MOLECULAR PHARMACOLOGY, 26:70–74

The Interaction of Sea Anemone and Scorpion Neurotoxins with Tetrodotoxin-Resistant Na^+ Channels in Rat Myoblasts

A Comparison with Na^+ Channels in Other Excitable and Non-excitable Cells

CHRISTIAN FRELIN, PAUL VIGNE, HUGUES SCHWEITZ, AND MICHEL LAZDUNSKI

Centre de Biochimie du Centre National de la Recherche Scientifique, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France

Received October 18, 1983; Accepted March 27, 1984

SUMMARY

The properties of interactions of several polypeptide neurotoxins isolated from sea anemone and scorpion venom with Na^+ channels of rat myoblasts, chick myotubes, neuroblastoma cells, and fibroblasts have been compared. Tetrodotoxin (TTX)-resistant Na^+ channels appear to be much more sensitive to the action of sea anemone toxins than TTX-sensitive Na^+ channels but have the same affinity for scorpion neurotoxins. This conclusion holds both for Na^+ channels that can be activated electrically and for silent forms of Na^+ channels. The sensitivity to sea anemone toxins of the different types of Na^+ channels that have been studied suggests the existence of multiple forms of Na^+ channels.

INTRODUCTION

The pharmacological properties of Na^+ channels have been studied in a variety of excitable and non-excitable cells. One class of these cells (neuroblastoma cells, chick cardiac and skeletal muscle cells, and various fibroblastic cell lines) possess functional Na^+ channels that have a high-affinity for TTX[1] (K_d near 1 nM), the classical blocker of the Na^+ channel (1–7). A second class of cells has Na^+ channels that are much less sensitive to inhibition by TTX (K_d between 0.1 and 1 μM). It includes C_9 cells, [a cell line isolated from a bladder metastasis of an *N*-ethyl-*N*-nitrosourea induced rat tumor (8)], rat cardiac cells, and rat skeletal myotubes (7–11).

TTX is only one of the numerous toxins specific for the Na^+ channel. There are four other classes of toxins which are also specific for this channel (1, 12). One well-known class of these toxins includes polypeptide neurotoxins isolated from sea anemone nematocysts and from scorpion venom. These toxic polypeptides specifically slow the inactivation of Na^+ channels in a variety of excitable cells (13, 14). They have been used extensively to analyze the molecular properties of Na^+ channels either in binding studies after having been radiolabeled (15–18) or in $^{22}Na^+$ flux studies in synergy with alkaloid toxins such as veratridine and batrachotoxin (1–6, 8, 10).

The purpose of this paper is (a) to analyze the interactions of several polypeptide neurotoxins with the TTX-resistant Na^+ channel of rat skeletal muscle myoblasts, which proved to be particularly sensitive to their action, and to compare them with the interactions of polypeptide neurotoxins with TTX-sensitive Na^+ channels of chick myotubes, and (b) to try to classify the different types of Na^+ channels that have been found in excitable and non-excitable cells according to their sensitivity to polypeptide neurotoxins.

EXPERIMENTAL PROCEDURES

Materials

Veratridine and ouabain were obtained from the Sigma Chemical Company. TTX was from Sankyo Chemical Company. Saxitoxin was obtained from the United States Food and Drug Administration (Washington, D. C.). Sea anemone toxins I, II, III, and V from *Anemonia sulcata* (AS_1, AS_2, AS_3, and AS_5); toxins I and II from *Anthopleura xanthogrammica* (AX_1 and AX_2); and toxins from *Stoichactis giganteus* and *Actinodendron plumosum* were prepared as previously described (19). Toxin II (Aa_2) from *Androctonus australis* was purified as described by Miranda *et al.* (20).

Toxin γ from *Tityus serrulatus* venom was kindly given to us by Dr. J. R. Giglio. Toxin M-10 from *Buthus eupeus* was a kind gift from Dr. V. Grishin.

$^{22}NaCl$ was purchased from the Commissariat à l'Energie Atomique (Saclay, France). Ham's F-12 culture medium, fetal calf serum, and horse serum were purchased from GIBCO (Grand Island, N. Y.).

