Isolation and characterization of two toxins from the Mexican scorpion Centruroides limpidus limpidus Karsch.

ARTICLE *in* COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY. B, COMPARATIVE BIOCHEMISTRY · FEBRUARY 1988

Impact Factor: 2.07 · DOI: 10.1016/0305-0491(88)90277-5 · Source: PubMed

Comp. Biochem. Physiol. Vol. 89B, No. 1, pp. 153–161, 1988
Printed in Great Britain

0305-0491/88 $3.00 + 0.00

ISOLATION AND CHARACTERIZATION OF TWO TOXINS FROM THE MEXICAN SCORPION *CENTRUROIDES LIMPIDUS LIMPIDUS* KARSCH§

A. C. ALAGÓN,* H. S. GUZMÁN,* B. M. MARTIN,† A. N. RAMÍREZ,* E. CARBONE‡ and L. D. POSSANI*‖

*Departamento de Bioquímica de Proteínas, Centro de Investigaciones sobre Ingeniería Genética y Biotecnología, UNAM, Apartado Postal 70-247, México D.F. 04510, Mexico
†National Institutes of Health, National Institute of Mental Health, Neuroscience Branch, Bethesda, Maryland 20892, USA
‡Dipartimento di Anatomia e Fisiologia Umana, Sezione Neuroscienze, 10125, Torino, Italy

(*Received* 16 *March* 1987)

Abstract—1. Several toxic polypetides were found in the venom of the scorpion *Centruroides limpidus limpidus*. Comparative studies of the potency of the venom in different strains of mice were conducted.

2. A new type of toxin (component II.9), specific for crustaceans (crayfish and isopods), was isolated from this scorpion and was shown to have the following *N*-terminal amino acid sequence: Lys-Lys-Asp-Gly-Tyr-Leu-Val-Asn-Lys-Tyr-Thr-Gly-Cys-Lys-Val-Asn-Cys-Tyr-Lys-Leu-Gly-Glu-Asn-Lys-Phe-Cys-Asn-Arg-Glu-.

3. A polypeptide toxic to mice (component II.6) from this venom was shown to have the following *N*-terminal sequence: Lys-Glu-Gly-Tyr-Leu-Val-Asn-His-Ser-Thr-Gly-Cys-Lys-Tyr-Glu-Cys-Tyr-Lys-Leu-Gly-Asp-Asn-Asp-Tyr-Cys-Leu-Arg-Glu-Cys-Lys-.

4. In cultured chick dorsal root ganglion cells, 1 μM of toxin II.6 was shown to reduce the size of sodium currents and to slow-down their activation–inactivation kinetics. The toxin had also a depressive action on the classical Ca^{2+} current activated at high membrane potentials (>0 mV).

INTRODUCTION

On the American Continent there are two genera of scorpions from the family Buthidae: *Centruroides* and *Tityus* which represent a medical problem and a life hazard to humans. From the genus *Centruroides* one of the most dangerous species is *Centruroides limpidus limpidus* from the state of Morelos and Guerrero (Mexico) and its venom is one of the least studied so far. A preliminary report concerning some toxic peptides from this scorpion has been published (Tato *et al.*, 1978), but no further biochemical or physiological characterization has since been presented (see review by Possani, 1984).

In the present communication we describe the chemical, biological and electrophysiological characterization of several components from this venom. A comparative study of the venom potency on different strains of mice was conducted. In addition a new toxin (component II.9) was discovered, which specifically affects crustaceans and not mice. Another, component II.6 is toxic to mice and chick (vertebrates) but had no effect on crustaceans. In avian sensory neurons, toxin II.6 acts upon the Na^+ and Ca^{2+} channels which are responsible for the action potential electrogenesis in these cells. The activation of both channels was strongly depressed by application of 1 μM of the toxin to the external bath.

MATERIALS AND METHODS

Source of venom

The scorpions were collected by us in the field and venom obtained by electrical stimulation of anesthetized animals as previously described (Dent *et al.*, 1980). Water solubilized venom was centrifuged at 15,000 *g* for 15 min in a Sorvall SS-34 rotor. The supernatant was freeze-dried and stored at −20°C until used.

Materials

All chemicals and reagents were analytical grade and were purchased from the companies indicated previously (Possani *et al.*, 1985). Sephadex G-50 (medium) was from

§Part of this publication was presented as a thesis dissertation for obtaining a College Degree in Biology by Hector S. Guzmán, School of Science, Universidad Nacional Autónoma de México. This work was supported in part by grants of the Mexican Council of Science and Technology numbers PVT/QF/NAL-85/2182 and PVT/AI/NAL-85/3029 to L.D.P. and Fondo Ricardo Zevada to A.C.A.

‖Correspondence should be sent to: L. D. Possani, Departamento de Bioquimica de Proteinas, UNAM Apartado Postal 70-247, Mexico D.F. 04510, Mexico.

Abbreviations used—AaH, *Androctonus australis* Hector; Cll, *Centruroides limpidus limpidus* Karsch; Clt, *Centruroides limpidus tecomanus* Hoffmann; CM-cellulose, carboxymethyl-cellulose; Cn, *Centruroides noxius* Hoffmann; CsE, *Centruroides sculpturatus* Ewing, Css, *Centruroides suffusus suffusus*; DRG, dorsal root ganglion; EGTA, ethylene glycol, Bis-(2-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Lqq, *Leiurus quinquestriatus quinquestriatrus*; mol-wt, molecular weight; *n*, *noxius*; *s.*, *serrulatus*; SDS, sodium dodecyl sulfate; *T.*, *Tityus*; TTX, tetrodotoxin; Ts, *Tityus serrulatus* Lutz and Mello.

Pharmacia Fine Chemicals (Uppsala, Sweden); Carboxymethyl-cellulose (CM-32) was from Whatman (Clifton, NJ, USA) and Bio-Rex 70 was from Bio-Rad Laboratories (Rockville Centre, NY, USA).

