Binding of Scorpion Toxins to Rat Brain Synaptosomal Fraction. Effects of Membrane Potential, Ions, and Other Neurotoxins†

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ABSTRACT: Neurotoxins from different scorpion venoms were shown to stimulate ^{86}Rb efflux from the rat brain synaptosomal fraction and to inhibit ^{86}Rb uptake. These effects were blocked by tetrodotoxin and mimicked by veratridine. Inhibition of ^{86}Rb uptake was used to measure the activities of several scorpion toxins. Toxin II from the scorpion *Androctonus australis* Hector was iodinated by using the lactoperoxidase method and the binding of ^{125}I-labeled toxin to synaptosomes was studied in a Na^+-free medium; a single class of noninteracting sites was described with a dissociation constant of 0.1–0.35 nM and a capacity of 40–200 fmol/mg protein. The presence of Na^+ in the medium reduced the toxin binding, and the Scatchard plot was curvilinear in these conditions, expressing a negative cooperativity. Scorpion toxin binding affinity was reduced by external K^+, veratridine, and gramicidin D, i.e., by membrane depolarization. Binding was inhibited by increasing external divalent cation concentration. Tetrodotoxin and tetracaine enhanced the number of scorpion toxin binding sites but had no effect on the membrane potential dependence. On the contrary, veratridine, even in a Na^+-free medium, induced a shift of $[K^+]$–scorpion toxin binding curves toward the higher $[K^+]$; this effect was not blocked by tetrodotoxin.

Neurotoxins purified from venoms of African scorpions are small basic proteins (Miranda et al., 1970; Rochat et al., 1979) which specifically block the inactivation of voltage-sensitive sodium channels in nerve (Romey et al., 1975). In mouse neuroblastoma cells in culture, they stimulate the sodium flux (Catterall, 1976), increase the action potential duration (Bernard et al., 1977), and bind to a single class of membrane receptor; the binding was demonstrated for two toxins, one from the scorpion *Leiurus quinquestriatus* (Catterall et al., 1976) and one from *Androctonus australis* Hector (Couraud et al., 1978). The affinity of scorpion toxins for their receptor is dependent on the membrane potential: depolarization of the cells decreases the affinity constant 10-fold every 31 mV (Catterall et al., 1976; Catterall, 1977a).

Synaptosomes, prepared from rat brain, are still able to retain resting membrane potential, and their sodium permeability increases on treatment with veratridine and scorpion venom (Blaustein & Goldring, 1975). These drugs stimulate calcium uptake and noradrenaline release (Blaustein, 1975). Toxin I from *A. australis* Hector has been shown to increase γ-aminobutyric acid release (Romey et al., 1976). We have demonstrated that ^{125}I-labeled toxin II from *A. australis* Hector binds to the rat brain synaptosomal fraction, and we have calculated the kinetic constants of the interaction (Jover et al., 1978). The binding of *L. quinquestriatus* toxin to rat synaptosomes was also described (Ray et al., 1978). In the present work, we have studied the effect of different scorpion toxins on rubidium-86 fluxes in the rat brain synaptosomal fraction and the binding of ^{125}I-labeled toxin II from *A. australis* Hector. We have analyzed the modulation of toxin affinity and membrane capacity with membrane potential, ions, and other neurotoxins.

Materials and Methods

Toxins and Chemicals. Toxins I and II from *A. australis* Hector (AaH I and AaH II), toxin V from *L. quinquestriatus quinquestriatus* (Lqq V), and toxin I from *Buthus occitanus tunetanus* (Bot I) were purified according to Miranda et al. (1970); toxin II from *Centruroides suffusus suffusus* (Css II) was purified according to Garcia (1976).

Tetrodotoxin and gramicidin D were from Boehringer (Mannheim, Germany), veratridine was from EGA Chemie (Steinheim, Germany), tetracaine, Tris, lactoperoxidase, bovine serum albumin (fraction V), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were from Sigma Chemical Co. (St. Louis, MO), and aconitine was from Fluka (Buchs, Switzerland). Rubidium-86 (1.6 mCi/mg) was provided from C.E.A. (Saclay, France) and iodine-125 carrier free (13–17 Ci/mg) was from the Radiochemical Center (Amersham, England).

