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Centruroides toxin, a selective blocker of surface Na^+ channels in skeletal muscle: Voltage-clamp analysis and biochemical characterization of the receptor

(scorpion toxin/muscle/excitation–contraction coupling/receptor binding)

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ABSTRACT **This paper describes the effects of a toxin from the scorpion *Centruroides suffusus suffusus* on frog skeletal muscle. The main findings are the following. (*i*) *Centruroides* toxin (Css_{II}) blocks the early phase of the inward sodium current in the muscle that arises from influx via Na^+ channels in the surface membrane, but it does not affect the late phase of the inward current that represents flux through Na^+ channels in the T-tubule membranes. (*ii*) Css_{II}, in marked contrast to tetrodotoxin, does not affect contraction of the muscle. (*iii*) Measurements of the binding of ^{125}I-labeled Css_{II} to a partially purified membrane preparation from the muscle indicate that the K_d of the Css_{II}–receptor complex is ≈0.4 nM. The half-life for the dissociation of this complex is 3 min at 22°C and 16 min at 2°C. Binding of the radiolabeled toxin varies markedly with pH and becomes insignificant at pH >8.5. Proteolytic digestion of the membrane preparation decreases its ability to bind Css_{II}, suggesting that the receptor is a protein. (*iv*) The number of binding sites for a radiolabeled derivative of tetrodotoxin on the membrane preparation was similar to that for Css_{II}. However, neither tetrodotoxin nor any of seven other neurotoxins and some local anesthetics that alter the functioning of the Na^+ channel have any effect on the binding of Css_{II} to the muscle membrane. These results therefore indicate that Css_{II} belongs to a different class of neurotoxins that has a different receptor on the Na^+ channel.**

In recent years, neurotoxins have become essential tools in neurobiological studies (1, 2). The Na^+ channel appears to be a privileged structure for the action of neurotoxic molecules of natural origin. Toxins that are specific for this channel include (*i*) tetrodotoxin (TTX) and saxitoxin, which block Na^+ entry through the Na^+ channel (1, 3); (*ii*) veratridine, batrachotoxin, aconitine, and grayanotoxin, all of which stabilize a permanently open form of the Na^+ channel and thereby provoke depolarization of excitable cells (1, 3–5); (*iii*) polypeptide toxins, such as those from the scorpion *Androctonus australis* Hector (1, 6) and from the sea anemone (1, 5, 7), which specifically slow down inactivation of the Na^+ channel; and (*iv*) pyrethroids, which also prolong the lifetime of the open form of the Na^+ channel but use a mechanism different from that of the polypeptide toxins (8, 9). All these toxins are useful in the investigation and interpretation of the structure, function, and differentiation of the Na^+ channel at the molecular level.

This paper describes the properties of action of a new type of polypeptide toxin extracted from the venom of the Mexican scorpion *Centruroides suffusus suffusus* (10). Two approaches have been used to study the mode of action of this toxin on muscle fibers. These are (*i*) an electrophysiological approach to determine the mechanism of action of the *Centruroides* toxin (Css_{II}) on the electrical activity and on the contraction of the muscle and (*ii*) a biochemical approach to investigate the specific binding properties of Css_{II} to the muscle membranes.

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MATERIALS AND METHODS

Electrophysiological Experiments. Single-twitch fibers isolated from the semitendinosus of *Rana esculenta* were mounted in a sucrose-gap device as described (11, 12). This system allows simultaneous recording of ionic currents and contraction and is of particular value for analysis of fast Na^+ currents (13). The physiological solution in which the experiments were carried out had the composition 110.5 mM NaCl/2.5 mM KCl/1.8 mM $CaCl_2$/2 mM $MgCl_2$/5 mM pyruvate/0.4 mM mannitol/3 mM Tris·HCl, pH 7.4. Isotonic mannitol (236 mM) was used instead of sucrose in the sucrose-gap device.

Preparation of Sarcolemma-Rich Membranes. Membranes were prepared from the leg muscles of the frog by gentle disruption of the fibers, elimination of contractile proteins with salt solution, and differential centrifugation, essentially as described for rat muscles by Schapira *et al.* (14).

