Differences in Steady-State Inactivation between Na Channel Isoforms Affect Local Anesthetic Binding Affinity

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ABSTRACT Cocaine and lidocaine are local anesthetics (LAs) that block Na currents in excitable tissues. Cocaine is also a cardiotoxic agent and can induce cardiac arrhythmia and ventricular fibrillation. Lidocaine is commonly used as a postinfarction antiarrhythmic agent. These LAs exert clinically relevant effects at concentrations that do not obviously affect the normal function of either nerve or skeletal muscle. We compared the cocaine and lidocaine affinities of human cardiac (hH1) and rat skeletal (μ1) muscle Na channels that were transiently expressed in HEK 293t cells. The affinities of resting μ1 and hH1 channels were similar for cocaine (269 and 235 μM, respectively) and for lidocaine (491 and 440 μM, respectively). In addition, the affinities of inactivated μ1 and hH1 channels were also similar for cocaine (12 and 10 μM, respectively) and for lidocaine (19 and 12 μM, respectively). In contrast to previous studies, our results indicate that the greater sensitivity of cardiac tissue to cocaine or lidocaine is not due to a higher affinity of the LA receptor in cardiac Na channels, but that at physiological resting potentials (−100 to −90 mV), a greater percentage of hH1 channels than μ1 channels are in the inactivated (i.e., high-affinity) state.

INTRODUCTION

Local anesthetics (LAs) block Na currents in excitable tissues such as nerve and muscle (Hille, 1992) by binding with voltage-gated Na channels. Tonic block occurs at the resting potential, as determined by infrequent stimulation, whereas use- and voltage-dependent block occurs during repetitive stimulation (Strichartz, 1973; Courtney, 1975). Channel state strongly influences the LA affinity of Na channels. In simple terms, resting channels have a low affinity for LAs, whereas open or inactivated channels have a higher affinity. The gradual changes in channel affinity for LAs between these two extremes reflect the complex nature of the LA-Na channel interaction. Two models of LA-Na channel binding have been used to explain the gradual variation between the low- and high-affinity channel states. The Modulated Receptor hypothesis (Hille, 1977; Hondeghem and Katzung, 1977) proposes that the changes in LA affinity can be ascribed to alterations in voltage-dependent channel conformation, so that membrane voltage modulates the affinity of the LA receptor. The Guarded Receptor hypothesis (Starmer, 1987) suggests that the LA affinity is governed by the voltage-dependent gates that permit LA access to a constant affinity receptor upon depolarization.

Although LAs block Na channels from all excitable tissues, the excitability of cardiac muscle is often affected by lower LA concentrations than those required to affect the excitability of peripheral nerve or skeletal muscle (Hille, 1978). The question of whether the cardiac Na channel isoform has a greater affinity for LAs than other Na channel isoforms has been difficult to answer because LA binding and affinity have been studied with experimental protocols specifically designed and optimized according to the preparation used (Strichartz, 1973; Schwarz et al., 1977; Crumb and Clarkson, 1990; Jia et al., 1993). In particular, the holding potential applied to preparations varies between −80 and −140 mV. With the advent of molecular cloning strategies, different Na channel isoforms can now be studied under identical conditions, and the sensitivities of individual isoforms to LAs can be compared.

In this study, we expressed rat skeletal muscle (μ1; Trimmer et al., 1989) and human heart muscle (hH1; Gellens et al., 1992) Na channels in HEK 293t cells to compare the voltage-dependent affinities of these channels for cocaine and lidocaine. (Note that μ1 and rSkM1 are both used as abbreviations for cloned rat skeletal muscle Na channel.) The primary sequences of Na channels from the same tissue of different species are far more similar to each other than are the primary sequences of Na channels from different tissues within the same species. For example, the amino acid sequence of μ1 is 92% identical to that of the human skeletal muscle (hSkM1) Na channel (George et al., 1992), whereas the amino acid sequences of hSkM1 and hH1 are only 59% identical. In the S6 segment of domain 4, where the putative LA receptor resides (Ragsdale et al., 1994), the amino acid sequences of μ1 and hSkM1 are 100% identical, and both μ1 and hSkM1 are 88% identical to hH1.

Do primary sequence differences within the LA binding site confer differences in LA binding affinity? Two recent reports suggest that the Na channels from cardiac tissue are

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intrinsically more sensitive to lidocaine than are the Na channels from skeletal muscle (Nuss et al., 1995; Wang et al., 1996b). In contrast, we find that μ1 and hH1 channels have a similar intrinsic resting affinity (at -180 to -160 mV) and a similar inactivated affinity (at -80 to -70 mV) for both cocaine and lidocaine. At intermediate voltages (-120 to -90 mV), hH1 channels are blocked more potently by cocaine or lidocaine than are μ1 channels, because a greater proportion of the hH1 channels are inactivated in this voltage range.

MATERIALS AND METHODS

Voltage-clamp procedures

The whole-cell method of the patch-clamp technique (Hamill et al., 1981) was used to study macroscopic Na currents at room temperature (23 ± 2°C). Electrode resistances ranged from 0.4 to 1.0 MΩ. Command voltages were programmed by pCLAMP software and delivered by a List EPC7 voltage clamp; data were filtered at 5 kHz. After gigaohm seal formation and establishment of whole-cell voltage clamp, the cells were dialyzed for 25–30 min before data were acquired. According to a previous report (Wang et al., 1996a), the time-dependent negative shift in the midpoint of the Na channel availability curve (h_∞) during the time frame of our experiments (~30–60 min after membrane rupture) would have been ~5–7 mV for each isoform. Because the negative shifts in the h_∞ curves of the two isoforms were of similar magnitude, any time-dependent alterations in anesthetic affinity would also have been similar. Data were acquired from a holding potential of -140 mV, where stable recordings could be maintained for up to 1.5 h. Most of the capacitative current was canceled by the EPC7 circuitry. Any remaining capacitative artifact and the leakage current were subtracted by the P/-4 method. The capacitative transient in response to a 20-mV hyperpolarization was used to calculate series resistance ($R_s = \tau/C_m$). R_s compensation of 30–50% typically resulted in voltage errors of ≤5 mV at +30 mV. Curve fitting was performed with Mirocal Origin software.

Solutions and chemicals

Cocaine hydrochloride was purchased from Mallinckrodt, Inc. (St. Louis, MO); lidocaine was purchased from Sigma Chemical Co. (St. Louis, MO). Cells were perfused with an extracellular solution containing (in mM) 65 NaCl, 85 choline Cl, 2 $CaCl_2$, and 10 HEPES (titrated with tetramethyl ammonium hydroxide to pH 7.4). The pipette (intracellular) solution consisted of (in mM) 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES (titrated with cesium hydroxide to pH 7.2). Because +30-mV test pulses were used to fully activate both channel isoforms, the reverse Na gradient produced easily measurable outward currents. For each experiment, a 10 mM solution of anesthetic was prepared in external bathing solution from a 200 mM stock (stored at −20°C). Final anesthetic concentrations were obtained by serial dilution.

