Isolation and molecular cloning of beta-neurotoxins from the venom of the scorpion Centruroides suffusus suffusus

ARTICLE *in* TOXICON · FEBRUARY 2011

Impact Factor: 2.49 · DOI: 10.1016/j.toxicon.2011.02.006 · Source: PubMed

Toxicon 57 (2011) 739–746

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Isolation and molecular cloning of beta-neurotoxins from the venom of the scorpion *Centruroides suffusus suffusus*☆

Gerardo Pavel Espino-Solis [a], Georgina Estrada [a], Timoteo Olamendi-Portugal [a], Elba Villegas [b], Fernando Zamudio [a], Sandrine Cestele [c], Lourival D. Possani [a], Gerardo Corzo [a,*]

[a] *Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, UNAM, Apartado Postal 510-3, Cuernavaca, Morelos 61500, Mexico*
[b] *Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Av. Universidad 2001, Cuernavaca, Morelos 62210, Mexico*
[c] *CNRS UMR 6097, IPMC-IN2M, 660 Route des Lucioles, 06560 Valbonne, France*

ARTICLE INFO

Article history:
Received 14 October 2010
Received in revised form 27 January 2011
Accepted 8 February 2011
Available online 15 February 2011

Keywords:
Beta-toxin
Centruroides suffusus suffusus
cDNA
Scorpion
Sodium channel

ABSTRACT

This communication reports the identification and characterization of two new toxins from the venom of the scorpion *Centruroides suffusus suffusus*, named: CssVIII and CssIX, according to the original nomenclature of toxins previously described for this scorpion. The isolation was obtained by means of two chromatographic steps, and a cDNA library was used to fully identify their precursors. CssVIII and CssIX contain signal peptides of 19 and 17 amino acid residues, and mature peptides of 66 and 65 residues, respectively. Intracranial injections into mice of both purified toxins showed toxicity results similar to those found for toxins CssII and CssIV. Additionally, they compete with the parent toxin CssIV, in binding and displacement experiments, conducted with brain synaptosomes showing nanomolar affinities. These results strongly support the conclusion that they are new β-neurotoxins and certainly would be of the interest of researchers in the field of venomics for studying sodium channels.

1. Introduction

Scorpion neurotoxins that affect the voltage-gated sodium channels (Na_v) are important molecules for discriminating Na_v sub-types, and they are also essential molecules to study the structure-function of Na_v (Catterall, 1992). Also, scorpion neurotoxins are the most important venom components responsible for human envenomation. For many years, the sting of *Centruroides suffusus suffusus* (*C. suffusus*) and other related scorpions have been a problem of Public Health in Mexico (Vega Franco and Lia Jaime, 1966). Several years ago, the soluble venom of the scorpion *C. suffusus* was analyzed, and seven neurotoxins (CssI to CssVII) were isolated (Martin et al., 1987) where CssII is the major toxic component of this venom (Estrada et al., 2007; Hernandez-Salgado et al., 2009). In this respect, from all described neurotoxins of this scorpion, only CssII and CssIV have been pharmacologically and structurally well characterized (Cestele et al., 1998; Cestele and Caterall, 2000; Cohen et al., 2005; Schiavon et al., 2006) and all of them belong to the classification of β-neurotoxins. Actually, CssII was the toxin taken as model for the definition of the β-type of scorpion toxins (β-ScTx), as originally described by (Jover et al., 1980a,b). The main effect of the β-ScTx is to allow the opening of Na_v channels at more negative potentials than that in normal conditions, in opposition to the toxins that prolong the action potential by maintaining the channels open for longer time, which were called α-scorpion toxins (Cestele et al., 1998; Cestele and Caterall, 2000).

Abbreviations: TFA, trifluoroacetic acid; ESI, electro spray ionization.

☆ The transcripts and protein sequences reported in this paper has been submitted to the Gene Bank Database under the accession numbers HQ262493 and HQ262494 for CssVIII and CssIX, respectively.

* Corresponding author. Tel.: +52 777 317 1209; fax: +52 777 317 2388.
E-mail address: corzo@ibt.unam.mx (G. Corzo).

0041-0101/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2011.02.006

The original method described for the purification of those seven peptides, from the soluble venom of *C. suffusus*, included five steps of chromatographic fractionation (Martin et al., 1987). Furthermore, a relative high quantity of starting scorpion venom (>1 g) was used for the isolation and biological identification of those neurotoxins (Martin et al., 1987). Here, a simplified strategy is described, using small amounts of soluble venom of this scorpion. Mass spectrometry guided-analysis and two-steps of HPLC provided a means of obtaining pure toxins: CssII and CssIV, which are well-studied neurotoxins from this venom, and they have been used as valuable tools for clinical and scientific research. The new strategy also provided the possibility of identifying two new toxins, here named CssVIII and CssIX, which certainly will be of the interest to those researchers working in the field of scorpion neurotoxins and searching for new Na_v ligands.

2. Materials and methods

2.1. Biological materials

The scorpions were collected from the state of Durango in Mexico and maintained alive in good conditions following the Mexican official requirements and licenses (SEMARNAT FAUT-0184 and MOR-IN-166-0704). *C. suffusus* crude venom was obtained by electrical stimulation, and it was frozen and stored at −20 °C until use.

