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Binding of [^{3}H]Batrachotoxinin A Benzoate to Specific Sites on Rat Cardiac Sodium Channels

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SUMMARY

Radiolabeled neurotoxins have been used to study the structure and function of sodium channels. We studied the binding of [^{3}H] batrachotoxinin A 20α-benzoate ([^{3}H]BTX-B) to specific sites on sodium channels on rat cardiac myocytes. Calcium-tolerant myocytes were prepared by collagenase dispersion of adult rat hearts and were 75–83% viable. As with the nerve channel, specific binding of [^{3}H]BTX-B to its receptor site was seen only in the presence of sea anemone toxin (ATX). The affinity of ATX for its binding sites may be estimated from its concentration-dependent stimulatory effect on [^{3}H]BTX-B binding. These results suggest that, in the presence of 5.4 mM KCl, the myocytes have two affinities for ATX with estimated dissociation constants of 0.52 μM and 12.9 μM. Depolarization of the myocytes with either 65 mM KCl or 0.1 mM $BaCl_2$ results in the loss of the 0.52 μM component, suggesting that it is voltage sensitive. The 0.52 μM and 12.9 μM components have maximal binding capacities corresponding to 4 and 11 sites/μm^2 of myocyte surface area, respectively. Scatchard analysis of [^{3}H]BTX-B binding in the presence of ATX demonstrates a single class of sites with a K_D of 25–35 nM. These results demonstrate that [^{3}H]BTX-B can be used as a radioligand probe of the adult rat sodium channel and will facilitate a biochemical approach to the study of the interaction between antiarrhythmic drugs and the sodium channel.

Our understanding of the mechanism by which antiarrhythmic drugs interact with the cardiac sodium channel has evolved from the concept that these drugs interact nonspecifically with the lipid bilayer of membranes to current models which involve binding of the drugs to a specific site(s) on the channel, thereby mediating electrophysiologic changes (1, 2). The characteristics of this binding process have been inferred from electrophysiologic data but only indirectly characterized. To complement these electrophysiologic approaches we use biochemical techniques to study the cardiac sodium channel.

These techniques, which draw upon pharmacological studies of the nerve sodium channel, involve the use of toxins which bind specifically and with high affinity to sites on the sodium channel. Sodium channels have at least four receptor sites for neurotoxins (3–7). Receptor site 1 binds tetrodotoxin and saxitoxin, which inhibit sodium channel influx through the channel. Receptor site 2 binds alkaloid toxins such as aconitine and batrachotoxin, which cause persistent activation of the channel. Receptor site 3 binds polypeptide toxins, such as ATX, which slow sodium channel inactivation and synergistically enhance the binding and action of the site 2 toxins through an allosteric mechanism. Site 4 binds toxins such as *Centruroides* toxin, which activates the sodium channel.

Radioactive derivatives of these toxins have proven to be useful probes in the study of nerve sodium channel structure and function and its interaction with various drugs. Of particular interest is the tritiated congener of batrachotoxin, [^{3}H]BTX-B (8). In the presence of scorpion toxin, [^{3}H]BTX-B binds to site 2 of synaptosomal sodium channels with a K_D of 82 nM. As well, local anesthetics bind at or near site 2 and inhibit [^{3}H]BTX-B binding in a stereospecific fashion at pharmacologically relevant concentrations, and with the same rank order of potency *in vivo* and *in vitro* (9, 10). This suggests that the pharmacologic effect of local anesthetics is mediated by their binding site on the nerve sodium channel.

Our interest in type I antiarrhythmic drugs, which are similar to local anesthetics, prompted a study of the binding of [^{3}H]BTX-B to cardiac sodium channels. Adult rat ventricular myocytes prepared by collagenase dispersion were used as an experimental model because of their ease of preparation (11, 12), resting membrane potential (13–15), and the presence of electrophysiologically active sodium channels on their surface (13–15). We asked whether [^{3}H]BTX-B binding was to a specific site with properties characteristic of site 2. These properties include [^{3}H]BTX-B binding which was stimulated by the site 3 toxin ATX, inhibited by other site 2 toxins, and whose stimulation by ATX was voltage sensitive.

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ABBREVIATIONS: ATX, sea anemone toxin II; [^{3}H]BTX-B, [^{3}H]batrachotoxinin A 20α-benzoate; MEM, Joklik's minimal essential medium for tissue culture; BSA, bovine serum albumin.

