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Differential Interaction of *R*-Mexiletine with the Local Anesthetic Receptor Site on Brain and Heart Sodium Channel α-Subunits

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ABSTRACT

Mexiletine is a class I antiarrhythmic drug with neuroprotective effects in models of brain ischemia attributable to inhibition of brain sodium channels. We compared effects of *R*-mexiletine on wild-type and mutant rat brain (rbIIA) and heart (rh1) sodium channel α-subunits transiently expressed in tsA-201 cells. *R*-mexiletine induced tonic and frequency-dependent block and bound with a 26-fold (brain) or 35-fold (heart) higher affinity to inactivated sodium channels. Affinities of both resting and inactivated channels for *R*-mexiletine block were approximately 2-fold higher for heart than for brain channels. Mutations in transmembrane segment IVS6 of heart (rhF1762A) and brain (rbF1764A and rbY1771A) channels, which reduce block by other local anesthetics, reduced high-affinity block of inactivated channels and frequency-dependent block of open channels by *R*-mexiletine and abolished the difference in affinity between brain and heart sodium channels. Unlike previous local anesthetics studied, the strongest effect was observed for mutation rbY1771A. Comparison of mutations of the homologous phenylalanine residue in brain and heart channels showed striking differences in the effects of the mutations. rbF1764A reduced drug block by slowing *R*-mexiletine binding to inactivated channels, whereas rhF1762A reduced block by increasing the rate of dissociation from inactivated and resting channels. Thus, rbF1764/rhF1762 is a critical determinant of affinity and tissue-specific differences in mexiletine block of brain and heart sodium channels, but its role in drug interaction differs in these two channel isoforms.

Inhibitors of voltage-gated sodium channels are widely used clinically. Blockers of sodium channels in the heart are potent antiarrhythmics, whereas the inhibition of neuronal sodium channels is useful for local anesthesia and treatment of epilepsy (Hondeghem and Katzung, 1984; Catterall, 1987; Butterworth and Strichartz, 1990; Ragsdale et al., 1991; Caron and Libersa, 1997). These overlapping actions bear risks and benefits. On the one hand, local anesthetics inadvertently injected into a blood vessel can cause severe cardiac arrhythmias. On the other hand, some antiarrhythmics, including mexiletine, penetrate the blood-brain barrier and have interesting neuroprotective properties (e.g., Stys and Lesiuk, 1996).

Voltage-gated brain sodium channels are heteromultimeric proteins consisting of a principal α-subunit of 260 kDa, as well as a β_1-subunit of 36 kDa and a β_2-subunit of 33 kDa (Catterall, 1992). Both α- and β_1-subunits also are expressed in cardiac myocytes (Qu et al., 1995a). The α-subunit consists of four homologous transmembrane domains (I–IV), each containing six transmembrane α-helical segments, called S1 through S6 (Catterall, 1992; Fozzard and Hanck, 1996). The principal electrophysiological functions are mediated by the α-subunit; the β-subunits have only minor effects when the channels are heterologously expressed in mammalian cells (Isom et al., 1995). The type IIA sodium channel α-subunit is a principal isoform expressed in the brain (Gordon et al., 1987; Beckh et al., 1989), and its heterologous expression in mammalian cells yields sodium currents with physiological and pharmacological properties that are similar to those observed in rat brain neurons (Ragsdale et al., 1991; West et al., 1992). The rh1 sodium channel α-subunit is the primary isoform expressed in the heart (Rogart et al., 1989; Kallen et al., 1990), and expression of this isoform in *Xenopus* oocytes or mammalian cells yields channels with physiological and pharmacological properties characteristic of heart sodium channels (Cribbs et al., 1990; White et al., 1991; Qu et al., 1994, 1995a).

Mutations of amino acid residues F1764 and Y1771 in transmembrane segment IVS6 of the rbIIA sodium channel

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[1] A longer version of this paper containing the results of additional kinetic analyses and control experiments can be found at http:/faculty.washington.edu/scheuer/.

ABBREVIATIONS: rb, rat brain; rh rat heart; WT, wild type; FF, fraction fast; τ_f, τ fast; τ_s, τ slow.