Iodination of Css_{II}. Toxin II from the venom of *C. suffusus suffusus* (15) was a gift from G. Garcia. Css_{II} was iodinated by the lactoperoxidase/H_2O_2 method (16). The iodinated mixture was passed through a Sephadex G-15 column (0.8 × 22 cm) equilibrated and eluted with 50 mM NaH_2PO_4, pH 7.4/200 mM NaCl/0.01% bovine serum albumin. The chromatography was followed by measurement of the radioactivity in each fraction and by measurement of the absorbance at 280 nm. Css_{II} concentrations were determined by using ε_{280} = 21,880 (15). The number of iodine atoms incorporated per mole of toxin was 1 ± 0.2 and specific radioactivities of 100–2,000 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels) were used in binding studies.

Binding Assays in Standard Conditions. The standard incubation buffer for ^{125}I-labeled Css_{II} ([^{125}I]Css_{II}) binding experiments to muscle membranes was 120 mM choline chloride/2.5 mM KCl/1.8 mM $CaCl_2$/0.1% bovine serum albumin/10 mM 4-morpholineethanesulfonic acid, pH 7. Standard binding experiments were carried out at 22°C. In equilibrium binding experiments, muscle membranes (0.3–0.6 mg of protein/ml) were incubated in 300 μl of standard incubation buffer in the presence of various concentrations of [^{125}I]Css_{II} for 20 min. Duplicate 100-μl aliquots were then filtered at reduced pressure using cellulose acetate filters (Sartorius SM 11106, 0.45-

Abbreviations: Css_{II}, *Centruroides suffusus suffusus* toxin II; [^{125}I]Css_{II}, radiolabeled derivative of Css_{II}; TTX, tetrodotoxin; [^3H]en-TTX, tritiated ethylenediamine derivative of tetrodotoxin.

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μm pore size). Radioactivity bound to the filters was assayed with an Intertechnique CG 4000 gamma counter. Nonspecific binding was determined in parallel experiments in the presence of excess unlabeled toxin (100 nM), which was incubated with the membranes for 15 min before adding the [^{125}I]Css$_{II}$. The same procedure was used for protection experiments and competition studies between [^{125}I]Css$_{II}$ and other toxins. The pH dependence of [^{125}I]Css$_{II}$ binding was studied between pH 5 and pH 8.5 by using appropriate mixtures of 4-morpholineethanesulfonic acid and Tris buffers.

The kinetics of association and dissociation of [^{125}I]Css$_{II}$ were followed by using the technique described for equilibrium binding experiments; aliquots were filtered after various incubation periods before and after addition of excess unlabeled toxin, respectively.

To study the susceptibility of the Css$_{II}$ receptor to protease digestion, muscle membranes (2.5 mg/ml) were incubated for 30 min at 22°C with various proteases in 0.1 M Tris·HCl, pH 7.4/0.5 mM EDTA/0.25 M sucrose. When papain was used, 1 mM dithiothreitol/10 mM cysteine was added to the incubation mixture.

The TTX receptor on the Na^+ channel was studied by using a radioactive derivative ([^{3}H]en-TTX) synthesized by coupling TTX with tritiated ethylenediamine (17). Binding experiments using [^{3}H]en-TTX were carried out as described (18).

RESULTS

In frog twitch-muscle fibers, the Na^+ inward current comprises two phases that have the same threshold and the same reversal potential (13). The rapid phase, corresponding to the early inward current, concerns the Na^+ conductance of the surface membrane and the smaller and slower phase, corresponding to the late inward current, concerns the Na^+ conductance of the T-system membrane (13, 19). For short and small depolarizations, for which the fast surface Na^+ current is inactivated and the outward delayed current is negligible, the tail current that is recorded at the end of the pulse represents mainly "deactivation" of the slow tubular Na^+ current (13).

