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Selective modification of the squid axon Na currents by *Centruroides noxius* toxin II-10*

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SUMMARY :

1° We have studied the effects of the purified toxin II-10, from the venom of the scorpion *Centruroides noxius* Hoffmann, on the Na and K currents of voltage clamped squid giant axons.

2° Extracellular applications of 10 μ M of toxin II-10 produced a selective depression of peak Na currents, with no significant effects on the time course of K currents.

3° On pharmacologically separated Na currents, low concentrations of toxin II-10 (0.28-1 μ M) caused a reversible decrease in inward and outward peak I_{Na} , with little effect on either the maintained level of the currents or their turning-off.

4° At high concentrations (> 3 μ M), toxin II-10 drastically reduced the peak conductance and increased both the level of the maintained conductance, and the time course of its turning-off.

5° It is suggested that, when applied extracellularly on squid axons, toxin II-10 primarily reduces the peak Na conductance by modifying the activation of fast-inactivating Na channels. At high concentrations (10 μ M), the toxin also modifies the rate constants of the transition from the inactivated to the second open state of the channel (CHANDLER and MEVES, 1970) thus producing an increased level of the maintained Na conductance. It is also very likely, however, that peak conductance and maintained conductance reflect two separate populations of Na channels on which toxin II-10 has a differentiated action. Under these conditions, toxin II-10 would be the first reported toxin which can pharmacologically separate the two types of channels.

Key-words : Na-channels. Squid axon. Scorpion toxins. Toxin purification.

INTRODUCTION

Neurotoxins have become increasingly important tools with which to investigate the molecular components that control membrane excitability (NARAHASHI, 1974 ; CATTERALL, 1980). Among them, scorpion toxins have recently been shown to interact specifically with either the Na channel of various cell preparations (ROMEY *et al.*, 1975 ; BERNARD *et al.*, 1977 ; MOZHAYEVA *et al.*, 1980 ; MEVES *et al.*, 1982 ; JAIMOVICH *et al.*, 1982 ; WANG and STRICHARTZ, 1982 ; BARHANIN *et al.*, 1983) or with the K channel of squid giant axons (CARBONE *et al.*, 1982 ; CARBONE *et al.*, 1983). Toxins acting on Na channels can be divided into two classes : those affecting Na activation and those affecting Na inactivation (HU *et al.*, 1983 ; WHEELER *et al.*, 1983). Very likely, different modes of action reflect different chemical structures of the various toxins. For instance, toxins acting on K channels appear to have rather similar molecular weight and amino acid sequence (POSSANI *et al.*, 1982), which differ considerably from those of toxins acting on Na channels (BABIN *et al.*, 1975 ; GARCÍA, 1976).

In this paper we present additional data on the purification and physiological characterization of toxin II-10 from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann. The major effect of the toxin is that it selectively reduces the peak Na conductance of squid axon membrane leaving the K-system almost unaltered. At concentrations greater than 3 μ M the peak decrement is usually accompanied by an increase in the maintained conductance, which was not clearly observed in previous studies (CARBONE *et al.*, 1982), where the concentrations used were lower than 1.4 μ M.

From its mode of action and partial amino acid sequence II-10 appears to be very similar to other toxins known to act on the activation machinery of other Na channels (JAIMOVICH *et al.*, 1982 ; MEVES *et al.*, 1982).

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METHODS

— **Axon preparation and voltage clamp.** All experiments were performed on internally perfused giant axons of the squid *Loligo vulgaris*, available in Camogli (Italy). The perfusion technique, voltage clamp apparatus and pulse protocol for measuring Na currents were similar to those previously described (WANKE *et al.*, 1979; CARBONE *et al.*, 1981). Axons were bathed in artificial sea water (ASW) containing (mM): 435 NaCl, 10 KCl, 10 CaCl₂, 40 MgCl₂, 20 Tris, pH₀ 8.0. The standard intracellular medium (50 Na-SIS) contained (mM): 267 CsF, 307 sucrose, 50 NaF, 45 K-phosphate, pH_i 7.2. When both Na and K currents were recorded, the intracellular medium was 400 K-SIS containing (mM): 317 KF, 307 sucrose, 45 K-phosphate buffer, pH_i 7.2. The fibres were held at a holding potential of -70 mV, and preconditioned to -90 mV for 80 ms before applying the depolarizing pulses. The temperature of the bath was maintained at 5 °C throughout all the experiments. Stock solutions of the purified toxin were added directly to the external bath by means of a Hamilton microsyringe to attain the desired final concentration.

