Stereoselective Block of Cardiac Sodium Channels by RAC109 in Single Guinea Pig Ventricular Myocytes

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The effects of the optical stereoisomers of the local anesthetic RAC109 (RAC109-I and RAC109-II) on sodium current in isolated guinea pig ventricular myocytes were investigated by use of the whole-cell variation of the patch-clamp technique. RAC109-I and RAC109-II produced similar levels of tonic block, but RAC109-I produced a significantly larger use-dependent block on repetitive pulsing to potentials positive to -60 mV. Definition of the time courses of block development at -20 mV and recovery at -140 and -160 mV indicated that RAC109-I had a higher affinity for activated and inactivated channels and dissociated more slowly at hyperpolarized potentials compared with RAC109-II. Removal of fast inactivation by α-chymotrypsin intensified tonic block but did not reduce use-dependent block by RAC109-I; this finding suggests that channel inactivation is not necessary for use-dependent block. The guarded-receptor model was used to calculate apparent rate constants of drug binding and unbinding. According to the model, RAC109-I and RAC109-II have significantly different unbinding rate constants for channels when they exist predominantly in rested, activated, or inactivated states, as well as significantly different binding rate constants when channels are activated. However, the apparent rates of drug binding to closed (rested and inactivated) channels are not significantly different for the two isomers; this finding indicates that drug binding to closed channels is not markedly stereospecific, in contrast to unbinding. The effects of RAC109 stereoisomers on cardiac sodium channels were also qualitatively similar to those previously reported in nerve; these findings suggest that the binding sites for local anesthetics in both tissue types have a similar structural topography. (Circulation Research 1989;65:1306-1323)
Isolation of Cardiac Myocytes

With scissors, and gently shaken at 37° C for 5 minutes. Ventricular myocytes used in this study is similar to 0.1 mg/ml streptomycin, and 100 units/ml penicillin.

Minimal Essential Medium supplemented with Earle's salts, L-glutamine (292 mg/ml), 3% horse serum, 0.28 mg/ml protease (5.6 units/mg; type XIV, Sigma Chemical, St. Louis, Missouri) and molded through 200 µm nylon mesh and by spinning the filtrate at 200-300 rpm in a table-top apparatus.

Hearts were initially perfused for 5 minutes at 10-15 ml/min with a calcium-free bicarbonate-buffered solution at 37° C, followed by an 8-minute perfusion with the same solution supplemented with 2 mg/ml collagenase (284 units/mg; type II, Worthington Biochemicals, Freehold, New Jersey) and 0.28 mg/ml protease (5.6 units/mg; type XIV, Sigma Chemical, St. Louis, Missouri). The ventricles were then cut away from the atria, placed in enzyme-free solution supplemented with 0.2 mM CaCl₂, minced with scissors, and gently shaken at 37° C for 5 minutes. Cells were harvested by filtering the suspension through 200 µm nylon mesh and by spinning the filtrate at 200-300 rpm in a table-top centrifuge to form a pellet. The supernatant was drawn off, and the pellet was resuspended in enzyme-free solution supplemented with 1 mM CaCl₂ and 1 mg/ml bovine serum albumin (Fraction V, Sigma Chemical). This suspension was also centrifuged to form a pellet, and the pellet was resuspended in Minimal Essential Medium supplemented with Earle’s salts, L-glutamine (292 mg/ml), 3% horse serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin.

The method for dissociation and isolation of ventricular myocytes used in this study is similar to that described by Mitra and Morad. Hearts from young guinea pigs (200-300 g) were removed under pentobarbital anesthesia and quickly mounted by the aorta on the cannula of a Langendorff perfusion apparatus. Hearts were initially perfused for 5 minutes at 10-15 ml/min with a calcium-free bicarbonate-buffered solution at 37° C, followed by an 8-minute perfusion with the same solution supplemented with 2 mg/ml collagenase (284 units/mg; type II, Worthington Biochemicals, Freehold, New Jersey) and 0.28 mg/ml protease (5.6 units/mg; type XIV, Sigma Chemical, St. Louis, Missouri). The ventricles were then cut away from the atria, placed in enzyme-free solution supplemented with 0.2 mM CaCl₂, minced with scissors, and gently shaken at 37° C for 5 minutes. Cells were harvested by filtering the suspension through 200 µm nylon mesh and by spinning the filtrate at 200-300 rpm in a table-top centrifuge to form a pellet. The supernatant was drawn off, and the pellet was resuspended in enzyme-free solution supplemented with 1 mM CaCl₂ and 1 mg/ml bovine serum albumin (Fraction V, Sigma Chemical). This suspension was also centrifuged to form a pellet, and the pellet was resuspended in Minimal Essential Medium supplemented with Earle’s salts, L-glutamine (292 mg/ml), 3% horse serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin.

Materials and Methods

Isolation of Cardiac Myocytes

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Solutions and Drugs

When electrical recordings were made on isolated cells, the tissue chamber was perfused with an “external” solution containing (mM) tetramethylammonium chloride 115, NaCl 25, CsCl 5, CoCl₂ 2, CaCl₂ 1.8, MgCl₂ 1.2, HEPES 20, and glucose 11 adjusted to a pH of 7.3 with tetramethylammonium hydroxide. The glass pipettes (electrodes) were filled with an internal solution containing (mM) CsF 125, CsCl 20, NaF 5.6, HEPES 5, and EGTA 5 adjusted to a pH of 7.2 with CsOH. The stereoisomers of RAC109 (RAC109-I and RAC109-II) were supplied by Ms. Jeanne Johnson (Astra Pharmaceutical Products, Westborough, Massachusetts). RAC109 stereoisomers have a pKₘ of 9.4. The estimated distribution coefficient of the base form of RAC109 in cod liver oil versus base plus cation in aqueous buffer at pH 7.4 is 2.6. α-Chymotrypsin (type II) was obtained from Sigma Chemical.

Voltage-Clamp Recording

At the beginning of each experiment, an aliquot of cells was transferred from a culture dish into a shallow chamber (~1.5-ml volume), which was mounted on the stage of an inverted microscope. The whole-cell variant of the patch-clamp method was used to measure Iₘ from isolated ventricular myocytes. Glass pipettes were pulled in two steps and fire-polished until tip openings were 3-4 µm in diameter. When filled with the standard internal solution and placed in the external solution, the cell membrane. The membrane patch under the pipette tip was then ruptured by applying a negative pressure transient, which created a continuity between the pipette (internal) solution and the cell cytoplasm. A thermistor was placed near the cell under study, and the temperature of the external solution was cooled to 16° C (±0.5° C) with a thermoelectric device (model 806-7243-01, Cambion/Midland Ross, Cambridge, Massachusetts).

An Axopatch amplifier (Axon Instruments, Burlingame, California) was used for whole-cell voltage clamping. Creation of voltage-clamp pulses and data acquisition was controlled by an IBM PC/AT computer running pClamp software (Axon Instruments) interfaced to the amplifier by an 80-kHz
Labmaster board (Tecmar, Solon, Ohio). Sodium currents were digitized at sample intervals of 20–200 μsec. Between pulse protocols, cells were maintained at a holding potential of −140 mV to remove fully resting inactivation.

**Evaluation of Method**

To study accurately drug effects on sodium current, contaminating ionic currents must be insignificant, and voltage-clamp errors related to uncompensated series resistance and voltage inhomogeneity must be reduced to within acceptable levels. In these experiments, the isolation of \( I_{Na} \) from calcium and potassium currents was accomplished by replacing all \( K^+ \) with \( Cs^+ \) and by blocking calcium channels with external \( Co^{2+} \) and internal \( F^- \) ions. Under these conditions, steady-state (leak) current at the test potential (−20 mV) was less than 0.1 nA, or less than 1–2% of peak \( I_{Na} \). No correction for leak current was made.

Capacitative transients evoked by 10-mV hyperpolarizing pulses were well-defined by single exponential functions with a mean time constant (\( \tau_c \)) of 85±8 μsec (n=13). Values of cell capacitance (\( C_m \)) were estimated from capacitative transients using the equation: \( C_m = Q/\Delta V \), where \( \Delta V \) is the amplitude of a voltage step, and \( Q \) is the total charge displaced during the voltage step. \( Q \) was estimated by integration of the capacitative transient. The mean value of cell capacitance for guinea pig myocytes from young animals was 66±4 pF (n = 13). The mean total series resistance (\( R_s \)) for the pathway between the pipette and cell membrane after rupture of the membrane seal was calculated from the equation \( \tau_c = R_s \times C_m \) to be approximately 1.3±0.1 MΩ (mean±SD, n=13) \(^{30} \) under the experimental conditions used in this study. Compensation for this series resistance was done empirically by applying electronic series resistance compensation to a maximum level of 60–80% before producing current oscillation. The mean amplitude of the peak \( I_{Na} \) near the peak of the current-voltage relation in this study was determined to be 9.2±4.2 nA (n=13) (holding potential, −140 mV; test potential, −20 mV). Under such conditions, the expected voltage drop across the uncompensated series resistance (\( \Delta V = I_{Na} \times R_s \)) is 2–5 mV. An attempt was made to confirm the size of the series resistance voltage error based on shifts in the sodium conductance-voltage relation produced by reductions in current amplitude. Reduction of peak \( I_{Na} \) by 44% after changes in the holding potential produced only a 1.6±0.5 mV shift of the conductance-voltage relation. \(^{30} \) These results indicate that measurement errors due to uncompensated series resistance were within acceptable limits under these experimental conditions. The estimated membrane length constant at the time of peak sodium conductance under these experimental conditions has also been calculated to be approximately 317 μm. \(^{30} \) This is more than five times the distance from the pipette tip to the end of a typical myocyte when the tip is placed centrally along the cell length; thus, problems due to nonuniformity are also within acceptable limits.