This work was supported by the Centre National de la Recherche Scientifique, the Commissariat à l'Energie Atomique and the Institut National de la Santé et de la Recherche Médicale.

[1] The abbreviations used are: TTX, tetrodotoxin; AS_2, *Anemonia sulcata* toxin II; AS_5, *Anemonia sulcata* toxin V; AX_1, *Anthopleura xanthogrammica* toxin I; AX_2, *Anthopleura xanthogrammica* toxin II; Aa_2, *Androctonus australis* toxin II.

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Cell Cultures

Primary cultures of skeletal muscle myoblasts were prepared from thigh muscle of newborn Wistar rats. Myoblasts were dissociated from the tissue as previously described (11) and seeded at a density of 4.10^5 cells/cm^2 in gelatin-coated 24-well Costar tissue culture clusters. The culture medium was Ham's F-12 medium supplemented with 10% fetal calf serum, 10% horse serum, penicillin (50 units/ml), and streptomycin (200 μg/ml). Under these culture conditions, myoblasts proliferate and the culture reaches confluency within a few days without any sign of morphological differentiation of the cells into myotubes. Experiments carried out in this study were conducted with 4-day-old cultures. Primary cultures of myogenic cells isolated from the breast muscle of 11- to 13-day-old chick embryos were prepared as previously described (3).

$^{22}Na^+$ uptake experiments: standard flux conditions. Cells were preincubated in a Na^+-free medium (140 mM choline chloride/5 mM KCl/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/5 mM glucose/25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid-Tris, pH 7.4) supplemented with 0.1 mM veratridine and polypeptide neurotoxin. After 15 min of incubation, which was sufficient to allow full equilibration of the toxins with the cells, cells were shifted to a medium containing 10 mM NaCl and 130 mM choline chloride and which was supplemented with neurotoxins, 1 mM ouabain, and $^{22}Na^+$ (1 μCi/ml). After 1 min of uptake at 37°, cells were quickly rinsed with a Na^+- and K^+-free medium (145 mM choline chloride buffered with 25 mM Tris-HCl, pH 7.4) and dissolved into 2 ml of 0.1 N NaOH. The radioactivity that was incorporated by the cells was determined with a gamma counter.

The rate of toxin-activated $^{22}Na^+$ uptake was obtained by subtracting the rate of $^{22}Na^+$ uptake measured in the absence of polypeptide neurotoxin from the rate of $^{22}Na^+$ uptake measured in the presence of toxin. Toxin-activated rates of $^{22}Na^+$ uptake are 3- to 5-fold higher than the basal rate of $^{22}Na^+$ uptake, depending on the cell type. Dose-response curves were fitted by means of the procedure described by Jacques *et al.* (6), which gave half-maximal values for toxin action ($K_{0.5}$) and the rate of $^{22}Na^+$ uptake that can be activated by a saturating concentration of toxin. In all figures the initial rate of $^{22}Na^+$ uptake at a given concentration of toxin is expressed relative to the maximal rate produced by a saturating concentration of the toxin.

In the protocol used, the (Na^+,K^+)ATPase is inhibited by ouabain to prevent the efflux of $^{22}Na^+$ that has entered the cells through the toxin-activated Na^+ channels. Since the (Na^+,K^+)ATPase of rat peripheral tissues is relatively insensitive to the inhibition by ouabain, it was important to determine which concentration of ouabain was required to block the activity of the Na^+ pump. The dose-response curve for ouabain inhibition of the initial rate of $^{86}Rb^+$ uptake catalyzed by the (Na^+,K^+)ATPase was determined as previously described for chick skeletal muscle cells (21). The half-maximal effect for ouabain inhibition was observed at 30 μM. Therefore, 1 mM ouabain was used in the routine procedure, this being large enough to block more than 95% of the activity of the Na^+ pump.

