Characterization of cocaine-induced block of cardiac sodium channels

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ABSTRACT Recent evidence suggests that cocaine can produce marked cardiac arrhythmias and sudden death. A possible mechanism for this effect is slowing of impulse conduction due to block of cardiac Na channels. We therefore investigated its effects on Na channels in isolated guinea pig ventricular myocytes using the whole-cell variant of the patch clamp technique. Cocaine (10–50 μM) was found to reduce Na current in a use-dependent manner. The time course for block development and recovery were characterized. At 30 μM cocaine, two phases of block development were defined: a rapid phase (τ = 5.7 ± 4.9 ms) and a slower phase (τ = 2.3 ± 0.7 s). Recovery from block at −140 mV was also defined by two phases: (τ_f = 136 ± 61 ms, τ_s = 8.5 ± 1.7 s) (n = 6). To further clarify the molecular mechanisms of cocaine action on cardiac Na channels, we characterized its effects using the guarded receptor model, obtaining estimated K_d values of 328, 19, and 8 μM for channels predominantly in the rested, activated, and inactivated states. These data indicate that cocaine can block cardiac Na channels in a use-dependent manner and provides a possible cellular explanation for its cardiotoxic effects.

INTRODUCTION

Whereas the mechanism(s) for cocaine-induced mortality remains poorly defined, it has been suggested that cocaine overdose may result in the production of cardiac arrhythmias and sudden death (Benchimol et al., 1978; Wetli and Wright, 1979; Nanji and Filipenko, 1984). Cocaine use has become a major medical problem in the United States, with overdose related deaths dramatically increasing over the past decade (Mittleman and Wetli, 1984; Pollin, 1985). Recent evidence suggests that blood levels of cocaine achieved in individuals who died from cocaine overdose range from 5–90 μM (Finkle and McCloskey, 1977; Mittelman and Wetli, 1984; Spiehler and Reed, 1985). Because cocaine is well known to have potent local anesthetic properties in nerve, one possible mechanism for cocaine cardiotoxicity is the production of cardiac conduction disturbances resulting from block of cardiac sodium (Na) channels. Because the cellular effects of cocaine on Na channels have not been well defined, we therefore investigated the effects of cocaine on Na channels in single cardiac myocytes isolated from guinea pigs using the whole-cell variant of the patch clamp technique (Hamill et al., 1981). We found cocaine to be a potent blocker of cardiac Na channels.

To further clarify the molecular mechanisms of cocaine action on cardiac Na channels, we characterized its effects using the guarded receptor model (Starmer et al., 1984; Starmer and Grant, 1985; Starmer et al., 1986; Starmer, 1987). Starmer et al. (1984) proposed the guarded receptor model as a simplification of the modulated receptor model. One of the virtues of the model is that it provides an analytical solution which allows the calculation of rate constants for drug-channel interactions. This model has been found to well describe the effects of local anesthetics on action potential upstroke velocity in heart tissue, but has not been adequately tested using direct measurements on Na currents. Therefore, our use of this model provides an additional test of the model as well as the estimation of dissociation constants for cocaine binding to channels in the rested, inactivated, or activated states. Our results provide experimental evidence that cocaine has channel-blocking properties that make it a potent blocker of cardiac Na channels, and suggests a possible cellular explanation for the cardiotoxic effects of cocaine. A preliminary report of this work has been previously submitted in abstract form (Crumb and Clarkson, 1989).

METHODS

Isolation of cardiac myocytes

The method for isolation of ventricular myocytes used in this study is similar to that described by Mitra and Morad (1985). Hearts from guinea pigs (200–300 g) were removed under pentobarbital anesthesia and mounted by the aorta onto the cannula of a Langendorff perfusion apparatus. Hearts were initially perfused for 5 min at 10–15 ml/min with a calcium-free bicarbonate buffered solution at 37°C followed by an 8-min perfusion with the same solution supplemented with 2 mg/ml collagenase (284 U/mg; Worthington Type II; Worthington Biochemi-

0006-3495/90/03/589/11 $2.00

cals, Freehold, NJ) and 0.28 mg/ml protease (5.6 U/mg; type XIV; Sigma Chemical Co., St. Louis, MO). The ventricles were then cut free, placed in an enzyme-free solution containing 0.2 mM $CaCl_2$, minced with scissors, and gently stirred at 37°C for 5 min. Cells were harvested from the suspension by filtration through a 200-μm nylon mesh, and the filtrate was then centrifuged at 200–300 rpm for 1.5 min to form a pellet. The supernatant was suctioned off and the pellet was resuspended in enzyme-free solution supplemented with 1 mM $CaCl_2$ and 1 mg/ml bovine serum albumin (fraction V; Sigma Chemical Co.). This suspension was also centrifuged to form a pellet, and this pellet was resuspended in minimal essential medium supplemented with Earle's salts, L-glutamine (292 μg/ml), 3% horse serum, 0.1 mg/ml streptomycin, and 100 U/ml penicillin (Gibco Laboratories, Grand Island, NY). This final suspension was poured into culture dishes and stored at 37°C in a CO_2 incubator until the cells were used in experiments (within 30 h after isolation). The cells used in experiments were of characteristic morphology (rod-shaped with well-defined striations) and lacked any visible blebs on the surface.

Solutions

The solution used for perfusing the heart and cell isolation consisted of Joklik modified minimum essential medium (Gibco Laboratories) adjusted to a pH of 7.2 with NaOH, gassed with 95% O_2:5% CO_2, and warmed to 37°C. When electrical recordings were made from isolated cells, the bath was perfused with an "external" solution that consisted of (in millimolars): 115 tetramethylammonium chloride, 25 NaCl, 5 CsCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 20 Hepes, 11 glucose; adjusted to a pH of 7.3 with tetramethylammonium hydroxide. Glass pipettes (electrodes) were filled with an "internal" solution that consisted of (in millimolars): 125 CsF, 20 CsCl, 10 NaF, 10 bis-Tris propane, 5 EGTA; adjusted to a pH of 7.2 with CsOH. The cocaine HCl was purchased from Sigma Chemical Co.

Voltage clamp recording

At the beginning of each experiment, an aliquot of cells was placed into a shallow tissue bath mounted atop an inverted microscope. Sodium currents from the isolated ventricular myocytes were measured using the whole-cell variant of the patch clamp method (Hamill et al., 1981). The glass pipettes used were pulled in two stages then fire polished until tip openings of 3–4 μm were obtained. Tip resistances were 0.3–0.5 MΩ when the pipettes were filled with the internal solution. Pipette tips were positioned medially along the long axis of the cell, and a gigohm seal was formed between the pipette tip and cell membrane. The membrane patch under the pipette tip was then ruptured by applying a suction transient, creating a continuity between the internal (pipette) solution and the cell interior. The bath temperature was measured by a thermistor placed near the cell under study and was maintained at 16°C ± 0.5°C with a thermoelectric device (model 806-7243-01; Cambion/Midland Ross, Cambridge, MA).