Lethality tests

The mouse lethality of various protein fractions was observed after intraperitoneal injection of different amounts of protein (usually from 5 to 100 μg) in 0.1–0.3 ml saline or buffer solutions, into adult 20–25 g mice (strain CD_1). To define the toxicity of various chromatographic components, three designations were used (Possani *et al.*, 1977). "Lethal" means that the component at the dose injected was enough to kill the tested mouse within 20 hr of injection. "Toxin" means that the mouse shows any of the following symptoms: excitability, salivation, temporary paralysis of rear limbs, dyspnoea, but recovered within 20 hr after injection. "Non-toxic" means normal behaviour similar to injection of 0.9% NaCl or buffer solutions.

Mice from the strains BALB/c, BALB/k, CD_1, C_{57}, DBA, NIH and Mexican SSA were used for the comparative LD_{50} determinations.

The method of Reed and Muench (1938) was applied to calculate the values of LD_{50} in the different mice strains for the soluble venom and sub-fractions of *C. l. limpidus* venom. Systematically we have used 10 mice for each dose of venom or fraction of venom. Several different doses were used for each LD_{50} at decreasing amount of protein (by a factor of two).

Two species of crustaceans were also used for toxicity tests: *Procambarus bouvieri* (crayfish) and *Oniscus* sp. ("cochinilla") a very common isopod found in gardens of Mexico City. This last animal was used based on the original report by Zlotkin *et al.* (1975) who described this technique. "Cochinillas" were injected in the dorsal part of the body between the cephalothorax and abdomen with a Hamilton microsyringe (10 μl capacity) not exceeding 5 μl total volume of injection. Toxicity or lethality was observed through the following symptoms; irregular movements and curvature of the body, paralysis of legs, immobilization in an upside-down position and finally death. The initiation of the symptoms was doses dependent, and the final results were taken after 12 hr of injection. Rare mechanical damage due to injection could clearly be differentiated from actual intoxication symptoms.

The crayfish were injected with a Hamilton microsyringe (50 μl capacity) in the dorsal cavity (heart). In crayfish the main symptoms of intoxication observed were interpreted as a generalized contraction (mainly the legs), followed by a half-turn over the body, sometimes in a complete up-side-down position and finally death within 20 hr of injection.

Crayfishes were used to corroborate the "cochinillas" results, but due to the difficulty of obtaining crayfish fewer animals were used.

Purification procedures

The soluble venom (835 mg) was divided into seven aliquots and applied independently to a Sephadex G-50 (medium) column. The toxin containing tubes were pooled (fraction number II), divided into five aliquots and chromatographed individually on a CM-cellulose column, equilibrated and run in 20 mM ammonium acetate buffer at pH 4.7. The most toxic fraction obtained (II.6) was subsequently purified by three further steps of ion exchange chromatography employing CM-cellulose and Bio-Rex-70 columns at different pHs. The conditions for the CM-cellulose columns are indicated below, in the footnotes of the figures. For the Bio-Rex-70 column (0.9 × 30 cm) a linear gradient of 250 ml of 50 mM sodium phosphate pH 6.4 and 250 ml of the same buffer containing 250 mM NaCl was applied. The column was previously equilibrated with the initial buffer. All columns were run at a flow rate of 40 ml/hr and 4 ml fractions were collected. Due to the heat-stability of the toxins and the absence of proteinases in this venom the columns were run at room temperature (20°C).

The crustacean toxin (most basic component of the venom, fraction II.9) was further purified in a CM-cellulose column (see Results).

Whenever it was necessary the fractions were dialysed against the appropriate buffers prior to rechromatography, using a Spectrapor 3M dialysis membrane (Spectrum Medical Industries, Los Angeles, CA). The purity of the fractions was followed by electrophoresis in polyacrylamide gel containing urea as described by Reisfeld *et al.* (1962), or in the presence of sodium dodecyl sulfate (SDS) according to Laemmli (1979).

Hyaluronidase activity was measured by the turbidimetric method of Tolksdorf *et al.* (1949), assuming that 1 unit of enzymatic activity is equal to the amount of enzyme required to reduce the concentration of hyaluronic acid (Sigma Chemical Co., St Louis, MO, grade I or grade III-P) from 0.2 to 0.1 mg/ml in 20 min at 25°C.

Chemical characterization of the toxins

Amino acid analysis was conducted using (after acid hydrolysis), a Durrum D-500 analyser. Duplicate samples were hydrolyzed *in vacuo* for 24 and 48 hr at 110°C in 6 N HCl. The values for serine and threonine were obtained by extrapolation to zero time, whereas the values for valine and leucine were obtained after 48 hr hydrolysis.

The *N*-terminal amino acid sequence of both toxins were determined by automatic Edman degradation (Edman and Beggs, 1967) in a Beckman 890M liquid-phase sequencer (Possani *et al.*, 1985), following reduction and pyridylethylation (Possani *et al.*, 1981a).

Electrophysiological experiments

The experiments were performed on primary cultures of dorsal root ganglion cells obtained from a 10 days old chick (*Gallus domesticus*). DRG cells were prepared as already described (Carbone and Lux, 1986) and used 6–12 hr following plating. During this period of incubation cells are of spherical shape, with no visible processes, and stick to the substrate. The experimental set-up, fabrication of patch electrodes and the I-V converter were similar to those described elsewhere (Carbone and Lux, 1984). Whole-cell clamp currents were measured according to the method of Hamill *et al.* (1981). Data were stored on a FM magnetic tape with a band width of 5 KHz. Analog recordings were digitized at a frequency of 12 KHz by a 12 bit A/D converter and analyzed by a LSI 11/23 minicomputer. Leakage and capacitative currents were reduced by analogue circuitry and the residual minimized by subtracting a scaled signal which was the average of ten hyperpolarizing pulses of appropriate amplitude.

Solutions were filtered through 0.22 μm Millipore filters before use. The external bath contained (mM): 120 NaCl, 3 KCl, 2 $CaCl_2$ and 10 Na-HEPES buffer solution (pH 7.3). The pipette filling solution was (mM): 130 CsCl, 10 tetraethylammonium chloride, 10 EGTA, 2 $MgCl_2$ and 10 Cs-HEPES buffer (pH 7.3). Stock solution of toxin II.6 was prepared by dissolving the lyophilized peptide in the external Ringer solution. Microliter amounts of the stock solution were then added directly to the bath via a Gilson pipette to the desired concentration. Since experiments were done only to test the effects of the toxins on cultured neurons, no attempts were made to verify recovery by washing the poisoned cells with appropriate buffers, as done previously (Carbone *et al.*, 1982, 1984).