Materials and Methods

Preparation of cardiac myocytes. Adult rat calcium-tolerant cardiac myocytes were prepared according to the method of Farmer *et al.* (11). Rats were sacrificed and the heart was rapidly excised and perfused in a Langendorff perfusion apparatus. The heart was perfused and later incubated with a series of solutions which were equilibrated with 95% O_2/5% CO_2 at 37°. The solutions were based on MEM. They sequentially included a rinse solution (MEM), a collagenase solution (MEM with 0.1% collagenase, 50 μM $CaCl_2$, and 0.1% BSA), a digestion solution (MEM with 0.1% collagenase, 1% BSA, and 50 μM $CaCl_2$), and an incubation solution (MEM with 50 μM $CaCl_2$ and 1% BSA). The heart first was perfused with rinse solution (12 ml/min for 3 min), then with collagenase solution (100 ml at 6 ml/min). The heart was removed from the cannula by cutting at the atrioventricular junction, then cut with fine scissors twice to the apex. The partly digested ventricle then was shaken gently with 7 ml of digestion solution and rinsed on a nylon filter with a pore size of 185 μm with 3 ml of incubation solution. The cells passed through the filter and were incubated at 21°. The larger tissue fragments were subjected to four or five more cycles of digestion and rinsing, until dispersion of the tissue was complete. The myocytes were collected by gentle centrifugation (40 × *g*), rinsed twice with incubation solution, and stored at 21° in incubation solution until used. They were used within 30 min.

[^{3}H]BTX-B radioligand binding experiments. Myocytes (6×10^6) in 50-μl incubation buffer were incubated with ATX and [^{3}H]BTX-B (50 Ci/mmol) for 45–60 min at 37° in the presence or absence of different concentrations of toxins and 0.13 mM tetrodotoxin. Tetrodotoxin was added to prevent depolarization induced by sodium influx. All assays were done in parallel with tubes containing no ATX or with 0.4 mM aconitine to determine nonspecific binding. The reaction was terminated by adding 10 ml of Krebs-Henseleit-BSA buffer (127 mM NaCl, 2.33 mM KCl, 1.30 mM KH_2PO_4, 1.23 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose, 50 μM $CaCl_2$, 1% BSA) equilibrated with 95% O_2/5% CO_2 and incubated at 37° for 1 min, then filtered through a Whatman GF-C 24-mm fiber-glass filter and washed four times at 20° with 5 ml of rinse buffer (25 mM Tris-Cl, pH 7.4, 130 mM NaCl, 5.5 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 μM $CaCl_2$). Fewer rinses resulted in a higher nonspecific background. The filters were then dried and counted in Econofluor scintillation fluid. The retained radioactivity represents [^{3}H]BTX-B bound to the myocytes.

Materials. Collagenase was purchased from Cooper Biomedical; [^{3}H]BTX-B was from New England Nuclear; and tetrodotoxin, aconitine, BSA, and ATX (sea anemone toxin II) were from Sigma. Batrachotoxin was a generous gift from Dr. E. Albuquerque (University of Maryland, Baltimore, MD).

Results

Myocyte preparation. Myocytes were prepared daily from adult rat ventricles with a yield of 8–10 × 10^6 myocytes/heart, of which 75–83% were viable rod-shaped cells. Virtually all of the viable cells were tolerant to 1 mM calcium. The cells did not contract spontaneously and had a resting membrane potential of at least −75 mV using a single-cell microelectrode suction pipette technique in the laboratory of Dr. W. Giles. About 60% of the cells remained viable at the end of the incubation period with the toxins. These data are similar to those of others and indicate that the cells are viable, selectively impermeable, and electrophysiologically active (11, 12).

[^{3}H]BTX-B binds to a saturable site. Binding of [^{3}H]BTX-B to nerve sodium channels is detectable in the presence but not the absence of ATX (8). Our initial experiments demonstrated that 1 μM ATX stimulated the total binding of [^{3}H]BTX-B to myocytes about 2- to 4-fold. We characterized the inhibition by batrachotoxin of [^{3}H]BTX-B binding to determine whether [^{3}H]BTX-B binds to a specific site. Fig. 1 shows that batrachotoxin inhibits ATX-stimulated [^{3}H]BTX-B binding. Control experiments showed that it had little effect on nonstimulated [^{3}H]BTX-B binding. The data indicate that batrachotoxin has an IC_{50} of 100 nM, which is similar to its affinity for other studied sodium channels (8). Furthermore, a Hill plot (16) of these data shows a Hill number of 0.91 suggesting a single class of binding sites for batrachotoxin. The results of three Hill plots yielded a Hill number of 1.01 ± 0.07 (SD). Thus, ATX-stimulated [^{3}H]BTX-B binding seems to be to a single class of specific, saturable binding sites.

The time course of association of [^{3}H]BTX-B with the binding site in the presence of ATX is shown in Fig. 2. Myocytes were incubated with [^{3}H]BTX-B in the presence or absence of ATX for various times and then filtered, and the bound radioactivity was counted. Specifically bound [^{3}H]BTX-B is the difference between the total bound in the presence of ATX and that bound in the absence of ATX (Fig. 2, A and B). The specifically bound [^{3}H]BTX-B increases with time, reaches a maximal level after 30 min, and is stable for at least 45 min. In contrast, the binding of nonspecifically bound [^{3}H]BTX-B is not dependent on time. All subsequent experiments were incubated at 37° for 60 min.