2.2. Isolation and sequencing procedures

C. suffusus lyophilized crude venom (10 mg) was dissolved in 500 μl of 0.1% aqueous TFA, and the insoluble material was removed by centrifugation at 14,000 g for 5 min. The soluble venom was used directly for high performance liquid chromatography (HPLC) fractionation using a reverse-phase semi-preparative C_{18} column ($5C_{18}$MS, 10 × 250 mm Nacalai-Tesque, Japan) equilibrated in 0.1% trifluoroacetic acid (TFA), and eluted with a linear gradient of acetonitrile from 0 to 60% in 0.1% TFA, run for 60 min at a flow rate of 2 ml/min. Effluent absorbance was monitored at 280 nm. Fractions were collected in 1.5 ml tubes and dried out under vacuum. The HPLC fractions of interest were further purified by cation-exchange chromatography on TSK-gel sulfopropyl column (SP-5PW, 4.6 × 75 mm, Tosoh, Japan). The fractions were diluted to 200 μl with 20 mM ammonium acetate in 1 M acetic acid pH 2.9, and they were further fractionated using a linear gradient of 2 M ammonium acetate in 1 M acetic acid pH 5.9 from 0 to 50%, in 50 min (1 ml/min). Eluted proteins were monitored by conductivity. If required, a final step purification, essentially for extra desalting, was performed in a C_{18} reverse-phase column (4.6 × 250 mm, Nacalai-Tesque, Japan) equilibrated in 0.1% TFA, and eluted with a linear gradient of acetonitrile from 20 to 60% in 0.1% TFA, run for 60 min at a flow rate of 1 ml/min. Effluent absorbance was monitored at 280 nm. The primary structure of peptides was determined by direct sequencing of the native toxin (Edman degradation) using an LF3000 Protein Sequencer (Beckman, CA, USA), as originally described for other peptides (Zamudio et al., 1992).

2.3. Enzymatic digestions

The peptides were subjected to enzymatic hydrolysis with type XVII-B endoproteinase Glu-C from *Staphylococcus aureus* V8 (Roche, Indianapolis, IN, USA), which was carried out in 0.1 M sodium bicarbonate buffer, pH 7.6 at 37 °C for 16 h, using a 1:20 (w/w) enzyme to substrate ratio. The endoproteinase digest was fractionated by reverse-phase HPLC using a C_{18} column (4.6 × 250 mm, Nacalai-Tesque, Japan) and a linear gradient of acetonitrile in 0.1% aqueous TFA. The endoproteinase fractions were analyzed by electro spray ionization mass spectrometry (ESI-MS) and sequenced by Edman degradation, as earlier described by our group (Corzo et al., 2008; Olamendi-Portugal et al., 2008).

2.4. Mass spectrometry

The mass identity of all isolated peptides and the peptide fragments from the enzymatic cleavage was verified by ESI-MS using a Finnigan LCQ^{DUO} ion trap mass spectrometer (San José, CA, USA).

2.5. cDNA library construction and gene cloning

2.5.1. RNA isolation

The total RNA from a single telson (last post-abdominal segment), which contains a pair of venom glands of a male scorpion of the species *C. suffusus* was extracted with the SV Total RNA Isolation System (Promega Co., Madison, WI, USA). The total RNA was obtained following the protocol described by the manufacturer. The RNA was treated with Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure from the full-length mRNA, leaving a 5′-monophosphate. A 45 length RNA-adapter oligonucleotide was ligated to the RNA population using the T4 RNA ligase. This procedure guarantees that the RNA-adapter could not be ligated to the dephosphorylated RNA because it lacks the 5′-phosphate necessary for RNA ligation. Furthermore, the total RNA was incubated with TAP at 37 °C for 1 h. Then, the TAP-treated RNA was ligated at the 5′RACE Adapter and the reaction was carried out with T4 RNA ligase (2.5 U/μl) and incubated at 37 °C for 1 h. The ligated RNA was treated with long template reverse transcriptase (Roche, Mannheim, Germany) to synthesize the cDNA using random hexamers. The reaction was carried at 42 °C for 1 h.

2.5.2. RACE 5′ (Rapid amplification of cDNA 5′ ends)

Single-stranded cDNA was synthesized from the scorpion glands TAP-treated RNA using random hexamers and a long template reverse transcriptase (Roche, Mannheim, Germany). The transcription reaction was extracted with phenol–chloroform and filtered through a G-50 spin column. The 5′-end was amplified by nested PCR using the complement 5′-Adapter Primer and the corresponding reverse primers (Table S1 in Supplementary Material Section).

2.5.3. RACE 3′ (Rapid amplification of cDNA 3′ends)

The 3′-end was amplified by nested PCR using the FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA) and the corresponding forward primers listed (Table S1 in Supplementary Material Section).

2.5.4. Gene cloning

Based on the amino acid sequences of the neurotoxins CssVIII and CssIX obtained by Edman degradation, specific oligonucleotides were designed for PCR reactions using cDNA material as template. A rapid amplification of the cDNA 3′ends was performed using the Fast Start High Fidelity PCR System (Roche, Mannheim, Germany) and 3′-adapter primers provided by the FirstChoice RLM-RACE kit (10 μM outer primer 5′-GCGAGCACAGAATTAATACGACT-3′ and 10 μM inner Primer 5′-CGCGGATCCGAATTAATACGAC TCACTATAGG-3′), (Ambion, Austin, TX, USA). Also specific forward distal and proximal primers were used (Table S1 in Supplementary Material Section). The PCR reaction was incubated at 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s per 35 cycles, followed by a 7 min final step at 72 °C. PCR products were purified by agarose gels. The amplified DNA was extracted with QIAquick Gel Extraction Kit (QIAGEN, USA), and then ligated into the pGEM®-T Easy Vector System (Promega, WI, USA). The ligation reaction was used to transform competent *E. coli* DH5-α cells. The cDNA of positive clones were sequenced by a rapid amplification of the cDNA 5′ ends. The complete clone was obtained using the DNA extracted from the 5′ end library as templates. The 5′-adapter primer and a specific reverse distal and proximal primers were used for the PCR amplification. The final cloning and sequencing were obtained as previously described (Espino-Solis et al., 2008; Corzo et al., 2009; Mendoza-Vargas et al., 2009).

