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Specific Binding of Toxin II from *Centruroides suffusus suffusus* to the Sodium Channel in Electroplaque Membranes†

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ABSTRACT: The binding of toxin II from the scorpion *Centruroides suffusus suffusus* (Css_{II}) to electroplaque membranes from *Electrophorus electricus* was studied with the use of a radiolabeled derivative of the toxin ($[^{125}I]Css_{II}$). Specific binding of the latter to the membranes required the protonation of a group, either in the membrane or in the toxin itself, with an apparent pK_a value of 7.5 and also the presence of a certain minimum concentration of ions, though there was no requirement for a specific ion. At 20 °C and pH 6 the second-order rate constant for formation of the $[^{125}I]Css_{II}$–membrane complex was about 5×10^6 M^{-1} s^{-1}, while the first-order constant for its dissociation was about 2×10^{-3} s^{-1}. Under equilibrium conditions specific binding of $[^{125}I]Css_{II}$ was a simple saturable function of $[^{125}I]Css_{II}$ concentration, characterized by a dissociation constant of 0.4–0.7 nM and a maximum capacity of 0.9–2.4 pmol of toxin/mg of membrane protein. The latter value was the same as the number of membrane sites that could specifically bind a radiolabeled derivative of tetrodotoxin. Unlabeled Css_{II} displaced bound $[^{125}I]Css_{II}$ with an apparent dissociation constant of about 1 nM. None of 19 other neurotoxins or local anaesthetics known to interact with Na^+ channels in excitable cells affected $[^{125}I]Css_{II}$ binding, but it was completely inhibited by toxin γ from the scorpion *Tityus serrulatus serrulatus*. These findings suggest that the Na^+ channel possesses a distinct class of binding sites to which these two scorpion toxins bind with high affinities. On the other hand, no Css_{II} receptor was detected in crab axonal membranes, indicating that it is not a characteristic feature of all Na^+ channels.

The primary importance of the Na^+ channel in the functioning of excitable cells has been emphasized by the discovery that a considerable number of naturally occurring toxins exert their effects by reacting specifically with the Na^+ channel, causing inhibition or modification of its normal behavior. Moreover, it is now clear that such neurotoxins do not all act on the Na^+ channel in the same way. Combined biochemical and electrophysiological studies have revealed the existence of a number of distinct binding sites for different toxins, together with at least four characteristically different ways of interference with the Na^+ channel's functioning (Narahashi, 1974; Ritchie & Rogart, 1977; Catterall, 1980; Howard & Gunderson, 1980; Lazdunski et al., 1980a; Rogart, 1981; Honerjäger, 1981). These latter findings are particularly important because they show how these neurotoxins can be used as specific probes for investigations at the molecular level of the structure, function, and differentiation of the Na^+ channel (Levinson & Ellory, 1973; Agnew et al., 1978; Barchi et al., 1980; Lazdunski et al., 1980b; Armstrong, 1981; Lazdunski & Renaud, 1982).

The fact that some of these neurotoxins interact with the Na^+ channel in different ways is not surprising in view of their radically different chemical compositions and molecular structures. But recent findings have indicated that some neurotoxins of apparently very similar composition and structure also produce characteristically different modifications of Na^+ channel functioning. In particular, the polypeptide toxin II extracted from the venom of the Mexican scorpion *Centruroides suffusus suffusus* has been found to act very differently from apparently similar polypeptide toxins derived

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from African scorpions and from the sea anemone. For example, Jover et al. (1980) showed that the *Centruroides* toxin (Css_{II})[1] binds to a specific site on rat brain synaptosomes that is clearly distinct from the specific binding site for a similar toxin derived from the African scorpion *Androctonus australis* Hector. They also demonstrated that, unlike the latter toxin, the binding of Css_{II} is not dependent on the membrane potential. More recently Jaimovich et al. (1982) found not only that Css_{II} binds specifically to frog skeletal muscle membranes but also that it selectively blocks the early phase of the inward Na^+ current in the muscle, without affecting the late phase. This observation indicates that Css_{II} can interact with Na^+ channels in the cell surface membrane but not with those in the T-tubule membranes. However, the specific binding site for Css_{II} on the muscle membrane was also shown to be different from those sites specific for eight other neurotoxins or local anaesthetics that alter the functioning of the Na^+ channel and that represent examples of all four established modes of action on the channel. Hence, Css_{II} appears to represent a fifth class of neurotoxins known to interact specifically with the Na^+ channel, and both because of its characteristic properties and because it can be iodinated to a very high specific radioactivity, Css_{II} should be an exceptionally useful tool for further attempts to study the Na^+ channel at the molecular level.

To try to gain more information about the properties of Css_{II} and to characterize further its specific binding to the Na^+ channel, we have investigated its interaction with membranes from other well-studied excitable tissues, the electroplaque of *Electrophorus electricus* and axons from *Cancer pagurus*.