RESULTS AND DISCUSSION

Characterization of the soluble venom

Electrical stimulation of the telsons of 3922 scor-

Table 1. LD_{50} values of the venom in different strains of mice

Mouse strain	LD_{50} (mg/kg)
BALB/c (white)	2.78
BALB/k (white)	3.31
CD_1 (white)	3.30
C_{57} (black)	1.20
DBA (brown)	1.20
NIH (white)	1.32
SSA (white)	0.61

The LD_{50} were calculated by the method of Reed–Meunch (1938).

pions gave 1364 mg of soluble venom (calculated by absorbance at 280 nm and assuming that 1 absorbance unit, in a quartz cell with 1 cm pathway, is equal to mg/ml protein), with a mean value of 348 μg of venom protein per scorpion.

The lethality tests conducted with the soluble venom from this scorpion showed a considerable variation according to the mice strain used for LD_{50} determination (Table 1). The electrophoretic behaviour of soluble venom from *C. l. limpidus* is shown in Fig. 1. At least ten components were discriminated on the basis of charges (Fig. 1A, left lane) and many more different components (approx. 20) were shown to be present, under denaturating conditions, with sodium dodecyl sulfate (SDS) polyacrylamide gels (Fig. 1B, left lane). It is worth mentioning the broad band at the mol. wt corresponding to scorpion toxins (number 4 in Fig. 1B), suggesting the presence of a heterogeneous population of low mol. wt peptides in this position of the gel (mol. wt = 7000).

The specific activity of the hyaluronidase present in the soluble venom was 43.5 ± 5.6 U/mg protein ($N = 6$).

Chromatographic purification of two toxins from this venom

Seven chromatographic separations of soluble venom in Sephadex G-50 columns gave essentially the same results (Fig. 2). The mean value for the total protein recovery in the columns of Fig. 2 was 85.4%. Tubes corresponding to four fractions were pooled as indicated by the horizontal bars. Only fraction II was toxic. Recoveries, enzymatic activity and lethality tests are indicated in Table 2. The hyaluronidase activity was eluted only in Fraction I of Fig. 2, and corresponded to 95.6% of the total activity applied to the column. Fraction II from Sephadex G-50 applied to a CM-cellulose column gave reproducible results (Fig. 3) and the final recovery was 90% (Table 2). Components II.4 to II.8 were all toxic to mice, but not toxic to crustaceans. Polyacrylamide gel electrophoresis of the toxic components showed from two to four bands in each fraction (data not shown). Component II.9 was obtained by washing the column with 1 M NaCl (Fig. 3) and was shown to be toxic only to crustaceans. The only other toxic component to crustaceans was fraction II-3 (Fig. 3), which at a dose of 80 to 200 μg was toxic to crayfish. The most potent toxin to mouse (component II.6) and the fraction

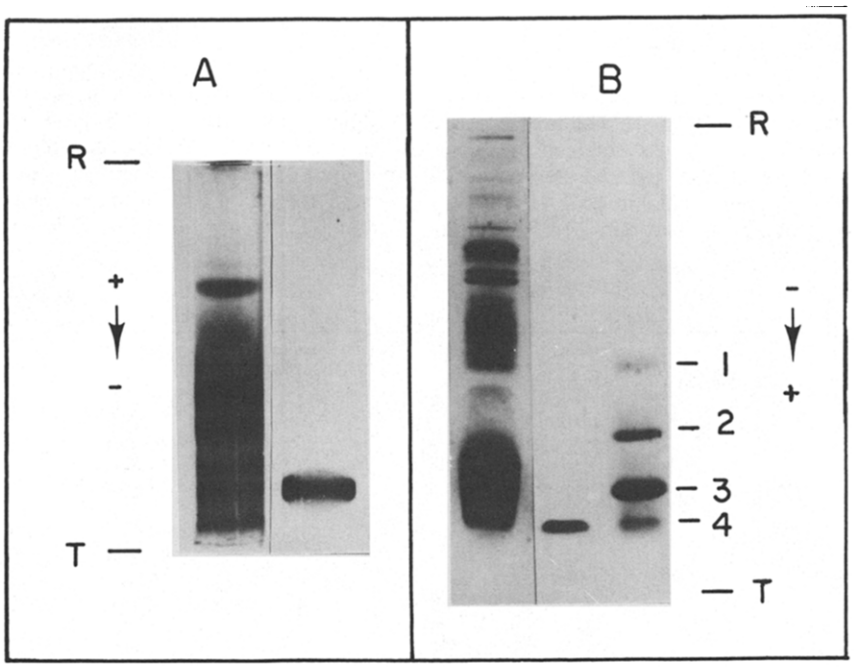

Fig. 1. Polyacrylamide gel electrophoresis separation of soluble venom and purified toxins from *C. l. limpidus*. A. Gel-electrophoresis separation in the β-alanine–acetate–urea system described by Reisfeld *et al* (1962). Left lane: 180 μg of soluble venom. The protein runs toward the cathode; approximately 10 bands were separated. Right lane: 13 μg of purified toxin II.6. B. Gel-electrophoresis in slab gel (15% acrylamide), containing SDS according to the technique of Laemmli (1970). Left lane: 140 μg of soluble venom. The proteins run toward the anode and at least 20 components were resolved. Middle lane: 10 μg of the crustacean toxin (II.9). Right lane: molecular weight markers: 1 (soy bean trypsin inhibitor, 21,500); 2 (lactoglobulin, 18,400); 3 (ribonuclease, 13,500); 4 (toxin II.9.2.2 from *C. noxius*, 7000).

