

## A new toxin from the sea anemone *Condylactis gigantea* with effect on sodium channel inactivation

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### Abstract

A new peptide toxin exhibiting a molecular weight of 5043 Da (av.) and comprising 47 amino acid residues was isolated from the sea anemone *Condylactis gigantea*. Purification of the peptide was achieved by a multistep chromatographic procedure monitoring its strong paralytic activity on crustacea (LD<sub>50</sub> approx. 1 µg/kg). Complete sequence analysis of the toxic peptide revealed the isolation of a new member of type I sea anemone sodium channel toxins containing the typical pattern of the six cysteine residues. From 11 kg of wet starting material, approximately 1 g of the peptide toxin was isolated. The physiological action of the new toxin from *C. gigantea* CgNa was investigated on sodium currents of rat dorsal root ganglion neurons in culture using whole-cell patch clamp technique ( $n = 60$ ). Under current clamp condition (CgNa) increased action potential duration. This effect is due to slowing down of the TTX-S sodium current inactivation, without modifying the activation process. CgNa prolonged the cardiac action potential duration and enhanced contractile force albeit at 100-fold higher concentrations than the *Anemonia sulcata* toxin ATXII. The action on sodium channel inactivation and on cardiac excitation-contraction coupling resemble previous results with compounds obtained from this and other sea anemones [Shapiro, B.I., 1968. Purification of a toxin from tentacles of the anemone *C. gigantea*. *Toxicon* 5, 253–259; Pelhate, M., Zlotkin, E., 1982. Actions of insect toxin and other toxins derived from the venom of scorpion *Androctonus australis* on isolated giant axons of the cockroach *Periplaneta americana*. *J. Exp. Biol.* 97, 67–77; Salgado, V., Kem, W., 1992. Actions of three structurally distinct sea anemone toxins on crustacean and insect sodium channels. *Toxicon* 30, 1365–1381; Bruhn, T., Schaller, C., Schulze, C., Sanchez-Rodriguez, J., Dannmeier, C., Ravens, U., Heubach, J.F., Eckhardt, K., Schmidt, H., Aneiros, A., Wachter, E., Béress, L., 2001. Isolation and characterization of 5 neurotoxic and cardiotoxic polypeptides from the sea anemone *Anthopleura elegantissima*. *Toxicon*, 39, 693–702]. Comprehensive analysis of the purified active fractions suggests that CgNa may represent the main peptide toxin of this sea anemone species.

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**Keywords:** Peptide toxin; Na<sup>+</sup>-channel inactivation; Positive inotropic effect; *Condylactis gigantea*; Sea anemone; Peptide purification

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✉In memoriam.

## 1. Introduction

Sea anemones are sessile coelenterates living on solid cliffs on the floor of the ocean. They have large tentacles that incorporate and eliminate substances for metabolism and that contain venom-producing nematocysts allowing them to prey on small crustaceans and defend themselves against predators. These tentacles are well-known sources for the isolation of toxic proteins including pore-forming cytotoxins (Wang et al., 2000; Anderluh and Macek, 2002), phospholipases (Grotendorst and Hessinger, 1999), Na<sup>+</sup>-channel toxins (Norton, 1991; Béress, 2004), K<sup>+</sup>-channel inhibitors (Kem, 1988; Aneiros et al., 1993; Castañeda et al., 1995; Schweitz et al., 1995; Diochot et al., 1998), and even proteinase inhibitors (Fritz et al., 1972; Antuch et al., 1993; Delfin et al., 1994). Several of the Na<sup>+</sup>-channel toxins exhibit a high paralytic activity against crustacea.

*Condylactis gigantea* is, among others, a large sea anemone found abundantly in the Bermuda region and in the Caribbean. From *C. gigantea* the first partially characterized polypeptide toxin was isolated consisting of a crab-paralyzing basic polypeptide fraction with a molecular weight of 10–15 kDa (Shapiro, 1968; Shapiro and Lilleheil, 1969). Later this polypeptide toxin was described to interact with nerve membrane ionic conductances (Narahashi et al., 1969) and with sodium conductance of crayfish giant axon membranes (Murayama et al., 1972). Subsequent attempts to reproduce the original toxin-isolation procedure (Yost and O'Brian, 1978) yielded in the isolation of different peptide toxins in the molecular range of 6 kDa, but their exact biochemical properties are still unknown. Salgado and Kem (1992) reported the isolation of a new Na<sup>+</sup>-channel toxin isolated from *C. gigantea*; however details about its molecular properties remain undescribed. Besides the well-characterized sea anemone toxins from *Anemonia sulcata*, *Anthopleura xanthogrammica* and *Anthopleura elegantissima* (reviewed by Norton, 1991; Béress, 2004), to date the molecular description of the main peptide toxins from *C. gigantea* remains unclarified. Here we describe the purification and present the complete primary structure of the main crab paralytic activity from *C. gigantea*. Its biological action on tetrodotoxin-sensitive sodium currents (TTX-S) of peripheral sensory neurons and on action potentials and force of contraction in mammalian myocardium is demonstrated.