An Axopatch 1-B amplifier (Axon Instruments, Burlingame, CA) was used for whole-cell voltage clamping. Creation of voltage clamp pulses and data acquisition were controlled by an IBM PC/AT computer running pClamp software (Axon Instruments). The computer was interfaced to the amplifier by an 80-kHz Labmaster board (Scientific Solutions, Inc., Solon, OH). Sodium currents were digitized at sample intervals of 20–200 μs. Cells were maintained at a holding potential of −140 mV between pulse protocols to fully remove inactivation.

Evaluation of methods

To accurately study drug effects on Na currents, other ionic currents must be insignificant, and voltage clamp errors related to uncompensated series resistance and voltage inhomogeneity reduced to within acceptable levels. In these experiments, Na currents were isolated from Ca^{++} and K^+ currents by replacing all K^+ with Cs^+, and eliminating Ca^{++} currents by blocking Ca^{++} channels with internal F^- ions. Under these conditions steady-state (leak) current at the test potential (−20 mV) was <0.1 nA, or <1–2% of peak Na current. Correction for leak current was not made. Values of cell capacitance were estimated from integration of capacitive transients, and had a mean value of 66 ± 4 pF (mean ± SD, n = 13). Following rupture of the membrane seal, the mean total series resistance (Rs) for the pathway between pipette and cell membrane was 1.3 ± 0.1 MΩ (mean ± SD, n = 13) under the experimental conditions used in this study. It was possible to electronically compensate for 60–80% of this series resistance in these cells. Experiments were performed in cells where the estimated voltage drop across the uncompensated series resistance was <5 mV.

Data analysis and mathematical modeling

An IBM-PC/AT using programs written in the ASYST language (Asyst Software Technologies, Inc., Rochester, NY) was used for curve fitting and mathematical modeling. A nonlinear least-squares algorithm using the Gauss-Newton method was used to fit exponential functions to experimental data.

To further define the effects of cocaine on cardiac Na channels, apparent rates of drug binding and unbinding to rested, open, and inactivated Na channels were calculated using analytical equations based upon the guarded receptor model (Starmer et al., 1984; Starmer and Grant, 1985; Starmer, 1987). According to the model, tonic and use-dependent block of Na channels observed during a train of depolarizing pulses results from drug interaction with a receptor site having diffusion pathways that are guarded by the position of channel activation and inactivation gates (Fig. 7). When all activation gates are closed, drugs cannot bind to the channel receptor since these gates prevent access to the receptor site via both hydrophilic and hydrophobic pathways. Therefore at very negative potentials, where the fraction of channels having open activation gates is small, only a relatively small tonic block is observed at low drug concentrations. When channels open, drug can bind and unbind from the receptor over both hydrophilic and hydrophobic pathways. Once the inactivation gate has closed and the hydrophilic pathway is obstructed, drug may continue to bind and unbind from the channel receptor via the hydrophobic (membrane) pathway. In a channel occluded by a charged drug, closing of either the activation or inactivation gates prevents drug dissociation until either the channel reopens, or as for a tertiary amine, the drug is converted to an uncharged (membrane permeant) form. Uncharged drugs are not trapped in rested or inactivated channels since they can diffuse into the lipid bilayer at any time. As a consequence of receptor guarding and drug trapping by channel gates, a pulse-by-pulse accumulation of channel block (use-dependent block) is observed when channels are repetitively cycled between hyperpolarized and depolarized potentials.

To estimate the apparent rate constants defining drug interaction with channels once they become inactivated, it was assumed that the slow component of time-dependent block characterized using a two-pulse protocol (Fig. 3 *A*) results from drug binding to a continuously accessible receptor (i.e., via the hydrophobic membrane pathway) as described by

$$[D] + U \underset{l_i}{\overset{k_i}{\rightleftharpoons}} B,$$

where U and B represent the fraction of unblocked and blocked channels, $[D]$ is the drug concentration, and k_i, l_i are apparent rates of drug binding and unbinding. According to this model, changes in the proportion of blocked channels observed during a long pulse is an exponential process defined by the equations:

$$\lambda_i = (k_i[D] + l_i); \quad \tau_i = \lambda_i^{-1} \tag{1}$$

$$I_\infty = k_i[D]/(k_i[D] + l_i) \quad \text{or} \quad I_\infty = k_i[D]\tau_i \tag{2}$$

$$k_i = I_\infty/(\tau_i[D]) \tag{3}$$

$$l_i = (1/\tau_i) - (k_i[D]), \tag{4}$$

where k_i and l_i reflect the apparent rates of drug binding and unbinding to inactivated channels, τ_i is the time constant for drug binding to inactivated channels, and I_∞ is the steady-state fraction of inactivated-blocked channels. These rate constants were calculated from estimates of the time constant (τ_i) and steady-state level (I_∞) of the slow phase of block development defined using a two-pulse protocol (Fig. 3 *A*).

The rate constants defining drug interaction with open and rested channel states were estimated from rate train data using an analysis scheme similar to that previously used by Starmer and co-workers (Starmer and Grant, 1985; Starmer et al., 1986; Starmer, 1987). However, because the experimental data indicated that cocaine can interact with both open and inactivated channels, the analytical equations of Starmer et al. were modified to incorporate the presence of three (vs. two) significant channel states (rested, open, and inactivated). The time course of use-dependent block onset during a train of repetitive pulses was described in terms of a recurrence relation (Starmer and Grant, 1985):

$$b_n = b_{ss} + (b_0 - b_{ss})e^{-n\lambda}, \tag{5}$$

where b_n is fraction of channels blocked during the nth pulse, b_0 is the fraction of channels initially blocked (e.g., after a 1-min rest at the holding potential), b_{ss} is the "steady-state" fraction of channels blocked after many pulses, n is the pulse number, and λ is the blocking rate constant. For a three-state model, the blocking rate constant λ can be further defined by

$$\lambda = \lambda_r t_r + \lambda_o t_o + \lambda_i t_i, \tag{6}$$

where λ_r, λ_o, and λ_i are the rates of drug interaction with channels when they are predominantly in rested, open, or inactivated states during the pulse protocol, t_r is the interpulse (recovery) interval, t_o is the channel mean open time, and t_i is the time interval channels spend in the inactivated state (Starmer and Grant, 1985). The steady-state level of channel block (b_{ss}) may also be defined by (Kojima and Ban, 1988; their Model I):

$$b_{ss} = O_\infty X_1 + I_\infty X_2 + R_\infty X_3, \tag{7}$$

where

$$X_1 = (1 - e^{-\lambda}o^t o)e^{-(\lambda_i t_i + \lambda_r t_r)}/(1 - e^{-\lambda}) \tag{8}$$