Experimental Procedures

Preparation of Electroplaque Membranes. Electric organs from *E. electricus* were stored at −70 °C. For a typical membrane preparation a piece of the tissue weighing about 125 g was chopped into pieces of about 1 cm^3, which were suspended in 600 mL of cold 50 mM K_2PO_4–KH_2PO_4 (pH 7.5) with the use of a Waring blender, operated at high speed for three consecutive periods of 20 s. The suspension was homogenized in a glass–Teflon homogenizer by using five strokes at 800 rpm and then filtered through gauze. The following fractionation was carried out at 4 °C. The homogenate was centrifuged at 2500*g* for 7 min, the supernatant was decanted and retained, and the pellet was resuspended in approximately 1.5 volumes of the phosphate buffer. The latter suspension was centrifuged as before, the supernatant added to the first supernatant and the mixture was centrifuged at 16600*g* for 60 min. The resulting supernatant was decanted and discarded. Two different procedures were then used to obtain the final membrane preparation. For the first method the pellet was homogenized in 50 mL of 0.32 M sucrose plus 5 mM Tris-HCl (pH 7.4) and the suspension centrifuged at 144000*g* for 60 min, giving a membrane pellet that was resuspended in 25 mL of choline medium (140 mM choline chloride, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 50 mM Hepes–Tris, pH 7.4). For the second method, the pellet from the 144000*g* centrifugation was resuspended in 5 mL of 0.32 M sucrose plus 5 mM Tris-HCl (pH 7.4), and 1-mL aliquots of this suspension were layered on top of discontinuous sucrose gradients in centrifuge tubes (Beckman SW27). Each gradient consisted of 10 mL of 1.2 M sucrose–5 mM Tris-HCl (pH 7.4), 10 mL of 0.98 M sucrose–5 mM Tris-HCl (pH 7.4), and 10 mL of 0.8 M sucrose–5 mM Tris-HCl (pH 7.4). The tubes were centrifuged at 25 500 rpm for 120 min, and the fraction that collected at the interface between the 1.2 and 0.98 M sucrose solutions was retained and diluted with about 5 volumes of choline medium.

Both the membrane fractions thus obtained were divided into small samples containing about 3 mg of protein and the membranes sedimented by centrifugation. The clear supernatants were decanted and discarded and the membrane pellets stored at −70 °C until required, when they were resuspended by homogenization in either the incubation medium or 0.3 M sucrose. Protein concentrations were measured according to the method described by Hartree (1972). Although some variation was observed from one preparation to another in the number of specific binding sites per milligram of protein for various toxins, no systematic difference was detected between the preparations obtained by the different fractionation methods. Hence, the different kinds of preparation are not distinguished in the following text. The number of TTX receptors, determined by measurement of the binding of [^{3}H]en-TTX, ranged from 1.9 to 3.7 pmol/mg of protein, and the (Na^+,K^+)-dependent ATPase content, determined by measurement of the binding of [^{3}H]ouabain, was 181–294 pmol/mg of protein, representing a purification of 5–8-fold over that of the tissue homogenate.

Preparation of Crab Axonal Membranes. Axonal membrane preparations from *Cancer pagarus* nerves were isolated and characterized according to the methods described previously (Balerna et al., 1975). Their [^{3}H]en-TTX binding capacity was about 8 pmol/mg of protein (M. Fosset, unpublished observation).

Css_{II} and [^{125}I]Css_{II}. The Css_{II} was a gift from Dr. Garcia, who had purified it from the venom (Garcia, 1977). The methods used were essentially those described in detail previously for other scorpion toxins (Miranda et al., 1970) and involved the following steps. (a) The first step was extraction of the crude venom with water and dialysis of the extract against water. (b) Gel filtration through Sephadex G-50 (4 × 200 cm column for the material from 2 g of venom) in 0.1 M ammonium acetate (pH 8.5) at 80 mL/h was the second step. (c) Third was recycling gel filtration through Sephadex G-50 (four columns of 3.2 × 100 cm in series) with 0.1 M ammonium acetate (pH 8.5) at 55 mL/h. Five cycles were used and the first two toxic fractions eluted during the last cycle were collected. (d) The fourth step was anion-exchange chromatography on DEAE-Sephadex A-50 (4 × 200 cm column for the material from 4 g of crude venom) eluted with 0.1 M ammonium acetate (pH 8.5) at 40 mL/h. (e) Fifth was cation-exchange chromatography on Amberlite CG-50 (4 × 200 cm column for the material from 6 g of venom) eluted with 0.2 M CH_3COONH_4 (pH 6.45) at 40 mL/h. The toxin was eluted in the fractions obtained after passage of between 3.5 and 4.3 L of buffer. A little more than 1 mg of toxin was obtained from 100 mg of venom. Purification was monitored by measurement of toxicity to mice and eluted fractions containing toxic activity were concentrated between each step by lyophilization (Miranda et al., 1970). The final product was shown to give a single band during polyacrylamide gel electrophoresis. Its amino acid composition and sequence were determined by conventional techniques, showing that Css_{II} consists of a single polypeptide chain of 66 amino acids with a molecular weight of 7544 (Garcia, 1977). Small samples of lyophilized toxin were stored at −70 °C until required, and

[1] Abbreviations: Css_{II}, *Centruroides suffusus suffusus* toxin II; [^{125}I]Css_{II}, radiolabeled derivative of the *Centruroides* toxin; TTX, tetrodotoxin; [^{3}H]en-TTX, tritiated ethylenediamine derivative of TTX; TMO, trimethyloxonium salt; TiTxx, *Tityus serrulatus serrulatus* toxin; TiTxγ, *Tityus serrulatus serrulatus* γ toxin.

