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Pharmacological Properties of Sodium Channels in Cultured Rat Heart Cells

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SUMMARY

The action of specific neurotoxins on sodium channels in cultured rat heart cells has been studied using ion flux methods under conditions where $^{22}Na^+$ influx is proportional to sodium permeability. Myocardial sodium channels are activated by batrachotoxin and veratridine. Batrachotoxin is a full agonist in this respect, whereas veratridine is a partial agonist. Aconitine and veratridine block the activation of sodium channels by batrachotoxin, consistent with their action as partial agonists at a common receptor site with batrachotoxin. The polypeptides scorpion toxin and sea anemone toxin II enhance persistent activation of sodium channels by alkaloid toxins. The concentration dependence of enhancement by scorpion toxin is hyperbolic with $K_{0.5}$ = 130 nM. In contrast, the enhancement of veratridine action by sea anemone toxin II is biphasic with $K_{0.5}$ values of 4 nM and 38 nM. Sea anemone toxin II increases the fraction of sodium channels activated by the partial agonists veratridine and aconitine and reduces $K_{0.5}$ for sodium channel activation by all three alkaloid toxins. Activation of sodium channels by batrachotoxin is not voltage-dependent, but enhancement of batrachotoxin activation of sodium channels by the polypeptide toxins is highly voltage-dependent. $K_{0.5}$ for sea anemone toxin II is increased 40-fold by depolarization of the cells in 135 mM K^+. Sodium channels activated by alkaloid and polypeptide neurotoxins are inhibited by saxitoxin with $K_{0.5}$ = 50 nM and tetrodotoxin with $K_{0.5}$ = 1 μM. The results show that, relative to neuronal sodium channels, myocardial sodium channels have greatly reduced toxin-binding affinity at the tetrodotoxin/saxitoxin receptor site and altered binding specificity at the polypeptide toxin receptor site. The pharmacological profile of myocardial sodium channels is essentially identical with that of the tetrodotoxin-insensitive sodium channels in denervated and uninnervated rat skeletal muscle. A pharmacological classification of sodium channel subtypes in the rat is proposed.

INTRODUCTION

Sodium channels in nerve, adult muscle, and cultured neuroblastoma cells have three separate neurotoxin receptor sites that have been well-characterized (reviewed in ref. 1). Receptor site 1 binds saxitoxin and tetrodotoxin, which inhibit sodium channel ion transport (1–3). Receptor site 2 binds grayanotoxin and the alkaloids aconitine, veratridine, and batrachotoxin, which alter the voltage-dependence of sodium channel activation and inactivation and cause persistent activation of sodium channels (1). The competitive interaction of these four toxins inferred from ion flux studies (4, 5) has recently been confirmed by direct binding studies with a ^3H-labeled batrachotoxin derivative (6, 7). Receptor site 3 binds the polypeptides scorpion toxin and sea anemone toxin. These toxins slow inactivation of sodium channels and enhance persistent activation of sodium channels by alkaloid toxins acting at receptor site 2 (1). The binding and action of the polypeptide toxins is highly voltage-dependent (1). In addition to these three well-defined sites of neurotoxin action, more recent work shows that certain classes of scorpion toxins (8) and a nonprotein toxin from the dinoflagellate *Ptychodiscus brevis* (9) may act at additional receptor sites on the sodium channel. These toxins provide sensitive probes of the structure, function, and voltage dependence of sodium channels.

Physiological studies reviewed in the accompanying paper (10) indicate that sodium channels in mammalian myocardium have 100-fold lower affinity for the inhibitory toxins tetrodotoxin and saxitoxin than do sodium channels in mammalian nerve and adult muscle. Similarly, sodium channels in mammalian myocardium have 100-fold lower affinity for these two toxins than do sodium channels in chicken or frog heart, the only two nonmammalian vertebrates that have been studied (reviewed in ref. 10). Therefore, it is of interest to examine the action of neurotoxins at the other neurotoxin receptor sites on mammalian myocardial sodium channels. Physiological studies indicate that grayanotoxin and the al-

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kaloid toxins have several effects on mammalian myocardium, including a positive inotropic effect at low concentration, genesis of arrhythmias, and depolarization of myocardial fibers at higher concentrations (11–14). The polypeptide toxins also cause both a positive inotropic effect at low concentrations and arrhythmias at higher concentrations (15–17). Quantitative analysis of the underlying effects on sodium channel properties have not been carried out.

Dissociated cultures of beating heart cells from newborn rats (18) have resting potentials and action potentials similar to those of heart cells *in situ* (19). These cells provide an excellent experimental preparation with which to study myocardial sodium channels by ion flux and neurotoxin binding methods. In this report, we describe ion flux studies of activation and inhibition of sodium channels in cultured rat heart cells by these neurotoxins. Our results show that rat myocardial sodium channels have 100-fold lower affinity for tetrodotoxin and saxitoxin than do sodium channels in nerve and adult muscle and, in addition, have altered toxin binding specificity for polypeptide toxins at receptor site 3. However, the mechanisms of neurotoxin action on these sodium channels are the same as those previously described for tetrodotoxin-sensitive sodium channels in nerve and adult muscle.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were obtained from the following sources: ^{22}NaCl from Amersham (Arlington, Ill.); [4,5-^{3}H] leucine from New England Nuclear Corporation (Boston, Mass.); veratridine from Aldrich Chemical Company (Milwaukee, Wisc.); aconitine from K and K Chemicals (Plainview, N. Y.); scorpion venom (*Leiurus quinquestriatus*), Type II crude trypsin, and ouabain from Sigma Chemical Company (St. Louis, Mo.); tetrodotoxin and calf-skin collagen from Calbiochem (San Diego, Calif.); saxitoxin from the National Institutes of Health (Bethesda, Md.); the Dulbecco-Vogt modification of Eagle's medium, fetal calf serum, and horse serum from Grand Biological Company (Grand Island, N. Y.); and *Anemonia sulcata* sea anemone toxin II from Ferring GmBH (Kiel, West Germany). Batrachotoxin was kindly provided by Dr. John Daly, Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases (Bethesda, Md.). Scorpion toxin was purified as described previously (20).

Cell cultures. Suspensions of single cells were prepared from 2- to 4-day-old rats by the repetitive trypsinization procedure of Harary *et al.* (18). The cell suspension in growth medium [10% (v/v) fetal calf serum, 10% (v/v) heat-inactivated horse serum, 80% Dulbecco-Vogt-modified Eagle's medium, penicillin (50 units/ml), and streptomycin (10 μg/ml)] was preplated in 100-mm Falcon plastic dishes for 2 hr at 36° to allow fibroblasts to attach to the plastic. Unattached cells were removed and the plates were rinsed to remove any loosely attached cells. [4,5-^{3}H]leucine was added to a final concentration of 0.2 μCi/ml and the cells were seeded at a density of 1.5×10^5 cells/cm^2 in collagen-coated multiwell plates (Costar). Cultures were used 3–6 days after preparation.