2.6. Binding and displacement experiments

2.6.1. Neuronal membrane preparation

Rat brain synaptosomes were prepared from adult albino Winstar rats, essentially as earlier described (Cestele et al., 1995, 1998). Homogenization of rat brain was performed in 0.32 M sucrose buffer containing 20 mM HEPES-Tris, pH 7.4. Following centrifugation at 1000 g for 5 min, the supernatant was re-centrifuged at 10,000 g for 20 min (*P*2 fraction). The pellet was resuspended in the buffer used for the homogenization.

2.6.2. Radioiodination

CssII and CssIV were radio-iodinated by lactoperoxydase as previously described (Cestele et al., 1998) using 1 nmol of toxin and 1 mCi of carrier-free $Na^{125}I$. The mono-iodotoxin was purified as described previously (Cestele et al., 1997).

2.6.3. Binding assays

Equilibrium competition assays were performed using increasing concentrations of unlabeled toxin in the presence of a constant low concentration of the radioactive toxin. The binding medium composition was; Choline-Cl, 130 mM; KCl, 5.4 mM; Glucose, 5.5 mM; HEPES 50 mM and BSA 2 mg/ml at pH 6.5. Rat brain synaptosomes were suspended in 0.2 ml binding buffer containing either ^{125}I-CssII or ^{125}I-CssIV. After incubation 1 h at room temperature, the reaction mixture was diluted with 3 ml ice-cold wash buffer and filtered through GF/C filters under vacuum. Filters were rapidly washed twice with additional 3 ml buffer. Non-specific binding was determined in the presence of either 70 nM unlabeled CssII or 70 nM unlabeled CssIV, and consists typically of 5–9% of the total binding. Each experiment was performed at least three times.

2.7. Biological activity

The protocol used for assaying the activity of these peptides in vivo, using the mice model, was followed according to the guidelines of our Institute Committee of Animal Welfare, keeping the number of animals to a minimum required to validate the experiments. Male mice (CD-1, 20 g body weight) were tested by intracranial injection. Pure peptides CssII, CssIV and CssVIII and CssIX were diluted up to 5 μl (for intracranial inoculation) with bovine serum albumin (BSA) solution (20 mg/ml in 0.9% NaCl). The injection, with a 10 μl microsyringe fitted with a glass capillary, was performed mid-way between the left eye and the left ear (intracranial). Negative controls were done with saline solution only and positive controls with the neurotoxic scorpion peptide LqhIV isolated in our laboratory (Corzo et al., 2001). Mice were observed for toxicity symptoms up to 24 h. The amounts used, to find out the LD_{100}, were 10, 20, 40, 60, 80 and 100 ng per mouse ($n = 2$, strain CD-1, 20 g body weight).

3. Results

3.1. Purification and amino acid sequence determination

The soluble venom of *C. suffusus* was fractionated using reverse-phase HPLC (Fig. 1). Protein fractions were manually collected and vacuum-dried. A complete proteomic analysis of each fraction separated from the HPLC column (sixty-two in total) was performed by mass spectrometry analysis in order to identify which of them could have molecular masses expected for the toxic peptides under study. Protein fractions numbers 35 and 40 (indicated #35 and #40 in Fig. 1) contained the molecular masses of 7537.6 and 7601.6 Da, which were the expected masses for CssII and CssIV, respectively. In the venom sample we used, none of the fractions containing the molecular masses of any of the other five neurotoxins CssI, CssIII, CssV, CssVI or CssVII previously reported by Martin et al. (1987), were found. In order to obtain homogeneous peptides, the two venom fractions containing the peptides CssII and CssIV were further separated by cation HPLC chromatography. The fraction containing CssII yielded three peptides, from which one of them has the molecular mass of 7537.6 Da as expected for this toxin (Fig. 1, inset A). The fraction containing CssIV yielded four additional pure peptides (Fig. 1, inset B). The first was the already known CssIV, the second was a peptide with a molecular mass of 13,596 Da, a protein of unknown function, the third one was a peptide with a mass of 7588.6 Da (named CssVIII), and the last one was a peptide with a mass of 7524.9 Da (named CssIX). The final yields of CssII, CssIV, CssVIII and CssIX were 2.3, 0.6, 0.8 and 0.8% of the whole soluble venom, respectively. The full amino acid sequence of these four peptides was obtained by a combination of direct Edman degradation, mass spectrometry analysis and nucleotide sequencing of the respective cloned genes, as shortly described below. The identification of CssII and CssIV was performed by direct N-terminal sequencing.