The inclusion of divalent cations commonly used to block calcium channels (including \( Co^{2+} \)) in the external solution has recently been reported to reduce \( I_{Na} \) amplitude. \(^{31} \) The hypothesis that cobalt block of sodium channels alters the blocking characteristics of RAC109 stereoisomers was considered. However, in a series of pilot experiments, the observed blocking effects of RAC109-I were not noticeably different when using an external solution without external \( Co^{2+} \). In four cells exposed to 50 μM RAC109-I in the absence of external \( Co^{2+} \), the mean amplitude of tonic block after a long rest at −140 mV was 0.27±0.10 of control \( I_{Na} \), and the steady-state amplitude of use-dependent block after a train of 15 pulses of 20-msec duration to −20 mV applied at 2 Hz was 0.71±0.06 of \( I_{Na} \) available after a long rest. These values are not significantly different from those observed in the presence of \( Co^{2+} \) (Table 4).

**Data Analysis and Mathematical Modeling**

Curve fitting and mathematical modeling were performed on an IBM-PC/AT with programs written in the ASYST language (ASYST Software Technologies, Rochester, New York). A nonlinear least-squares algorithm using the Gauss-Newton method was used to fit exponential functions to experimental data. When the time courses of block development and recovery were defined, an F test based on the residual error was used to compare the goodness of fit for single and double exponential functions. A double exponential function was accepted if it provided a significantly better fit (p<0.05).

A “two-state” version of the guarded-receptor hypothesis \(^{23-26} \) was used to describe the effects of RAC109 on cardiac sodium channels and to calculate apparent association and dissociation rate constants for drug interaction with channels at the holding (−140 mV) and test (−20 mV) potentials. The theoretical basis for the parameter estimation method has been extensively described by Starmer and coworkers. \(^{24-26} \) According to the model, tonic and use-dependent block of sodium channels that is observed during a train of short depolarizing pulses results from drug interaction with the receptor site having diffusion pathways that are guarded by the position of channel activation and inactivation gates. Drug cannot bind to a channel having all its activation gates closed since activation gates guard access to the receptor site via both hydrophilic and hydrophobic pathways. Thus, at strongly negative holding potentials, where the fraction of channels having open activation gates (\( m^3 \) in a Hodgkin-Huxley model) is small, only a relatively small “tonic” block is observed at low drug concentrations. When a channel is open, a drug can bind and unbind from the channel receptor via both hydrophilic and hydrophobic pathways. In addition, drug may continue to bind and unbind from the channel
receptor via the hydrophilic (membrane) pathway once the hydrophilic pathway becomes obstructed by a closed inactivation gate. When the channel activation or inactivation gates close in a channel occluded by a charged drug, drug dissociation cannot occur until the channel reopens or, as for a tertiary amine, the dipole moment of the drug and the membrane electric field. For charged hydrophilic drugs, Starmer and Grant also defined $k_\sigma$ or $k_\sigma = kF/m^3$, and $l_\sigma$ or $l_\sigma = l$ since such agents can only bind to channels once the activation gates open but can escape into the lipid bilayer at any time.

Tertiary amines such as RAC109 may not behave simply as charged or uncharged drugs since they can interconvert between charged and uncharged drug forms by protonation and deprotonation at any time. It has been proposed that the drug-channel interaction in the presence of a tertiary amine may, therefore, be more completely characterized by the following scheme24,25:

Uncharged interaction: $\text{[D]}_u + \text{U} \xrightarrow{k_u/m^3} \text{B}_u$

Charged interaction: $\text{[D]}_c + \text{U} \xrightarrow{k_{m^3}h} \text{B}_c$

where $k_p$ and $l_p$ represent the protonation and deprotonation rates of drug within the blocked channel, $D_c$ and $D_u$ indicate the concentration of charged (c) and uncharged (u) drug near the receptor site, $B_c$ and $B_u$ are the fraction of channels blocked by charged and uncharged drug forms, and $k_{c}$, $k_u$, $I_c$, and $I_u$ are the rates of binding and unbinding for charged and uncharged drug forms, which may not be equal.23 A mathematical description for channel block by drug mixtures based on the guarded receptor model has been derived25 and predicts a biexponential time course for use-dependent accumulation of block and recovery from block when both the charged and uncharged drug forms contribute significantly to both channel block and unblock. However, as proposed by Hille,28 block of open or activated channels may be predominantly or exclusively due to binding of the charged form over an aqueous pathway, whereas the lipid soluble neutral form may be the predominant drug form responsible for interacting with closed (rested or inactivated) channels. Based on these assumptions, the time course of use-dependent block during a pulse train may be modeled as a sequence of bimolecular binding reactions similar to that predicted for a drug of fixed charge; the major caveat is that the apparent rate constants derived for drug interaction with open channels may reflect the binding properties of a different drug form (i.e., charged) than that responsible for drug interaction with closed channels (i.e., uncharged). In addition, since RAC109 stereoiso-
mers are highly charged drugs with protonated lifetimes of several seconds, it is quite possible that the deprotonation process may be rate limiting in determining the rate of drug egress from closed channels. In this case, the apparent rate constants defining unbinding from rested and inactivated channels (l_i and l_f) would primarily reflect the rate of drug deprotonation rather than the actual rate of drug dissociation from the receptor. Further experimental information on the rate of deprotonation of bound RAC109 molecules is required to test this hypothesis. In this study, only the composite "voltage-dependent" or "state-dependent" rate constants are defined since neither the proper gating model for cardiac sodium channels nor the conversion rates from charged to uncharged RAC109 within the channel were known.

When assuming a two-state model to simulate drug interaction during a train of pulses, the guarded-receptor model predicts that the time course of use-dependent block onset during a train of repetitive pulses can be described in terms of a recurrence relation 24:

\[ b_n = b_n + (b_0 - b_n) e^{-\lambda t} \]  

where \( b_n \) is the fraction of channels blocked during the \( n^{th} \) pulse, \( b_n \) is the fraction of channels initially blocked (e.g., after a 3-minute rest at the holding potential), \( b_0 \) is the "steady-state" fraction of channels blocked after many pulses, \( n \) is the pulse number, and \( \lambda \) is the blocking rate constant. The blocking rate constant \( \lambda \) can be further defined by

\[ \lambda = \lambda_a + \lambda_u \]  

where \( \lambda_a \) and \( \lambda_u \) are the blocking rates associated with the activation and recovery intervals during the pulse protocol, \( t \) is the interpulse (recovery) interval, and \( t_5 \) is the mean receptor access time. 24,26 For analysis, \( t_5 \) was assumed to be equal to 1 msec (i.e., similar to reported values of the mean open time for single cardiac sodium channels). 32,33

Least-squares fits of peak sodium currents during pulse trains to Equation 3 were used to obtain estimates of the parameters \( \lambda \), \( b_0 \), and \( b_n \). Estimates of \( \lambda_a \) and \( \lambda_u \) were then computed according to equation 4 by linear regression of \( \lambda \) versus \( t \) by use of data from pulse trains elicited at four to five different interpulse intervals. The equilibrium levels of block at the holding (\( R_h \)) and test potential (\( A_u \)) were then computed by linear regression of \( b_n \) (obtained from Equation 3) versus \( \alpha \) according to the equations:

\[ b_n = A_u + \alpha (R_s - A_u) \]  

where \( \alpha \) is the blocking parameter and

\[ \alpha = (1 - e^{-\lambda t})/(1 - e^{-\lambda t}) \]  

From calculated values of \( \lambda_a \), \( \lambda_u \), \( R_s \), and \( A_u \), the forward \( (k_f \) and \( k_u \)) and reverse \( (l_i \) and \( l_f \)) rate constants defining drug interaction with channels during the recovery and activation intervals were calculated by use of the following equations:

\[ k_f = \lambda_f R_s / [D] \]  

\[ k_u = \lambda_u A_u / [D] \]  

\[ l_i = \lambda_i (1 - R_s) \]  

\[ l_f = \lambda_f (1 - A_u) \]  