— **Biochemical characterization of toxin II-10.** The venom from the Mexican scorpion *Centruroides noxius* Hoffmann was previously fractionated by Sephadex G-50 gel filtration followed by ion exchange chromatography on carboxymethylcellulose column in 20 mM ammonium acetate buffer pH 4.7 (POSSANI *et al.*, 1981). Another chromatographic step in CM-cellulose column equilibrated with 50 mM sodium phosphate buffer, pH 6.0, was introduced (see Results). The homogeneity of the toxin was verified by polyacrylamide gel electrophoresis in the acetate-urea-β-alanine system of REISFELD *et al.*, (1962) and by amino acid sequencing. The pure toxin was reduced and carboxymethylated according to the procedure described (POSSANI *et al.*, 1981), and its N-terminal amino acid sequence was determined by Edman degradation in a Beckman 890 C sequencer following the method of EDMAN and BEGG (1967). The identification of the phenylthiohydantoin amino acids (PTH) was performed by high liquid pressure chromatography and by amino acid analysis (Durrum D-500) of the PTH amino acids back hydrolyzed, as already published (POSSANI *et al.*, 1981).

RESULTS

A - Purification and partial sequence determination of toxin II-10

The toxin component II-10 from the venom of the scorpion *Centruroides noxius* (lethal to mice) was completely purified by a second ion exchange chromatographic step (Fig. 1). From the three toxic fractions (b, c and d) the second one was shown by amino acid analysis and by voltage clamp experiments to correspond to our previous toxin II-10, which affects the peak Na conductance of squid giant axons (CARBONE *et al.*, 1982).

The N-terminal amino acid sequence of the reduced and carboxymethylated toxin II-10 (c) (\approx 50 nmoles) was shown to be: Lys-Glu-Gly-Tyr-Leu-Val Asn-Leu-Tyr-Thr-Gly-Cys-Lys-Tyr-Glu-Cys-Phe-Lys-Leu-Gly-Asp Asn Asp-Tyr-Cys-Leu-.... Only one amino acid was identified at each step of the sequence with an over-all

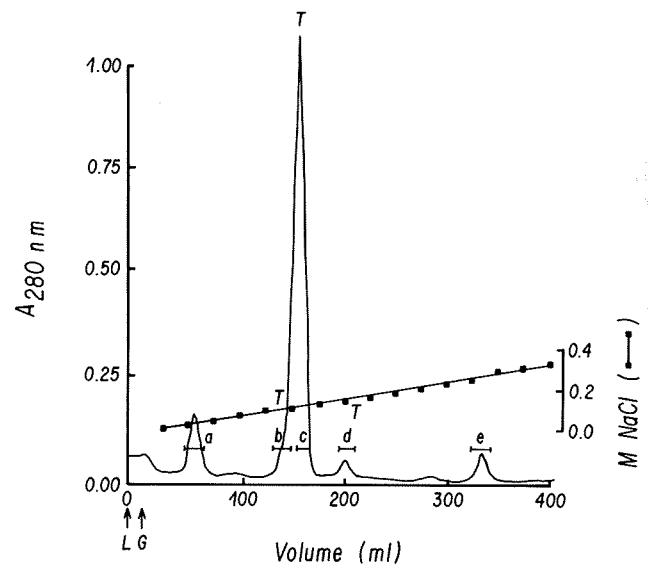


FIG. 1. — Carboxymethylcellulose chromatography of component II-10. Component II-10 from previous chromatography (POSSANI *et al.*, 1981) was loaded (20.2 mg in 27 ml) to a CM-cellulose column (0.9 × 32 cm), equilibrated and eluted with 50 mM sodium phosphate buffer, pH 6.0 with a linear gradient (250 ml each) of sodium chloride from 0 to 0.38 M in the same buffer. Aliquots of 2.5 ml were collected at the flow rate of 30 ml/h and pooled as fractions II-10 (a) to II-10 (e) (horizontal bars). The final column recovery was approximately 92 % of the applied sample and fractions (a) through (e) correspond respectively to 9.2, 11.7, 51.5, 3.1 and 3.5 % recovery. Effluent during loading (L) and side-tubes during gradient run (G) accounted for 21 % of the remaining material. (T) denotes toxic to mice (intraperitoneally injection of 20 to 40 μg protein per 20 g mouse). Only one band in gel electrophoresis was obtained with component II-10 (c), while toxin II-10 (b) had an important contamination of toxin II-10 (c) (data not shown).

yield of 95 %. These results corroborate the purity of the toxin II-10 (c) (same as II-10 from CARBONE *et al.*, 1982). Toxin from this preparation was used for the present study.