Records of currents obtained for different depolarizations lasting 3 ms, both in the absence and in the presence of Css$_{II}$ (20 nM), after 6 min of incubation are shown in Fig. 1. Voltage-clamp data obtained in control Ringer's solution in the absence of toxin show the different phases of inward current corresponding to the surface and to the tubular Na^+ currents (13). In the presence of Css$_{II}$, the rapid phase of the inward current was almost completely abolished whereas the second (slower) phase of the Na^+ current was still observed.

A simultaneous recording of the effects of Css$_{II}$ on both inward Na^+ currents and contraction obtained for a depolarization of 50 mV is shown in Fig. 2*A*. When the surface Na^+ current that constitutes the fast phase of the inward current disappears, the slower tubular Na^+ current becomes more visible. An interesting observation is that the tail current recorded at the end of the pulse that corresponds to deactivation of the tubular Na^+ current (13) was not modified by the polypeptide toxin. Another interesting observation is that the Css$_{II}$ that abolished all the fast Na^+ current coming from the surface membrane had no effect on the amplitude of contraction of the fiber.

Fig. 2*B* shows that the action of Css$_{II}$ was fast. After 1.5 min of incubation with Css$_{II}$, about half of the maximal effect was already obtained.

Fig. 2 *C* and *D* is presented to compare the action of Css$_{II}$ with that of TTX on both inward Na^+ currents and contraction. TTX rapidly blocked both phases of inward Na^+ current. Unlike Css$_{II}$, TTX both drastically changed the tail current and considerably reduced contraction.

Fig. 3 shows results of binding experiments in which various concentrations of [^{125}I]Css$_{II}$ were added to a fixed concentration of muscle membranes. The specific binding is the difference between total and protected (nonspecific) binding. The maximal binding capacity of the muscle membranes was 220 fmol of [^{125}I]Css$_{II}$/mg of protein. Half-maximal binding (K_d) was observed at 0.4 nM of the free toxin.

Typical kinetics of association between [^{125}I]Css$_{II}$ (0.4 nM) and muscle membranes is shown in Fig. 3*B Inset*. Fig. 3*B* shows the kinetic properties of the displacement of [^{125}I]Css$_{II}$ bound to muscle membranes by unlabeled Css$_{II}$ at 50 nM, which is much higher than the K_d of the toxin–receptor complex. The half-life of dissociation of [^{125}I]Css$_{II}$ from its receptor was ≈3 min at 22°C and 16 min at 2°C. Equilibrium is reached after 5 to 6 min under these conditions.

Fig. 4 shows that higher concentrations of unlabeled Css$_{II}$ gradually inhibited [^{125}I]Css$_{II}$ binding to the specific toxin receptor in the frog muscle membranes. Half-maximal inhibition