The rate of dissociation of the toxin-binding site complex was studied by first incubating the myocytes with [^{3}H]BTX-B in the presence or absence of ATX for 60 min at 37°, then diluting the cells 200-fold into Krebs-Henseleit buffer at 37° (Fig. 2C). Samples were withdrawn at various times and assayed. Fig. 2 shows that specifically bound [^{3}H]BTX-B dissociates from its binding site with a half-life of about 12 min. From this we estimate that about 6% of specifically bound [^{3}H]BTX-B is lost under our standard filtration conditions (see Materials and Methods).

Myocytes have two affinities for ATX. ATX from *Anemonia sulcata* was used to stimulate [^{3}H]BTX-B binding. Fig. 3 illustrates the effects of various concentrations of ATX on [^{3}H]BTX-B binding. There is little stimulation with less than 65 nM ATX. However, at concentrations above 65 nM, ATX causes a progressively increasing stimulation of [^{3}H]BTX-B binding. Although the effect of ATX on [^{3}H]BTX-B binding nears saturation over the concentration range studied, the K_D for ATX binding cannot be inferred directly from these data.

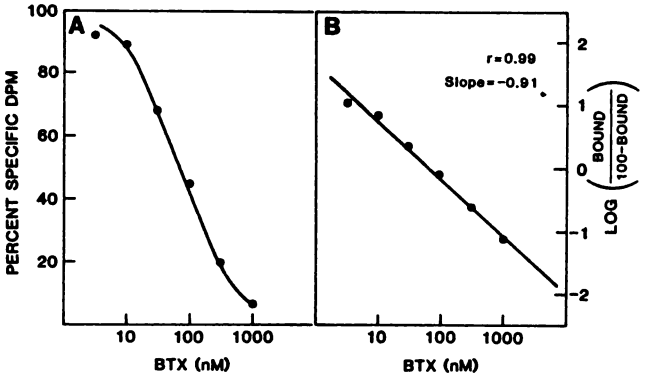

Fig. 1. Inhibition of ATX-stimulated [^{3}H]BTX-B binding by batrachotoxin. A. Binding to myocytes of 13 nM [^{3}H]BTX-B was measured in the presence of 1.3 μM ATX and increasing concentrations of unlabeled batrachotoxin. Nonspecific binding in the absence of ATX has been subtracted. B. Pseudo-Hill plot of the specific data from A. Specific binding is calculated as the difference between total binding and binding in the absence of ATX.

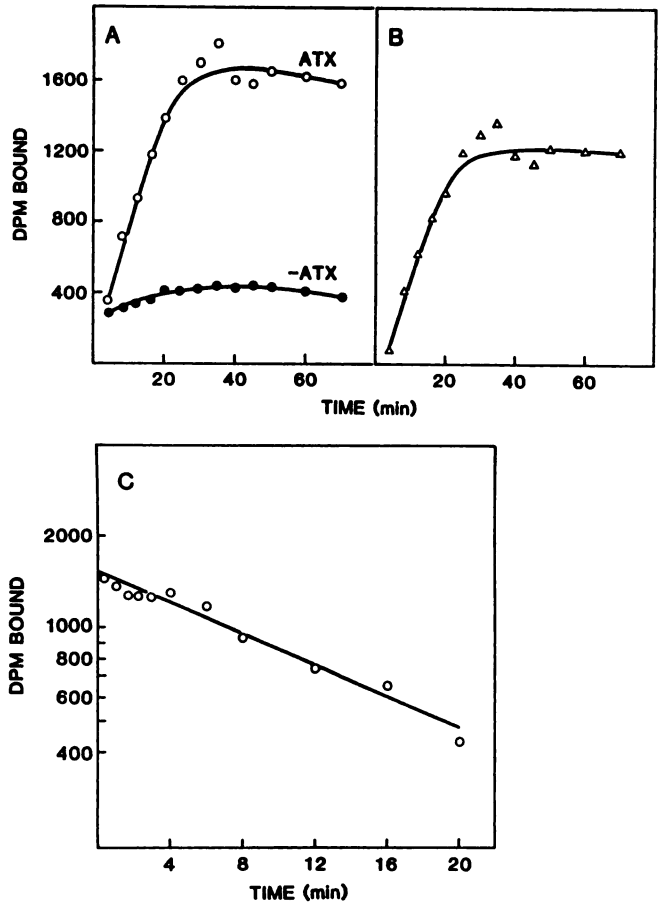

Fig. 2. Time course of formation and dissociation of the [^{3}H]BTX-B-myocyte complex. A. Myocytes were incubated with 13 nM [^{3}H]BTX-B in the presence (○) or absence (●) of 1.3 μM ATX, and bound [^{3}H]BTX-B was determined as described under Materials and Methods. B. Specifically bound [^{3}H]BTX-B was calculated as the difference between the binding in the presence and absence of ATX. C. Kinetics of dissociation of the [^{3}H]BTX-B-myocyte complex. Myocytes were incubated with 13 nM [^{3}H]BTX-B in the presence or absence of 1.3 μM ATX for 60 min, then diluted 200-fold into KHS buffer at 37°. Samples were withdrawn at the indicated times, filtered, and counted. Specific binding is the difference between binding in the presence and absence of ATX.