$^{22}Na^+$ flux measurements. Sodium channels were studied using the $^{22}Na^+$ flux approach initially described by Catterall and Nirenberg (21) as modified in more recent work (5, 20). Cell cultures were incubated in sodium-free standard binding medium (see accompanying paper, ref. 10) at 36° for 2–10 min to allow neurotoxins to bind to their sites of action without altering ionic gradients across the cell membrane. After equilibration with toxins, the standard binding medium was removed by aspiration and the cells were incubated for 15 or 30 sec in assay medium consisting of 4 mM NaCl, 126 mM choline chloride, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 5 mM ouabain, 0.8 mM $MgSO_4$, 5.4 mM KCl, and ^{22}NaCl (1 μCi/ml). Uptake of $^{22}Na^+$ was terminated by washing three times with sodium-free wash medium (see accompanying paper, ref. 10). Cells were suspended in 0.4 M NaOH and radioactivity was determined as described previously (4, 5). Results are presented as nanomoles of $^{22}Na^+$ influx per minute per cell culture. The results from individual cultures within a single experiment were normalized for varying recovery of cells using the recovered counts per minute of [4,5-^{3}H]leucine in each culture.

In some experiments, the effect of membrane potential on the binding and action of neurotoxins was studied. In these experiments, the indicated concentrations of KCl were substituted for choline chloride in the preliminary incubation in standard binding medium. The assay and wash media were not altered.

RESULTS

Relationship between $^{22}Na^+$ influx and sodium permeability. Preliminary experiments showed that $^{22}Na^+$ influx is increased by veratridine and batrachotoxin and that this effect is enhanced by sea anemone toxin II and scorpion toxin. The time course of $^{22}Na^+$ influx is illustrated in Fig. 1. In this experiment, heart cells were incubated with sea anemone toxin II in standard binding medium for 10 min and then the initial rate of $^{22}Na^+$ influx was measured in a second incubation of 10–90 sec in assay medium containing both sea anemone toxin II and veratridine. The initial rate of $^{22}Na^+$ influx into treated cells (○) is 10-fold greater than into control cells (●). The initial rate of influx is maintained for 30 sec and then is reduced as equilibrium is approached. In subsequent experiments, an incubation time of 15 sec was used when nearly maximal activation of sodium channels was expected, whereas an incubation time of 30 sec was used under conditions giving partial activation.

The fraction of sodium channels activated by neurotoxins is directly proportional to the increase in sodium permeability (P_{Na}). The initial rate of $^{22}Na^+$ influx is directly proportional to sodium permeability if the flux measurements are carried out at constant membrane potential. Thus, it is important to verify that measured influx is indeed proportional to P_{Na}. Equation 2.4 of Hodgkin and Katz (22) predicts that $^{22}Na^+$ influx is proportional to P_{Na} if influx is linearly proportional to Na^+ concentration. Experimental conditions under which this criterion is fulfilled were developed for studies of neuroblastoma cells (5, 20) and have been used in these

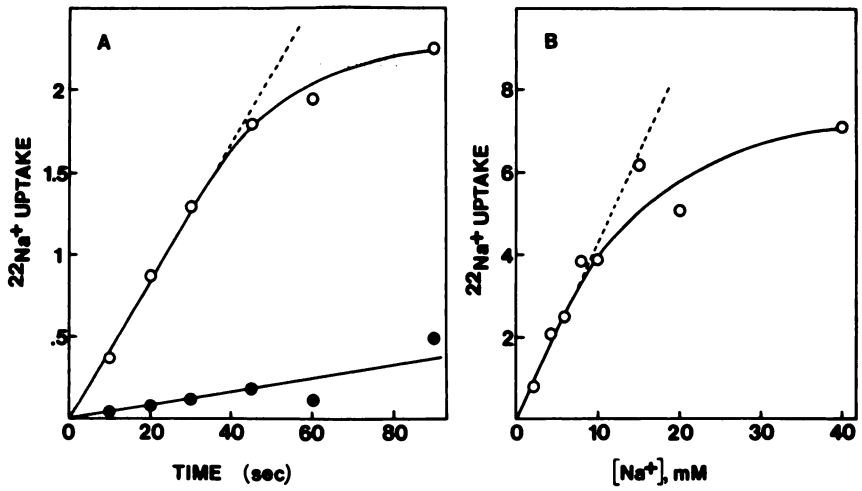

FIG. 1. *Dependence of $^{22}Na^+$ influx on time and $[Na^+]_{out}$*

A. Heart cells were incubated for 15 min at 36° in standard binding medium with no additions (●) or with 300 nM sea anemone toxin II (○) and then $^{22}Na^+$ influx was measured for the times indicated in assay medium with no additions (●) or with 200 μM veratridine and 300 nM sea anemone toxin II (○) as described under Experimental Procedures.

B. Heart cells were incubated for 15 min at 36° in standard binding medium containing 300 nM sea anemone toxin II and then $^{22}Na^+$ influx was measured for 30 sec in assay medium containing 300 nM sea anemone toxin II, 200 μM veratridine, and the indicated concentrations of Na^+. $^{22}Na^+$ influx into control cells incubated without toxins has been subtracted from the data.

experiments. Cells are incubated with neurotoxins in sodium-free medium to allow equilibration with receptor sites without accumulation of intracellular sodium. Initial rates of $^{22}Na^+$ influx are then measured in medium of low sodium concentration to prevent depolarization during the flux measurement. Using this approach (see Experimental Procedures), the initial rate of $^{22}Na^+$ influx increases linearly with sodium concentration up to 9 mM (Fig. 1B). A standard concentration of 4 mM Na^+ was used in subsequent experiments. Under these conditions, the initial rate of $^{22}Na^+$ influx provides a valid measure of the fraction of sodium channels activated by neurotoxins.