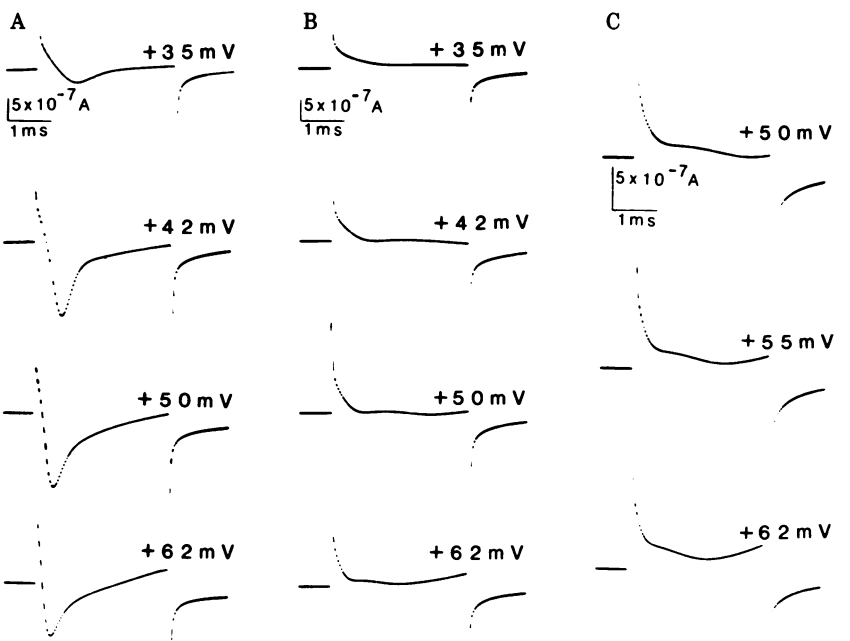

FIG. 1. Effect of Css$_{II}$ on inward Na^+ currents. Positive values of potential on the recordings correspond to depolarizations imposed from the resting potential (E_M = 90 mV). (*A*) Control: Ringer's solution, no Css$_{II}$. (*B*) Currents recorded in Ringer's solution/20 nM Css$_{II}$ after 6 min of action. (*C*) Currents recorded in Ringer's solution/20 nM Css$_{II}$ after 8 min of action at a higher gain than in *B*. Experiments were carried out at 22°C.

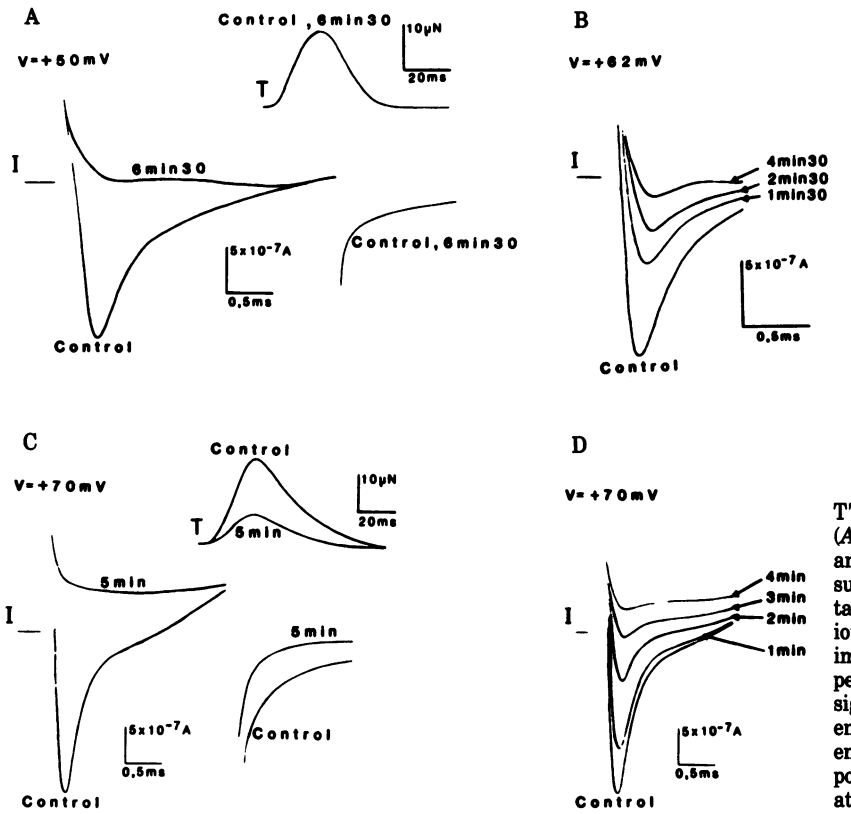

FIG. 2. Effects of 20 nM Css_{II} (*A* and *B*) and 6 nM TTX (*C* and *D*) on Na^+ currents (I) and contraction (T). (*A* and *C*) I and T obtained in control Ringer's solution and after 6.5 min of action by Css_{II} or 5 min by TTX are superimposed. Note the absence of effect of Css_{II} on the tail current. (*B* and *D*) Na^+ currents obtained after various times in the presence of Css_{II} or TTX are superimposed. The fast inward current completely disappeared after 6 min at this potential (not shown). No significant shift of the time to peak occurred in the presence of TTX, but a slight one was observed in the presence of Css_{II}: V is the depolarization from the resting potential (E_M = 90 mV). Experiments were carried out at 22°C.

of $[^{125}I]Css_{II}$ binding occurred at $K_{0.5} = 1.1 \times 10^{-9}$ M.