to alanine substantially reduced block by the local anesthetic etidocaine (Ragsdale et al., 1994) and by a range of local anesthetic, anticonvulsant, and antiarrhythmic drugs (Ragsdale et al., 1996; Wang et al., 1998; Wright et al., 1998), defining a local anesthetic receptor site in this region of the channel structure. Mutation of F1762 in the rh1 sodium channel, which is homologous to rbF1764, to alanine also resulted in loss of block of the rh1 channel by the quaternary lidocaine analog QX314 (Qu et al., 1995b). Mexiletine is a class I antiarrhythmic that inhibits both brain and heart sodium channels in vitro and in vivo (Stys and Lesiuk, 1996; Wang et al., 1997). In the experiments described here, we compared the effects of *R*-mexiletine on cloned rat brain and heart sodium channel α-subunits heterologously expressed in a common cellular background, and we examined the effects of mutations in the local anesthetic receptor site on mexiletine block of these two channel types. Our results show that heart sodium channels have an intrinsically higher affinity for block by *R*-mexiletine than do brain channels when expressed in the same cellular background. Surprisingly, although mutations of the homologous phenylalanine residue in brain and heart channels (rhF1762A/rbF1764A) reduced block by *R*-mexiletine and abolished the difference in affinity between the two channel isoforms, the effect was primarily on drug association rate for rbIIA and on drug dissociation rate for rh1. Thus, although this phenylalanine residue is essential for mexiletine block of both heart and brain sodium channel isoforms and is responsible for the difference in mexiletine affinity between them, its role in drug binding is fundamentally different in the two channel backgrounds. This finding suggests that there are important differences in the interaction of local anesthetics and related compounds with brain and cardiac sodium channels. These differences may be valuable in the development of novel, subtype-selective antiarrhythmic drugs.

Materials and Methods

Cell Maintenance and Transient Transfection for Electrophysiological Recording. tsA-201 cells, which are a subclone of HEK293 cells expressing simian virus 40 *t*-antigen, were a kind gift from Dr. Robert Dubridge (Cell Genesys, Foster City, CA). Cells were maintained in Dulbecco's modified Eagle's medium/F12 media (Gibco Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 25 U/ml penicillin and 25 μg/ml streptomycin (Sigma, St. Louis, MO.). An *Eco*RV fragment containing mutants F1764A and Y1771A of RIIA in pVA2580 (Ragsdale et al., 1996) was subcloned into pCDM8 containing the remainder of the rIIA sodium channel α-subunit, as described (Linford et al., 1998). rH1 (Rogart et al., 1989) and rH1 mutant F1762A in pCDM8 were described previously (Qu et al., 1995b). tsA-201 cells were transiently transfected with wild-type (WT) or mutant α-subunits and a vector encoding the human CD8 cell surface protein (EBO-pCD-leu2; American Type Culture, Rockville, MD) for cell recognition, as described (Margolskee et al., 1993). Successfully transfected cells were labeled to recognize them for recording using anti-CD8-coated polystyrene microspheres (Dynabeads M-450 CD8; Dynal, Great Neck, NY), as described (Jurman et al., 1994).

Electrophysiological Recording. Sodium currents were recorded from transiently transfected tsA-201 cells in the whole-cell voltage-clamp configuration (Hamill et al., 1981) at 22°C, as described (Qu et al., 1996). The extracellular solution contained 140 mM NaCl, 5 mM CsCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4, adjusted with NaOH). The intracellular solution contained 90 mM CsF, 50 mM CsCl, 10 mM CsEGTA, 10 mM NaF, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4, adjusted with CsOH). Recording pipettes had resistances of 0.8 to 1.8 MΩ when filled with intracellular solution. Mexiletine is a racemate of *S*($\pm$)- and *R*($-$)-enantiomers, which have differential effects on sodium channels (De Luca et al., 1995). In this study, only the ($-$)-enantiomer (R-mexiletine) was used to avoid differential effects of enantiomers on the sodium channel mutants. Purity of the *R*-enantiomer of mexiletine was >98%. The compound was synthesized at the Department of Pharmaceutical Chemistry at Boehringer Ingelheim KG (Ingelheim, Germany). *R*-mexiletine was dissolved in extracellular solution at the highest concentration to be used in an experiment, and diluted with extracellular solution to each of the other concentrations. The cells were bathed with the effluent of a gravity-driven "sewer-pipe" perfusion system consisting of a series of parallel tubes with each tube containing either control solution or a solution containing *R*-mexiletine. Solutions were changed by translating the array of tubes so that the tube containing the appropriate concentration was bathing the cell. The entire Petri dish was perfused continuously with control extracellular solution. Solution changes were complete within 2 s. Currents were recorded using an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA). Voltage-clamp commands were delivered and currents recorded using PClamp 6 controlling a Digidata 1200 interface (Axon Instruments). Whole-cell capacitance was compensated using the internal voltage-clamp circuitry and ~80% of series resistance was compensated. Residual linear leakage and capacitance were subtracted using a P/4 protocol when appropriate (Bezanilla and Armstrong, 1977). Data analysis and curve fitting were performed using Sigma Plot (SPSS, Chicago, IL) or Prism (GraphPad Software, San Diego, CA). All data points are the means of three to six experiments, and grouped data are reported as $\pm$S.D.

Unless otherwise indicated, holding and test potentials were -100 and 0 mV for rbIIA WT and F1764A, -120 and 0 mV for Y1771A, and -120 and -20 mV for rH1 heart sodium channels.

