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Block of Human Heart hH1 Sodium Channels by the Enantiomers of Bupivacaine

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Background: **S(−)-bupivacaine reportedly exhibits lower cardiotoxicity but similar local anesthetic potency compared with *R*(+)-bupivacaine. The bupivacaine binding site in human heart (hH1) Na^+ channels has not been studied to date. The authors investigated the interaction of bupivacaine enantiomers with hH1 Na^+ channels, assessed the contribution of putatively relevant residues to binding, and compared the intrinsic affinities to another isoform, the rat skeletal muscle (μ1) Na^+ channel.**

Methods: **Human heart and μ1 Na^+ channel α subunits were transiently expressed in HEK293t cells and investigated during whole cell voltage-clamp conditions. Using site-directed mutagenesis, the authors created point mutations at positions hH1-F1760, hH1-N1765, hH1-Y1767, and hH1-N406 by introducing the positively charged lysine (K) or the negatively charged aspartic acid (D) and studied their influence on state-dependent block by bupivacaine enantiomers.**

Results: **Inactivated hH1 Na^+ channels displayed a weak stereoselectivity with a stereopotency ratio (+/−) of 1.5. In mutations hH1-F1760K and hH1-N1765K, bupivacaine affinity of inactivated channels was reduced by ~ 20- to 40-fold, in mutation hH1-N406K by ~ sevenfold, and in mutations hH1-Y1767K and hH1-Y1767D by ~ twofold to threefold. Changes in recovery of inactivated mutant channels from block paralleled those of inactivated channel affinity. Inactivated hH1 Na^+ channels exhibited a slightly higher intrinsic affinity than μ1 Na^+ channels.**

Conclusions: **Differences in bupivacaine stereoselectivity and intrinsic affinity between hH1 and μ1 Na^+ channels are small and most likely of minor clinical relevance. Amino acid residues in positions hH1-F1760, hH1-N1765, and hH1-N406 may contribute to binding of bupivacaine enantiomers in hH1 Na^+ channels, whereas the role of hH1-Y1767 remains unclear. (Key words: Cardiotoxicity; local anesthetic binding site; point mutations.)**

LOCAL anesthetics (LAs) block voltage-gated Na^+ channels. The potency of LAs is modulated by channel state, with open and inactivated states being favored over resting states. The known side effects of LAs in the cardiovascular system are, in part, a result of the blockade of cardiac Na^+ channels. On the other hand, interaction with cardiac Na^+ channels is the feature exploited in the use of some LAs as antiarrhythmics. Bupivacaine and lidocaine represent typical examples, respectively, of a cardiotoxic LA and an antiarrhythmic LA. This divergence is a result of the slowed recovery of cardiac Na^+ channels from block by bupivacaine resulting in an accumulation of block during diastole and consequently in slowed conduction that favors reentry-induced arrhythmias, whereas recovery from block by lidocaine is faster.[1] Apparently, LAs show a slightly higher potency to block cardiac than neuronal or skeletal muscle Na^+ channels.[2] However, there is controversy about whether cardiac Na^+ channels have an intrinsic higher LA affinity or whether the different inactivation gating properties of these isoforms are responsible for the different blocking potencies of LAs.

Na^+ channels consist of a pore-forming α subunit and one or two β subunits. The α subunit is formed by four homologous domains (D1–D4), each containing six α-helical transmembrane segments (S1–S6).[3] Parts of the LA binding sites have been identified in D4-S6 as phenylalanine (F1764) and tyrosine (Y1771) of rat brain IIA Na^+ channels (rIIA),[4] residues that correspond to F1760 and Y1767 in human heart (hH1) Na^+ channels. A D4-S6 mutation in position rIIA-N1769 (hH1-N1765) was also shown to influence LA binding but might do so through indirect effects.[4] In addition to these residues in D4-S6, asparagine (N) in position N434 of D1-S6 in rat skeletal muscle Na^+ channels (μ1; hH1-N406)[5] and residues in the channel's selectivity filter[6] have been shown to participate in LA binding. All of these residues are conserved in cardiac, neuronal, and muscle Na^+ channel isoforms. However, their contribution to LA binding in hH1 Na^+ channels has not yet been studied.

Bupivacaine exists in two stereoisomeric forms. Stereoselectivity of Na^+ channels toward *R*(+)- and *S*(−)-bupivacaine enantiomers is weak,[7,8] although stereoselectivity in hH1 Na^+ channels has not yet been demonstrated. The apparently lower cardiotoxicity of *S*(−)-bupivacaine[9] together with a similar clinical LA potency of R(+)- and *S*(−)-bupivacaine,[10] however, has led to the introduction of the homochiral drug *S*(−)-bupivacaine into clinical anesthesia. The aims of this study were (1) to investigate the interaction of bupivacaine enantiomers with hH1 Na^+ channels, (2) to assess contribution of the residues hH1-F1760, hH1-N1765, hH1-Y1767, and hH1-N406 to binding in hH1 Na^+ channels, and (3) to compare the intrinsic affinities of hH1

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Na^+ channels for bupivacaine enantiomers with those of the rat skeletal muscle (μ1) Na^+ channel isoform.

Materials and Methods

Site-directed Mutagenesis and Transient Expression

Human heart cDNA[11] was obtained from Dr. Roland Kallen (University of Pennsylvania, Philadelphia, PA). Mutagenesis of the hH1 clone was performed with the Transformer Site-Directed Mutagenesis Kit (Clontech, Inc., Palo Alto, CA). Two primers (a mutagenesis primer and a restriction primer) were synthesized and used to generate the desired mutants. The restriction primer had a sequence of 5′-GGAATTCTGCAGAATTCCATCACACTGG-3′, in which the restriction site EcoRV in the polylinker region has been changed to SacI. *In vitro* synthesis was performed for 4 h, with one addition of deoxyribonucleatide 5′-triphosphates (dNTPs) and T4-DNA polymerase during the reaction. The potential mutants were identified as EcoRV-resistant plasmids and confirmed by DNA sequencing, using appropriate primers near the mutated region.

The culture of HEK293t cells and their transfection by the calcium phosphate precipitation method have been described previously.[12] Cells were grown to 50% confluence before transfection. After transfection of hH1 (5–10 μg) and reporter plasmid CD8-pih3m (1 μg) for 15 h, cells were replated in 35-mm culture dishes. Transfected cells were used within 4 days. Transfection-positive cells were identified by immunobeads (CD-8 Dynabeads; Dynal, Lake Success, NY).