To estimate the apparent rate constants defining drug interaction with channels once they become inactivated during a long pulse at -20 mV, it was assumed that the slow component of time-dependent block characterized by use of the two-pulse protocol (Figure 4) results from drug binding to a continuously accessible receptor (i.e., via the hydrophobic membrane pathway) as described by

\[ [D] + U \rightarrow k_1 \rightarrow B \]  

where \( k_1 \) and \( l_i \) are apparent rates of drug binding and unbinding, respectively. During a long depolarizing pulse, changes in the proportion of blocked channels is an exponential process defined by the rate equations:

\[ \lambda_i = (k_f [D] + l_f) \]  

\[ B_n = k_f [D] / (k_f [D] + l_f) \]  

\[ \lambda_i = k_u / (k_f [D]) \]  

\[ l_i = (1/\tau_i) - (k_f [D]) \]  

Statistical Analysis

Comparisons between two groups of data were evaluated for statistical significance by paired or unpaired two-tailed Student's t test. Comparisons between more than two groups were evaluated by one-way ANOVA and Scheffe's test for critical difference. Data analyzed by linear regression were evaluated by calculation of the Pearson product-moment correlation coefficient (\( r \)) and a t test for significance of the linear trend. Differences were considered significant if values of \( p < 0.05 \) were obtained. Results are expressed as mean±SD.

Results

Stereospecifity of Tonic and Use-Dependent Block

The effects of RAC109 isomers RAC109-I and RAC109-II on cardiac INa were similar to most other local anesthetic agents in that block could be empirically divided into two primary components, a tonic block and a use-dependent block. As illustrated in Figures 1B and 1C, on application of a train of depolarizing pulses at 1 Hz after a long rest, there was no change in sodium current in the absence of drug. In contrast, after a 15-minute exposure to 50 \( \mu \)M of either RAC109 stereoisomer, there was a reduction of INa amplitude during the first pulse after...
a long rest (tonic block) and an additional use-dependent block of current that became progressively larger during each successive pulse until a steady state was approached after 10–15 pulses.

The mean levels of tonic and use-dependent block at different stimulation rates are shown in Figure 1C. In the absence of drug, there was no significant change in INa amplitude as the stimulation rate was increased up to 5 Hz. In the presence of 50 μM RAC109-I or RAC109-II, only a stable tonic block was observed when preparations were stimulated infrequently (e.g., once per 3 minutes), and the levels of tonic block were not significantly different for the two drugs (RAC109-I, 0.30±0.08, n=10; RAC109-II, 0.24±0.10, n=12) (Figure 1C). However, at stimulation rates greater than 0.1 Hz, there was a marked use-dependent block that became progressively larger at higher stimulation rates with RAC109-I producing a significantly greater block compared with RAC109-II at all stimulation rates between 0.1 and 5 Hz (p<0.05).

Voltage-Dependence of Use-Dependent Block

Block of sodium channels in nerve by local anesthetic drugs, including RAC109,34,35 has been shown to be a strongly voltage-dependent process. The voltage dependence of sodium channel block by RAC109 in cardiac myocytes was characterized by use of the pulse protocol shown in Figure 2A. Thirty pulses of 2-msec duration were applied to various conditioning voltages at 3 Hz to produce a steady-state level of block. The level of block produced by the conditioning train was then determined by a test pulse after a 300-msec recovery interval to allow unblocked channels to recover from inactivation. In the absence of drug, the conditioning train had little effect on the test INa (Figure 2B). However, after addition of either stereoisomer, channel block was a steep function of membrane voltage between -60 and +40 mV. As indicated by the smooth curves in Figure 2B, the voltage dependence of block could be well described by an equation that has been previously used to describe block of open channels by local anesthetics in nerve35,36:

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

(15)

where B is the steady-state level of sodium current remaining at positive potentials, \(V_c\) is the membrane potential during the conditioning train, \(V_{mid}\) is the membrane potential at which drug block is half maximal, and S is the slope of the voltage-dependent block. Least-squares fits of Equation 15 to data obtained in the presence of 50 μM drug indicated that RAC109-I reduced \(I_{Na}\) to a significantly smaller steady-state level at positive potentials (B=0.32±0.06; n=6) compared with RAC109-II (B=0.72±0.05 mV; n=5) and that the slope factor for block by RAC109-I (S=13.7±1.9) was significantly larger than for RAC109-II (S=17.6±3.3 mV; p<0.05). The midpoints for voltage-dependent block (\(V_{mid}\)) were not significantly different (-37.9±3.9 for RAC109-I vs. -34.2±6.7 mV for RAC109-II).

The parameters defining the voltage dependence of channel block were also similar, but not identical, to those of channel opening or conductance (\(G_{Na}\)) with \(G_{Na} = I_{Na}/(V_m-V_{na})\), \(V_m\) is membrane potential, and \(V_{na}\) is sodium equilibrium potential. \(G_{Na}\) was calculated from either peak \(I_{Na}\) or integrals

![FIGURE 1. Tonic and use-dependent inhibition of the peak sodium current (\(I_{Na}\)) by RAC109-I and RAC109-II in guinea pig ventricular myocytes. Panel A: Diagram of pulse protocol. A train of depolarizing pulses to -20 mV was applied at different stimulation rates after a 3-minute rest at the holding potential (-140 mV). Panel B: Superimposed recordings of \(I_{Na}\) obtained during a train of pulses applied at 1 Hz before and during exposure to 50 μM RAC109-I or RAC109-II. For the controls, currents for only the first and fifteenth 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 10–15 pulses. The arrows at the beginning of each family of tracings indicate the zero current level. Panel C: Graph showing the mean level of peak sodium current after 15 pulses at selected stimulation rates under control conditions (○) and in the presence of 50 μM RAC109-I (■) and 50 μM RAC109-II (●). Peak \(I_{Na}\) amplitudes have been normalized to the amplitude of control \(I_{Na}\) obtained after a rest of 10 seconds or more. RAC109-I produced a significantly greater block compared with RAC109-II at all stimulation rates between 0.1–5 Hz (p<0.05). Data values represent mean±SD (n=4–10).]
produced by RAC109 stereoisomers. Panel A: Diagram showing use-dependent block produced by a train of 30 conditioning pulses of 2-msec duration at 3 Hz to selected membrane potentials (V<sub>c</sub>). The amplitude of the peak sodium current (I<sub>Na</sub>) available after the conditioning train was determined with a test pulse after a recovery interval of 300 msec. Panel B: Graph showing the relation between peak I<sub>Na</sub> and conditioning potential. Peak current was normalized to its value after a long rest at -140 mV. Data values represent mean±SD. ○, control (n = 11); ■, RAC109-I (n=6); ●, RAC109-II (n=5). The amplitude of block produced by RAC109-I was significantly greater than RAC109-II at all voltages between -60 and +40 mV (p<0.05). The smooth curves represent least-squares fits to a Boltzmann relation (equation 15). The best-fit values were B=0.32, S=13.6 mV, and V<sub>mid</sub> = -38 mV for RAC109-I and B=0.73, S=16.3 mV, and V<sub>mid</sub> = -34.2 mV for RAC109-II, where B is the steady-state level of I<sub>Na</sub> remaining at positive potentials, S is the slope of the voltage-dependent block, and V<sub>mid</sub> is the membrane potential at which drug block is half-maximal.

FIGURE 2. Voltage dependence of use-dependent block produced by RAC109 stereoisomers. Panel A: Diagram showing use-dependent block produced by a train of 30 conditioning pulses of 2-msec duration at 3 Hz to selected membrane potentials (V<sub>c</sub>). The amplitude of the peak sodium current (I<sub>Na</sub>) available after the conditioning train was determined with a test pulse after a recovery interval of 300 msec. Panel B: Graph showing the relation between peak I<sub>Na</sub> and conditioning potential. Peak current was normalized to its value after a long rest at -140 mV. Data values represent mean±SD. ○, control (n = 11); ■, RAC109-I (n=6); ●, RAC109-II (n=5). The amplitude of block produced by RAC109-I was significantly greater than RAC109-II at all voltages between -60 and +40 mV (p<0.05). The smooth curves represent least-squares fits to a Boltzmann relation (equation 15). The best-fit values were B=0.32, S=13.6 mV, and V<sub>mid</sub> = -38 mV for RAC109-I and B=0.73, S=16.3 mV, and V<sub>mid</sub> = -34.2 mV for RAC109-II, where B is the steady-state level of I<sub>Na</sub> remaining at positive potentials, S is the slope of the voltage-dependent block, and V<sub>mid</sub> is the membrane potential at which drug block is half-maximal.