$$X_2 = (1 - e^{-\lambda_i t_i})e^{-\lambda_r t_r}/(1 - e^{-\lambda}) \tag{9}$$

$$X_3 = (1 - e^{-\lambda_r t_r})/(1 - e^{-\lambda}). \tag{10}$$

Eq. 7 may also be simplified as

$$b_{ss} = O_\infty X_1 + \delta, \tag{11}$$

where

$$\delta = I_\infty X_2 + R_\infty X_3. \tag{12}$$

To estimate the rate constants for drug binding and unbinding to rested and open-channel states, estimates of the parameters λ, b_o, and b_{ss} were first obtained by fits of Eq. 5 to peak Na currents obtained during pulse trains at five to six different interpulse intervals. The estimated value of b_o was taken as an estimate of the equilibrium level of block at the holding potential (R_∞). Estimates of λ_r and λ_o were then computed according to Eq. 6 by linear regression of λ vs. t_r (λ_r = slope, λ_o = [λ-intercept $- \lambda_i t_i$]/t_o). For analysis, t_o was assumed to be equal to 1 ms (i.e., similar to reported values of the mean open time for single cardiac Na channels) (Fozzard et al., 1987; Kunze et al., 1985) and t_i was assumed to be equal to the duration of the test pulse at -20 mV $- t_o$. The equilibrium level of open channel block at the test potential (O_∞) was estimated from Eq. 11 by linear regression of b_{ss} vs. X_1 (O_∞ = slope).

From calculated values of λ_r, R_∞, and O_∞, the apparent forward (k_r, k_o) and reverse (l_r, l_o) rate constants defining drug interaction with rested and open channels were then calculated using the following equations (Starmer, 1987)

$$k_r = \lambda_r R_\infty/[D] \tag{13}$$

$$k_o = \lambda_o O_\infty/[D] \tag{14}$$

$$l_r = \lambda_r(1 - R_\infty) \tag{15}$$

$$l_o = \lambda_o(1 - O_\infty). \tag{16}$$

The guarded receptor model predicts that these state-dependent rate constants can theoretically be broken down further into guard and trap functions and state-independent binding and unbinding rates once the model for channel gating and deprotonation/protonation rates for bound drug are known (Starmer and Grant, 1985; Starmer and Courtney, 1986). However, in this study, only the apparent "state-dependent" rate constants were defined because neither the proper gating model for cardiac Na channels, nor the conversion rates between charged and uncharged cocaine within the channel were known.

Statistical analysis

Paired and unpaired two-tailed Student's t tests were used to evaluate significance between two groups of data. Comparisons between more than two groups was accomplished by one-way analysis of variance (ANOVA). Data analyzed by linear regression were evaluated by calculation of the Pearson product-moment correlation coefficient (r) and a t test for significance of linear trend. If values of $P < 0.05$ were obtained, the differences were considered significant. Results are expressed as mean ± SD.

RESULTS

Tonic and use-dependent block

Cocaine reduced cardiac Na current in a manner similar to many other local anesthetics. This reduction in current amplitude could be divided into two components: tonic and use-dependent block. As indicated in Fig. 1 *B* and *C*, upon application of a train of depolarizing pulses at 5 Hz after a long rest, there was no noticeable change in Na current amplitude in the absence of drug. After a 10–20 min exposure to either 10, 30, or 50 μM cocaine, there was a reduction in Na current amplitude during the first pulse after a long rest (tonic block) as well as an additional

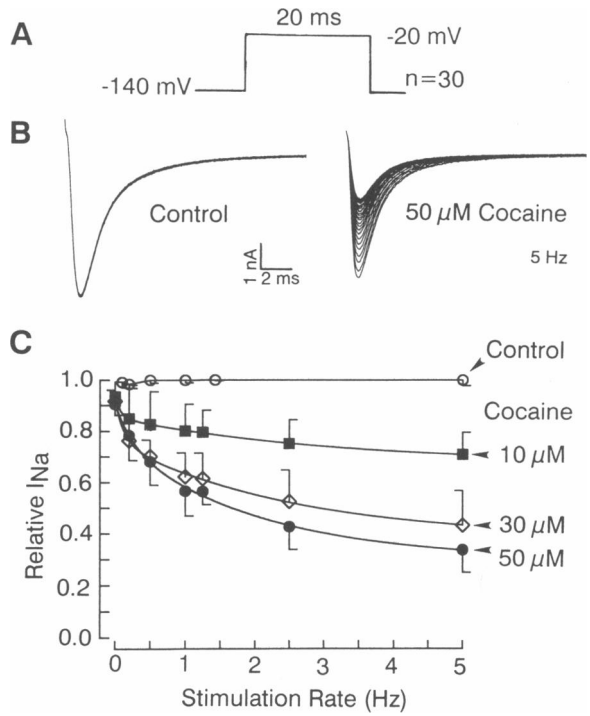

FIGURE 1 Tonic and use-dependent inhibition of the peak sodium (Na) current by cocaine in guinea pig ventricular myocytes. (*A*) Pulse protocol. A train of depolarizing pulses to −20 mV was applied at different stimulation rates after a 1 min rest at the holding potential (−140 mV). (*B*) Superimposed records of Na current obtained during a train of pulses applied at 5 Hz before and during exposure to 50 μM cocaine. For the controls, currents for only the 1st and 30th pulses are shown. There was no noticeable decrease in peak current during repetitive pulsing under control conditions, whereas in the presence of drug there was both an initial (tonic) block observed on the first pulse, as well as a use-dependent block that approached a steady-state within 30 pulses. (*C*) Shows the mean level of peak Na current after 30 pulses at selected stimulation rates under control conditions (*open circles*), in the presence of 10 μM cocaine (*closed squares*), 30 μM cocaine (*open diamonds*), and 50 μM cocaine (*closed circles*). Peak Na current amplitudes have been normalized to the amplitude of control Na current after a >10-s rest. Data values represent mean ± SD (n = 4–6).