Preparation of the Synaptosomal Fraction. The synaptosomal fraction was prepared by using the method of Gray & Whittaker (1962) slightly modified by Blaustein & Ector (1976). Cortex, striatum, and hypothalamus were dissected from Wistar rats and homogenized with a Teflon pestle in a 0.32 M sucrose solution (0.1 g of tissue per mL). The homogenate was centrifuged 10 min at 750*g*, and the resulting supernatant was centrifuged 25 min at 10000*g*. Then the pellet, washed once in 0.32 M sucrose solution and sedimented 25 min at 10000*g*, was suspended in a medium containing 140 mM choline chloride, 5 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM glucose, 25 mM Hepes, and Tris base to pH 7.2. Protein was measured by a modified Lowry method (Lowry et al., 1951).

Measurement of Rubidium-86 Uptake. The synaptosomal fraction was diluted in sodium medium containing 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM glucose, 25 mM Hepes, 0.25% bovine serum albumin, and Tris base to pH 7.2; the final concentration was 0.5–1 mg of protein per mL. The suspension was distributed (200 μL/tube) and preincubated 15 min at 37 °C. The incubation was started with the addition of 50 μL of the same medium containing rubidium-86 (5 μCi/mL) and different neurotoxins. The

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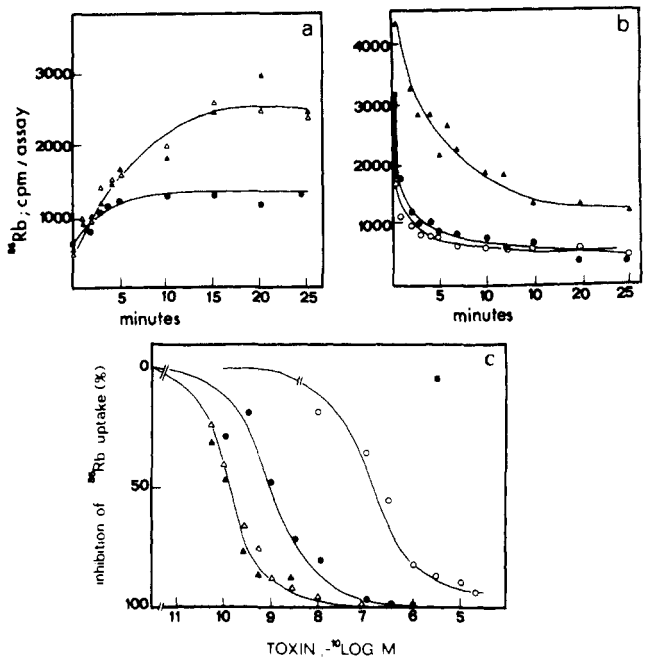

FIGURE 1: Effect of scorpion neurotoxins on rubidium-86 fluxes. (a) Kinetics of rubidium-86 uptake measured as described under Materials and Methods in the absence (▲) or in the presence of 1 μM AaH II (●) or of 1 μM AaH II and 1 μM tetrodotoxin (△). (b) Kinetics of rubidium-86 efflux measured as described under Materials and Methods in the absence (▲), in the presence of 1 μM AaH II (●), or in the presence of 50 μM veratridine (○). (c) Dose–response curves on rubidium-86 uptake measured as described under Materials and Methods during 20 min in the presence of the indicated concentrations of AaH II (△), [^{125}I]AaH II (▲), AaH I (●), Bot I (○), and Css II (■). Results were expressed as percentage of the maximum scorpion toxin inhibition of ^{86}Rb uptake, obtained in the presence of 1 μM AaH II.

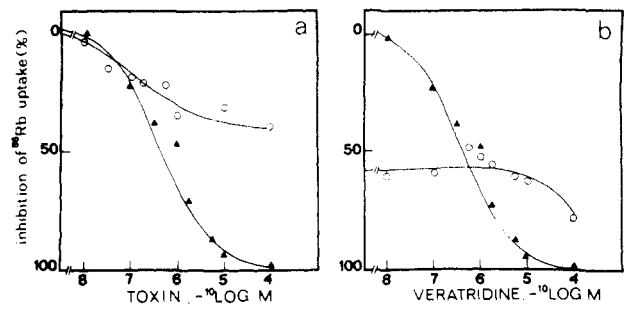

FIGURE 2: Effects of veratridine and aconitine on rubidium-86 uptake. (a) The ^{86}Rb uptake was measured as described under Materials and Methods during 20 min in the presence of the indicated concentrations of veratridine (▲) and aconitine (○). Results were expressed as percentage of the maximum veratridine inhibition of ^{86}Rb uptake, obtained in the presence of 100 μM veratridine. (b) Same experiment as described in (a) with the indicated concentrations of veratridine, in the absence (▲) or the presence (○) of 50 μM aconitine.

incubation was stopped by filtration (Millipore HAWP 25) of 200 μL of suspension. The filters, washed at 4 °C with the sodium medium (3 × 1.5 mL), were counted in 5 mL of Unisolve 1 by using a liquid scintillation counter (Packard).

