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Binding of Scorpion Neurotoxins to Chick Embryonic Heart Cells in Culture and Relationship to Calcium Uptake and Membrane Potential†

François Couraud,* Hervé Rochat, and Serge Lissitzky

ABSTRACT: Stimulation of ^{45}Ca uptake by scorpion neurotoxins in cultured chick embryonic heart cells has been shown to be directly linked to their effect on sodium channels. This property was used to compare the activity of 15 neurotoxins from five different species to their lethal effect in the mouse and immunological properties. As scorpion neurotoxins, the alkaloid neurotoxin veratridine enhanced ^{45}Ca uptake, and an apparent positive cooperativity between the two drugs was observed. ^{125}I-Labeled toxin II from the scorpion *Androctonus australis* Hector was shown to bind to chick heart cells specifically, saturably, and reversibly with high affinity (K_D = 1–3 nM in sodium-free medium) and low capacity (10–20 fmol/mg cell protein). As shown by ^{45}Ca uptake and radioactive toxin binding experiments, the affinity of scorpion neurotoxin to heart cell receptors was dependent on external K^+ concentration. Toxin binding was lowered by increasing Na^+ concentration in the medium and was abolished by veratridine in a sodium (140 mM) containing medium. As previously reported for neuroblastoma cells, all these results are in agreement with the membrane potential dependence of scorpion neurotoxin affinity for its membrane receptor.

Neurotoxins isolated from African scorpion venoms are small basic proteins (Miranda et al., 1970; Rochat et al., 1979) which modify the action potential in nerve (Romey et al., 1975) and in heart cell (Coraboeuf et al., 1975) by blocking the inactivation of the sodium channel. In cultured neuroblastoma cells, they stimulate sodium transport (Catterall, 1976), increase the duration of action potential (Bernard et al., 1977), and bind to a specific membrane receptor (Catterall et al., 1976; Couraud et al., 1978). The affinity of scorpion toxin for its receptor depends upon membrane potential (Catterall et al., 1976; Catterall, 1977).

Cultured chick embryonic heart cells provide a useful system to study electrical phenomena in cardiac muscle in a nerve-free environment. Scorpion toxins were shown to induce an increase of cell beat frequency with contracture at high concentrations (Fayet et al., 1974). They stimulate the passive Na^+ and Ca^{2+} uptake (Couraud et al., 1976) and increase the duration of action potential of chick embryonic heart cells (Bernard & Couraud, 1979).

In the present work, we have investigated the effects of several toxins from five different scorpion venoms on calcium

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uptake and the apparent cooperativity of scorpion toxins and veratridine on Ca^{2+} transport. We have also studied the binding of toxin II from the venom of *Androctonus australis* Hector to cultured heart cells and its modulation by membrane potential.

Materials and Methods

Chemicals. Chemicals were obtained from the following sources: veratridine from E.G.A. Chemie (Steinheim, West Germany); tetrodotoxin, bovine serum albumin (fraction V), lactoperoxidase, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), and Tris from Sigma Chemical Co. (St. Louis, MO); ouabain and ovomucoid from Boehringer (Mannheim, West Germany); crude trypsin from Institut Pasteur (Paris, France); fetal calf serum and Eagle's minimal essential medium from GIBCO (Paisley, Scotland); NCTC 109 medium from Eurobio (Paris, France); $^{45}CaCl_2$ (40 mCi/mg) from the CEA (Saclay, France); iodine-125 carrier free (13–17 mCi/μg) from the Radiochemical Center (Amersham, England).

Toxins. Toxins from the venom of the scorpions *A. australis* Hector (AaH I, II, and III), *Buthus occitanus tunetanus* (Bot I, II, III, IV, V, VIII, IX, and XI), *Leiurus quinquestriatus quinquestriatus* (Lqq IV and V), and *Androctonus mauretanicus mauretanicus* (Amm III and V) were purified as described by Miranda et al. (1970) and toxin II was purified from the scorpion *Centruroides suffusus suffusus* (Css II) as described by Garcia (1976). Rabbit antiserum against toxin II of *A. australis* Hector was the generous gift of Dr. Delori.