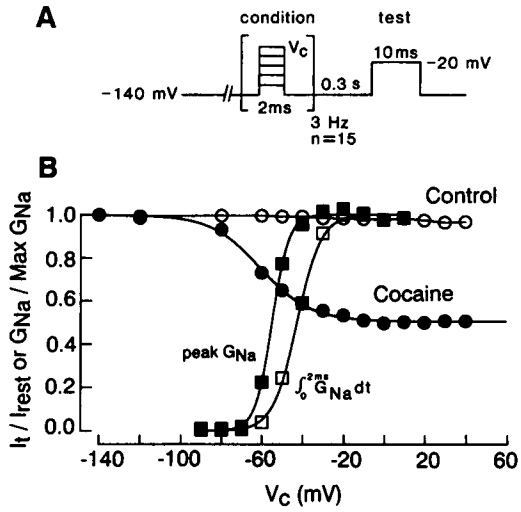

FIGURE 2 Voltage-dependence of use-dependent block produced by 30 μM cocaine. (*A*) Pulse protocol. Use-dependent block was produced by a train of 15 pulses of 2 ms duration at 3 Hz to selected conditioning potentials (V_c). The amplitude of the peak Na current available after the conditioning train was determined with a test pulse after a recovery interval of 300 ms. (*B*) Shows the relationship between peak Na current and the conditioning potential (V_c). Peak current was normalized to its value after a long rest at −140 mV. Open circle is control, closed circle is 30 μM cocaine. The relation between membrane potential and G_{Na} for the same cell is also shown for comparison. G_{Na} was calculated using both peak Na current (*closed squares*) and the integral of Na current over a 2-ms interval (*open squares*).

reduction in current amplitude which became progressively larger with each successive pulse (use-dependent block), reaching a steady state after 15–30 pulses. This steady-state level of block was frequency dependent.

Mean levels of both tonic and use-dependent block are shown in Fig. 1 *C*. In the absence of drug, there was no noticeable effect of pulsing on Na current amplitude as stimulation rate was increased to 5 Hz. In contrast, with the addition of 10, 30, or 50 μM cocaine there was up to a 10% reduction in Na current amplitude upon the first pulse. In addition there was a significant use-dependent block which developed upon repetitive stimulation at rates above 0.2 Hz ($P < 0.05$).

Voltage dependence

The relationship between membrane potential and Na channel block was determined by using the pulse protocol shown in Fig. 2 *A*. A conditioning train consisting of 15 pulses of 2-ms duration was applied to various voltages to produce a steady-state level of block. After a 300-ms recovery interval, a test pulse was given to determine the amount of block produced by the conditioning train. The 300-ms recovery interval was long enough to allow unblocked channels to recover from inactivation, but short enough to allow minimal recovery from cocaine-induced block.

In the absence of drug, the conditioning train had little effect on the amplitude of the Na current recorded during the test pulse (Fig. 2 *B*). However the addition of either 10, 30, or 50 μM cocaine resulted in a voltage-dependent channel block. As indicated by the smooth curve in Fig. 2 *B* the voltage dependence of block could be well described by an equation used to describe open channel block by local anesthetics in nerve (Cahalan, 1978; Cahalan and Almers, 1979):

$$I_{Na}\,(\text{drug})/I_{Na\,(\text{control})} = (1 - B)/(1 + \exp[V_c - V_{mid}/S]) + B, \quad (15)$$

where B is the asymptotic level of Na current remaining after conditioning trains to positive potentials, V_c is the membrane potential during the conditioning train, V_{mid} is the membrane potential at half-maximal block, and S is the slope of the voltage dependent block. Estimated mean values of these variables obtained from least squares fits to Eq. 15 using data obtained in the presence of 10–50 μM cocaine ($n = 5$) were -51.1 ± 6.9 mV for the midpoint for voltage-dependent block (V_{mid}) and 13.7 ± 3.1 mV for the slope factor for block (S).

To determine whether the voltage dependence of channel block was similar to channel opening, channel block was compared with Na channel conductance (G_{Na}). G_{Na} was calculated from peak I_{Na}, and from integrations of I_{Na} during the initial 2 ms or over the entire 50 ms of depolarizing pulses to potentials from -90 to $+10$ mV. The 2- and 50-ms integrals were selected because they closely resemble either the conditioning pulse duration (Fig. 2 A) or an integral of I_{Na} over a time period during which the Na current is almost completely inactivated (50 ms). Peak G_{Na} fits obtained using a Boltzman equation of the form $G_{Na} = G_{max}/[1 + \exp(V_{mid} - V_m)/S]$ had a mean half-maximal G_{Na} value (V_{mid}) at -55.6 ± 7.1 mV ($n = 5$) and a mean slope factor (S) of 4.4 ± 1.7 mV ($n = 5$). Thus although both channel block and peak G_{Na} were half maximal at similar potentials, the slope factor for peak G_{Na} was significantly smaller ($P < 0.001$) than that for channel block, indicating that channel opening had a steeper voltage dependence than channel block. The slope factors for time integrals of G_{Na} were also significantly smaller than that of channel block [for a 2-ms integral: $S = 6.1 \pm 0.7$ ($P < 0.001$), $V_{mid} = -43.3 \pm 6.6$; and for a 50-ms integral: $S = 2.5 \pm 2.6$ ($P < 0.01$), $V_{mid} = -63.5 \pm 5.3$]($n = 5$).

Block development

To characterize the ability of cocaine to bind to Na channels during a depolarizing pulse a two-pulse protocol was employed to determine the time course of block development. As illustrated in Fig. 3 A, the protocol consisted of a conditioning pulse of variable duration at -20 mV followed by a test pulse after a 300-ms recovery interval. The recovery interval was long enough for most drug-free channels to recover from inactivation, but short enough to allow only a minimal (<4%) amount of recovery from channel block. In the absence of drug, test current amplitude was not substantially depressed even with conditioning pulse durations of up to 10 s. However, with the addition of 30 μM cocaine, test current amplitude progressively decreased as the conditioning pulse duration was increased. The time course of block development consisted of two distinguishable phases (fast and slow) which could be well approximated by the following

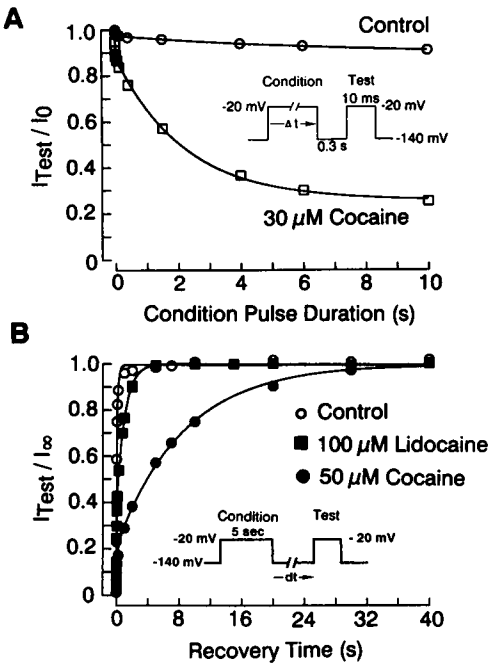

FIGURE 3 (A) Biphasic time course of block development at -20 mV. (*Inset*) Pulse protocol. Block development was determined by a single test pulse applied 300 ms after a conditioning pulse of variable duration. The interval between each trial of the pulse protocol was 1 min. Panel A shows the time course of block development at -20 mV. Under control conditions (*open circles*) there was little change in peak current amplitude following conditioning pulses of up to 10 s duration. In the presence of 30 μM cocaine, peak Na current was inhibited in two distinct phases. Test currents have been normalized to their value after a long rest in the absence of a prepulse (I_o). (B) Time course of recovery from block at the holding potential (-140 mV). (*Inset*) Pulse protocol. A steady-state level of block was produced by a conditioning pulse of 5 s duration to -20 mV. The kinetics of recovery from block were then defined by measuring the fraction of peak Na current (I_{Test}) available with a test pulse elicited after a variable recovery interval. Panel B shows the time course of recovery of peak Na current under control conditions (*open circles*), 50 μM cocaine (*closed circles*), and for comparison 100 μM lidocaine (*closed squares*). The test currents have been normalized to their steady-state rested values (I_∞). In the presence of 50 μM cocaine, recovery from block consisted of two phases and was well fit by the sum of two exponentials. The slow phase of recovery with 50 μM cocaine had a time constant (τ_s) of 8.5 ± 1.7 s as opposed to that of 100 μM lidocaine where τ_s is 1.0 ± 0.4 s.