Measurement of Rubidium-86 Efflux. The synaptosomal fraction was diluted in the sodium medium (1 mg of protein per mL) and distributed (225 μL/tube). Rubidium-86 in the sodium medium (25 μL) was added to obtain a radioactivity of 10 μCi/mL. The synaptosomes, incubated 20 min at 37 °C, were centrifuged and the pellet was suspended in the same medium free of rubidium-86 but containing neurotoxins. The incubation, carried out at 37 °C, was stopped by filtration of a 200-μL suspension. Filters were treated as described for uptake measurements.

Measurement of Scorpion Toxin Binding. ^{125}I-Labeled AaH II was prepared as previously described (Rochat et al., 1977). Specific radioactivities of 700 Ci/mmol were routinely obtained.

The unlabeled neurotoxins (25 μL) were added to synaptosomal suspension (200 μL) maintained at 4 °C. The incubation, for 30 min at 37 °C, started by addition of 25 μL of an [^{125}I]AaH II solution, was stopped by centrifugation (1 min at 11000*g*). The pellet was washed twice at 4 °C with 1 mL of medium, and the radioactivity was evaluated in a γ spectrometer (Beckman). In each experiment the same medium was used to prepare neurotoxin solutions and synaptosomal suspension and to wash the pellet. In experiments leading to the determination of characteristic parameters of the binding, a choline medium was used: 140 mM choline chloride, 5 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM glucose, 25 mM Hepes, 0.25% bovine serum albumin, and Tris base to pH 7.2. When necessary, other media were used that are defined in the legends to the figures.

Results

Effect of Scorpion Toxins on Rubidium-86 Fluxes. The uptake of rubidium-86 by the synaptosomal fraction reached a plateau within 10–15 min. In the presence of 1 μM AaH II the plateau was lowered and reached within 5–10 min (Figure 1a). This effect was abolished by 1 μM tetrodotoxin. The lowering of the plateau was dependent on the scorpion toxin concentration, the half-maximum effect being obtained for 0.15 nM AaH II (Figure 1c). The kinetics of rubidium-86 efflux was increased by addition of 1 μM AaH II or 50 μM veratridine; a plateau was reached that was lower than in the test experiment (Figure 1b). The increase of rubidium-86 efflux measured after 2-min incubation depended on the AaH II concentration, the half-maximum effect being obtained for 0.28 nM AaH II (not represented). The inhibition of rubidium-86 uptake by three other scorpion toxins was studied; the $K_{0.5}$ values were 1.5 nM (AaH I) and 140 nM (Bot I), but 3 μM Css II was still inactive (Figure 1c). When tested in regard to its acitivity toward rubidium-86 uptake, [^{125}I]AaH II gave results not significantly different from those of the native toxin (Figure 1c). The effect of scorpion toxin on rubidium-86 uptake was mimicked by veratridine ($K_{0.5}$ = 0.5 μM) and only to a small extent by aconitine (Figure 2a). However, aconitine in high concentration (50 μM) blocked the veratridine inhibition of rubidium-86 uptake (Figure 2b), which is in good agreement with a competition of both alkaloids for the same site. Similar results have been obtained with cultured neuroblastoma cells (Catterall, 1977b).