Activation of sodium channels by alkaloid neurotoxins. As in previous studies with neuroblastoma cells (23) and muscle cells (24), we found that activation of sodium channels by veratridine was rapid, reaching equilibrium at the earliest time tested (1 min), whereas activation by batrachotoxin was slower, reaching equilibrium after 20 min (data not shown). Equilibrium concentration-effect curves for activation of sodium channels by batrachotoxin, veratridine, and aconitine are illustrated in Fig. 2. Batrachotoxin is the most potent of the three alkaloids, giving half-maximal activation at 0.7 μM as compared with 20 μM for veratridine. Aconitine does not activate a detectable fraction of the sodium channels. Batrachotoxin causes the greatest increase in $^{22}Na^+$ influx at saturation, consistent with its designation as a full agonist as in neuroblastoma cells (5, 23). Veratridine is a partial agonist, activating only 22–35% as many sodium channels as batrachotoxin at saturation.

Previous ion flux studies of alkaloid toxin action in neuroblastoma cells have shown that the partial agonists veratridine and aconitine competitively inhibit activation of sodium channels by the full agonist batrachotoxin, as expected if these toxins act at a common receptor site (5, 23). More recently, direct studies of binding of [^{3}H]batrachotoxinin A 20-α-benzoate have confirmed the conclusion that all three alkaloid toxins act at a common receptor site at which batrachotoxin is a full agonist and aconitine and veratridine are partial agonists (6, 7). In view of these results, we studied the effect of veratridine and aconitine in the presence of 1 μM batrachotoxin. We found that both veratridine (data not shown) and aconitine (Fig. 3) reduce the level of $^{22}Na^+$ influx caused by batrachotoxin, as expected if these toxins act at a com-

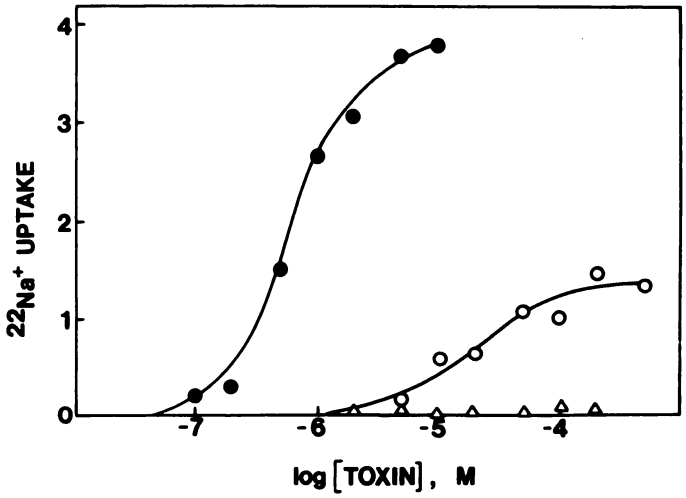

FIG. 2. *Concentration dependence of sodium channel activation by batrachotoxin, veratridine, and aconitine*

Heart cells were incubated for 15 min at 36° in standard binding medium containing the indicated concentrations of batrachotoxin (●), veratridine (○), or aconitine (△). $^{22}Na^+$ influx was then measured for 15 sec (●) or 30 sec (△, ○) at 36° in assay medium containing the same concentrations of neurotoxins. $^{22}Na^+$ influx into control cells incubated without neurotoxins has been subtracted from the data.

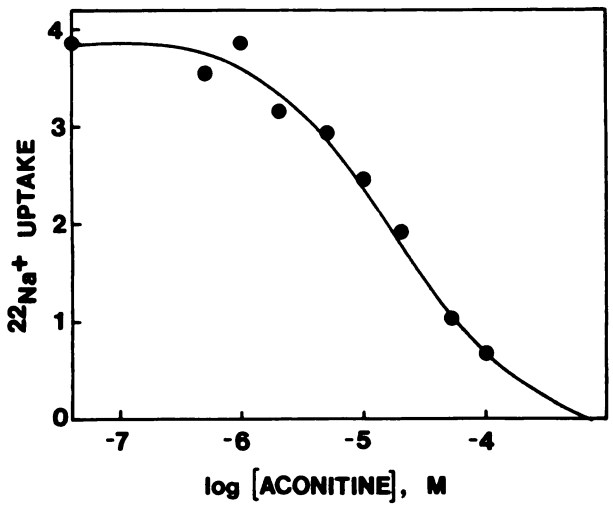

FIG. 3. *Inhibition of batrachotoxin activation by aconitine*

Heart cells were incubated for 10 min at 36° in standard binding medium containing 1 μM batrachotoxin and the indicated concentrations of aconitine. $^{22}Na^+$ influx of control cells incubated without neurotoxins has been subtracted from the data.

mon receptor site in heart cells as well. Assuming a competitive interaction, the data of Fig. 3 give an apparent K_D of 8.2 μM for aconitine.

These results show that interaction of neurotoxins with receptor site 2 on sodium channels in cultured rat heart cells is essentially identical with interaction with sodium channels in neuroblastoma cells. Values for apparent K_D for activation by alkaloid toxins are nearly identical. In addition, as in neuroblastoma cells, batrachotoxin is a full agonist, veratridine is a partial agonist, and aconitine is a very weak partial agonist (see below) in activating sodium channels.

Enhancement of alkaloid toxin action by scorpion toxin and sea anemone toxin II. In addition to their action to slow sodium channel inactivation in nerve and muscle (1), the polypeptide toxins enhance persistent activation of sodium channels by alkaloid neurotoxins (1, 4, 5) owing to an allosteric interaction between receptor sites 2 and 3. Binding of polypeptide toxins at receptor site 3 increases the affinity for alkaloid toxins at receptor site 2 (5) by stabilizing the active states of the sodium channel to which the alkaloid toxins bind with high affinity. The time course of sea anemone toxin II enhancement of veratridine activation of sodium channels in rat heart cells is illustrated in Fig. 4A. Heart cells were incubated with 0 (●), 20 (○), or 300 (△) nM sea anemone toxin II for 2–30 min and then $^{22}Na^+$ influx was measured in the presence of the same sea anemone toxin II concentration plus 200 μM veratridine. Both concentrations of sea anemone toxin II significantly increased the fraction of sodium channels activated by this saturating concentration of veratridine. The maximal effect was observed within 10 min and $^{22}Na^+$ influx declined thereafter. Thus, sea anemone toxin II acts rapidly to enhance activation of sodium channels in cultured rat heart cells by veratridine. The effect of sea anemone toxin II is also rapidly reversible. When the cultured heart cells were first incubated with 20 nM sea anemone toxin II for 10 min and then washed and incubated further in the absence of sea anemone toxin II before assay in 200 μM veratridine, the enhancement of veratridine activation was reversed with a half-time of less than 2 min (Fig. 4B). Similar experiments with scorpion toxin also showed that scorpion toxin action was complete within 10 min and was reversed with a half-time of less than 2 min. These results contrast with those on cultured neuroblastoma cells which show a slow reversal of scorpion toxin binding and action at the resting membrane potential (29).