Results

Mutations F1764A and F1771A Inhibit Tonic and Frequency-Dependent Block of Brain Sodium Channels by *R*-Mexiletine. The principal characteristics of sodium channel block by *R*-mexiletine are illustrated by the current traces in Fig. 1 (inset). tsA-201 cells expressing rbIIA sodium channel α-subunits were voltage-clamped at a holding potential of -100 mV, and a control current trace was recorded in response to a 10-ms depolarization to 0 mV. The cell was then exposed to *R*-mexiletine (100 μM) in the absence of pulses, followed by a 5 Hz train of 100 pulses (10 ms duration) to 0 mV. Approximately 20% of the current was tonically blocked in the first test pulse at this concentration, and an additional 25% block was observed at the steady state during the 5-Hz train (Fig. 1A, inset). The frequency-dependent component of block reached steady state rapidly, as illustrated for 300 μM *R*-mexiletine in Fig. 1A. Similar experiments were performed in different *R*-mexiletine concentrations, yielding concentration-response curves with IC_{50} values of 305 and 165 μM for tonic and frequency-dependent block, respectively (Fig. 1B, WT).

Mutants F1764A and Y1771A were studied similarly. Frequency-dependent block during a 5-Hz train of impulses was reduced substantially for F1764A and virtually abolished for Y1771A compared with WT (Fig. 1A). The IC_{50} values were shifted to 610 and 410 μM for tonic and frequency-dependent block, respectively, for F1764A, and to 717 and 616 μM for Y1771A (Fig. 1B).

Effects of Mutations F1764A and Y1771A on Drug Binding to the Resting and Inactivated States of Brain Sodium Channels. The tonic block of sodium current observed at a holding potential of −100 mV reflects a combination of binding to resting and inactivated sodium channels, because drug binding favors the inactivated state. Therefore, to estimate the affinity for block of resting channels, we determined the IC_{50} value for block of sodium channels at increasingly negative membrane potentials where the contribution of the inactivated state would be progressively reduced. Limiting values for the IC_{50} were reached at −140 mV (data not shown[1]), corresponding to block of the resting state with an IC_{50} value of 547 ± 107 μM for WT channels, 816 ± 224 μM for F1764A, and 642 ± 75 μM for Y1771A (Fig. 2, solid lines). Thus, no significant effect of mutations on resting affinity was observed.

To estimate the affinity for block of inactivated sodium channels, we first investigated the kinetics of *R*-mexiletine binding at depolarized potentials to determine the time required for steady-state block. Drug block reached steady state within 1 s and had two phases, a rapid phase corresponding in time to the opening of sodium channels and a slow phase coinciding with channel inactivation (data not shown[1]). To measure drug block after 1 s at −40 mV, where >97% of channels were inactivated, the membrane potential was returned to the holding potential of −100 mV for 10 ms before the test pulse to allow recovery of drug-free (but not drug-blocked) channels from inactivation. Concentration-response curves obtained using this protocol showed that *R*-mexiletine inhibited WT channels with an IC_{50} value of 20.7 ± 2.0 μM (Fig. 2, dashed line). Inactivated mutant channels had substantially reduced affinity for block by *R*-mexiletine, with IC_{50} values of 157 ± 12 μM and 317 ± 57 μM for F1764A and Y1771A channels, respectively (Fig. 2; $p < .001$ for each pair of conditions). Thus, the mutation F1764A disrupted block of the inactivated state by 7.6-fold, and the mutation Y1771A caused a 15.3-fold disruption of inactivated state block. For WT channels, the ratio between the affinity for the resting and inactivated states was 26.4. In contrast, for mutant F1764A this ratio was only 5.2, and for mutant Y1771A was 2.0. Thus, these mutations affect *R*-mexiletine affinity for inactivated channels much more than they affect affinity for resting channels and thereby reduce the difference in affinity between these channel states.

It is surprising that the effects of mutation of Y1771 on block by mexiletine are greater than those for mutation of F1764, in contrast to previous results with other structurally similar local anesthetics such as lidocaine and etidocaine (Ragsdale et al., 1994, 1996). Evidently, the strength of the molecular contacts of these structurally similar drugs with the amino acid residues in the local anesthetic receptor site is significantly different, suggesting a highly specific interaction of these drugs with their receptor site.

Block of WT and F1762A Mutant Rat Heart Sodium Channels by *R*-Mexiletine. We studied the block of cloned and expressed rh1 sodium channel α-subunits by *R*-mexiletine to compare the potency and mechanism of block to brain channels. Tonic and frequency-dependent block were initially examined during a train of 100 depolarizations using a protocol analogous to that used in Fig. 1 (Fig. 3A). For these experiments, the holding and test potentials were 20 mV negative to those used for brain channels to compensate for the more negative activation and inactivation properties of the rat heart channel (Fozzard and Hanck, 1996; T.W. and T.S., unpublished observations). As with brain channels, frequency-dependent block was observed at 5 Hz for WT and was much impaired for mutant rhF1762A (Fig. 3A). Concentration–response curves (Fig. 3B) obtained using this protocol gave IC_{50} values of 165 and 52 μM for tonic (closed circles) and frequency-dependent (open circles) block of WT rh1 channels, and IC_{50} values of 748 and 619 μM for rhF1762A.

