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Class I Antiarrhythmic Drug Receptor: Biochemical Evidence for State-Dependent Interaction with Quinidine and Lidocaine

ROGER J. HILL, HENRY J. DUFF, and ROBERT S. SHELDON

Cardiovascular Research Group, Department of Medicine, University of Calgary, Calgary, Alberta, Canada

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SUMMARY

The state-dependent binding of class I antiarrhythmic drugs to a receptor associated with the cardiac sodium channel was assessed using [^{3}H]batrachotoxinin A 20-α-benzoate ([^{3}H]BTXB) binding. [^{3}H]BTXB binds specifically to and stabilizes activated states of the sodium channel. Quinidine (IC_{50} = 40 μM) and lidocaine (IC_{50} = 61 μM) inhibited equilibrium [^{3}H]BTXB binding to sodium channels present on freshly isolated rat cardiac myocytes. Scatchard analysis of [^{3}H]BTXB binding in the presence of quinidine and lidocaine revealed two apparent patterns of inhibition. Quinidine (33 μM) increased the K_D but had no significant effect on the B_{max}, whereas lidocaine (91 μM) reduced the B_{max} but had no significant effect on the K_D. To address drug binding to activated and nonactivated states, we exploited the state-specific binding of [^{3}H]BTXB. Drugs that increase the rate of dissociation (k_{-1}) of [^{3}H]BTXB must bind to sodium channels to which [^{3}H]BTXB is already bound (i.e., activated channels). Therefore, drug-mediated increases in k_{-1} measure drug binding to activated states. Both quinidine and lidocaine increased the k_{-1} of [^{3}H]BTXB, indicating drug binding to and destabilization of activated sodium channels. However, the minimal affinities of quinidine and lidocaine for activated channels (K_D^{act}) were estimated to be 433 and 455 μM, respectively, concentrations much higher than the equilibrium IC_{50} values. Drugs that allosterically decrease the rate of association (k_{+1}) of [^{3}H]BTXB must bind to sodium channels to which [^{3}H]BTXB is not already bound (i.e., nonactivated channels). Therefore, drug-mediated decreases in k_{+1} measures drug binding to nonactivated states. Quinidine and lidocaine decreased the k_{+1} of [^{3}H]BTXB, indicating drug binding to and stabilization of nonactivated sodium channels. The affinity of quinidine and lidocaine for nonactivated channels (K_D^{non}) was estimated to be 10 and 35 μM, respectively, concentrations close to the equilibrium IC_{50} values. The markedly different K_D^{act} and K_D^{non} values for both quinidine and lidocaine indicate state-dependent binding of quinidine and lidocaine to the class I receptor on the cardiac sodium channel. Both drugs destabilize activated channels and stabilize nonactivated channels. The Scatchard results suggest that quinidine and lidocaine may have different mechanisms of allosteric inhibition of [^{3}H]BTXB binding.

The antiarrhythmic effect of class I drugs is believed to be mediated by the interaction of the drugs with a receptor associated with the cardiac sodium channel (1–3). Drug binding to this site is thought to decrease both the maximum rate of rise of the action potential upstroke (4) and the conduction velocity. These electrophysiologic effects of class I drugs have been shown to be both frequency and voltage dependent (1, 4, 5). Two major hypotheses have been proposed to explain the electrophysiologic characteristics of class I drugs in terms of state-dependent drug binding to a channel-associated receptor. The modulated receptor hypothesis (1, 6) proposes a receptor with state-dependent binding affinities. In contrast, the guarded receptor hypothesis (2) proposes a constant affinity receptor in which drug access to the receptor is affected by the state of the channel. Notably, both hypotheses predict state-dependent binding of class I antiarrhythmic drugs to a channel-associated receptor.

However, there are significant electrophysiologic differences between class I drugs that may reflect the manner in which they interact with the sodium channel. For example, the frequency- and voltage-dependent characteristics are quite distinct for quinidine and lidocaine (7–9). Quinidine, when compared with lidocaine, produces a significantly slower development of sodium channel blockade and has a significantly longer half-time of recovery from frequency-dependent block. Both models explain these differences on the basis of different state-dependent interactions between the drugs and their receptor. Restated in biochemical terms, both models presuppose a state-dependent interaction between small ligands (drugs) and a receptor (associated with the sodium channel).

Adopting a biochemical approach, we have recently used [^{3}H]BTXB, a sodium channel-specific toxin, to study the interaction of class I antiarrhythmic drugs with a receptor associated with sodium channels present on rat cardiac myocytes (3, 10–13). Class I drugs inhibit [^{3}H]BTXB binding at pharmacologi-

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ABBREVIATIONS: [^{3}H]BTXB, [^{3}H]batrachotoxinin A 20-α-benzoate; ATX, sea anemone toxin II.

cally relevant concentrations with the same rank order of potency *in vitro* as *in vivo*, suggesting that binding to this site is relevant to clinical effect. BTXB might prove useful in studying state-dependent drug binding because it binds specifically to and stabilizes an activated state of the channel (14–16). This state-specific binding by BTXB has previously been exploited to evaluate state-dependent binding by class I antiarrhythmic drugs, local anesthetics, and anticonvulsants to the sodium channel (3, 17–19). Two basic approaches have been employed. First, the general nature of drug inhibition of [^{3}H]BTXB binding has been assessed at steady state using Scatchard analysis of concentration-dependent [^{3}H]BTXB binding. This analysis has shown that lidocaine (3) and amiodarone (20) both reduce the B_{max} for [^{3}H]BTXB binding, suggesting that these drugs allosterically inhibit [^{3}H]BTXB binding by stabilizing a nonactivated state of the channel and, thereby, decreasing the number of activated channels available for [^{3}H]BTXB binding.

The second basic approach has been a kinetic analysis of [^{3}H]BTXB binding, which again exploits the state-specific binding of [^{3}H]BTXB to activated channels. For example, in the nerve sodium channel, local anesthetics and anticonvulsants increase the rate of dissociation of [^{3}H]BTXB from activated sodium channel complexes (17, 19), indicating that the drugs must bind to activated sodium channel complexes that already contain [^{3}H]BTXB. Thus, the concentration dependence of a drug-mediated increase in the rate of dissociation of [^{3}H]BTXB binding reflects the affinity of drugs for activated states of the sodium channel. In contrast, drugs that decrease the rate of association of [^{3}H]BTXB binding most likely do so by binding to sodium channels to which [^{3}H]BTXB is not already bound and by stabilizing a state to which [^{3}H]BTXB cannot bind (i.e., nonactivated channels). Therefore, the concentration dependence of a drug-mediated decrease in the rate of association of [^{3}H]BTXB binding to sodium channels may reflect the affinity of the drugs for nonactivated states of the sodium channel. For example, (+)-neopseudococaine significantly decreases the rate of association of [^{3}H]BTXB with nerve sodium channels (21). The authors concluded that this drug effect most likely reflects the binding of cocaine to a nonactivated state of the sodium channel, thereby inhibiting [^{3}H]BTXB binding to sodium channels.

In the present study, we have used [^{3}H]BTXB as a molecular probe to evaluate state-dependent binding of class I antiarrhythmic drugs to cardiac sodium channels. We have used steady state and kinetic binding techniques to compare the interaction of quinidine and lidocaine with their receptor on freshly isolated rat cardiac myocytes.

Materials and Methods

Myocyte preparation. Cardiac myocytes were isolated from adult male Sprague-Dawley rats (200–250 g) by collagenase dispersion, using the method of Kryski *et al.* (22), as previously described (3). This method routinely yielded about 60 mg (dry weight) of myocytes, which corresponds to 1.2×10^7 cells (22–23). The cells were 80–85% viable rod-shaped cells that excluded trypan blue and were tolerant of 1 mM calcium.