B - Effects of the purified toxin II-10 on Na and K currents.

The effects of 10 μM of toxin II-10 on the time course of Na and K currents are shown in Fig. 2. Toxin II-10 produces a rapid decrease of peak I_{Na} at 0 mV without altering either the steady-state level of I_K or the time course of the tail currents recorded on repolarization to -70 mV. Closer inspection shows that the depression of peak currents is accompanied by a slight prolongation of the time to peak, and by a sizeable slow down of the outward current component (Fig. 2 trace T). The latter effect, observed in two other axons, can be due either to a prolongation of the time course of the outward K currents or to a slowing down of the Na inactivation process. As shown below the

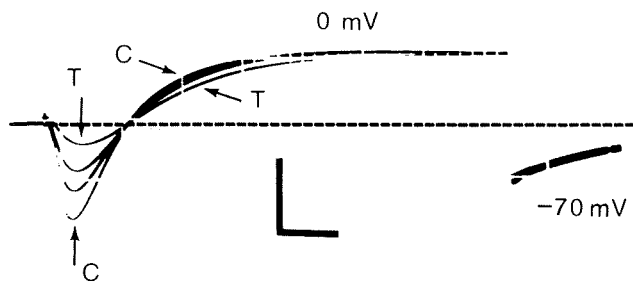


FIG. 2. — Selective modification of Na currents after external application of $10 \mu\text{M}$ toxin II-10. The records were obtained on step depolarization to 0 mV from an axon maintained near physiological conditions. Trace (c) was taken before application of the toxin. Successive records (showing progressively smaller peak inward currents) were recorded at 1 min intervals. Trace (T) was the last before the recovery started. Note the constancy of the steady-state level of I_K at the end of the pulse, and the little effect of the toxin on the time course of tail currents on repolarization to -70 mV. Bars : 2 mA/cm², 2 ms. In : 400 K-SIS. Out : ASW. Axon C12FB82. Temperature : 5°C .

second possibility can account for most of these effects, suggesting only a weak interaction, if any, of II-10 with the K channel kinetics. This point, however, was not further investigated.

C - Effects of II-10 on pharmacologically separated Na currents.

In axons internally perfused with 217 mM CsF to block K outward currents, addition of 280 nM of toxin II-10 (Fig. 3, left) reduces by 60 % the size of peak I_{Na} at 0 mV with no significant effects on : (1) the time course of Na activation and inactivation, (2) the maintained level of currents at $+100$ mV, and (3) the time course of tail currents on step repolarization. At this concentration the action is fully reversible (Fig. 3, right), and requires approximately 10 min to be complete. Larger doses of toxin (up to $1 \mu\text{M}$) speed up the reduction of peak currents and increase the extent of

the depression (see also Fig. 2d in CARBONE *et al.*, 1982).

At large concentrations ($> 3 \mu\text{M}$) the action of toxin II-10 becomes somewhat more complex. At $10 \mu\text{M}$ (Fig. 4) the depression of peak I_{Na} is more pronounced (87 % at 0 mV) while the level of the maintained currents increases by 60 % at $+100$ mV. Comparing the records at 0 mV taken before (C) and after 8 min addition of the toxin (T), it is evident that II-10 prolongs the time to peak of I_{Na} , as well as the time constant of Na inactivation. The effects of the toxin are even more striking on the kinetics of Na channel closing. Tail currents recorded on repolarization to -70 mV become larger and markedly slower in the presence of the toxin. Usually, at these toxin concentrations leakage currents show a slight and progressive increase which, in any case, never contribute more than 15 % to the level of peak outward current at $+100$ mV. Under these conditions, recovery was usually largely uncomplete.