The equation for $K_{0.5}$ is

$$K_{0.5} = K_d[1 + ([^{125}I]Css_{II})_{0.5}/K_d^*],$$

where $([^{125}I]Css_{II})_{0.5}$ is the concentration of free labeled ligand at half-displacement and K_d^* and K_d are values for complexes formed between muscle membranes and $[^{125}I]Css_{II}$ and unlabeled Css_{II}, respectively. K_d was 0.4 nM (see above) and $([^{125}I]Css_{II})_{0.5}$ was 0.56 nM under the experimental conditions of Fig. 4. Thus, the value of K_d was 0.45 nM.

Comparison of Figs. 1 and 2 shows that there are both differences and similarities between the actions of TTX and Css_{II}. Therefore, the first question that arose was whether these toxins share a common binding site. As shown in Fig. 4, TTX concentrations as high as 10 μM had no effect on $[^{125}I]Css_{II}$ binding to its specific site and, as shown in Fig. 4 (*Inset*), concentrations of Css_{II} up to 50 nM have no effect on [^{3}H]en-TTX binding to the TTX receptor.

Fig. 5 shows that binding of $[^{125}I]Css_{II}$ to its receptor was pH dependent and was abolished at alkaline pH. The variation in the binding capacity (measured with 0.2 nM $[^{125}I]Css_{II}$) with pH was biphasic: a first phase was observed at pH 5–7 and a second one was observed at pH 7–9. The pH profile of $[^{125}I]Css_{II}$ binding suggests that there are two ionizable groups with pK_a values near 6 and 8 that are important for the association of the toxin with its receptor. Whether these ionizable groups are on the toxin itself or on the toxin receptor is not known at present.

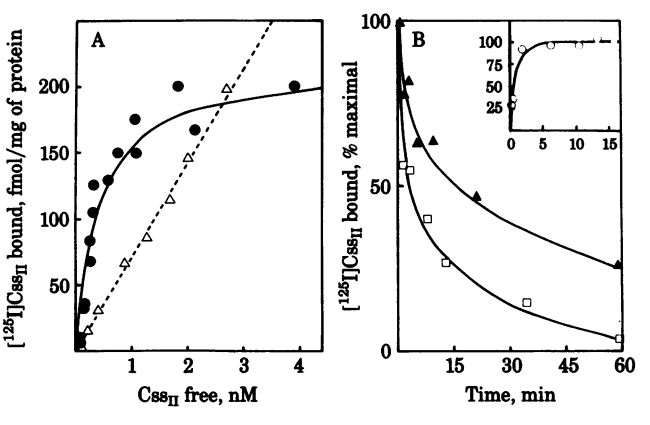

FIG. 3. (*A*) Equilibrium binding of $[^{125}I]Css_{II}$ to frog muscle membranes. ●, Specific; △, nonspecific. (*B*) Dissociation and association (*Inset*) kinetics for binding of $[^{125}I]Css_{II}$ to muscle membranes. For association kinetics, muscle membranes (0.5 mg/ml) were incubated with 0.4 nM $[^{125}I]Css_{II}$. The free $[^{125}I]Css_{II}$ concentration varied <5% during the course of the association process. □, At 22°C; ▲, at 2°C.

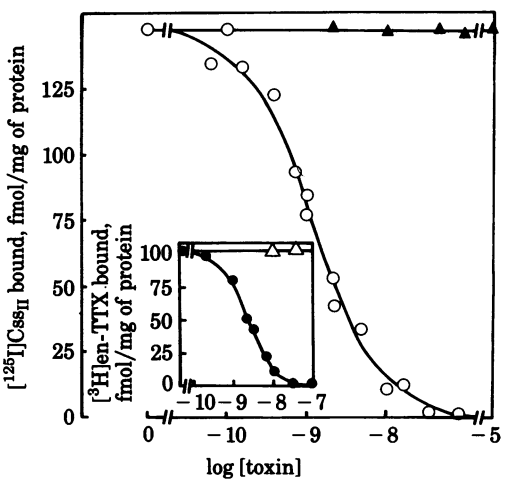

FIG. 4. Competition between $[^{125}I]Css_{II}$ and unlabeled Css_{II} under standard conditions (○) and between $[^{125}I]Css_{II}$ and TTX (▲). (*Inset*) Competition between [^{3}H]en-TTX (5 nM, 27 Ci/mmol) and TTX (●) and between [^{3}H]en-TTX and Css_{II} (△).