Equilibrium binding assays. Equilibrium binding assays were performed as described previously (3). Myocytes (6×10^5/assay) in 50 μl of incubation buffer were incubated with 1.3 μM ATX, 13 nM [^{3}H]BTXB (50 Ci/mmol), and 0.13 mM tetrodotoxin for 45–60 min at 37° (see Ref. 24 for a review of sodium channel toxins). Tetrodotoxin was added to prevent depolarization induced by sodium influx; without tetrodotoxin, no specific binding is observed (10, 19). Various concentrations of drugs and toxins were included in the incubations. Assays were done in parallel with tubes containing 0.4 mM aconitine to define nonspecific binding. Reactions were terminated by adding 10 ml of Krebs-Henseleit-bovine serum albumin 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 $CaCl_2$, and 1% bovine serum albumin) that was equilibrated with 95% O_2/5% CO_2, incubated at 37° for 1 min, filtered through a Whatman GF-C 24 mm filter, and washed four times with 5 ml of rinse buffer (25 mM Tris·HCl, pH 7.4, 130 mM NaCl, 5.5 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 50 μM $CaCl_2$. The filters were then dried and counted in Econofluor scintillation fluid. The retained radioactivity represents [^{3}H]BTXB bound to myocytes.

The rationale for the incubation and filtration conditions have previously been described (10). The conditions provide a maximal reduction in background and scatter with a minimal reduction in specific binding. The total wash time is 45 sec. Initial control experiments showed that under these conditions less than 10% of the specifically bound [^{3}H]BTXB dissociated from the complex. Under these reaction conditions (13 nM [^{3}H]BTXB, 0.13 mM tetrodotoxin, and 1.3 μM ATX), about 60–75% of the total radioactivity retained on the filters is bound specifically to the [^{3}H]BTXB binding site.

Kinetic binding assays. The rate of dissociation of [^{3}H]BTXB was measured by preincubating myocytes (6×10^5/assay) for 55 min with 13 nM [^{3}H]BTXB, 1.3 μM ATX, and 0.13 mM tetrodotoxin at 37°. Dissociation of [^{3}H]BTXB was initiated by volumetric dilution (1:5) with incubation buffer containing 1.3 μM ATX, 0.13 mM tetrodotoxin, and 0.4 mM aconitine, in the absence or presence of quinidine or lidocaine. An excess of aconitine (an alkaloid competitive inhibitor of [^{3}H]BTXB) was included in the dissociation solution in order to block reassociation of [^{3}H]BTXB with its toxin binding site, which is a standard procedure for this type of measurement (17, 25, 26). Incubation was continued at 37° and reactions were terminated by rapid filtration at successive times thereafter, followed by estimation of bound [^{3}H]BTXB as described above. Assays were carried out in parallel containing 0.4 mM aconitine throughout the experiment, to determine nonspecific binding at each time point.

The rate of association of [^{3}H]BTXB was measured by preincubating myocytes with 1.3 μM ATX and 0.13 mM tetrodotoxin for 30 min at 37°, in the absence or presence of antiarrhythmic drug. [^{3}H]BTXB (13 nM) was then added and myocytes (6×10^5/assay) were incubated for various amounts of time at 37°. Using this method, there was no lag time in the onset of [^{3}H]BTXB binding (Fig. 4B), indicating that binding of other toxins had reached equilibrium before the addition of [^{3}H]BTXB. Assays were carried out in parallel with tubes containing 0.4 mM aconitine throughout the experiment, to determine nonspecific binding at each time point. Incubations were terminated at successive time points by rapid filtration and [^{3}H]BTXB bound to myocytes was estimated as described above.

Class I antiarrhythmic drugs have been shown to interact with the cardiac sodium channel with time constants on the order of 1 msec to 10 sec (1). Therefore, we assume that the effects of quinidine and lidocaine on the kinetics of [^{3}H]BTXB binding are not time dependent.

Data analysis. Definitions are as follows: k_{-1}, dissociation rate constant for [^{3}H]BTXB in the absence of antiarrhythmic drugs; k_{-1}^M, measured dissociation rate constant in the presence of drugs; k_{+1}, association rate constant for [^{3}H]BTXB in the absence of antiarrhythmic drugs; k_{+1}^M, measured association constant for [^{3}H]BTXB in the presence of drugs.

The dissociation rate constant, k_{-1} of [^{3}H]BTXB binding was calculated from the equation $\ln(SB_t/SB_0) = -k_{-1}t$ (27), where SB_t = specific binding of [^{3}H]BTXB at time t and SB_0 = specific binding of [^{3}H]BTXB at time zero. A plot of $\ln(SB_t/SB_0)$ versus t, in the absence or presence of antiarrhythmic drug, was linear with a slope of k_{-1}. Initially, the effects of quinidine and lidocaine on k_{-1} (Fig. 5) were assessed using multiple time points ranging from 0 to 90 min.

To measure the concentration-dependent effects of quinidine or lidocaine on k_{-1} (Fig. 6), duplicate samples were withdrawn at 60 min in the absence and presence of each drug concentration, an approach that has recently been adopted in the nerve sodium channel by Reith *et al.* (21). This is possible because plots of $\ln(SB_t/SB_0)$ versus t, in the absence or presence of drugs, were linear, with a slope of $-k_{-1}$ (Figs. 4A and 5). Thus, k_{-1} was independent of t. The k_{-1} of [^{3}H]BTXB in the absence of drug and for each concentration of drug was then calculated, using the equation described above. The concentration-dependent changes are plotted as k_{-1} versus [drug], where [drug] represents drug concentration. These data may allow an estimation of the affinity of drugs for activated sodium channels, K_I^{act} (see Appendix).

The association rate constant, k_{+1}, of [^{3}H]BTXB binding was calculated from the equation: $\ln(SB_{eq}/SB_{eq} - SB_t) = ([L]k_{+1} + k_{-1})t$ (27), where SB_{eq} = specific binding of [^{3}H]BTXB at equilibrium time (55 min), SB_t = specific binding of [^{3}H]BTXB at time t, $[L]$ = concentration of [^{3}H]BTXB (nM), and k_{-1} = rate of dissociation of [^{3}H]BTXB at the ambient drug concentration. The 55-min time period was sufficient to achieve equilibrium conditions both in the absence (Fig. 4B) and presence of antiarrhythmic drugs (Fig. 1). A plot of $\ln(SB_{eq}/SB_{eq} - SB_t)$ versus t, in the presence or absence of antiarrhythmic drug, was linear, with a slope of $([L]k_{+1} + k_{-1})$. Because $[L]$ and k_{-1} were known, k_{+1} could be calculated. Initially, the effects of quinidine and lidocaine on k_{+1} (Fig. 7) were assessed using multiple time points ranging from 4 to 55 min.

To measure the concentration-dependent effects of quinidine and lidocaine on k_{+1} (Fig. 8), duplicate samples were withdrawn at 10 min in the absence and presence of each drug concentration. Duplicate samples at 55 min (equilibrium) were also included for the absence of drug and for each drug concentration. This is possible because plots of $\ln(SB_{eq}/SB_{eq} - SB_t)$ versus t, in the absence or presence of drugs, were linear, with a slope of $([L]k_{+1} + k_{-1})$ (Figs. 4B and 7). Thus k_{+1} was independent of t. The k_{+1} in the absence of drug and for each concentration of drug was then calculated using the equation described above. The concentration-dependent changes are plotted as $\%(k_{+1_{drug}}/k_{+1_{drug\text{-}free}})$ versus [drug], where [drug] represents drug concentration. These data allow an estimation of the affinity of drugs for nonactivated sodium channels, K_I^{non} (see Appendix).