Chemicals and Solutions

Bupivacaine enantiomers were gifts from Chiroscience Ltd. (Cambridge, United Kingdom) and Dr. Rune Sandberg (Astra Pain Control, Södertälje, Sweden). The drugs were dissolved in aqueous solution to give 100 mM stock solutions and stored at −20°C. Experiments were performed with an external solution containing 65 mM NaCl, 85 mM choline Cl, 2 mM $CaCl_2$, and 10 mM HEPES (adjusted to pH 7.4 with tetramethyl ammonium hydroxide [TMA-OH]), and a pipette solution containing 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES (adjusted to pH 7.2 with CsOH). The reversed Na^+ gradient was used to minimize the series resistance artifact, which is less serious with outward than with inward currents.[13] After a gigaohm seal and a whole cell voltage-clamp were established, the cells were dialyzed for a minimum of 20 min before data were acquired. Control solutions as well as test solutions were applied with a multiple-barrel perfusion system.

Electrophysiologic Technique and Data Acquisition

Na^+ currents transiently expressed in HEK293t cells were recorded at room temperature (23 ± 2°C) with the whole cell configuration of the patch clamp method.[14] Patch pipettes were pulled from borosilicate glass tubes (TW150F-3; World Precision Instruments, Sarasota, FL) and heat polished at the tip to give a resistance of 0.6–1.0 MΩ. Currents were recorded with an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA), filtered at 5 kHz, sampled at 20 kHz, and stored on the hard disk of an IBM-compatible computer. All experiments were conducted during capacitance cancellation and series resistance compensation. Voltage errors were approximately ≤ 5 mV on average after compensation. Leakage currents were subtracted by the P/4 method. Liquid junction potentials were less than 3 mV and were not corrected. The time-dependent negative shift in the midpoint potential of peak Na^+ current activation ($E_{0.5}$) and inactivation ($V_{0.5}$) between 30 and 60 min after membrane rupture is approximately 5–7 mV.[15] Corresponding data for wild-type and all mutant channels were acquired within corresponding time frames.

pCLAMP 6.0 (Axon Instruments) was used for acquisition and analysis of currents. Microcal Origin (Microcal Software, Northampton, MA) was used to perform least-squares fitting and to create figures. Data are presented as mean values ± SE or fitted value ± SE of the fit. An unpaired Student *t* test (SigmaStat, Jandel Scientific Software, San Rafael, CA) was used to evaluate the significance of changes in mean values. *P* values < 0.05 were considered statistically significant.

Results

Block of Human Heart Na^+ Channels by Bupivacaine Enantiomers

To initially assess state-dependent block of hH1 Na^+ channels by bupivacaine enantiomers, we estimated the binding affinities of resting and inactivated channels using the following pulse protocol: a 10-s conditioning prepulse ranging from −180 to −50 mV was applied to allow LA binding to reach steady state; a 100-ms interval at the holding potential of −140 mV was then inserted before the test pulse of +30 mV to allow drug-free channels to recover from fast inactivation. Block of hH1 Na^+ channels by 10 μM and 100 μM *R*(+)- or *S*(−)-bupivacaine reached a plateau at conditioning prepulses of ≤ −160 mV and ≥ −80 mV, corresponding to block of resting and inactivated channels, respectively. Therefore, we chose conditioning prepulses to −180 mV and −70 mV to estimate affinities of resting and inactivated channels in a concentration–inhibition experiment (fig. 1). The 50% inhibitory concentrations (IC_{50}) for block by *R*(+)- and *S*(−)-bupivacaine were 63.8 ± 5.5 and 76.4 ± 7.9 μM, respectively, for resting channels and 3.03 ± 0.09 and 4.45 ± 0.14 μM, respectively, for inactivated channels. The Hill coefficients were close to unity, suggesting a single binding site for bupivacaine enantiomers

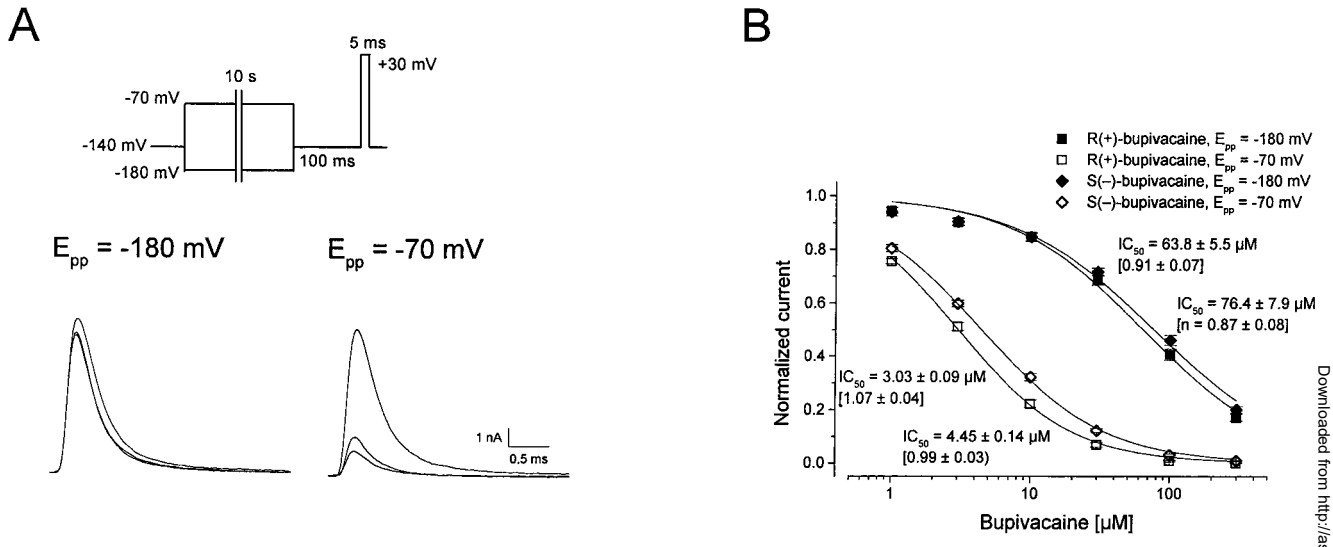


Fig. 1. State-dependent block of human heart (hH1) Na^+ channels by bupivacaine enantiomers. (*A*) hH1 wild-type Na^+ currents in control and in the presence of 10 μM *R*(+)- or *S*(−)-bupivacaine. The pulse protocol is shown in inset. (*B*) Concentration dependence of block of resting and inactivated hH1 wild-type Na^+ channels by *R*(+)-bupivacaine (squares) and *S*(−)-bupivacaine (diamonds). The same pulse protocol as described in (*A*) was used with conditioning prepulses to −180 mV for block of resting channels (solid symbols) and −70 mV for block of inactivated channels (open symbols). Pulses were delivered at 30-s intervals. The peak amplitudes of Na^+ currents were measured in different drug concentrations, normalized with respect to the peak amplitude in control, and plotted against the drug concentration. Solid lines represent fits to the data with the Hill equation. Hill coefficients are given in brackets.