aqueous solutions of 1–10 μM Css_{II} were stored in polyethylene tubes at 4 °C for up to 1 month. More dilute solutions were made in 0.1% bovine serum albumin solution and used immediately. [^{125}I]Css_{II} was prepared by the lactoperoxidase–H_2O_2 method (Morrison & Bayse, 1970) as described previously (Jaimovich et al., 1982) and stored at 4 °C in a solution containing 200 mM NaCl, 0.01% bovine serum albumin, and 50 mM $NaHPO_4$–Na_2HPO_4 (pH 7.4). The concentrations of both Css_{II} and [^{125}I]Css_{II} were calculated from measurements of the absorption of their solutions at 280 nm with the use of E_M^{280} = 21 880. (The use of the same extinction coefficient for the monoiodinated toxin is reasonable because each molecule contains two tryptophan and seven tyrosine residues, each of the latter contributing only 6.4% of the total absorption.)

Standard Binding Assays. As a routine samples of electroplaque membranes were incubated at 20 °C in 300 μL of a solution containing 140 mM choline chloride, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, 20 mM Mes–Tris (pH 6 at 20 °C), and the required concentrations of [^{125}I]Css_{II}. The final concentration of membranes was 0.02–0.3 mg of protein/mL. For measurement of nonspecific binding unlabeled Css_{II} was also present at a final concentration of 30–100 nM. Variations from these conditions for individual experiments are noted in the legends to the figures and tables.

The [^{125}I]Css_{II} was always added last, to start the incubations, which were from 20 to 40 min for standard assays of equilibrium binding. The incubations were stopped by rapid filtration of 100-μL samples of the incubation mixture through cellulose acetate filters (Sartorius 5 M 11106, 0.45-μm pore size) under reduced pressure. The filters were immediately washed twice with 4 mL of a solution containing 150 or 200 mM choline chloride and 0.1% bovine serum albumin. The filters were soaked in a sample of the washing solution for at least 30 min before being used, and each was washed once with 4 mL of washing solution immediately before the incubation sample was filtered. Duplicate assays were made for each condition, and then a 50-μL sample of the incubation mixture was also taken for measurement of the total [^{125}I]Css_{II} present. The radioactivity in the latter sample and that bound to the filter was measured with an Intertechnique CG 4000 γ counter.

Assay of [^{3}H]en-TTX Binding. A tritiated ethylenediamine derivative ([^{3}H]en-TTX) of tetrodotoxin (Chicheportiche et al., 1980) was used to assay the specific receptor for TTX in the electroplaque membranes under conditions previously described (Lombet et al., 1981).

Other Materials. Fraction V bovine serum albumin, trypsin, and α-chymotrypsin were obtained from Sigma, Pronase was from Boehringer, trypsin inhibitor was from Serva, and trimethyloxonium tetrafluoroborate was from Fluka. All other compounds were normal commercial products for analytical use. Solutions were made in deionized water.

Results

Specific Binding of [^{125}I]Css_{II} to Electroplaque Membranes. Preliminary experiments showed that the amount of [^{125}I]Css_{II} that bound to the membranes increased during the first 15 min of incubation at 20 °C but then reached a plateau value, which remained constant for at least 1 h. Hence, incubations of 20–60 min at 20 °C were suitable for measurement of binding under equilibrium conditions, and Figure 1A illustrates such binding as a function of the concentration of [^{125}I]Css_{II}, in the presence and absence of a much higher concentration of unlabeled Css_{II}. The nonspecific binding that persisted in the

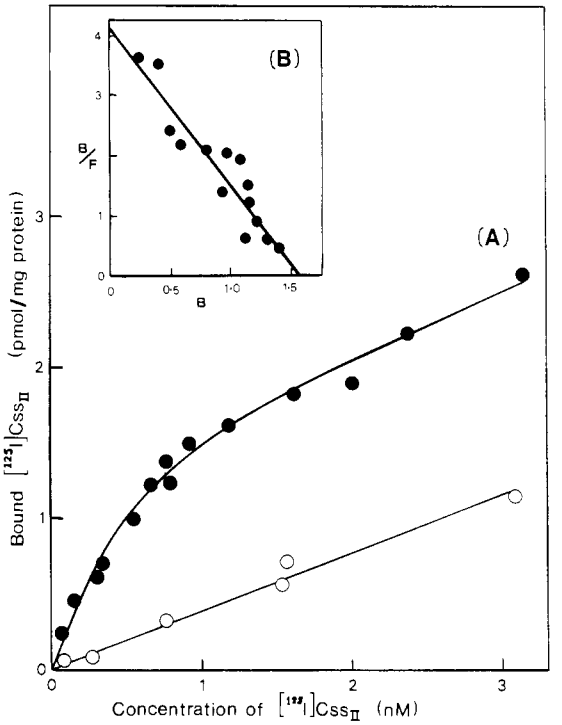

FIGURE 1: Effect of the concentration of [^{125}I]Css_{II} on its binding to electroplaque membranes. Binding was measured under equilibrium conditions as described in the text, with the indicated concentrations of [^{125}I]Css_{II}. (A) Total binding in the absence (●) or presence (○) of 100 nM unlabeled Css_{II}. (B) Scatchard plot of specific binding. B = bound (pmol/mg of protein) and F = free (nM).