## 2. Materials and methods

### 2.1. Toxin extraction and isolation

*C. gigantea* was collected from the Caribbean sea close to Havana. The entire sea anemone body was washed with sea water to free them from attached sand and mud, immediately cooled to 0 °C, and later frozen at –20 °C. For peptide extraction, 11 kg of the sea anemone were mixed with 11 L of ethanol and 110 mL of glacial acetic acid was added. The mixture was homogenized portionwise in a Waring blender, heated to 65 °C for denaturation of enzymes and structural proteins. After centrifugation at room temperature (3000 rpm), the supernatant was collected, and the remaining pellet was re-homogenized and re-extracted with 11 L of ethanol. The supernatants obtained were combined and a protein precipitation was started by adding 15 L ethanol to the 22-L anemone extract and left overnight at room temperature. The solution was filtered on a Seitz K 1000 filter and the filtrate was concentrated at reduced pressure (Rotavapor, Büchi, Essen, Germany) to a final volume of 2 L. A 10-fold amount of acetone was added to the concentrate by stirring and the mixture was left overnight at –20 °C in order to complete the precipitation of proteins and peptides. After 12 h the acetone supernatant was discarded, and the precipitate was re-dissolved in 2.5 L distilled water, filtered, and centrifuged at 5000 rpm.

*Step 1 of purification:* The resulting peptide-containing solution was applied to a Serdolit AD-2 column (5 × 50 cm i.d., Serva, Heidelberg, Germany) at a flow rate of 10 mL/min. The column was washed with 2.7 L one molar ammonium acetate, pH 5.4 and afterwards with 2.7 L water. Gradient elution was carried out using subsequently 2.1 L 1.2% (v/v) acetic acid/water; 3 L of 12.5% (v/v) acetic acid/water; and 4.7 L gradient volume from 12.5% to 50% (v/v) acetic acid/water; stepwise elution was carried out by 1.5 L 50% (v/v) acetic acid/water, 1.8 L 80% (v/v) acetic acid/water, and finally using 1.8 L 100% (v/v) acetic acid. Crab paralyzing activity was tested in all fractions.

All fractions were concentrated at reduced pressure (Rotavapor, Büchi) to 1/30 of their original volume.

*Step 2 of purification:* A 50 mL concentrate of the toxin fraction No. 9 was applied to a Sephadex G50 column (7 × 110 cm i.d., Pharmacia, Uppsala, Sweden) with a flow rate of 5 mL/min. The toxin

was eluted in the 5 kDa peptide fraction No. 4 using 1 M acetic acid as eluent. The fraction was concentrated and freeze dried, yielding about 8 g of protein.

*Step 3 of purification:* In total, 4.5 g of the toxic fraction (No. 4 of step 2) was dissolved in 4.5 L water and applied to cation-exchange chromatography on a SP-Sephadex C25 column (2 × 28 cm i.d., Pharmacia). After rinsing with water at pH 5.4, elution was carried out with distilled water and thereafter with an ammonium acetate buffer at a flow rate of 10 mL/min using a three-step gradient: from 0.01 to 0.1, 0.1 to 0.3 and 0.3 to 1 M. The single eluates were concentrated in an evaporator up to a few milliliter and each fraction was desalted on a Sephadex G25 column by elution with 0.3 M acetic acid. After concentration at reduced pressure the fractions became freeze dried.

*Step 4 of purification:* Final purification of the active fraction (No. 5 of step 3) was performed on an analytical RP HPLC column (Jupiter C18, 4.6 × 250 mm i.d., 5 μm, 30 nm, Phenomenex, Torrance, CA, USA) using 0.1% (v/v) TFA in water as eluent A and A + 80% (v/v) acetonitrile as eluent B. The flow rate was 0.7 mL/min with the following gradient: 0–15% B within 5 min, 15–45% B within 60 min, and from 45% to 100% B within the last 1.5 min. The toxic fraction eluted in a sharp peak at 33% (v/v) B and was subjected to mass spectrometric and sequence analysis.