Expression of μ1 and hH1 in HEK 293t cells

Transformed human embryonic kidney (HEK 293t) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with taurine (1%), penicillin/streptomycin (1%), and heat-inactivated fetal bovine serum (10%). Cells were split twice per week. Cloned μ1 and hH1 Na channels were transiently expressed in HEK 293t cells as follows (Cannon and Strittmatter, 1993): 1 μg of CD8 (cell surface antigen) and 2–10 μg of Na channel cDNA clones in the pcDNA1/amp vector (Invitrogen, San Diego, CA) were prepared in 250 mM $CaCl_2$, added to a test tube containing 0.36 ml of Hanks' balanced salt (2×) solution (in mM: 274 NaCl, 40 HEPES, 12 dextrose, 10 KCl, 1.4 Na_2HPO_4, pH 7.05), and incubated at 22°C for 20 min. The DNA solution was then dripped over a cell culture (30–50% confluence in a TI-25 flask) containing 7 ml of DMEM. The transfected cells were replated 15 h later onto 35-mm culture dishes (which also served as recording chambers) containing 2 ml of fresh DMEM; cells were used within 3 days of plating. Cells that expressed the CD8 cell surface protein, as determined by binding beads (Dynabeads M-450 CD8; Dynal A.S., Oslo, Norway) coated with a monoclonal antibody (ITI-5C2) specific for CD8 antigen, were selected for patch-clamp experiments.

RESULTS

Voltage-dependent activation and inactivation of μ1 and hH1 Na channels

Both μ1 and hH1 Na channels are well expressed in HEK 293t cells, with peak outward currents of >1 nA at +30 mV. Fig. 1 *A* shows sodium currents through μ1 channels (*upper traces*) and hH1 channels (*lower traces*). The hH1 Na current activated at less depolarized voltages and had a slower inactivation rate than did the μ1 Na current. The differences between μ1 and hH1 in voltage-dependent activation and inactivation rate were consistent with the corresponding differences between the hSkM1 (human skeletal muscle) Na channel isoform and hH1 (Wang et al., 1996a). We delivered 10-ms depolarizations ranging from -100 mV to +50 mV to determine the current-voltage and conductance-voltage (Fig. 1 *B*) relations of the two isoforms. With the Na^+ gradient used, the sodium current reversed near -10 mV at both μ1 and hH1 Na channels. The hH1 Na channels activated near -70 mV, whereas the μ1 Na channels activated near -60 mV.

Fig. 1 *B* plots the normalized membrane conductance for μ1 and hH1 Na channels. These data were fitted with a Boltzmann equation $\{1/[1 + \exp((V_{0.5} - V)/k)]\}$ to determine the midpoints of activation, where $V_{0.5}$ is the midpoint voltage (50% of the maximum conductance) of the function and k is the slope factor (in mV/*e*-fold change in current). The average midpoints of activation for μ1 and hH1 channels were -32.8 ± 2.8 mV (SEM, $k = 9.3 \pm 1.5$ mV) and -48.0 ± 1.8 mV ($k = 9.4 \pm 0.9$ mV), respectively. The difference between the midpoints of μ1 and hH1 Na channel activation was statistically significant ($p < 0.05$, *t*-test), whereas the difference between the slope factors was not significant ($p > 0.05$).

Fig. 1 *C* shows representative examples of outward Na current through μ1 and hH1 Na channels in response to test depolarizations to +30 mV. For these and other experiments that utilized a conditioning test-pulse protocol, we used test depolarizations to +30 mV to ensure that both channel isoforms were maximally activated (see Fig. 1 *B*). From a holding potential of -140 mV, we delivered 100-ms conditioning pulses between -160 and -35 mV in 5-mV increments and measured the relative amount of Na channel inactivation (h_∞) at the test pulse. The labeled current traces in Fig. 1 *C* indicate the membrane potential of the conditioning pulse that preceded the test pulse to +30 mV. For both μ1 and hH1 Na channels, the Na current at the test pulse decreased as the amplitude of the conditioning pulse