Experimental design of kinetic binding assays. Experiments were designed to compare the concentration-dependent effects of drugs on the association kinetics of [^{3}H]BTXB with their concentrations-dependent effects on equilibrium [^{3}H]BTXB binding. When comparing K_I^{non} values with IC_{50} values (Fig. 8), experiments were carried out in parallel using common preparations of cells, toxins, and drugs on the same day. This paired design eliminates any artifactual differences that might be produced by day-to-day variability of cell preparations and toxin and drug concentrations.

Materials. The incubation buffer was Jokliks minimal essential medium supplemented with 1.2 mM $MgSO_4$ and 1 mM *dl*-carnitine, with 1 mM $CaCl_2$ and 1% fatty acid-free bovine albumin.

[^{3}H]BTXB was purchased from New England Nuclear; collagenase from Cooper Biomedical; tetrodotoxin, aconitine, albumin, and ATX from Sigma; quinidine from Aldrich Chemical Company; and lidocaine from Astra.

Results

Inhibition of equilibrium [^{3}H]BTXB binding to myocytes. Class I antiarrhythmic drugs inhibit [^{3}H]BTXB binding to sodium channels on rat cardiac myocytes (3). Fig. 2 displays representative curves that show that both quinidine and lidocaine inhibit [^{3}H]BTXB binding in a dose-dependent manner, with IC_{50} values of 28 and 69 μM, respectively. The mean IC_{50} values (± SD) were 40 ± 12 μM (six experiments) for quinidine and 61 ± 25 μM (five experiments) for lidocaine. The mean pseudo-Hill coefficients (±SD) were 0.89 ± 0.1 for quinidine and 0.87 ± 0.1 for lidocaine.

The nature of drug-mediated inhibition of [^{3}H]BTXB binding was assessed at steady state using Scatchard analysis (28) of concentration-dependent [^{3}H]BTXB binding. In the absence of drugs, [^{3}H]BTXB bound to a single class of binding sites with a mean K_D (±SD) of 18 ± 5 nM and a mean number of binding sites (B_{max}) (±SD) of 17 ± 5 fmol/10^5 cells (12 experiments), similar to results reported previously (3). The effects of quinidine and lidocaine on Scatchard plots of [^{3}H]BTXB binding are shown in Fig. 3. Quinidine (33 μM) increased the K_D of [^{3}H]BTXB from 23 to 41 nM but had no significant effect

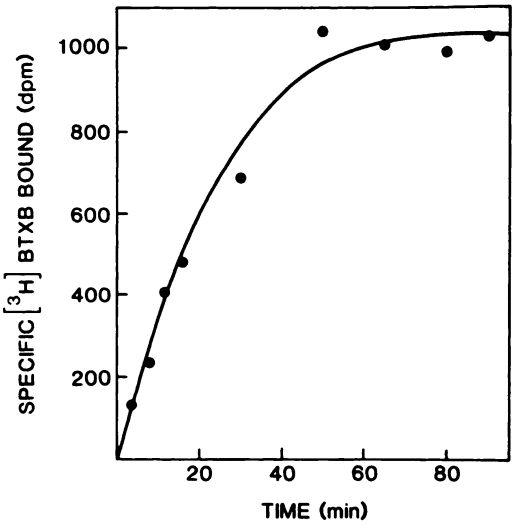

Fig. 1. Association kinetics of [^{3}H]BTXB binding to myocytes in the presence of lidocaine. Myocytes were preincubated with 1.3 μM ATX, 0.13 mM tetrodotoxin, and 50 μM lidocaine for 30 min. At time 0, [^{3}H] BTXB (10 nM) was added and myocytes (6 × 10^5 assay) were incubated for the indicated amounts of time. Specifically bound [^{3}H]BTXB was measured at each time point as described under Materials and Methods.

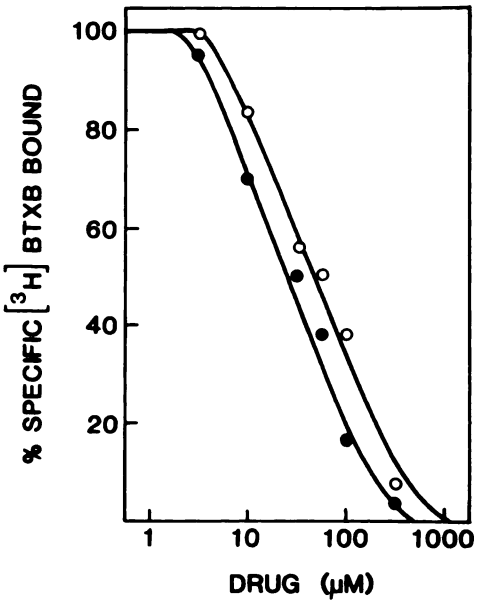

Fig. 2. Effect of quinidine and lidocaine on [^{3}H]BTXB binding to myocytes. Myocytes (6 × 10^5/assay) were incubated for 55 min with 13 nM [^{3}H] BTXB, 0.13 mM tetrodotoxin, 1.3 μM ATX, and various concentrations of quinidine (●) and lidocaine (○). Specifically bound [^{3}H]BTXB was measured as described under Materials and Methods.

on B_{max} (Fig. 3A). In contrast, lidocaine (91 μM) reduced B_{max} from 12 to 4 fmol of [^{3}H]BTXB but had no significant effect on K_D (Fig. 3B), in keeping with previously reported allosteric noncompetitive inhibition of [^{3}H]BTXB binding by lidocaine (3).

Association and dissociation kinetics of [^{3}H]BTXB binding to myocytes. The dissociation rate constant (k_{-1}) of [^{3}H]BTXB from its binding site was first determined in the absence of antiarrhythmic drugs. Fig. 4A shows that the dissociation of [^{3}H]BTXB occurred exponentially and the dissociation rate constant k_{-1} (Fig. 4A, *inset*) was 6.0×10^{-3} min^{-1}. The mean k_{-1} (±SD; 10 experiments) for [^{3}H]BTXB binding was $9.8 \pm 2.5 \times 10^{-3}$ min^{-1}.

We then determined the association rate constant (k_{+1}) of [^{3}H]BTXB with its binding site in the absence of antiarrhythmic drug. Fig. 4B shows that [^{3}H]BTXB binding reaches equilibrium by 55 min. The association rate constant k_{+1} (Fig. 4B, *inset*) was 3.6×10^{-3} nM^{-1} min^{-1}. The mean k_{+1} (±SD; seven experiments) of [^{3}H]BTXB binding was estimated to be $3.5 \pm 0.7 \times 10^{-3}$ nM^{-1} min^{-1}.