presence of the unlabeled toxin increased linearly with the concentration of [^{125}I]Css_{II}, whereas the specific binding, defined as the difference between total binding and nonspecific binding, was a saturable function of [^{125}I]Css_{II} concentration. Figure 1B shows that the results were consistent with specific binding to a single class of sites and gives values of 1.6 pmol/mg of protein for the number of sites available (B_{max}) and 0.4 nM for the equilibrium dissociation constant (K_D). Similar experiments with different preparations of membranes and labeled toxin gave slightly different values for these constants, the range being 0.9–2.4 pmol/mg of protein for B_{max} and 0.4–0.7 nM for K_D.

Association and Dissociation Kinetics. The amount of [^{125}I]Css_{II} specifically bound to the membranes was measured as a function of time until equilibrium was reached. The rate of dissociation of the toxin–membrane complex was then followed after (a) the addition of excess unlabeled Css_{II} and (b) a 20-fold dilution of the incubation mixture (Figure 2A). Since the equilibrium binding constants for the membrane preparation had already been determined (B_{max} = 0.9 pmol/mg of protein and K_D = 0.4 nM), these rate curves could be used to calculate the rate constant of association (k_{+1}) as well as of dissociation (k_{-1}). The plot in Figure 2B shows that the data for association were consistent with second-order kinetics, while Figure 2C shows that dissociation followed first-order kinetics after the first 0.5 min (Weiland & Molinoff, 1981). The values of the kinetic constants calculated from the plots in Figure 2B,C were $k_{+1} = 4.8 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 2.2 \times 10^{-3}$ s^{-1}, so that $K_D = k_{-1}/k_{+1} = 0.5$ nM. Thus, the estimates of K_D from equilibrium binding (0.4 nM) and from these kinetic constants (0.5 nM) were consistent.

Specificity of the Binding Site for Css_{II}. The effects of relatively high concentrations of a number of other neurotoxins and some local anaesthetics on the binding of [^{125}I]Css_{II} to electroplaque membranes are summarized in Table I. Al-

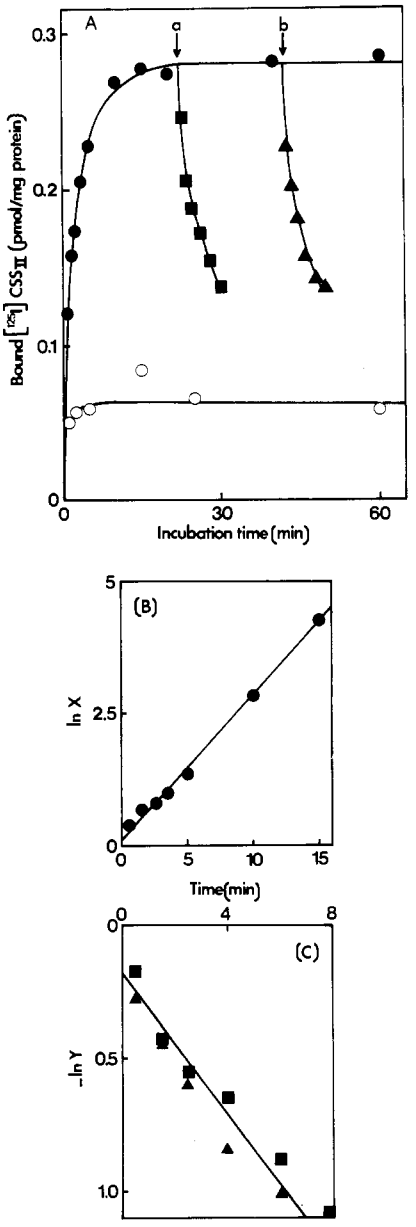

FIGURE 2: Kinetics of formation and dissociation of the [^{125}I]-Css_{II}–membrane complex. (A) The concentration of the [^{125}I]-Css_{II}–membrane complex formed in the absence (●) or presence (○) of 100 nM unlabeled Css_{II} was measured by sampling the incubation mixture after the indicated times of incubation at 20 °C. The concentration of [^{125}I]Css_{II} was 0.28 nM, and the membrane concentration was 0.2 mg of protein/mL. After equilibrium had been reached the rate of dissociation of the complex was monitored following (a) addition of unlabeled Css_{II} (final concentration 100 nM) and (b) dilution of 1 volume of the incubation mixture with 19 volumes of the choline medium. (B) The data for specific binding during the first 15 min are plotted as ln X vs. time, where $X = [(1 - [LR][LR]_E)/([R_T]\cdot[L_T])]/(1 - [LR][LR]_E)$, [LR] is the concentration of the [^{125}I]-Css_{II}–membrane complex at the indicated time, $[LR]_E$ is its concentration at equilibrium, and $[R_T]$ and $[L_T]$ are the total concentrations of specific binding sites and [^{125}I]Css_{II}, respectively. (C) The data for dissociation are plotted as $-\ln Y$ against time, where $Y = [LR]/[LR]_E$, (▲) after dilution and (■) in the presence of 100 nM Css_{II}.

though each compound was present at a concentration well above its own K_D value, only unlabeled Css_{II} and the scorpion toxin TiTxγ inhibited the binding of [^{125}I]Css_{II} to a significant extent.