Cell Culture. Cells were isolated from the hearts of chick embryos aged 11 days as previously described (Couraud et al., 1976) except that ovomucoid (10 μg/mL) was added to the spinner solution to stop the effect of trypsin. Cells (1.5×10^6/mL) were suspended in NCTC 109 medium containing 10% fetal calf serum, penicillin G (100 units/mL), and streptomycin sulfate (50 μg/mL) and were seeded in 24 dish COSTAR plates (ϕ 16 mm) with 0.5 mL of suspension per dish. Incubation was at 36 °C in 95% air–5% CO_2.

Measurement of Calcium Uptake. Uptake of calcium-45 was measured as previously described (Couraud et al., 1976) with slight modifications. Heart cells were used after 3 or 4 days of culture without medium change. Culture medium was removed and replaced by incubation medium (Eagle's minimal essential medium containing 25 mM Hepes, pH 7.2, and 1% fetal calf serum) 15 min before the addition of $^{45}Ca^{2+}$. Incubation medium contained 145 mM Na^+ and 1.8 mM Ca^{2+}. At time 0, medium was replaced by fresh incubation medium containing 0.5 μCi/mL $^{45}CaCl_2$, 0.5 mM ouabain, and appropriate toxins as described in the text and legends of the figures. Incubation temperature was 35 °C. Uptake measurements were stopped after 10 min by aspirating off the radioactive incubation medium and washing the cell layer 3 times at room temperature. Total washing time was 15 s. For measurement of the effect of membrane potential on scorpion toxin affinity, cells were preincubated in a medium containing 5 mM or 145 mM K^+. The preincubation medium was Na^+ free so that sodium permeability and thus membrane potential could not be changed by scorpion toxin addition during preincubation. Its composition was 140 mM choline chloride, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM glucose, 0.25% bovine serum albumin, 25 mM Hepes, and Tris base to obtain pH 7.2 (choline medium). In order to depolarize the cells during preincubation, we used K^+ in place of choline so that [K^+] plus [choline] was 145 mM.

Iodination of Toxin II from A. australis Hector (AaH II). AaH II was iodinated by using the lactoperoxidase method of iodide-125 oxidation and purified by immunoprecipitation with a monospecific antiserum prepared against the native toxin according to the method described by Rochat et al. (1977). Specific radioactivities of 700 Ci/mmol were routinely obtained. As shown by injection into mice, the iodinated toxin showed no toxicity loss.

Measurement of Toxin Binding. Culture medium was discarded and replaced by the choline medium. After 5 min at 35 °C, this medium was substituted by the same medium containing [^{125}I]AaH II and other effectors as described in the legends of the figures. At the end of the incubation time, the medium was removed and cells were washed 4 times with the same medium at 4 °C. Total washing time did not exceed 20 s. Protein concentration was determined in dishes washed in the absence of bovine serum albumin by a modified Lowry method (Lowry et al., 1951).

Results

Effect of Scorpion Toxin on Calcium Uptake. Incubated in the presence of 0.5 mM ouabain, the internal Na^+ level of cultured heart cells was shown to rise to 150 mM instead of 20 mM in its absence as a consequence of the blockade of Na^+,K^+-ATPase activity (Couraud et al., 1976). Ouabain also induced a stimulation of calcium uptake probably due to the lack of a Na^+ gradient. Addition of scorpion neurotoxin in the presence of ouabain enhanced the rate of ^{45}Ca uptake, a maximum effect occurring between 10 and 20 min instead of 60 min in the absence of toxin. This effect was blocked by tetrodotoxin, a specific inhibitor of sodium channel in nerve (Narahashi et al., 1964), was dependent on extracellular Na^+ concentration, and was not modified by D600 at doses where this drug blocks Na^+–Ca^{2+} channels and not the fast Na^+ channel in cultured embryonic heart cells (Bernard & Couraud, 1979). Thus, stimulation of calcium uptake seems to be directly linked to the effect of scorpion toxin on sodium channels.

In the present studies, stimulation of Ca^{2+} uptake in the first 10 min in chick embryonic heart cells was related to scorpion toxin concentration and used to study the relative activities of several scorpion toxins. In each experiment a dose–response curve with AaH II was introduced as a reference. The $K_{0.5}$ values (concentration of toxin giving half-maximum stimulation) are given in Table I and compared to lethality of the toxins in the mouse (LD_{50}). The maximum effect was the same for all scorpion toxins tested except for Css II which was inactive.