Scorpion Toxin Binding. Binding of [^{125}I]AaH II to the synaptosomal fraction prepared from striata in the choline medium was a saturable phenomenon (Figure 3a). The Scatchard plot (Figure 3b) revealed a single class of noninteracting binding sites with a dissociation constant K_D* of 0.2 nM. The calculated Hill number was 0.98. From 10 independent experiments, the values of K_D* varied between 0.1 and 0.35 nM and the membrane capacity varied between 40 and 200 fmol/mg protein. The dissociation constant of the native toxin, K_D, calculated by displacement of [^{125}I]AaH II by increasing concentrations of AaH II, was very similar: 0.3 nM (Figure 3c). Iodinated AaH II was also displaced by increasing concentrations of three other scorpion toxins, Lqq V, AaH I, and Bot I; $K_{0.5}$ values were, respectively, 1.0, 4.5, and 600 nM (Figure 3c) and calculated K_D values were 0.5, 2.2, and 300 nM. Concerning Css II no effect was obtained with concentrations up to 3 μM. Using [^{125}I]AaH II, we measured

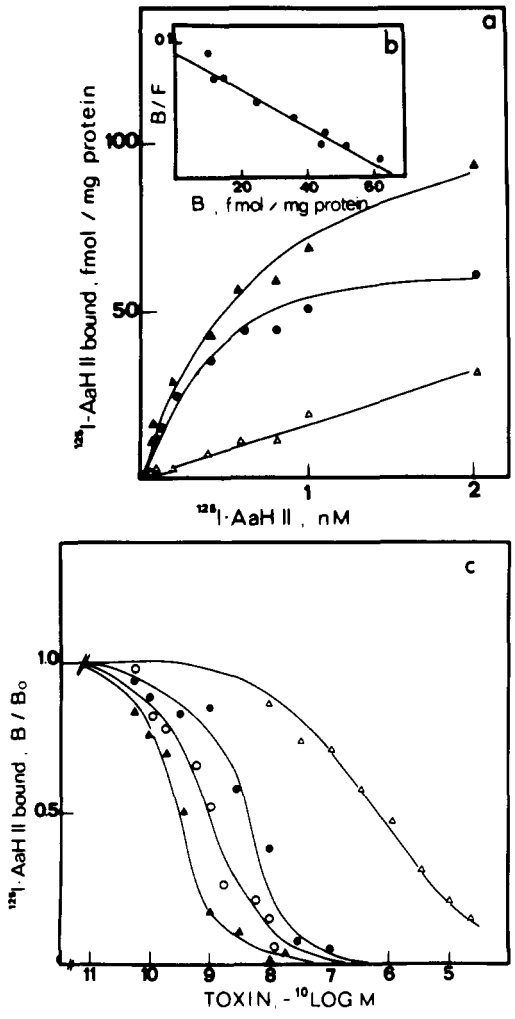

FIGURE 3: Scorpion toxin binding in the choline medium. (a) Synaptosomal fraction (0.6 mg of protein per mL) was incubated 30 min at 37 °C, in the choline medium, with the indicated concentrations of [^{125}I]AaH II in the presence (△) or the absence (▲) of 0.2 μM native AaH II. Specific binding (●) was the difference between the two curves. (b) Scatchard plot of specific binding. (c) Displacement of [^{125}I]AaH II bound to the synaptosomal fraction by native scorpion toxins: synaptosomes (0.5 mg of protein per mL) were incubated, 30 min at 37 °C, in the choline medium with 0.2 nM [^{125}I]AaH II and increasing concentrations of native AaH II (▲), AaH I (●), Lqq V (○), Bot I (△), and Css II (■). B_0 is the binding of [^{125}I]AaH II in the absence of native toxin and B the binding in the presence of the indicated concentration of native toxin. The nonspecific binding has been subtracted.

Table I: [^{125}I]AaH II Binding to the Synaptosomal Fraction Prepared from Different Parts of the Brain[a]

tissue	K_D* (nM)	R_T (fmol/mg protein)
cortex	0.25	135
hypothalamus	0.30	189
striatum	0.45	139

[a] The affinity of the toxin (K_D*), as the capacity in receptors of the synaptosomes (R_T), was calculated by using Scatchard plots of data obtained in the course of saturation experiments as shown in Figure 3.

both the affinity and the number of sites in the synaptosomal fraction prepared from different regions of brain (striatum, hypothalamus, and cortex); the variations were very low (Table I).