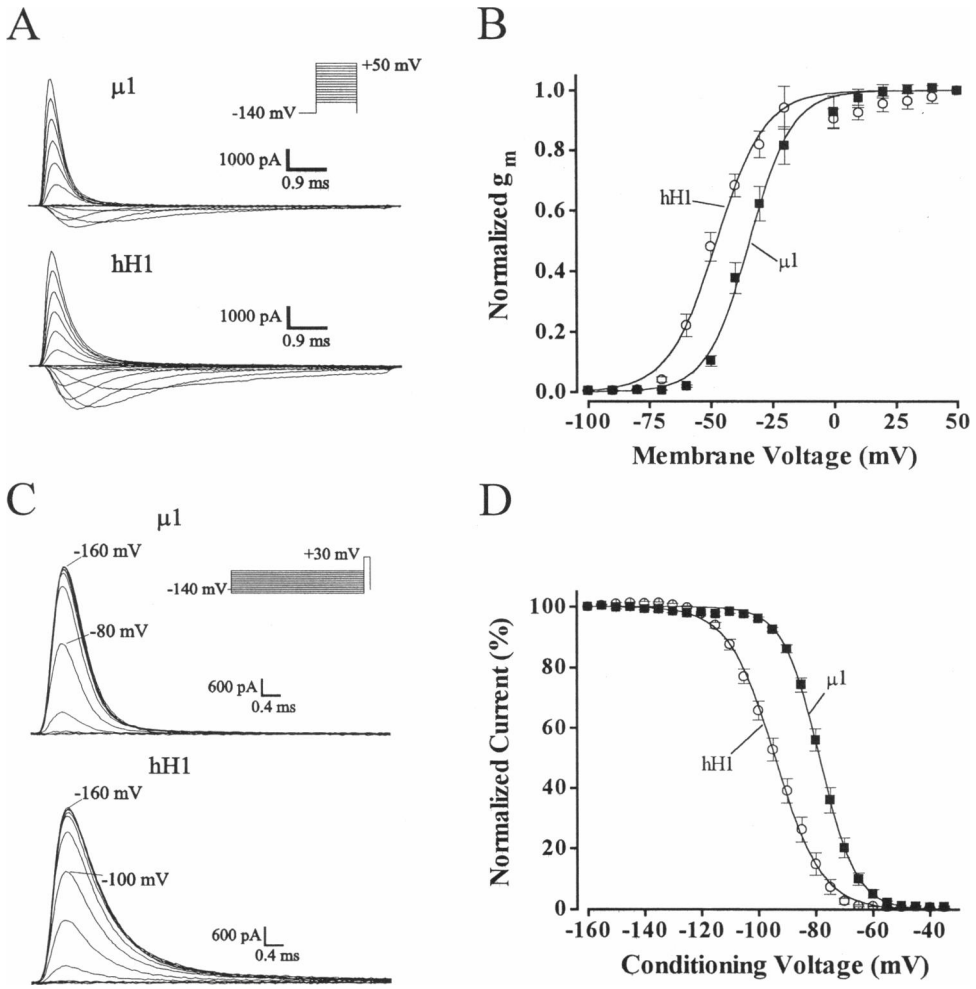

FIGURE 1 Activation (*A,B*) and inactivation (*C,D*) kinetics of μ1 and hH1 Na currents. (*A*) Na currents through μ1 channels (*upper traces*) and hH1 channels (*lower traces*) evoked by 10-ms pulses ranging from −100 mV to +50 mV in 10-mV increments from a holding potential of −140 mV. (*B*) Normalized membrane conductance (g_m) plotted versus the amplitude of the 10-ms voltage command. g_m at a given voltage step was calculated from the equation $g_m = I_{Na}/(E_m - E_{Na})$, where I_{Na} is the current, E_m is the amplitude of the voltage step, and E_{Na} is the reversal potential of the Na current. The data points were fitted with a Boltzmann function ($1/[1 + \exp((V_{0.5} - V)/k)]$). The average midpoint voltage ($V_{0.5}$) and slope factor (k) for the μ1 data (■, $n = 7$) were −32.8 ± 2.8 mV and 9.3 ± 1.5 mV, respectively. The corresponding values for the hH1 data (○, $n = 6$) were −48.0 ± 1.8 mV and 9.4 ± 0.9 mV, respectively. (*C*) Na currents from μ1 channels (*upper traces*) or hH1 channels (*lower traces*) evoked in response to a 5-ms test pulse to +30 mV. The test pulses were preceded by 100-ms conditioning pulses between −160 and −35 mV in 5-mV increments (10-mV increments are shown for clarity). The pulse protocol is shown in the μ1 panel. Test currents evoked after conditioning pulses to −80 mV (μ1) and −100 mV (hH1) are labeled. Pulses were delivered at 5-s intervals. (*D*) Normalized Na current availability of μ1 channels (■, $n = 8$) and hH1 channels (○, $n = 9$) plotted as a function of the conditioning pulse voltage. The average midpoint (50% reduction) and slope factor of the Boltzmann function ($1/[1 + \exp((V - V_{0.5})/k)]$) fitted to the μ1 data were −78.5 ± 1.0 mV and 6.1 ± 0.3 mV, respectively; for the hH1 data the average values were −94.1 ± 1.4 mV and 7.7 ± 0.1 mV, respectively.

became more positive. Decreases in the hH1 test current began at more negative conditioning voltages than did decreases in the μ1 current.

Fig. 1 *D* plots the relative amount of current evoked at the +30-mV test pulse as a function of the conditioning pulse voltage for μ1 channels and hH1 channels. The data were normalized by dividing the peak test current evoked after each conditioning potential by the peak test current evoked after the conditioning pulse to −160 mV. Each experiment was fitted with a Boltzmann function $\{1/[1 + \exp((V - V_{0.5})/k)]\}$, and the average midpoint voltage and slope factor of the μ1 data were −78.5 ± 1.0 and 6.1 ± 0.3 mV, respectively, whereas these values for the hH1 data were −94.1 ± 1.4 and 7.7 ± 0.1 mV, respectively. The difference in the midpoint voltages of inactivation between μ1 and hH1 channels was significant ($p < 0.05$) and was similar to the differences in the midpoint voltages between hSkM1 and hH1 Na channels (Wang et al., 1996a).

Cocaine dose-response experiments

We performed dose-response experiments to determine the IC_{50} values (50% tonic block) of μ1 and hH1 channels for

cocaine at three conditioning membrane potentials (Fig. 2). A 10-s conditioning pulse to −100, −140, or −180 mV was delivered from a holding potential of −140 mV. Increasing the duration of the conditioning pulses to 30 s had little or no effect on cocaine binding. After conditioning pulses, cells were returned to −140 mV for 100 ms before delivery of a test pulse to +30 mV to allow drug-free channels to recover from fast inactivation (−100-mV conditioning pulses) or to reset channel activation (−180-mV conditioning pulses). A 30-s rest at the holding potential between each conditioning test-pulse pair allowed drug-blocked channels to recover completely. The current traces shown in the left panels of Fig. 2, *A–C*, are from single experiments that are representative of the averaged data. The left sets of traces are from the same μ1 cell, and the right sets of traces are from the same hH1 cell. Fig. 2, *A*, *B*, and *C*, shows the Na currents evoked by test pulses to +30 mV when preceded by a conditioning pulse to −100 mV, −140 mV and −180 mV, respectively. With conditioning pulses to −100 mV, cocaine blocked the hH1 Na current much more potently than it blocked the μ1 Na current. In contrast, cocaine blocked the μ1 and hH1 Na current with similar potency when a conditioning pulse to −180 mV preceded the test pulse. The Na currents at both isoforms recovered nearly completely (~98%) after 3–4 min of washing with control saline (data not shown).

The right panels in Fig. 2, *A–C*, show the dose-response curves of the averaged data for μ1 channels and hH1