When heart cells were preincubated in a medium containing either 5 or 145 mM K^+, the dose–response curves for AaH II on the stimulation of $^{45}Ca^{2+}$ uptake gave $K_{0.5}$ values of 3 and 40 nM, respectively (Figure 1). This result is in good agreement with the hypothesis that scorpion toxin binding to heart cells is a membrane potential dependent phenomenon and that its affinity is higher for the polarized (K^+ = 5 mM) than for the depolarized (K^+ = 145 mM) membrane. The factor between the two affinities was between 10 and 30. Similar results were obtained with AaH I (Figure 1).

Effect of Veratridine on Calcium Uptake. Veratridine, an activator of the sodium channel in nerve (Ulbricht, 1969), was shown to act cooperatively with scorpion venom on the sodium flux in cultured neuroblastoma cells (Catterall, 1975). Calcium uptake by chick embryonic heart cells was stimulated by veratridine: the $K_{0.5}$ values were 3.3 and 4 μM after preincubation in 5 and 145 mM K^+ medium, respectively (Figure 1). Experiments carried out without preincubation gave very similar results ($K_{0.5}$ between 3 and 10 μM). It thus appears that the binding of veratridine to heart cells membrane

Table I: Effects of Different Scorpion Toxins on Calcium Uptake by Heart Cells in Culture and on Mouse Lethality[a]

toxin	immunologic group	$K_{0.5}$ heart cells (nM)	LD_{50} mouse (μg/kg)
AaH I	1	10 000	17
AaH III	1	10 000	23
Amm III	1	1 500	53
AaH II	2	20	9
Lqq V	2	50	25
Amm V	2	50	17
Bot III	2	600	21
Bot XI	2	1 500	810
Lqq IV	3	4 000	70
Bot I	4	500	91
Bot II	4	600	144
Bot VIII	4	600	211
Bot IV plus Bot V	4	400	500
Bot IX	4	200	34
Css II	5	inactive at 10 000	25

[a] $K_{0.5}$ is the concentration of toxin giving half-maximum stimulation of $^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake was measured in 10 min at 35 °C in the standard incubation medium containing 0.5 mM ouabain without preincubation.

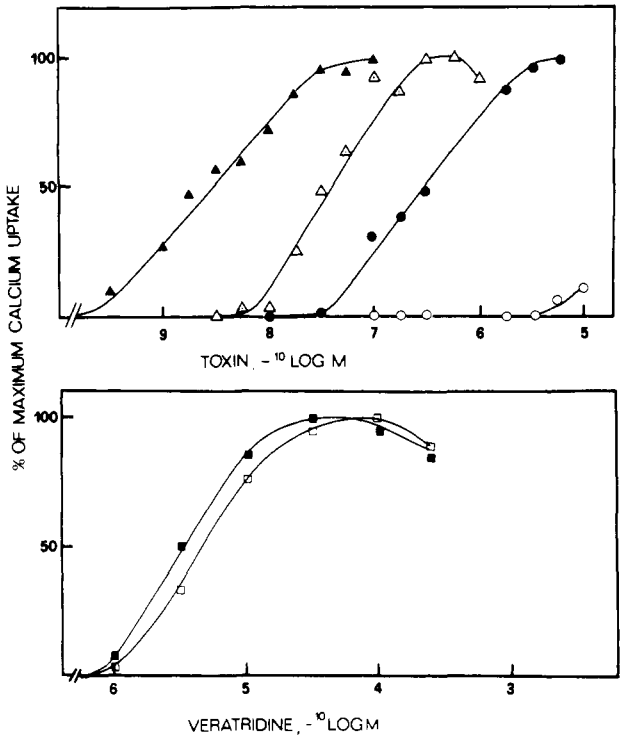

FIGURE 1: Effect of membrane potential on scorpion toxin and veratridine-stimulated calcium uptake. Heart cells were preincubated for 20 min in the choline medium containing either 140 mM choline and 5 mM K^+ (▲, ●, and ■) or 0 mM choline and 145 mM K^+ (Δ, ○, and □) and increasing concentrations of AaH II (▲ and Δ), AaH I (● and ○) or veratridine (■ and □) and then incubated for 4 min in the standard incubation medium containing the toxins at the same concentrations and 0.5 mM ouabain.

does not depend on the membrane potential.