equation: $A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s} + A_\infty$. The amplitudes and time constants for block development in the presence of 30 μM cocaine were: $A_f = 0.14 \pm 0.08$, $\tau_f = 5.68 \pm 4.92$ ms, $A_s = 0.64 \pm 0.06$, $\tau_s = 2.29 \pm 0.68$ s, and $A_\infty = 0.23 \pm 0.03$ ($n = 5$); and those for 50 μM cocaine (not shown in Fig. 3 A) were: $A_f = 0.07 \pm 0.01$, $\tau_f = 2.88 \pm 1.47$ ms, $A_s = 0.79 \pm 0.01$, $\tau_s = 1.46 \pm 0.61$ s, and $A_\infty = 0.13 \pm 0.02$ ($n = 4$). The time constants for both phases and the amplitude of the fast phase were not significantly different at either concentration, however, the amplitudes of

the slow phase and the steady-state level of block were significantly different ($P < 0.001$).

Recovery from block

The time course of recovery from block was characterized using the two-pulse protocol depicted in Fig. 3 *B*. A steady-state level of block was produced by a single conditioning pulse to −20 mV for 5 s. The time course of recovery from block was then determined by a test pulse after a variable recovery interval. In the absence of drug, recovery from inactivation at −140 mV was described by a double exponential function having a large fast component with an amplitude (A_f) of 0.87 ± 0.05 and a time constant (τ_f) of 13 ± 4.0 ms, and a small slow component with an amplitude (A_s) of 0.18 ± 0.05 and a time constant (τ_s) of 246 ± 163 ms ($n = 8$). In the presence of 50 μM cocaine, recovery of the Na current after the conditioning pulse displayed two phases (Fig. 3 *B*): a small fast phase ($A_f = 0.13 \pm 0.03$, $\tau_f = 136 \pm 61$ ms), which probably reflects a combination of recovery of drug-free channels as well as rapid unblocking of drug from open channels; and a large slow phase ($A_s = 0.85 \pm 0.03$, $\tau_s = 8.5 \pm 1.7$ s) ($n = 6$), which reflects recovery of drug-associated channels from a rested state. Alteration of the recovery potential over a 40-mV range (from −120 to −150 mV) did not significantly alter the time constant for recovery from block (Fig. 4).

Mathematical modeling of cocaine block

The apparent rate constants defining drug binding (k_i) and unbinding (l_i) from inactivated channels were calculated using Eqs. 1–4 from estimates of the steady-state level of block (tonic + time-dependent) at −20 mV (I_∞), and the time constant for the slow phase of channel block obtained using a two pulse protocol (Fig. 3 *A*). Mean values of k_i and l_i are shown in Table 1. To calculate the apparent rates of drug binding to rested and open (activated) channels, data from pulse train protocols (Fig. 5 *A*) were analyzed using a parameter estimation procedure based on the guarded receptor model (see Methods). A nonlinear least squares algorithm was used to obtain fits

TABLE 1 State-dependent rate constants for cocaine HCl*

Parameter	Value
$k_r(M^{-1}s^{-1})$	274 ± 217
$l_r(s^{-1})$	0.1 ± 0.05
$K_{dr}(\mu M)$	328 ± 156
$k_o(M^{-1}s^{-1})$	$1.13 \times 10^6 \pm 2.5 \times 10^5$
$l_o(s^{-1})$	20 ± 10.2
$K_{do}(\mu M)$	18.5 ± 10.2
$k_i(M^{-1}s^{-1})$	$1.2 \times 10^4 \pm 5.0 \times 10^3$
$l_i(s^{-1})$	0.1 ± 0.05
$K_{di}(\mu M)$	7.9 ± 1.4

*Data are grouped mean ± SD ($n = 10$), derived from rate constants for 30 and 50 μM cocaine. Rate constants are apparent binding and unbinding rates to channels rested at −140 mV, activated at −20 mV, and inactivated at −20 mV. No attempt was made to factor out receptor guard and trap functions (see Methods). State-dependent dissociation constants (K_d) are the ratio of l/k.

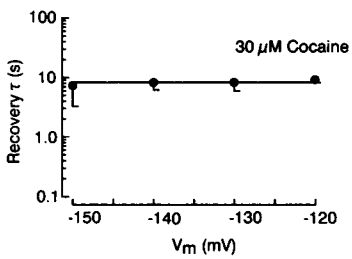

FIGURE 4 Voltage dependence of recovery from block by 30 μM cocaine. A steady-state level of block was produced with a 5 s pulse to −20 mV. The voltage dependence of cocaine unblocking was defined by measuring the slow component of recovery from block at different holding potentials (V_m) (−150 mV = 7.4 ± 4.1 s, −140 mV = 8.2 ± 2.3 s, −130 mV = 8.4 ± 2.4 s, and −120 mV = 9.3 ± 1.1 s) ($n = 3$–8). The equation of best fit was $\tau = 8.4\ e^{(0.21\ V_m)}$. Values are given as mean ± SD.