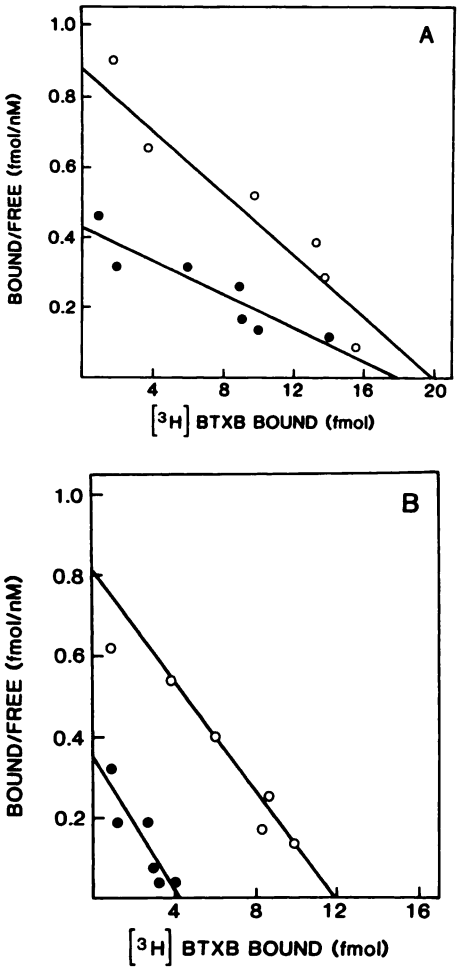

Fig. 3. Scatchard analysis of the effect of quinidine (A) and lidocaine (B) on [^{3}H]BTXB binding to myocytes. A, Myocytes (6×10^5/assay) were incubated with 0.13 mM tetrodotoxin, 1.3 μM ATX, and various concentrations of [^{3}H]BTXB in the absence (○) or presence (●) of 33 μM quinidine. Specifically bound [^{3}H]BTXB was measured as described under Materials and Methods and subjected to Scatchard analysis. Linear regression best fit values were B_{max} of 20 fmol and K_D of 23 nM in the absence of quinidine ($r = 0.90$), and B_{max} of 18 fmol and K_D of 41 nM in the presence of quinidine ($r = 0.92$). B, Experimental conditions and procedures were the same as above except that myocytes were incubated in the absence (○) or presence of 92 μM lidocaine (●). Linear regression best fit values were B_{max} of 12 fmol and K_D of 14 nM in the absence of lidocaine ($r = 0.96$) and B_{max} of 4 fmol and K_D of 11 nM in the presence of lidocaine ($r = 0.84$).

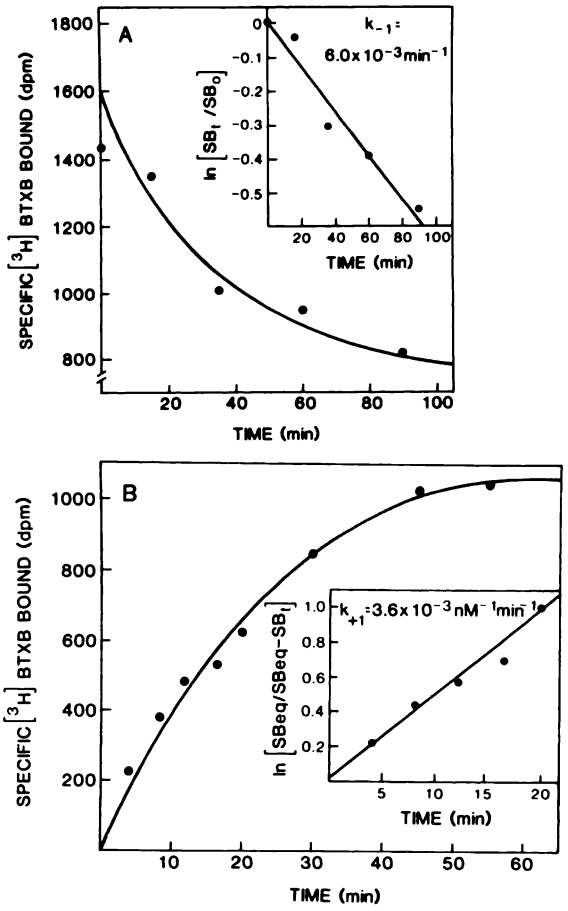

Fig. 4. Dissociation and association kinetics of [^{3}H]BTXB binding to myocytes. A, Myocytes (6×10^5/assay) were incubated for 55 min with 13 nM [^{3}H]BTXB, 1.3 μM ATX, and 0.13 mM tetrodotoxin and, at time 0, dissociation was initiated by volumetric dilution (1:5) with incubation buffer containing 1.3 μM ATX, 0.13 mM tetrodotoxin, and 0.4 mM aconitine. Incubation was continued and specifically bound [^{3}H]BTXB at indicated time points was measured as described under Materials and Methods. The dissociation rate constant (k_{-1}) was estimated from the slope of the semilogarithmic plot (*inset*) by linear regression ($r = 0.96$). B, Myocytes were preincubated with 1.3 μM ATX and 0.13 mM tetrodotoxin for 30 min. At time 0, [^{3}H]BTXB (10 nM) was added and myocytes (6×10^5/assay) were incubated for the indicated amounts of time. Specifically bound [^{3}H]BTXB was measured at each time point as described under Materials and Methods. The association rate constant (k_{+1}) was estimated from the slope of the semilogarithmic plot (*inset*) by linear regression ($r = 0.99$).

Effect of quinidine and lidocaine on the dissociation kinetics of [^{3}H]BTXB binding from myocytes. The effects of quinidine and lidocaine on the dissociation rate constant of [^{3}H]BTXB binding is shown in Fig. 5, A and B, respectively. Quinidine (250 μM) increased k_{-1} for [^{3}H]BTXB binding from 7.6×10^{-3} min^{-1} to 21.3×10^{-3} min^{-1}, whereas lidocaine (100 μM) increased k_{-1} from 6.0 to 10^{-3} min^{-1} to 10.0×10^{-3} min^{-1}. Thus, both quinidine and lidocaine increased the rate of dissociation of [^{3}H]BTXB binding from cardiac sodium channels.

The effect of a full range of concentrations of both quinidine and lidocaine on the k_{-1} of [^{3}H]BTXB binding is shown in Fig. 6. These curves were generated as described in Material and

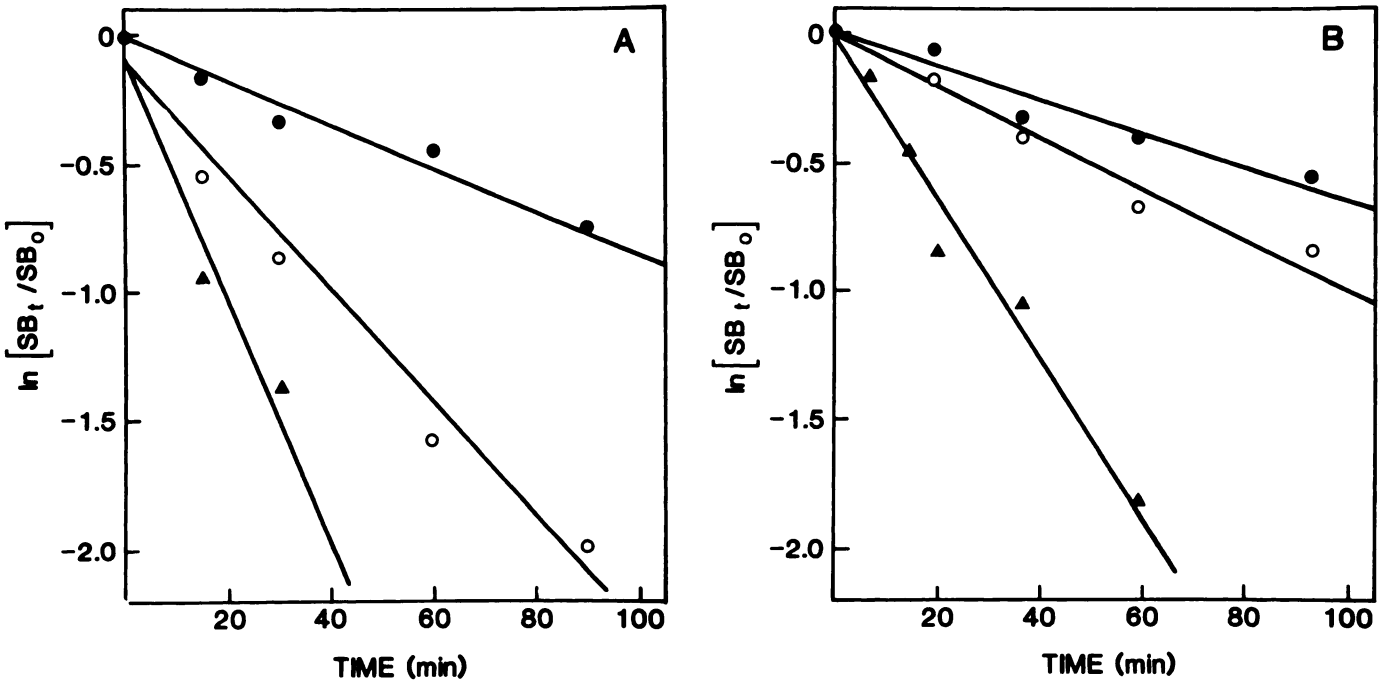

Fig. 5. Effect of quinidine (A) and lidocaine (B) on the dissociation kinetics of [^{3}H]BTXB binding from myocytes. Experimental conditions and procedures were the same as those described under Fig. 4A, except that, at time 0, dissociation was initiated in the absence (●) or the presence of quinidine at 250 μM (○) or 500 μM (▲) (A) and the absence (●) or the presence of lidocaine at 100 μM (○) or 1000 μM (▲) (B). The dissociation rate constant (k_{-1}) of [^{3}H]BTXB in the absence of drug and for each concentration of drug was estimated from the slope of the semilogarithmic plot by linear regression. Each *point* is the mean of duplicate determinations.