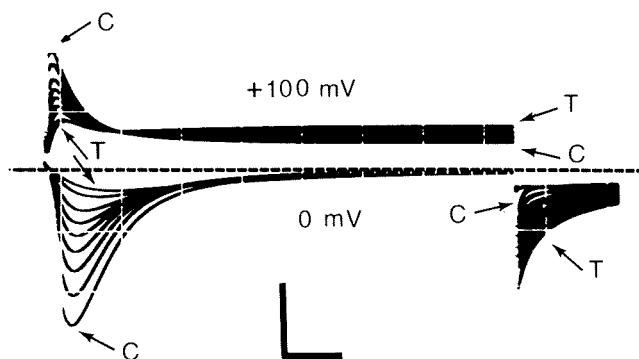


FIG. 4. — Effects of high concentrations of toxin II-10 on Na currents. Lower records were taken at 0 mV; upper records at $+100$ mV. Traces (C) were recorded before the addition of $10 \mu\text{M}$ of toxin II-10. Successive traces, showing progressively smaller peak amplitudes, were taken after toxin application at intervals of 1 min. Traces T were the last records before recovery (not shown). Note the increase of maintained currents at the end of the $+100$ mV pulse and the pronounced increase in tail current amplitude during successive repolarization to -70 mV. Bars : 1 mA/cm², 2 ms. In : 50 Na-SIS. Out : ASW. Axon B12FB82. Temperature : 5°C .

Toxin

Washout

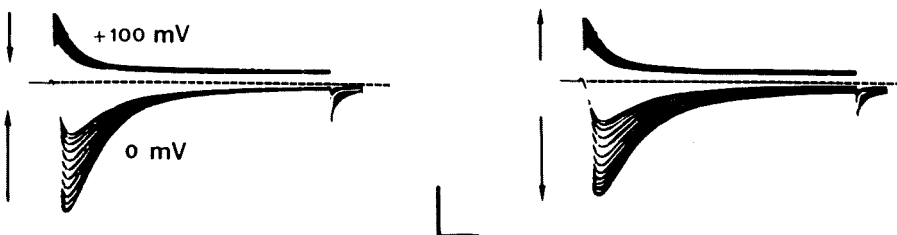


FIG. 3. — Reversible action of toxin II-10 on Na currents. The records on the left were obtained during application of 280 nM of toxin II-10, those on the right during washout. Lower traces were recorded on depolarization to 0 mV; upper traces to $+100$ mV. Pulses to 0 mV and $+100$ mV were separated by 3 s, and repeated each minute. Arrows indicate the sequential order in which the voltage clamp currents were recorded. Repolarizations were to -70 mV. Bars : 1 mA/cm², 2 ms. In : 50 Na-SIS. Out : ASW. Axon 26JN82. Temperature : 5°C .

DISCUSSION

Our results show that II-10 acts on the squid axon membrane by selectively decreasing the peak Na currents. The action of the toxin is fast, develops at low concentrations (at 280 nM Na currents are nearly halved at +100 mV) and is fully reversible (Fig. 3). Thus, the present data confirm our previous findings obtained with a similar toxin, which has been shown to affect the peak Na-conductance in a dose-dependent manner (CARBONE *et al.*, 1982). From these observations it was concluded that toxin II-10 might interact specifically with the transiently open Na channel by fully blocking it in a one to one ratio. Such interpretation, however, is now in contrast to the present results. At large depolarizations, the block of the peak conductance is largely incomplete and the level of the maintained Na-conductance increases in the presence of 10 μ M toxin (Fig. 4).

From the above arguments it is tempting to suggest that toxin II-10 acts on the Na-conductance mechanism by increasing the number of channels which go from the peak conductance state into the maintained conductance state (CHANDLER and MEVES, 1970; GILLESPIE and MEVES, 1980). But it is also possible that peak conductance and maintained conductance reflect two separate populations of Na channels (one with fast activation and inactivation, and one with slow activation and no inactivation) and that II-10 transforms one type of channel into the other (MATTESON and ARMSTRONG, 1982). In the second case, however, we can not exclude the possibility that toxin II-10 has a differentiated action on the two channels: partially blocking those fast-inactivating and decreasing the rate of closing of those slowly activating. The latter effect

would account for most of the increased level of the maintained conductance as well as for the drastic slow down of tail currents during membrane repolarizations. A better discrimination between the various mechanisms of action presently requires further experimental work.