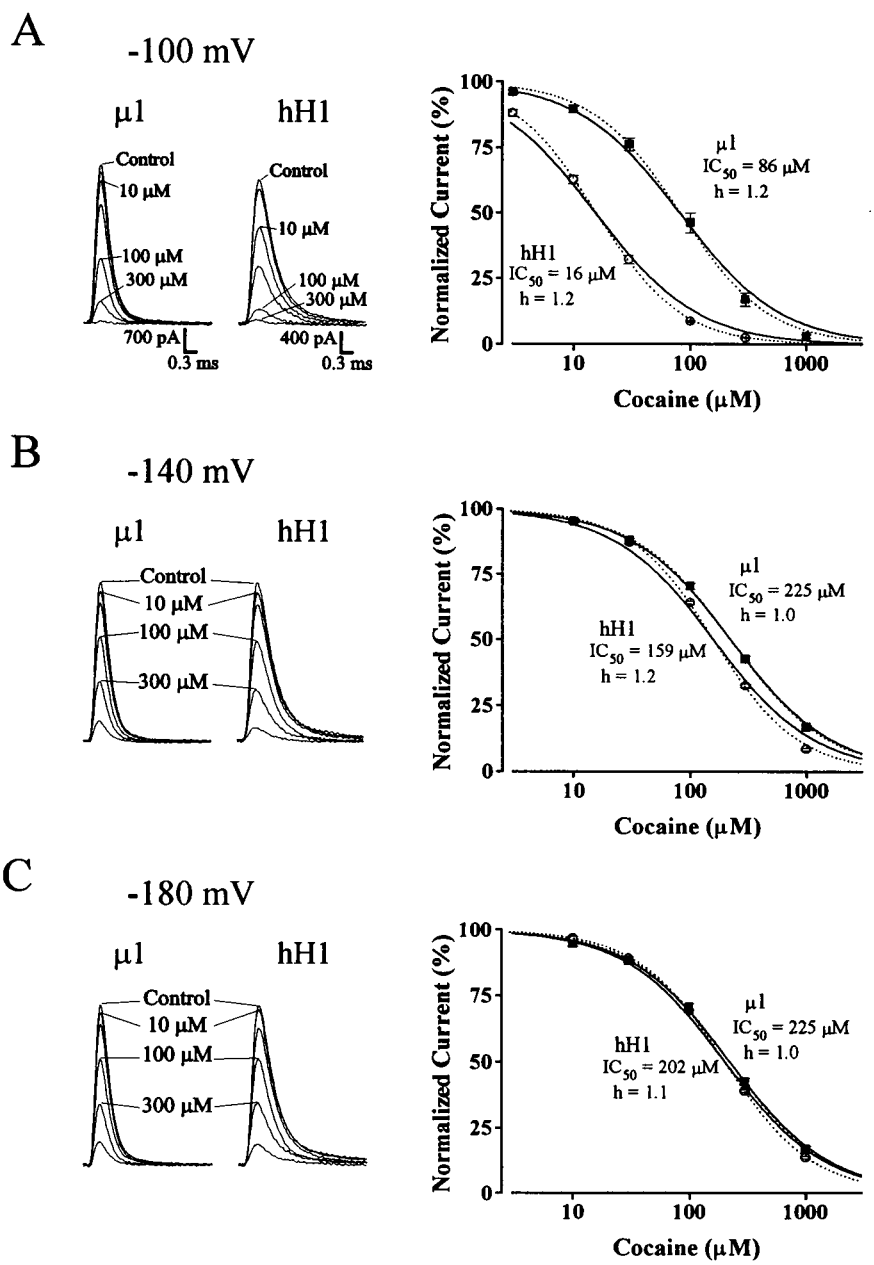

FIGURE 2 Cocaine dose-response experiments. μ1 and hH1 Na channels were stepped to +30 mV after 10-s conditioning pulses to (*A*) −100, (*B*) −140, and (*C*) −180 mV. The membrane potential was returned to −140 mV for 100 ms before delivery of the test pulse. The Na current traces in the left panels of *A–C* are from a μ1 cell (*left*) and an hH1 cell (*right*) that received all three conditioning pulse amplitudes. Conditioning test-pulse pairs were delivered at 30-s intervals. The amplitudes of the control test currents evoked after conditioning pulses to −180 mV (*C*) are scaled to the same amplitude. This scaling factor is the same for the test currents shown in *A* and *B*. The control test currents and the currents evoked in the presence of 10, 100, and 300 μM cocaine are labeled. The right panels show the normalized dose-response curves. The dotted lines are fits to the data with the Hill equation. IC_{50} is the concentration producing 50% block, and *h* (*dotted lines*) is the slope of the function. The solid lines are the fits with a constrained slope of 1. (*A*) Conditioning pulse to −100 mV. The average IC_{50} and slope for the μ1 data (■, n = 7) were 86.2 ± 10.8 μM and 1.19 ± 0.03, respectively. The average IC_{50} and slope for the hH1 data (○, n = 7) were 15.8 ± 0.9 μM and 1.22 ± 0.02, respectively. (*B*) Conditioning pulse to −140 mV. The average IC_{50} and slope for the μ1 data (■, n = 11) were 225.3 ± 10.4 μM and 1.04 ± 0.02, respectively. The average IC_{50} and slope for the hH1 data (○, n = 9) were 159.4 ± 4.0 μM and 1.19 ± 0.01, respectively. (*C*) Conditioning pulse to −180 mV. The average IC_{50} and slope for the μ1 data (■, n = 6) were 224.8 ± 9.7 μM and 1.04 ± 0.03, respectively. The average IC_{50} and slope for the hH1 data (○, n = 6) were 201.8 ± 2.1 μM and 1.14 ± 0.02, respectively.

channels. For each cell, the data were normalized by dividing the peak Na current measured at each cocaine concentration by the peak Na current measured in control saline. The cocaine affinities of μ1 and hH1 channels became more similar with increasingly negative conditioning pulses. Fits of the data from both channel isoforms with the Hill equation gave a stoichiometry of drug molecule per channel of ~1:1.

Dependence of cocaine binding affinity on channel state

Because the conditioning voltage had such a strong effect on cocaine affinity in the dose-response experiments, we further examined the relative affinities of μ1 and hH1 Na channels for cocaine (Fig. 3) and for lidocaine (Fig. 4), using 10-s conditioning pulses of various amplitudes. The binding affinities of cocaine and lidocaine were studied during exposure to 30 and 300 μM of each LA. Fig. 3 *A* shows the Na currents evoked by a +30-mV test depolarization in each concentration of cocaine after 10-s conditioning pulses ranging from −180 mV to −80 mV. A 100-ms interval to −140 mV inserted between the conditioning and test pulses allowed drug-free channels to recover from fast inactivation; 30 s separated each conditioning test-pulse pair. Selected test current traces are labeled with the amplitude of the conditioning pulse that preceded them. The test currents evoked after the conditioning pulse to −180 mV were scaled to the same amplitude to allow comparison between μ1 and hH1 currents at subsequent conditioning voltages.

Fig. 3 *B* shows the average percentage of Na current remaining at the test pulse when cells were given 10-s conditioning pulses ranging from −180 to −70 mV. The control data for μ1 and hH1 are the combined averages of the control data obtained before exposure to either 30 μM or 300 μM cocaine. In 30 μM cocaine, the amplitude of the test Na current after the conditioning pulse to −180 mV was ~90% of the control amplitude for both μ1 and hH1 channels. Cocaine at 300 μM reduced the amplitude of the μ1 and hH1 test current to ~45% of the control amplitude when preceded by a conditioning pulse to −180 mV. As the amplitude of the conditioning pulse became more positive, cocaine block of the hH1 channels began at more negative voltages than did block of the μ1 channels. In general, cocaine blocked the μ1 and hH1 channels to a similar extent when the conditioning pulse was to −180 mV; the percentage of block after conditioning pulses to −70 mV was more pronounced, but was again relatively similar for the two isoforms. More hH1 current was blocked when the conditioning pulse was between −130 and −90 mV.

The cocaine data in Fig. 3 *B* were each fitted with a Boltzmann function to determine the midpoint voltage and slope factor of the relationship. In 30 μM cocaine, the midpoint voltage of the hH1 data was 23 mV more negative than that of the μ1 data. In the presence of 300 μM cocaine, the midpoint voltage of the hH1 data was 29 mV more negative than that of the μ1 data. The differences between the midpoints of the Boltzmann functions in 30 μM and 300 μM cocaine for each channel, as well as those between the two channel isoforms, were all significant ($p < 0.05$). A concentration-dependent shift in the midpoint of block has also been reported for the steady-state lidocaine block of cardiac Na channels (Bean et al., 1983).