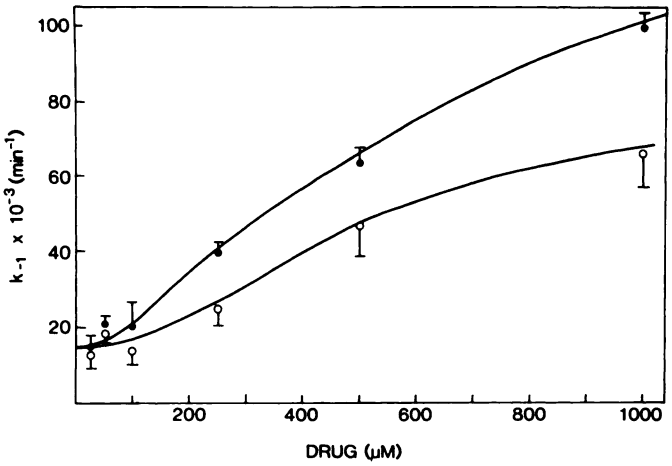

Fig. 6. Concentration-dependent effect of quinidine and lidocaine on the k_{-1} of [^{3}H]BTXB binding to myocytes. Experimental conditions and procedures were the same as those described for Fig. 4A, except that, at time 0, dissociation was initiated in the presence or absence of various concentrations of quinidine (●) and lidocaine (○). Incubation was continued and specifically bound [^{3}H]BTXB was measured in duplicate at 60 min. The dissociation rate constant (k_{-1}) in the absence of drug and for each drug concentration was estimated as described under Materials and Methods. Each *point* is the mean ± standard deviation of two to four determinations. The mean value of k_{-1} in the absence of drug was $13 \pm 3 \times 10^{-3}$ min^{-1}.

Methods and the results are plotted as k_{-1} versus [drug], where [drug] represents drug concentration. Both quinidine and lidocaine increased k_{-1} in a concentration-dependent manner. The limited solubility of supramillimolar concentrations of quinidine at physiologic pH precluded the determination of the saturability of this effect for quinidine. However, assuming a minimum value of 100×10^{-3} min^{-1} for the maximum drug-associated rate constant for [^{3}H]BTXB in the presence of quinidine, these data may allow an estimation of the affinity of quinidine for activated sodium channels, K_D^{act} (see Appendix). The K_D^{act} for quinidine was estimated to be at least 433 μM. Similarly, assuming an minimum value of 70×10^{-3} min^{-1} for the maximum drug-associated rate constant for [^{3}H]BTXB in the presence of lidocaine, the K_D^{act} for lidocaine was estimated to be at least 455 μM. Significantly, the minimum estimated K_D^{act} values for quinidine and lidocaine are 11-fold and 8-fold greater than their respective mean IC_{50} values for inhibition of equilibrium [^{3}H]BTXB binding. Although quantitatively the estimated K_D^{act} values may not be exact, it is clear from Fig. 6 that, at concentrations less than 100 μM, these drugs have little or no effect on the k_{-1} of [^{3}H]BTXB binding.

Effect of quinidine and lidocaine on the association kinetics of [^{3}H]BTXB binding to myocytes. The effect of a single concentration of quinidine and lidocaine on the association rate constant of [^{3}H]BTXB binding is shown in Fig. 7, A and B, respectively. Quinidine (33 μM) reduced k_{+1} from 3.0 $\times 10^{-3}$ nM^{-1} min^{-1} to 1.6×10^{-3} nM^{-1} min^{-1}, whereas lidocaine (65 μM) reduced k_{+1} from 2.3×10^{-3} nM^{-1} min^{-1} to 0.6×10^{-3} nM^{-1} min^{-1}. Thus, both quinidine and lidocaine, at concentrations close to their IC_{50} values, decreased the rate of association of [^{3}H]BTXB binding to cardiac sodium channels. Experiments were then designed to compare the abilities of quinidine and lidocaine to decrease the rate of association of [^{3}H]BTXB to its binding site with their potencies for inhibition of equilibrium [^{3}H]BTXB binding. These experiments were carried out in parallel using a paired design, as described under Materials and Methods.

In the presence of increasing concentrations of quinidine, k_{+1}

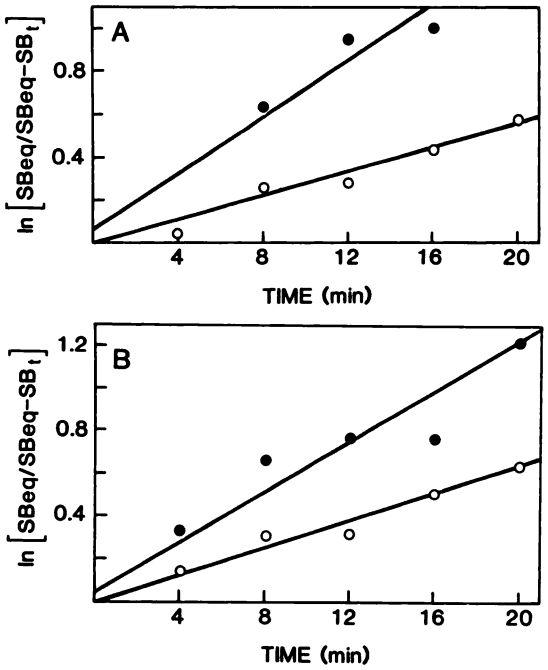

Fig. 7. Effect of quinidine (A) and lidocaine (B) on the association kinetics of [^{3}H]BTXB binding to myocytes. Experimental conditions and procedures were the same as those described for Fig. 4B, except that myocytes were preincubated for 30 min in the absence (●) or presence (○) of 33 μM quinidine (A) and the absence (●) or presence (○) of 65 μM lidocaine (B). Each *point* is the mean of duplicate determinations.

was decreased in a concentration-dependent manner (Fig. 8A) and is plotted as $\%(k_{+1_{quinidine}}/k_{+1_{drug\text{-}free}})$ versus [quinidine] (see Materials and Methods). These data allow an estimation of the affinity of quinidine for nonactivated sodium channels, K_I^{non} (see Appendix). The K_I^{non} for quinidine was estimated to be 10 μM. In the same experiment, quinidine inhibited equilibrium [^{3}H]BTXB binding in a dose-dependent manner, with an IC_{50} of 20 μM (Fig. 8A). Thus, the K_I^{non} of quinidine is similar to its IC_{50} for inhibition of equilibrium [^{3}H]BTXB binding.

In the presence of increasing concentrations of lidocaine, k_{+1} was also decreased in a concentration-dependent manner (Fig. 8B) and is plotted as for quinidine above. The K_I^{non} of lidocaine was estimated to be 35 μM. In the same experiment, lidocaine inhibited equilibrium [^{3}H]BTXB binding in a dose-dependent manner, with an IC_{50} of 60 μM (Fig. 8B). Thus, the K_I^{non} of lidocaine is similar to its IC_{50} for inhibition of equilibrium [^{3}H]BTXB binding.