of I<sub>Na</sub>·dt during the initial 2 msec or 25 msec of depolarizing pulses to potentials from -70 to +20 mV. The 2-msec and 25-msec integrals were selected on the basis that they closely resembled either the conditioning pulse duration (Figure 2A) or an infinite integral of I<sub>Na</sub>·dt since I<sub>Na</sub> was almost fully inactivated within 25 msec at most potentials. Fits of peak G<sub>Na</sub> with a Boltzmann equation of the form G<sub>Na</sub> = G<sub>max</sub>/(1 + exp(V<sub>c</sub>-V<sub>mid</sub>/S)) (where G<sub>max</sub> is the maximum G<sub>Na</sub>) had a mean slope factor (S) of 6.0±1.2 mV and a half-maximal value (V<sub>mid</sub>) of -34.4±7.9 mV (n=9). Thus, although both channel block and peak G<sub>Na</sub> were half-maximal at similar potentials, the slope factor for peak G<sub>Na</sub> was significantly smaller (p<0.05) than that for channel block; thus, the channel opening had a steeper voltage dependence than channel block. Similar differences in the slope factors were also obtained when comparing channel block with time integrals of G<sub>Na</sub> for a 2-msec integral: S=7.3±0.8, V<sub>mid</sub>= -21.3±7.5; and for a 25-msec integral: S=4.3±1.1, V<sub>mid</sub>= -42.1±7.4; (n=9).

**Time Course of Recovery From Use-Dependent Block**

The time course of recovery from use-dependent block was characterized using the pulse protocol shown in Figure 3A. A steady-state level of block was first produced by a train of pulses of 50-msec duration to +20 mV at 3 Hz, and the time course of recovery from block was defined by use of a single test pulse evoked after a variable recovery time. In absence of drug, recovery of I<sub>Na</sub> from fast inactivation at -140 mV was well described by a single exponential function having a time constant of 7.2±3.1 msec (n=16). In contrast, in the presence of 50 μM of either RAC109-I or RAC109-II, recovery of I<sub>Na</sub> after the conditioning train exhibited two distinct phases: a fast phase and a slow phase (Figures 3B and 3C). In the presence of either stereoisomer, the fast phase had a time constant similar, although significantly larger, than control (Table 1). The time constant of the slow phase of recovery from block at both -140 and -160 mV was significantly larger in the presence of RAC109-I compared with RAC109-II (p<0.001) (Table 1). The slow phase of recovery in the presence of drug was assumed to reflect the time course with which drug unbinds or escapes from rested channels. The fast phase of recovery in the presence of drug may reflect a combination of recovery from inactivation of drug-free channels as well as some unblocking from open channels at -20 mV after block induced by pulses at +20 mV. Hyperpolarization of the membrane by -20 mV during the recovery period (from -140 to -160 mV) did not significantly alter the time constant for recovery from block (Table 1).

**Time Course of Block Development**

To determine how RAC109 stereoisomers differ in their binding to sodium channels during a depolarizing pulse, the time course of block development was defined by use of a two-pulse protocol. As illustrated in Figure 4A, the membrane was first conditioned by a clamp of variable duration to -20 mV. The level of block produced by this conditioning pulse was then determined with a test pulse to -20 mV after a fixed recovery interval to allow drug-free channels to recover from inactivation. Figure 4B shows the mean time course of block development in the presence of 50 μM RAC109-I and RAC109-II. In the absence of drug, there was little change in test current amplitude after conditioning pulses of up to 15-second duration. However, after addition of either RAC109 isomer, there was a progressive decrease in I<sub>Na</sub> amplitude as the conditioning pulse duration was increased. Two definable phases of time-dependent...
block were observed, which could be well approximated by the sum of two exponentials and a constant \( A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s} + A_0 \). The amplitudes \( A_f \) (fast phase; \( A_s \), slow phase; \( A_0 \), steady state) and time constants \( \tau_f \) (fast phase; \( \tau_s \), slow phase) for time-dependent block by RAC109-I were \( A_f = 0.11 \pm 0.03 \), \( \tau_f = 2.1 \pm 1.5 \) msec, \( A_s = 0.74 \pm 0.10 \), \( \tau_s = 14.1 \pm 4.6 \) sec, and those for RAC109-II were \( A_f = 0.10 \pm 0.05 \), \( \tau_f = 2.7 \pm 1.8 \) msec, \( A_s = 0.54 \pm 0.11 \), \( \tau_s = 15.1 \pm 3.4 \) sec, and \( A_0 = 0.36 \pm 0.09 \) (n=7). The time constants for both phases and the amplitude of the fast phase were not significantly different for RAC109-I versus RAC109-II, in contrast to the amplitude of the slow phase and the steady-state level of block, which were significantly different for the two isomers \((p<0.05)\).

The very large time constant (14-15 seconds) observed for the slow phase of block development for both stereoisomers indicates that this component of channel block cannot contribute significantly to the cumulative use-dependent block previously defined by use of trains of brief pulses (Figures 1 and 2). In addition, since use-dependent block were observed, which could be well approximated by the sum of two exponentials and a constant \( A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s} + A_0 \). The amplitudes \( A_f \) (fast phase; \( A_s \), slow phase; \( A_0 \), steady state) and time constants \( \tau_f \) (fast phase; \( \tau_s \), slow phase) for time-dependent block by RAC109-I were \( A_f = 0.11 \pm 0.03 \), \( \tau_f = 2.1 \pm 1.5 \) msec, \( A_s = 0.74 \pm 0.10 \), \( \tau_s = 14.1 \pm 4.6 \) sec, and those for RAC109-II were \( A_f = 0.10 \pm 0.05 \), \( \tau_f = 2.7 \pm 1.8 \) msec, \( A_s = 0.54 \pm 0.11 \), \( \tau_s = 15.1 \pm 3.4 \) sec, and \( A_0 = 0.36 \pm 0.09 \) (n=7). The time constants for both phases and the amplitude of the fast phase were not significantly different for RAC109-I versus RAC109-II, in contrast to the amplitude of the slow phase and the steady-state level of block, which were significantly different for the two isomers \((p<0.05)\).

The very large time constant (14-15 seconds) observed for the slow phase of block development for both stereoisomers indicates that this component of channel block cannot contribute significantly to the cumulative use-dependent block previously defined by use of trains of brief pulses (Figures 1 and 2). In addition, since use-dependent

<table>
<thead>
<tr>
<th>Parameter</th>
<th>-140 mV RAC109-I</th>
<th>-160 mV RAC109-I</th>
<th>-140 mV RAC109-II</th>
<th>-160 mV RAC109-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A_f )</td>
<td>0.30±0.15</td>
<td>...</td>
<td>0.65±0.14</td>
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</tr>
<tr>
<td>( \eta ) (msec)</td>
<td>19.5±6.6*</td>
<td>...</td>
<td>13.0±6.2*</td>
<td>...</td>
</tr>
<tr>
<td>( A_s )</td>
<td>0.65±0.10</td>
<td>0.66±0.09</td>
<td>0.32±0.05†</td>
<td>0.31±0.04†</td>
</tr>
<tr>
<td>( \tau_s ) (sec)</td>
<td>26.8±4.3</td>
<td>20.5±3.8</td>
<td>15.1±4.6†</td>
<td>11.9±1.0†</td>
</tr>
<tr>
<td>( n )</td>
<td>9</td>
<td>6</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are mean±SD. \( A_f \), amplitude of fast phase; \( \eta \), time constant for fast phase; \( A_s \), amplitude of slow phase; \( \tau_s \), time constant for slow phase. \( A_f \) and \( A_s \) represent the fraction of total available current recovering at the recovery potential.

*Significantly different from control time constant: 7.2±3.1 msec (n=16) \((p<0.05)\).
†Significantly different from RAC109-I at the same recovery potential \((p<0.05)\).
Effects of RAC109 on \(I_{\text{Na}}\) Time Course

Although 50 \(\mu M\) of either RAC109 isomer could produce a large use-dependent block of \(I_{\text{Na}}\) during repetitive pulsing to \(-20 \text{ mV}\), neither isomer produced a significant effect on the time course of \(I_{\text{Na}}\) at this potential (Table 2). In the absence of drug, at \(-20 \text{ mV}\) the decay of \(I_{\text{Na}}\) after its inward peak can be well described as the sum of a fast and slow exponential function plus a small constant. After a 15-minute exposure to 50 \(\mu M\) of either RAC109 stereoisomer, neither the time constants nor the relative fractions of available current that decayed with fast or slow time constants were significantly affected by exposure to drug (Table 2). Consistent with the lack of effect on sodium current kinetics, neither isomer produced a significant change in the time to peak current after the onset of pulses to \(-20 \text{ mV}\) when compared with control (RAC109-I: \(-0.06\pm0.26 \text{ msec}, n=11\); RAC109-II: \(-0.01\pm0.14 \text{ msec}, n=12\)). These results suggest that, if RAC109 stereoisomers bind to open channels during a depolarizing pulse, they must do so with a rate that is slower than that for channel inactivation.