Dependence of lidocaine binding affinity on channel state

We used the same protocol to study the block of μ1 and hH1 channels during exposure to 30 μM or 300 μM lidocaine (Fig. 4 *A*). As with cocaine block, voltage-dependent lidocaine block was similar for μ1 and hH1 channels after conditioning pulses between −180 and −160 mV. However, in contrast to cocaine block, lidocaine block of the two isoforms was not similar after conditioning pulses to −70 mV. After conditioning pulses to −180 mV, 30 μM lidocaine reduced both μ1 and hH1 test currents to ~94% of the control amplitude. After conditioning pulses to −70 mV, 30 μM lidocaine blocked the hH1 test current ($n = 6$) more than the μ1 test current ($n = 7$). After conditioning pulses to −180 mV, 300 μM lidocaine reduced the amplitudes of both μ1 and hH1 test currents to ~60% of the control amplitude. After conditioning pulses to −70 mV, 300 μM lidocaine blocked more hH1 current ($n = 7$) than μ1 current ($n = 7$). Each data set in Fig. 4 was fitted with a Boltzmann function. In 30 μM lidocaine, the midpoint voltage of the hH1 data was 19 mV more negative than that of the μ1 data. In the presence of 300 μM lidocaine, the midpoint voltage of the hH1 data was 26 mV more negative than that of the μ1 data. The differences between the μ1 and the hH1 midpoint voltages were significant ($p < 0.05$) in both 30 μM and 300 μM lidocaine.

Because recovery from lidocaine block typically occurs with a τ of a few hundred milliseconds at strongly negative recovery potentials (Bean et al., 1983; Clarkson and Hondeghem, 1985; Jia et al., 1993), significant amounts of lidocaine unbinding from inactivated channels could have occurred during the 100-ms gap between the conditioning pulse and test pulse. In addition, if lidocaine unbinds from μ1 and hH1 channels with different time constants (τ), as has been shown previously for μ1 and hH1 channels coexpressed in oocytes with the rat brain β-subunit (β_1) (Nuss et al., 1995), this could further confound the estimation of block of the inactivated channels at −70 mV. To determine the percentage of block of the inactivated channels, we examined the recovery time course from block by 30 μM lidocaine (Fig. 4 *B*). For both μ1 and hH1 channels, recovery from lidocaine block was best fitted by the sum of two exponentials. The fast components of recovery had τ values of a few milliseconds, whereas the slow components of recovery had τ values of hundreds of milliseconds. The τ values of the fast components of recovery for μ1 and hH1

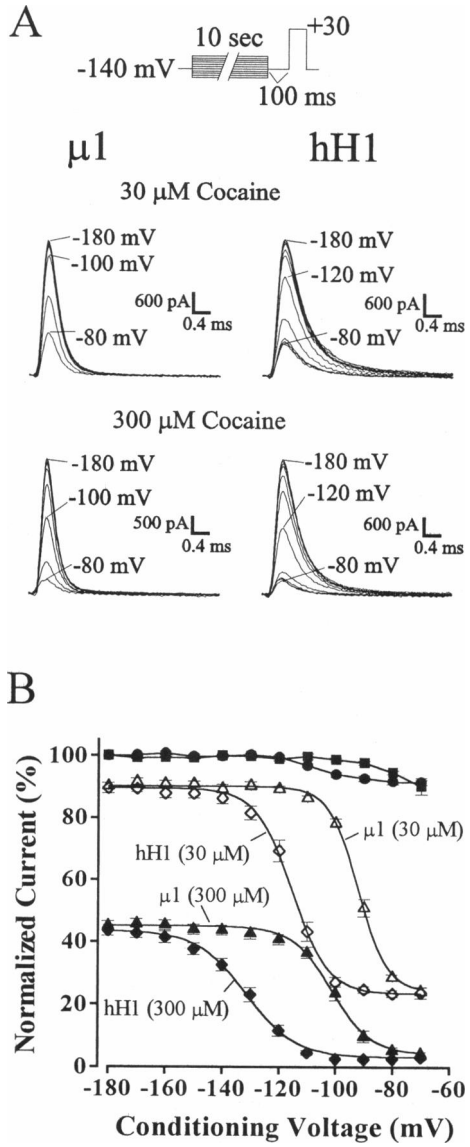

FIGURE 3 Steady-state cocaine block of μ1 and hH1 Na channels. Cells were held at −140 mV and received 10-s pulses ranging in amplitude from −180 to −70 mV. A 100-ms interval to −140 mV preceded a test pulse to +30 mV to allow drug-free channels to recover from fast inactivation. (*A*) Na currents through μ1 channels (*left*) and hH1 channels (*right*) recorded during a test pulse to +30 mV in the presence of either 30 μM cocaine (*upper traces*) or 300 μM cocaine (*lower traces*). Pulses were delivered at 30-s intervals to allow full recovery of drug-blocked channels. The data for μ1 and hH1 are scaled to approximately the same amplitude to allow comparison of cocaine block after conditioning pulses (as labeled) of different amplitude. The 30 μM and 300 μM data for each isoform are from different cells. (*B*) Plot of normalized Na current as a function of conditioning-pulse amplitude. Data obtained in control saline (μ1, ■; hH1, ●), in the presence of 30 μM cocaine (μ1, △; hH1, ◇), and in the presence of 300 μM cocaine (μ1, ▲; hH1, ◆) are shown. The control data for each isoform from experiments with 30 and 300 μM cocaine were combined. The average midpoint and slope of the Boltzmann function fitted to the μ1 data in the presence of 30 μM cocaine (n = 7) were −92.1 ± 0.8 mV and 5.0 ± 0.2 mV, respectively. In the presence of 300 μM cocaine (n = 10), the μ1 data had midpoint and slope values of −101.8 ± 1.5 mV and 6.2 ± 0.7 mV, respectively. In 30 μM cocaine (n = 6), hH1 channels had midpoint and slope values of −115.4 ± 1.2 mV and 6.1 ± 0.2 mV, respectively. In 300 μM cocaine (n = 11); hH1 channels had midpoint and slope values of −131.1 ± 1.5 mV and 8.7 ± 0.4 mV, respectively.