Cooperative Effects of Scorpion Toxins and Veratridine on Calcium Uptake. When dose–response studies were carried out with AaH II in the presence of increasing concentrations of veratridine, the $K_{0.5}$ for scorpion toxin was progressively displaced toward the lower values: $K_{0.5}$ values were 20, 8, 5, 2.5, 1.8, and 0.7 nM for 0, 0.1, 0.25, 0.5, 1.0, and 3.0 μM veratridine, respectively (Figure 2a). Fairly symmetrical results were obtained when dose–response assays for veratridine

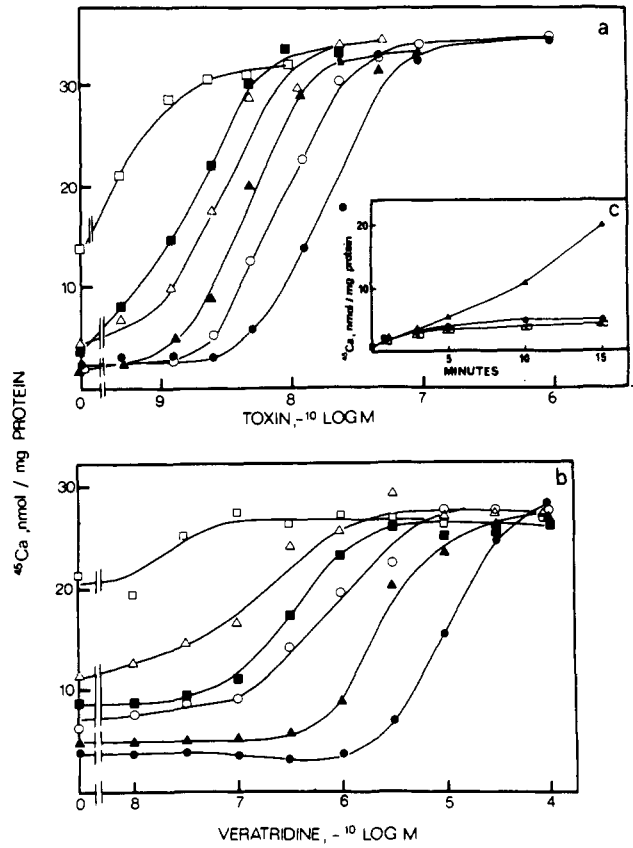

FIGURE 2: Apparent positive cooperativity between veratridine and scorpion toxin. (a) Effect of veratridine on the dose–response curve for AaH II. $^{45}Ca^{2+}$ uptake was measured after 10 min in the standard incubation medium containing 0.5 mM ouabain, increasing concentrations of AaH II, and several different fixed concentrations of veratridine: none (●); 0.1 (○); 0.25 (▲); 0.5 (Δ); 1 (■); 3 μM (□). (b) Effect of AaH II on the dose–response curve for veratridine. $^{45}Ca^{2+}$ uptake was measured after 10 min in the standard incubation medium containing 0.5 mM ouabain, increasing concentrations of veratridine, and several different fixed concentrations of AaH II: none (●); 0.5 (▲); 2.5 (○); 5 (■); 10 (Δ); 100 nM (□). (c) Effect of veratridine and AaH II on ^{45}Ca uptake kinetics. ^{45}Ca uptake was measured after the indicated time in the standard incubation medium containing 0.5 mM ouabain and no effector (□), 2 nM AaH II (●), 1 μM veratridine (Δ), or 2 nM AaH II plus 1 μM veratridine (▲).

were done in the presence of increasing concentrations of AaH II: $K_{0.5}$ values for veratridine were 10, 2.5, 0.65, 0.3, and 0.16 μM in the presence of 0, 0.5, 2.5, 5, and 10 nM AaH II, respectively (Figure 2b). In both types of experiments, veratridine or scorpion toxin did not enhance the maximum calcium uptake. An example of the kinetics of this cooperative effect is shown in Figure 2c; when two subliminal concentrations of AaH II and veratridine were added together, stimulation of $^{45}Ca^{2+}$ uptake was observed.

The displacement of the $K_{0.5}$ for AaH II by veratridine was also obtained when a preincubation in the choline medium containing AaH II, veratridine and 5 mM K^+ was done (not illustrated). Moreover, a similar effect was obtained with AaH I.