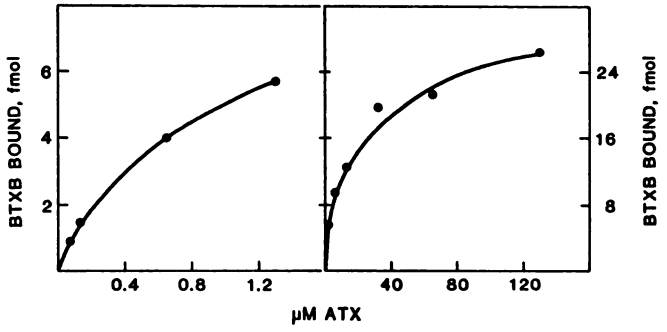

Fig. 3. Enhancement of specific [^{3}H]BTX-B binding by ATX. [^{3}H]BTX-B binding to myocytes was measured in the presence of 13 nM [^{3}H]BTX-B and various concentrations of ATX. Nonspecific binding (in the absence of ATX) has been subtracted.

In order to obtain a more accurate estimate of the K_D for ATX binding to myocytes, we have subjected the data for the stimulation of [^{3}H]BTX-B binding by ATX to Scatchard analysis (16). The rationale for this analysis is presented in the Appendix.

Fig. 4 presents a Scatchard analysis of the concentration-dependent stimulation by ATX of [^{3}H]BTX-B binding in the presence of 5.4 mM KCl. The data can be resolved into two linear components, indicating two affinities for ATX with estimated dissociation constants of 0.52 μM and 12.9 μM. The maximal binding capacities (B_{max}) for the two components in this experiment are 7.6 fmol and 19.4 fmol for the 0.52 μM and 12.9 μM sites, respectively. Therefore, in the presence of 5.4 mM KCl, the B_{max} of the 0.52 μM component is about 28.5% of the overall B_{max}.

Voltage-sensitive binding of ATX. Studies with the nerve sodium channel (4, 5, 8) have shown that ATX binding to the channel is voltage sensitive. Depolarization of the channel greatly weakens the affinity of the channel for ATX. This suggests that if we are indeed studying the cardiac sodium channel, then the binding of ATX to the tighter 0.52 μM component should be voltage sensitive. To test this, we examined the concentration-dependent effect of ATX on [^{3}H]BTX-B binding to cardiac myocytes in the presence of 65 mM KCl. Fig. 5A shows that, under these conditions, the 0.52 μM component of binding is abolished but that the 12.9 μM component persists. This suggests that the 0.52 μM component of ATX binding is voltage sensitive. We have also examined the binding of ATX to myocytes in the presence of 0.1 mM $BaCl_2$. Barium blocks the inward rectifier and causes depolarization of the myocytes (17). In the presence of barium the 0.52 μM component is abolished, whereas the 12.9 μM component persists (Fig. 5B). Thus, two independent methods of depolarization both eliminate the 0.52 μM component, demonstrating its voltage sensitivity. This is consistent with the notion that binding of ATX to at least the 0.52 μM component represents binding to the sodium channel.

Alkaloid toxins bind to a single class of sites. The two components of ATX binding raised the possibility that alkaloid toxin binding to site 2 might also be heterogeneous. If the affinity of alkaloid toxins is different for each of the components identified by ATX binding, then one would expect that the K_D for [^{3}H]BTX-B would depend upon the concentration of ATX. Fig. 6A depicts the results of an experiment in which we examined binding of [^{3}H]BTX-B to myocytes in the presence of 1.3 μM ATX. At this concentration about 75% of ATX binding is to the 0.52 μM component and 25% to the 12.9 μM