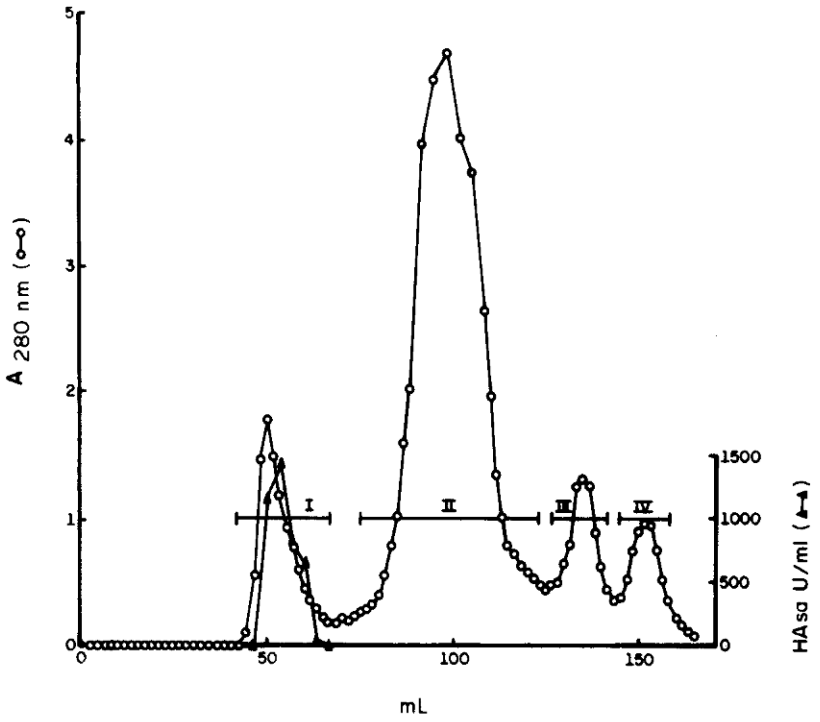

Fig. 2. Sephadex G-50 gel filtration of soluble venom. The column (0.9 × 200 cm) equilibrated with 0.02 M ammonium acetate buffer, pH 4.7, was loaded with 115.5 mg of *C. l. limpidus* venom and eluted with the same buffer at a flow rate of 20 ml/hr. Tubes containing 1.66 ml were pooled as shown by the horizontal bars (I to IV) according to the absorbance at 280 nm. Hyaluronidase activity is indicated in the figure.

II.9, toxic to crustaceans were further purified to homogeneity. For fraction II.6 three successive columns were used, whereas for fraction II.9 only one more steps was necesary (Fig. 4). The profile of the column shown on Fig. 4A was obtained in 50 mM ammonium acetate buffer, pH 5.6. Several components were separated; the main peak (II.6.2) was further chromatographed in a Bio-Rex 70 column (data not shown). The major component (II.6.2.2) corresponding to 66% of the material applied to the Bio-Rex 70 column, was finally separated on a CM-cellulose column as shown (Fig. 4B and right lane of Fig. 1A). Pure toxin II.6.2.2.2 represents approximately 1.1% of the protein present in the soluble venom and had a LD_{50} of 0.333 mg/kg mouse of the strain CD_1. This component injected at a dose of

Table 2. Recovery and lethality of chromatographic fractions

Column	Protein content (mg)	% Recovery*	Lethality LD_{50}†
Sephadex G-50	Soluble venom (835.0)	100	3.3
(Fig. 2)	Fraction I (36.0)	4.3	Not toxic‡
	II (555.0)	66.5	2.9
	III (90.3)	10.8	Not toxic
	IV (32.0)	3.8	Not toxic
	Protein recovered	85.4	
CM-cellulose	Fraction II (490.0)	100.0	2.9
(Fig. 3)	II.1 (48.8)	9.9	Not toxic
	II.2 (53.6)	10.9	Not toxic
	II.3 (28.7)	5.8	Not toxic
	II.4 (169.6)	34.6	13.58
	II.5 (22.9)	4.7	0.89
	II.6 (56.4)	11.5	0.53
	II.7 (18.8)	3.8	0.66
	II.8 (23.8)	4.8	0.60
	II.9 (14.7)	3.0	Not toxic
	Protein recovered	90.0	

*The values reported are percentages calculated from the number of absorbancy units at 280 nm, as described in Materials and Methods.

†LD_{50} were determined by the method of Reed–Muench (1938).

‡Not toxic means that doses of 150 μg protein of the fraction per 20 g mouse weight was uneffective.

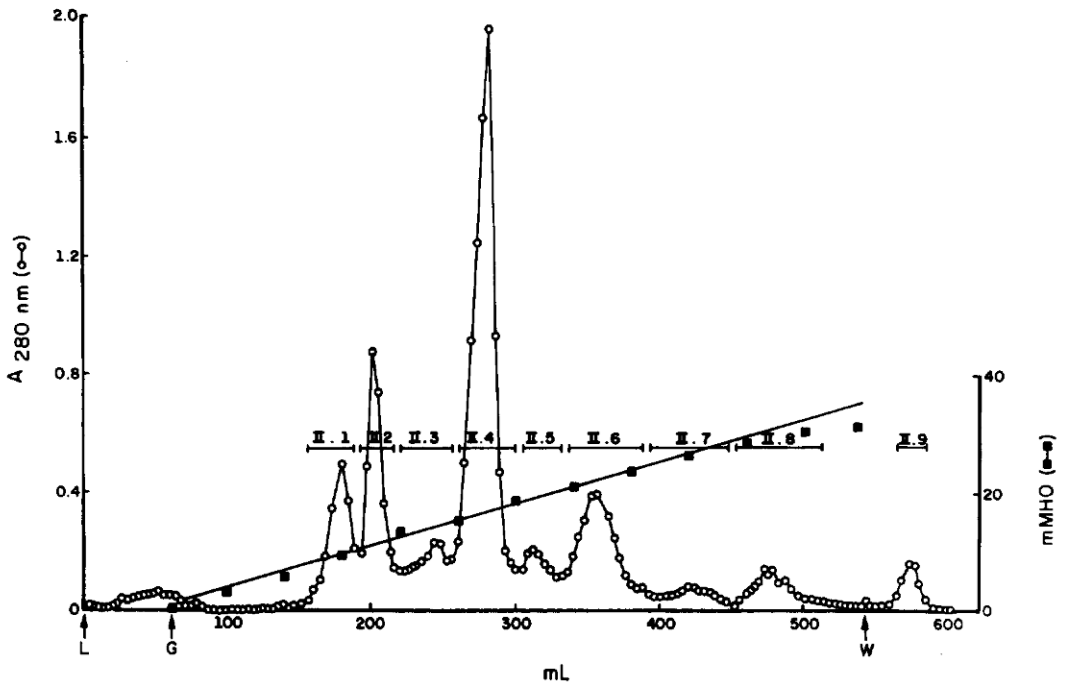

Fig. 3. Ion-exchange chromatography of fraction II. The toxic fraction from Sephadex G-50 was separated in a CM-cellulose column (0.9 × 30 cm) equilibrated and run with 0.02 M ammonium acetate buffer, pH 4.7. Fraction II (100 mg) was applied to the column and eluted with salt at a flow rate of 40 ml/hr; a linear gradient was formed by mixing 250 ml of buffer in 0 M NaCl with 250 ml of buffer in 0.55 M NaCl. Fractions of 3.9 ml were collected and pooled according to the absorbance at 280 nm (horizontal bars). The arrow with L means loading sample; G means starting point of the gradient; W means washing with 1 M NaCl.