Measurement of [^{125}I]AaH II Binding. By use of increasing concentrations of [^{125}I]AaH II, binding to chick embryonic heart cells was a saturable phenomenon although the percentage of specific vs. total binding was low (Figure 3). Fluctuations were important and, consequently, the dissociation constant (K_D*) and the binding capacity were difficult to measure accurately by this method. Thus, saturation curves were obtained by incubation of [^{125}I]AaH II in the presence of increasing concentrations of native AaH II (Figure 4a). A

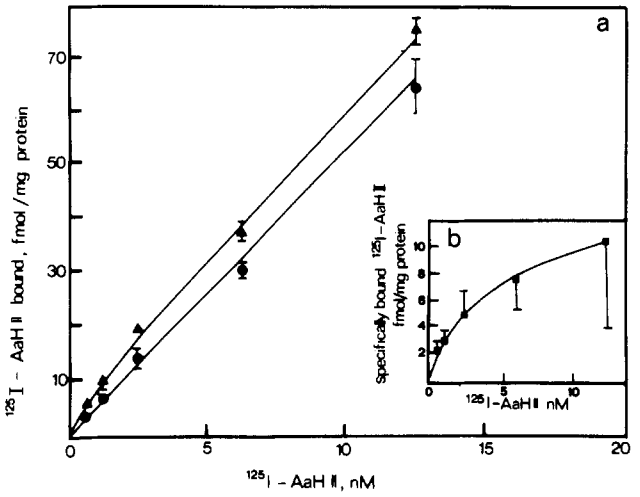

FIGURE 3: Binding of [^{125}I]AaH II to embryonic heart cells. (a) Total binding; heart cells were incubated for 30 min at 35°C in the choline medium containing increasing concentrations of [^{125}I]AaH II in the absence (▲) or in the presence (●) of 1 μM native AaH II. (b) Specific binding; the difference between the two curves represents the specific binding (■).

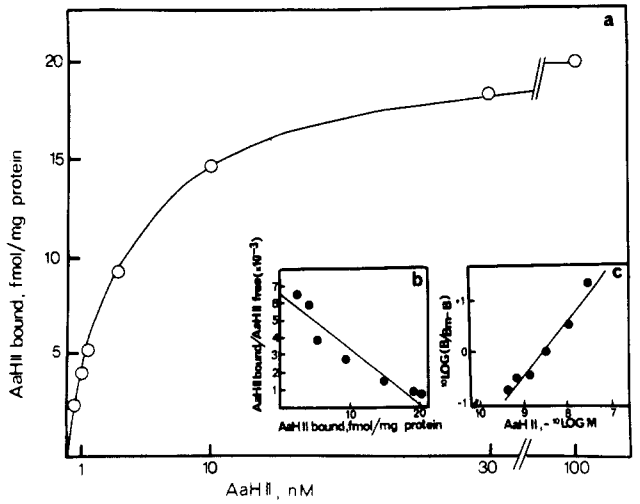

FIGURE 4: Binding of [^{125}I]AaH II to embryonic heart cells. (a) Specific binding; cells were incubated for 30 min at 35 °C in the choline medium containing 0.2 nM [^{125}I]AaH II and increasing concentrations of unlabeled AaH II. Specific binding was calculated by subtracting nonspecific binding (i.e., binding in the presence of 1 μM native AaH II). (b) Scatchard plot calculated from data of (a). The coefficient r^2 for the regression curve was 0.92. (c) Hill plot calculated from data of (a). The coefficient r^2 for the regression curve was 0.97.

Scatchard plot of the data revealed a single class of noninteracting sites (Figure 4b) with a dissociation constant (K_D) of 2.5 nM and a binding capacity (R_T) of 20 fmol/mg protein. The Hill number was 1.08 (Figure 4c). From independent experiments, K_D was between 1 and 3 nM and R_T was between 10 and 20 fmol/mg protein. All these experiments were done in the choline medium so that no depolarization was induced by addition of scorpion toxin. There was no specific binding on cardiac fibroblasts.