Effects of Reducing Channel Inactivation

Since sodium channels are both activating and inactivating within the first few milliseconds of a pulse to \(-20 \text{ mV}\), the possibility was considered that the fast phase of RAC109 block could result from drug binding to a rapidly occupied inactivated state. To test this hypothesis, cardiac myocytes were first pretreated with \(\alpha\)-chymotrypsin (0.7 mg/ml applied intracellularly by addition to the pipette solution), which has been reported to markedly reduce or eliminate fast inactivation in neuroblastoma cells. After exposure of the internal membrane surface of cardiac myocytes to \(\alpha\)-chymotrypsin for 30–60 minutes, there was only a very slow and incomplete decay of \(I_{\text{Na}}\) detectable during pulses of 20–100-msec duration at potentials ranging from \(-60 \text{ mV}\) to \(+60 \text{ mV}\) (Figures 5C and 6). In addition, as illustrated in Figure 5C, RAC109-I was still fully capable of producing use-dependent block in cells in which fast inactivation was virtually abolished by enzyme pretreatment. At a 1-Hz stimulation rate, the amplitude of use-dependent block produced by 50 \(\mu M\) RAC109-I was similar in both normal (0.50±0.10; \(n=10\)) and inactivation-modified cells.
(0.59±0.09; n=5) (p=0.05) (compare Figures 5B and 5C). However, the rate of onset of use-dependent block during a train of pulses was significantly accelerated in inactivation-modified cells (Figures 5B and 5C): the pulse constant for the onset of use-dependent block for RAC109-I at 1 Hz was 5.48±1.10 pulses in normal cells (n=10) and 1.88±0.37 pulses in inactivation-modified cells (n=5) (p<0.001). These results suggest that inactivation is not essential for use-dependent block and that the presence of an intact inactivation gate may actually retard its development. In contrast to use-dependent block, the amplitude of tonic block produced by RAC109-I was significantly increased in inactivation-modified cells (0.54±0.11; n=5) compared with normal cells (0.30±0.08; n=10) (p<0.01).

In addition to producing a use-dependent decrease in peak current amplitude, exposure to 50 μM RAC109-I also induced a noticeable time-dependent decay of I_{Na} in inactivation-modified cells (which was not definable in cells with intact inactivation) (Figures 5C and 6). The kinetics of this time-dependent block of channels were defined by fitting the ratio of current in drug over the control current obtained during 50–100-msec pulses to −20 mV (Figure 6C). (Although fitting raw current traces or difference currents provided similar results, fitting of the current ratio was considered to more accurately reflect the drug-induced time-dependent block since most cells had a small amount of slowly inactivating current before drug exposure [see Figure 6B] and there was some overlap in the kinetics of channel activation and channel block.) By use of this approach, the time course of drug-induced block could be well fit by a single exponential function and a constant (e.g., I_{Na} ratio=Ae^{-t/T}+B, where t=time) (Figure 6C). The mean amplitude of time-dependent block (A) was 0.37±0.05, the mean time constant (T) of channel block was 34.0±10.0 msec, and the fraction of unblocked current at steady state (B) was 0.14±0.03 (n=6). The instantaneous level of current (A+B) provides an additional estimate of channel block before channel activation and had a mean amplitude of 0.50±0.05 (n=6).

Mathematical Modeling of RAC109 Block

To gain further insight into the molecular mechanisms responsible for the stereoselectivity of use-dependent and tonic block produced by RAC109-I, data from pulse train protocols (Figure 7A) were analyzed by a parameter estimation procedure based on the guarded-receptor model (see “Materials and Methods”). A nonlinear least-squares algorithm was used to obtain fits of equation 3 to values of peak I_{Na} recorded during trains of 20-msec pulses to −20 mV (Figure 7B). From these fits, values of blocking rate (A), tonic block (b_{t}), and the steady-state level of use-dependent block (b_{s}) were estimated for four to five different interpulse intervals (t). A linear regression of blocking rate (A) against recovery interval (t) was then performed by use of Equation 4 to calculate values of A_{t} (the intercept) and λ_{t} (the slope) (Figure 7C). A 1-msec value for the activation interval (t_{a}) consistent with estimates of sodium channel mean open time,22,23 was assumed to calculate λ_{a}. Values of the blocking parameter (α) were then computed by use of Equation 6, and a linear regression of steady-state block (b_{α}) against α was performed by use of Equation 5 to estimate the equilibrium levels of channel block at the holding potential (R_{h}) and at −20 mV (A_{h}) (Figure 7D). Both types of relations (λ vs. t_{a} and b_{α} vs. α) were found to be well described by a simple linear function (r>0.9, p<0.05) (Figures 7C and 7D), consistent with the theoretical model. These estimated model parameters were then used to calculate the apparent binding and unbinding rates according to Equations 7–10. Mean values of the apparent binding and unbinding rate constants k_{b}, k_{u}, k_{s}, and l_{s} obtained from 10 experiments in RAC109-I and nine experiments in RAC109-II are given in Table 3. Since the estimates of k_{a} and l_{a} were based on an assumed value for t_{a}, assumption of a different access interval for calculation of λ_{b} will result in reciprocal changes in estimated values of k_{a} and l_{a} from Equations 8 and 10 (e.g., assuming t_{a}=2 msec results in values of k_{a} and l_{a} half those shown in Table 3).

The apparent rate constants defining drug binding and unbinding from inactivated channels (k_{b} and l_{b}) were calculated from Equations 11–14 by use of estimates of the steady-state level of block ( tonic+time-dependent) at −20 mV (B_{α}) and the time constant for the slow phase of channel block obtained by use of a two-pulse protocol (Figure 4). Mean values for k_{b} and l_{b} are shown in Table 3.

FIGURE 5. Effect of inactivation modification on sodium channel block by RAC109-I. Panel A: Diagram showing a train of depolarizing pulses to −20 mV applied at 1 Hz after a 3-minute rest at the holding potential (−140 mV). Panel B: Tracings showing tonic and use-dependent block produced by 50 μM RAC109-I in a typical cell with intact inactivation. Panel C: Tracings showing tonic and use-dependent block produced in a cell pretreated with 0.7 mg/ml α-chymotrypsin. Although inactivation was reduced to a negligible level, RAC109-I still produced a tonic and use-dependent block qualitatively similar to that observed in cells with intact inactivation. The horizontal arrows indicate the level of zero current.
A statistical comparison of the apparent rate constants for RAC109-I and RAC109-II (Table 3) indicates that the two stereoisomers have significantly different unbinding rates from sodium channels when they exist primarily in rested, activated, or inactivated states \( (k_u, k_a, \text{and } k_i) \). In addition, the two stereoisomers differ significantly in their apparent rates of binding to rested or inactivated channels \( (k_u, k_i) \) (Table 3). The ability of the model to predict and simulate channel block by use of the estimated mean rate constants was tested by comparing computed model predictions with experimentally determined values of tonic and use-dependent block, as well as the kinetics of development and recovery from use-dependent block for both stereoisomers. As shown in Table 4, all of the model predictions fall within a standard deviation from the mean experimental data; thus, the model provides a reasonably accurate description of the blocking effects of RAC109 stereoisomers on cardiac sodium channels.

Discussion

Stereospecificity of Channel Block

One of the main conclusions from this study is that block of cardiac sodium channels by tertiary amine local anesthetic drugs can be stereoselective. This provides additional support for the hypothesis that tertiary amine local anesthetic agents and class I antiarrhythmic drugs block cardiac sodium channels by binding to a specific receptor site.\(^{38-40}\) In addition, the demonstration of a marked difference in drug-channel interaction between two agents that differ only in their geometric conformation indicates that drug shape can be an important determinant of drug binding to its receptor, in addition to other factors such as molecular size and lipid solubility.\(^{41,42}\)

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Effect of RAC109-I on sodium current time course in a cell pretreated with α-chymotrypsin. Panel A: Diagram showing a series of nine pulses of 100-msec duration of \(-20\) mV applied at a cycle length (CL) of 3 minutes during the onset of exposure to 50 μM RAC109-I. Panel B: Tracings showing the effect of RAC109-I on sodium current time course in a cell pretreated with 0.7 mg/ml α-chymotrypsin to reduce channel inactivation. Comparison of current tracings recorded in the presence of RAC109-I (third to ninth pulse) to control indicates that RAC109 blocks activated channels in a time-dependent manner. Panel C: Tracing showing the ratio of current recorded in RAC109-I (ninth pulse) to control (first pulse). The smooth curve indicates a least-squares fit of the current ratio to a single exponential equation: 

\[
0.385 e^{-t/0.155}, \text{where } t \text{ is time. The horizontal arrows indicate the level of zero current.}
\]