Equilibrium concentration-effect curves for sea anemone toxin II and scorpion toxin are illustrated in Fig. 5A. Both toxins increase $^{22}Na^+$ influx in the presence of 200 μM veratridine up to 5-fold. A half-maximal effect of sea anemone toxin II is observed at 20 nM, whereas 130 nM scorpion toxin is required. These results show that the binding specificity of receptor site 3 on sodium channels in rat heart cells is reversed from that in nerve, where $K_{0.5}$ for scorpion toxin action is 1 nM and for sea anemone toxin II action is 100 nM.

The same data are presented in the form of an Eadie-Hofstee plot in Fig. 5B. The data for scorpion toxin (○) describe a straight line, indicating a single class of scorpion toxin receptor sites with an apparent K_D of 130 nM. In contrast, the data for sea anemone toxin II (●) are curvilinear, indicating either two classes of receptor sites for sea anemone toxin II or negatively cooperative interactions among receptor sites. If these data represent the contributions of two distinct receptor sites, the apparent K_D for the high-affinity site would be 4 nM and that for the low-affinity site 38 nM. Since scorpion toxin and sea anemone toxin II give similar levels of $^{22}Na^+$ influx at saturation (Fig. 5A and B), these data are consistent with the conclusion that sea anemone toxin II and scorpion toxin act at a common class of receptor sites but that sea anemone toxin II distinguishes two subclasses among

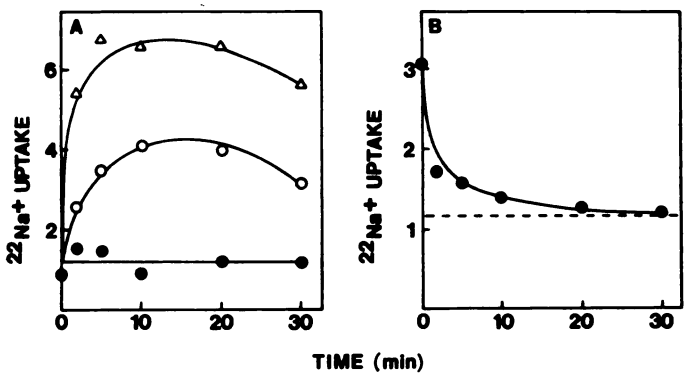

FIG. 4. *Time course of onset and reversal of sea anemone toxin II action*

A. Heart cells were incubated at 36° for the indicated times in standard binding medium containing 0 (●), 20 (○), or 300 (△) nM sea anemone toxin II. $^{22}Na^+$ influx was then measured for 30 sec at 36° in assay medium containing 200 μM veratridine and the same concentration of sea anemone toxin II. $^{22}Na^+$ influx of control cells incubated without neurotoxins has been subtracted from the data.

B. Heart cells were incubated for 15 min at 36° with standard binding medium containing 20 nM sea anemone toxin II. The cells were then rinsed to remove unbound sea anemone toxin II and incubated for the indicated times at 36° in standard binding medium. Finally, $^{22}Na^+$ influx was measured for 30 sec at 36° in assay medium containing 200 μM veratridine. $^{22}Na^+$ influx of control cells incubated without neurotoxins has been subtracted from the data. - - - represents the rate of $^{22}Na^+$ influx of untreated cells assayed in the presence of 200 μM veratridine.

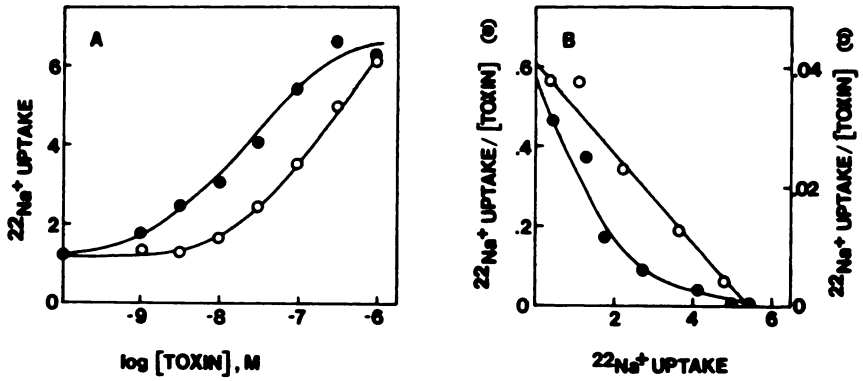

FIG. 5. *Concentration dependence of polypeptide toxin enhancement of sodium channel activation by veratridine*

A. Heart cells were incubated for 10 min at 36° in standard binding medium containing the indicated concentrations of sea anemone toxin II (●) or scorpion toxin (○). $^{22}Na^+$ influx was then measured for 30 sec at 36° in assay medium containing 200 μM veratridine and the same concentration of polypeptide toxin. $^{22}Na^+$ influx of control cells incubated without toxins has been subtracted from the data.

B. $^{22}Na^+$ influx in the presence of veratridine alone (ordinate in A) was subtracted from the data and the increment in influx due to the polypeptide toxins was plotted in the form of an Eadie-Hofstee plot. Note the different ordinate values for the two sets of data.

these sites or induces negatively cooperative interactions not observed with scorpion toxin. In this respect, the action of sea anemone toxin and scorpion toxin on sodium channels in cultured heart cells resembles their action in neuroblastoma cells and muscle cells (24–26).

The effect of a saturating concentration (300 nM) of sea anemone toxin II on the concentration-effect curves for batrachotoxin, veratridine, and aconitine is illustrated in Fig. 6A–C. With batrachotoxin (Fig. 6A), $K_{0.5}$ for sodium channel activation is reduced 37-fold from 1.2