The distinct effects of toxin II-10 on the peak and maintained Na-conductance (Fig. 3, 4) are typical of other toxins known to act on Na channels. For instance, the action on the peak conductance observed at 280 nM (Fig. 3) is similar to that of toxin II of the venom *Centruroides suffusus suffusus*, *C.s.s.II* (JAIMOVICH *et al.*, 1982) and of toxin- γ from the venom *Tityus serrulatus*, *TiTx γ* (VIJVERBERG and LAZDUNSKI, 1983). In the case of *C.s.s.II*, 20 nM of toxin are sufficient to fully block the peak Na currents of frog skeletal muscles, while 100 nM of *TiTx γ* block only 50% of the inward Na currents in neuroblastoma cells. As for II-10, the action of the two toxins is shown to be selective for the Na channel, but there is no proof of their reversibility of action. In contrast to that, the effects on the maintained conductance observed above 3 μ M (Fig. 4) resemble more the actions of the venoms *Centruroides sculpturatus* Ewing and *Leiurus quinquestriatus* on the Na channels of squid giant axons (GILLESPIE and MEVES, 1980) and of mammal toxin I, *MTI*, from the venom *Androctonus australis* Hector on the same preparation (PICHON, 1983). In both cases, applications of 0.1 to 0.5 mg/ml of the venom *C.s.E* (or micromolar aliquotes of *MTI*) produce a sizeable increase in the maintained Na-conductance and a slow down of its turning-off kinetics. Fig. 5 shows that similar effects can also be seen when 1 mg/ml of the venoms *Centruroides suffusus suffusus* and *Centruroides noxius* Hoffmann are applied on perfused

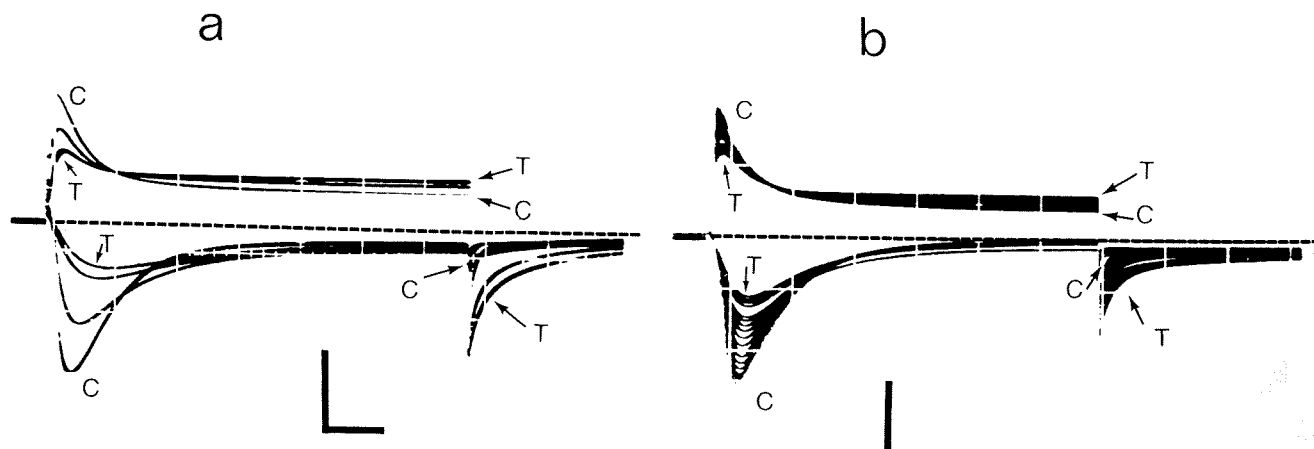


FIG. 5. — Effects on Na currents of — a : Venoms from the scorpions *Centruroides noxius* Hoffmann. — b : *Centruroides suffusus suffusus*. Lower traces were recorded with pulses to 0 mV and upper traces to +100 mV. (C) traces were taken before the addition of 1 mg/ml of the venoms. (T) were the last records before recovery (not shown). In a the voltage clamp currents were recorded every 3 min ; in b every min. Repolarizations to -70 mV. Bars : 1 mA/cm², 2 ms. In : 50 Na-SIS. Out : ASW. Axons : A26MR82 and BO3FB82. Temperature : 5 °C.

TABLE I. — Comparison of the N-terminal amino acid sequence and physiological action of toxins from scorpions of the genus *Centruroides*. * Total amino acid sequence known (see reference). Abbreviations according to the scientific names of the scorpions were used : C.n. : *Centruroides noxius* ; C.s.E : *C. sculpturatus* Ewing ; C.s.s. : *C. suffusus suffusus* ; C.e. : *C. elegans* ; C.l.t. : *C. limpidus tecomanus*. NTX means noxius-toxin. Dash (—) was introduced in order to enhance similarities in the sequences. X means unknown amino acid.