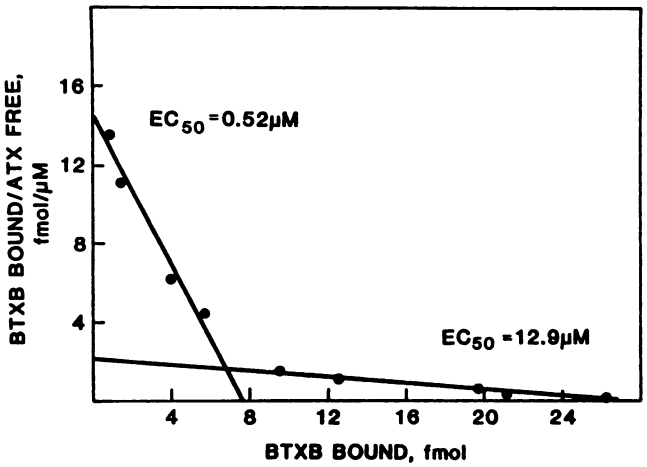

Fig. 4. Scatchard analysis of ATX stimulation of [^{3}H]BTX-B binding. The data from Fig. 3 were analyzed by plotting bound [^{3}H]BTX-B (fmol)/free ATX (μM) versus bound [^{3}H]BTX-B (fmol).

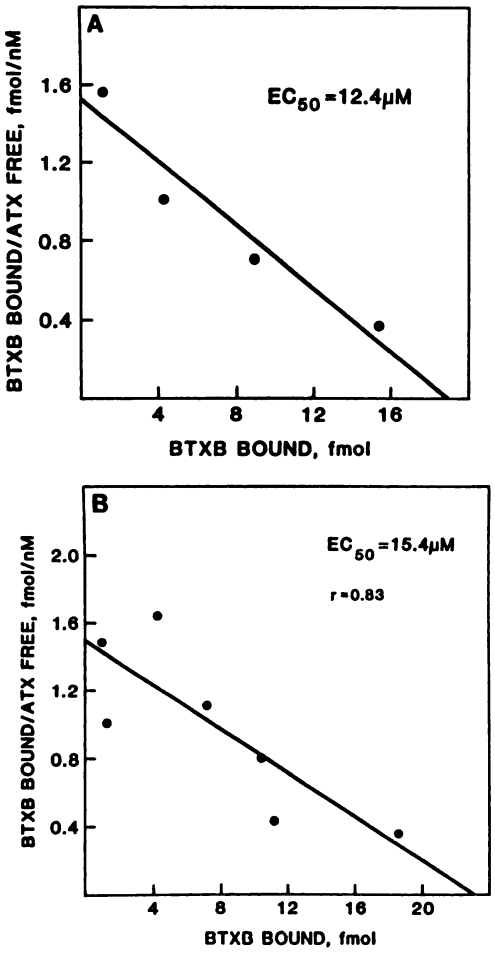

Fig. 5. Effect of depolarization on ATX stimulation of [^{3}H]BTX-B binding. A. Myocytes were incubated with 13 nM [^{3}H]BTX-B and various concentrations of ATX in the presence of 65 mM KCl. B. Myocytes were incubated with 13 nM [^{3}H]BTX-B and various concentrations of ATX in the presence of 0.1 mM $BaCl_2$. Results are presented in the form of Scatchard plots.

site. Binding of [^{3}H]BTX-B increases in a concentration-dependent and saturable fashion. The data are presented in a Scatchard analysis in Fig. 6B. The data indicate that binding of [^{3}H]BTX-B is to a single class of binding sites with a K_D of 24 nM. Had [^{3}H]BTX-B binding been to two different classes of binding sites, then one would expect to see two binding isotherms, as with ATX binding. Fig. 7 presents data from a similar experiment in which [^{3}H]BTX-B binding was examined in the presence of 52 μM ATX. Under these conditions about 32% of ATX binding is to the 0.52 μM component and 68% to the 12.9 μM component. Again, the data indicate that [^{3}H]BTX-B binds to a single class of sites with a K_D of 38 nM. The results of four similar experiments at each concentration indicate that the K_D (±SD) for BTX-B in the presence of 1.3 μM ATX is 24.7 ± 3.3 nM, and in the presence of 52 μM ATX is 35.3 ± 3.5 nM. These data demonstrate that the affinities of [^{3}H]BTX-B for myocytes do not depend greatly upon ATX concentration and suggest that [^{3}H]BTX-B binds to a single class of alkaloid-binding sites.

If there is a single class of alkaloid-binding sites, as suggested by the preceding experiment, then the affinities of myocytes for two other alkaloid toxins—batrachotoxin and aconitine—should also be independent of ATX concentration. To test this, we measured the ability of these toxins to inhibit [^{3}H]BTX-B binding to myocytes in the presence of either 1.3 μM or 52 μM ATX. The results in Fig. 8 show that the IC_{50} of aconitine is almost identical at 1.3 μM and 52 μM ATX, with similar results being obtained with batrachotoxin. Thus, the affinities of three alkaloid toxins for their binding site are virtually independent of ATX concentration. Taken together, the data are most consistent with the notion that there is a single class of alkaloid toxin-binding sites whose affinity for alkaloid toxins is the same at 1.3 μM or 52 μM ATX.