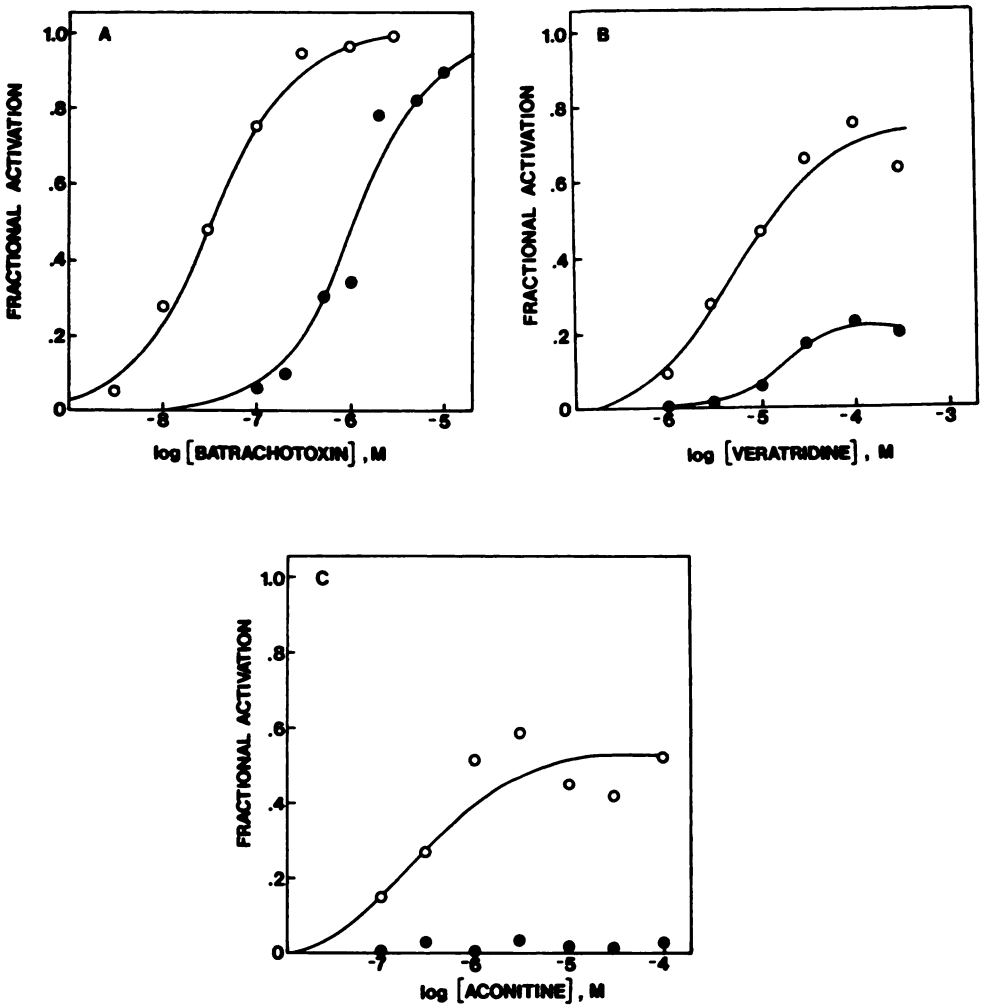

FIG. 6. *Effect of sea anemone toxin II on concentration-effect curves for alkaloid toxins*

Heart cells were incubated for 10 min at 36° in standard binding medium containing the indicated concentrations of alkaloid neurotoxin in the presence (○) or absence (●) of 300 nM sea anemone toxin II. $^{22}Na^+$ influx was then measured for 15 sec (A) or 30 sec (B and C) in assay medium containing the same neurotoxin concentrations. $^{22}Na^+$ influx of control cells incubated without neurotoxins has been subtracted from the data.

μM to 0.032 μM without a significant effect on the maximal $^{22}Na^+$ influx at saturation. These results support the designation of batrachotoxin as a full agonist which activates all of the sodium channels in the heart cells at saturation. On this assumption, we have set the value of $^{22}Na^+$ influx at 3 μM batrachotoxin plus 300 nM sea anemone toxin II equal to a fractional sodium channel activation of 1.0. The activation of sodium channels by partial agonists in different experiments is normalized to this value (Fig. 6B and C).

In this series of experiments, veratridine caused a fractional activation of 0.23 at saturation (Fig. 6B) with $K_{0.5}$ = 17 μM. Addition of 300 nM sea anemone toxin II increased fractional activation to 0.76 and reduced $K_{0.5}$ to 6.5 μM. Aconitine alone did not activate a detectable fraction (<5%) of sodium channels but caused a fractional activation of 0.52 with a $K_{0.5}$ of 0.6 μM in the presence of 300 nM sea anemone toxin II (Fig. 6C). Thus, sea anemone toxin II shifted the concentration-effect curve for the full agonist batrachotoxin to the left with no change in maximal flux and both reduced $K_{0.5}$ and increased the maximal fractional activation for the partial agonists veratridine and aconitine. Similar results were obtained with scorpion toxin (1 μM) and veratridine or batrachotoxin (data not shown). These results are similar to those obtained previously with neuroblastoma cells and muscle cells (24–26) and indicate that the allosteric interactions between the alkaloid toxins and polypeptide toxins are the same with sodium channels in neuroblastoma cells, muscle cells, and heart cells. Thus, the binding specificity of receptor site 3 is altered in myocardial sodium channels, but the allosteric interactions of this receptor site with receptor site 2 in causing persistent activation of sodium channels is not altered.

Inhibition of sodium channels in rat heart cells by tetrodotoxin and saxitoxin. The physiological studies and [^{3}H]saxitoxin binding studies presented in the accompanying report (10) indicate that sodium channels in cultured rat heart cells would be expected to have relatively low affinity for tetrodotoxin and saxitoxin. Fig. 7 illustrates inhibition of persistently activated sodium channels by these toxins. Saxitoxin blocks $^{22}Na^+$ influx with $K_{0.5}$ = 50 nM and tetrodotoxin with $K_{0.5}$ = 960 nM. Thus, as expected, myocardial sodium channels are relatively insensitive to these toxins. Since the K_D for tetrodotoxin or saxitoxin is increased by elevated temperature, reduced pH, or increased concentration of alkali metal cations, it is important to compare values for binding and ion flux experiments under identical conditions. The sodium-deficient medium used for the binding and ion flux experiments is essentially the same. Most of the binding experiments were carried out at 0° (10). However, measurements at 36° gave K_D = 7 nM for saxitoxin and K_D = 10 nM for tetrodotoxin (10). Thus, the sodium channels observed in ion flux experiments in cultured rat heart cells have toxin-binding properties very different from those observed in toxin-binding experiments in heart homogenates. These results are consistent with the conclusion that a major fraction of the high-affinity tetrodotoxin/saxitoxin receptor sites in heart homogenates is associated with nerve endings (10).

To investigate this point further, we tested homogenates of rat heart cell cultures for high-affinity saxitoxin receptor sites. Since nerve endings will not survive in cell culture without the cell bodies, the only neuronal elements in our heart cell cultures should be the small complement of parasympathetic neurons in the cardiac ganglia. Therefore, if the high-affinity saxitoxin receptor sites in the heart are associated primarily with nerve endings, there should be many fewer sites in the cell cultures. Comparative binding experiments showed that cultured heart cells have only 23.8 fmoles of high-affinity saxitoxin receptor sites per milligram of protein compared with 169.2 fmoles/mg for heart homogenates of adult rats (Table 1). These data also are consistent with the conclusion that the high affinity saxitoxin receptor sites are associated with nerve endings.