$^{22}Na^+$ uptake experiments under polarized or depolarized membrane conditions. In experiments requiring the equilibration of cells in the presence of a toxin under depolarized membrane conditions, cells were first equilibrated with the toxin in a Na^+-free, 145 mM KCl medium. After 15 min of equilibration, cells were quickly rinsed with a Na^+- and K^+-free medium to remove external K^+ and unbound toxin and were further incubated for 1 min in a 10 mM Na^+/5 mM K^+ medium supplemented with 1 mM ouabain, $^{22}NaCl$ (1 μCi/ml), and 0.1 mM veratridine. Cells were then handled as described above for standard flux conditions. Depolarizing membrane conditions were also obtained in cultures of rat myoblasts by equilibrating muscle cells with the toxin in the presence of 0.1 mM veratridine in a 140 mM Na^+ medium. Initial rates of $^{22}Na^+$ uptake were then determined as described above. To study the effect of membrane polarization, cells were equilibrated with the toxin in a Na^+-free, 5 mM KCl medium for 15 min and then handled as described above.

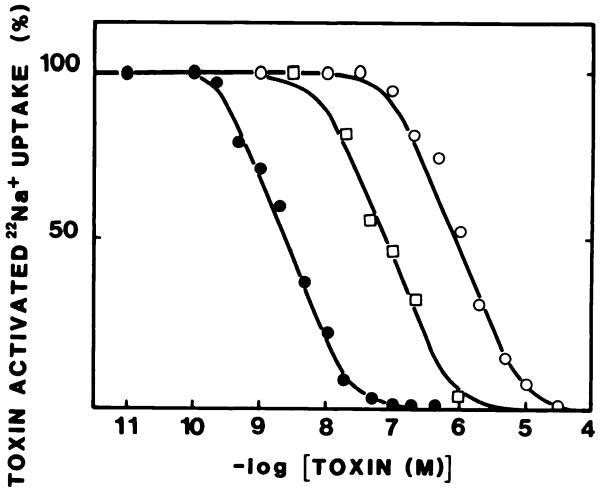

FIG. 1. *Inhibition of the rates of toxin-activated $^{22}Na^+$ uptake by TTX and saxitoxin in cultures of rat myoblasts and of chick myotubes*

Dose-response curves for TTX (●, ○) or saxitoxin (□) inhibition of the initial rate of $^{22}Na^+$ uptake stimulated by 0.1 μM AS_2 and 0.1 mM veratridine [rat myoblasts (○, □)] or by 10 μM AS_2 and 0.1 mM veratridine [chick myotubes (●)]. Standard flux conditions were used. Each point represents the mean of two determinations.

RESULTS

Rat myoblasts used in this study are electrically excitable cells that generate action potentials[2] which are blocked with 10 μM TTX. At concentrations below 10 nM, TTX did not block the electrical activity of rat myoblasts.[2] Mixtures of veratridine and AS_2 stabilize an open conformation of the Na^+ channel and, as a consequence, increase the membrane permeability to Na^+ ions in cultures of excitable cells (1–11). The dose-response curves for TTX inhibition of the initial rate of $^{22}Na^+$ uptake through toxin-activated Na^+ channels of chick myotubes and rat myoblasts are presented in Fig. 1. Half-maximal inhibition of toxin-activated $^{22}Na^+$ uptake occurred with about 2 μM TTX in rat myoblasts, compared with about 3 nM for chick myotubes (Fig. 1). Results of binding experiments with a tritiated derivative of TTX were in agreement with these observations, showing high-affinity binding sites for TTX (K_d = 1 nM) in homogenates prepared from cultures of chick myotubes (3, 7), but not in rat myoblasts (11). The toxin-activated $^{22}Na^+$ influx in rat myoblasts is also inhibited by saxitoxin, with a $K_{0.5}$ value of 0.1 μM (Fig. 1). In cultures of rat myoblasts, saxitoxin appears to be 10 times more potent than TTX for inhibiting Na^+ channels, whereas in cultures of chick myotubes, TTX and saxitoxin have similar potencies (22).

Inset A of Fig. 2 shows the time course of equilibration of AS_2 with its receptor site on the Na^+ channel of rat myoblasts. It indicates that a 15-min incubation of the cells in a Na^+-free medium is sufficient to produce maximal rates of toxin-activated $^{22}Na^+$ uptake. Equilibrium is also reached within 15 min for all of the other polypeptide toxins described later in this paper. The main panel of Fig. 2 compares the dose-response curves for

[2] C. Frelin, H. Vijverberg, G. Romey, P. Vigne, and M. Lazdunski, in preparation.