Effects of Membrane Potential and Ions on Scorpion Toxin Binding. The substitution of choline by sodium or lithium in

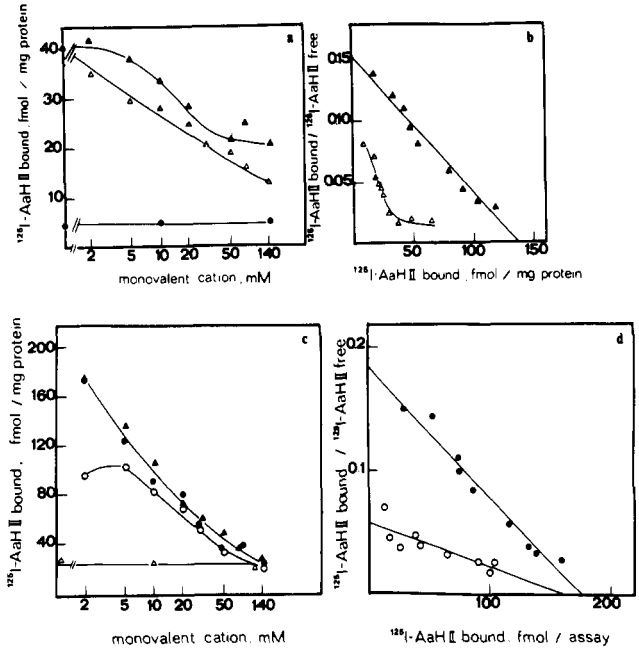

FIGURE 4: Effect of monovalent cations on [^{125}I]AaH II binding. (a) The synaptosomal fraction (0.6 mg of protein per mL) was incubated, 30 min at 37 °C, with 0.2 nM [^{125}I]AaH II in a medium containing sodium (▲) or lithium (△) in place of choline so that [choline] plus either [Na^+] or [Li^+] was 140 mM. The binding was measured in the presence (●) or in the absence (▲ and △) of 0.2 μM native AaH II. (b) Scatchard plot of specific binding of [^{125}I]AaH II in the choline medium (▲) and in the sodium medium (△). Specific binding was determined as described in Figure 3. (c) Synaptosomal fraction (0.15 mg of protein per mL) was incubated, 30 min at 37 °C, with 0.2 nM [^{125}I]AaH II in the choline medium free of K^+, containing K^+ (▲), Rb^+ (●), or Cs^+ (○) in place of choline so that [choline] plus either [K^+] or [Rb^+] or [Cs^+] was 145 mM. The binding was measured in the presence (△) or in the absence (▲, ●, and ○) of 0.2 μM native AaH II. (d) Scatchard plot of [^{125}I]AaH II specific binding in 5 mM K^+ medium (●) and 15 mM K^+ medium (○). Specific binding was determined as described in Figure 3.

the assay medium reduced the binding of [^{125}I]AaH II to rat synaptosomes (Figure 4a); however, the inhibition was not complete. The Scatchard plot, obtained when the medium was 140 mM sodium, was curvilinear with an upward concavity (Figure 4b). In this medium the displacement of bound [^{125}I]AaH II by increasing concentrations of native AaH II gives a $K_{0.5}$ of 0.2 nM and a Hill number of 0.69 (not represented).

Replacement of choline by potassium showed a complete inhibition of [^{125}I]AaH II specific binding (Figure 4c). Scatchard plots obtained when [K^+] was 5 or 15 mM showed a decrease of the affinity but no modification of the number of sites (Figure 4d). Replacement of choline by rubidium or cesium gave similar results (Figure 4c).

When choline was replaced by divalent cations (one cation for two cholines), we observed a complete inhibition of scorpion toxin binding with $K_{0.5}$ 4, 10, 10, 10, and 25 mM, respectively, for Ba^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+}.

Effects of Tetrodotoxin and Veratridine on Scorpion Toxin Binding. Tetrodotoxin, which specifically inhibits sodium permeability in nerve (Narahashi et al., 1964), enhanced scorpion toxin specific binding in both media, 0 and 140 mM [Na^+]; the $K_{0.5}$ for tetrodotoxin was 10 nM (Figure 5a). The affinity of scorpion toxin for its receptor was not modified whereas membrane capacity was increased (Figure 5b). Similar results were observed with tetracaine (not represented), a local anesthetic which was also shown to block sodium permeability in nerve.