## 2.2. Peptide analysis

Mass analysis of purified peptides and fractions was performed with Voyager-De MALDI-TOF-MS (Applied Biosystems, Darmstadt, Germany) using standard conditions as recommended by the manufacturer. Peptides were sequenced on a 473 A gas-phase sequencer (Applied Biosystems, Weiterstadt, Germany) by Edman degradation with on-line detection of phenylthiohydantoin amino acids using the standard protocol recommended by the manufacturer.

To analyze the complete peptide sequence, proteolytic cleavage of the reduced and carboxamidomethylated peptide was performed by the endoproteases trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and Asp-N (EC 3.4.24.33) (sequencing grade from Roche Diagnostics, Mannheim, Germany).

The lyophilized, native peptide was reduced with 0.3 mg/mL dithiothreitol (DTT; incubation for

45 min at 50 °C) and alkylated at cysteine residues with iodoacetamide (0.3 mg/mL; incubation for 45 min at room temperature) by means of standard conditions. The carboxamidomethylated peptides were purified by reversed-phase HPLC and analyzed by mass spectrometry.

For tryptic cleavage, the carboxamidomethylated peptide was incubated using a peptide/enzyme ratio of 1/100 (w/w) for 12 h at 37 °C in buffer (pH 7.8, 25 mM NH<sub>4</sub>HCO<sub>3</sub>) as recommended by the manufacturer.

For chymotryptic cleavage, the carboxamidomethylated peptide was incubated using a peptide/enzyme ratio of 1/100 (w/w) for 8 h at 25 °C in Tris-HCl buffer (pH 7.8, 100 mM) as recommended by the manufacturer.

For Asp-N cleavage, incubation of the carboxamidomethylated was performed using a peptide/enzyme ratio of 1/100 (w/w) for 10 s at 37 °C in sodium phosphate buffer (pH 8.0, 50 mM) according to the manufacturer's instructions. The reaction was stopped by adding 200 μl 0.1% (v/v) TFA.

The resulting fragments were separated by analytical C18 RP-HPLC and characterized by ESI-MS, MALDI-MS, and sequence analysis.

## 2.3. Toxin assay and physiological studies

Animal care and procedures were carried out in accordance with the Declaration of Helsinki. The number of animals used for this work was kept to the minimum necessary for a meaningful interpretation of the data.

The assay for toxicity was carried out on the shore crab *Carcinus maenas* as described by Béress and Béress (1971). Sample solutions were injected into the crabs at the junction between the body and the walking leg. Envenomated crustacea exhibited a severe paralysis characterized by an initial spastic and tetanic phase, and a later rigid phase. Depending on the toxin concentration injected, the spastic and tetanic reactions could be observed within seconds or within a few minutes and were followed by paralysis and death of the crab.

Toxicities were proven by serial dilutions (1/10 up to 1/1000) of the samples until a dosage that paralyzed the crab significantly was reached.

### 2.3.1. Electrophysiology in neurons

To determine the action of CgNa toxin on Na<sup>+</sup> currents the patch clamp technique was used. For this purpose rat dorsal root ganglion neurons

(DRGs) were isolated and cultured from young Wistar rats (P5–9) of either sex following the procedure described by Salceda et al. (2002). In current clamp experiments designed to evaluate the effect of CgNa on action potential extracellular standard solutions containing (in mM): 140 NaCl, 5.4 KCl, 3.6 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 10 HEPES at pH = 7.4 (osmolarity = 308 mOsm) were used. The intracellular standard solution was composed of (in mM) 3 NaCl, 145 KCl, 0.1 CaCl<sub>2</sub>, 5 HEPES at pH 7.4 (osmolarity = 303 mOsm).

Whole cell recording was carried out with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). Command pulse generation and data sampling were controlled by the Pclamp 8.0 software (Axon Instruments) using a 16-bit data acquisition system (Digidata 1320A, Axon Instruments). Intracellular modified solution contained (in mM): 10 NaCl, 100 CsF, 30 CsCl, 10 tetraethylammonium chloride (TEA-Cl), 8 EGTA and 5 HEPES. The pH of this solution was adjusted to 7.3 with CsOH. The osmolarity was adjusted to 300 mOsm. The extracellular modified solution to record TTX-S sodium currents contained (in mM): 20 NaCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 45 TEA-Cl, 70 choline chloride, 10 4-aminopyridine (4-AP) and 5 HEPES. The pH of this solution was adjusted to 7.4 with HCl. Osmolarity was adjusted to 290 mOsm using dextrose. Recordings of tetrodotoxin-resistant (TTX-R) sodium currents were made in the presence of TTX (300 nM) added to the modified extracellular solution. The inactivation time constant ( $\tau_h$ ) was calculated adjusting the inactivation time course of TTX-S sodium currents with an exponential function over the following 10 ms after the peak current. A detailed description of methods and data analysis was shown by Salceda et al. (2002).