As for brain sodium channels, we measured the affinities of the resting and inactivated states of rh1 WT and mutant channels for *R*-mexiletine (Fig. 4). Resting rh1 WT channels had an IC_{50} value of 325 ± 54 μM at −160 mV, whereas mutant rhF1762A had an IC_{50} value of 1330 ± 504 μM ($p < .05$). *R*-mexiletine inhibited inactivated rhWT channels at −60 mV with an IC_{50} value of 9 ± 3 μM, whereas a concen-

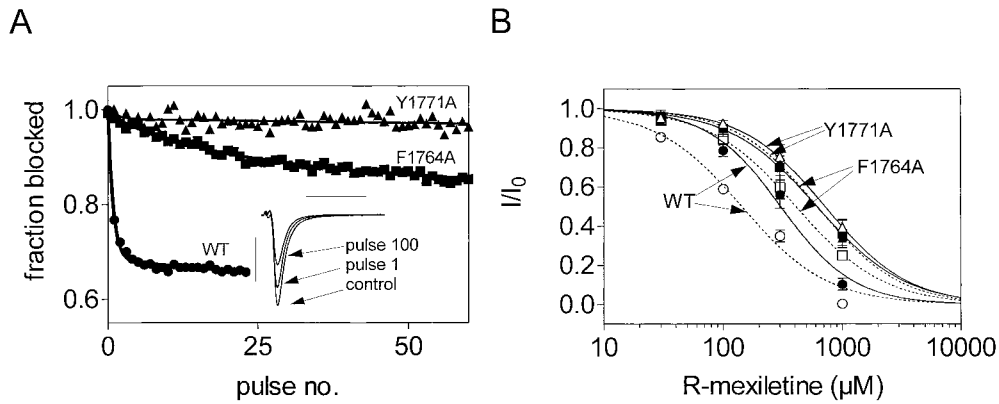

Fig. 1. Mutations in the IVS6 region of brain-type sodium channel α-subunits influence tonic and frequency-dependent block by mexiletine. A, a single pulse to 0 mV was applied in the absence of drug. Then *R*-mexiletine was washed into the bath for 30 s to 3 min without pulsing. Control experiments showed that equilibrium *R*-mexiletine block was reached within seconds. Finally, a 5-Hz train of 100 depolarizations (10-ms duration) to 0 mV was applied to voltage-clamped tsA-201 cells that had been transfected with rat brain sodium channel α-subunits. Inset, current traces evoked by the single pulse in drug-free solution (control) and by pulse 1 and pulse 100 of the train in the presence of 100 μM *R*-mexiletine. Scale bars: 2 ms, 2 nA. A, the additional frequency-dependent block observed during 5-Hz trains of pulses in the presence of 300 μM *R*-mexiletine for WT (●), F1764A (■), and Y1771A (▲) channels. B, the inhibition in response to pulse 1 is referred to as tonic block, and to pulse 100 as frequency-dependent block. Concentration-response curves are plotted for tonic (▲, ■, ●) and frequency-dependent (△, □, ○) block of rbWT (○, ●), rb F1764A (□, ■), and rbY1771A (△, ▲). The lines are fits to the data for each construct with IC_{50} values of 305 and 148 μM for tonic and frequency-dependent block of rbWT channels, 614 and 410 μM for rbF1764A, and 718 and 617 μM for Y1771A. Solid lines, tonic block; dotted lines, frequency-dependent block.

tration of 151 ± 25 μM ($p < .001$) was necessary for half-maximum block of rhF1762A channels (Fig. 4). Thus, rh1 WT channels have higher intrinsic affinity for *R*-mexiletine than do brain channels, consistent with the higher sensitivity of heart channels to frequency-dependent block by *R*-mexiletine. Interaction with rhF1762 is crucial for high-affinity drug block of the rh1 channels, and mutation of this residue reduces substantially the affinity for both resting and inactivated states.

Comparison of Affinity and Kinetic Mechanism of Block of Brain and Cardiac Sodium Channels by Mexiletine. WT heart sodium channels were more sensitive to frequency-dependent block at high frequencies than were WT brain sodium channels (Fig. 5A). Similarly, the IC_{50} values for the inhibition of resting and inactivated WT heart channels were approximately 2-fold lower than those for WT brain channels (Fig. 5B). In contrast, the concentrations for half-maximum block of inactivated rhF1762A and rbF1764A were similar (151 versus 157 μM; Fig. 5B), indicating that differences in interaction between bound mexiletine and F1764/F1762 are responsible for the difference in overall affinity for mexiletine block of the two channels.