Inhibition of [^{125}I]Css_{II} Binding by Unlabeled Css_{II} and TiTxγ. The relative inhibitory effects of various concentrations of unlabeled Css_{II} and TiTxγ on the equilibrium binding

Table I: Binding of [^{125}I]Css_{II} to Electroplaque Membranes in the Presence of Other Neurotoxins or Local Anaesthetics[a]

addition	concn (M)	[^{125}I]Css_{II} bound (% of control)
none		100
Centruroides suffusus suffusus II toxin	5×10^{-8}	16
Tityus serrulatus serrulatus γ toxin	10^{-8}	17
Tityus serrulatus serrulatus toxin	10^{-7}	84
Androctonus australis Hector toxin	10^{-7}	96
Anemonia sulcata II toxin	10^{-5}	95
Anemonia sulcata V toxin	10^{-5}	84
Anthopleura xanthogrammica toxin	10^{-6}	82
apamin	10^{-7}	88
tetrodotoxin	10^{-5}	92
saxitoxin	10^{-6}	105
aconitine	10^{-4}	100
ervatamine	10^{-5}	96
epiervatamine	5×10^{-5}	84
batrachotoxin	5×10^{-5}	86
veratridine	10^{-3}	95
grayanotoxin (GH401)	10^{-3}	89
pyrethroid (Dcis)	10^{-5}	100
pyrethroid (Ru_{15})	10^{-5}	100
procaine	10^{-3}	89
tetrocaine	10^{-3}	76
lidocaine	10^{-3}	88

[a] Electroplaque membranes (0.2 mg of protein/mL) were incubated at 20 °C for 10–15 min in standard choline medium containing the neurotoxins or local anaesthetic as indicated. Then [^{125}I]Css_{II} (0.3 nM) was added and its binding to the membranes under equilibrium conditions assayed as described in the text. Appropriate control incubations were run to check the effects of solvents (ethanol or dimethyl sulfoxide) in which some compounds were dissolved and all necessary corrections made to the apparent binding values.

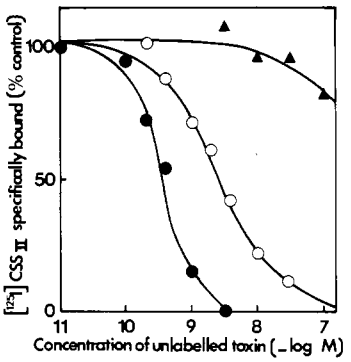

FIGURE 3: Inhibition of [^{125}I]Css_{II} binding by unlabeled Css_{II} or TiTxγ. Binding of [^{125}I]Css_{II} to the membranes was measured under equilibrium conditions in the presence of the indicated concentrations of (○) Css_{II}, (●) TiTxγ, or (▲) TiTxx, as described in the text.

of labeled Css_{II} to electroplaque membranes are illustrated in Figure 3. From these curves and the previously measured values of K_D and B_{max} for [^{125}I]Css_{II} binding to these membranes, K_D values for unlabeled Css_{II} and TiTxγ were calculated according to the relationship

$$K_D = F[[C_T]/([R_T] - [RL] - F) - 1]$$

where $F = K_D^*[RL]'/([L_T] - [RL])$ and K_D^* is the equilibrium dissociation constant for the [^{125}I]Css_{II}–membrane complex, $[R_T]$ is the total concentration of specific binding sites, $[L_T]$ is the total concentration of [^{125}I]Css_{II}, [RL] is the concentration of [^{125}I]Css_{II} bound in the presence of unlabeled toxin at a concentration $[C_T]$, and [RL]′ is the corresponding concentration of specifically bound [^{125}I]Css_{II}. For Css_{II} the K_D values obtained in this way were 1.0 and 1.1 nM, while

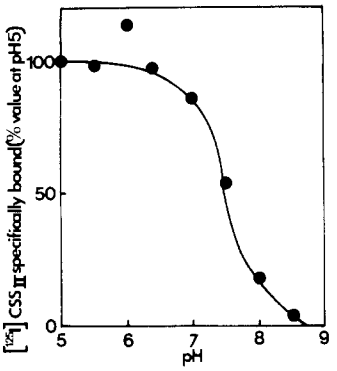

FIGURE 4: Effect of pH on $[^{125}I]Css_{II}$ binding. The concentration of the $[^{125}I]Css_{II}$–membrane complex was measured under equilibrium conditions at the indicated pH values, which were obtained with the use of appropriate mixtures of Mes (20 mM) and Tris buffers.

for TiTxγ the value was 0.1 nM.

The difference between these K_D values for Css_{II} and those obtained for $[^{125}I]Css_{II}$ (0.4–0.7 nM) is probably not significant, and it is clear that the affinities of the labeled and unlabeled toxin for the membrane receptor are about equal.