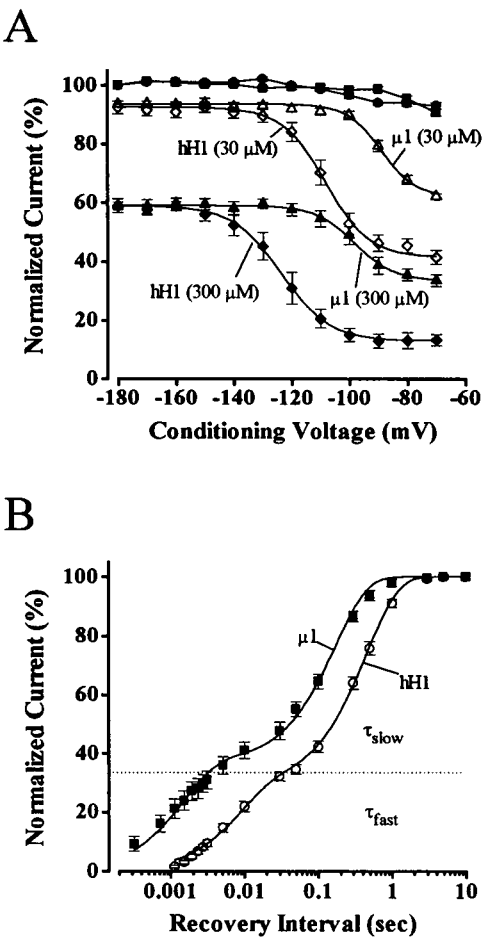

FIGURE 4 Lidocaine block of μ1 and hH1 channels. (*A*) The pulse protocol used was the same as that described for cocaine block in Fig. 3. Data obtained in control saline (μ1, ■; hH1, ●), in the presence of 30 μM lidocaine (μ1, △; hH1, ◇), and in the presence of 300 μM lidocaine (μ1, ▲; hH1, ◆) are shown. The average midpoint and slope factor of the Boltzmann function fitted to the μ1 data in the presence of 30 μM lidocaine (n = 7) were −88.9 ± 0.7 mV and 5.7 ± 0.3 mV, respectively. In 300 μM lidocaine (n = 7), the μ1 data had midpoint and slope values of −98.2 ± 1.7 mV and 6.2 ± 0.8 mV, respectively. In 30 μM lidocaine (n = 6), hH1 channels had midpoint and slope values of −108.2 ± 1.2 mV and 7.4 ± 0.2 mV, respectively. In 300 μM lidocaine (n = 7), hH1 channels had midpoint and slope values of −124.1 ± 3.1 mV and 7.2 ± 0.5 mV, respectively. (*B*) Time dependence of recovery from block by 30 μM lidocaine. Cells were depolarized to −70 mV for 10 s from a holding potential of −140 mV, and the recovery from lidocaine block was determined by giving a test pulse to +30 mV from the holding potential at various times after the conditioning pulse. The data for each cell were normalized according to the amplitude of the test pulse at the 10-s recovery interval. The data were fitted by the sum of two exponentials (y_0 + A1*[1 − exp(−x/τ1)] + A2*[1 − exp(−x/τ2)]). μ1 channels (■; n = 8) recovered with fast and slow time constants of 1.8 ± 0.4 and 179.0 ± 16.6 ms, respectively. hH1 channels (○; n = 8) recovered with fast and slow time constants of 9.3 ± 1.2 and 459.8 ± 39.4 ms, respectively. The fractional amplitudes of the fast phase of recovery (approximated by the *horizontal dotted line*) for μ1 and hH1 were 38% and 29%, respectively.

were similar to the τ values of recovery from fast inactivation in control saline (data not shown), whereas the slower components of recovery presumably reflected the unbinding of lidocaine from drug-blocked channels. For μ1, the τ of

the fast component of recovery was 1.8 ms, and the fractional amplitude was ~38% of the control test current. For hH1, the τ of the fast component of recovery was 9.3 ms and the fractional amplitude was ~29% of the control test current. The slow phases of recovery from lidocaine block had time constants of 179 and 460 ms for μ1 and hH1, respectively. Because the τ values of the fast components were ~100-fold (μ1) and ~50-fold (hH1) faster than the τ values of the slow components, we assumed that the percentages of block at completion of the fast phase represented the percentages of block of the inactivated channels. The similarities in the percentage of blocked current at completion of the fast components of recovery show that inactivated μ1 and hH1 channels indeed have a similar affinity for lidocaine.

Cocaine and lidocaine affinities (K_d) of resting and inactivated channels

We estimated the K_d of resting and inactivated channels for cocaine (Fig. 5; *upper*) and for lidocaine (*lower*) by taking the percentages of block at −180 mV and −70 mV and calculating the apparent K_d according to the Langmuir isotherm {% block = [LA]/([LA] + K_d)}, where [LA] is the concentration of local anesthetic. This calculation assumes that the LA binds to the Na channels with a 1:1 stoichiometry. For both drugs we used the percentage block by both the 30 μM and 300 μM concentrations to calculate resting channel affinities. For inactivated channels, we used the 30 μM and 300 μM data for calculating the K_d of cocaine and used the 30 μM data for calculating the K_d of lidocaine. (The current was completely blocked by 300 μM lidocaine, thereby making it impossible to distinguish the time course of recovery of any drug-free channels.) The K_d of cocaine at μ1 and hH1 channels were 268.6 ± 17.3 and 235.2 ± 14.5 μM ($p > 0.05$), respectively, for resting channels and 11.9 ± 1.4 and 9.6 ± 0.5 μM ($p > 0.05$), respectively, for inactivated channels. The K_d of lidocaine at μ1 and hH1 channels were 490.8 ± 55.5 and 439.8 ± 44.3 μM ($p > 0.05$), respectively, for resting channels and 18.5 ± 1.6 and 12.4 ± 0.8 μM ($p < 0.05$), respectively, for inactivated channels.

Open-channel block by cocaine or lidocaine

The macroscopic current transient represents the ensemble average of single-channel openings (Hille, 1992), and LA could bind with open channels at various time points of the transient. If either of these isoforms were more susceptible to open channel block, then some additional block of the current during the 5-ms test pulse would have caused us to overestimate the affinity of the resting and inactivated channels for that isoform. We examined whether there were differences between μ1 and hH1 in open channel block in the presence of either 300 μM cocaine or 300 μM lidocaine by comparing the percentages of block of the peak current with the block of the current time integral (nA · ms) (Wang et al., 1996b) during the 5-ms pulse. Lidocaine blocked the μ1 current integral significantly ($p < 0.05$) more than it blocked the peak current. Cocaine and lidocaine both blocked the hH1 current integral significantly ($p < 0.05$) more than they blocked the peak current. We compared the magnitudes of the increased block at each isoform and found that cocaine block of the current integral was 1.8 ± 0.5% ($n = 20$) greater than the block of the peak current at μ1 channels and was 2.9 ± 0.4% ($n = 19$) greater at hH1 channels. Lidocaine block of the current integral was 8.0 ± 0.8% ($n = 7$) greater at μ1 channels and was 10.5 ± 1.3%