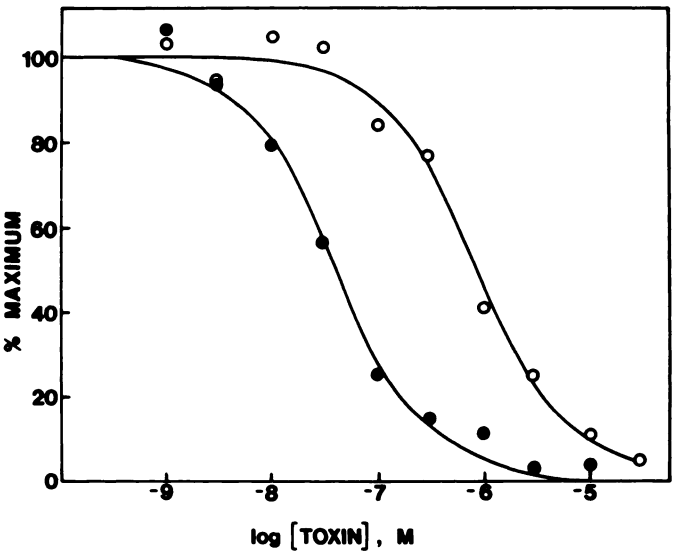

FIG. 7. *Inhibition of myocardial sodium channels by tetrodotoxin and saxitoxin*

Heart cells were incubated for 10 min at 36° in standard binding medium containing 300 nM sea anemone toxin II and the indicated concentrations of saxitoxin (●) or tetrodotoxin (○). $^{22}Na^+$ influx was then measured for 30 sec at 36° in assay medium containing 200 μM veratridine and the same concentrations of saxitoxin, tetrodotoxin, and sea anemone toxin II.

Voltage dependence of neurotoxin action. In nerve and muscle, the binding of neurotoxins to receptor sites 1 and 2 is not voltage-dependent in the voltage range between the resting membrane potential and 0 mV, whereas the binding of sea anemone toxin II and scorpion

TABLE 1

Saxitoxin binding to homogenates of cultured heart cells

Total particulate fractions were prepared from cultured heart cells and from adult rat heart, and specific [^{3}H]saxitoxin binding was measured as described in the accompanying report (10) at a concentration of 15 nM [^{3}H]saxitoxin. Protein in the whole homogenate was determined by the method of Lowry *et al.* (28).

Tissue	Specific binding	%
	fmol/mg	
Adult rat heart	169.2	100
Cultured heart cells	23.8	14

toxin to neurotoxin receptor site 3 is highly voltage-dependent in this range (1, 25, 29). In ion flux experiments, we study the voltage dependence of neurotoxin action by changing the K^+ concentration during the preliminary incubation in standard binding medium. Neurotoxins therefore equilibrate with their receptor sites at different membrane potentials. Then, $^{22}Na^+$ influx is measured in the absence of neurotoxins in assay medium containing 5.4 mM K^+. Under these conditions, the driving force for $^{22}Na^+$ influx remains constant even though the neurotoxins have been allowed to bind at different membrane potentials. This approach is accurate if the half-time for reversal of toxin action is long compared with the assay time (15 sec) so that dissociation and reversal of the toxin effect during the assay can be neglected. For batrachotoxin, this is a good approximation, since the half-time for reversal of the toxin effect is longer than 30 min (data not shown), as in neuroblastoma cells (23). Figure 8 illustrates the effect of depolarization with K^+ on batrachotoxin action (△). The cells were incubated for 20 min with 2 μM batrachotoxin in standard binding medium with the indicated K^+ concentration and rinsed, and $^{22}Na^+$ influx was measured in medium with 5.4 mM K^+ and no batrachotoxin. The results indicate a slight increase (<25%) in $^{22}Na^+$ influx when the cells are depolarized to 0 mV with 135 mM K^+. These data indicate, at most, a minor effect of membrane potential on batrachotoxin action in this membrane potential range.

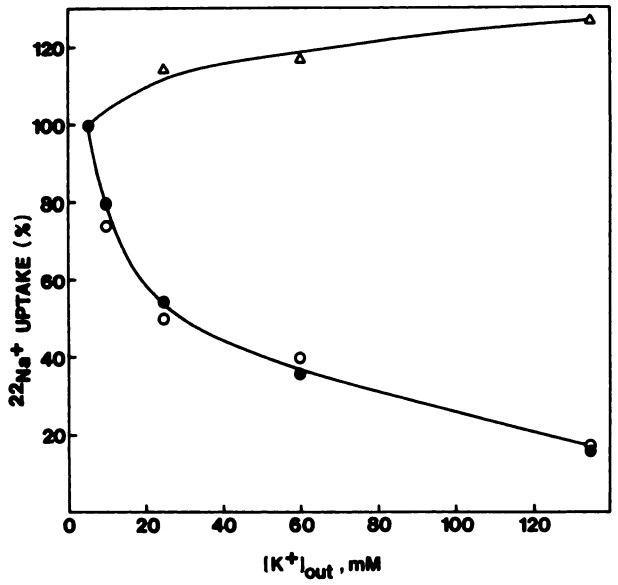

FIG. 8. *Voltage dependence of neurotoxin action*

Heart cells were incubated for 10 min at 36° in standard binding medium containing 2 μM batrachotoxin (△) and the indicated concentrations of K^+ or for 10 min at 36° in standard binding medium containing 100 nM sea anemone toxin I (○) or 100 nM scorpion toxin (●) and the indicated concentrations of K^+ followed by 3 min at 36° in the same medium plus 0.3 μM batrachotoxin. $^{22}Na^+$ influx of all cultures was then measured for 30 sec at 36° in assay medium. $^{22}Na^+$ influx of cells incubated with 0.3 μM batrachotoxin alone has been subtracted from the data for scorpion toxin (●) and sea anemone toxin II (○) so that the values presented represent the increment due to polypeptide toxin. $^{22}Na^+$ influx of control cells incubated without toxins has been subtracted from the data for batrachotoxin (△).