### 2.3.2. Action potentials and force of contraction mammalian myocardium

Thin right ventricular papillary muscles were excised from the hearts of male guinea pigs (body weight 280–350 g) and mounted in a small chamber perfused with oxygenated Tyrode solution (composition in mM: 126.7 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 1.05 MgCl<sub>2</sub>, 22 NaHCO<sub>3</sub>, 0.42 NaHPO<sub>4</sub>, 5 glucose. After equilibration with carbogen, 95% (v/v) O<sub>2</sub> and 5% (v/v) CO<sub>2</sub>, the pH of the solution was set to 7.4 at 37 °C. The preparations were stimulated electrically (1 Hz, 10% above threshold) and force of contraction and action potentials were recorded with conventional force transducer and glass microelec-

trodes, respectively (Ravens, 1976). *Anemonia sulcata* toxin (ATXII) and *C. gigantea* toxin (CgNa) were dissolved in water as concentrated stock solutions to yield the final bath concentrations of 0.01–10  $\mu$ M. The muscles were allowed to equilibrate for at least 90 min before intracellular action potentials were recorded with conventional glass pipettes filled with 2 M KCl solution (tip resistances of 10–20 M $\Omega$ ). Action potentials from stable impalements were recorded 30–45 min after drug addition before the concentration was increased. Action potentials and force signals were stored on a personal computer and the following parameters were analyzed off-line: resting membrane potential (RMP), action potential amplitude (APA), action potential duration at 20%, 50%, and 90% of repolarization (APD<sub>20</sub>, APD<sub>50</sub> and APD<sub>90</sub>), maximum upstroke velocity ( $dV/dt_{max}$ ). All data acquisition and analysis were carried out with the ISO 2 system (MFK, Niedernhausen, Germany).

## 3. Results

### 3.1. Extraction and isolation of CgNa

The toxin CgNa was isolated from an ethanol/water/acetic acid extract prepared from the whole body of the sea anemone *C. gigantea*. In the supernatant of the ethanol/acetic acid extract a strong crab-paralyzing activity was detected. After concentration and acetone precipitation of the protein/peptide fraction, this activity was observed in the precipitate and its further enrichment was achieved by subsequent chromatographic steps (Fig. 1A–D). Each purification step was accompanied by determination of the crab-paralyzing activity in the obtained chromatographic fractions. In the final reversed-phase purification step (Fig. 1D) a sharp, symmetric peak was obtained and was subjected to mass spectrometric analyses, demonstrating molecular weights of 5027 and 5043 Da, respectively (Fig. 1E).

### 3.2. Primary structure analysis of CgNa

Originally 38 amino acids of the N-terminal sequence were obtained by Edman degradation of the native peptide: GVPCRCDSGDGPSVHGNTLSGTVWVGSCASGWHKCNDEY....

Cysteine residues were not identified but supposed by homology search. To obtain the complete amino acid sequence, the native toxin CgNa was