![Figure 7](http://circres.ahajournals.org/)

**Figure 7.** Mathematical modeling of channel block by RAC109-I according to the guarded-receptor hypothesis. Panel A: Diagram showing a train of depolarizing pulses to \(-20\) mV applied at different stimulation rates after a 3-minute rest at the holding potential (\(-140\) mV). Panel B: Graph showing peak sodium current (\(I_{Na}\)) recorded during pulse trains at different rates. These currents were fit to equation 3 to estimate uptake rates \((\lambda)\) and steady-state amplitudes of block \((b_n)\). The peak current amplitude for control was 5.37 nA. For recovery (interpulse) times of 0.18, 0.48, 0.98, 1.98, and 4.98 seconds, the associated blocking rates \((\lambda)\) for RAC109-I were per 0.132, 0.155, 0.163, 0.197, and 0.257 pulses. The associated steady-state levels of block \((b_n)\) were 0.82, 0.71, 0.69, 0.57, and 0.52. Panel C: Graph showing a fit of the relation between blocking rate \((\lambda)\) and the interpulse recovery time (\(t_i\)) according to equation 4. The best fit was \(\lambda=0.025 t_i+0.138 (r=0.99, p<0.01)\). Panel D: Graph showing a fit of the relation between \(b_n\) and the blocking parameter (\(\alpha\)) according to equation 5. The best fit was \(b_n=0.784-0.573\alpha (r=0.92, p<0.05)\). The observed curves are in agreement with the theoretical predictions of an exponential relation between \(I_{Na}\) amplitude and pulse number, as well as linear relations between \(\lambda\) vs. \(t_i\) and \(b_n\) vs. \(\alpha\).
Table 3. State-Dependent Rate Constants for RAC109 Stereoisomers

<table>
<thead>
<tr>
<th></th>
<th>( k_i ) (M/msec)</th>
<th>( l_i ) (msec)</th>
<th>( K_{ar} ) (( \mu )M)</th>
<th>( k_{ar} ) (M/msec)</th>
<th>( l_{ar} ) (msec)</th>
<th>( K_{a0} ) (( \mu )M)</th>
<th>( k_x ) (( \mu )M)</th>
<th>( l_x ) (msec)</th>
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<tbody>
<tr>
<td>RAC109-I</td>
<td>Mean</td>
<td>2.09 ( \times ) 10(^{-3})</td>
<td>2.24 ( \times ) 10(^{-3})</td>
<td>143</td>
<td>2.52 ( \times ) 10(^1)</td>
<td>2.86 ( \times ) 10(^{-2})</td>
<td>12.3</td>
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<tr>
<td></td>
<td>SD</td>
<td>1.02 ( \times ) 10(^{-3})</td>
<td>0.55 ( \times ) 10(^{-3})</td>
<td>104</td>
<td>0.68 ( \times ) 10(^1)</td>
<td>1.33 ( \times ) 10(^{-2})</td>
<td>6.0</td>
<td>0.36</td>
</tr>
<tr>
<td>RAC109-II</td>
<td>Mean</td>
<td>4.62 ( \times ) 10(^{-1})</td>
<td>6.08 ( \times ) 10(^{-1})</td>
<td>610</td>
<td>1.52 ( \times ) 10(^1)</td>
<td>8.00 ( \times ) 10(^{-2})</td>
<td>60.1</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>5.63 ( \times ) 10(^{-1})</td>
<td>3.14 ( \times ) 10(^{-1})</td>
<td>987</td>
<td>0.82 ( \times ) 10(^1)</td>
<td>3.50 ( \times ) 10(^{-2})</td>
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<td>( p )</td>
<td>NS</td>
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<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.0005</td>
<td>NS</td>
<td>&lt;0.03</td>
</tr>
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</table>

Rate constants indicate apparent binding (k) and unbinding (l) rates to channels rested at -140 mV (r), activated at -20 mV (a), and inactivated at -20 mV (i). No attempt was made to factor out receptor guard and trap functions (see 'Materials and Methods').

This conclusion is also supported by the results of several recent structure-activity studies, which have shown that physiochemical factors (e.g., pK\(_a\), lipid solubility, and molecular weight) alone can only partly account for the ability of different drugs to block sodium channels. 43-45 Interestingly, although use-dependent block by RAC109 was markedly stereoselective, the level of tonic block produced by the two stereoisomers was not significantly different (Figure 1 and Table 4). As discussed below, this similarity in tonic block can be attributed to a lack of stereospecificity in the binding (association) rates (\( k_i \)) for RAC109 enantiomers to channels at the holding potential (Table 3).

Two Components of Block Development

Recent voltage-clamp studies on cardiac sodium channels have provided strong evidence that use-dependent block produced by tertiary amine local anesthetics can result from drug binding to multiple channel states (e.g., activated and inactivated). 30,46,47 Therefore, experiments were performed to clarify further the molecular basis underlying the stereospecificity of RAC109 block.

Characterization of the time course of RAC109 block development at -20 mV by use of a two-pulse protocol (Figure 4) revealed that block associated with depolarization consists of two definable components: a fast component (\( \tau = 2-3 \) msec) and a very slow component (\( \tau = 14-15 \) sec). This suggests that RAC109 stereoisomers have a relatively high affinity for more than one channel state occupied during a depolarizing pulse. Since virtually all sodium channels are completely inactivated within less than a hundred milliseconds at -20 mV, the simplest explanation for the slow component of block is that it reflects very slow drug binding to inactivated channels. Furthermore, the slow component develops so slowly that it cannot significantly contribute to the use-dependent block measured by use of brief trains of short (2-20-msec) pulses (Figures 1 and 2). Therefore, the use-dependent block defined by use of trains of short pulses must reflect a pulse-by-pulse accumulation of channels blocked via the

Table 4. Comparison of Model Predictions With Experimental Data

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Model</th>
<th>Experiment</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ( \mu )M RAC109-I</td>
<td></td>
<td>50 ( \mu )M RAC109-II</td>
<td></td>
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<tr>
<td>Tonic block</td>
<td>0.30 ± 0.08</td>
<td>0.32</td>
<td>0.24 ± 0.10</td>
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<tr>
<td>Steady-state block</td>
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<td>5 Hz</td>
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<td>0.74</td>
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<td>0.71</td>
<td>0.44 ± 0.09</td>
</tr>
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<td>1 Hz</td>
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<td>0.5 Hz</td>
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<td>0.2 Hz</td>
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<tr>
<td>( \tau ) (sec)</td>
<td>26.8 ± 4.3</td>
<td>30.4</td>
<td>15.1 ± 4.6</td>
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</table>

Experimental data are mean ± SD (n=4-10). Values are model predictions computed from the rate constants shown in Table 3. Steady-state block was produced by a train of 15 pulses of 20-msec duration to -20 mV (holding potential = -140 mV). \( \lambda \), rate of use-dependent block onset; \( \tau \), time constant for recovery from use-dependent block at -140 mV.
fast component. This conclusion is supported by the observation that the amplitude of channel block produced by a series of brief pulses (Figures 1 and 2) is much greater than the amplitude of the fast component produced by a single pulse of variable duration (Figure 4, inset). The observation of a component of drug binding that develops rapidly but does not reach steady state during a single depolarizing pulse indicates that RAC109 isomers must bind to a channel state that is only transiently occupied on membrane depolarization and that the time constant for drug binding is slower than the mean dwell time of the channel in this high-affinity state.

Since sodium channels fluctuate through a number of conformational states during the first few milliseconds of a suprathreshold depolarization, the identity of the high-affinity activated state is not readily obvious. However, current theories suggest that activation-related block may be primarily due to the charged (cationic) form of local anesthetics selectively binding to open sodium channels. Because both RAC109 enantiomers (pKₐ=9.4) exist almost exclusively in the charged form at the pH used (7.2-7.3) and the time course of the observed fast component overlaps that of channel opening, the possibility was considered that this component results from block of open channels (which have a mean dwell time of only 1-2 msec at 10°-20° C). Drugs believed to block open channels in nerve, including RAC109, have been shown to block sodium channels in a voltage-dependent manner.

Characterization of the voltage dependence of RAC109 block in cardiac myocytes (Figure 2) revealed that the fast component of block had a voltage dependence that was similar to channel opening in that both phenomena developed and saturated over a similar voltage range and had similar voltage midpoints (-34 to -38 mV). In contrast, the voltage dependence of block differed from that of channel inactivation, which develops and saturates over a voltage range of -120 to -60 mV under these conditions. Furthermore, the amplitude of use-dependent block was not reduced even after enzymatic modification of channels, which prevented inactivation development during short pulses (Figure 5C). In fact, the rate of use-dependent block onset was accelerated in inactivation-modified cells; this occurrence suggests that the closure of the inactivation gate actually retards the development of the fast component of block. These results indicate that the fast component of RAC109 block cannot be attributed to drug binding to inactivated channels and must result from binding to either the open state or a preopen closed state that is closely associated with the open state (i.e., also transiently occupied at depolarized potentials and is not removed by proteolytic enzymes).