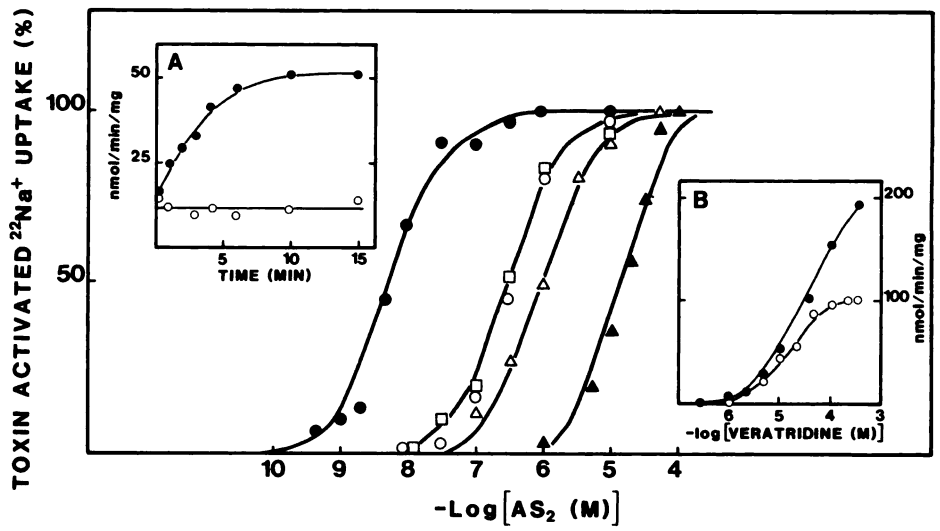

FIG. 2. *Action of AS_2 on Na^+ channels of rat myoblasts and chick myotubes*

Inset A. The time course of AS_2 equilibration with the Na^+ channel of rat myoblasts. Cells were equilibrated in the presence of 10 nM AS_2 in a Na^+-free medium for the times indicated and then shifted to a medium that contained 10 mM Na^+, 10 nM AS_2, 0.1 mM veratridine, and 1 mM ouabain in the presence (○) or in the absence (●) of 10 μM TTX. Time of uptake was 1 min.

Main panel. Dose-response curve for AS_2 action on the initial rate of $^{22}Na^+$ uptake by cultures of rat myoblasts (●, ○, □) or of chick myotubes (▲, △). Experiments were carried out in the presence of 0.1 mM veratridine under normal (●, △) or depolarized membrane conditions (○, □, ▲). Depolarizing membrane conditions were obtained in cultures of rat myoblasts either by equilibrating muscle cells with AS_2 in a 145 mM K^+ medium (○) or with AS_2 and veratridine in a 140 mM Na^+ medium (□). Maximal rates of toxin-activated $^{22}Na^+$ uptake were 105 nmoles/min/mg of protein and 190 nmoles/min/mg of protein for rat myoblasts incubated under normal and depolarized membrane conditions, respectively, and 60 and 85 nmoles/min/mg of protein for chick myotubes.

Inset B. Dose-response curves for veratridine action on the initial rate of TTX-sensitive $^{22}Na^+$ uptake by rat myoblasts. Experiments were performed in the presence of 0.1 μM AS_2 under normal (○) or depolarized membrane conditions (●).

AS_2 activation of the initial rate of $^{22}Na^+$ uptake in cultures of rat myoblasts and chick myotubes. Two conditions have been studied: (a) equilibration of the toxin with the cells under normal conditions of membrane polarization (5 mM K^+) and (b) equilibration of the toxin with depolarized cells (145 mM K^+). The half-maximal effect for AS_2 action was observed at 5 nM in cultures of rat myoblasts. Depolarization with 145 mM K^+ decreased the effect of AS_2 on the Na^+ channel and increased the concentration for half-maximal effect to 300 nM. Membrane depolarization can also be obtained by first equilibrating rat myoblasts for 15 min with 0.1 mM veratridine in high external Na^+ conditions (140 mM Na^+); in this case, the half-maximal effect for AS_2 action was also observed at 300 nM (Fig. 2). In cultures of chick myotubes, the $K_{0.5}$ value for AS_2 action was 1 μM under conditions in which the membrane was polarized. Depolarizing membrane conditions increased this to 20 μM (Fig. 2). In contrast to the results obtained with AS_2, we found that the $K_{0.5}$ value (15 μM) for veratridine activation of $^{22}Na^+$ uptake by rat myoblasts incubated in the presence of 0.1 μM AS_2 was not modified by changing the state of membrane polarization (Fig. 2, *inset B*).