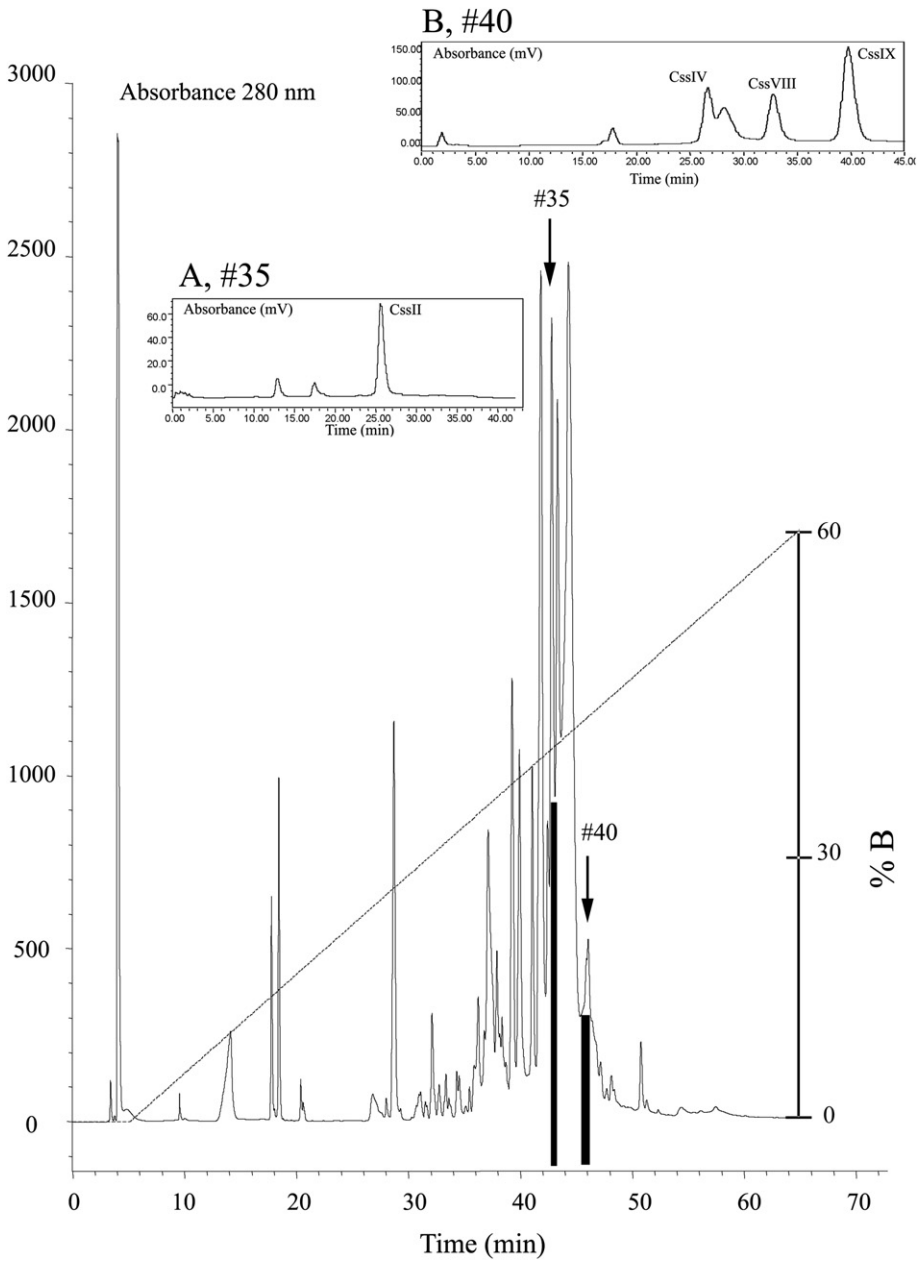

Fig. 1. Chromatographic separation of *C. suffusus* venom. The soluble venom obtained after dissolving and separating 10 mg of crude venom from *C. suffusus* was applied to a semi-preparative C_{18} reverse-phase column, and eluted with a linear gradient. Inset A, cation-exchange HPLC profile of the reverse-phase fraction number 35 containing the β-neurotoxin CssII. Inset B, cation-exchange HPLC profile of the reverse-phase of fraction number 40 containing the β-neurotoxins CssIV, CssVIII and CssIX (see separation conditions in the section of Material and Methods).

The first 15 amino acid residues were identified given the sequence expected for both peptides, which together with the molecular masses found by mass spectrometry results, were taken as sufficient indication that these two peptides were obtained in pure form. Apart from the confirmation of the presence of CssII and CssIV in the sample used for this work, the complete amino acid sequence of the new CssVIII and CssIX neurotoxins were determined. For this purpose, pure peptides were alkylated and sequenced by Edman degradation. The amino acid sequence found for the first 25 residues of the novel CssVIII and CssIX peptides gave the primary amino acid composition expected from the respective cloned genes. Further confirmation of the full sequence was obtained by sequencing sub-peptides obtained by HPLC after digestion with Glu-C or Lys-C endopeptidases, which gave unequivocal identification of amino acids at different positions (Table 1). The extend of direct sequencing with identical amino acids as expected from the cloned genes and the exact molecular mass found by mass spectrometry results was taken as sufficient evidence that indeed the correct primary structure was obtained for both CssVIII and CssIX.

Table 1
Direct and partial N-terminal sequences of CssVIII and CssIX after enzymatic digestion.

Peptide	Sequence
CssVIII	KEGYLVNSYTG**C**KFE**C**FKLGDNDY**C**KRE**C**KQQYGKGSGGY**C**YAFG**C**XXXXXXXQAVVWPLXXXXX
Direct	KEGYLVNSYTG**C**KFE**C**FKLGDNDY**C**KRE**C**KQQYGKG
Glu-C	**C**FKLGDNDY**C**KR **C**KQQYGKGSGGY**C**YAFG**C** QAVVWPL
CssIX	KDGYPMDHKG**C**KIS**C**VINNKY**C**ETE**C**VTVLKGXXGY**C**YFXXLA**C**YCEGLPNWAKVWDRXXXXXXX
Direct	KDGYPMDHLG**C**KIS**C**VINXKY**C**ETE**C**VTVLKGXXGY
Glu-C	TE**C**VTV GLPNWAKVIDR
Lys-C	IS**C**VIN Y**C**ETE GY**C**YF LA**C**Y**C**EGLPN

The underlined letter X has been introduced to show unidentified amino acids during N-terminal sequencing.

3.2. Gene cloning

The cDNA library prepared from the venom glands of one scorpion (it is worth recalling that one telson has a pair of venom glands) was used to clone the genes responsible for the peptides isolated and chemically characterized, especially those of CssVIII and CssIX. Since stretches of the amino acid sequences of CssVIII and CssIX were known by Edman degradation, degenerate oligonucleotides were designed and used to amplify the nucleotide sequence of each peptide transcript. Several sequences with the expected size were obtained from PCR reactions using such cDNA library. To find out the complete clones of CssVIII and CssIX, a random screening of positive colonies was conducted. The CssVIII and CssIX clones were found in several bacterial colonies. After proper nucleotide sequencing, it was found that the messenger coding for CssVIII codifies for 87 amino acid residues from which 19 residues are part of the signal peptide, and 67 residues correspond to the mature protein, from which the last one is removed after posttranslational modification of CssVIII, given a total of 66 amino acids. Therefore, for CssVIII the amino acid residue in position 67 is the donor of the amino group for the last one, which is asparagine amide (Fig. 2). Table 2 shows the complete 66 amino acid sequence of the mature peptide CssVIII, thus matching the expected molecular mass (7589.6 Da) with that experimentally determined (7588.6 Da). It can be seeing that the molecular mass found for the pure peptide has 1.0 unit of mass less than expected, supporting the idea of a C-terminal asparagine amidated. It is also worth noting that the amino acid before the stop codon in position 67 is glycine, known to be a donor of amides for amidation of peptides at the C-terminal region (Possani et al., 2000). The molecular mass found is consistent with the idea that CssVIII is folded by four disulfide bridges, as seen in other active multiple cysteine-containing peptide toxins.