in hH1 Na^+ channels. The IC_{50} values for inactivated but not for resting channels showed a statistically significant difference ($P < 0.05$), giving a ratio [*R*(+)/*S*(−)] of 1.5. These results demonstrate a similar weak stereoselectivity of bupivacaine enantiomers for inactivated hH1 Na^+ channels to that known for other Na^+ channel isoforms.[5,8,17]

Development of Block and Recovery from Block of Human Heart Na^+ Channels by Bupivacaine Enantiomers

To characterize the stereoselective block of inactivated states of hH1 Na^+ channels, we studied the time course of development and recovery from block by bupivacaine enantiomers (fig. 2). The development of block was determined by applying conditioning prepulses to −70 mV of variable duration followed by a 100-ms interval at the holding potential of −160 mV and a test pulse to +30 mV to evoke Na^+ currents (fig. 2A, inset). Block by 10 μM *R*(+)- and *S*(−)-bupivacaine at −70 mV developed with similar time constants of 1.8 ± 0.1 and 1.5 ± 0.1 s, respectively (fig. 2A; $P > 0.05$).

The recovery from block was determined by applying a test pulse to +30 mV from a holding potential of −160 mV at various times after a 10-s conditioning prepulses to −70 mV (fig. 2B, inset). Currents during control conditions and in the presence of *R*(+)- and *S*(−)-bupivacaine recovered with fast (T_1) and slow time constants (T_2). Control currents showed fast and slow time constants of 2.8 ± 0.2 ms and 0.06 ± 0.01 s, respectively. In the presence of 10 μM *R*(+)-bupivacaine, hH1 channels recovered with fast and slow time constants of 7.3 ± 1.1 ms and 2.1 ± 0.1 s, respectively, and in 10 μM *S*(−)-bupivacaine with respective values of 7.2 ± 1.1 ms and 1.3 ± 0.1 s. The fractional amplitudes of the slow phase of recovery for *R*(+)- and *S*(−)-bupivacaine were 77% and 66%, respectively, because of the slow dissociation of the drug from inactivated channels that were blocked during the conditioning prepulse. The T_2 values for *R*(+)- and *S*(−)-bupivacaine were significantly different ($P < 0.05$) and gave a ratio of 1.6. Thus, the stereoselective actions of bupivacaine enantiomers in hH1 Na^+ channels seem to be mainly based on differences in recovery kinetics.

To assess the contribution of the residues hH1-F1760, hH1-N1765, hH1-Y1767, and hH1-N406 in bupivacaine binding, we substituted these residues, one at a time, with positively charged lysine (K). This approach has been proven useful in μ1 Na^+ channels for LA binding interactions.[18]

Activation and Inactivation Kinetics of Human Heart Wild-type and Mutant Na^+ Channels

First, we briefly characterized activation and inactivation properties of hH1 wild-type and mutant Na^+ channels with standard pulse protocols (fig. 3, insets). The midpoint potentials of peak Na^+ conductance ($E_{0.5}$) for mutations hH1-F1760K and hH1-N406K showed no statistically significant difference compared with hH1 wild-type channels. The $E_{0.5}$ values for hH1-N1765K and hH1-Y1767K were shifted to more positive potentials by 14.1 and 8.2 mV, respectively (fig. 3A and table 1). Regarding the steady state inactivation, only the midpoint potential of inactivation ($V_{0.5}$) for mutation hH1-N1765K showed a statistically significant difference compared with hH1 wild-type channels, with a positive shift of 17.1 mV (fig.