Figure 3A presents the data obtained with the three sea anemone toxins which have been previously described to be the most active on the Na^+ channel of neuroblastoma cells (19). The $K_{0.5}$ value for AS_5 action was 2 nM on rat myoblasts and 500 nM on chick myotubes. Depolarizing membrane conditions shift these $K_{0.5}$ values to 90 nM and 10 μM, respectively.

Figure 3B presents the dose-response curves for the action of toxins I and II from another sea anemone species, *Anthopleura xanthogrammica*, on rat myoblasts. $K_{0.5}$ values for AX_1 and AX_2 were 3 nM and 0.15 nM, respectively. Depolarization increases these values to 800 nM and 60 nM, respectively, which are still lower than the $K_{0.5}$ values for chick myotubes measured under normal conditions (1 μM and 0.3 μM, respectively) (Table 1).

Toxins from other sea anemone species, which are known to be more toxic to invertebrates than they are to mammals (12), were also assayed. Toxin I from *Anemonia sulcata* (AS_1) stimulated the rate of $^{22}Na^+$ uptake by cultures of rat myoblasts when in the presence of 1 mM veratridine (instead of 0.1 mM veratridine for the previous toxins). AS_1 caused very little stimulation of $^{22}Na^+$ uptake when tested with 0.1 mM veratridine. The $K_{0.5}$ value for AS_1 was about 3 μM. Neurotoxins isolated from other sea anemone species (*Stoichactis giganteus* and *Actinodendron plumosum*) and toxin III from *A. sulcata* at concentrations up to 10 μM had no influence on $^{22}Na^+$ uptake by rat myoblasts in the presence of 1 mM veratridine. These latter toxins had no action on the TTX-sensitive Na^+ channel of mouse neuroblastoma cells (19).

Dose-response curves for the action of neurotoxin II isolated from the venom of the scorpion *Androctonus australis* (Aa_2) on the rates of $^{22}Na^+$ uptake by cultures of rat myoblasts and chick myotubes are presented in Fig. 4. The $K_{0.5}$ value for Aa_2 action was observed at 0.2 nM in cultures of rat myoblasts. This value was shifted to 80 nM by depolarizing membrane conditions. In cul-

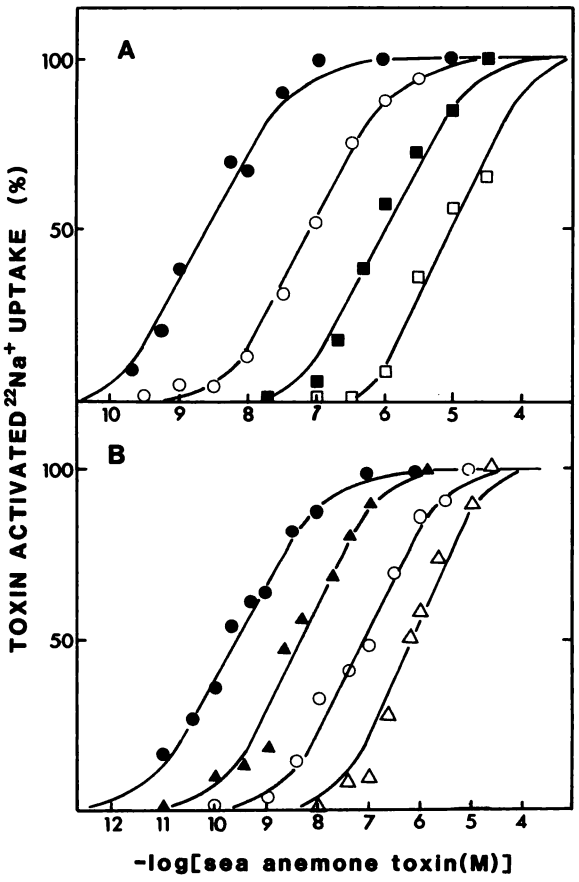

FIG. 3. *Action of AS_5, AX_1, and AX_2 on Na^+ channels of rat myoblasts and chick myotubes*

A. Dose-response curves for AS_5 action on the initial rate of $^{22}Na^+$ uptake by rat myoblasts (●, ○) or chick myotubes (■, □). Experiments were performed in the presence of 0.1 mM veratridine under normal (●, ■) or depolarized membrane conditions (○, □).