Drugs that bind preferentially to the inactivated state of sodium channels shift steady-state inactivation curves toward more negative potentials (Hille, 1977; Hondeghem and Katzung, 1984). For a given mexiletine concentration, the shift in $V_{0.5}$ for inactivation was more pronounced for rhWT channels than for their rbWT counterparts (Fig. 5C, open symbols). The shift was reduced for the rhF1762A channels and was comparable with the corresponding shift for rbF1764A (Fig. 5C, filled symbols). These results also are consistent with the conclusion that interaction with F1762/F1764 is responsible for the difference in drug binding to the two channels.

The difference in the effects of mutations on the affinity for *R*-mexiletine block of brain and heart sodium channels could be caused by different effects on the kinetics of *R*-mexiletine action. The rate of onset of block in response to depolarization in mutant rbF1764A was much slower than that for rbWT, as demonstrated when the time courses for onset of block are normalized and superimposed (Fig. 6A). In contrast, the kinetics of the development of block of rhF1762A were not dramatically affected, as illustrated by the normalized and superimposed curves in Fig. 6B. To compare the kinetics of recovery from block, frequency-dependent block was induced by a train of 10 conditioning pulses to 0 mV (−20 mV for rh1 channels), and recovery was assayed by a test pulse to 0 mV (−20 mV for rh channels) after repolarizations to −100 mV (−120 mV for rh channels) of increasing duration. Recovery occurred with two exponential components: a rapid compo-

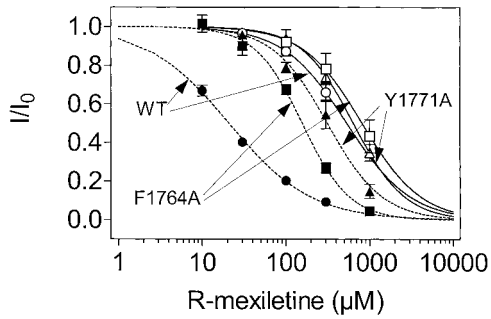

Fig. 2. Mutations rbF1764A and rbY1771A reduce block of resting and inactivated sodium channels. The inhibition of inactivated sodium channels was investigated using a 1-s depolarizing prepulse to −40 mV (−60 mV for rbY1771A) to inactivate most channels and allow *R*-mexiletine to bind to the inactivated channels. The membrane potential then was returned to the holding potential of −100 mV (−120 mV for Y1771A) for 10 ms to allow drug-free channels to recover from inactivation. A test pulse to 0 mV then was applied. *R*-mexiletine block of peak test pulse current is plotted as a function of *R*-mexiletine concentration for WT (●), rbF1764A (■), and rbY1771A (▲) channels. The dotted lines are fits of a logistic equation to the data with IC_{50} values of 20.7 μM (WT), 156 μM (rbF1764A), and 309 μM (rbY1771A), with slopes of 0.9, 1.5, and 1.3, respectively. Resting channel block (solid lines) IC_{50} values were 533 μM (○, WT), 813 μM (□, F1764A), and 655 μM (△, Y1771A), with slopes of 1.2, 1.2, and 1.4, respectively. Varying slope values are expected from allosteric models of drug binding in which binding is distributed among multiple states with varying affinities.

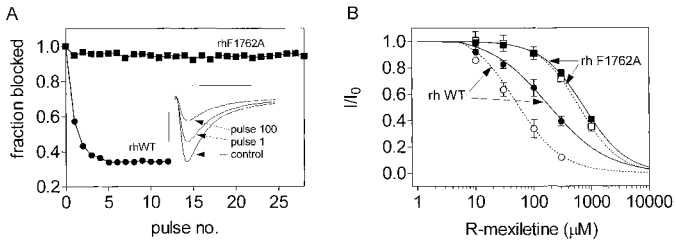

Fig. 3. Tonic and frequency-dependent inhibition of WT and mutant rat heart sodium channels. A (inset), current recorded during test pulses to −20 mV in control and during the 1st and 100th pulses of a 5-Hz train in the presence of 100 μM *R*-mexiletine in a cell expressing rhWT channels. Scale bars: 2 ms, 5 nA. A, the pulsewise development of frequency-dependent block using 5-Hz stimulation in the presence of 300 μM R-mexiletine was measured for WT (●) and rhF1762A (■) sodium channels. The protocol was the same as in Fig. 1, except that holding and test potentials were −120 mV and −20 mV, respectively, to account for the more negative voltage dependence of the heart channel. B, Concentration–response curves for tonic (■, ●) and frequency-dependent (□, ○) block of rhWT (●, ○) and rhF1762A (■, □) by *R*-mexiletine determined from pulse 1 and pulse 100 of trains as described in the legend to Fig. 1. The solid lines are fits of a logistic equation, with IC_{50} values for rhWT of 165 and 52 μM for tonic and phasic block, respectively, and for rhF1762A of 748 and 618 μM.