Although the fast component of channel block by RAC109 isomers developed and saturated over a similar voltage range as that of channel opening (e.g., peak G Na), the voltage dependence for the two phenomena were not identical in that channel block was consistently less steep than that of peak G Na. The explanation for this difference is not clear but might possibly be due to a competitive interaction between RAC109 and monovalent cations (e.g., Na⁺) for a common binding site. Previous studies in nerve have shown that the presence of external sodium ions can markedly reduce the slope for voltage-dependent block for QX-314 as well as some analogues of disopyramide. However, additional studies are needed to confirm this for RAC109.

Although the results seem consistent with block of open channels, the voltage dependence of block is much steeper than predicted by previously proposed models that account for voltage-dependent open-channel block in terms of an effect of the transmembrane field on the passive diffusion of cationic drug to a blocking site within the sodium channel. According to this type of model, the slope parameter defining the steepness of voltage-dependent block (e.g., S in Equation 15) can be defined as S=RT/zΔF, where R, T, and ΔF have their usual meaning, z is the drug molecule valence, and ΔF is the equivalent electrical distance of the binding site across the membrane field (0-1) from the inside. In this study, the experimental conditions were such that RT/ΔF=25 mV and ΔF=1. Therefore, the minimal value of S predicted by this theory (i.e., when ΔF=1) is 25. In contrast, RAC109-I and RAC109-II had mean slope factors of 13.6±1.7 and 17.6±3.3 mV, respectively. Similarly, slope factors have also been recently reported for block of sodium channels by lidocaine in cardiac myocytes and for block of sodium channels in the squid giant axon by analogues of disopyramide. These results indicate that the dependence of fast channel block on membrane voltage (Figure 2) may be due primarily to a voltage dependence of the availability of a high-affinity channel state, rather than a voltage dependence of the binding and/or unbinding rate constants (kₐ or kᵢ).
(Figure 7B), and linear relations were observed between both the uptake rate (A) and interpulse recovery time (t), as well as the stimulus parameter \( \alpha \) and \( b_{m} \) \((r>0.9, \ p<0.05)\) (Figures 7C and 7D). In addition, state-dependent rate constants calculated from the guarded-receptor parameter estimation procedure provided an accurate description of both the kinetics and amplitude of block by RAC109 stereoisomers (Table 4).

When comparing the model rate constants estimated for drug interaction with rested, activated, and inactivated channels (Table 3), one pattern that becomes apparent is that the rates of both drug binding and unbinding from activated channels \((k_{b} \text{ and } l_{b})\) are three to four orders of magnitude larger than those defining drug interaction with channels that exist predominantly in rested \((k_{r} \text{ and } l_{r})\) or inactivated \((k_{i} \text{ and } l_{i})\) states. Such differences in the state-dependent rate constants are at least qualitatively consistent with the guarded-receptor model, which predicts that closure of either the activation or inactivation gates can greatly impede access and egress of drug from its receptor site.23-26 However, when one attempts to account for the differences in the state-dependent rate constants more quantitatively by factoring out estimated guard and trap functions \((m^{3} \text{ and } h)\) to derive the state-independent rate constants, some inconsistencies emerge from the analysis. Since the guarded-receptor model assumes that tonic block at strongly hyperpolarized potentials results from drug binding to infrequently opening channels23-26 and that \(k_{T}\) reflects drug binding to open channels, then estimates of \(k_{T}\) for RAC109 stereoisomers should be approximately equal to \(k_{T}m^{3}h_{T}\). This prediction was tested by estimating the voltage dependence of \(m^{3}\) and \(h_{T}\) from least-squares fits of the peak \(G_{max} \text{ vs } V_{m}\) relation (where peak \(G_{max}=m^{3}\)) and sodium current availability \((h_{T})\) curves to Boltzmann equations.30 The mean midpoints and slope factors for these relations were \(V_{m}=-34±8\ \text{mV} \text{ and } S=6.0±1.2\ \text{mV} \text{ (n=9)}\) for the peak \(G_{max} \text{ vs } V_{m}\) relation and \(h_{T}=-93±3\ \text{mV} \text{ and } S=5.0±0.5\ \text{mV} \text{ (n=16)}\) for the \(h_{T} \text{ vs } V_{m}\) relation. From these values, the estimated levels of \(m^{3}\) and \(h_{T}\) at \(-140\ \text{mV}\) are calculated to be \(m^{3}=2×10^{-8}\) (or \(m^{3}=1×10^{-9}\) when estimated from an infinite time integral of \(G_{max}\)) and \(h_{T}=1.0\). Multiplying these values for \(m^{3}\) and \(h_{T}\) by the values for \(k_{T}\) shown in Table 3 yields predicted values of \(k_{S}\) for RAC109-I and RAC109-II of \(5×10^{-2}\) and \(3×10^{-2}\) M/msec, which are four orders of magnitude smaller than the values of \(k_{S}\) calculated from the experimental data (0.2 and 0.5/M/msec). This appreciable discrepancy suggests that there is an error in either the formulation of the model or in the estimation of the gating parameters. Although a Boltzmann equation can accurately describe the voltage dependence of \(G_{max}\) (m3) at potentials where the macroscopic current can be accurately measured (i.e., positive to \(-60\ \text{mV}\)), it is not clear whether the same equation can accurately predict the frequency of channel opening at much more hyperpolarized potentials. More precise measurements of the frequency of channel openings based on single channel recordings may provide a more accurate estimation of the guard parameter \((m^{3})\) and would help to clarify whether the difference between the predicted and calculated values of \(k_{T}\) are due to inaccuracies in model assumptions or inaccurate estimates of the guard parameter \((m^{3})\). Values of \(k_{r}\) larger than that predicted by use of an \(m^{3}\) guard parameter could also result if drug access to channels via the lipid bilayer pathway was possible after only partial channel activation, for example, after opening of a single \((m)\) or pair \((m^{2})\) of activation gates. A recent structural model of the excitatory sodium channel has postulated that the channel activation gates are located on four homologous transmembrane segments that are concentrically arranged around the channel lumen.50 Therefore, it seems reasonable to postulate that rearrangement of only one or two activation gates neighboring a drug positioned within the membrane may be sufficient to permit lateral diffusion of drug into an obstructive position within the channel lumen, whereas entrance of charged drug into the channel lumen via the aqueous pathway might still require all activation gates to be open. This variant of the guarded-receptor model could account for the presence of significant tonic block \((large \ k_{T})\) produced by lipid soluble drugs at hyperpolarized potentials where the frequency of channel opening may be very low. Comparison of \(k_{T}\) values for other lipid soluble and lipid insoluble drugs would provide one means of testing this hypothesis. Finally, it is difficult to rule out the possibility that large values for \(k_{T}\) could reflect the existence of a separate receptor for tonic block34,43 or a receptor having a state-dependent conformation as postulated by the modulated-receptor models of Hille38 and Hondeghem and Katzung.39 Indeed, without firm evidence for a quantitative relation between the apparent rate constants and channel gating variables for a variety of drugs, a modulated-receptor explanation for the state-dependent rate constants seems equally defensible. Even though there is more than one possible molecular explanation for the state-dependent rate constants, the close agreement between the experimental data and model predictions (Table 4) suggests that the procedure for calculating rate constants (Equations 1-14) provides a sufficiently accurate description of use-dependent block and as such may provide useful insight into how local anesthetic agents differ in their state-dependent interaction with cardiac sodium channels.

**Stereo-selectivity of Model Rate Constants**

Comparison of the estimated rate constants for RAC109-I and RAC109-II interaction with cardiac sodium channels indicates that the stereo-selectivity of use-dependent block observed by use of trains of brief pulses results from two factors: significantly different affinities and rate constants \((k_{T} \text{ and } l_{T})\) defining drug interaction with activated channels.
during the test pulse and significantly different rates of unbinding ($t_u$) during the interpulse interval (Table 3). Both of these factors contribute to the difference in observed potency of use-dependent block induced by brief pulse trains (Figures 1 and 2).