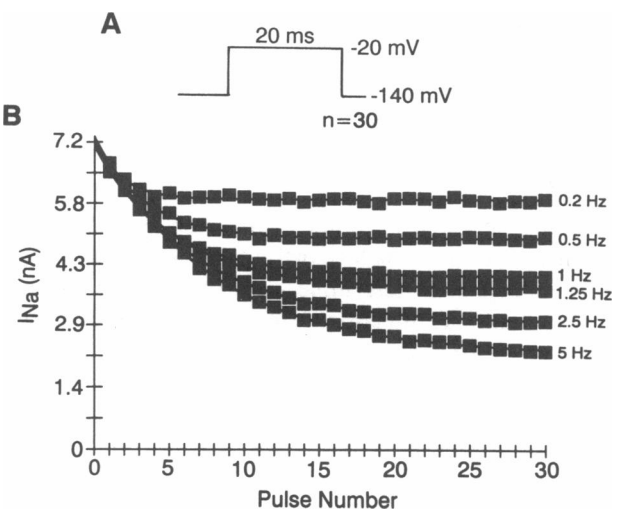

FIGURE 5 Guarded receptor analysis of use-dependent block of cardiac Na channels by cocaine. (*A*) Pulse protocol. A train of depolarizing pulses to −20 mV was applied at different stimulation rates following a 1-min rest at the holding potential (−140 mV). (*B*) Peak Na currents recorded during pulse trains at different rates were fit to Eq. 5 to estimate uptake rates (λ) and steady-state amplitudes of block (b_∞).

of Eq. 5 to peak Na current values recorded during trains of 20 ms pulses to −20 mV (Fig. 5 *B*). From these fits, values of tonic block (b_o), blocking rate (λ), and steady-state level of use-dependent block (b_{ss}) were estimated for 5–6 different interpulse intervals (t_r). A linear regression of blocking rate (λ) against recovery interval (t_r) was then performed using Eq. 6 to calculate values of λ_r and λ_o (λ_r = slope, λ_o = [λ-intercept − $\lambda_i t_i$]/t_o)(Fig. 6 *A*). Values of the blocking parameter X_1 were then computed using Eq. 8, and a linear regression of steady-state block (b_{ss}) against X_1 was performed using Eq. 11 to estimate the equilibrium levels of channel block at −20 mV (slope = O_∞) (Fig. 6 *B*). The relationships between both λ vs. t_r and b_{ss} vs. X_1 were found to be well described by a simple linear function ($r > 0.9$, $P < 0.05$) (Fig. 6 *A* and *B*), consistent with the theoretical model. These estimated model parameters were then used to calculate the apparent binding and unbinding rates according to Eqs. 13–16. Mean values for k_r, l_r, k_o, and l_o are given in Table 1. Since the estimates of k_o and l_o were based upon an assumed value for t_o (1 ms), assumption of a different access interval for calculation of λ_o will result in reciprocal changes in estimated values of k_o and l_o from Eqs. 14 and 16 (e.g., assuming a value of t_o = 2 ms results in values of k_o and l_o half those appearing in Table 1).

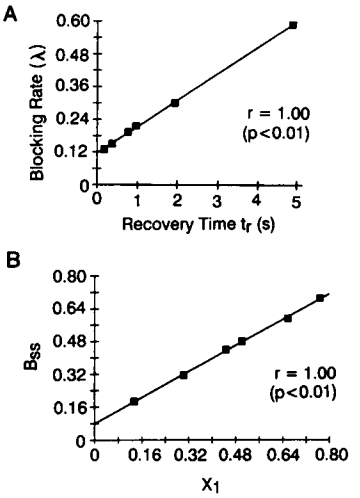

FIGURE 6 Mathematical modeling of channel block by cocaine according to the guarded receptor model. (*A*) Shows a fit of the relationship between blocking rate (λ) and the interpulse recovery time (t_r) according to Eq. 6. (*B*) Shows a fit of the relationship between b_{ss} and X_1 according to Eq. 7. The observed curves are in agreement with the theoretical predictions of an exponential relationship between Na current amplitude and pulse number (Fig. 5 *B*), as well as linear relationships between λ vs. t_r and b_{ss} vs. X_1.

DISCUSSION

Block development

The time course of cocaine block of cardiac Na channels during a depolarizing pulse was characterized using a two-pulse protocol (Fig. 3 *A*). As with other local anesthetics (Clarkson et al., 1988), cocaine block consisted of two definable phases: a rapid phase (τ_f = 3–6 ms) and a slow phase (τ_s = 1.5–2.3 s), indicating that cocaine exhibits a relatively high affinity for multiple channel states occupied during a depolarizing pulse. During the first few milliseconds of a suprathreshold depolarizing pulse, Na channels fluctuate through multiple conformational states, thus making it difficult to accurately identify the channel states occupied during the rapid phase of block. Previous studies on other local anesthetics have attributed a rapid phase of channel block to the charged (cationic) form of the drug binding to the open-state of the Na channel (Strichartz, 1973; Hille, 1977; Hondeghem and Katzung, 1977; Starmer et al., 1984). Under the conditions of these experiments (pH = 7.2–7.3, temperature = 15–16°C), cocaine (pKa = 8.6) should exist largely (95–96%) in its charged form. The fact that cocaine exists primarily in the charged (cationic) form and has a component of block development that overlaps that of channel opening lead us to further test the hypothesis that this component of block was due to block of open Na channels in cardiac myocytes.

A recent single channel study of cocaine's effects on neuronal Na channels in planar lipid bilayers (Wang, 1988) indicates that cocaine can block open neuronal Na channels in a voltage-dependent manner. Using a pulse protocol (Fig. 2 *A*) similar to that previously used to define the voltage dependence of local anesthetic block in nerve (Clarkson et al., 1988), we found that the rapid component of channel block had a voltage dependence that was similar to that of channel opening: both peak G_{Na} and channel block developed and reached an asymptote over the same voltage range and had voltage midpoints that were not significantly different (−55.6 vs. −51.1 mV) ($P > 0.3$). This differs from the voltage dependence of inactivation which under the conditions of this experiment develops and saturates over the voltage range of −120 to −60 mV (Follmer et al., 1987; Clarkson et al., 1988). Although the voltage-dependence of the fast component of block developed and saturated over a similar voltage range as that of channel opening, the two were not identical in that channel block was consistently less steep than channel opening. The explanation for this difference is not clear but could possibly be due to competitive interactions between cocaine and monovalent cations (e.g., Na^+) at a common binding site, as recently reported in lipid bilayers (Wang, 1988). Wang found that as external Na^+ was increased from 100 to 300 mM, there

was an increase in drug dissociation rate resulting in a sixfold increase in the calculated K_d value for drug interaction with open channels. It therefore seems reasonable to speculate that an increase in the external Na^+ from that used in this study (25 mM) to more physiologic concentrations might similarly increase the cocaine dissociation rate and the estimated K_d value for cocaine interaction with open channels in the heart as well.

The voltage-dependence of cocaine block was not adequately fit by a previously proposed model describing the voltage dependence of channel block to the effect of changes in the transmembrane voltage gradient on the passive movement of drug to a binding site within the channel (Strichartz, 1973; Cahalan, 1978). This type of model predicts the slope parameter (S) which defines the steepness of voltage-dependent block should be equal to $RT/z\delta F$, where F, R, and T have their usual meanings, z is the drug molecule's valence, and δ is the equivalent electrical distance of the binding site across the membrane field (0–1) from inside to out (Cahalan, 1978). Our experimental conditions were such that $RT/F = 25$ mV and $z = 1$. Therefore the minimal value of S predicted by this model is 25 (when $\delta = 1$). This contrasts with the mean slope values of 13.7 ± 3.1 mV for cocaine block. These results indicate that the observed dependence of the fast component of cocaine block on membrane voltage cannot be attributed to movement of charged drug across the membrane. An alternative explanation is that the voltage dependence of channel block results indirectly from the voltage dependent availability of a channel state to which drug binds with relatively high affinity. The similarity between the voltage-dependence of channel block and peak G_{Na} suggests that the high-affinity state responsible for the rapid component of block is either the open state, or a closely associated "activated" channel state.

