., Amara, S., Stábeli, R.G., Giglio, J.R., Arantes, E.C., Soares, A. M., 2005. Biological and enzymatic activities of *Micrurus* sp. (Coral) snake venoms. Comp. Biochem. Physiol. A Mol. Integr. Physiol 140, 125–134.

Daltry, J.C., Wüster, W., Thorpe, R.S., 1996. Diet and snake venom evolution. Nature 379, 537–540.

Dokmetjian, J.C., Canto, S., Vinzón, S., Bonino, M.B.J., 2009. Biochemical characterization of the *Micrurus pyrrhocryptus* venom. Toxicon 53, 375–382.

Francis, B.R., Jorge da Silva, N., Seebart, C., Casais e Silva, L.L., Schmidt, J., Kaiser, I.I., 1997. Toxins isolated from the venom of the Brazilian coral snake (*Micrurus frontalis frontalis*) include hemorrhagic type phospholipases A2 and postsynaptic neurotoxins. Toxicon 35, 1193–1203.

Gutiérrez, J.M., Rojas, G., Jorge da Silva Jr., N., Nuñez, J., 1992. Experimental myonecrosis induced by the venoms of South American *Micrurus* (coral snakes). Toxicon 30, 1299–1302.

Hodgson, W.C., Wickramaratna, J.C., 2002. *In vitro* neuromuscular activity of snake venom. Clin. Exp. Pharmacol. Physiol. 29 (9), 807–814.

Jorge da Silva, N., Aird, S.D., 2001. Prey specificity, comparative lethality and compositional differences of coral snake venoms. Comp. Biochem. Physiol. C 128, 425–456.

Katz, B., Thesleff, S., 1957. On the factors which determine the amplitude of the miniature end-plate potential. J. Physiol. 137, 267–278.

Kini, R.M., 2002. Molecular moulds with multiple missions: functional sites in three-finger toxins. Clin. Exp. Pharmacol. Physiol. 29, 815–822.

Leão, L.I., Ho, P.L., Junqueira-de-Azevedo, I.L.M., 2009. Transcriptomic basis for an antiserum against *Micrurus corallinus* (coral snake) venom. BMC Genomics 10, 112.

Menez, A., 1998. Functional architectures of animal toxins: A clue to drug design? Toxicon 36, 1557–1572.

Nirthanan, S., Charpantier, E., Gopalakrishnakone, P., Gwee, M.C.E., Khoo, H.E., Cheah, L.S., Bertrand, D., Kini, R.M., 2002. Candoxin, a novel toxin from *Bungarus candidus*, is a reversible antagonist of muscle (alphabetagammadelta) but a poorly reversible antagonist of neuronal alpha 7 nicotinic acetylcholine receptors. J. Biol. Chem. 277 (20), 17811–17820.

Nirthanan, S., Charpantier, E., Gopalakrishnakone, P., Gwee, M.C.E., Khoo, H.E., Cheah, L.S., Kini, R.M., Bertrand, D., 2003. Neuromuscular effects of candoxin, a novel toxin from the venom of Malayan krait (*Bungarus candidus*). Br. J. Pharmacol. 139, 832–844.

Ogay, A., Rzhevsky, D.I., Murashev, A.N., Tsetlin, V.I., Utkin, Y.N., 2005. Weak neurotoxin from *Naja kaouthia* cobra venom affects haemodynamic regulation by acting acetylcholine receptors. Toxicon 45, 83–99.

Oliveira, J.S., Silva, A.R.B.P., Soares, M.B., Stephano, M.A., Dias, W.O., Raw, I., Ho, P.L., 2000. Cloning and characterization of an α-neurotoxin-type protein specific for the coral snake venom *Micrurus corallinus*. Biochem. Biophys. Res. Commun. 267(3), 887–891.

Oliveira, F.A., Silveira, P.E., Lopes, M.J., Kushmerick, C., Naves, L.A., 2007. Angiotensin II increases evoked release at the frog neuromuscular junction through a receptor sensitive to A779. Brain Res. 1175, 48–53.

Olamendi-Portugal, T., Batista, C.V.F., Restano-Casulini, R., Pando, V., Villa-Hernandez, O., Zavaleta-Matínez-Vargas, A., Salas-Arruz, M.C., Veja, R. C.R., Becerril, B., Possani, L.D., 2008. Proteomic analysis of the venom from the fish eating coral snake *Micrurus surinamensis*: novel toxins, their function and phylogeny. Proteomics 8, 1919–1932.

Rosso, J.P., Vargas-Rosso, O., Gutiérrez, J.M., Rochat, H., Bougis, E.P., 1996. Characterization of alpha-neurotoxin and phospholipase A2 activities from *Micrurus* venoms. Eur. J. Biochem. 238, 231–239.

Roze, J.A., 1996. Coral Snakes of the Americas. Biology, Identification and Venoms. Struik Publishing Co., Malabar, FL.

Serafim, F.G., Reali, M., Cruz-Höfling, M.A., Fontana, M.D., 2002. Action of *Micrurus dumerilli carinicauda* coral snake venom on the mammalian neuromuscular junction. Toxicon 40, 167–174.

Servent, D., Winckler-Dietrich, V., Hu, H.Y., Kessler, P., Drevet, P., Bertrand, D., Ménez, A., 1997. Only snake curaremimetic toxins with fifth disulfide bond have high affinity for the neuronal a7 nicotinic receptor. J. Biol. Chem. 272 (39), 24279–24286.

Servent, D., Antil-Delbeke, S., Gaillard, C., Corringer, P., Changeux, J.P., Ménez, A., 2000. Molecular characterization of the specificity of interactions of various neurotoxins on two distinct nicotinic acetylcholine receptors. Eur. J. Biochem. 393, 197–204.

St Pierre, L., Fischer, H., Adams, D.J., Schenning, M., Lavidis, N., de Jersey, J., Masci, P.P., Lavin, M.F., 2007. Distinct activities of novel neurotoxins from Australian venomous snakes for nicotinic acetylcholine receptors. Cell. Mol. Life Sci. 64, 2829–2840.

Tsetlin, V., 1999. Snake venom alpha-neurotoxins and other 'three-finger'proteins. Eur. J. Biochem. 264, 281–286.

Utkin, Y.N., Kaukhtina, V.V., Kryukova, V., Chiodini, F., Bertrand, D., Methfessel, C., Tsetlin, V.I., 2001a. Weak Toxin from *Naja kaouthia* is a nontoxic antagonist of a7 and muscle-type nicotinic acetylcholine receptors. J. Biol. Chem. 276 (19), 15810–15815.

Utkin, Y.N., Kaukhtina, V.V., Maslennikov, I.V., Eletsky, A.V., Starkov, V.G., Weise, C., Franke, P., Hucho, F., Tsetlin, V.I., 2001b. First tryptophan-containing weak neurotoxin from cobra venom. Toxicon 39, 921–927.

Vital-Brazil, O., Fontana, M.D., 1983/1984. Ações pré-juncionais e pós-juncionais da peçonha da cobra coral *Micrurus corallinus* na junção neuromuscular. Mem. Inst. Butantan 47/48, 13–26.

Vital-Brazil, O., 1987. Coral snake venoms: mode of action and pathophysiology of experimental envenomation. Rev. Inst. Med. Trop. São Paulo 29 (3), 119–126.