Figure 3 also shows that *Tityus serrulatus serrulatus* toxin, unlike TiTxγ, was unable to displace bound $[^{125}I]Css_{II}$ to a significant extent.

Variation of Binding with pH and Ionic Strength. Binding of $[^{125}I]Css_{II}$ to electroplaque membranes varied markedly with variation of pH of the incubation medium, decreasing as the pH increased from pH 5 to pH 8.5. Nonspecific binding decreased by about half over this pH range (data not shown) while specific binding fell almost to zero. The relationship between specific binding and pH resembled a simple titration curve (Figure 4), indicating that the specific binding required protonation of a group with an apparent pK_a value of about 7.5.

Both nonspecific binding and specific binding of the labeled toxin were affected by the concentration of ions in the incubation medium but in different ways. With low concentrations of monovalent ions (10 mM Mes–Tris, pH 6; 1–20 mM NaCl) specific binding was very low and sometimes undetectable, whereas nonspecific binding was very high (Figure 5). Addition of 50 mM NaCl (together with 10 mM Mes–Tris) gave approximately maximal specific binding, with no further significant change in the presence of NaCl concentrations up to 150 mM. In contrast, nonspecific binding decreased as the NaCl concentration was raised from 20 to 150 mM (Figure 5). Choline chloride mimicked these effects of NaCl (Figure 5), and 150 mM KCl also had the same effect as 150 mM NaCl (inset to Figure 5).

That these effects were caused by the ionic concentrations rather than by the accompanying osmotic pressure changes was shown by the actions of sucrose and of low concentrations of $CaCl_2$ and $MgCl_2$. With 300 mM sucrose and 20 mM Mes–Tris (pH 6), specific binding was low and nonspecific binding high (inset to Figure 5). The addition of only 2.8 mM $CaCl_2$ plus 1.3 mM $MgCl_2$, in the presence or absence of 300 mM sucrose, increased specific binding to the maximum value, with no significant change in nonspecific binding. However, addition of $CaCl_2$–$MgCl_2$ to 150 mM KCl produced no significant change in either type of binding (inset to Figure 5).

Other experiments established that the addition of similar low concentrations of $CaCl_2$ and $MgCl_2$, separately or together, had no effect on the binding measured in the presence of 150 mM choline chloride, 150 mM NaCl, 140 mM choline chloride plus 5.4 mM KCl, or 140 mM NaCl plus 5.4 nM KCl (data not shown).

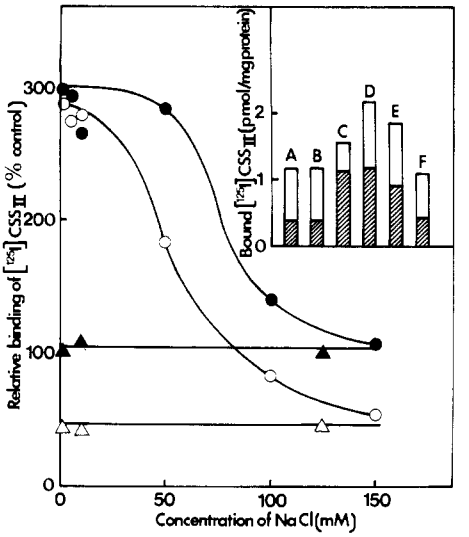

FIGURE 5: Variation of $[^{125}I]Css_{II}$ binding with the concentration of ions in the incubation medium. (Main panel) Amount of $[^{125}I]Css_{II}$ bound under equilibrium conditions during incubation in a medium containing 10 mM Mes–Tris (pH 6) and the indicated concentrations of NaCl, either alone (○, ●) or with appropriate concentrations of choline chloride to maintain the ionic strength constant (△, ▲). Total binding is indicated by filled symbols and nonspecific binding by open symbols. (Inset) Total binding and nonspecific binding (hatched area) during incubation in a solution containing 20 mM Mes–Tris (pH 6) and (A) 150 mM NaCl, (B) 150 mM KCl, (C) 300 mM sucrose, (D) 2.8 mM $CaCl_2$ and 1.3 mM $MgCl_2$, (E) 300 mM sucrose plus 2.8 mM $CaCl_2$ and 1.3 mM $MgCl_2$, and (F) 150 mM KCl plus 2.8 mM $CaCl_2$ and 1.3 mM $MgCl_2$.

Table II: Effect of Treatment of Membranes with Trimethyloxonium Ions[a]

preliminary treatment of the membranes	specific binding (% of control) of [3H]en-TTX	specific binding (% of control) of $[^{125}I]Css_{II}$
(A) none (control)	100	100
(B) plus TMO	31	98
(C) plus TTX and TMO	74	117

[a] For condition B, 1 mL of electroplaque membrane suspensions (3.6 mg of protein) in 50 mM Hepes-Tris buffer, pH 7.1, was added to 1.7 mg of trimethyloxonium fluoroborate (TMO). The mixture was stirred continuously at about 2 °C, and small volumes of 2 M Tris were added to maintain the pH approximately constant. Sample C was the same except for the addition of TTX (5 μM) to the membrane suspension 15 min before addition to the TMO. Sample A was simply kept on ice for the same period of time. Each sample was then diluted with 1 mL of the same buffer solution and the membranes were sedimented by centrifugation (20 000*g*, 10 min, 4 °C). The membranes were washed twice by resuspension in 2 mL of buffer solution and centrifugation, and then each pellet was resuspended in 2 mL of 0.3 M sucrose. The protein content of each suspension was measured, and the specific binding of $[^{125}I]Css_{II}$ and [3H]en-TTX to the membranes was assayed as described in the text. Nonspecific binding was about 27% of total binding for $[^{125}I]Css_{II}$ and about 3% for [3H]en-TTX.