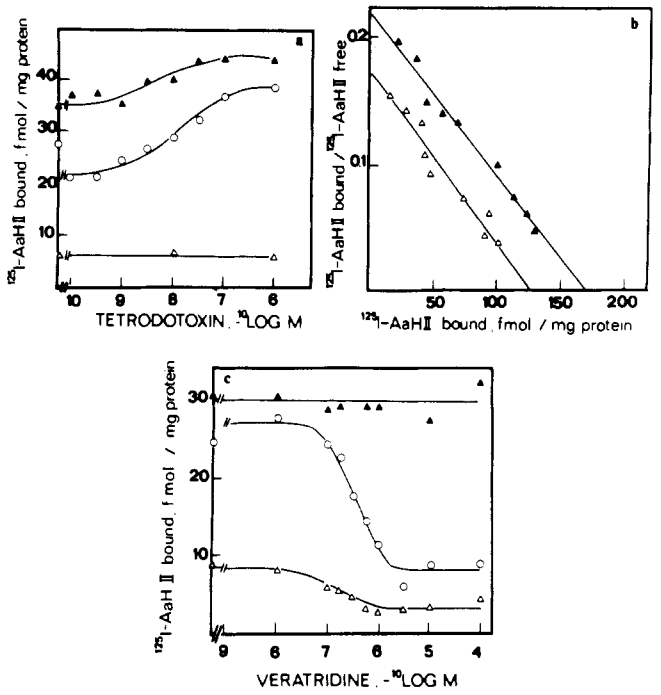

FIGURE 5: Effects of tetrodotoxin and veratridine on [^{125}I]AaH II binding. (a) The synaptosomal fraction (0.6 mg of protein per mL) was incubated, 30 min at 37 °C, with 0.2 nM [^{125}I]AaH II either in the choline medium (▲) or in the sodium medium (○) in the presence of the indicated concentrations of tetrodotoxin. Nonspecific binding was measured in the presence of 0.2 μM native AaH II (△). (b) Scatchard plot of specific binding of [^{125}I]AaH II in the choline medium in the presence (▲) or in the absence (△) of 1 μM tetrodotoxin. Specific binding was determined as described in Figure 3. (c) The synaptosomal fraction (0.4 mg of protein per mL) was incubated, 30 min at 37 °C, with 0.2 nM [^{125}I]AaH II in the choline medium containing none (▲), 30 (○), or 140 mM sodium (△) in place of choline so that [choline] plus [sodium] was 140 mM, in the presence of the indicated concentrations of veratridine. Nonspecific binding was subtracted.

Veratridine had no action on scorpion toxin binding in the choline medium. However, as the sodium concentration increased, we observed an inhibition of [^{125}I]AaH II binding by veratridine: $K_{0.5}$ values were, respectively, 0.30 and 0.15 μM for 30 and 140 mM sodium (Figure 5c). Similar results were obtained with aconitine.

Tetrodotoxin did not modify the dependence of [^{125}I]AaH II binding on external [K^+], i.e., on membrane potential, whereas 0.4 mM veratridine displaced the curve of [^{125}I]AaH II binding toward higher [K^+]. This effect occurred in the choline medium and was not blocked by 1 μM tetrodotoxin (Figure 6).

Discussion

The effects of scorpion toxins and veratridine on rubidium-86 fluxes can be summarized as follows: there was no great modification of the initial rate of rubidium uptake but the level of the plateau is lowered; on the other hand, the initial rate of the rubidium-86 efflux increases greatly (Figure 1b). Measurement of the inhibition of rubidium-86 uptake proved to be a useful test for measuring the activities of scorpion toxins on synaptosomal potassium flux. The efflux was a more complex phenomenon than a single exponential. As for the lowering of the influx plateau in the presence of the toxin (Figure 1a), it may be due to the increase of the efflux rate. Three mechanisms to explain this increase. (1) Rubidium-86 may have used the voltage-sensitive K^+ channel: in that case, the effect should have been blocked by TEA, which we did not observe. (2) Rubidium may have used the opened Na^+

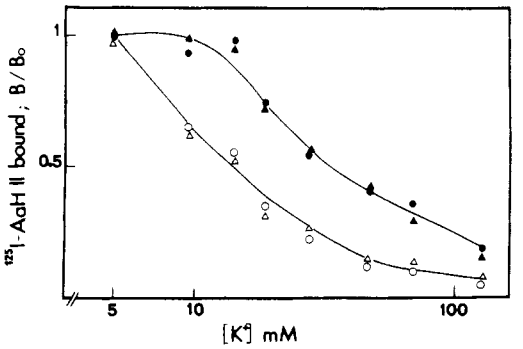

FIGURE 6: Effect of tetrodotoxin and veratridine on [K^+]-dependent [^{125}I]AaH II binding. The synaptosomal fraction was incubated with 0.2 nM [^{125}I]AaH II in the choline medium containing increasing K^+ in place of choline so that [choline] plus [K^+] was 145 mM in the presence of no effector (△), 1 μM tetrodotoxin (○), 0.4 mM veratridine (▲), or 1 μM tetrodotoxin plus 0.4 mM veratridine (●). Results were expressed as the ratio between specific binding at various [K^+] and at 5 mM K^+.