Toxin	Sequence			Reference	Action	Reference
	1	10	20			
C.n. II-10	KEGYLVNLYTGCKYECFKLGDNDY	- -	CL...	This work	Na activation	
C.n. II-9.2.2	KEGYLVDKNTGCKYECLKLGNDY	- -	CL...	POSSANI <i>et al.</i> , 1981	No effects	CARBONE <i>et al.</i> (see text)
C.n. II-13	KEGYI VDYHDGCKYXCYKLGNDY	- -	XL...	POSSANI <i>et al.</i> , 1981	No effects	CARBONE <i>et al.</i>
C.n. II-14	KDGYLVDAK- GCKKNCYKLGKNDY	- -	CN...	POSSANI <i>et al.</i> , 1981	Na activation	CARBONE <i>et al.</i>
C.s.E. I*	KDGYLVEKT- GCKKTCYKLGENDF	- -	CN...	BABIN <i>et al.</i> , 1975	Na activation	MEVES <i>et al.</i> , 1982
C.s.E. V1*	KEGYLVKKS DGCKYDCFWL GKNEHNTCE	BABIN <i>et al.</i> , 1974	Na activation	MEVES <i>et al.</i> , 1982
C.s.E. V2*	KEGYLVNKS TGCKYGLKGENE GNKCE	BABIN <i>et al.</i> , 1974	Na inactivation	MEVES <i>et al.</i> , 1982
C.s.E. V3*	KEGYLVKKS DGCKYGLKLGNEG-	- -	CD...	FONTECILLA-CAMPS <i>et al.</i> , 1980	Na inactivation	MEVES <i>et al.</i> , 1982
C.s.s. I	KEGYLVSKSTGCKYECLKLGNDY	- -	CL...	GARCIA, 1976	Na inactivation	
C.s.s. II*	KEGYLVSKSTGCKYECLKLGNDY	- -	CL...	GARCIA, 1976	Na activation	JAIMOVICH <i>et al.</i> , 1982
C.e. II-6.3	KZGYLVBHSTGCKYZCFKLGBBBY	- -	CL...	RAMIREZ <i>et al.</i> , 1981	Na activation	
C.l.t. II-9.3	KZGXLVBHXTGC	POSSANI <i>et al.</i> , 1980	Na activation	
C.n. NTX*	TI I NVKCTSPKQCS KPCKELYGSS AGAK	POSSANI <i>et al.</i> , 1982	K activation	CARBONE <i>et al.</i> , 1982
C.n. II-10.2	TFI DVKCGSSKECXP	POSSANI <i>et al.</i> , 1982		

axons, indicating that the increase in the maintained conductance and related phenomena are a general property of most scorpion venoms when applied to the giant axon of the squid. It is noteworthy that the venoms *C.s.E* and *L.q.* applied to myelinated fibres produce rather different effects (CAHALAN, 1975 ; MEVES *et al.*, 1982). The reason for this has still to be clarified.

Finally we would like to comment on the above physiological data with respect to the information available on the primary structure of the toxins from the venom of the scorpion *Centruroides*. In Table I we have reported the partial N-terminal amino acid sequence of 14 toxins from the scorpions of the genus *Centruroides*. As shown, toxins acting on Na channels appear to have very similar structure, which differ remarkably from that of K-toxins : NTX and *C.n.II-10.2* (POSSANI *et al.*, 1982). Among Na-toxins the similarity between II-10, *C.s.s.II* and *C.n.II-14* is remarkable. The latter, at high concentrations was observed to have a slight effect on the peak Na-conductance, while toxins *C.n.II-9.2.2* and *C.n.II-13* were found to be insensitive to both Na and K currents of the squid giant axon (CARBONE, PRESTIPINO, WANKE, POSSANI and MAELICKE, unpublished observations).

A more detailed discussion of the effects of toxin II-10 compared to other Na-toxins requires of course more work and the full knowledge of its amino acid sequence. Since it is very likely that minor changes or deletions condition the fine mechanism of action of each toxin, it is conceivable that their identification will allow for a better understanding of the mode of action of the toxins and of the structure of their receptors at the

channel site. In this respect, toxin II-10 seems to have all the prerequisites for such an approach : selectivity of action and high affinity for the channel.

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