Density of binding sites on myocyte surface. An estimate of the density of toxin-binding sites can be derived from these data. The binding data of 23 separate experiments at different ATX and [^{3}H]BTX-B concentrations were used to estimate a B_{max} for each experiment and then pooled. The results indicate B_{max} at infinite ATX and [^{3}H]BTX-B concentrations to be 32.7 ± 10.6 (SD) fmol/10^5 cells. Using Avogadro's number, these results suggest that each myocyte carries about 2.04 ± 0.66 × 10^5 [^{3}H]BTX-B-binding sites. Although the myocyte surface area is not known with precision, Severs *et al.* (18) have estimated it to be 13,800 μm^2. Together, these data

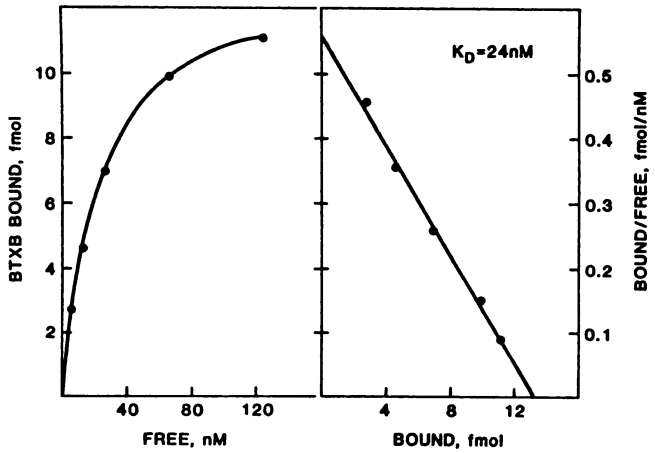

Fig. 6. [^{3}H]BTX-B binding in the presence of 1.3 μM ATX. Myocytes were incubated with 1.3 μM ATX and various concentrations of [^{3}H]BTX-B. A. Specifically bound [^{3}H]BTX-B is plotted versus free [^{3}H]BTX-B. B. Scatchard analysis of the data in A.

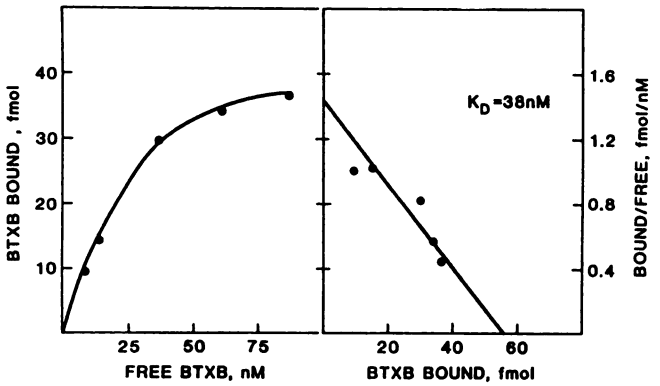

Fig. 7. [^{3}H]BTX-B binding in the presence of 52 μM ATX. Myocytes were incubated with 52 μM ATX and various concentrations of [^{3}H]BTX-B. A. Specifically found [^{3}H]BTX-B is plotted versus free [^{3}H]BTX-B. B. Scatchard analysis of the data in A.

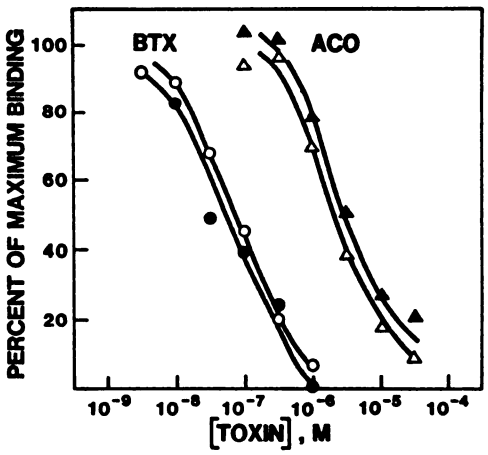

Fig. 8. Competitive inhibition of [³H]BTX-B binding by aconitine and batrachotoxin. Myocytes were incubated with 13 nM [³H]BTX-B and 1.3 μM ATX (●, ▲) or 26 μM ATX (○, △) as well as various concentrations of aconitine (△) or batrachotoxin (○). Data are expressed as percentage of specifically bound [³H]BTX-B versus toxin concentration.

suggest that there are 14.8 ± 4.8 [³H]BTX-B binding sites/μm² of myocyte membrane.