Discussion

A receptor for class I antiarrhythmic drugs. There is substantial electrophysiologic evidence that class I antiarrhythmic drugs interact with a specific receptor associated with the cardiac sodium channel. As well, there is evidence that class I drugs such as lidocaine and quinidine interact with the channel with different electrophysiologic patterns (7–9). For example quinidine, when compared with lidocaine, produces a significantly slower development of sodium channel blockade and has a significantly longer half-time of recovery from frequency-dependent block. The modulated receptor hypothesis (1, 6) and the guarded receptor hypothesis (2) propose that the different electrophysiologic profiles of class I drugs are the result of state-dependent drug binding to a receptor associated with the sodium channel. In biochemical terms, both hypotheses presuppose an interaction between small ligands (drugs) and a receptor (on the sodium channel). However, the hypothesis of state-dependent binding of class I drugs has not been directly assessed using biochemical techniques.

The purpose of this study was to assess state-dependent antiarrhythmic drug binding, using a radioligand assay involving [^{3}H]BTXB. BTXB is a sodium channel-specific toxin that causes persistent activation of the sodium channel by binding specifically to and stabilizing the activated state of the channel (14–16). The development of a radioactive derivative of BTXB, [^{3}H]BTXB, has provided a biochemical approach for the study of the interaction of drugs with sodium channel-associated receptors (3, 19). We have recently used this approach to identify a receptor for class I antiarrhythmic drugs that is associated with the cardiac sodium channel (3). Class I drugs

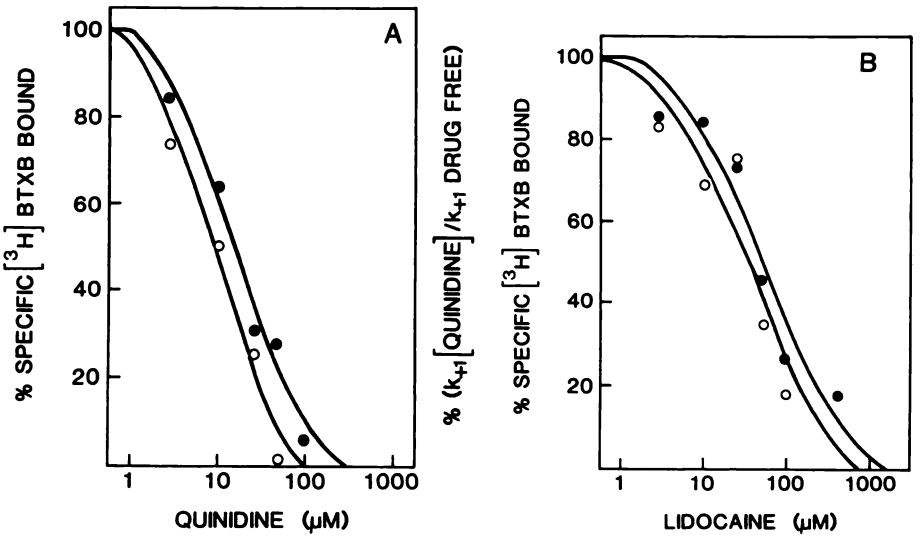

Fig. 8. Comparison of the concentration-dependent effect of quinidine and lidocaine on the k_{+1} of [^{3}H]BTXB with their inhibition of equilibrium [^{3}H]BTXB binding. *Curves* were generated in parallel using a common preparation of cells. ○, Myocytes were preincubated for 30 min with 1.3 μM ATX, 0.13 mM tetrodotoxin and various concentrations of quinidine (A) and lidocaine (B). At time 0, [^{3}H]BTXB was added and myocytes (6×10^6/assay) were incubated further. Specifically bound [^{3}H]BTXB was measured in duplicate at 10 and 55 min and the association rate constant (k_{+1}) in the absence of drug and for each drug concentration was estimated as described under Materials and Methods. Each *point* is the mean of duplicate determinations. ●, Myocytes (6×10^5/assay) were incubated for 55 min with 13 nM [^{3}H]BTXB, 0.13 mM tetrodotoxin, 1.3 μM ATX, and various concentrations of quinidine (A) and lidocaine (B). Specifically bound [^{3}H]BTXB was measured as described under Materials and Methods. Each *point* is the mean of duplicate determinations.

inhibit [^{3}H]BTXB binding in a fashion consistent with their binding to a specific receptor site. This drug effect is saturable, reversible, and stereospecific and occurs at pharmacologically relevant concentrations with similar rank orders of potency *in vitro* and *in vivo*, suggesting that binding to this site is relevant to the clinical effect of the drugs. The specific binding of [^{3}H]BTXB to activated channels suggested a biochemical approach that we have used to characterize the state-dependent interaction of quinidine and lidocaine with their receptor on freshly isolated rat cardiac myocytes.

[^{3}H]BTXB binding. In equilibrium experiments, [^{3}H]BTXB binds saturably and with high affinity to an apparently homogeneous receptor population (Fig. 3). The fact that only a single binding affinity (K_D, 18 nM) is seen suggests that the affinity of [^{3}H]BTXB for the activated state is sufficiently low that it does not contribute significantly to [^{3}H]BTXB binding under our experimental conditions. This agrees with estimates of 400–700 nM for the K_D of batrachotoxin for nonactivated nerve sodium channels (14, 29). Kinetic analysis indicates that [^{3}H]BTXB rapidly associates with and dissociates from this receptor population. The mean k_{-1} for [^{3}H]BTXB binding to the rat cardiac sodium channel (9.8×10^{-3} min^{-1}) is in good agreement with reported values for binding to mouse brain channels (Ref. 17, 9.0×10^{-3} min^{-1}; Ref. 21, 6.8×10^{-3} min^{-1}; Ref. 30, 9.4×10^{-3} min^{-1}). Similarly, the mean k_{+1} for [^{3}H]BTXB binding to the rat cardiac channel (3.5×10^{-3} nM^{-1} min^{-1}) agrees well with estimates of k_{+1} for the rat nerve sodium channel (Ref. 17, 7.0×10^{-3} nM^{-1} min^{-1}). Thus, the association and dissociation rate constants for [^{3}H]BTXB binding to rat cardiac sodium channels agree with the results for binding to mouse nerve sodium channels.

Scatchard analysis: allosteric inhibition. Both quinidine and lidocaine inhibited equilibrium [^{3}H]BTXB binding, with mean IC_{50} values of 40 and 61 μM, respectively, and Hill numbers of 0.89 ± 0.1 and 0.87 ± 0.1, respectively, values similar to those reported previously (3). However, Scatchard analysis of [^{3}H]BTXB binding in the presence of quinidine and lidocaine indicated two apparent patterns of inhibition. Scatchard analysis showed that quinidine had little effect on B_{max} but increased the K_D of [^{3}H]BTXB binding (Fig. 3A). This pattern of inhibition does not clearly distinguish between competitive and noncompetitive inhibition in receptor systems (31). However, there are two lines of evidence that suggest that quinidine is an allosteric competitive inhibitor of [^{3}H]BTXB binding. First, quinidine accelerates the dissociation of the [^{3}H]BTXB-sodium channel complex, providing direct evidence for quinidine binding to a site distinct from that of the [^{3}H]BTXB site. As well, quinidine and local anaesthetics have a mixed competitive/noncompetitive pattern of inhibition for the sodium channel partial agonist veratradine (32). This mixed pattern of inhibition strongly suggests that the drugs are allosteric competitive inhibitors. As well, in studies with nerve sodium channels, local anaesthetics (19), class I antiarrhythmic drugs (32), anticonvulsants (17), and cocaine congeners (21) behave similarly to quinidine. Thus, quinidine is an allosteric competitive inhibitor of [^{3}H]BTXB binding to the cardiac sodium channel.