B. Dose-response curves for AX_1 (▲, △) and AX_2 (●, ○) action on the initial rate of $^{22}Na^+$ uptake by rat myoblasts. Experiments were performed in the presence of 0.1 mM veratridine under normal (●, ▲) or depolarized membrane conditions (○, △). Depolarized membrane conditions were obtained by equilibrating cells in a 145 mM K^+ medium.

tures of chick myotubes, these values are 0.7 nM and 200 nM, respectively. Neurotoxins isolated from the venoms of *Buthus eupeus* (toxin M-10, used at concentrations up to 0.1 μM), *Leiurus quinquestriatus* (at concentrations up to 50 nM) and *Tityus serrulatus* (γ toxin used at concentrations up to 50 nM) did not stimulate the rate of $^{22}Na^+$ uptake by cultures of rat myoblasts in the presence or in the absence of 0.1 mM veratridine.

Table 1 summarizes $K_{0.5}$ values obtained for the different polypeptide neurotoxins used in this study for rat myoblasts and chick myotubes and compares them with the corresponding $K_{0.5}$ values obtained for other TTX-resistant cells such as C_9 cells or TTX-sensitive cells such as neuroblastoma cells and CCl_{39} fibroblasts.

The order of affinities of the sea anemone toxins in all systems investigated was $AX_2 > AS_5 > AX_1 \geq AS_2$.

DISCUSSION

There are different classes of Na^+ channels in excitable cells. One of the properties that differentiate these

TABLE 1

Compared properties of interaction of TTX, sea anemone, and scorpion toxins with the Na^+ channel in different cell types

$K_{0.5}$ values were determined from toxin-activated rates of $^{22}Na^+$ uptake measured in the presence of 0.1 mM veratridine.

Toxin	$K_{0.5}$					
	Rat myoblasts		Chick myotubes	C_9	N1E-115	CCl_{39}
	Polarized	Depolarized				
	nM					
TTX	1000	2000	4[a]	300[b]	5[c]	5[e]
AS_2	5	300	1000[a]	38[b]	150[d]	600[e]
AS_5	2	90	500[a]	5[f]	15[d]	100[f]
AX_1	3	800	1000	20[f]	47[d]	400[f]
AX_2	0.15	60	300	2[f]	7[d]	60[f]
Aa_2	0.20	80	1[a]	5[b]	0.20[c]	50[e]

[a] Ref. 3.
[b] Ref. 8.
[c] Ref. 6.
[d] Ref. 19.
[e] Ref. 4.
[f] Unpublished data determined under standard flux conditions.

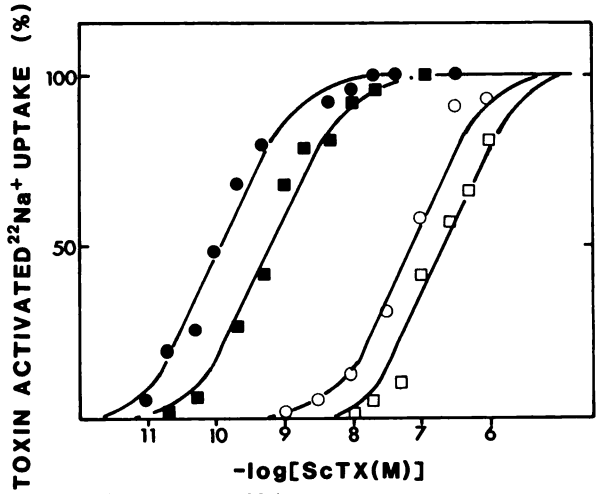

FIG. 4. *Action of Aa_2 on Na^+ channels of rat myoblasts and chick myotubes*

Dose-response curves for Aa_2 action on the initial rate of $^{22}Na^+$ uptake by rat myoblasts (●, ○) or chick myotubes (■, □). Experiments were performed in the presence of 0.1 mM veratridine under normal (■, ●) or depolarized membrane conditions (□, ○). Depolarized membrane conditions were obtained by equilibrating cells in a 145 mM K^+ medium.