0.75 μg per "cochinilla" was not toxic, giving essentially the same results as the control animal injected with saline. The toxin at a dose of 73 μg per crayfish was also not toxic.

The crustacean toxin was obtained in pure form after the CM-cellulose column shown (Fig. 4C and middle lane of Fig. 1B). Component II.9.2 corresponds to approximately 0.9% of the initial soluble venom. Eight out of ten "cochinillas" injected with 0.50 μg of toxin II.9.2 died within the first 12 hr after injection. Three crayfish with 25 g body weight injected with 25, 50 and 125 μg of toxin II.9.2 died with all the symptoms of intoxication, within 12 hr of injection. The same component II.9.2 injected at a dose of 150 μg/20 g mouse weight was not toxic. In order to simplify notation toxic components II.6.2.2.2 and II.9.2 will be called simply toxin II.6 and II.9 respectively.

Chemical charaterization of toxins II.6 and II.9

The apparent mol. wt of both toxins by SDS polyacrylamide gel was near 7000 (see Fig. 1B). The amino acid composition of the toxins shown in Table 3 revealed the lack of isoleucine and methionine in either toxin. The latter amino acid (Met) is absent in most scorpion toxins (Possani *et al.*, 1977). The crustacean toxin II.9 is a slightly larger molecule than most mammalian scorpion toxins, generally having 60–65 amino acid residues. Toxin II.9 has approx. 70 amino acid residues, similar to the insect toxin from *Androctonus australis* Hector which has 71 amino acids (Darbon *et al.*, 1982).

The *N*-terminal amino acid sequence of toxins II.6 and II.9 are compared in Table 4 with some representative toxins from other known scorpion species. Toxins II.6 and II.9 are different not only with respect to the biological activity: mammalian and crustacean specificity respectively, but also with regard to their amino acid composition and number of residues (Table 3) as well as differences in the amino acid sequence (Table 4). However, both toxins have 65% of the positions occupied by identical amino acid residues at the *N*-terminal sequences including the cysteine residues, which are important for disulfide bridge formation and hence for stabilization of the three dimensional structure of the molecules.

If we compare the mammalian toxin II.6 from *C. l. limpidus* with similar toxins from other *Centruroides* scorpions (Table 4) the similarity is even greater. Toxin *C. s. suffusus* II shows 90% identity, toxin *C. sculpturatus* I shows 70% identity and toxin *C. noxius* II-14 has 65% identity. All the cysteine residues are conserved at equivalent positions. If we compare toxin C11 II.6 with toxin γ from the Brazilian scorpion *T. serrulatus* we find less similarities (50% identity); but if the comparison is made with mammalian toxins from the North African scorpion from the genus *Androctonus* and *Leiurus* the difference is greater, and also some of the cysteine residues are not conserved at the same position. The insect toxin (IT)