The time course of [^{125}I]AaH II binding to heart cells is shown in Figure 5. This interaction can be analyzed as a pseudo-first-order reaction by the equation

$$\ln [B_{eq}/(B_{eq} - B)] = (Lk_1 + k_{-1})t$$

in which B_{eq} is the concentration of bound ligand at equilibrium, B is the concentration of the bound ligand at a given time t, L is the concentration of ligand, k_1 is the rate constant of association, and k_{-1} is the rate constant of dissociation.

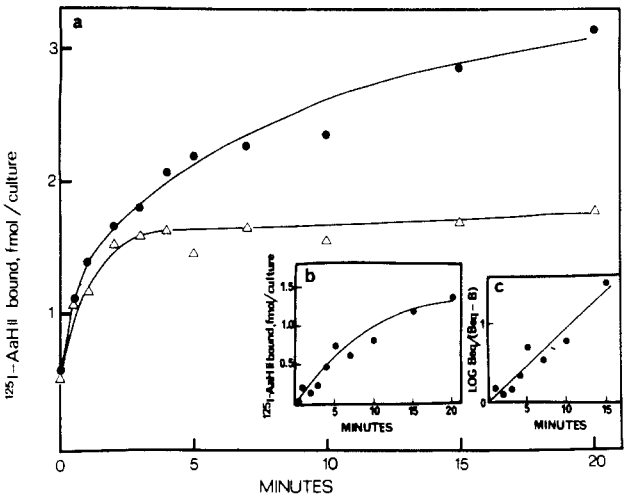

FIGURE 5: Time course of [^{125}I]AaH II binding to embryonic heart cells. (a) Total binding; 0.8 nM [^{125}I]AaH II was incubated at 35 °C with heart cells in the absence (●) or in the presence (△) of 1 μM unlabeled AaH II for the indicated time in the choline medium. (b) Time course of the specific binding. (c) The kinetics of specific binding was linearized according to the equation of a pseudo first order as explained in the text.

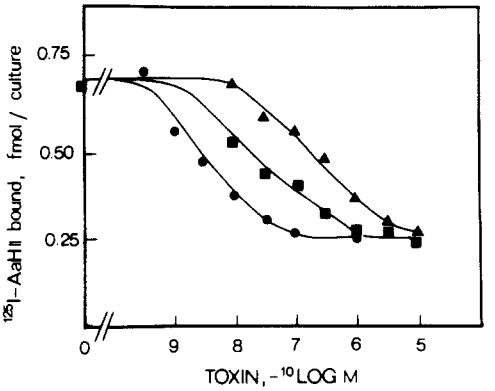

FIGURE 6: Displacement of [^{125}I]AaH II bound to heart cells by native AaH II (●), Bot I (■), and AaH I (▲). Cells were incubated for 30 min at 35 °C in the choline medium containing 0.2 nM [^{125}I]AaH II and the indicated concentrations of native scorpion toxins.

When ln $[B_{eq}/(B_{eq} - B)]$ was plotted as a function of time (Figure 5c), a straight line with a slope value of $(0.8 \times 10^{-9})k_1 + k_{-1} = 1.57 \times 10^{-3}$ s^{-1} was obtained.

From the value of $K_D = 2.5 \times 10^{-9}$ M obtained by equilibrium experiments, we calculated $k_1 = 5 \times 10^5$ M^{-1} s^{-1}, $k_{-1} = 1.25 \times 10^{-3}$ s^{-1}, and a $t_{1/2}$ of 550 s (9 min). Due to the importance of nonspecific binding, this result was difficult to verify by dissociation experiments.

Displacement of [^{125}I]AaH II by Other Scorpion Toxins. [^{125}I]AaH II was displaced by increasing concentrations of native toxins, AaH II, Bot I, and AaH I; the $K_{0.5}$ values (i.e., the concentrations of native toxin which gave half-maximum displacement) were, respectively, 2, 50, and 400 nM (Figure 6). Since concentrations of [^{125}I]AaH II (0.2 nM) were low compared to the dissociation constant of [^{125}I]AaH II (2 nM), $K_{0.5}$ values were very near K_D values.

Effect of Sodium and Potassium on [^{125}I]AaH II Binding. Substitution in the choline medium of choline by sodium induced a progressive decrease of the specific binding of [^{125}I]AaH II without affecting the nonspecific binding. Suppression of toxin binding was not complete at 140 mM sodium (Figure 7). Because of the important nonspecific binding, we could not analyze this effect and reveal if it was related to a change in toxin affinity or in membrane capacity.