Concerning the gene coding for the other peptide, CssIX, it was found that it codifies for an 82 amino acid residues sequence, from which 17 residues are part of the signal peptide, and 65 residues correspond to the mature protein (Fig. 2). The molecular weight theoretically expected for the first 65 amino acids of the mature peptide CssIX is 7524.8 Da, in close agreement with the experimental value found, which was 7524.9 Da. For this peptide the C-terminal amino acid (alanine) is not amidated (Table 2). The 8 cysteine found are forming 4 disulfide bridges, based on the molecular mass results.

CssVIII shares high identity (89–98%) to five neurotoxins from the species of the genus *Centruroides,* whereas CssIX shows low identity (56%) to neurotoxins from the genus *Centruroides*. The sequence similarity of CssIX is greater (59–62%) when compared with toxins from scorpions of the species belonging to the genus *Tityus* (Table 3).

3.3. Biological activity in vitro

Initially, lethality tests were performed by injecting mice, via intracranial, with all four isolated neurotoxins CssII, CssIV, CssVIII and CssIX. The calculated LD_{100} by intracranial injection for CssII, IV, VIII and IX were 60, 60, 60 and 80 ng per 20 g of mice strain CD-1, respectively.

In order to figure out if the novel two neurotoxins belong to the pharmacological classification of β-neurotoxins, competitive binding and displacement experiments were performed. β-neurotoxins bind to the site-4 of Na_v; therefore, the β-neurotoxin CssIV, the most studied toxin from this venom, was used as a probe for binding to brain synaptosomal preparations, a rich source of Na channels. It is worth recalling that this was the prototype toxin and the experimental protocol initially used for the definition of β-ScTX (Jover et al., 1980a,b). CssIV was radio-labeled with I^{125} and competitive binding experiments were performed with the four native (non labeled) peptides CssII, CssIV, CssVIII and CssIX. The IC_{50} values were 30.90 ± 0.80 nM for CssII, 0.33 ± 0.05 nM for CssIV, 0.08 ± 0.02 nM for CssVIII and 0.15 ± 0.06 nM for CssIX, as estimated from the results of Fig. 3. These data are distinctive from the IC_{50} value found for CssII (Fig. 3). One of the particularities of the β-neurotoxins from *C. suffusus* is their specificity toward subtypes of Na_v ion-channels. CssIV binds with high affinity to the three sodium channels isoforms present in rat brain synaptosomes: $Na_v1.1$, $Na_v1.2$ and $Na_v1.6$, whereas it has been demonstrated than CssII binds specifically to $Na_v1.6$ (Schiavon et al., 2006; Estrada et al., 2007). Those data explain why the apparent IC_{50} of CssII is two orders of magnitude higher than the cold CssIV. CssII competes with ^{125}I-CssIV bound on $Na_v1.6$ channels present on rat brain synaptosomes but not, or with very low affinity, to $Na_v1.1$ and $Na_v1.2$. Our binding data show that the new toxins CssVIII and CssIX compete with high affinity with ^{125}I-CssIV bound on rat brain synaptosomes. The data obtained support the assumption that CssVIII and CssIX bind to receptor site-4, and they are classified as β-scorpion toxins. Additionally, the data shown here suggest that CssVIII and