Lack of Effect of Methylation of Membrane Carboxyl Groups. In parallel to previous findings (Reed & Raftery, 1976) exposure of the electroplaque membranes to trimethyloxonium ions and to methylate-exposed carboxyl groups, inhibited their ability to bind [3H]en-TTX, and the addition of a relatively high concentration of unlabeled tetrodotoxin provided partial protection against this action of trimethyloxonium ions (Table II). In contrast, the binding of $[^{125}I]Css_{II}$ to the electroplaque membranes was unaffected by their treatment with trimethyloxonium ions (Table II).

Table III: Effects of Partial Proteolytic Digestion of the Membrane[a]

after digestion with	[^{125}I]Css_{II} bound (pmol/mg of protein) total	nonspecific	specific
none (control)	1.29	0.31	0.98
α-chymotrypsin	0.72	0.22	0.50
Pronase	0.34	0.13	0.21
papain	0.25	0.13	0.12
trypsin	0.23	0.09	0.14

[a] Samples of electroplaque membranes suspended in a solution containing 250 mM sucrose, 0.5 mM EDTA, and 100 mM Tris-HCl (pH 7.4) were incubated for 30 min at 25 °C with α-chymotrypsin (0.5 mg/mL), Pronase (0.1 mg/mL), papain (0.1 mg/mL), or trypsin (0.1 mg/mL). The total volume for each incubation was 0.2 mL, and each sample contained membranes at a final concentration of 1 mg of protein/mL. The sample with papain also contained cysteine (10 mM) and dithiothreitol (1 mM). Two control incubations containing no proteases were run at the same time. After the incubation trypsin inhibitor was added at a final concentration of 1 mg/mL to the samples containing trypsin and chymotrypsin and to one of the controls. Then trypsin (0.1 mg/mL) was added to the latter mixture. All samples were quickly diluted by the addition of 1 mL of the cold sucrose-buffer solution and the membranes washed 3 times by centrifugation and resuspension at 4 °C. Each pellet was finally resuspended in 0.5 mL of 0.3 M sucrose, and samples of the suspensions were used for measurement of protein concentration and of [^{125}I]Css_{II} binding under the standard conditions described in the text. There was no significant difference in [^{125}I]Css_{II} binding to the two control samples.

Effect of Partial Proteolytic Digestion of the Membranes. Partial digestion of the electroplaque membranes with a number of different proteases dimished their ability to bind [^{125}I]Css_{II}, specific binding being inhibited to a greater extent than nonspecific binding (Table III).

Lack of Binding to Crab Axonal Membranes. In contrast to the findings described above, no specific binding of [^{125}I]-Css_{II} to crab axonal membranes was observed under the standard incubation conditions.

Discussion

There is a marked parallelism between the results described above for [^{125}I]Css_{II} binding to electroplaque membranes and those obtained previously for [^{125}I]Css_{II} binding to frog muscle membranes (Jaimovich et al., 1982). Specific binding of the labeled toxin to both types of membranes exhibited similar K_D values, the K_D values calculated for the binding of unlabeled Css_{II} in competition with [^{125}I]Css_{II} were not significantly different, the effect of pH on the specific binding was similar for both kinds of membranes, and [^{125}I]Css_{II} binding to both membrane preparations was equally resistant to displacement by a number of other potent neurotoxins but similarly sensitive to the action of proteases. In addition, the total number of receptors on the electroplaque membranes that were specific for [^{125}I]Css_{II} (0.9–2.4 pmol/mg of protein) was approximately the same as the total number specific for [^{3}H]en-TTX binding (1.9–2.6 pmol/mg of protein) (A. Lombet, unpublished results), as was also found with the frog membranes (Jaimovich et al., 1982).

The specific binding of [^{125}I]Css_{II} by rat brain synaptosomes was characterized by somewhat higher K_D values (Jover et al., 1980). On the other hand, the total number of binding sites on the synaptosomes specific for [^{125}I]Css_{II} was 6–10 times greater than the total number specific for the binding of iodinated *A. australis* Hector toxin (Jover et al., 1980), which means that once again the capacity for Css_{II} binding must have been about the same as for TTX binding (Lazdunski et al., 1980a). Also, none of the other polypeptide neurotoxins tested, which did not include TiTxγ, could displace [^{125}I]Css_{II} from the synaptosomes (Jover et al., 1980).

A potentially important indicator of the nature of Css_{II}'s interaction with its membrane receptor at the molecular level is provided by the effect of pH variation on the specific binding (Figure 4). Clearly a group containing a dissociable proton must be involved, and its pK_a value is about 7.5. It will be important to find out whether this group is in the membrane or in the toxin itself; but, unfortunately, such experiments will require relatively large quantities of toxin.