channel as it has been suggested for veratridine-stimulated rubidium-86 efflux in neuroblastoma cells (Palfrey & Littauer, 1976). (3) Increase of rubidium efflux was due to a depolarization of the membrane provoked by the toxins. As we were unable to measure the potential of synaptosomal membranes, we have no definite evidence to make a final choice between the two hypotheses. However, the results of scorpion toxin binding in the presence of sodium suggest that the depolarization hypothesis was the most probable.

We obtained a good correlation between the activities of several scorpion toxins on rubidium-86 uptake and their affinities for synaptosomes as measured by binding experiments. It is important to note that the affinity of AaH II was 10-fold greater than that of AaH I whereas a ratio of 2 was obtained when comparing mouse lethalities (Miranda et al., 1970). Similar results were observed with chick embryonic heart cells (Couraud et al., 1980).

We confirmed that scorpion toxin binding affinity depends on membrane potential: increase in external K^+ concentration and addition of veratridine or gramicidin D in the presence of sodium, which induce membrane depolarization, inhibited scorpion toxin binding. The inhibition of toxin binding by sodium was another confirmation: the curvilinear Scatchard plot obtained in 140 mM sodium medium (Figure 4b) was interpreted as the expression of a negative cooperativity, i.e., the binding of scorpion toxin to its receptor increased the activity of sodium channels leading to a depolarization of synaptosomal membrane and finally to a diminution of the scorpion toxin affinity for its receptor. The negative cooperativity was confirmed by a Hill number of 0.69. Lithium had a similar effect on scorpion toxin binding; this is in good agreement with the results of Blaustein & Goldring (1975) that have proved similar effects of lithium and sodium on synaptosomal membrane potential. Again, the effects of Rb^+ and K^+ on scorpion toxin binding were identical, as they behave in a similar way on membrane potential. On the contrary, the effects of Cs^+ were different (Figure 4c); an explanation may be that Cs^+, contrary to K^+, was not able to stimulate the activity of the Na^+,K^+-ATPase whose blocking would induce a membrane depolarization.

Divalent cations inhibit scorpion toxin binding. The order of activity was $Ba^{2+} > Ca^{2+} = Mn^{2+} = Mg^{2+} > Co^{2+}$. Their effects may be explained by two hypotheses: either divalent cations induced a membrane depolarization or they bound to the scorpion toxin receptor, inhibiting toxin binding in a competitive manner. In that last case, scorpion toxins and

divalent cations could bind to a negative surface charge whose existence near sodium channels was proposed by Hille et al. (1975).

The dependence on membrane potential of scorpion toxin affinity was not modified by tetrodotoxin which induced an apparent increase of the number of receptors. Two mechanisms are possible. (1) The first is an effect on synaptosomes population; a fraction of synaptosomes was depolarized by spontaneous activation of sodium channels even in a sodium-free medium. The binding of scorpion toxin could not be measured on this fraction. In the presence of tetrodotoxin this depolarization was blocked and the apparent number of receptors increased. (2) The second is a direct interaction between scorpion toxin and tetrodotoxin receptors which could lead to the unmasking of scorpion toxin receptors. First of all, this second hypothesis is not probable because it has been found that tetrodotoxin has no effect on scorpion toxin binding in neuroblastoma cells (Catterall, 1977a; F. Couraud, unpublished results); second, effects of tetracaine, which were very similar to that of tetrodotoxin, are in favor of the first mechanism.

Veratridine seemed to have a direct effect on scorpion toxin binding as previously shown for batrachotoxin on the same preparation (Ray et al., 1978). It induced a shift of the curves membrane potential–scorpion toxin binding toward the less negative potentials; this effect was not inhibited by tetrodotoxin which is an argument for a direct interaction between veratridine and scorpion toxin receptors.

Thus, these results show that scorpion toxins may be used to study the properties of membrane molecules whose conformation depends on membrane potential and which are related to the inactivation process of sodium channel.

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