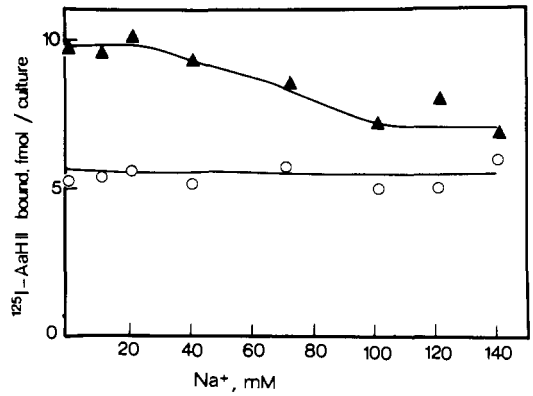

FIGURE 7: Effect of sodium on [^{125}I]AaH II binding to embryonic heart cells. Heart cells were incubated for 30 min at 35 °C in the choline medium in which choline was replaced by Na^+ so that [choline] plus [Na^+] was 140 mM and with 4 nM [^{125}I]AaH II in the presence (○) or the absence (▲) of 1 μM unlabeled AaH II.

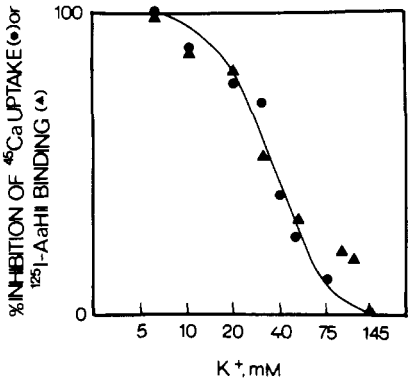

FIGURE 8: Effect of potassium on [^{125}I]AaH II binding and AaH II stimulated calcium uptake. [^{125}I]AaH II binding: heart cells were incubated for 30 min at 35 °C in the choline medium where choline was substituted by K^+ so that [choline] plus [K^+] was 145 mM with 4 nM [^{125}I]AaH II in the presence or the absence of 1 μM unlabeled AaH II. The results are expressed as the percent of maximum specific binding obtained when K^+ was 5 mM (▲). Effect on calcium uptake: heart cells were preincubated for 20 min at 35 °C in the same medium as for [^{125}I]AaH II binding but containing 10 nM unlabeled AaH II and then incubated for 4 min in the standard incubation medium containing 0.5 mM ouabain and 10 nM AaH II. Results are expressed as the percent of maximum AaH II stimulated calcium uptake (●).

Substitution of choline by potassium also inhibited [^{125}I]-AaH II specific binding. The inhibition was complete at 145 mM K^+ and the half-maximum effect was obtained for a concentration of 30–40 mM (Figure 8). The inhibition of toxin binding correlated well with the inhibition of toxin-stimulated calcium uptake by K^+ (Figure 8).

Effect of Veratridine and Tetrodotoxin on [^{125}I]AaH II Binding. Addition of 1 μM tetrodotoxin in the incubation medium containing 140 mM Na^+ stimulated the binding of [^{125}I]AaH II (Figure 9). The K_D calculated in these conditions was 3 nM. On the contrary, in the choline medium the effect of tetrodotoxin on the scorpion toxin binding was very low (not illustrated). Addition of 100 μM veratridine in the sodium medium (Na^+ = 140 mM) completely abolished [^{125}I]AaH II binding (Figure 9) whereas it had no effect in the choline medium.

Discussion

The scorpion toxins used in these investigations were purified from the venoms of five different scorpions, three of them from North Africa (AaH, Amm, and Bot), one from East Africa (Lqq), and one from Mexico (Css). Measurement of $^{45}Ca^{2+}$ uptake by chick embryo heart cells in culture allowed us to