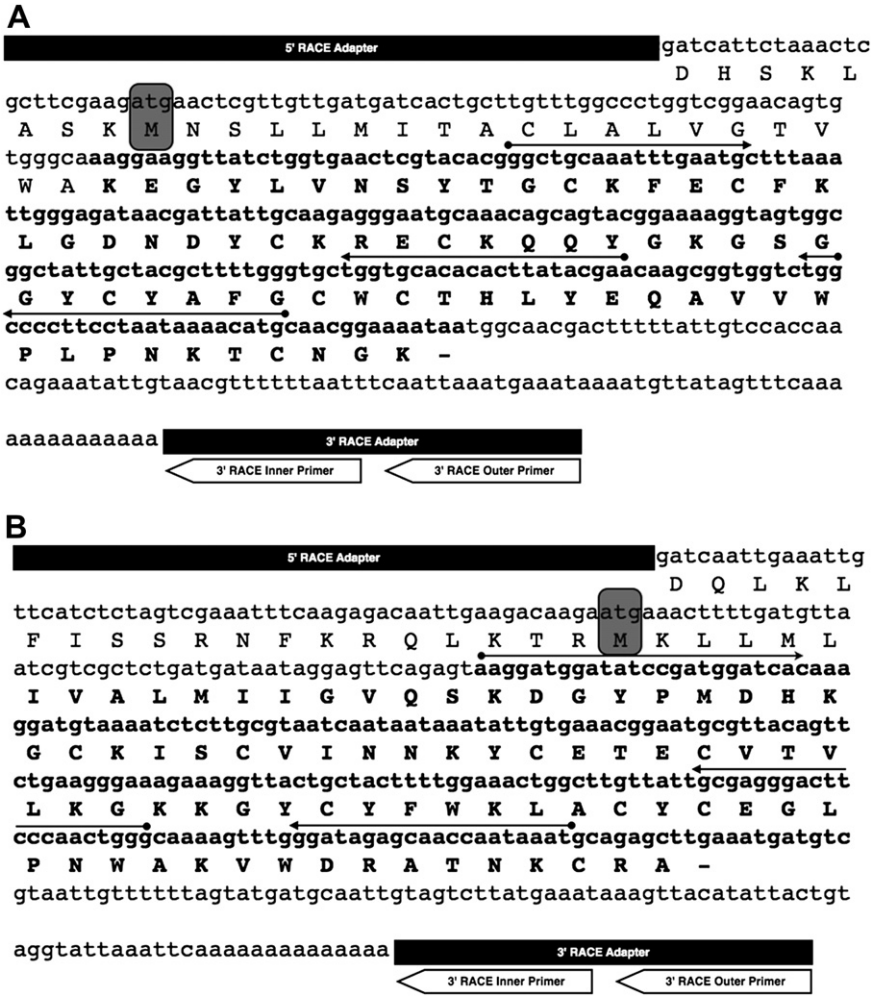

Fig. 2. The cDNA and deduced amino acid sequences of CssVIII and CssIX. A) The cDNA of CssVIII encodes a mature peptide of 66 residues (bold capital letters). The last Asn–Gly pattern (residues 66–67) of the mature peptide are required for posttranslational processing of the C-terminal amidation of Asn. B) The cDNA of CssIX encodes a mature peptide of 65 residues (bold capital letters). Amino acids are indicated by one-letter symbols. The black bars at the ends of the sequences indicate the position of adapter primers that were used to isolate the genes. The sequences of the gene as well as mature protein are highlighted in black, while the untranslated regions (UTR's) of the gene are shown in normal text. The ATG codon (methionine) is highlighted in gray indicating the start of the signal peptide. The stop codons TAA (A) and TGA (B) are shown by a dash. Arrows on the nucleotide sequences indicate the position, direction and sequence of primers used to isolate each gene, in combination with 3′ and 5′ adapter primers shown in white arrows.

CssIX, as CssIV may bind with high affinity to the three sodium channels isoforms present on rat brain synaptosomes.

4. Discussion

The discovery of novel ligands capable of binding to sodium channels is fundamental for comparative studies aimed at elucidating the important amino acids implicated in the binding surface of toxins to ion-channels. Usually, these studies are carried out by means of the double mutant analysis strategy, where substitutions are made in amino acids corresponding to different positions of both: the toxins and the corresponding ion-channels (Miller, 1995; Rodriguez de la Vega et al., 2003). However, the

Table 2
Sequences of mammalian β-neurotoxins from the venom of *C. suffusus*.

Peptide	Sequence						MWexp.	MWcalc	Accs
CssII	KEGYLVSKST	G**C**KYE**C**LKLG	DNDY**C**LRE**C**K	QQYGKSSGGY	**C**YAFA–**C**W**C**TH	LYEQAVVWPL	PNKT**C**N*	7537.6	7538.6	P08900
CssIV	KEGYLVNSYT	G**C**KFE**C**FKLG	DNDY**C**LRE**C**R	QQYGKGSGGY	**C**YAFG–**C**W**C**TH	LYEQAVVWPL	PNKT**C**N*	7601.6	7602.6	P60266
CssVI	KEGYLVNSYT	G**C**KFE**C**FKLG	DNDY**C**K̄RE**C**K̄	QQYGKSSGGY	**C**YAFG–**C**W**C**TH	LYEQAVVWPL	PNKT**C**N*	NF	7619.6	P60267
CssVIII	KEGYLVNSYT	G**C**KFE**C**FKLG	DNDY**C**KRE**C**K	QQYGKGSGGY	**C**YAFG–**C**W**C**TH	LYEQAVVWPL	PNKT**C**N*	7588.6	7589.6	HQ262493
CssIX	KDGYPMDHK-	G**C**KIS**C**VINN	–KY**C**ETE**C**V	TVLKG-K̄KGY	**C**YFWKLA**C**Y**C**EG	LPNWAKVWDR	ATNK**C**RA	7524.9	7524.8	HQ262494
The sequences of CssI, CssIII CssV, CssVII were not available										

Gaps (-) have been introduced to enhance similarities. *Means amidated C-terminal. NF means not found. Acss means accession number.

Table 3
Identity of neurotoxins CssVIII and CssIX to known neurotoxins from the venom of scorpions from the genus *Centruroides* and *Tityus*.

Toxin	Sequence	Identity (%)	Accs
CssVIII	**KEGYLVNSYTGCKFECFKLGDNDYCKRECKQQYGKGSGGYCYAFGCWCTHLYEQAVVWPLPNKTCN**	100	HQ262493
SCX6_CENSU	-------SY----F--F--------K---K-----SS----------PN---N	98	P60267
SCX4_CENSU	-------SY----F--F--------L---R-----GS----------PN---N	96	P60266
SCX4_CENNO	-------SY----Y--F--------L---K-----GA----------KN---NGK	93	P45662
SCX1_CENLI	-------HS----Y--F--------L---R-----GA----------PN---S	89	P18926
SCX1_CENII	-------HS----Y--Y--------L---K-----GA----------PK---N	89	P59897
CssIX	**KDGYPMDHKGCKISCVIN-NKYCETECVTVLKGKKGYCYFWKLACYCEGLPNWAKVWDRATNKCRA**	100	HQ262494
SCX1_TITST	KE--LMDHE---LS-FIRPSG--GRE-TLKK--SS----A--P----Y-LPNWV-V-DRA--K-GKK	62	P56612
SCX7_TITBA	KE--LMDHE---LS-FIRPSG--GSE-KIKK--SS----A--P----Y-LPNWV-V-DRA--K-GKK	62	P56611
SCX7_TITSE	KE--LMDHE---LS-FIRPSG--GRE-GIKK--SS----A--P----Y-LPNWV-V-DRA--K-GKK	62	P15226
B8XH11_BUTOS	AD--LKGHD---LA-VVN-NK--NKE-QAEG--NY---YK--L----E-LSE-K-T-KPE--K-RSD	59	B8XH11
SCX7_TITCO	KE--AMD-E---LS-FIRPSG--GR--GYKK--SS----A--P----Y—PNWV--V-ERA--R-GKK	59	Q5G8B8
SCXB_CENNO	RD--PVDEK---LS-LIN-DKW-NSA-HSRG---Y----TGGL----EAVPDNV-V-TYE--T-	56	P58296