The results of Scatchard analysis of the effect of lidocaine on [^{3}H]BTXB binding are novel, in that they are characterized by a decrease in B_{max} with little effect on the K_D of [^{3}H]BTXB (Fig. 3B). This pattern of allosteric noncompetitive inhibition has not been reported in studies with nerve sodium channel. It is seen with irreversible inhibitors, but we have previously shown that lidocaine is a reversible inhibitor of [^{3}H]BTXB binding (3). However, it is characteristic of allosteric binding in a number of other receptor systems, including the β-adrenergic receptor (33), dopamine receptor (34), 5-hydroxytryptamine receptor (35), and the enkephalin receptor (36, 37).

Drug binding to activated channels. To test the hypothesis that the receptor can exist in at least two states with state-specific drug binding constants (38), we have developed assays that reflect drug binding to both activated and nonactivated channels. The concentration-dependent effect of drugs on k_{-1}^M allows an estimation of their affinity for activated sodium channels, K_D^{act} (see Appendix). Both quinidine and lidocaine increased the rate of dissociation of [^{3}H]BTXB from its binding site in a concentration-dependent manner (Fig. 6), indicating that quinidine and lidocaine must bind to activated sodium channel-[^{3}H]BTXB complexes. This demonstrates that the binding sites for [^{3}H]BTXB and class I drugs must be different. However, the minimum estimated K_D^{act} values of quinidine and lidocaine for this effect were 11-and 8-fold greater, respectively, than their IC_{50} values for inhibition of equilibrium [^{3}H]BTXB binding (Table 1). Although quantitatively the estimated K_D^{act} values may not be exact, it is clear from Fig. 6 that, at concentrations less than 100 μM, these drugs have little or no effect on the k_{-1} of [^{3}H]BTXB binding. Therefore, the drug-mediated increase in k_{-1}^M does not appear to be the major determinant of inhibition of [^{3}H]BTXB binding by therapeutic concentrations of quinidine and lidocaine.

Drug binding to nonactivated channels. The concentration-dependent effect of drugs on k_{+1}^M allows an estimation of their affinity for nonactivated sodium channels, K_D^{non} (see Appendix). Although we recognize that class I drugs may decrease k_{+1}^M by directly competing with [^{3}H]BTXB, this would imply two drug binding sites, the drug receptor for the k_{-1}^M effect and the [^{3}H]BTXB receptor. This seems less likely than a single-site model of drug binding. Both quinidine and lidocaine decreased the k_{+1}^M of [^{3}H]BTXB binding, at concentrations similar to those that inhibit equilibrium [^{3}H]BTXB binding (Fig. 8; Table 1). This indicates that the drug-mediated decrease in k_{+1}^M is a major determinant of inhibition of [^{3}H]BTXB binding by quinidine and lidocaine. This in turn suggests that a major determinant of drug effect is stabilization of a nonactivated state of the sodium channel.

The results of the present study are in keeping with a previously proposed allosteric heterotropic model for the sodium channel (17, 19). This model was originally developed to account for the interaction of local anesthetics and anticonvulsants with the nerve sodium channel. Taken together with our previous biochemical studies, the results of this study support the conclusion that quinidine and lidocaine exhibit state-de-

TABLE 1

Comparison of the effects of quinidine and lidocaine on the characteristics of [^{3}H]BTXB binding to myocytes

K_D^{act}, Minimum estimated affinity of drugs for activated sodium channels; K_D^{non}, affinity of drug for nonactivated sodium channels. K_D^{non} values have been normalized to mean IC_{50} values to facilitate direct comparison.

Drug	IC_{50}	K_D^{act}	K_D^{non}	Effect on B_{max}
		μM		
Quinidine	40	433	20	No change
Lidocaine	61	455	36	Decreased

pendent binding to a receptor associated with the cardiac sodium channel, which results in allosteric inhibition of [^{3}H]BTXB binding. These drugs appear to bind to and stabilize nonactivated states of the sodium channel.

Limitations. A potential limitation is that we have only shown that antiarrhythmic drugs effect the association and dissociation rates of [^{3}H]BTXB. Because ATX synergistically enhances the binding of [^{3}H]BTXB to sodium channels (10, 32), it is possible that the effects of antiarrhythmic drugs on [^{3}H]BTXB binding could be secondary to an inhibition of ATX binding. However, both we (3) and Postma and Catterall (19) have shown previously that antiarrhythmic drugs do not significantly effect ATX or scorpion toxin binding. Thus, we feel that the major effect of antiarrhythmic drugs is on the association and dissociation kinetics of [^{3}H]BTXB binding.

It is also important to appreciate the potential limitations of this biochemical approach in accounting for electrophysiologic findings. One such limitation is that the toxin-activated state is similar to but not identical with the electrophysiologically activated state. For example, it has been shown that the toxin-associated state differs from the voltage-activated state in ion selectivity (39). However, voltage-clamp studies have indicated that batrachotoxin binds to channels that have been electrophysiologically activated and that one of the major effects of alkaloid toxins such as batrachotoxin is to greatly prolong the duration of the activated state and slow inactivation (40, 41). Thus, it seems likely that [^{3}H]BTXB does bind with high affinity to channels that correspond to electrophysiologically activated channels. However, there is less certainty as to the electrophysiologic correlate of sodium channels to which [^{3}H]BTXB has not bound. These channels could be rested, inactivated, or existing in an activated state that is not recognized by the toxins. This may account for the discrepancy between the value of K_D^{act} for quinidine reported here and values previously reported from electrophysiologic studies (1). Quinidine may stabilize an activated state that is not recognized by the toxins. Although this issue has not been directly addressed in this study, the simplest hypothesis is that class I drugs interact with high affinity with a single state of the sodium channel. Given that there is relatively little electrophysiologic evidence that drugs interact with rested channels (42), this suggests that the biochemical state to which drugs bind is one of the inactivated states.

Summary. In summary, we have presented biochemical evidence that both quinidine and lidocaine exhibit state-dependent binding to a receptor associated with the cardiac sodium channel. This report substantially confirms electrophysiologic theories that postulate state-dependent interactions of quinidine and lidocaine with the cardiac sodium channel (7–9). Both drugs destabilize activated channels and stabilize nonactivated channels. As well, the data show that class I drugs are allosteric inhibitors of [^{3}H]BTXB binding and suggest the presence of a separate class I drug-binding protein.

Acknowledgments

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Appendix I

Drug K_D for Activated Channels

We have shown that antiarrhythmic drugs increase the rate of dissociation of [^{3}H]BTXB from activated sodium channels. In this section, we demonstrate that the affinity of drugs for activated sodium channels (K_D^{act}) may be estimated by determining the effect of the drug on increasing the measured global dissociation rate constant k_{-1}^M of [^{3}H]BTXB-channel complexes. We will first derive the relationship between k_{-1}^M and the individual dissociation rate constants of specific [^{3}H]BTXB-channel complexes and then derive the relationship between k_{-1}^M and K_D^{act}.

Dissociation rate constants. The global rate of dissociation of [^{3}H]BTXB from activated channels is

$$-\frac{d[SB]}{dt} = k_{-1}^M[SB] \tag{1}$$

where SB = specifically bound [^{3}H]BTXB. However, SB represents the sum of two types of [^{3}H]BTXB-channel complexes. Suppose that a drug (D) and [^{3}H]BTXB (B) can both bind to activated sodium channels. Then B can dissociate from two types of complexes:

$$\begin{array}{ccc} RB + D & \underset{k_{-2}}{\overset{k_{+2}}{}} & RDB \\ k_{-11} & & k_{-12} \\ R + B & & RD + B \end{array} \tag{2}$$

Both RB and RDB will be detected as specifically bound dpm (SB) on the filters. Thus

$$SB = RB + RDB = R_{act} \tag{3}$$

where R_{act} = the total population of [^{3}H]BTXB-activated sodium channel complexes.