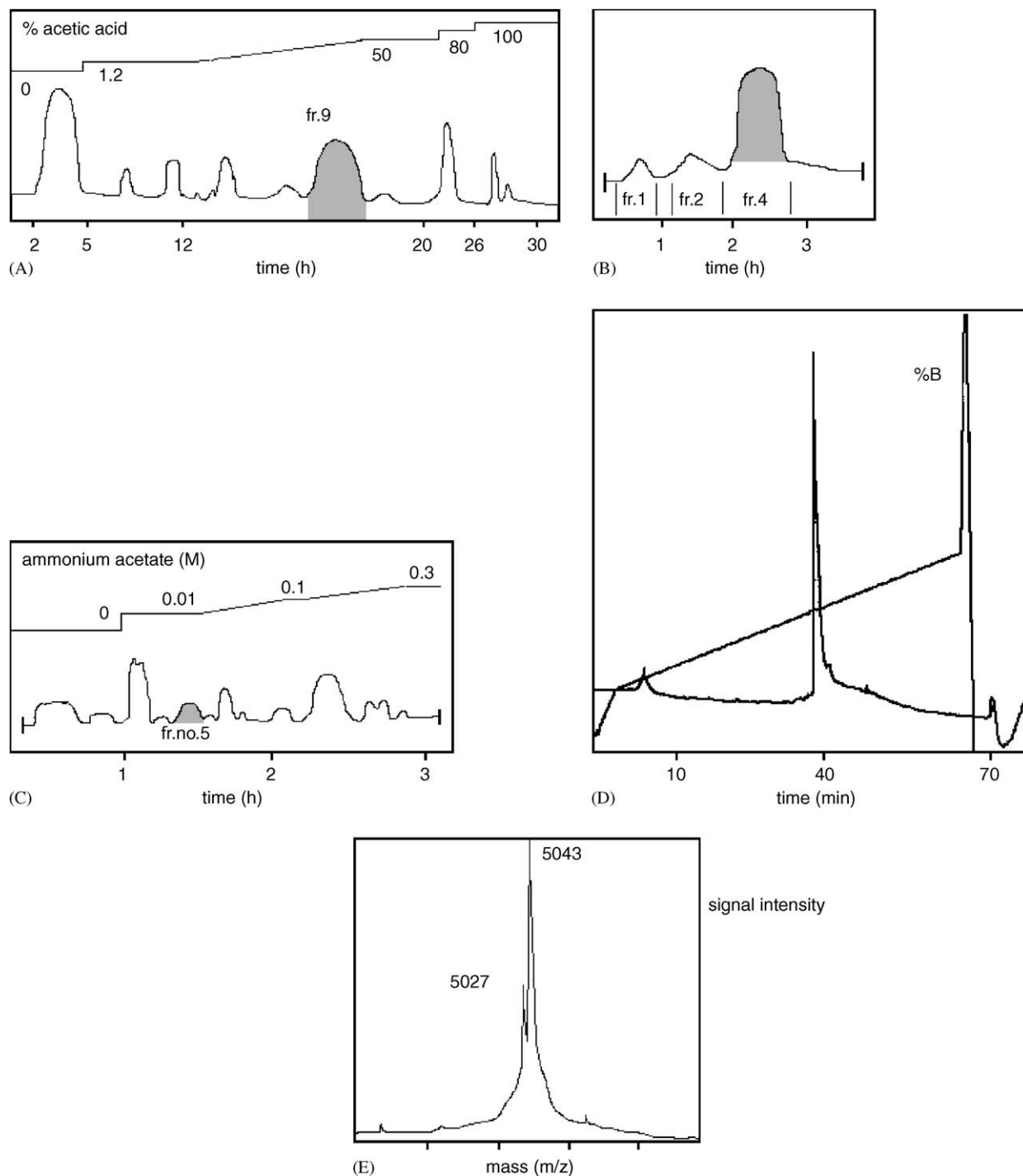


Fig. 1. Isolation of CgNa from acetic acid/ethanol/acetone extract of 11 kg tentacles of *C. gigantea*. (A) The crude extract was first separated on a Serdolit AD-2 column using an acetic acid gradient. The crab-paralyzing activity eluted in fraction 9 and was further enriched by three consecutive chromatographic steps. (B) Purification of the active fraction on a size exclusion chromatography (Sephadex G50) column yielded an enrichment of the activity in fraction no. 4. (C) This active fraction was further separated on cation-exchange chromatography on a SP-Sephadex C25 column and yielded in active fraction no. 5. (D) Final purification step of CgNa on an analytical RP column. (E) Mass spectrometric analysis by MALDI-MS revealed molecular masses of 5027 and 5043 Da indicating the occurrence of a single oxidated form as the major isolation product.

GVPCRCDSGDGPSVHGNTLSGTVWVGSCASGWHKCNDEYNIAYECCKE (native; MW 5043 Da)  
 GVPCRCDSGDGPSVHGNTLSGTVWVGSCASGWHKCN (Cys-CAM; MW 5392 Da)  
 HKCNDEY (Chymotrypsin; MW 965 Da)  
 CNDEYNIAYECCKE (Chymotrypsin; MW 1739 Da)  
 DEYNIAYECCKE (Asp-N; MW 1596 Da)

Fig. 2. Sequence analysis of CgNa by analysis of the reduced, carboxamidomethylated CgNa and of overlapping fragments obtained by cleavages with endoproteinases chymotrypsin and Asp-N. Prior cleavage, CgNa was reduced and carboxamidomethylated at cysteine residues (Cys-CAM). The fragments obtained were analyzed by ESI-MS and *N*-terminal Edman sequence analysis.

reduced, and carboxamidomethylated and cleaved by the endoproteinases trypsin, chymotrypsin, and Asp-N. Trypsin leads to three major fragments comprising major parts of the toxin sequence. Chymotrypsin generated five smaller peptide fragments comprising 10 amino acid residues. All fragment sequences confirmed the initially obtained sequence information. The sequence information could be completed by cleavage with endoproteinase Asp-N yielding one fragment exhibiting a molecular weight of 1594 Da and comprising the sequence DEYNIAYECCKE which corresponds to the C-terminal portion of CgNa (Fig. 2). The molecular mass of the native and of the reduced and carboxamidomethylated CgNa determined by ESI-MS was 5046 and 5394 Da, respectively, matching exactly to the theoretical masses of the corresponding peptides, assuming the occurrence of one hydroxyproline in the CgNa sequence. Therefore the complete CgNa sequence comprises the following 47 amino acid residues:

GVHypCRCDSGDGPSVHGNTLSGTVWVGSCASGWHKCNDEYNIAYECCKE

CgNa has a theoretical pI of 5.05. The difference of the calculated mass (5030.5 Da) and the measured mass (5046 Da) is 16 Da which corresponds to one oxygen. The major peptide exhibits a molecular weight of 5046 Da (oxidized form). The non-oxidized peptide with a molecular weight of 5030 Da was also detected (see Fig. 1E). By sequence analysis, we found that the major portion of the peptide contains one hydroxyproline instead of proline in position three of the sequence. Hydroxyproline was recently found in the related sea anemone peptide toxin Am II from *Antheopsis maculata* (Honma et al., 2005).

### 3.3. Physiological examinations

Under current clamp conditions action potentials were evoked by passing depolarizing current pulses of 1 nA, for 5 ms at a stimulus rate of 0.125 Hz. External application of 10  $\mu$ M of the CgNa toxin for about 1 min induced an evident prolongation of the evoked action potential of about 463% ( $n = 3$ ), measured at the midpoint of the action potential amplitude as depicted in Fig. 3A.

Sensory neurons of dorsal root ganglion are known to express tetrodotoxin-resistant (TTX-R) Na<sup>+</sup> channels and tetrodotoxin-sensitive (TTX-S) Na<sup>+</sup> channels. The effects of the CgNa toxin on the Na<sup>+</sup> currents were studied by a single-step voltage protocol in which from a holding potential of  $-100$  mV a 40 ms test pulse to  $-20$  mV was applied with an interpulse interval of 8 s. The CgNa toxin had no effect on TTX-R Na<sup>+</sup> channels. As it is shown in the Fig. 3B that illustrates a typical experiment, 10  $\mu$ M CgNa slowed down the inactivation process of TTX-S Na<sup>+</sup> current rendering it incomplete. The inactivation time constant changed from  $0.5 \pm 0.7$  ms in control condition to  $0.85 \pm 0.15$  ms after toxin application ( $n = 9$ ). The maximum effect was reached within 1–2 min depending of toxin concentration. This effect was reversible after repeated washing of the preparation.

Like ATXII, *C. gigantea* toxin prolonged the action potential duration and enhanced force of contraction in guinea pig papillary muscles, albeit at 100-fold higher concentrations (Fig. 4). After 20 min of exposure to 10  $\mu$ M CgNa, action potential duration at 90% repolarization had markedly increased and force of contraction was enhanced to a similar extent as with 1  $\mu$ M ATXII with no statistically significant difference. All effects were completely reversible after washing in toxin-free solution.