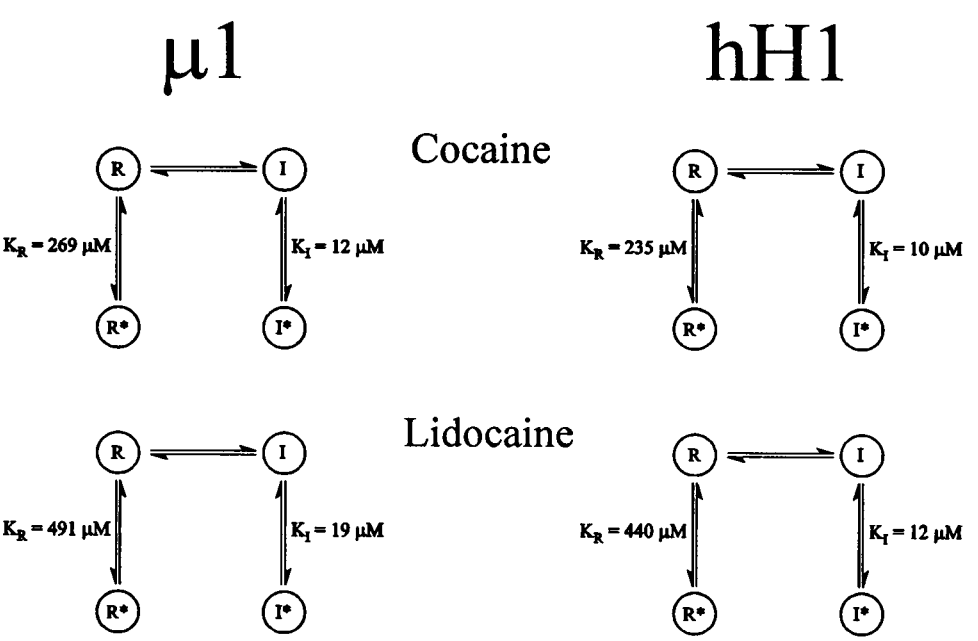

FIGURE 5 Simplified modulated receptor model for cocaine (*upper*) and lidocaine (*lower*) binding to μ1 (*left*) and hH1 (*right*) Na channels. R and I are the resting and inactivated channels, respectively, and * denotes an LA-bound channel. The dissociation constants of both cocaine and lidocaine were similar for μ1 and hH1 channels when either resting (K_R) or inactivated (K_I) channel affinities were compared.

($n = 7$) greater at hH1 channels. The difference between μ1 and hH1 in the increased block of the current integral by either drug was not significant ($p > 0.05$). These data indicate that open μ1 and hH1 channels have similar affinities for these two LAs and that the increased block would not affect the overall interpretation of our data. Any increases in block of the peak current as a result of open-channel block would have to occur in the 300 μs that precede the peak current to affect our estimates of resting and inactivated channel affinity. Therefore, the overestimation of the lidocaine affinity at resting and inactivated channels would be much less than 10%.

DISCUSSION

The net effects of a cardiotoxic (cocaine) or an antiarrhythmic (lidocaine) LA rely on a combination of physiological conditions, including heart rate, diastolic membrane potential, and central and sympathetic nervous system actions. In conjunction with these conditions, interaction of the LAs with cardiac Na channels is believed to be a major determinant of cardiotoxic (Crumb et al., 1990; Simkovich et al., 1994) or antiarrhythmic (Grant et al., 1984) manifestation.

In this study we used cloned voltage-gated Na channels from human heart and rat skeletal muscle to determine if the affinities for cocaine and lidocaine varied between tissue isoforms. We used a different approach to assess LA block of Na channels than has been used previously. Many studies of LA binding have examined the negative shift in the h_∞ curve as a determination of state-dependent anesthetic block as set out by Hille (1977). Conditioning pulses of 500 ms or longer have often been used with the h_∞ protocol to ensure that steady-state binding was reached (Bean et al., 1983). For our studies, we chose instead to change the membrane potential for 10 s at each conditioning pulse to allow binding to reach a steady state. We also inserted a 100-ms gap at the holding potential before delivery of the test pulse to allow drug-free channels to recover from fast inactivation. Although this protocol has its own drawbacks (see below), it has some advantages over using the standard h_∞ protocol. First, our initial studies with cocaine indicated that 10 s was required for steady-state binding with inactivated channels. If we had used conditioning pulses of shorter duration, then we would have seriously underestimated the cocaine affinity of inactivated channels. Second, conditioning pulses of 500 ms or longer in the h_∞ protocol would not allow us to distinguish the fast inactivated channels from the inactivated and blocked channels. The 100-ms gap at the holding potential showed that slow inactivation was minimal, even at -70 mV ($\sim$10%), and permitted drug-free channels to recover from fast inactivation.

The three principal findings of this study are as follows: 1) Resting μ1 and hH1 channels have similar intrinsic affinities for cocaine and lidocaine. 2) Inactivated μ1 and hH1 channels also have similar affinities for cocaine and lidocaine that are $\sim$20-fold higher than the affinities of the resting channels. 3) The modulation of hH1 channel affinity for cocaine and lidocaine occurs over a more negative voltage range than does the modulation of μ1 channel affinity, and therefore reflects the differences between the two channel isoforms in steady-state inactivation. Thus, compared to skeletal muscle Na channels at normal resting potentials (-100 to -90 mV), the greater sensitivity of cardiac muscle Na channels for cocaine and lidocaine is due to the larger proportion of cardiac channels in the inactivated, high-affinity state.

State dependence of cocaine or lidocaine binding to μ1 and hH1 Na channels

A significant feature of the binding studies with cocaine (Fig. 3) and lidocaine (Fig. 4) is that blockade saturates at the most negative and most positive conditioning voltages. Saturation at each extreme shows that the Na channels possess two distinct binding states for cocaine or lidocaine: a low-affinity state at -180 to -160 mV and a high-affinity state at -80 to -70 mV. The increases in percentage block that occur as the conditioning voltage becomes more positive than -160 mV are determined by the voltage-dependent transition of the channel population from the low-affinity state at -180 mV to the high-affinity state at -70 mV. This phenomenon is consistent with the Modulated Receptor hypothesis proposed by Hille (1977) and by Hondeghem and Katzung (1977). According to this hypothesis, inactivated channels have a greater affinity than resting channels for LA. As the conditioning pulse voltage becomes more depolarized, the proportion of inactivated channels increases, as does channel affinity and the percentage of block. This idea is supported by the findings of Bennett et al. (1995), who showed that removal of fast inactivation in Na channels, either by mutation of the interdomain sequence connecting domains 3 and 4 of the Na channel α-subunit or by intracellular proteolytic treatment, markedly reduced resting channel affinity for lidocaine.

The estimated K_d of cocaine and lidocaine at μ1 and hH1 channels (Fig. 5) reported here are comparable to the K_d reported in previous studies on cardiac Na channels. Guinea pig ventricular myocytes were shown to bind cocaine with K_d of 328 μM and 8 μM at resting and inactivated channels, respectively (Crumb and Clarkson, 1990). In human cardiac cells, the K_d of lidocaine at resting and inactivated channels were estimated to be 291 μM and 17 μM, respectively (Jia et al., 1993). In our comparison of the μ1 and hH1 affinities for cocaine and lidocaine, the estimated K_d were not statistically different from each other, with the exception of the affinities of inactivated channels for lidocaine. However, the difference between inactivated μ1 and hH1 channels in affinity for lidocaine was modest and cannot alone account for the antiarrhythmic activity of lidocaine.