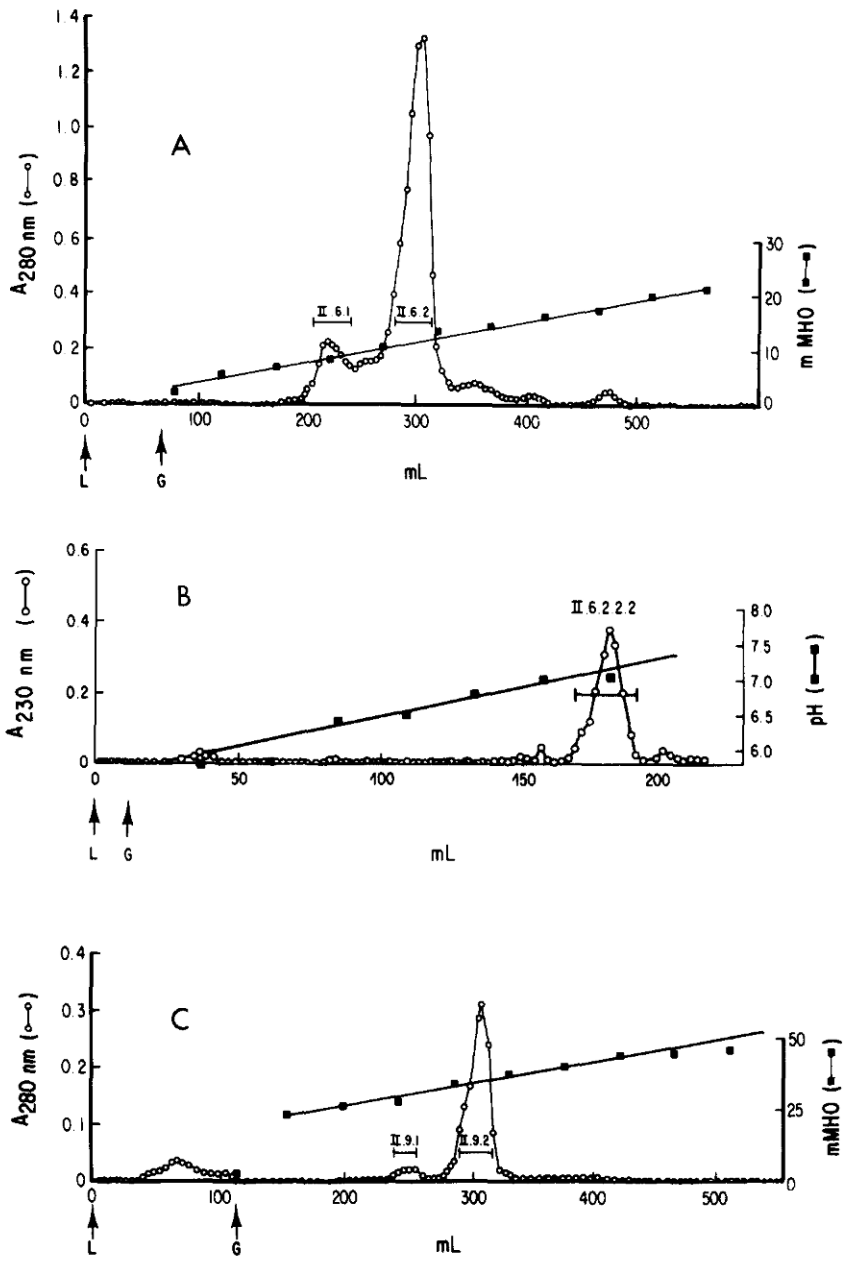

Fig. 4. Ion-exchange chromatographic separation of toxic sub-fractions. A. Fraction II.6 from Fig. 3 (51.5 mg) was applied to a CM-cellulose column (0.9 × 30 cm) equilibrated and run in 0.05 M ammonium acetate buffer, pH 5.6, at a flow rate of 40 ml/h with a salt gradient from 0–0.4 M NaCl (250 ml each). Tube fractions of 4.6 ml volume each were collected and pooled according to the horizontal bars. Toxin II.6.2 corresponded to 25 mg protein, 48.5% of the material applied to the column. B. Final purification of fraction II.6.2.2 from Bio-Rex 70 (see Results). A CM-cellulose column (0.9 × 30 cm) was equilibrated in 0.075 M ammonium acetate buffer, pH 5.8. Toxin (5.5 mg) was applied to the column and eluted at flow rate of 10 ml/h with a linear pH gradient (total volume 200 ml) from 5.8 to 8.0, in 2.42 ml fractions. The absorbance was read at 230 nm and the fractions corresponding to toxin II.6.2.2.2 were pooled, as indicated by the horizontal bar. Purity of sample is shown in Fig. 1A. C. Final purification of the crustacean toxin (II.9.2) in a CM-cellulose column (0.9 × 30 cm) equilibrated and run with 0.02 M ammonium acetate buffer, pH 4.7 at a flow rate of 40 ml/hr. Fraction II.9 (11.7 mg) was applied to the column and eluted with a linear gradient from 250 ml buffer containing 0.4 M NaCl to 250 ml of the same buffer containing 1.0 M NaCl. Fractions of 4 ml each were collected and pooled as indicated by the horizontal bars. Purity of sample is indicated in Fig. 1B.

Table 3. Comparison of amino acid composition of pure toxins II.6 and II.9 with other American scorpion toxins*

Amino acid	Cll† II.6	Cll† II.9	Clt II.9.3	Cn II.9.2.2	Cn II.14	Ts gamma	CsE I	Css II
Asp	5	7	5	6	8	4	6	5
Thr	3	3	3	2	4	1	5	3
Ser	2	4	2	1	3	4	2	4
Glu	7	5	7	7	3	3	5	7
Pro	1	2	2	2	2	3	3	2
Gly	8	10	8	7	9	8	9	6
Ala	3	3	3	4	1	3	0	3
1/2Cys	nd‡	nd	8	8	8	8	8	8
Val	2	2	3	2	1	2	1	3
Met	0	0	0	0	1	1	0	0
Ile	0	0	1	1	0	2	1	0
Leu	5	3	4	6	3	3	4	6
Tyr	6	5	6	6	7	5	6	7
Phe	1	2	2	1	1	1	2	1
His	3	2	2	1	1	1	1	1
Lys	5	7	6	8	8	6	8	7
Trp	nd	nd	1	1	1	3	2	2
Arg	1	3	2	2	3	3	1	1
Total	nd	nd	65	65	64	61	64	66

*Cll II.6 and II.9 are from *C. l. limpidus*, this work; Clt II.9.3 from *C. l. tecomanus*, Possani *et al.* (1980); Cn II.9.2.2 and II.14 from *C. noxius*, and Ts gamma from *T. serrulatus*, Possani *et al.* (1985); CsE I from *C. sculpturatus*, Babin *et al.* (1975); Css II from *C. s. suffusus*, Garcia (1976).

†Cll, nearest integer number of amino acid, obtained by acid hydrolysis at 24 and 48 hr, as described in Materials and Methods.

‡nd means not determined.

from *A. australis* has 40% identity with C11 II.6, while *L. q. quinquestriatus* IV has 27%, and toxin *A. australis* I has only 20% identity (Table 4). These results indicate that the mammalian toxins from the North American scorpions are more closely related to each other than the South American or North African scorpion toxins. They also show that the similarity between the North American and the Brazilian scorpion toxins are greater than to the North African, which confirms results previously found when comparing sequence homologies of scorpion toxins from these three distinct geographical localizations by the more rigorous method of metric analysis (see Possani *et al.*, 1985, Erickson and Sellers, 1983). If we compare the crustacean toxin C11 II.9 with toxin γ from *T. serrulatus* the number of identical positions is 38% (data not shown) with conserved cysteine residues, but it is only 27% identical when compared with both North African AaH I and Lqq IV, without conservation of one of the cysteine positions. The insect toxin IT from *A. australis* showed more homology to crustacean toxin C11 II.9 (38%), but also with one residue out of register.