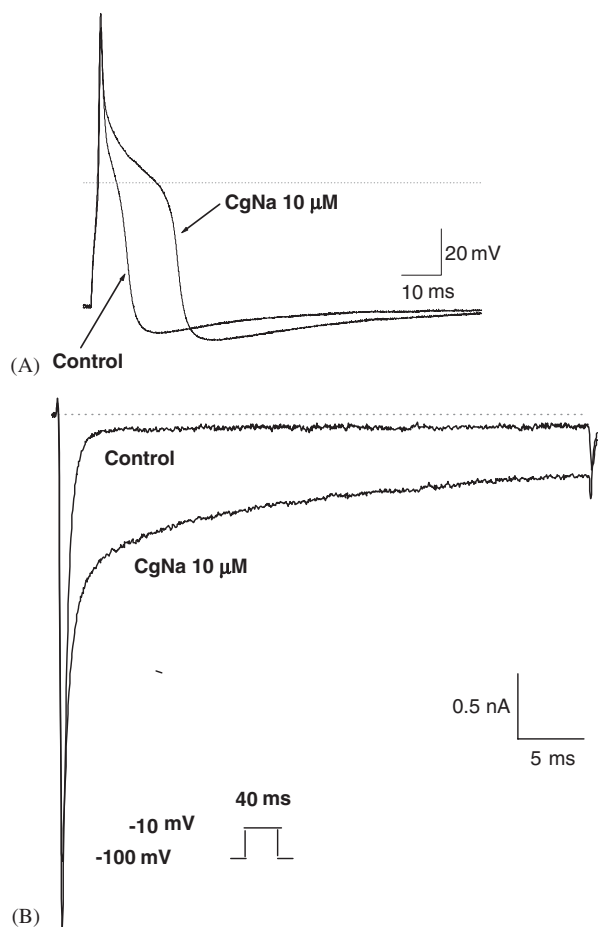


Fig. 3. Effect of toxin CgNa on current clamp and voltage clamp condition. (A) Effect of CgNa ( $10\ \mu\text{M}$ ) under current clamp condition on action potentials of rat dorsal root ganglion neurons. Action potentials were elicited by  $1\ \text{nA}$ ,  $2.5\ \text{ms}$  depolarizing stimuli and recorded in the absence (control) and in the presence of toxin CgNa. The neurons were stimulated at a frequency of  $0.125\ \text{Hz}$ . (B) The records represent superimposed traces before and after application of CgNa ( $10\ \mu\text{M}$ ). Notice that the toxin CgNa produced a marked slowing of the inactivation process of the TTX-S sodium current. The inactivation time constant changed from  $0.40\ \text{ms}$  in control condition to  $0.75\ \text{ms}$  after toxin CgNa application. It represents a change of about 87%.

#### 4. Discussion

According to their primary structure and disulfide bonding pattern, sea anemone sodium channel toxins can be classified into three main types: Type 1 and type 2 toxins consisting of 46–49 amino acid residues connected by three disulfide bonds and type 3 toxins of 27–31 residues (Norton, 1991). They are generally more toxic to crustaceans than to mam-

mals. The CgNa toxin presented in this study clearly belongs to type I toxins sharing the closest sequence homology to ATXII (Wunderer et al., 1976) and ATXV (Scheffler et al., 1982) from *Anemonia sulcata* as well as to ApC from *Anthopleura elegantissima* (Bruhn et al., 2001) (Fig. 5). Further members of this group include AftI and AftII from *Anthopleura fuscoviridis*, ApA and ApB from *Anthopleura xantogrammica* and BgII and BgIII from *Bunodosoma granulifera* (Norton, 1981; Kelso and Blumenthal, 1998; Goudet et al., 2001; Salceda, et al., 2002).

Comprehensive examination of all peptide-containing fractions prepared from *C. gigantea* using the applied toxin assay and mass spectrometry revealed that the CgNa toxin represents the main peptide toxin occurring in this sea anemone species.

The type I toxins share a considerable similarity in electrophysiological activity. They target receptor site 3 on mammalian sodium channels and their main effect seems to delay sodium channel inactivation leading to neurotoxic (repetitive firing) and cardiotoxic (arrhythmia) effects (Caterall 1995; Rogers et al., 1996).

Since the early work of Shapiro (1968), on a partially purified toxin from the tentacles of the sea anemone *C. gigantea*, showing an increase in action potential duration in lobster giant axons, different studies were developed in various laboratories. Subsequent studies of this material using voltage clamp techniques in crayfish giant axons showed that the above-mentioned effect was due to the slowing of the rate of inactivation of  $\text{Na}^+$  channel (Narahashi et al., 1969; Murayama et al., 1972).

Moreover, the effect of a compound from *C. gigantea* was studied under current clamp and voltage clamp conditions on single giant axons of cockroach (Pelhate et al., 1979). This compound prolonged the falling phase of the  $\text{Na}^+$  current associated with a delay in the inactivation process of this current and with a decrease in the potassium conductance. According to Salgado and Kem (1992), the results obtained for the pure toxin CgII from this sea anemone resembled the above described effects. This toxin was particularly lethal to crustaceans, moderately toxic to insect (cockroach) and essentially non-toxic to mammal (mouse). This peptide produced a qualitatively similar action at the  $\text{Na}^+$  channel of the three models. Qualitatively similar effects of CgNa and ATXII were also observed with respect to prolonging of the action potential and positive