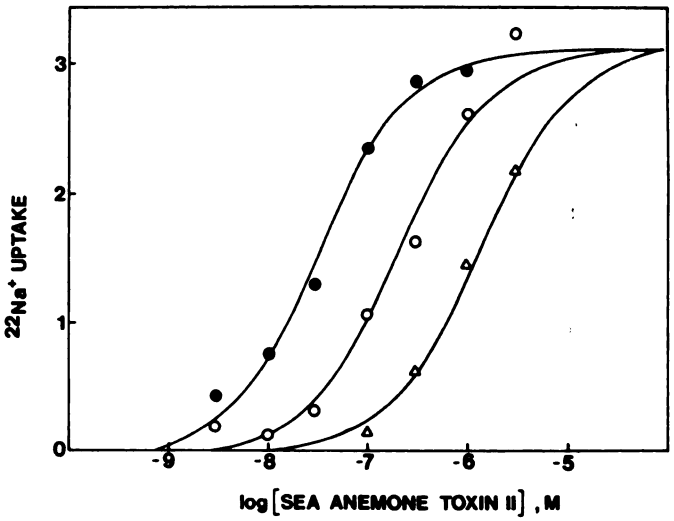

FIG. 9. *Effect of depolarization on the concentration-effect curve for sea anemone toxin II*

Heart cells were incubated for 10 min at 36° in standard binding medium containing 5.4 mM (●), 25 mM (○), or 135 mM (△) K^+ and the indicated concentrations of sea anemone toxin II followed by a second incubation of 3 min at 36° in the same medium plus 0.3 μM batrachotoxin. $^{22}Na^+$ influx was then measured for 30 sec at 36° in assay medium with no toxins. $^{22}Na^+$ influx of control cells incubated without neurotoxins has been subtracted from the data.

Concentration-effect curves (not shown) suggested that this small effect was on V_{max} for $^{22}Na^+$ influx rather than on $K_{0.5}$.

The action of the polypeptide toxins is rapidly reversible (Fig. 4B). However, if batrachotoxin and a polypeptide toxin are incubated with heart cells, the polypeptide toxin enhances the binding of batrachotoxin (Fig. 6A) and the increased activation of sodium channels due to the presence of the polypeptide toxin is slowly reversible with a half-time greater than 30 min (data not shown) because the binding of batrachotoxin is slowly reversible. Thus, the voltage dependence of polypeptide toxin action can be examined by incubating cells with a subsaturating concentration of batrachotoxin (0.3 μM) plus polypeptide toxin in medium with increased K^+, rinsing the cells, and measuring $^{22}Na^+$ influx in 5.4 mM K^+ in the absence of neurotoxins. Figure 8 illustrates the results of such an experiment with fixed concentrations of scorpion toxin (300 nM, ○) and sea anemone toxin II (100 nM, ●). Depolarization with K^+ reduces $^{22}Na^+$ influx up to 83% with both polypeptide toxins. These data show that polypeptide toxin action on myocardial sodium channels is inhibited by membrane depolarization as in neuroblastoma cells (25, 29) and muscle cells (24).

The voltage dependence of sea anemone toxin II action is examined further in the experiment illustrated in Fig. 9. Heart cells were incubated with 0.3 μM batrachotoxin and increasing concentrations of sea anemone toxin II in standard binding medium containing 5.4 mM (●), 25 mM (○), and 135 mM (△) K^+. The cells were then rinsed and $^{22}Na^+$ influx was measured in medium with 5.4 mM K^+. The results show that depolarization causes a shift of the concentration-effect curves for sea anemone toxin II action to the right consistent with an increase in the $K_{0.5}$

TABLE 2

Summary of neurotoxin affinities for sodium channels in the rat

Receptor site	Ligand	Synaptosomes (ref.)	$K_{0.5}$ for toxin action in		
			Adult muscle (ref.)	Cultured muscle cells (ref.)	Cultured heart cells
		M	M	M	M
1	Tetrodotoxin	5×10^{-9} (35)	1.2×10^{-8} (36)	1×10^{-6} (24)	1×10^{-6}
	Saxitoxin	5×10^{-9} (35)	$2–5 \times 10^{-9}$ (36–38)	3×10^{-7} (34)	5×10^{-8}
2	Batrachotoxin	0.5×10^{-7} (35)	1.5×10^{-6} (39)	1.7×10^{-6} (24)	0.7×10^{-6}
	Veratridine	1.3×10^{-5} (35)		4×10^{-5} (34)	2×10^{-5}
	Aconitine	1.4×10^{-5} (35)		1.3×10^{-5} (24)	0.9×10^{-5}
3	Scorpion toxin	2×10^{-9} (35)		6×10^{-8} (24)	1.3×10^{-7}
	Sea anemone toxin II	2×10^{-7} (35)		2×10^{-8} (24)	2×10^{-8}

for toxin action without a major change in the maximal stimulation of $^{22}Na^+$ uptake. The voltage dependence of polypeptide toxin action in cultured heart cells is therefore likely to be due to a progressive increase in the K_D for toxin binding as described previously in nerve and muscle cells (24, 25, 29).

DISCUSSION

Pharmacological classification of sodium channels in the rat. Our results show that the sodium channels in cultured rat heart cells are relatively insensitive to inhibition by tetrodotoxin and saxitoxin. In this respect, these sodium channels resemble the tetrodotoxin-insensitive sodium channels in denervated skeletal muscle *in vivo* (30, 31) and in uninnervated skeletal muscle cells cultured *in vitro* (24, 32–34) and differ from sodium channels in nerve and adult skeletal muscle which have high affinity for these toxins. Table 2 summarizes the apparent binding constants and sites of action for neurotoxins on sodium channels in rat brain synaptosomes, adult rat muscle, cultured rat skeletal muscle cells, and cultured rat heart cells. Interactions of neurotoxins at each of the three neurotoxin receptor sites on the sodium channel are considered and compared in the following paragraphs.

In nerve and adult muscle, tetrodotoxin and saxitoxin bind to sodium channels with K_D values of 5–12 nM (Table 2). In contrast, in cultured skeletal muscle and heart muscle cells, the apparent binding constants for saxitoxin are 10-fold higher (50–300 nM) and for tetrodotoxin are more than 100-fold higher (1 μM). In nerve (40, 41), adult muscle (42), and cultured muscle cells (24), the binding of tetrodotoxin and saxitoxin is not voltage-dependent. In heart, voltage-clamp experiments with rabbit Purkinje fibers (43) indicate that the binding of these inhibitory toxins is also independent of membrane potential. It is likely that the voltage dependence of tetrodotoxin action observed with dV/dt measurements (44) reflects an effect of membrane potential on inactivation of sodium channels which alters the relationship between the fraction of sodium channels activated and dV/dt (43). Thus, the mechanism of tetrodotoxin and saxitoxin action on sodium channels in these various excitable cells seems similar, but the sodium channels in heart and cultured muscle cells have much lower affinity for tetrodotoxin and saxitoxin.