Accs means accession number, Dashes (-) mean identical residue.

expression of toxins having four disulfide bridges is not always a straightforward procedure that gives correctly folded peptides with activity, when using heterologous expression in microorganisms (Estrada et al., 2007). This makes difficult to obtain mutants of these toxins by means of recombinant DNA techniques. For the specific case of scorpion venom components and Na_v, it turned out that the venom of the scorpion C. *suffusus* was an excellent source of such natural ligands, well folded and biologically active. If the two novel peptides are taken into consideration plus the already known ones, thus far nine different peptides have been identified and studied concerning their ability to recognize sodium channels. The fact that they show different amino acid sequences and sizes could suggest that they might recognize different sub-types of ion-channels, which was in fact shown recently (Schiavon et al., 2006). However, all of them are considered to be β-ScTxs that bind to site-4 of voltage-dependent Na^+-channels, being excellent tools to study the relationships of toxin and Na_v.

In this work a two-step chromatographic method for the purification of four such peptides from *C. suffusus* venom (CssII, CssIV, CssVIII and CssIX) is described. The two last ones are novel toxins, whose primary structures were determined. During proteomic analysis of the fractions obtained from this scorpion, using our source of venom, the previously described neurotoxins CssI, CssIII, CssV, CssVI and CssVII were not found. In this regard, it is worth noting that the pioneer work done with this venom, several years ago (Martin et al., 1987) report the primary structure of only three peptides (CssII, CssIV and CssVI), as it can be found in the Universal Protein Resource (UniProt) protein database (i.e. P08900, P60266, P60267). In the present study the strategy previously described was simplified. In order to identify the expected peptides using the system shown in Fig. 1, the theoretical molecular masses of CssII, CssIV and CssVI were calculated and used to identify the protein that could contain these peptides. None of the molecular masses found matches with the expected sequence for toxin CssVI. Eventually, the lack of CssVI could be due to toxin content variations within samples of venom from species collected in different places. It is known from previously publications that there is toxin polymorphism in scorpions of the Old World (El Ayeb and Rochat, 1985). Since the complete primary structure of CssI, CssIII, CssV and CssVII were not reported (Martin et al., 1987) we could not use the same strategy to locate these peptides in our sixty-two chromatographic fractions. However, two novel peptides were found and denominated CssVIII and CssIX, according to the nomenclature proposed by Martin et al. (1987). Fortunately the two main toxins, already described (CssII and CssIV) were clearly identified and confirmed. Toxin CssII is the most important component in terms of quantity, but also in terms of lethality tests, as mentioned here and elsewere (Martin et al., 1987; Estrada et al., 2007; Hernandez-Salgado et al., 2009).

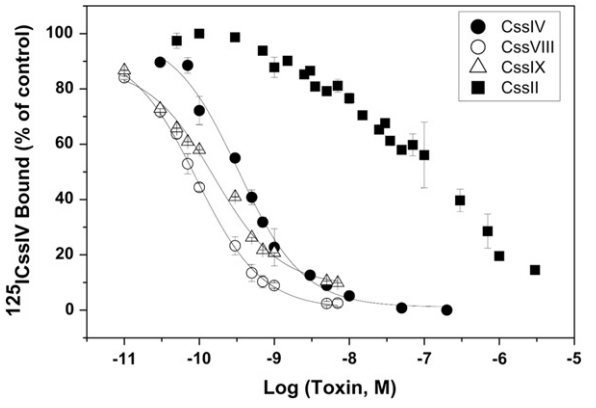

Fig. 3. Binding and displacement experiments of scorpion β-toxins. Radiolabeled CssIV was used for comparative binding and displacements using native peptides CssIV, CssVIII and CssIX. Experiments were performed by three independent experiments.

The amino acid sequence of CssIV is quite similar to that of CssVIII with only two changes at position L26 for K26, and R30 for K30, respectively. Concerning the amino acid sequence of CssIX, it is an interesting peptide that has amino acid similarities to the neurotoxins found in the venom of the scorpion species *Centruroides sculpturatus* and *Tityus serrulatus* from North- and South-America, respectively (Table 3). CssIX has low identity to the neurotoxins found in the same venom of *C. suffusus*; therefore, CssIX could be an interesting peptide ligand to study Na_v sub-types.

In conclusion, this manuscript reports two novel peptides from the venom of the Mexican scorpion *C. suffusus,* whose structure and function were elucidated, increasing

the number and diversity of these interesting ligands for further studies on the relationship between scorpion toxins and sodium channels.

Ethical approval

The protocol used for assaying the activity of these peptides in vivo, using the mice model, was followed according to the guidelines of our Institute Committee of Animal Welfare, keeping the number of animals to a minimum required to validate the experiments.

Conflicts of interest

None.

Acknowledgments

We are very grateful to Dr. Blanca Ines García Gómez and M.Sc. Alfredo Mendoza Vargas for their valuable advice during the cDNA library construction. This work was supported by grants from *Dirección General de Asuntos del Personal Academico* of the National Autonomous University of Mexico, IN220809 DGAPA to GC and IN204110 to LDP.

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.toxicon.2011.02.006.

References

Catterall, W.A., 1992. Cellular and molecular biology of voltage-gated sodium channels. Physiol. Rev. 72, S15–S48.

Cestele, S., Ben Khalifa, R.B., Pelhate, M., Rochat, H., Gordon, D., 1995. Alpha-scorpion toxins binding on rat brain and insect sodium channels reveal divergent allosteric modulations by brevetoxin and veratridine. J. Biol. Chem. 270, 15153–15161.