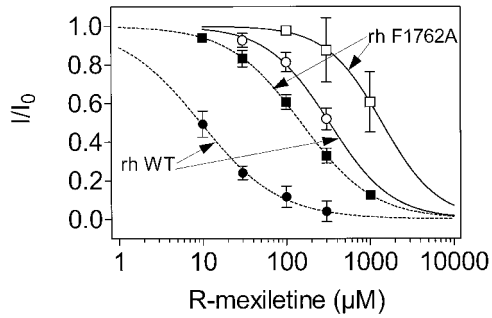

Fig. 4. Mutant rhF1762A reduces block of resting and inactivated sodium channels. The inhibition of inactivated sodium channels was investigated using a 1-s depolarizing prepulse to −60 mV. The membrane potential then was returned to the holding potential of −120 mV for 10 ms, and a test pulse to −20 mV was applied. The stimulus sequence was applied once in the presence of each drug concentration. *R*-mexiletine block of peak test pulse current is plotted as a function of *R*-mexiletine concentration for rhWT (●) and rhF1762A (■). The dotted lines are fits of a logistic equation to the data for inactivated channels, with IC_{50} values of 9.4 μM (rhWT) and 149 μM (rhF1762A) and slope values of 0.93 (rhWT) and 1.0 (rhF1762A). To measure block of resting channels, the indicated concentrations of *R*-mexiletine were applied to rhWT or rhF1762A channels at a holding potential of −160 mV, and block was measured during a test depolarization to −20 mV. The concentration–response curve for rhWT (○) and rhF1762A (□) reaches a limiting affinity at this membrane potential, as determined from parallel measurements at −100 mV, −120 mV, −140 mV, and −160 mV. Fit of logistic equations to the mean data (solid lines) for WT are IC_{50} = 325 μM, slope = 1.2, and for rhF1762A are IC_{50} = 1389 μM, slope = 1.3.

nent representing recovery of unblocked channels and a slow component representing drug dissociation from blocked channels. These components could be clearly separated (data not shown[1]). Figure 6, C and D, illustrates the normalized single exponential time course of recovery of the drug-blocked channels. For rbWT channels (Fig. 6C), the time constant for drug dissociation was 530 ± 15 ms, compared with 540 ± 157 ms for rbF1764A. For rhWT channels, the time constant for recovery was 671 ± 71 ms (Fig. 6D). In contrast, blocked rhF1762A channels recovered with a time constant of 170 ± 7 ms, 4-fold faster than that with the rhWT channel (Fig. 6D). Surprisingly, these results indicate that the reduced affinity of mutant rbF1764A channels is caused primarily by a reduced association rate, whereas the reduced affinity of mutant rhF1762A channels is caused primarily by an increased rate of dissociation of the drug-channel complex. These results point to different interactions of the analogous F1764 and F1762 residues with bound mexiletine in these two channel types.

Discussion

***R*-Mexiletine Block of Native and Heterologously Expressed Heart Sodium Channels Is Similar.** *R*-mexiletine has been described as a fast-onset, frequency-dependent blocker of heart sodium channels. Using rat ventricular myocytes, Yatani and Akaike (1985) found an IC_{50} value of 28 μM for tonic inhibition. Those data were obtained using a holding potential of −80 mV, which caused ~50% channel inactivation under their experimental conditions. In the same preparation, Ono et al. (1994) reported half-maximum inhibition of depolarized channels to be 15 μM. Heterologous expression of WT human heart sodium channels resulted in steady-state block of inactivated channels with an IC_{50} value of 15 μM (Wang et al., 1997). These data are consistent with the IC_{50} value of 9.4 μM that we obtained for *R*-mexiletine block of expressed rat heart sodium channels under depolarized conditions in the present study. Thus, the data on rat heart sodium channel α-subunits presented here are in good agreement with those obtained previously. Although these results were obtained with channels formed by sodium channel α-subunits alone, coexpression of sodium channel β_1- and β_2-subunits in mammalian cells cause only minor quantitative effects on channel function. Thus, we expect block of WT and mutant channels formed by the full complement of subunits to be qualitatively similar to that determined here with α-subunits alone.

Higher Intrinsic Affinity for Block of Heart Versus Brain Sodium Channels. The best estimate of affinity for resting channels is to determine block of peak current using increasingly negative holding potentials. Block by *R*-mexiletine reaches a limiting affinity that is not reduced as the holding potential is made more negative. This limiting IC_{50} value is 547 μM in the brain channel and 325 μM in the heart channel. Thus, when elicited from the fully resting state, the heart sodium channel is ~1.7 times more sensitive to block than is the brain channel. Likewise, our estimates for block of inactivated channels obtained using a depolarizing prepulse indicates approximately 2-fold higher sensitivity of inactivated heart channels to block by *R*-mexiletine. These findings contrast with a recent report comparing lidocaine sensitivity for resting brain and heart sodium channels, which indicated that there is little intrinsic difference in sensitivity of the isoforms to block of resting channels by lidocaine (but see Wang et al., 1996; Wright et al., 1997). Preferential block of heart sodium channels likely contributes to the selective therapeutic effect of mexiletine on cardiac arrhythmias.