The slow component of channel block can be most easily explained by drug binding to inactivated channels. This explanation seems plausible because virtually all Na channels become inactivated within 100 ms at −20 mV. Other tertiary amines have also been shown to produce a slow phase of channel block in cardiac tissue (Bean et al., 1983; Clarkson et al., 1988).

Recovery

As indicated in Fig. 3 *B*, recovery of Na current in the presence of a high concentration of cocaine exhibits two phases: (*a*) a small rapid phase which may be due to either recovery of channels which were never blocked or rapid open-channel unblocking of cocaine-blocked channels during the test pulse and, (*b*) a large slow phase. Two possible explanations for the slow phase of cocaine unbinding include: (*a*) trapping of drug in an inactivated-blocked state due to a drug-induced hyperpolarizing shift in the voltage dependence of the inactivation gating mechanism (Hille, 1977; Hondeghem and Katzung, 1977; Schwarz et al., 1977; Hondeghem and Katzung, 1980; Gintant and Hoffman, 1984) or (*b*) trapping of charged cocaine molecules behind closed activation gates (Strichartz, 1973; Courtney, 1975; Hille, 1977; Yeh and Tanguy, 1985; Starmer et al., 1986).

We believe that the slow component of drug unbinding cannot be explained in terms of drug trapping in an inactivated-blocked state at the holding potentials studied. For example, using the equation of Bean et al. (1983) ($\Delta V_{h\infty} = kln[K_{dI}/K_{dR}]$, where k is the slope factor of the inactivation curve), one arrives at a value of ≈ −22 mV for the cocaine-induced voltage shift of inactivation (assuming K_{dI} and K_{dR} values shown in Table 1, $k = 6$ mV) (Clarkson et al., 1988). The midpoint of the inactivation (h_∞) curve was estimated to be −93 ± 3 mV ($n = 16$). Therefore, clamping the membrane to potentials negative to −120 mV would be expected to completely surmount a drug-induced shift in inactivation of this magnitude. Hyperpolarization to potentials that surmount a voltage shift should result in both rapid recovery of Na current at the holding potential as well as an abolishment of use-dependent block when drug trapping in an inactivated-blocked state is the rate-limiting step regulating channel unblocking (Hondeghem and Katzung, 1980). This was not seen experimentally (Fig. 1), even when the membrane was clamped to −160 mV. Thus the trapping of channels in an inactivated-blocked state cannot explain the slow rate of cocaine unbinding at hyperpolarized potentials (−120 to −160 mV).

An alternative explanation for slow unblocking at hyperpolarized potentials is trapping of charged drug behind closed activation gates (Starmer and Grant, 1985; Yeh and Tanguy, 1985; Starmer et al., 1986). At very negative potentials (i.e., negative to −120 mV), the activation gates in most channels are closed. Closed activation gates may regulate the rate of drug efflux and influx to the channel receptor (Starmer and Grant, 1985; Yeh and Tanguy, 1985; Starmer et al., 1986). However, explaining slow unbinding in terms of activation gate trapping is only applicable if the drug molecule exists primarily in the charged form, and/or cannot readily escape from the channel lumen by diffusion into the lipid membrane. Although cocaine (pK = 8.6) exists primarily in the cationic form at the pH used in these experiments (7.2–7.3), because it is a tertiary amine, it is expected to undergo periodic deprotonation to an uncharged (lipid soluble) form. This suggests that the rate of drug efflux from closed channels at the holding potential could be rate-limited by the kinetics of drug deprotonation to the

lipid soluble form rather than the rate of infrequent channel openings. Assuming a protonation rate (k_p) of $5 \times 10^8\ M^{-1}s^{-1}$ (Schwarz et al., 1977), the lifetime of the cationic form of cocaine ($1/l_p$) is predicted from ($k_p/l_p = 10^{pKa}$) (Schwarz et al., 1977; Starmer and Courtney, 1986) to be ~0.8 s. Because the time constant for channel unblocking observed experimentally was an order of magnitude larger (~8 s) it is apparent that the deprotonation rate predicted for cocaine in aqueous solution cannot solely explain the slow rate of drug unbinding, and other variables may be involved. A previous study by Woodhull (1973) has suggested that hyperpolarized potentials can attract H^+ from the extracellular solution into the channel lumen. An increased proton concentration within the channel would be predicted to increase the probability of a proton-cocaine collision and the probability of drug protonation. An increased probability of drug protonation would be expected to prolong the existence of the cationic species within the channel. In addition, the pKa of cocaine within the channel lumen could be different from that of cocaine within the bulk solution (Starmer and Courtney, 1986). Taking this into consideration, the rate of unbinding can be more closely approximated if a pKa of 9.6 (vs. 8.6) is assumed for cocaine molecules within the channel lumen. Therefore, although the slow rate of cocaine unbinding from rested channels seems consistent with activation gate trapping, it is possible that changes in local pH within the channel and/or changes of drug pKa within the channel may also modulate the kinetics of drug unbinding. Alternatively, the possibility exists that the rate of exit of the neutral form of the drug through the channel wall and into the surrounding bilayer could also be rate-limiting.

As indicated by Fig. 4, the time course of recovery from cocaine block does not significantly differ over a voltage range of −120 to −150 mV. This suggests that the probability of activation gate opening and/or the lifetime of the charged species in the channel over this voltage range are relatively constant. Experimental conditions did not allow discerning the importance of these two mechanisms.

Mathematical modeling

Previous studies using upstroke velocity measurements in cardiac tissue have suggested that the guarded receptor model can adequately describe the blocking effects of local anesthetic agents on cardiac Na channels (Starmer and Grant, 1985; Starmer, 1987). Guarded receptor equations have also been recently shown to well describe the blocking effects of RAC109 stereoisomers on cardiac Na current (Clarkson, 1989). In the present study, equations based upon the guarded receptor model were found to adequately simulate the use-dependent effect of cocaine on cardiac Na channels. As predicted by the model, the time course of use-dependent block was well described by a single exponential function (Fig. 5 *B*), and linear relationships were observed between both the uptake rate (λ) and the interpulse recovery time (t_r), as well as the stimulus parameter X_1 and steady-state block (b_{ss}) ($r > 0.9$, $P < 0.01$) (Fig. 6, *A* and *B*). In addition, both the kinetics and amplitude of block by cocaine were closely described by "state-dependent" rate constants calculated from the guarded receptor parameter estimation procedure (Table 2).