classes of Na^+ channel structures is their sensitivity to TTX (7, 10). Rat myoblasts have only Na^+ channels belonging to the low-affinity type for TTX (11), whereas differentiated rat myotubes, like denervated rat muscle (23), have both high- and low-affinity binding sites for TTX (11, 24). For that reason, rat myoblasts (instead of rat myotubes) were chosen to study the interaction of polypeptide neurotoxins with TTX-resistant Na^+ channels. The low-affinity binding site for TTX of the Na^+ channels in rat myoblasts is characterized by a $K_{0.5}$ value of 1 μM (10, 11). This apparent affinity is 1000 times lower than the affinity found for TTX-sensitive Na^+ channels in chick myotubes (3, 22) or in mouse neuroblastoma cells (1, 6). A similar difference is seen with saxitoxin. High-affinity binding sites for saxitoxin cor-

respond to $K_{0.5}$ or K_d values near 1 nM (10), whereas the low-affinity binding sites correspond to $K_{0.5}$ or K_d values near 0.1 μM (Fig. 1).

Polypeptide toxins that are listed in Table 1 and which have been extracted from sea anemone nematocysts or from scorpion venoms are known to slow the inactivation of Na^+ channels in a variety of excitable cells (13, 14). All of these toxins bind to the Na^+ channels of rat myoblasts with a high affinity. Half-maximal concentrations for the effect on $^{22}Na^+$ uptake range from 0.15 to 5 nM (Table 1). The most potent sea anemone toxin (AX_2) is as potent as toxin Aa_2, which is one of the most potent Na^+ channel toxins thus far described.

The affinity of sea anemone toxins for the Na^+ channels of rat myoblasts is strongly influenced by the state of polarization of the membrane as first described by Catterall *et al.* (15) for the scorpion neurotoxins, although all of the toxins are not identical in this respect. Depolarization decreases the activity of AS_2 and AS_5 by a factor of 45–60 and by a factor of 250–400 for AX_1 and AX_2. The scorpion toxin Aa_2 has nearly the same potency as the sea anemone toxin AX_2, and also shows the same dependence on membrane potential.

The main conclusions that come from the results in Table 1 are the following: (a) Na^+ channels that have a low affinity for TTX also are highly sensitive to sea anemone toxins, the highest potencies of these toxins being observed in rat myoblasts. (b) Large differences in sensitivity to TTX and sea anemone toxins among different types of cultured cells, such as rat myoblasts, chick myotubes, and neuroblastoma cells, do not extend to scorpion toxin Aa_2. This result suggests that the change(s) in the structural properties of the Na^+ channel which are responsible for the changes in affinities at both the TTX and sea anemone toxin receptors are not perceptible at the level of the scorpion toxin receptor. (c) As observed with excitable cells, changes in the affinity for TTX are also accompanied by changes in the affinity for sea anemone toxins in electrically inexcitable cells with silent Na^+ channels. (d) Although the TTX sensitivity of Na^+ channels suggests the existence of two categories, TTX-sensitive ($K_{0.5}$ = 1 nM) and TTX-resistant ($K_{0.5}$ = 0.1 to 1 μM) (24), the sensitivity to sea anemone toxins of these Na^+ channels shows that each cell type has a channel with a different affinity for these toxins. This could imply the existence of more than two types of Na^+ channels.

ACKNOWLEDGMENTS

We are grateful to M. T. Ravier, N. Boyer, and M. Valetti for expert technical assistance; to Dr. J. R. Giglio, to Dr. V. Grishin for the gift of scorpion neurotoxins; and to Dr. M. Starzack for a careful reading of the manuscript.

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Send reprint requests to: Dr. Christian Frelin, Centre de Biochimie du Centre National de la Recherche Scientifique, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France.