Sodium channels in all four cell types have essentially the same affinities (Table 2) for the alkaloid neurotoxins acting at receptor site 2. In addition, the relative efficacy of these three toxins in activating sodium channels is the same. In each case, batrachotoxin is a full agonist, veratridine a partial agonist, and aconitine a weaker partial agonist. Finally, in each case, batrachotoxin action is voltage-independent in the voltage range between the resting membrane potential and 0 mV. By these criteria, the affinities and mechanisms of action of neurotoxins at receptor site 2 are identical in tetrodotoxin-sensitive sodium channels in nerve and adult muscle and tetrodotoxin-insensitive sodium channels in heart and cultured muscle cells.

In nerve, the polypeptides scorpion toxin and sea anemone toxin II bind at receptor site 3 with K_D values of 2 nM and 200 nM, respectively (Table 2). They slow inactivation of sodium channels (1) and enhance persistent activation of sodium channels by alkaloid toxins (1, 4, 5, 25, 26). In cultured rat skeletal muscle cells and heart cells, these toxins also enhance persistent activation of sodium channels by alkaloid toxins (ref. 24; Fig. 5). Both toxins increase V_{max} and reduce $K_{0.5}$ for the partial agonists veratridine and aconitine, and they reduce $K_{0.5}$ without effect on V_{max} for the full agonist batrachotoxin. However, the tetrodotoxin-insensitive sodium channels in both cultured muscle cells and heart cells have markedly altered affinity for scorpion toxin and sea anemone toxin II (ref. 24, Table 2). $K_{0.5}$ for sea anemone toxin II is decreased 10-fold and $K_{0.5}$ for scorpion toxin is increased 60-fold relative to rat brain synaptosomes. Thus, the binding specificity of receptor site 3 on tetrodotoxin-insensitive sodium channels is reversed.

Although the binding specificity of receptor site 3 is altered, the mechanism of action of neurotoxins at this receptor site is apparently unchanged. In all three cell types studied, the binding and action of scorpion toxin is well fit by a hyperbolic saturation curve, whereas concentration-effect curves for sea anemone toxin II are consistently shallower than hyperbolic, indicating multiple receptor sites or a complex mechanism of toxin action (20, 24, 25, 26) (Fig. 5). In addition, in all three cell types studied, binding and action of both polypeptide toxins is voltage-dependent (24, 25, 29). Thus, by these criteria, the mechanism of polypeptide toxin action at receptor site 3 is the same on tetrodotoxin-sensitive and

tetrodotoxin-insensitive sodium channels, but the binding specificity of the receptor site is altered.

The pharmacological profiles of sodium channels in the four rat cell types studied indicate that these channels can be classified into two groups. The tetrodotoxin-sensitive sodium channels in nerve and adult muscle have a common pharmacological profile and may represent a single molecular species (Table 2), although the values for K_D for the polypeptide toxins in adult rat muscle are not available at present. The tetrodotoxin-insensitive sodium channels in cultured skeletal muscle cells and heart cells also have a common pharmacological profile (Table 2) that is distinctly different from that of the tetrodotoxin-sensitive channels. Thus, these sodium channels may also represent a single class of macromolecules. Examination of the species distribution of tetrodotoxin-insensitive sodium channels in heart and skeletal muscle also lends support to the hypothesis that these channels are closely related. As reviewed in the accompanying report (10), physiological studies indicate that mammalian myocardium has tetrodotoxin-insensitive sodium channels, whereas myocardial sodium channels in nonmammalian vertebrates are tetrodotoxin-sensitive. As expected from this, sodium channels in cultured chick heart cells are tetrodotoxin-sensitive (45–48) and the polypeptide toxin receptor site binds scorpion toxin with high affinity (48, 49) and sea anemone toxin II with lower affinity (50). Similarly, denervation of mammalian muscle leads to appearance of tetrodotoxin-insensitive sodium channels (30, 31), whereas denervation of nonmammalian vertebrate muscle does not lead to appearance of tetrodotoxin-insensitive channels (27, 51). As expected from this, uninnervated rat skeletal muscle cells in culture have only tetrodotoxin-insensitive sodium channels (24, 32–34), whereas chick skeletal muscle cells in culture have tetrodotoxin-sensitive channels (51, 52). We conclude from these results that tetrodotoxin-insensitive sodium channels are characteristic of mammalian myocardium and denervated or uninnervated mammalian skeletal muscle and that the tetrodotoxin-insensitive channels in heart and skeletal muscle may represent a single class of macromolecules.

We propose that sodium channels may be classified on the basis of pharmacological profile in the same manner that receptors for hormones and neurotransmitters have been classified. The data on sodium channels in excitable tissues of the rat that are available at present indicate that there are two pharmacologically distinct subtypes of sodium channels: tetrodotoxin-sensitive and tetrodotoxin-insensitive. These channel subtypes have characteristic differences in toxin-binding specificity at two of the three neurotoxin receptor sites that have been studied. It will be of interest to determine whether these pharmacological differences are accompanied by major differences in the molecular structure of the channel.

Neurotoxins as biochemical probes of sodium channels in mammalian myocardium. In order to use neurotoxins as biochemical probes of sodium channel structure and to identify and purify protein components of sodium channels, it is essential that the neurotoxins bind with high affinity and specificity to the sodium channels of interest. Our results and previous physiological studies show that tetrodotoxin and saxitoxin are unlikely to be useful for this purpose in the mammalian heart because mammalian myocardial sodium channels have low affinity for these toxins, whereas sodium channels associated with nerve endings in the mammalian heart have high affinity for them. Similarly, myocardial sodium channels have low affinity for scorpion toxin whereas neuronal sodium channels have high affinity. Therefore, of the toxins studied, only sea anemone toxin II has the high affinity and specificity required for biochemical studies of myocardial sodium channels. The apparent K_D for this toxin for the high-affinity component of $^{22}Na^+$ influx is 4 nM (Fig. 5B). Sea anemone toxin II has an average $K_{0.5}$ for activation of myocardial sodium channels that is 10-fold lower than that for neuronal channels (Table 2). Specific binding of ^{125}I-labeled sea anemone toxin II to tetrodotoxin-insensitive sodium channels in cultured rat skeletal muscle cells has been successfully measured and studied in detail (53). Thus, sea anemone toxin II appears to be the toxin of choice for biochemical studies of mammalian myocardial sodium channels.

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