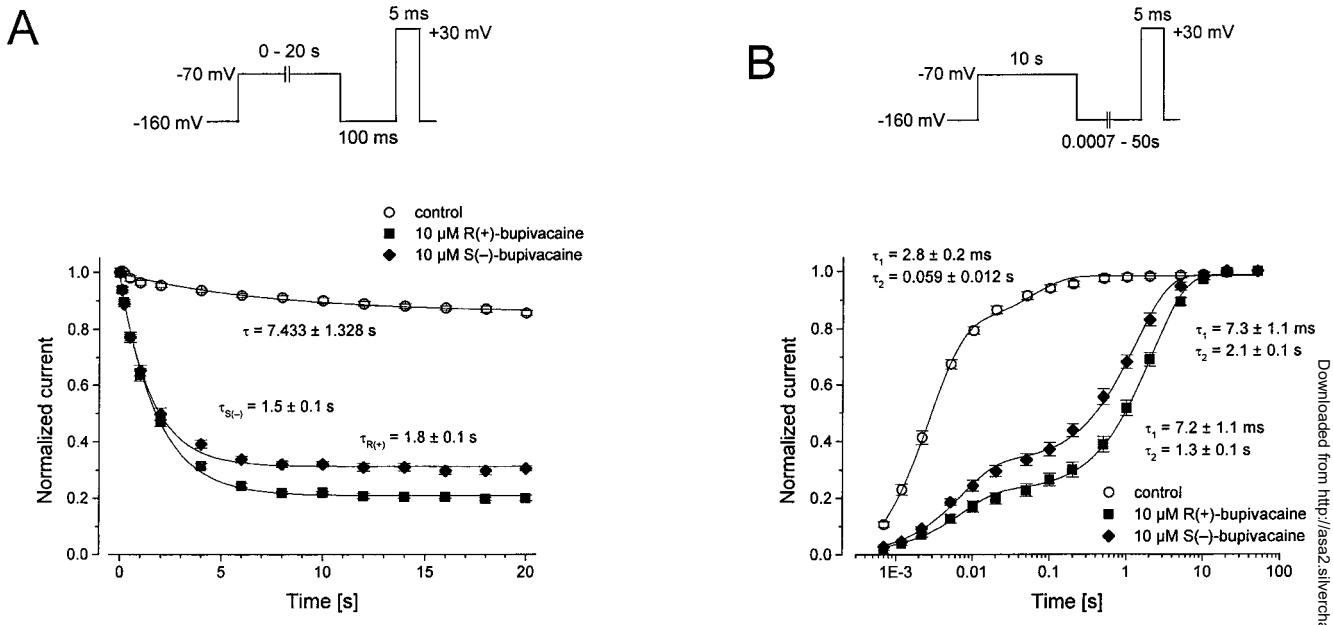


Fig. 2. Development and recovery of inactivated human heart (hH1) channels from block by bupivacaine enantiomers. (*A*) A conditioning pulse to −70 mV of variable duration was applied. A 100-ms interval at the holding potential of −160 mV was then inserted before delivery of the test pulse to +30 mV to evoke the Na^+ currents. Control currents (open circles) and currents in the presence of 10 μM *R*(+)- (filled squares) and *S*(−)-bupivacaine (filled diamonds) were normalized to the current obtained without a preceding conditioning pulse to −70 mV. Solid lines represent fits of the data to a single exponential function. (*B*) Recovery from block of inactivated hH1 Na^+ channels by bupivacaine enantiomers. Cells were conditioned with a 10-s depolarizing pulse to −70 mV from a holding potential of −160 mV. Recovery was determined by applying a test pulse to +30 mV at various times after the conditioning pulse. The data were normalized to the amplitude of the test pulse obtained after 50-s recovery time. The data were best fitted by the sum of two exponentials. The control data (open circles) for experiments with *R*(+)- and *S*(−)-bupivacaine were combined. hH1 wild-type currents in the absence of drugs (open squares) recoverd with fast (T1) and slow time constants (T2) of 2.8 ± 0.2 ms and 0.06 ± 0.01 s, respectively. Inactivated hH1 wild-type channels recovered from block by 10 μM *R*(+)-bupivacaine (filled squares; n = 5) with T1 = 7.3 ± 1.1 ms and T2 = 2.1 ± 0.1 s and from block by *S*(−)-bupivacaine (filled diamonds; n = 5) with T1 = 7.2 ± 1.1 ms and T2 = 1.3 ± 0.1 s.

3B and table 1). This mutation showed a small noninactivating current component up to conditioning pulses of −15 mV. These results demonstrate that the mutant channels remained functional, and most of them showed only slight alterations in gating properties.

State-dependent Block of Human Heart Wild-type and Mutant Na^+ Channels by Bupivacaine Enantiomers

We compared state-dependent block of hH1 wild-type and mutant Na^+ channels by bupivacaine enantiomers at a concentration of 100 μM as described in figure 4. As shown in figures 4A and 4B, block of hH1 wild-type Na^+ channels by 100 μM *R*(+)- or *S*(−)-bupivacaine reached a plateau at conditioning prepulses of ≤ −160 mV and ≥ −90 mV, corresponding to block of resting and inactivated channels, respectively. In mutations hH1-F1760K and hH1-N1765K, block of resting channels (E_{pp} ≤ −160 mV) was significantly reduced compared with hH1 wild-type channels. More importantly, high-affinity binding to inactivated channels (E_{pp} ≥ −90 mV) was virtually eliminated. Interestingly, in both mutations these reductions in affinities were greater for *S*(−)-bupivacaine, resulting in an increased stereoselectivity that remained constant at all conditioning prepulse potentials.

In mutation hH1-Y1767K, prepulses more positive than −160 mV began to induce slow inactivation, resulting in a decrease in peak currents in control solution. However, block of resting channels by bupivacaine enantiomers (E_{pp} ≤ −170 mV) was nearly unchanged, and block of inactivated channels (E_{pp} ≥ −100 mV) was only slightly decreased compared with hH1 wild-type channels. As in wild-type and hH1-Y1767K channels, block of hH1-N406K channels by bupivacaine enantiomers reached plateaus at negative potentials (E_{pp} ≤ −140 mV) and less negative potentials (E_{pp} ≤ −80 mV). However, block of resting and inactivated channels were significantly reduced compared with hH1 wild-type channels and more so for *S*(−)- than for *R*(+)-bupivacaine, resulting in an increased stereoselectivity.

The IC_{50} values estimated from these data for bupivacaine block at −180 mV and −70 mV are given in table 2.

Recovery from Block of Inactivated Human Heart Mutant Na^+ Channels by Bupivacaine Enantiomers

To study the blocking kinetics, we compared recovery from block of inactivated channels by 100 μM *R*(+)- and *S*(−)-bupivacaine (fig. 5). The fast and slow time constants of recovery (T_1 and T_2) for wild-type and mutant