Electrophysiological studies of toxin II.6

Figure 5a shows a family of membrane currents recorded from a whole cell clamped chick DRG bathed in a Ringer solution containing 120 mM NaCl, 2 mM $CaCl_2$ and internally perfused with 130 mM CsCl, 10 mM tetraethylammonium chloride. Under these conditions, outward potassium currents are minimized and the major portion of membrane currents flow inward through open Na and Ca channels (see Fig. 3 in Carbone and Lux, 1986). At positive potentials, Na currents (I_{Na}) activate quickly and inactivate within 8 ms (at temperature of 18°C). Calcium currents (I_{Ca}) activate also rather quickly but inactivate very slowly in the presence of a strong intracellular Ca^{2+} – buffer (10 mM EGTA). Thus, for pulses lasting 12 ms, I_{Na} can be roughly identified

Table 4. Comparison of the *N*-terminal amino acid sequence of toxins II.6 and II.9 of *C.l.limpidus* with other known scorpion toxins*

	1	5	10	15	20	25	30	(%)†
Cll II.6	– – K E G Y L V N H S T G C K Y E C Y K L G D N D Y C L R E C K...							100
Css II	– – K E G Y L V S K S T G C K Y E C L K L G D N D Y C L R E C K...							90
CsE I	– – K D G Y L V E – K T G C K K T C Y K L G E N D F C N R E C K...							70
Cn II.14	– – K D G Y L V D A K – G C K K N C Y K L G K N D Y C N R E C R...							66
Cll II.9	– K K D G Y L V N K Y T G C K V N C Y K L G E N K P C N R E ...							65
Ts γ	– – K E G Y L M D H – E G C K L S C F I R P S G – Y C G R E C G...							50
AaH IT	– K K N G Y A V D – S S G K A P E C L – L – S N – Y C N N Q C T...							40
AaH I	– K R D G Y I V Y P N N – C V Y H C V P P C D – G L C K K N G G...							20
Lqq IV	G V R D A Y I A D D K N – C V Y Y T C – – – G S N S Y C N T E C T...							27

*Cll II.6 and Cll II.9 toxins from *C.l. limpidus*, this work; Css II is a toxin from *C.s.suffusus*, Garcia (1976); CsE I is from *C.scupturatus*, Babin *et al.* (1975); Cn II.14 from *C. noxius* and toxin γ from *T.serrulatus* (Possani *et al.* (1985); AaH IT and AaH I are toxins from *A. australis*, Darbon *et al.* (1982) and Rochat *et al.* (1970), respectively; Lqq IV is from *L.q.quinquestriatus*, Kopeyan *et al.* (1982).

†Percentage of identity was calculated by taking the number of identical amino acids in the same equivalent position and dividing by the number of total amino acids under comparison. Note that blank spaces were included "–" in order to increase homology. Alignments of cysteines were taken as guide-lines for the introduction of the blank spaces.

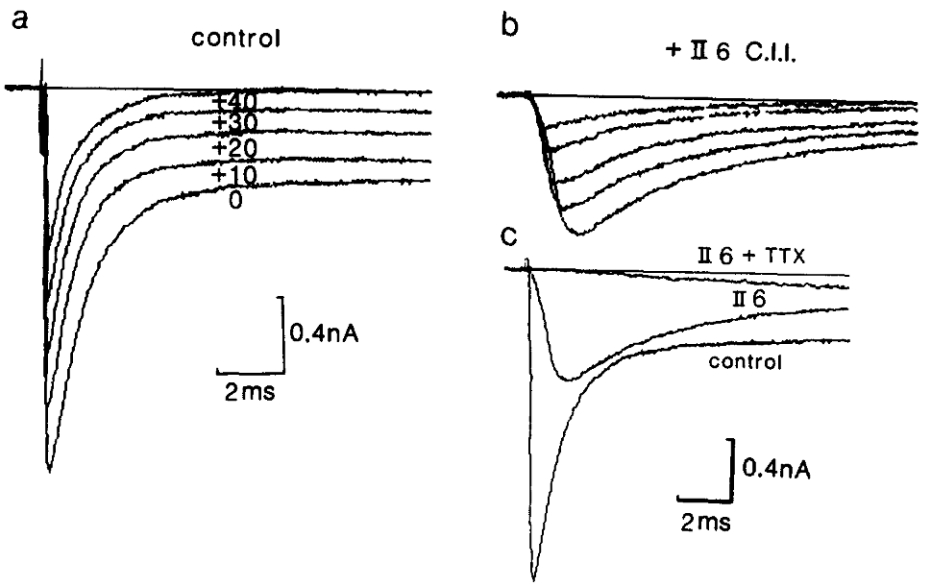

Fig. 5. Electrophysiological effects of pure toxins on DRG cells. Action of toxin II.6 on Na^+ and Ca^{2+} currents recorded from a chick dorsal root ganglion cell at the membrane potentials indicated. (a), (b): inward current records obtained before (control) and after 8 min addition of 1 μM C11 II.6 to the bath. In (c) are shown the current traces recorded at 0 mV before (control) and during application of 1 μM of toxin II.6 superimposed with the membrane current remaining after addition of 0.3 μM Tetrodotoxin (TTX) to the bath. V_h; −80 mV. Ionic solutions as described in Materials and Methods. Temp. 18°C.

with the transient phase of the current and I_{Ca} with the steady-state level reached near the end of the pulse.

As shown in Fig. 5b, application of 1 μM of toxin II-6 to the external bath caused a drastic modification of the time course of both Na and Ca currents. In the potential range examined, the following features were consistently observed: (i) the peak current amplitude (I_{Na}) decreased by about 60% (58 ± 12% in five cells), (ii) the steady state level of the current (I_{Ca}) nearly halved, (iii) the time to peak and the time constant of inactivation increased by a factor of 3 and 6, respectively, (iv) the toxin-modified membrane currents were almost fully blocked by addition of 0.3 μM of Tetrodotoxin (TTX) to the external bath (Fig. 5c). All this suggested that toxin II-6 exerts a depressive action on both Na and Ca currents. Ca currents activated at positive potentials (Carbone and Lux, 1984) appear to be almost completely blocked by toxin II-6 while Na currents are only partially reduced. The time course of the toxin-modified Na current show strong prolongations.

Our data do not allow more quantitative conclusions, but it is interesting to point out that the action of toxin II-6 on the membrane currents of chick DRG has some similarity with those of other scorpion toxins with close amino acid sequence. Thus, toxin *C. s. suffusus* II (which has 90% identity with our toxin II-6, has been shown to reduce the size of Na currents in skeletal muscle at concentrations below 1 μM (Jaimovich *et al.*, 1982). Toxin *C. sculpturatus* I and toxin γ from *T. serrulatus* (which show 70 and 50% identity, respectively) have been reported to affect the voltage-dependent probability of Na channel opening in various preparations (Meves *et al.*, 1982; Vijverberg, 1984). Finally, we found that 1 μM of toxin *C. noxius* II-14 (66% identity) causes also a marked reduction of Na and Ca currents in chick DRG neurons (Carbone and Possani, unpublished).