The rates of dissociation of the two activated channel-[^{3}H]BTXB complexes RB and RDB can be obtained from Eq. 2:

$$\frac{-d[RDB]}{dt} = k_{-12}[RDB] + k_{-2}[RDB] - k_{+2}[RB]\,[D] \tag{4}$$

and

$$\frac{-d[RB]}{dt} = k_{-11}[RB] + k_{+2}[RB]\,[D] - k_{-2}[RDB] \tag{5}$$

Combining Eqs. 3, 4, and 5, the global rate of dissociation of RB and RDB is:

$$\frac{-d[SB]}{dt} = k_{-11}\,[RB] + k_{-12}[RDB] \tag{6}$$

Because Eqs. 1 and 6 both equal $\frac{-d[SB]}{dt}$, then

$$k_{-1}^M[SB] = k_{-11}[RB] + k_{-12}[RDB] \tag{7}$$

However, the relative distribution of RB and RDB depends upon the ambient drug concentration [D] and upon the K_D^{act} of the drugs for their receptors that are associated with activated channels. Thus, the rate of decay of SB at various drug concentrations is described by measured k_{-1}^M values of [^{3}H]BTXB dissociation, which reflect the ambient [D] and K_D^{act} of drugs for activated channels.

Relationship between K_D^{act} and k_{-1}^M. The K_D^{act} for drug binding is the concentration of D that occupies half the drug receptors associated with activated channels. Thus, at K_D^{act}, [RB] = [RDB] = 0.5[R_{act}] = 0.5[SB]. Then, from Eq. 6

$$\begin{aligned} \frac{-d[SB]}{dt} &= k_{-11}\,[SB]de2 + k_{-12}\frac{[SB]}{2} \\ &= \frac{[SB]}{2}\,(k_{-11} + k_{-12}) \end{aligned} \tag{8}$$

Integrating and rearranging Eq. 8, we get

$$\ln[SB_t/SB_o] = -\,(0.5)\,(k_{-11} + k_{-12})t \tag{9}$$

where SB_t = specifically bound [^{3}H]BTXB at time t and SB_0 = specifically bound [^{3}H]BTXB at time 0.

Similarly, integrating and rearranging Eq. 1 we obtain

$$\ln[SB_t/SB_o] = -k^M_{-1}t$$

Thus, the measured k^M_{-1} of [^{3}H]BTXB dissociation from activated sodium channels at the K_D^{act} concentration for drug binding to activated channels is

$$k^M_{-1} = (0.5)\ (k_{-11} + k_{-12}) \tag{10}$$

All three rate constants, k^M_{-1}, k_{-11}, and k_{-12}, can be directly measured. k_{-11} is the drug-free rate constant for [^{3}H]BTXB dissociation and k_{-12} is the maximal dissociation rate constant for [^{3}H]BTXB dissociation in the presence of drugs. Thus, the drug concentration at which $k^M_{-1} = 0.5(k_{-11} + k_{-12})$ is the K_D^{act} for drug interaction with activated sodium channels.

Drug K_D for Nonactivated Channels

The results show that antiarrhythmic drugs decrease the rate of association of [^{3}H]BTXB with sodium channels. In this section, we demonstrate that the affinity of drugs for nonactivated sodium channels (K_D^{non}) may be estimated by determining the concentration-dependent effect of the drug D on the measured global association rate constant k^M_{+1} of [^{3}H]BTXB with its receptor. We will derive the relationship between k^M_{+1}, k^M_{-1}, and the individual association rate constants of [^{3}H]BTXB with specific channel complexes and then derive the relationship between k^M_{+1} and K_D^{non}.

Association rate constants. The global rate of formation of [^{3}H]BTXB-channel complexes may be expressed (27) as:

$$\frac{d[SB]}{dt} = (K^M_{+1})\ [R_{non}][B] - k^M_{-1}[SB] \tag{11}$$

where SB = specifically bound dpm, k^M_{+1} = experimentally determined global association rate constant, R_{non} = nonactivated sodium channels, B = [^{3}H]BTXB, and k^M_{-1} = experimentally determined dissociation rate constant at drug concentration [D]. (R_{non} in fact represents sodium channels to which ATX is bound and are, therefore, able to bind [^{3}H]BTXB.)

However, B can bind to two types of R_{non} complexes, R and RD.

$$\begin{array}{ccc} R + B & & RD + B \\ k_{+11} \quad k_{-11} & & k_{+12} \quad k_{-12} \\ & k_{+2} & \\ D + RB & & RDB \\ & k_{-2} & \end{array} \tag{12}$$

The overall rate of association of B with R and RD to form both specifically bound complexes SB may be expressed as:

$$\frac{d[RB]}{dt} = k_{+11}[R][B] - k_{-11}[RB] + k_{-2}[RDB] - k_{+2}[RB][D] \tag{13}$$

$$\frac{d[RDB]}{dt} = k_{+12}[RD][B] - k_{-12}[RDB] + k_{+2}[RB][D] - k_{-2}[RDB] \tag{14}$$

However, $[SB] = [RB] + [RDB]$. Thus, by combining Eqs. 13 and 14, we obtain

$$\frac{d[SB]}{dt} = [B]\ (k_{+11}[R] + k_{+12}[RD]) - (k_{-11}[RB] + k_{-12}[RDB] \tag{15}$$

However, from Eq. 7

$$k^M_{-1}[SB] = k_{-11}[RB] + k_{-12}[RDB]$$

Thus, Eq. 15 may be simplified to

$$\frac{d[SB]}{dt} = [B]\ (k_{+11}[R] + k_{+12}[RD]) - k^M_{-1}[SB] \tag{16}$$

Both Eqs. 11 and 16 equal $\frac{d[SB]}{dt}$.

Thus

$$k^M_{+1}[R_{non}] = k_{+11}[R] + k_{+12}[RD] \tag{17}$$

However, the relative distribution of R and RD depends upon the ambient drug concentration [D] and upon the K_D^{non} of the drug for its receptor associated with nonactivated channels. Thus, the rate of formation of SB at various drug concentrations is described by measured k^M_{+1} values for [^{3}H]BTXB association, which reflect the ambient [D] and the K_D^{non} of drugs for nonactivated channels.

Relationship between K_D^{non} and k^M_{+1}. The K_D^{non} for drug binding to nonactivated channels is the concentration of drug that occupies half the drug receptors associated with nonactivated channels. At this drug concentration, $[R] = [RD] = 0.5[R_{non}]$ and Eq. 17 simplifies to:

$$k^M_{+1}[R_{non}] = 0.5[R_{non}](k_{+11} + k_{+12})$$

However, in the Results, we show that $k_{+11} \gg k_{+12}$. Thus, at drug concentration K_D^{non}, $k^M_{+1} = 0.5\ k_{+11}$.

To estimate k^M_{+1}, Eq. 11 must first be integrated to

$$ln\ \frac{[SB]_{eq}}{[SB]_{eq} - [SB]} = ([B]\ k^M_{+1} + k^M_{-1})t \tag{18}$$

where $[SB]_{eq} = [SB]$ at equilibrium. From a plot of $\ln[SB]_{eq}/[SB]_{eq} - [SB]_t$ versus t at known $[B]$ and k^M_{-1}, one can estimate k^M_{+1}. Thus, the drug concentration at which $k^M_{+1} = 0.5k_{+11}$ is the K_D^{non} for drug interaction with nonactivated channels.

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Send reprint requests to: Dr. Roger J. Hill, Department of Medicine, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta T2N 4N1, Canada.