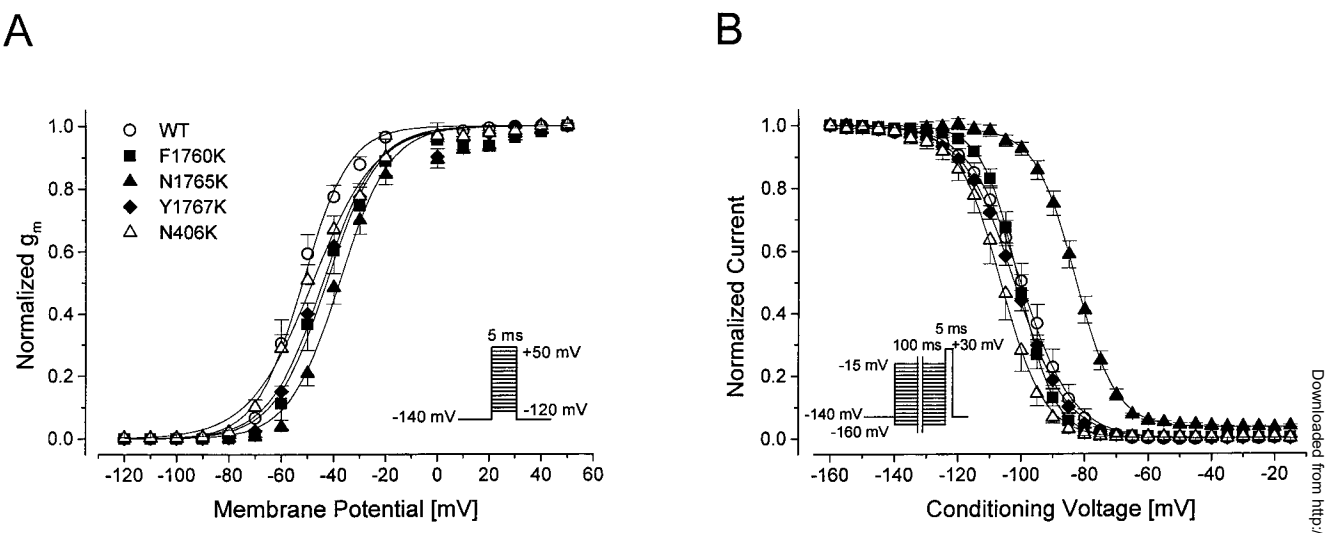


Fig. 3. Activation and inactivation kinetics of human heart (hH1) wild-type and mutant Na^+ channels. (*A*) Wild-type and mutant Na^+ currents were evoked by 5-ms pulses ranging from −120 mV to +50 mV (see inset). Holding potential was −140 mV. Pulses were delivered at 20-s intervals. Holding potential was −140 mV. Normalized membrane conductance (g_m) was calculated at a given voltage step from the equation $g_m = I_{Na}/(E_m - E_{Na})$, where I_{Na} is the current, E_m is the amplitude of the voltage step, and E_{Na} is the reversal potential of the Na current. The data were fitted with a Boltzmann function $(1/[1 + \exp((V_{0.5} - V)/k_E)])$. The average midpoint voltage ($E_{0.5}$) and slope factors (k_E) are given in table 1. (*B*) Voltage dependence of fast inactivation. Wild-type and mutant Na^+ currents were evoked by a 5-ms test pulse to +30 mV after 100-ms conditioning prepulses (V_{PP}) between −160 and −15 mV in 5-mV increments (see inset). Pulses were delivered at 20-s intervals. Holding potential was −140 mV. The currents were normalized with respect to the current obtained after a prepulse to −160 mV, plotted against the prepulse potential, and fitted to a Boltzmann equation $\{1/1 + \text{epx}[(V_{PP} - V_{0.5})/k_V]\}$.

channels are given in table 3. All mutant channels showed decreased fractional amplitudes of the slow phase of recovery and accelerated slow time constants of T_2 compared with wild-type channels. These changes were the largest in mutations hH1-F1760K and hH1-N1765K, moderate in mutation hH1-N406K, and small in mutation hH1-Y1767K. The changes in T_2 values parallel those of the estimated IC_{50} values for block of inactivated states as shown in figures 6A and 6B.

Mutation hH1-Y1767K did not have profound effects on either state-dependent block or recovery of inactivated channels from block by bupivacaine enantiomers. We also examined mutation hH1-Y1767D with a negatively charged aspartic acid. This mutation showed a 1.6- and 1.4-fold reduced resting affinity and a 2.1- and 2.5-fold inactivated affinity for *R*(+)-and *S*(−)-bupivacaine, respectively (fig. 7A). Thus, affinity of inactivated channels was reduced to a similar degree as in mutation hH1-Y1767K. However, recovery from block of inactivated channels was 1.4- and 2-fold faster for the respective enantiomer than in mutation hH1-Y1767K (fig. 7B).

Comparison of Block of Human Heart and Rat Skeletal Muscle Na^+ Channels by Bupivacaine Enantiomers

To compare their intrinsic affinities, we assessed state-dependent block of μ1 wild-type Na^+ channels by bupivacaine enantiomers during identical experimental conditions as for hH1 Na^+ channels. Conditioning prepulses to −180 and −70 mV were chosen to estimate affinities of resting and inactivated channels in concentration-inhibition experiments (figs. 8A and 8B). Resulting IC_{50} values for block by *R*(+)- and *S*(−)-bupivacaine were 81.2 ± 3.4 and 80.2 ± 3.8 μM, respectively, for

Table 1. Parameters for Activation and Inactivation Kinetics of hH1 Wild-type and Mutant Na^+ Channels

	Activation			Inactivation		
	$E_{0.5}$ [mV]	k_E [mV]	n	$V_{0.5}$ [mV]	k_V [mV]	n
hH1 WT	−52.0 ± 2.5	8.3 ± 0.4	5	−100.1 ± 2.2	7.8 ± 0.1	6
F1760K	−42.6 ± 3.4	8.9 ± 0.9	6	−100.8 ± 1.3	5.4 ± 0.2*	7
N1765K	−37.9 ± 1.9*	9.1 ± 0.7	4	−83.0 ± 1.3*	6.0 ± 0.2*	5
Y1767K	−43.8 ± 1.7*	10.4 ± 0.8*	4	−102.2 ± 1.0	8.1 ± 0.4	5
N406K	−48.4 ± 2.4	11.2 ± 0.6*	8	−106.7 ± 2.2	6.1 ± 0.4*	7

The midpoint voltages of activation ($E_{0.5}$) and inactivation ($V_{0.5}$) and the corresponding slope factors K_E and k_V were determined as described in figures 3A and B.
* $P < 0.05$ compared with the wild-type.

resting channels and 4.67 ± 0.14 and 5.99 ± 0.19 μM, respectively, for inactivated channels. The Hill coefficients were close to unity. As in hH1 channels, the IC_{50} values for inactivated channels showed a small but statistically significant difference ($P < 0.05$), giving a stereopotency ratio [*R*(+)/*S*(−)] of 1.3.

It is known that hH1 wild-type channels activate and inactivate at more negative potentials than μ1 wild-type channels. The midpoint potential of inactivation $V_{0.5}$ for hH1 wild-type channels found in this study was −100.1 ± 2.2 mV, whereas $V_{0.5}$ for μ1 channels was previously demonstrated to be −82.1 ± 1.4 mV.[5] Differences in the voltage dependence of steady state inactivation elicit corresponding shifts in the voltage dependence of steady state block but do not affect LA affinities of resting and inactivated channels.[16] To confirm whether conditioning prepulses to −180 mV and −70 mV were appropriate for both channel isoforms to estimate affinities of resting and inactivated channels, respectively, we further assessed state-dependent block of hH1 and μ1 wild-type channels by bupivacaine enantiomers at a concentration of 10 μM by applying conditioning prepulses to various potentials ranging in amplitude from −180 to −50 mV. The results are shown in figures 8C and 8D. As predicted by Wright *et al.*,[19] transitions from block of resting to inactivated channels occur at a potential range that is approximately 30 mV more negative for hH1 than for μ1 wild-type channels. Coexpression of the β1 subunit produces shifts in steady state inactivation of Na^+ channel isoforms, yet this β1 addition does not affect LA affinity of resting and inactivated channels. We therefore did not include the β1 subunit in this study. At −180 and −70 mV, however, block of both isoforms approached a plateau, so that these prepulses are deemed appropriate to compare resting and inactivated affinities. Moreover, because both channels showed a small degree of slow inactivation at −70 mV, a different contribution of slow inactivated channels to block between the isoforms is not to be expected. Surprisingly, with our approach we could indeed detect slight differences in block of resting and inactivated channels by bupivacaine enantiomers, with hH1 wild-type channels showing higher affinities. The estimated IC_{50} values gave affinity ratios (hH1/μ1) of 1.3 ($P = 0.025$) and 1.0 ($P = 0.7$) for block of resting channels and of 1.5 ($P < 0.001$) and 1.3 ($P < 0.001$) for block of inactivated channels by *R*(+)- and *S*(−)-bupivacaine, respectively.