Interactions with rbF1764/rhF1762 Are Responsible for the Difference in Affinity for Brain and Heart Sodium Channels. The greater affinity of heart sodium channels for block by *R*-mexiletine was largely abolished by the mutations F1764A and F1762A. The affinity of the inactivated states of these two mutants was not markedly different (K_I = 157 and 151 μM, respectively), and IC_{50} values at different potentials also were similar for these two mutants. These results suggest that interactions with this key phenylalanine could account for the higher affinity of heart sodium channels for *R*-mexiletine.

Homologous Mutations F1764A and F1762A Affect *R*-Mexiletine Block in Different Ways. In spite of the similarities in steady-state block of the mutant brain and heart channels, the mutations at F1764/1762 disrupted *R*-mexiletine block in strikingly different ways. Mutation F1764A of the brain channel had a pronounced effect on the rate of association of the drug with the channel, but there

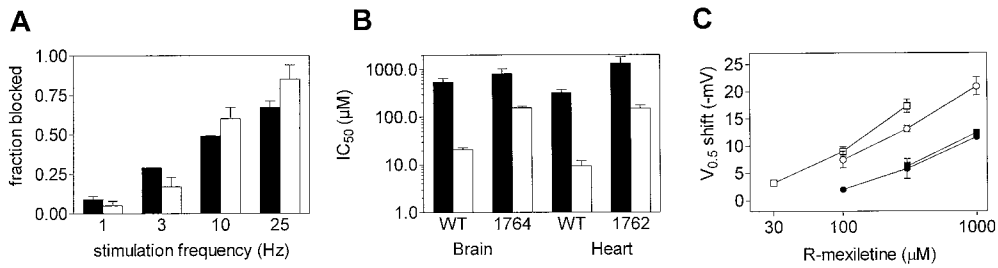

Fig. 5. Comparison of mexiletine block of brain and heart sodium channels. A, cells expressing rbWT or rhWT sodium channels were stimulated with trains of 15 depolarizations (10-ms duration) to 0 mV (brain, filled bars) or −20 mV (heart, open bars) at the indicated frequencies in the presence of 100 μM mexiletine. The fraction of unblocked current was determined as the peak current evoked by pulse 15 as a fraction of the peak current evoked by pulse 1 (the tonically blocked channel) for each stimulus frequency. The fraction of blocked current (1-fraction of unblocked current) was plotted as a function of stimulus frequency. Frequency-dependent block in control solutions was <5% at the frequencies tested. B, comparison of IC_{50} values for block of resting channels (filled bars) and of inactivated channels (open bars), for the indicated sodium channel types. C, hyperpolarizing shifts of steady-state inactivation curves in response to *R*-mexiletine. Steady-state inactivation of sodium channels was investigated using 1-s prepulses to a variable potential, followed by a test pulse to 0 mV (for rb channels) or −20 mV (for rh channels). The control curve was completed and curves then were obtained in progressively increasing *R*-mexiletine concentrations. The shift in the voltage of half-inactivation at a given concentration was greater for WT heart channels (□) than for WT brain channels (○). In contrast, in rhF1762A (■) and rbF1764A channels (●), a given concentration of *R*-mexiletine induced comparable shifts.

was little effect of the mutation on drug dissociation. In contrast, mutation of the analogous amino acid in the heart sodium channel accelerated drug dissociation with little effect on the rate of drug binding. The simplest interpretation of these findings is that in the brain channel, F1764 facilitates rapid drug binding. In contrast, in the WT heart channel, F1762 stabilizes the drug in its binding site but plays little role in association. Thus, these findings suggest that the conserved F1764/1762 residues in transmembrane segment IVS6 play fundamentally different roles in mexiletine binding in the brain and heart sodium channel backgrounds.

Common and Divergent Effects of IVS6 Mutations on Block of Brain Sodium Channels by *R*-Mexiletine and Other Local Anesthetic/Antiarrhythmic Compounds.