In conclusion, the structural identities outlined here might support close biological and physiological actions reported among various toxins of different scorpions.

Acknowledgements—The authors are greatly indebted to Mr Fredy Coronas Valderrama, Mrs Georgina B. Gurrola and Ms Denise Merkle-Lehman for technical assistance.

REFERENCES

Babin D. R., Watt D. D., Goos S. M. and Mlejnek R. V. (1975) Amino acid sequence of neurotoxin I from *Centruroides sculpturatus* Ewing. *Archs Biochem. Biophys.* **166,** 125–134.

Carbone E., Wanke E., Prestipino G., Possani L. D. and Maelicke A. (1982) Selective blockage of voltage-dependent K^+ channels by a novel scorpion toxin. *Nature* **296,** 90–91.

Carbone E., Prestipino G., Franciolini F., Dent M. A. R. and Possani L. D. (1984) Selective modification of the squid axon Na currents by *Centruroides noxius* toxin II-10. *J. Physiol.* (Paris) **79,** 179–184.

Carbone E. and Lux H. D. (1984) A low-voltage activated calcium conductance in embryonic chick sensory neurons. *Biophys. J.* **46,** 413–418.

Carbone E. and Lux H. D. (1986) Na^+-channels in cultured chick dorsal root ganglion neurons. *European Biophys J.* **13,** 259–271.

Darbon H., Rochat H., Kopeyan C. and Rietschoten J. V. (1982) Covalent structure of the insect toxin of *Androctonus australis* Hector. *Toxicon* **20,** 64.

Dent M. A. R., Possani L. D., Ramirez G. A. and Fletcher

Jr., P. L. (1980) Purification and characterization of two mammalian toxins from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann. *Toxicon* **18,** 343–350.

Edman P. and Beggs G. (1967) A protein sequenator. *Eur. J. Biochem.* **1,** 80–91.

Erickson B. W. and Sellers P. H. (1983) In *Time Warps, String Edits, and Macromolecules* (Edited by Sankoff D. and Kruskal J. D.), pp. 59–91. Addison-Wesley Publishing Co, Reading.

Garcia G. P. (1976) Etude des neurotoxines du venin du scorpion Mexican "*Centruroides suffusus suffusus*". Ph.D. thesis, Université de Nice, France.

Hamill O. P., Marty A., Neher E., Sakmann B. and Sigworth E. F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **39,** 85–100.

Jaimovich E., Ildefonse M., Barhanin J., Rougier D. and Lazdunski M. (1982) *Centruroides* toxin, a selective blocker of surface Na^+ channels in skeletal muscle: voltage clamp analysis and biochemical characterization of the receptor. *Proc. natn. Acad. Sci.* (USA) **79,** 3896–3900.

Kopeyan C., Martinez G. and Rochat H. (1982) Primary structure of toxin IV of *Leiurus quinquestriatus quinquestriatus* and characterization of a new group of scorpion neurotoxins. *Toxicon* **20,** 71.

Laemmli U. K. (1979) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Meves H., Rubly N. and Watt D. D. (1982) Effect of toxins isolated from the venom of the scorpion *Centruroides sculpturatus* on the Na currents of the node of Ranvier. *Pfluegers Arch.* **393,** 56–62.

Possani L. D., Alagon A. C., Fletcher Jr., P. L. and Erickson B. W. (1977) Purification and properties of mammalian toxins from the venom of the Brazilian scorpion *Tityus serrulatus* Lutz and Mello. *Archs Biochem. Biophys.* **180,** 394–403.

Possani L. D., Fletcher Jr., P. L., Alagon A. B. C., Alagon A. C. and Julia J. Z. (1980) Purification and characterization of a mammalian toxin from venom of the Mexican scorpion, *Centruroides limpidus tecomanus* Hoffman. *Toxicon* **18,** 175–183.

Possani L. D., Martin B. M., Mochca-Morales J. and Svendsen I. (1981a) Purification and chemical characterization of the major toxins from the venom of the Brazilian scorpion *Tityus serrulatus* Lutz and Mello. *Carlsberg Res. Commun.* **46,** 195–205.

Possani L. D., Steinmetz W. E., Dent M. A. R., Alagon A. C. and Wuthrich K. (1981b). Preliminary spectroscopic characterization of six toxins from Latin American scorpions. *Biochim. biophys. Acta* **669,** 183–192.

Possani L. D. (1984) Structure of scorpion toxins. In *Handbook of Natural Toxins* (Edited by Anthony T. Tu), pp. 513–550. Marcel Dekker, New York.

Possani L. D., Martin B. M., Svendsen I., Rode G. S. and Erickson B. W. (1985) Scorpion toxins from *Centruroides noxius* and *Tityus serrulatus*. Primary structures and sequence comparison by metric analysis. *Biochem. J.* **229,** 739–750.

Reed L. J. and Muench H. (1938) A simple method of estimating fifty percent end points. *Am. J. Hyg.* (Baltimore) **27,** 493–497.

Reisfeld R. A., Lewis U. J. and Williams D. E. (1962) Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* **195,** 281–283.

Rochat H., Rochat C., Miranda F., Lissitzky S. and Edman P. (1970) The amino acid sequence of neurotoxin I of *Androctonus australis* Hector. *Eur. J. Biochem.* **17,** 262–266.

Tato P., Gavilanes M., Munoz L., Fletcher P. and Molinari J. L. (1978) Epidemiological aspects of scorpionism in Mexico. 1. Purification of neurotoxins from *Centruroides limpidus limpidus* venom. *Toxicon* (*Suppl.*), **1,** 639–646.

Tolksdorf S., McCready M., McCullagh D. and Schwenk E. (1949) The turbidimetric assay of Hyaluronidase. *J. Lab. Clin. Med.* **34,** 74–89.

Vijverberg P. M., Pauron D. and Lazdunski M. (1984) The effect of *Tityus serrulatus* scorpion toxin γ on Na channels in neuroblastoma cells. *Pfluegers Arch.* **401,** 297–303.

Zlotkin E., Martinez G., Rochat H. and Miranda F. (1975) A protein toxic to crustacea from the venom of the scorpion *Androctonus australis. Insect. Biochem.* **5,** 243–250.