Discussion

The model. In this paper we have characterized the binding of both ATX and [³H]BTX-B to freshly isolated rat cardiomyocytes. Our long-term goal is to develop a radioligand assay which describes the interaction of type I antiarrhythmic drugs with the cardiac sodium channel. However, no radiolabeled type I drug exists with a sufficiently tight affinity for the channel to enable the use of the drug in a ligand-binding assay. [³H]BTX-B was chosen because of its known tight affinity for the channel, and because previous work with the nerve sodium channel suggested that [³H]BTX-B binding to the cardiac channel would be antagonized by the binding of type I drugs to the channel (9, 10, 19). For these reasons we have characterized the binding of ATX and [³H]BTX-B to the cardiac sodium channel.

Rat cardiac myocytes were prepared daily by collagenase dispersion. This method was chosen because we wished to study a viable preparation which maintains a low resting membrane potential and is stable during the course of the experiment. Furthermore, the electrophysiologic characterization of freshly isolated myocytes is well under way in other laboratories (13, 14). The myocytes prepared for this study are 75–83% viable, are virtually all calcium tolerant, and have a low resting membrane potential (−75 mV). As well, about 60% remain viable at the end of the incubation period. They are therefore a suitable material for physiologically meaningful radioligand binding experiments.

[³H]BTX-B binds to cardiac sodium channels. We have shown that toxins bind to a preparation of cardiac myocytes. It is formally possible that this binding is to macromolecules other than the sodium channel (20), or to sodium channels on nerve cells present in the ventricles. These possibilities seem unlikely. The binding of toxins described here has several features characteristic of toxin binding to sodium channels (3). First, both ATX and [³H]BTX-B are known sodium channel toxins and ATX stimulation of alkaloid toxin binding is characteristic of the sodium channel. Second, the dissociation constants of the alkaloid toxins here are very similar to their dissociation constants for the nerve sodium channel (Table 1) estimated by Catterall *et al.* (8) using a similar [³H]BTX-B assay. Third, at least 28% of the binding sites for ATX in this assay are voltage sensitive, as is ATX binding to synaptosomal sodium channels (8). Thus, it seems very likely that the toxin binding described in the study is to sites on sodium channels.

It seems equally likely that the sodium channel is on the myocytes. First, voltage-sensitive toxin binding requires an intact, relatively impermeable biological preparation such as synaptosomal or cultured muscle or nerve cells. We have never observed subcellular material or cells other than myocytes which exclude the vital stain trypan blue. Thus, myocytes appear to be the only impermeable structures in the preparation. Second, we have shown that type I antiarrhythmic drugs bind to this channel,[1] with affinities which correlate well with known effective concentrations of antiarrhythmic drugs when treating cardiac arrhythmias in man. Therefore, we conclude that ATX and [³H]BTX-B bind to specific sites on the sodium channel of cardiac myocytes.

Surface density of toxin-binding sites. We have established that the surface density of toxin-binding sites for either [³H]BTX-B or ATX is about 15 sites/μm² of myocyte membrane. To interpret this estimate one must consider the nature of the binding sites and the origin of the estimates for myocyte surface.

ATX binds to two components with estimated affinities of 0.52 μM and 12.9 μM. The tighter 0.52 μM component is voltage sensitive and is very likely to correspond to the fast sodium channel. The nature of the 12.9 μM component is less clear. It might reflect sodium channels in depolarized myocytes or sodium channel-like structures on myocytes (6, 20) or a combination of both. It is clear, however, that the two components of ATX binding are linked to very similar—perhaps identical—binding sites for alkaloid toxins. If the 0.52 μM component alone represents the sodium channel, then there are only about 4 sodium channels/μm².

The estimate of a 13,800-μm² surface area/myocyte includes the area of membranes in T-tubules and caveoli (18). There is no evidence regarding the presence or absence of sodium channels in these structures in ventricular myocytes, which include about 44% of the area of myocyte surface membranes. However, sodium channels are present in skeletal muscle T-tubules (7). If channels are not present in these structures in heart myocytes, then the estimate of surface density on non-T-tubule plasmalemma of the channels would be about 80% higher.

We have not corrected the estimate of surface density for the fraction of viable cells because both cell viability and binding vary with time, and because the issue of the necessity of cell viability for toxin binding has not been systematically addressed.

TABLE 1

Comparison of K_D values for alkaloid toxins for nerve and cardiac sodium channels

Preparation	BTX-B	Batrachotoxin	Aconitine
Synaptosomes	0.08	0.05	1.2
Cardiomyocytes	0.03	0.07	1.2

Values of K_D (μM) for binding to cardiomyocytes measured in this study were compared to those for binding to synaptosomes described by Catterall *et al.* (8).

[1] R. Sheldon, N. J. Cannon, and H. J. Duff, submitted for publication.