The apparent lack of any requirement for a specific ion makes the effects of ions on the binding of [^{125}I]Css_{II} less useful. Effects of ion concentrations in experiments with such membrane preparations are complicated by the existence of membrane vesicles, which pose questions concerning the orientation of the membrane surfaces, the relative accessibility of the two surfaces, and possible osmotic effects. However, the results in Figure 5 show that [^{125}I]Css_{II} binding is affected by the ionic concentration in a way that cannot be attributed either to an osmotic effect or to just a decrease in nonspecific binding. Specific binding seems to require a certain minimum concentration of ions, and salts of the divalent cations Mg^{2+} and Ca^{2+} are clearly more effective than salts of the monovalent cations, such as Na^+ and K^+, because only about 4 mM ($CaCl_2$ plus $MgCl_2$) gave the same level of binding as about 50 mM NaCl (Figure 5).

Since electrophysiological experiments with frog muscle confirmed that Css_{II} inhibits the Na^+ channel function (Jaimovich et al., 1982), it seems safe to conclude from the similarities described above that Css_{II} also binds to Na^+ channels in electroplaque and synaptosome membranes. However, it is obvious that the Css_{II} receptor must be different from that for TTX, both because neither TTX nor saxitoxin interfered with [^{125}I]Css_{II} binding (Table I) and because exposure of the electroplaque membranes to trimethyloxonium ions affected [^{3}H]en-TTX binding but not [^{125}I]Css_{II} binding (Table II). In addition, the absence of a specific receptor for Css_{II} in crab axonal membranes shows that this receptor is not a characteristic feature of Na^+ channels in all excitable membranes. The electrophysiological effects of Css_{II} on muscle similarly indicated that the toxin discriminates between Na^+ channels in the cell surface and T-tubule membranes (Jaimovich et al., 1982).

The finding that of all the other neurotoxins and local anaesthetics tested only *T. serrulatus serrulatus* γ toxin (TiTxγ) could displace specific binding of [^{125}I]Css_{II} is particularly interesting. No electrophysiological effects of TiTx have been reported, but it has been tentatively classified with Css_{II} on the basis of its amino acid sequence (Rochat et al., 1979). Since the results described above support the conclusion that Css_{II} represents a class of neurotoxins that interact with the Na^+ channel at a site distinct from those previously established for other toxins, this interaction between TiTxγ and [^{125}I]Css_{II} obviously also suggests that TiTxγ may be classified together with Css_{II}. Moreover, the data obtained here show that TiTxγ has an even higher affinity for the receptor than does Css_{II} (Figure 3), which indicates that it could be a particularly powerful experimental tool for more detailed investigations of the Na^+ channel at the molecular level.

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Lactose–Proton Symport by Purified *lac* Carrier Protein†

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ABSTRACT: The *lac* carrier protein of *Escherichia coli* was purified by an improved procedure and its activity assayed by a rapid filter method. Following reconstitution of the carrier by octyl glucoside dilution, proteoliposomes were concentrated by filtration on a microporous filter. Lactose accumulation by adsorbed or entrapped proteoliposomes is driven by an artificially imposed pH gradient (interior alkaline), by a membrane potential (interior negative), or by a combination of both forces. Activity is almost completely abolished by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone or by the competitive inhibitor thiodigalactoside. Addition of lactose to proteoliposomes under appropriate conditions results in alkalinization of the external medium. This effect is not observed with liposomes devoid of *lac* carrier or in the presence of proton conducting agents. The results provide a strong indication that the *lac y* gene product is the only protein in the cytoplasmic membrane of *Escherichia coli* required for lactose–proton symport.

During the last decade, a large body of evidence has accumulated supporting Mitchell's hypothesis (Mitchell, 1961, 1963, 1968) that chemiosmotic phenomena play a central role in active transport [cf. Rosen & Kashket (1978) for a review]. According to the hypothesis, the uptake of many solutes is mediated by specific polypeptide carriers that couple the uphill translocation of substrate across the cytoplasmic membrane to the simultaneous downhill movement of a cation such as H^+ or Na^+ (i.e., symport or antiport). Thus, the concentration gradients of many substrates across the cell membrane are maintained as a consequence of an electrochemical ion gradient generated by the action of various cationmotive pumps (e.g., membrane-bound respiratory chains, proton-translocating ATPases, or Na^+/K^+-ATPases).

The development of facile methods to reconstitute and assay the activity of transport systems is crucial to their isolation and study. Most of the methods described to date are time consuming and poorly suited to the analysis of the large number of fractions typically encountered during the course of a purification. Recently, two transport systems from *Escherichia coli* were solubilized and reconstituted in functional form by octyl β-D-glucopyranoside (octyl glucoside)[1] dilution (Newman & Wilson, 1980; Tsuchiya et al., 1982).[2] Although

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[1] Abbreviations: octyl glucoside, octyl β-D-glucopyranoside; ΔpH, transmembrane pH gradient; ΔΨ, transmembrane electrical potential; $\Delta\bar{\mu}_{H^+}$, transmembrane electrochemical proton gradient; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; [^{3}H]NPG, 4-nitro[2-^{3}H]phenyl α-D-galactopyranoside.

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