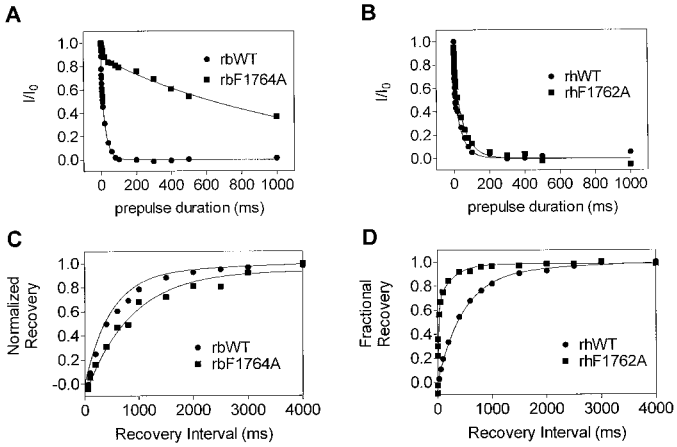

Fig. 6. Kinetics of onset and recovery from use-dependent block for WT and mutant channels. A, the time course of onset of *R*-mexiletine block during depolarization was studied using a 0-mV prepulse of variable duration followed by a test pulse to the same potential. A 45-ms interval was present between prepulse and test pulse to allow drug-free channels to recover from fast inactivation. Peak test pulse current in the presence 300 μM *R*-mexiletine was normalized to control values obtained using the same protocol and plotted versus prepulse duration. The resulting data points were fit with the sum of two exponentials with WT time constants (τ) of 1.0 ms and 25 ms with the fraction slow = 0.5; for F1764A, τ = 11 and 1140 ms, with fraction slow = 0.89. This slower time constant represents a minimum value attributable to the failure to reach steady state at the longest depolarization used (1000 ms). The baseline of the fits was subtracted from both the data and the fit for each construct. The resulting values then were normalized with respect to the first data points to compare the kinetics of development of block for rbWT (●) and rbF1764A (■). B, onset of *R*-mexiletine block of rhWT (●) and rhF1762A (■) channels studied as in A, except that prepulses and test pulses were to −20 mV. The effect of mutant rbF1764A on the onset of block was much stronger than was the effect of the equivalent heart mutant, rhF1762A. C, inhibition by 300 μM mexiletine was generated by applying a 10-Hz conditioning train of 10 pulses to 0 mV. The membrane potential then was returned to −100 mV, and a test pulse to 0 mV was applied after a recovery interval of variable duration. The rates of recovery for rbWT (●) and rbF1764A (■) were generated by plotting the peak test pulse current normalized to its value after 5000-ms recovery as a function of recovery interval. The data were fitted by double-exponential functions, where the faster time constant reflected the recovery from inactivation of unblocked channels, and the slower time reflected the recovery from block by mexiletine. The fast component of recovery was subtracted from the data, and the normalized slow component of recovery representing drug dissociation was plotted. The mean values obtained from the fits were for WT (mean ± S.E.): fraction fast (FF) = 0.36 ± 0.02 ms, τ fast (τ_f) = 15 ± 2 ms, τ slow (τ_s) = 530 ± 15 ms; for F1764A: FF = 0.91 ± .03, τ_f = 3.3 ± 0.4 ms, τ_s = 540 ± 157 ms. Recovery time courses for rhWT (●) and rhF1762A (■) sodium channels using the protocol in of C, except that conditioning and test pulses were to −20 mV and the holding and recovery potentials were −120 mV. The mean values obtained from the fits were for rhWT: FF = 0.32 ± 0.02, τ_f = 26 ± 8 ms, τ_s = 671 ± 71 ms; for rhF1762A: FF = 0.63 ± 0.05, τ_f = 6 ± 0.5 ms, τ_s = 170 ± 7 ms.

Each point mutation in transmembrane segment IVS6 that was known to reduce block by other local anesthetic, anticonvulsant, and antiarrhythmic drugs also reduced frequency-dependent *R*-mexiletine block of rbIIA sodium channels (Ragsdale et al., 1994; Qu et al., 1995b). Frequency- and voltage-dependent block of brain sodium channels by etidocaine is reduced in mutants F1764A and Y1771A, and F1764 is more important for binding than is Y1771 (Ragsdale et al., 1994). Although these two mutations also reduced the affinity for *R*-mexiletine, Y1771A reduced *R*-mexiletine block more profoundly than did F1764A. Similarly, Ragsdale et al. (1996) found that effects of a range of sodium channel inhibitors on mutants F1764A and Y1771A were all reduced compared with WT channels, but the rank order of potencies for frequency-dependent block was different for the compounds tested. Block by lidocaine was reduced more by mutant F1764A than by mutant Y1771A, in contrast to our results with mexiletine, for which binding is more affected in Y1771A. Thus, sodium channel modulators of subtly different chemical structure may interact in different ways with the local anesthetic receptor site in segment IVS6 of the sodium channel.

Overall this study demonstrates that mexiletine has higher affinity for binding to the local anesthetic receptor site of heart versus brain sodium channel subtypes. Mutations of equivalent amino acids have strikingly different effects on the kinetics of inhibition in these channel subtypes. These findings suggest differing roles for equivalent amino acids in the brain and heart channel subtypes and suggest that mexiletine, and perhaps other local anesthetics, interacts with brain and heart sodium channels in fundamentally different ways. They also highlight the importance of careful kinetic analysis in examining the role of specific amino acid residues in drug block. The differences in affinity and mechanisms of drug interaction provide a rationale for the development of tissue-specific sodium channel blockers with defined kinetic properties.

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