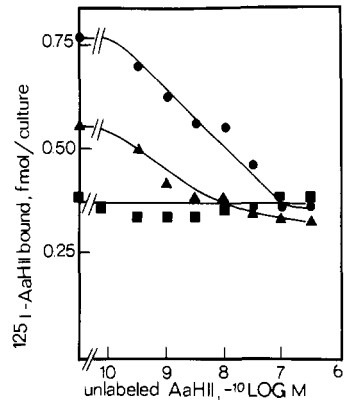

FIGURE 9: Effect of tetrodotoxin and veratridine on [^{125}I]AaH II binding to embryonic heart cells. Heart cells were incubated for 30 min at 35 °C in a medium containing 140 mM Na^+, 0.5 nM [^{125}I]AaH II, and increasing concentrations of native AaH II in the absence of any other effector (▲) and in the presence of either 1 μM tetrodotoxin (●) or 100 μM veratridine (■).

test the activities of 15 scorpion toxins. Addition of 0.5 mM ouabain at the beginning of the incubation induced a very rapid (less than 3 min) and almost complete depolarization of the cells after which the membrane potential remained stable. This stabilization of the potential accounts for the low fluctuations observed in the results of toxin affinities that are very close to those obtained after a preincubation step in a depolarizing medium. Scorpion toxins have been classified according to their antigenic properties in five groups showing no cross antigenicity (P. Delori, unpublished experiments). Moreover, toxins in each immunologic group show important analogies of sequence. Toxins from group 1 have the same toxicity as toxins from group 2 in the mouse, whereas they are 500 times less active in chick embryonic heart cells in culture. This difference may be species specific. In group 2, Bot XI is less active than the other toxins of the group both in the mouse and in the chick heart cells. In this toxin, valine was found at position 67 instead of lysine in the other toxins of the group (Sampieri & Habersetzer-Rochat, 1978). Css II is completely inactive on calcium uptake and does not displace [^{125}I]AaH II bound to heart cells whereas it is very toxic to the mouse. Two hypotheses may explain these results: the receptor for Css II is different from that for AaH II and other toxins from African scorpions or the affinity of Css II for AaH II receptor is very low in heart cells. Very similar results obtained in mouse neuroblastoma cells (unpublished results) and in rat brain synaptosomes (Jover et al., 1980) favor the first explanation.

We confirmed that veratridine stimulates $^{45}Ca^{2+}$ uptake by embryonic heart cells as previously shown by Fosset et al. (1977), and we showed that veratridine affinity was not dependent on membrane potential. Three possible explanations could account for the apparent cooperative effect of scorpion toxin and veratridine on $^{45}Ca^{2+}$ uptake. First, an effect on membrane potential must be considered: the addition of ouabain induces a very rapid and complete depolarization of the membrane. In fact, electrophysiological experiments have shown that veratridine did not noticeably modify the rate and the kinetics of depolarization (P. Bernard, personal communication). It is thus unlikely that veratridine acts through modification of membrane potential. Another possibility is that veratridine could induce a decrease of scorpion toxin receptor dependence upon membrane depolarization as was demonstrated in rat brain synaptosomes (Ray et al., 1978; Jover et al., 1980). This possibility cannot be completely excluded, but the fact that veratridine shifted the dose–response

curves of the scorpion toxin even when measured in polarized conditions (preincubation with 5 mM K^+) is not in agreement with this hypothesis. The last explanation is the existence of an apparent cooperativity on $^{45}Ca^{2+}$ uptake without cooperativity on neurotoxins binding because veratridine did not modify scorpion toxin binding in the Na^+-free choline medium.

One result of this work was to show that, as in neuroblastoma cells (Catterall et al., 1976) and in rat brain synaptosomes (Ray et al., 1978; Jover et al., 1980), the binding affinity of scorpion toxin depends on the membrane potential of chick embryo heart cells. This is proved by the effect of extracellular K^+ concentration on scorpion toxin binding and on stimulation of $^{45}Ca^{2+}$ uptake by scorpion toxin. Veratridine (100 μM) completely depolarized heart cells in a medium containing 140 mM sodium and completely abolished scorpion toxin binding. Similarly, in a medium containing 140 mM Na^+ and 1.8 mM Ca^{2+}, i.e., in a medium in which scorpion toxin depolarized the cell by increasing the action potential duration, scorpion toxin binding was not detectable. The effect of sodium on toxin binding is probably due to a modification of the membrane potential induced by the addition of scorpion toxin; this effect is antagonized by tetrodotoxin which blocks the scorpion toxin effect. A similar effect was shown in rat brain synaptosomes (Jover et al., 1980).

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