Interestingly, although the two stereoisomers have different binding rates to activated channels ($k_a$), there is no significant stereoselectivity in the apparent rates of drug binding to channels when they are held in predominantly rested or inactivated states ($k_r$ and $k_t$) (Table 3). This observation may be accounted for by Hille’s hypothesis that local anesthetics interact with closed versus open channels via different pathways: charged local anesthetics selectively block open channels by accessing the receptor via a hydrophilic pathway, whereas uncharged drug forms can interact with closed channels by diffusing to the receptor via a hydrophobic pathway through the lipid bilayer. Although RAC109 stereoisomers exist primarily in their charged form in aqueous solution (>99% at pH 7.2-7.3), the partition coefficient (P) of the uncharged form is fairly high (P=260), so that, from the equation: Q/P=(1+10$^{pK_a-pH}$), at pH 7.2-7.3, the amount of uncharged RAC109 dissolved in the membrane is expected to be 1.6-2 times higher than the total amount of drug present in the aqueous phase. Therefore, it seems reasonable to expect that RAC109 stereoisomers could interact with their receptor in both uncharged and charged forms. Based on this assumption, one possible explanation for the observed difference in stereoselectivity of $k_r$ and $k_t$ versus $k_a$ is that both $k_r$ and $k_t$ reflect binding of uncharged drug to closed-channel states (pre-open or inactivated), whereas $k_a$ reflects binding of charged drug over the aqueous pathway. Charged and uncharged drugs might be expected to have different conformations or orientations when presented to the drug receptor; thus, different stereoselectivity of binding would result.

Comparison of the ratio of unbinding rates for the two stereoisomers (RAC109-I and RAC109-II) for all three channel states (0.37, 0.36, and 0.44 for rested, activated, and inactivated states) indicates that there is relatively little state-dependent change in stereoselectivity of the unbinding rates. This is consistent with the guarded-receptor model, which assumes that the receptor conformation is static and that differences in unbinding rates for different channel states result primarily from changes in the positions of channel gates (drug trapping), which should affect both stereoisomers equally.

Role of Channel Activation and Inactivation Gates in Recovery From Block

For use-dependent block to occur on application of depolarizing pulses, there must be increased drug binding during each depolarizing pulse as well as slow and incomplete unbinding between each pulse at the holding potential. Two possible molecular mechanisms for slow unbinding at the holding potential include 1) trapping of charged drug within the channel by closed activation gates, as previously postulated by numerous investigators, or 2) trapping of drug in an inactivated-blocked state due to a drug-induced hyperpolarizing shift in the voltage dependence of the inactivation gating mechanism.

Several pieces of evidence indicate that the slow rate of RAC109 unbinding at the holding potentials studied (140 to 160 mV) cannot be accounted for by drug trapping in an inactivated-blocked state. First, estimation of the voltage shift of inactivation for RAC109 stereoisomers by use of the equation of Bean et al. ($\Delta V_{in} = k\ln(K_d/K_a)$, where $k$ is the slope factor for the inactivation curve and $K_d$ and $K_a$ are dissociation constants in activated and in resting states, respectively) predicts a drug-induced voltage shift of only -19 to -21 mV (assuming $K_d$ and $K_a$ values shown in Table 3 and $k=6 mV^{32}$). Under the experimental conditions, the midpoint of the inactivation (h) curve was estimated to be $-93\pm3 mV$ ($n=16$). Therefore, clamping the membrane to potentials between -140 to -160 mV would be predicted to completely surmount a drug-induced voltage shift of this magnitude. Hyperpolarization to potentials that surmount a voltage shift should result in rapid recovery of sodium current at the holding potential and abolishment of use-dependent block when drug trapping in an inactivated-blocked state is the rate-limiting step regulating channel unblocking. Such effects were not observed experimentally (Figure 1 and Table 1). In addition, use-dependent block remained marked even after removal of fast inactivation by $\alpha$-chymotrypsin (Figure 5C). These results indicate that trapping of channels in an inactivated-blocked state is not responsible for use-dependent accumulation of block when pulsing from strongly hyperpolarized potentials.

In contrast, the observed increase in block on application of depolarizing pulses, slow recovery from block at strongly hyperpolarized potentials, and persistence of use-dependent block after removal of fast inactivation can be explained in terms of receptor guarding and trapping by the channel activation gates. At strongly hyperpolarized potentials, most channels will have their activation gates closed; this condition will permit only a relatively small tonic block to develop even after several minutes rest at -140 mV. Closed activation gates will also act to trap charged drug within the channel lumen. Nevertheless, since RAC109 stereoisomers are tertiary amines, the lifetime of drug within the channel at hyperpolarized holding potentials may not be regulated by the low frequency of channel openings since these drugs may deprotonate to an uncharged lipid soluble form while trapped within the channel. Assuming a protonation rate ($k_p$) of $5\times10^4/M/sec^5$, the lifetime of cationic RAC109 (1/4) is predicted (from $k_p/\mu=10^{6-7}$) to be ~5 seconds. However, the lifetime of cationic RAC109
may actually be longer than this if the charged microenvironment of the channel increases the pK_a of bound drug. The protonation-deprotonation reactions could be significantly affected by such factors as local potential fields or proton access paths or lowering of local pH due to attraction of charged H^+ ions into the channel from the extracellular solution on hyperpolarization. Thus, the slow rate of RAC109 unbinding from rested channels seems consistent with trapping of drug within the channel by closed activation gates although it is necessary to assume the presence of a higher pK_a for both stereoisomers when they are bound within the channel.

The conclusion that RAC109 stereoisomers can become trapped behind closed activation gates at strongly hyperpolarized potentials is consistent with the conclusions of several previous studies that investigated the blocking behavior of other highly charged drugs on V_max in cardiac tissue and sodium current in nerve. Thus, although strong hyperpolarization may result in relief of block for some tertiary amine drugs (e.g., lidocaine and quinidine), it may not do so for others (e.g., RAC109 stereoisomers and penticainide).

**Effects of α-Chymotrypsin on Channel Block**

In addition to a lack of removal of use-dependent block (Figure 5), several other effects of RAC109 stereoisomers observed after enzyme treatment also seem consistent with predictions made by the guarded-receptor model. For example, recent studies have shown that removal of channel inactivation by agents such as α-chymotrypsin, trypsin, and N-bromoacetamide produce both a several-fold increase in the single channel mean open time at the holding potential (i.e., m^3.>0), such an enzyme-induced shift of channel activation to more negative potentials would be expected to increase significantly the level of tonic block. An enzyme-induced increase in mean open time (t_o) would also be expected to increase the onset of use-dependent block (Equation 4). Both of these effects were observed (Figure 5). Hypothetically, if the mean open time were to become infinitely long (which seems unlikely), then the time constants for drug-induced decay should approach the predicted time constant for activated-state block (1/k_s=6 msec). The observation of a significantly longer time constant is expected if there is significant channel flickering between open (activated) and closed states, as has been observed after treatments that reduce channel inactivation.

The conclusions drawn from experiments carried out in cells pretreated with α-chymotrypsin to remove channel inactivation are based largely on the assumption that α-chymotrypsin has a selective effect on removing fast inactivation in cardiac cells, similar to its effect in neuroblastoma cells. Although other effects cannot be ruled out, the observation that cardiac sodium currents still have a qualitatively similar sigmoidal onset on depolarization and brusk deactivation kinetics on repolarization (Figures 5 and 6) suggests that the function of the channel activation gates in cardiac tissue may be relatively unaffected after a digestion sufficient to virtually abolish fast channel inactivation in cardiac cells. However, it is difficult to rule out the possibility that proteolytic digestion may not also directly modify the drug receptor or induce structural changes to the sodium channel that alter drug access and egress from the receptor, as has been suggested to occur after pronase treatment in squid giant axons. For example, in earlier experiments using concentrations of trypsin that reduced sodium conductance twofold to threefold, little if any time-dependent block by RAC109 could be defined during 20-msec pulses to -20 mV (although use-dependent block was still observable). This contrasts with the results observed in the present study where a milder digestion using α-chymotrypsin was performed, and both use-dependent and time-dependent block were clearly observable during 20-msec pulses (Figure 5C). Thus, strong digestion, or different agents, may produce slightly different effects on the drug-receptor reaction. A similar conclusion was reached by Wang et al., who found that removal of channel inactivation by chloramidine T and pronase can affect local anesthetic block differently in squid giant axons. Nevertheless, the observation of use-dependent block after abolition of fast inactivation indicates that channel inactivation is not obligatory for block of cardiac sodium channels by local anesthetics and that it does not regulate channel unblocking at strongly hyperpolarized potentials.

**RAC109 Has Similar Blocking Characteristics in Nerve and Heart**

There are many close similarities between the effects of RAC109 stereoisomers in cardiac myocytes and in nerve. In both tissues RAC109-I has been found to produce two to three times more use-dependent block compared with RAC109-II at equimolar concentrations (Figure 1) although both stereoisomers produce similar levels of tonic block (Figures 1). In addition, use-dependent block by both stereoisomers is strongly voltage-dependent in both tissues; RAC109-I has the steeper voltage dependence (Figure 2). Finally, recovery from use-dependent block by RAC109-I occurs with a time constant several times slower than for RAC109-II in both tissues (Figure 3). These findings suggest that the local anesthetic binding site in sodium channels of nerve and heart may have a similar structural topography.
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