Cestele, S., Caterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 82, 883–892.

Cestele, S., Kopeyan, C., Oughideni, R., Mansuelle, P., Granier, C., Rochat, H., 1997. Biochemical and pharmacological characterization of a depressant insect toxin from the venom of the scorpion *Buthacus arenicola*. Eur. J. Biochem. 243, 93–99.

Cestele, S., Qu, Y., Rogers, J.C., Rochat, H., Scheuer, T., Catterall, W.A., 1998. Voltage sensor-trapping: enhanced activation of sodium channels by beta-scorpion toxin bound to the S3-S4 loop in domain II. Neuron 21, 919–931.

Cohen, L., Karbat, I., Gilles, N., Ilan, N., Benveniste, M., Gordon, D., Gurevitz, M., 2005. Common features in the functional surface of scorpion beta-toxins and elements that confer specificity for insect and mammalian voltage-gated sodium channels. J. Biol. Chem. 280, 5045–5053.

Corzo, G., Diego-Garcia, E., Clement, H., Peigneur, S., George, O., Tytgat, J., Possani, L.D., Alagon, A., 2008. An insecticidal peptide from the theraposid *Brachypelma smithi* spider venom reveals common molecular features among spider species from different genera. Peptides 29, 1901–1908.

Corzo, G., Prochnicka-Chalufour, A., García, B.I., Possani, L.D., Delepierre, M., 2009. Solution structure of Cn5, a crustacean toxin found in the venom of the scorpions *Centruroides noxius* and *Centruroides suffusus suffusus*. BBA. Proteins Proteomics 1794, 1591–1598.

Corzo, G., Villegas, E., Nakajima, T., 2001. Isolation and structural characterization of a peptide from the venom of scorpion with toxicity towards invertebrates and vertebrates. Prot. Pept. Lett. 8, 385–393.

El Ayeb, M., Rochat, H., 1985. Polymorphism and quantitative variations of toxins in the venom of the scorpion *Androctonus australis* Hector. Toxicon 23, 755–760.

Espino-Solis, G.P., Osuna-Quintero, J., Possani, L.D., 2008. Molecular cloning and characterization of the alphaX subunit from CD11c/CD18 horse. Vet. Immunol. Immunopathol. 122, 326–334.

Estrada, G., Garcia, B.I., Schiavon, E., Ortiz, E., Cestele, S., Wanke, E., Possani, L.D., Corzo, G., 2007. Four disulfide-bridged scorpion beta neurotoxin CssII: heterologous expression and proper folding in vitro. Biochim. Biophys. Acta 1770, 1161–1168.

Hernandez-Salgado, K., Estrada, G., Olvera, A., Coronas, F.I., Possani, L.D., Corzo, G., 2009. Heterologous expressed toxic and non-toxic peptide variants of toxin CssII are capable to produce neutralizing antibodies against the venom of the scorpion *Centruroides suffusus suffusus*. Immunol. Lett. 125, 93–99.

Jover, E., Couraud, F., Rochat, H., 1980a. Two types of scorpion neurotoxins characterized by their binding to two separate receptor sites on rat brain synaptosomes. Biochem. Biophys. Res. Commun. 95, 1607–1614.

Jover, E., Martin-Moutot, N., Couraud, F., Rochat, H., 1980b. Binding of scorpion toxins to rat brain synaptosomal fraction. effects of membrane potential, ions, and other neurotoxins. Biochemistry 19, 463–467.

Martin, M.F., Garcia y Perez, L.G., el Ayeb, M., Kopeyan, C., Bechis, G., Jover, E., Rochat, H., 1987. Purification and chemical and biological characterizations of seven toxins from the Mexican scorpion, *Centruroides suffusus suffusus*. J. Biol. Chem. 262, 4452–4459.

Mendoza-Vargas, A., Olvera, L., Olvera, M., Grande, R., Vega-Alvarado, L., Taboada, B., Jimenez-Jacinto, V., Salgado, H., Juarez, K., Contreras-Moreira, B., Huerta, A.M., Collado-Vides, J., Morett, E., 2009. Genome-wide identification of transcription start sites, promoters and transcription factor binding sites in E. coli. PLoS ONE 4, e7526.

Miller, C., 1995. The charybdotoxin family of K+-channel-blocking peptides. Neuron 15, 5–10.

Olamendi-Portugal, T., Batista, C.V.F., Restano-Cassulini, R., Pando, V., Villa-Hernández, O., Zavaleta-Martínez-Vargas, A., Salas-Arruz, M.C., Rodríguez de la Vega, 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.

Possani, L.D., Merino, E., Corona, M., Bolivar, F., Becerril, B., 2000. Peptides and genes coding for scorpion toxins that affect ion-channels. Biochimie 8, 861–868.

Rodriguez de la Vega, R.C., Merino, E., Becerril, B., Possani, L.D., 2003. Novel interactions between K+ channels and scorpion toxins. Trends Pharmacol. Sci. 24, 222–227.

Schiavon, E., Sacco, T., Cassulini, R., Gurrola, G., Tempia, F., Possani, L.D., Wanke, E., 2006. Resurgent current and voltage sensor trapping enhanced activation by a beta-scorpion toxin solely in Nav1.6 channel. significance in mice Purkinje neurons. J. Biol. Chem. 281, 20326–20337.

Vega Franco, L., Lia Jaime, M., 1966. Epidemiologic considerations on scorpion stings in Durango city. Rev. Invest. Salud Publica 26, 7–21.

Zamudio, F., Saavedra, R., Martin, B.M., Gurrola-Briones, G., Herion, P., Possani, L.D., 1992. Amino acid sequence and immunological characterization with monoclonal antibodies of two toxins from the venom of the scorpion *Centruroides noxius* Hoffmann. Eur. J. Biochem. 204, 281–292.