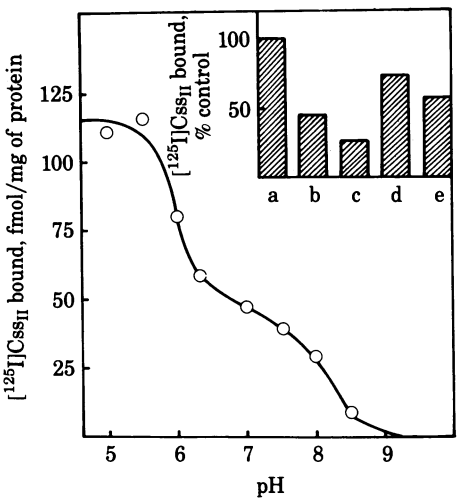

FIG. 5. Effect of pH and of various proteases on [^{125}I]Css$_{II}$ (0.2 nM) binding to muscle membranes. (*Inset*) [^{125}I]Css$_{II}$ binding to control membranes (bar a) is compared with that obtained with membranes treated with Pronase at 0.1 mg/ml (bar b), trypsin at 0.1 mg/ml (bar c), chymotrypsin at 0.5 mg/ml (bar d), and papain at 0.1 mg/ml (bar e) for 30 min at 22°C. Proteolysis was stopped by washing the membranes three times with ice-cold buffer.

Fig. 5 *Inset* shows that binding of [^{125}I]Css$_{II}$ to its receptor site was drastically decreased after treatment of the muscle membranes with trypsin or Pronase; chymotrypsin and papain also degrade the Css$_{II}$ receptor but less effectively than trypsin.

In addition to TTX, a large number of toxic compounds affect the Na^+ channel. The compounds investigated as possible competitors with [^{125}I]Css$_{II}$ for binding to the Na^+ channel are listed in Table 1. None of them had any effect on [^{125}I]Css$_{II}$ binding to its receptor site.

DISCUSSION

Na^+ channels and Na^+-dependent action potentials are present in both the surface membrane and the T-system membrane of muscle fiber (20–22). The action potential of the T system contributes a large part of the total mechanical output of normal isotonic twitch (21). Caillé *et al.* (13) have shown that the surface Na^+ conductance and the T-system Na^+ conductance can be separately identified in voltage-clamp experiments on the frog twitch-muscle membrane by using a double sucrose gap apparatus. The surface Na^+ channel generates an early inward current whereas the tubular Na^+ channel generates a late inward current that is TTX sensitive and is responsible for a large part of the contraction (ref. 13 and Fig. 2).

Table 1. Classes of toxins acting on the Na^+ channel and their effects on [^{125}I]Css$_{II}$ binding

Toxin	Physiological effect	Protection of [^{125}I]Css$_{II}$ binding	
		%	Conc., μM
TTX	Blocks Na^+ currents	0	10
Saxitoxin		0	1
Veratridine	Causes persistent acti-	0	10
Batrachotoxin	vation of Na^+ channels	0	0.1
Androctonus australis toxin	Specifically slows down Na^+ current	0	0.2
Anemonia sulcata toxin II	inactivation	0	10
Decis (pyrethroid)	Transforms fast Na^+ channels into slower ones	0	10
Lidocaine	Local anesthetic,	0	1,000
Tetracaine	blocks Na^+ currents	0	1,000
Procaine		0	1,000
Unlabeled Css$_{II}$	Blocks the early Na^+ current in muscle	100	0.01

All concentrations used were high enough to saturate the sites for the different toxins. Conc., concentration.