Upon inspection of Table 1, it is evident that the model rate constants defining drug binding (k_o) and unbinding (l_o) from activated channels are two to four orders of magnitude larger than those for drug binding and unbinding from rested or inactivated channels at concentrations of 30 and 50 μM cocaine. As illustrated in Fig. 7, binding and unbinding may be regulated by the position of the activation and inactivation gates (Starmer et al., 1984; Starmer and Grant, 1985; Starmer et al., 1986; Starmer, 1987), thus accounting for these differences in state-dependent rate constants.

Although the guarded receptor equations can adequately simulate most of our experimental data, this observation does not indicate that other models could not describe the data equally well. Nevertheless, the close agreement between model predictions and experimental data (Table 2) suggest that the model may be used to

TABLE 2 Comparison of model predictions with experimental data

	10 μM Cocaine		50 μM Cocaine	
	Experiment*	Model‡	Experiment*	Model‡
Tonic block	0.94 ± 0.06	0.95	0.91 ± 0.04	0.78
Steady-state§ block at:				
5.0 *Hz*	0.77 ± 0.14	0.75	0.34 ± 0.09	0.33
2.5 *Hz*	0.81 ± 0.14	0.79	0.43 ± 0.09	0.40
1.25 *Hz*	0.85 ± 0.13	0.83	0.56 ± 0.05	0.50
1.0 *Hz*	0.86 ± 0.14	0.85	0.57 ± 0.1	0.53
0.5 *Hz*	0.83 ± 0.13	0.89	0.68 ± 0.09	0.63
Use-dependent block rate (λ) at:				
5.0 *Hz*	0.05 ± 0.02	0.05	0.12 ± 0.02	0.11
2.5 *Hz*	0.07 ± 0.02	0.07	0.14 ± 0.02	0.13
1.25 *Hz*	0.12 ± 0.06	0.10	0.18 ± 0.05	0.18
1.0 *Hz*	0.11 ± 0.08	0.12	0.21 ± 0.05	0.20
0.5 *Hz*	0.20 ± 0.14	0.21	0.29 ± 0.03	0.31

*Experimental data are mean ± SD (n = 3–6).
‡Values are model predictions computed from the mean rate constants shown in Table 1 (n = 10).
§Block produced by a train of 30 pulses of 20 ms duration to −20 mV (V_h = −140 mV).

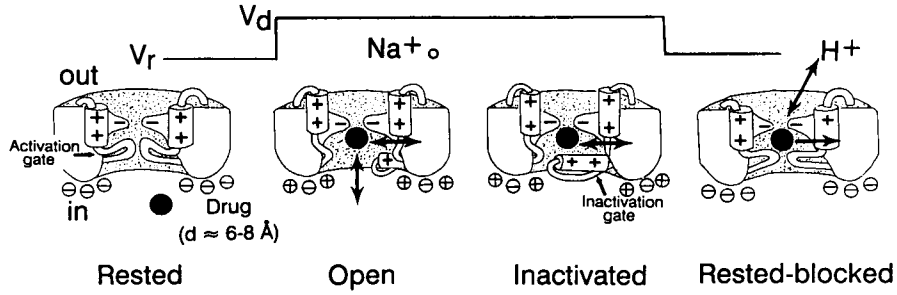

FIGURE 7 An illustration of the guarded receptor model indicating the proposed mechanism by which the activation and inactivation gates regulate binding and unbinding of drug (*dark sphere*). The model assumes that local anesthetic agents bind to a receptor site in the Na channel for which drug access and egress (indicated by arrows) are regulated by the position of channel gates. There are two pathways for drug access and egress from the channel receptor: a hydrophilic pathway from the cell interior, and a hydrophobic pathway through the lipid bilayer. (*Resting block*) When channels are held at a hyperpolarized holding potential (e.g., −140 mV) most channels have their activation gates closed, resulting in guarding of drug access to the receptor over both hydrophilic and hydrophobic pathways, and little net channel block (small tonic block). (*Open channel block*) When the activation gates open during a depolarizing pulse, drug can bind and unbind rapidly, resulting in measureable channel block observed after one or more brief depolarizing pulses. (*Inactivated channel block*) Within ≈1 ms after a channel opens, it enters another closed (inactivated) state due to closure of an "inactivation" gate. Closure of the inactivation gate prevents drug access and egress over the hydrophilic pathway only. In inactivated channels, lipid soluble drugs have continuous access to the channel receptor via the lipid bilayer pathway, resulting in a slow component of channel block observed with prolonged depolarization. Egress from inactivated channels is accomplished by drug escaping into the lipid bilayer, which for cocaine would involve deprotonation. The illustration is adapted from the molecular model proposed by Guy (1988) depicting a cross section of a Na channel revealing two of the activation gates and the inactivation gate. Positively charged gating segments linked to each activation gate move in response to changes in transmembrane voltage. Outward movement of the gating segments pull the flexible activation gates close to the walls of the channel, permitting ion flow through the channel lumen, as well as unrestricted drug access and egress to the channel receptor. Drug is represented by a dark sphere with a diameter of 6–8 Å according to Courtney (1984). Protons (H^+) have access and egress pathways (indicated by arrow) to and from the drug binding site as proposed by Woodhull (1973).

define rate constants that can adequately predict use-dependent block of cardiac Na current.

Clinical relevance

Cocaine abuse has become a major medical problem in the United States with cocaine related deaths increasing over the past decade (Mittleman and Wetli, 1984; Pollin, 1985). Case reports suggest a cardiovascular etiology (Young and Glauber, 1947; Jonsson et al., 1983; Kossowsky and Lyon, 1984; Nanji and Filipenko, 1984; Isner et al., 1986) with some implicating conduction block (Young and Glauber, 1947; Jonsson et al., 1983; Nanji and Filipenko, 1984), as the underlying cause. Because cocaine has been shown to be a local anesthetic in nerve, one possible mechanism for cocaine cardiotoxicity is the alteration of normal conduction within the heart. The present study has shown that cocaine is a potent cardiac Na channel blocker. A comparison of the recovery time constant of cocaine (τ_s = 8.5 s) with that of lidocaine (τ_s = 1.0 ± 0.4 s) indicates cocaine unbinds approximately eight times slower than lidocaine (Fig. 3 *B*). However, the K_{dI} of cocaine (7.9 μM) is similar to that of lidocaine (≈10 μM) (Bean et al., 1983). This suggests that at equimolar concentrations, cocaine may produce a much greater cumulative block compared with lidocaine due to its slower rate of unbinding. This behavior would be expected to result in disturbances of cardiac conduction, and production of conduction block and/or reentrant arrhythmias. Experiments to test this hypothesis in vivo are now in progress.

This work was supported by a grant from National Institutes of Health (HL-36096).

Received for publication 20 July 1989 and in final form 10 November 1989.

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