Discussion

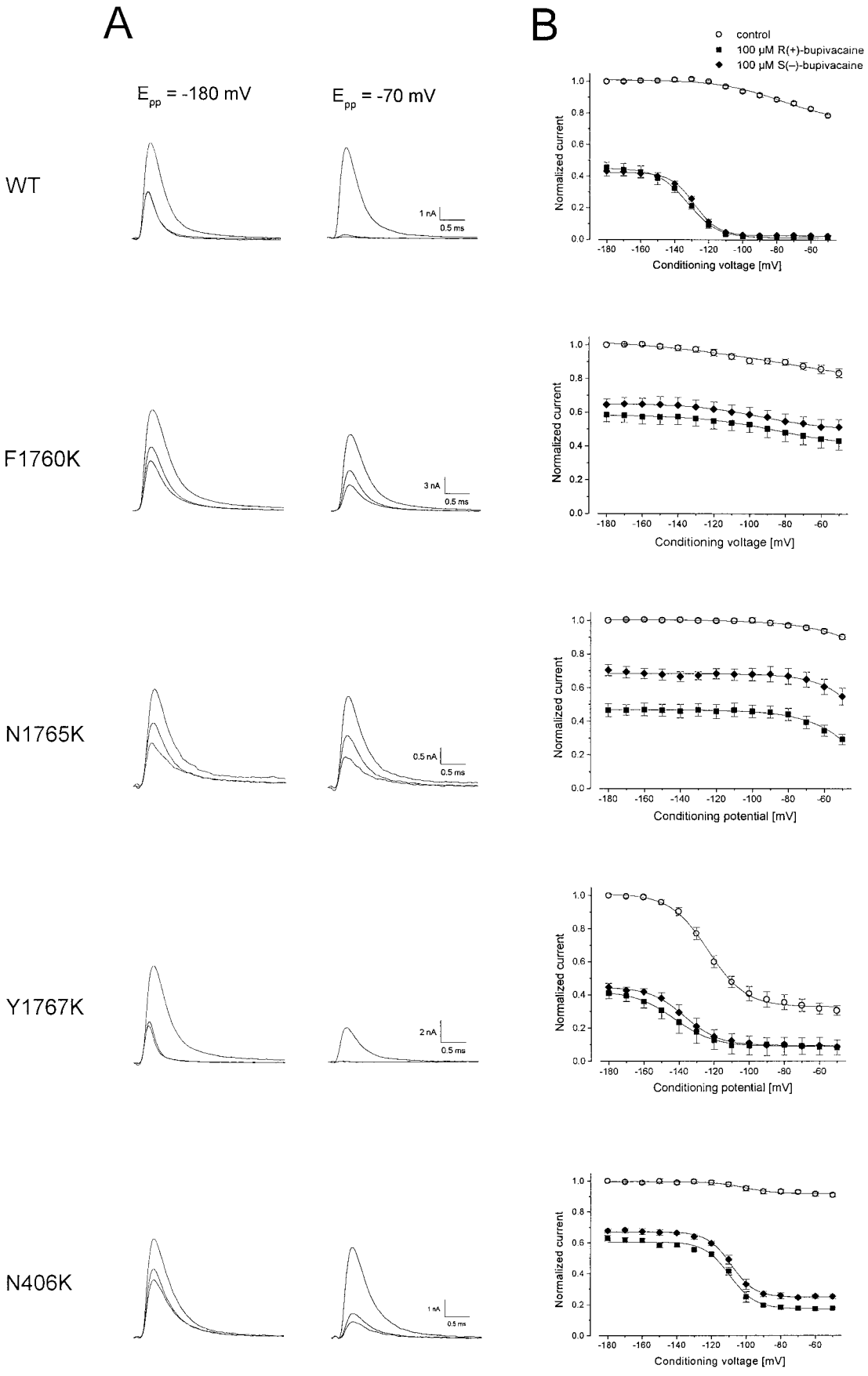
In this study we showed that (1) hH1 Na^+ channels expressed in HEK293t cells display a weak stereoselectivity for block by bupivacaine enantiomers with a stereopotency ratio (+/−) of 1.5 for the inactivated state. Block of resting channels is not stereoselective. (2) Mutants with lysine point mutations at positions putatively contributing or near the LA binding site in D4-S6 and D1-S6 remained functional. Mutations hH1-F1760K, hH1-N1765K, and hH1-N406K showed a twofold to threefold reduction in affinity of resting channels, and mutations hH1-F1760K and hH1-N1765K a 20- to 40-fold and mutation hH1-N406K a sevenfold reduction in affinity of inactivated channels. In mutations hH1-Y1767K, resting channel affinity was unchanged, and inactivated channel affinity was reduced by about threefold; in mutation hH1-Y1767D, resting channel affinity was reduced by approximately 1.5-fold, and inactivated channel affinity was reduced by approximately twofold. (3) Changes in recovery of inactivated mutant channels from block by bupivacaine enantiomers paralleled those of inactivated-channel affinity. (4) Inactivated hH1 Na^+ channels exhibited a slightly higher intrinsic affinity for bupivacaine enantiomers than inactivated μ1 isoforms.

Stereoselectivity of Human Heart Na^+ Channels Toward Bupivacaine Enantiomers

The weak stereoselectivity found in hH1 is similar to the reported weak stereoselectivity of other Na^+ channel isoforms for bupivacaine enantiomers. The stereopotency ratio [*R*(+)/*S*(−)] of 1.5 for the inactivated state is in good agreement with results from μ1 Na^+ channels (1.4)[5] and Na^+ channels in guinea pig ventricular myocytes (1.7)[8] and in GH_3 cells (1.6).[17]

Amino Acid Residues of Human Heart Na^+ Channels Contributing to Binding of Bupivacaine Enantiomers

At the molecular level, our data support the importance of the amino acid phenylalanine (F) in position F1760 in the interaction of hH1 Na^+ channels with bupivacaine enantiomers. It was proposed that aromatic phenylalanine interacts with the positively charged moiety of tertiary amine LAs through an electrostatic attraction between the quadrupole of an aromatic group and a positive charge.[4,20] Another study suggested that F1760 interacts with LA in two different modes depending on the channel conformation: for the resting state, hydrophobic interactions were more important, whereas for the inactivated state π electron–cation interactions were dominant.[21] Our data appear to agree with these interpretations. Substitution of phenylalanine by the positively charged residue lysine leads to an electrostatic repulsion of the positively charged moiety of bupivacaine enantiomers and consequently to a decrease in affinity that is more pronounced for inactivated than for resting channels. Moreover, recovery of inactivated hH1-F1760K channels from block by *R*(+)- and *S*(−)-bupivacaine was accelerated by 12- and 7-fold, respectively, compared with wild-type channels. Thus, the interaction of F1760 with the positively charged moiety of bupivacaine might be predominant for the "fast-in-slow-out"