The density of sodium channels or myocyte surface membranes has also been estimated from patch-clamp data and from [^{3}H]saxitoxin radioligand binding data. Fozzard *et al.* (21), using patch-clamp data, estimated that there are 2–10 channels/μm^2 of rat or rabbit ventricular myocyte membrane. Doyle and Page (22) examined [^{3}H]saxitoxin binding to rat ventricular hemogenates. Saxitoxin binds to the tetradotoxin receptor. The data suggest that there are 8.5 sodium channels/μm^2 of total plasmalemma, which includes both external membrane and T-tubules. Thus, three different techniques—[^{3}H]BTX-B binding, patch-clamp analysis, and [^{3}H]saxitoxin binding—all suggest that there are about 10 sodium channels/μm^2 of myocyte membrane surface area.

In conclusion, we have demonstrated that ATX and [^{3}H]BTX-B bind to the sodium channel on adult rat cardiac myocytes. A portion of the binding is voltage sensitive. This method will enable us to study the interactions of antiarrhythmic drugs[2] with the channel and also opens the possibility of studying the metabolism and response to ischemia of the cardiac sodium channel.

Acknowledgments

We wish to thank Dr. W. Giles for the microelectrode measurements, Dr. K. McCannell for his encouragement, and Mr. G. Douglas for invaluable help with the manuscript.

Appendix

The binding of ATX was characterized by its concentration-dependent stimulation of [^{3}H]BTX-B binding to myocytes because no direct measure of ATX binding was available. Below we show that this approach is amenable to Scatchard analysis.

Scatchard analysis is a method of examining binding of a ligand, L, to its receptor, R, to form receptor-ligand complexes with a dissociation constant, K_D. Thus,

$$K_D = \frac{[R][L]}{[RL]} \tag{1}$$

If the total number of receptors is R_T, then

$$R_T = R + RL \tag{2}$$

The Scatchard rearrangement of Eqs. 1 and 2 is

$$\frac{[RL]}{[L]} = \frac{R_T}{K_D} - \frac{[RL]}{K_D} \tag{3}$$

This is commonly expressed as

$$\frac{\text{Bound ligand}}{\text{Free ligand}} = \frac{B_{max}}{K_D} - \frac{\text{Bound ligand}}{K_D} \tag{4}$$

We make two assumptions. The first is that specific binding of [^{3}H]BTX-B to myocytes does not occur in the absence of ATX. Specific binding of [^{3}H]BTX-B in the absence of ATX has not been observed under our conditions. Thus, our experimental data confirm that [^{3}H]BTX-B binding is dependent upon prior ATX binding. The second assumption is that the affinity of ATX for its binding site is not affected to a large extent by the presence of bound [^{3}H]BTX-B (23). The nature of our method precludes directly testing this.

In our analysis ATX is the ligand and R the receptor for ATX on the sodium channel. Let R_T represent the total number of sodium channels, R the channels without bound toxins, R_A the channels to which ATX is bound, R_B the channels to which [^{3}H]BTX-B is bound, and R_{AB} the channels to which both toxins are bound. However, $R_B \rightarrow 0$. Then, by analogy with Eq. 2:

$$R_T = R_A + R_{AB} + R \tag{5}$$

Also, by analogy with Eq. 1:

$$K_D = \frac{[R][A]}{[R_A + R_{AB}]} \tag{6}$$

By rearranging Eqs. 5 and 6 to the Scatchard form we obtain (see Eq. 3):

$$\frac{[R_A + R_{AB}]}{[A]} = \frac{[R_T]}{K_D} - \frac{[R_A + R_{AB}]}{K_D} \tag{7}$$

Notice that since ATX is considered to be the ligand, then $[R_A + R_{AB}]$ represents *bound* ATX, $[A]$ represents *free* ATX, and R_T represents B_{max}. Thus, Eq. 7 takes the familiar form of Eq. 4:

$$\frac{\text{Bound}}{\text{Free}} = \frac{B_{max}}{K_D} - \frac{\text{Bound}}{K_D}$$

The term $[R_A + R_{AB}]$ represents channels to which ATX is bound, but only R_{AB} is radioactively labeled. At higher concentrations of [^{3}H]BTX-B, the fraction of ATX-bound channels labeled will approach 1, whereas at lower concentrations of [^{3}H]BTX-B, that fraction will approach 0.

Therefore, plots of $\frac{[R_{AB}]}{[A]}$ versus $[R_{AB}]$ will give a family of curves whose slopes are the same and reflect the K_D for ATX binding but whose B_{max} values reflect the [^{3}H]BTX-B concentration in the assay. Our data are plotted using the equation

$$\frac{[R_{AB}]}{[A]} = \frac{B_{max}}{K_D} - \frac{[R_{AB}]}{K_D} \tag{8}$$

Thus, a plot of $\frac{[R_{AB}]}{[A]}$ versus $[R_{AB}]$ yields an estimate of the K_D for ATX and a B_{max} at the ambient concentration of [^{3}H]BTX-B.

[2] R. Sheldon, N. J. Cannon, and H. J. Duff, submitted for publication.

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