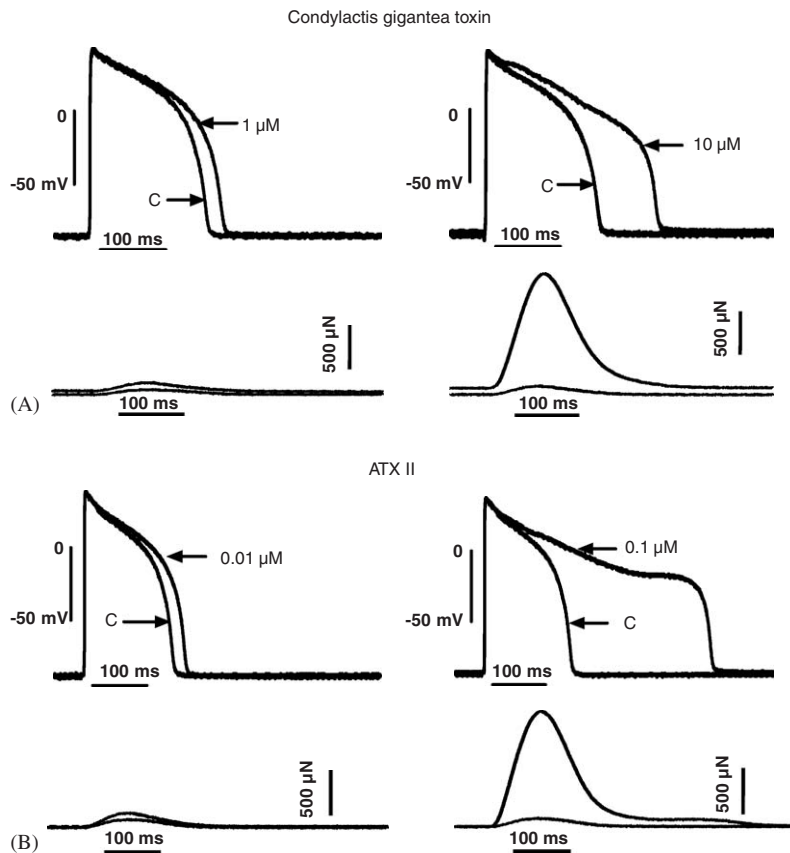


Fig. 4. Effects of *C. gigantea* (CgNa, A) and *A. sulcata* toxin (ATXII, B) on action potentials and force of contraction of guinea pig right ventricular papillary muscles. (C) Control tracing, tracings after 20 min of exposure to toxin are indicated by the respective concentration. Rate of stimulation 1 Hz. Mean values for control recordings ( $n = 6$ ): Action potential amplitude  $126 \pm 0$  mV, resting membrane potential  $-87.2 \pm 0.3$  mV,  $APD_{90}$   $167.7 \pm 2.1$  ms,  $APD_{50}$   $145.8 \pm 2.2$  ms,  $APD_{20}$   $82.2 \pm 3.5$  ms,  $dV/dt_{max}$   $243.3 \pm 6.4$  V/s, force of contraction  $75.0 \pm 9.9$   $\mu$ N.

CgNa GVP**C**R**C**DS**D**SGPSVHGNTLSGTVVWVSGSCASGWHKCNDEYNIAYE**C**CKE  
 ATXII GVP**C**L**C**DS**D**SGPSV**R**GN**T**LSG**I**I**W**L**A**F**C**PSGWH**N**CK**K**H**G**P**T**I**G**W**C**CK**Q**  
 ATXV GVP**C**L**C**DS**D**SGPSV**R**GN**T**LSG**I**L**W**L**A**G**C**PSGWH**N**CK**K**H**K**P**T**I**G**W**C**CK  
 AP-C GVP**C**L**C**DS**D**SGPSV**R**GN**T**LSG**I**L**W**L**A**G**C**PSGWH**N**CK**A**H**G**P**T**I**G**W**C**CK**Q**

Fig. 5. Amino acid sequence alignment of CgNa with analogous peptide toxins. ATXII and ATXV are from *Anemonia sulcata*, AP-C is from *Anthopleura elegantissima*. Identical residues with CgNa are in black color, residues different from CgNa are in red color, and the conserved cysteines are highlighted in blue. The first proline in the CgNa sequence was determined to be a hydroxyproline.

inotropic effects in the mammalian heart. Loading of cardiac cells with  $Na^+$  via non-inactivating sodium channels increases the content of intracellular  $Ca^{2+}$  stores thereby allowing more  $Ca^{2+}$  release and hence stronger contractile activation during an action potential (Bruhn et al., 2001).

In addition to the above-mentioned compounds, others such as a phospholipase A acting preferentially as a blocker of  $Na^+$  currents in snail neurons (Garateix et al., 1990), a high molecular weight compound with anticholinergic action in mice and snail neurons (Garateix et al., 1992) and cytolisins (Bernheimer et al., 1982) were reported.

CgNa slowed the inactivation kinetics of the sodium current cultured isolated neurons from rat dorsal root ganglion. This effect is in agreement with previous results using extracts or semi-purified compounds from the sea anemone (Shapiro, 1968; Pelhate and Zlotkin, 1982; Salgado and Kem, 1992) and with the classical action of sea anemone type I  $Na^+$  channel toxins. The actions of CgNa on neuronal sodium currents is probably caused by altering the gating of the S4 segment of domain IV, as has been proposed for other site-3 toxins



(Chen et al., 2000). Further studies of the structure and function relationship will try to clarify the molecular mode of action of the CgNa toxin.

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