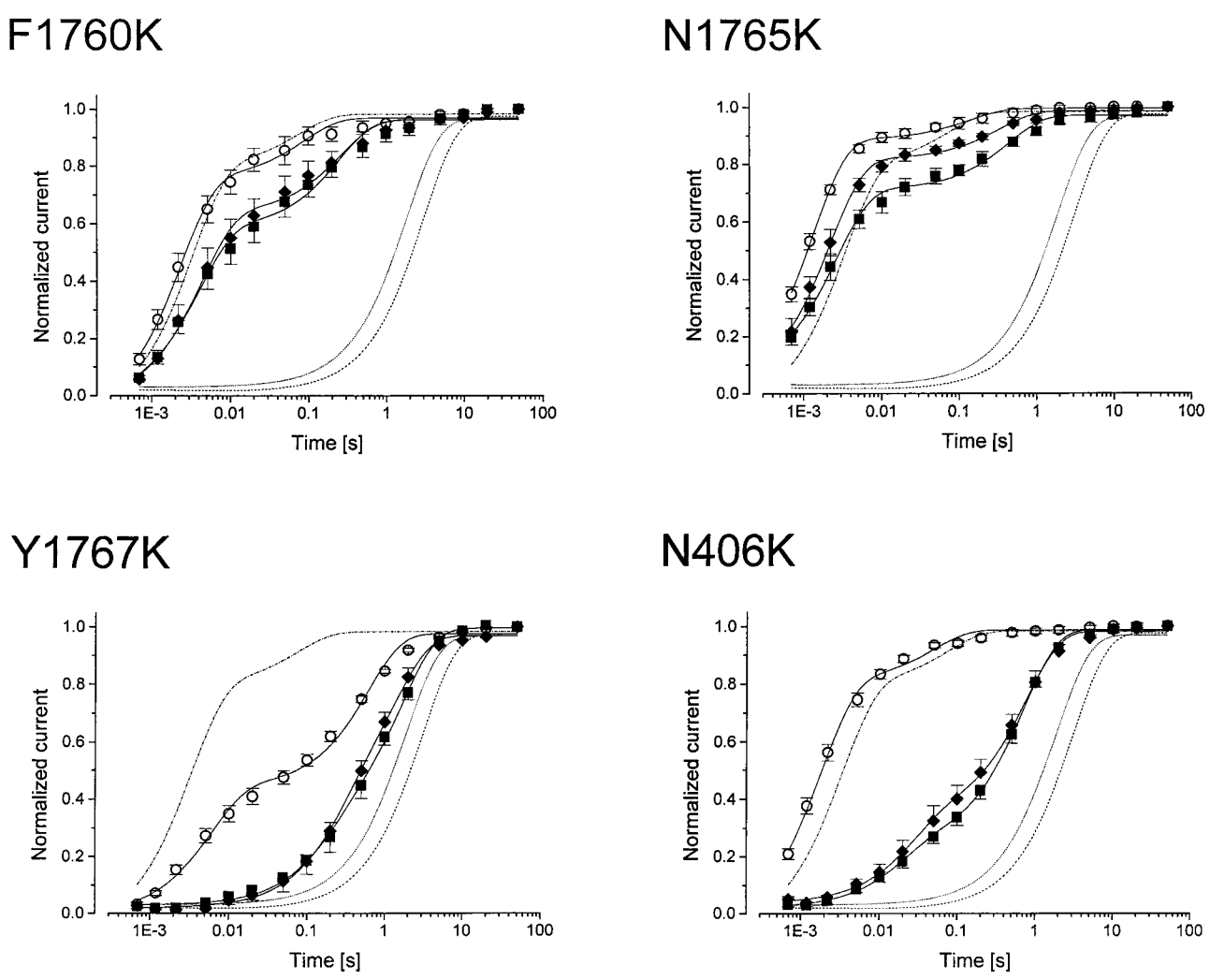


Fig. 5. Recovery from block of inactivated human heart (hH1) mutant Na^+ channels by *R*(+)- (filled squares) and *S*(−)-bupivacaine (filled diamonds) at a concentration of 100 μM. Cells were conditioned with a 10-s depolarizing pulse to −70 mV from a holding potential of −160 mV. Recovery was determined by applying a test pulse to +30 mV at various times after the conditioning pulse. The data were normalized to the amplitude of the test pulse obtained after 50-s recovery time. The data were best fitted by the sum of two exponentials. The control data (open circles) for experiments with *R*(+)- and *S*(−)-bupivacaine were combined. The data for recovery of hH1 wild-type currents from block by 100 μM *R*(+)- (dashed lines) and *S*(−)-bupivacaine (dotted lines) are given for comparison. For fast and slow time constants of recovery (T1 and T2) for wild-type and mutant channels, see table 3.

block of Na^+ channels by bupivacaine, which was proposed to be one of the responsible mechanisms for the severe and extremely difficult-to-reverse cardiac conduction depression by bupivacaine.[1]

Similar to hH1-F1760 in D4-S6, position hH1-N406 in D1-S6 was previously proposed to interact directly with the positively charged moiety of LAs, at least in inactivated channels, suggesting a domain-interface binding site for LAs between D4-S6 and D1-S6.[5,22] This proposal was based on data obtained from μ1 Na^+ channels. Results in the present study obtained with mutation hH1-N406K are consistent with an electrostatic repulsion of the positively charged moiety of bupivacaine enantiomers by the lysine substitution at position hH1-

Fig. 4. State-dependent block of human heart (hH1) mutant and wild-type Na^+ channels by bupivacaine enantiomers. (*A*) Mutant and wild-type Na^+ currents in control (larger currents in each set of traces) and in the presence of 10 μM *R*(+)- and *S*(−)-bupivacaine (smaller currents in each set of traces). The same pulse protocol as described in figure 1A was used with conditioning prepulses to −180 mV for block of resting channels (E_{PP} = −180 mV, *left column*) and −70 mV for block of inactivated channels (E_{PP} = −70 mV, *right column*). (*B*) Normalized hH1 wild-type and mutant Na^+ currents as a function of conditioning prepulse potential. Ten-second conditioning prepulses ranging in amplitude from −180 to −50 mV were applied; 100-ms intervals at the holding potential of −140 mV were then inserted before delivery of the test pulses to +30 mV to evoke the Na^+ currents. Pulses were delivered at 30-s intervals. Control currents (open circles) were normalized to the current obtained with a prepulse to −180 mV. The control data for experiments with *R*(+)- (filled squares) and *S*(−)-bupivacaine (filled diamonds) were combined. Currents obtained in the presence of 100 μM *R*(+)- or *S*(−)-bupivacaine were normalized to the current obtained in control with the corresponding prepulse potential. Solid lines represent fits of the data to a Boltzmann function.