The polypeptide toxin Css$_{II}$ has the following properties of interaction with the frog muscle fiber. (*i*) Css$_{II}$ at 20 nM completely blocks the fast inward current corresponding to the surface Na^+ conductance after a few minutes; (*ii*) Css$_{II}$ does not seem to affect the late Na^+ inward current; this is particularly well observed on tail currents that correspond to deactivation of the tubular Na^+ conductance and that are not affected by Css$_{II}$. (*iii*) Css$_{II}$ does not change the characteristics of contraction. This last observation agrees well with the interpretation that Css$_{II}$ is without effect on the tubular Na^+ channel. This toxin seems to be a powerful tool to block selectively surface Na^+ channels without impairing contraction.

There are two possible reasons to explain the lack of effect of Css$_{II}$ on tubular Na^+ channels: (*i*) the toxin does not penetrate (or penetrates very slowly) into the tubules; (*ii*) the affinities of surface and tubular Na^+ channels for Css$_{II}$ are different, the tubular Na^+ channel having low affinity or no affinity at all for the toxin. [^{125}I]Css$_{II}$ binds to its receptor site on the Na^+ channel to form a complex that has a K_d of 0.4 nM. The K_d for the complex formed with the unlabeled toxin is very similar (0.45 nM). There is a good agreement between the electrophysiological and the biochemical data since a fast and complete block of the surface Na^+ channels of the muscle fiber has been obtained with 20 nM toxin, a saturating concentration 50 times greater than the K_d for the receptor.

Css$_{II}$ binds to a protein receptor that is degraded by proteases. This observation is not really unexpected since the TTX receptor is also known to be a protein (4). Css$_{II}$ binding is pH dependent and it will be interesting to identify the essential ionizable groups that control toxin binding and to establish whether they belong to the polypeptide toxin structure or to its receptor.

The maximal number of binding sites for [^{3}H]en-TTX found with the enriched plasma membrane preparation from muscle was ≈230 fmol/mg of protein, very near the maximal value for [^{125}I]Css$_{II}$ binding (Fig. 3A). Therefore, there seems to be a 1:1 stoichiometry for the Css$_{II}$ binding site and the TTX binding site at the Na^+ channel.

Molecules that alter the normal function of the Na^+ channel and that have been tested for their action on [^{125}I]Css$_{II}$ binding are separated into six classes in Table 1. None of the molecules in the first five classes has any effect on [^{125}I]Css$_{II}$ binding to its receptor. This is particularly true in the case of TTX (Fig. 4). Therefore, Css$_{II}$ constitutes another class of toxin. More work remains to be carried out to establish the exact mechanism of action for Css$_{II}$ on the Na^+ channel. It has been reported (23, 24) that both the venom and some of the purified toxins of the scorpion *Centruroides sculpturatus* transform some of the fast Na^+ channels in the frog myelinated fiber into slower ones. The properties of the interaction of Css$_{II}$ with the muscle fiber are different. Possible explanations for these differences are (*i*) that Na^+ channels in the node of Ranvier and in the surface muscle membrane have different pharmacological properties with respect to Css$_{II}$ and (*ii*) that *C. sculpturatus* and *C. suffusus suffusus* venoms are different in their toxin composition.

The low K_d of the Css$_{II}$–receptor complex (0.4 nM) is comparable with that found for the TTX– (or saxitoxin)–receptor complex (1, 3–5). However, radiolabeled Css$_{II}$ may become more interesting than labeled TTX derivatives, or than

[^{3}H]saxitoxin, in structural, functional, and developmental studies of the Na^+ channel because it can be prepared with a specific radioactivity of up to 2,000 Ci/mmol, which is ≈50 times greater than that of the most radioactive TTX derivatives and >100 times higher than that of [^{3}H]saxitoxin. Css_{II} may also be more useful than the other categories of polypeptide toxins that are specific for the inactivation of the fast Na^+ channel. Iodinated derivatives of these toxins can be obtained with a high specific radioactivity but either the toxins have a relatively low affinity for the channel (sea anemone toxins; ref. 25) or the binding is voltage dependent and disappears when the membrane is depolarized (other scorpion toxins; ref. 26). Css_{II}, like TTX and saxitoxin, has the advantages of binding well to isolated plasma membranes and of forming a complex that has a relatively long half-life, which should be useful both for autoradiographic studies and for purification of the sodium channel.

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