Most studies of cardiac Na channels are performed at holding potentials of -120 to -140 mV (Jia et al., 1993; Chahine et al., 1992; Crumb and Clarkson, 1992; O'Leary

and Horn, 1994; Wang et al., 1996a,b), where removal of inactivation is presumed to be complete according to h_∞ data obtained in control saline. In the presence of cocaine, however, block becomes evident for hH1 channels near −150 mV (see Fig. 3 *B*) when the conditioning pulse is 10 s in duration. This perhaps suggests that 1–2% of the channels may enter the inactivated state at −150 mV, but are not detectable in the h_∞ protocol. Any hH1 channels that had entered the inactivated state would bind LA, become blocked, and be unavailable at the test pulse. Nuss et al. (1995) used a holding potential of −100 mV and coexpressed the Na channels with the rat brain β_1 subunit in *Xenopus* oocytes to show that the lidocaine affinity of resting hH1 channels was greater than that of μ1 channels. It is difficult to directly compare our results to those of Nuss et al. (1995), because both μ1 and hH1 channels have markedly different activation and inactivation kinetics when they are expressed in oocytes. First, steady-state activation and inactivation begin ~20 mV more negative in HEK cells compared to activation and inactivation in oocytes. Second, the macroscopic current of hH1 channels decays more slowly than does the μ1 current in HEK cells, whereas the μ1 current decays more slowly than the hH1 current in oocytes, even when the channels are coexpressed with the rat brain β_1 subunit. These kinetic differences between μ1-β_1 and hH1-β_1 channels in the oocyte expression system could account for the apparent greater affinity of resting hH1 channels for lidocaine. Wang et al. (1996b) found that hH1 channels had a higher affinity than hSkM1 for lidocaine when the HEK cells were held at −120 mV. Our data indicate that hH1 channels also have an apparent higher affinity than μ1 channels for cocaine and lidocaine at −120 mV and at −140 mV. However, conditioning pulses to −160 to −180 mV lower the percentage of blocked hH1 current, indicating that some of the hH1 channels are in the high-affinity state at −120 mV.

For hH1 channels, when conditioning pulses that are more negative than the holding potential are used, some rebinding of the LA could occur with any channels that may become inactivated during the 100-ms gap at the −140-mV holding potential. This is clearly not a question for the μ1 isoform because the percentage of block by cocaine or lidocaine remains constant for conditioning pulses between −180 and −120 mV. Because block of the hH1 isoform at −140 mV was greater than the block at −180 mV, any channels that may enter the inactivated state during the 100-ms gap at −140 mV have the opportunity to rebind LA. However, significant amounts of rebinding are unlikely, because the development of inactivation at −140 mV would be very slow. Moreover, if some rebinding does occur during the 100-ms gap at −140 mV, then we have in fact overestimated the resting affinity of hH1 channels, as determined with the conditioning pulses from −180 to −160 mV.

At −80 to −70 mV, where steady-state inactivation of both channel isoforms nears completion, the cocaine and lidocaine affinities are both more than 20-fold greater than the affinities at resting μ1 and hH1 Na channels. For cocaine, the pulse protocol we used was sufficient to determine the affinities of the two isoforms at −70 mV because the recovery from cocaine block has a τ of several seconds (Crumb and Clarkson, 1990). To determine the affinities of the inactivated channels for lidocaine, we had to examine the recovery from lidocaine block because some of the blocked channels had recovered during the 100-ms interval at the holding potential. The apparent difference between μ1 and hH1 in lidocaine block at −70 mV was in fact due to more rapid recovery from fast inactivation and to more rapid unbinding of lidocaine from the μ1 channels during the 100-ms interval to −140 mV. Nuss et al. (1995) have also shown that μ1 channels recover from lidocaine block more rapidly than hH1 channels. According to the recovery time courses from lidocaine block (Fig. 4 *B*), the percentages of block of inactivated μ1 and hH1 channels were indeed similar after recovery of the drug-free inactivated channels.

The similar resting (−180 mV) affinities of μ1 and hH1 channels for cocaine or lidocaine, and the differences between these isoforms in the proportion of resting:inactivated channels between −120 and −90 mV have two important implications. First, the intrinsic resting affinities (for cocaine or lidocaine) of the LA receptors in μ1 and hH1 Na channels are very similar, whereas the differences in the percentage of block between −120 and −90 mV resemble to some extent the differences between μ1 and hH1 in their h_∞ curves. Therefore, there is no need to postulate that cardiac Na channels have a greater intrinsic affinity for cocaine/lidocaine as an explanation for the cardiotoxic/antiarrhythmic effects of these LAs. Second, the block of hH1 channels by 30 μM cocaine is similar in extent to the block of μ1 channels by 300 μM cocaine after a conditioning pulse to −100 mV, which is near the resting membrane potential (−90 to −100 mV) of mammalian cardiac Purkinje fibers and skeletal muscle fibers (Guyton, 1986). This 10-fold difference between μ1 and hH1 in cocaine sensitivity at −100 mV is undoubtedly due to the greater amount of inactivation of the hH1 isoform. The estimated K_d of cocaine at inactivated hH1 channels is ~10 μM, which is the lower limit of circulating cocaine concentrations (10–50 μM) after fatal cocaine overdose in humans (Virmani et al., 1988). The estimated K_d of lidocaine at inactivated hH1 channels is ~12 μM, which is within the effective therapeutic range (6–25 μM) of the lidocaine dosage used to treat postinfarction arrhythmias (Bigger and Hoffman, 1985).

Because LAs often act as cardiotoxic or antiarrhythmic agents at concentrations that do not obviously affect the normal physiology of nerve or skeletal muscle, several reports have concluded that the LA receptor of cardiac Na channels is intrinsically more sensitive to LAs than are the LA receptors of other Na channel isoforms (Nuss et al., 1995; Wang et al., 1996b). To our knowledge, this is the first study to show that mammalian isoforms of cardiac and skeletal muscle Na channels have a similar intrinsic resting

affinity for cocaine or lidocaine. We note that there may well be differences in affinity between μ1 and hH1 channels for other LAs, and that other regions in addition to the S6 segment of domain 4 may influence LA binding (Bennett et al., 1995). The differences in LA affinity between cardiac and skeletal muscle Na channels near the resting potential are most likely due to channel regions that control steady-state inactivation. As predicted by the Modulated Receptor hypothesis, the cocaine or lidocaine affinity of each isoform is strongly modulated by membrane voltage. We can conclude that the greater sensitivity of cardiac tissue to cocaine or lidocaine is due to the greater proportion of cardiac Na channels that are inactivated at normal resting potentials, rather than to a greater intrinsic affinity of the cardiac LA receptor.

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