Table 2. Estimated Values of 50% Inhibitory Concentrations (IC_{50}) for Block of hH1 Wild-type and Mutant Na^+ Channels by Bupivacaine Enantiomers

	$IC_{50,\ R}$ (μM) R(+)-bupivacaine	n	$IC_{50,\ I}$ (μM) R(+)-bupivacaine	n	$IC_{50,\ R}$ (μM) S(−)-bupivacaine	n	$IC_{50,\ I}$ (μM) S(−)-bupivacaine	n
hH1 WT	63.8 ± 5.5	4	3.03 ± 0.09	4	76.4 ± 7.9	4	4.45 ± 0.14	4
F1760K	152.6 ± 29.8*	5	95.1 ± 22.4*	5	184.9 ± 33.6*	4	121.4 ± 22.5*	4
N1765K	90.5 ± 15.5	4	67.3 ± 11.3*	4	253.9 ± 49.4*	4	202.8 ± 47.7*	4
Y1767K	71.6 ± 10.4	4	13.6 ± 6.3	4	91.3 ± 18.3	4	11.0 ± 6.8	4
N406K	172.9 ± 15.1*	4	21.5 ± 1.3*	4	209.9 ± 12.6*	4	32.4 ± 1.6*	4

The IC_{50} values for block of wild-type channels were derived from concentration–inhibition experiments as described in figure 1B. The IC_{50} values for block of mutant channels were estimated as described in figure 6A.

* Indicates a statistically significant change in IC_{50} value compared with wild-type ($P < 0.05$).

N406 as well. Therefore, we now extend the model of the domain-interface binding site for LAs between D4-S6 and D1-S6 to hH1 Na^+ channels. However, quantitative comparison of the effects of lysine mutations in positions F1760 and N406 clearly underline the predominant role of the aromatic phenylalanine in the interaction with bupivacaine enantiomers.

More puzzling are the contribution of positions hH1-N1765K and hH1-Y1767K to bupivacaine binding as revealed in this study. Position hH1-N1765 is oriented away from the face in the α-helical D4-S6 that contains hH1-F1760 and hH1-Y1767 and thus was initially not considered to contribute directly to LA binding.[4] In the present study, however, lysine substitution at hH1-N1765 reduced resting and especially inactivated affinity for *R*(+)-bupivacaine, to a similar degree as in hH1-F1760, and even exceeded this effect over that of hH1-F1760 for *S*(−)-bupivacaine. The result is an increase in stereoselectivity with a stereopotency ratio [*R*(+)/*S*(−)] of approximately 3 for both inactivated and resting conformations. The residue at position hH1-N1765 thus also determines LA affinity. One possible way to do so is that the lysine mutation at hH1-N1765 (and possibly also at hH1-F1760) hinders the LA site from entering the high-affinity conformation without preventing inactivation of the channel protein. Clearly, further studies are needed to show whether the underlying mechanisms are of a direct or indirect allosteric nature.

Mutation hH1-Y1767K caused only a slight reduction in affinity of inactivated channels for bupivacaine enantiomers. If tyrosine at this position in wild-type channels indeed interacts with the aromatic moiety of LA through aromatic–aromatic or hydrophobic interactions, as suggested previously,[4] a substituted lysine could do so through cation-π electron interactions, resulting in a comparable affinity with wild-type channels. However, a substituted negatively charged residue should decrease LA affinity through electrostatic repulsion. Contrary to this expectation, mutation hH1-Y1767D with the negatively charged aspartic acid at position hH1-Y1767, exhibited a binding affinity of inactivated channels not different from mutation hH1-Y1767K. However, recovery from block of inactivated channels hH1-Y1767D was 1.4-fold and 2-fold faster for *R*(+)- and *S*(−)-bupivacaine than in mutation hH1-Y1767K. Together, these results seem to conflict with an early report based on alanine mutations that considers position hH1-Y1767 as a direct participant in LA binding[4] but is consonant with recent observations based on mutations of different size, hydrophobicity, and aromaticity that questions a direct contribution of this position to LA binding.[22] Thus, the where

Table 3. Recovery from Block of Inactivated Channels

	Control		100 μM R(+)		100 μM S(−)	
	τ_1 [ms]	τ_2 [s]	τ_1 [ms]	τ_2 [s]	τ_1 [ms]	τ_2 [s]
hH1 WT	3.2 ± 0.3	0.065 ± 0.016	—	3.07 ± 0.09	—	1.84 ± 0.07
	[0.85 ± 0.03]	[0.20 ± 0.03]		[0.96 ± 0.01]		[0.93 ± 0.01]
F1760K	2.4 ± 0.3	0.089 ± 0.028	4.0 ± 0.8	0.251 ± 0.058*	4.4 ± 0.7	0.271 ± 0.064*
	[0.83 ± 0.05]	[0.22 ± 0.03]	[0.60 ± 0.04]	[0.25 ± 0.06]*	[0.66 ± 0.04]	[0.33 ± 0.03]*
N1765K	1.3 ± 0.1*	0.142 ± 0.029*	2.7 ± 0.3	0.447 ± 0.087*	2.2 ± 0.2	0.341 ± 0.075*
	[0.90 ± 0.03]	[0.11 ± 0.01]*	[0.65 ± 0.04]	[0.26 ± 0.02]*	[0.80 ± 0.03]	[0.17 ± 0.01]*
Y1767K	6.1 ± 1.0*	0.613 ± 0.070*	175 ± 37	1.70 ± 0.15*	239 ± 56	1.43 ± 0.21*
	[0.44 ± 0.03]*	[0.54 ± 0.02]*	[0.26 ± 0.04]	[0.71 ± 0.04]*	[0.35 ± 0.08]	[0.60 ± 0.08]*
N406K	1.8 ± 0.2*	0.050 ± 0.014	19 ± 3	0.711 ± 0.025*	26 ± 4	0.767 ± 0.048*
	[0.86 ± 0.04]	[0.18 ± 0.02]	[0.22 ± 0.01]	[0.74 ± 0.01]*	[0.30 ± 0.02]	[0.65 ± 0.02]*

The fast and slow time constants of recovery (τ_1 and τ_2) were determined as described in figure 5. The fractional amplitudes of the fast and slow phase of recovery are given in brackets. Because a large fraction of inactivated hH1 channels are blocked by either 100 μM R(+)- or S(−)-bupivacaine (> 90%), no reliable τ_1 could be estimated.

* $P < 0.05$ compared with the wild-type.

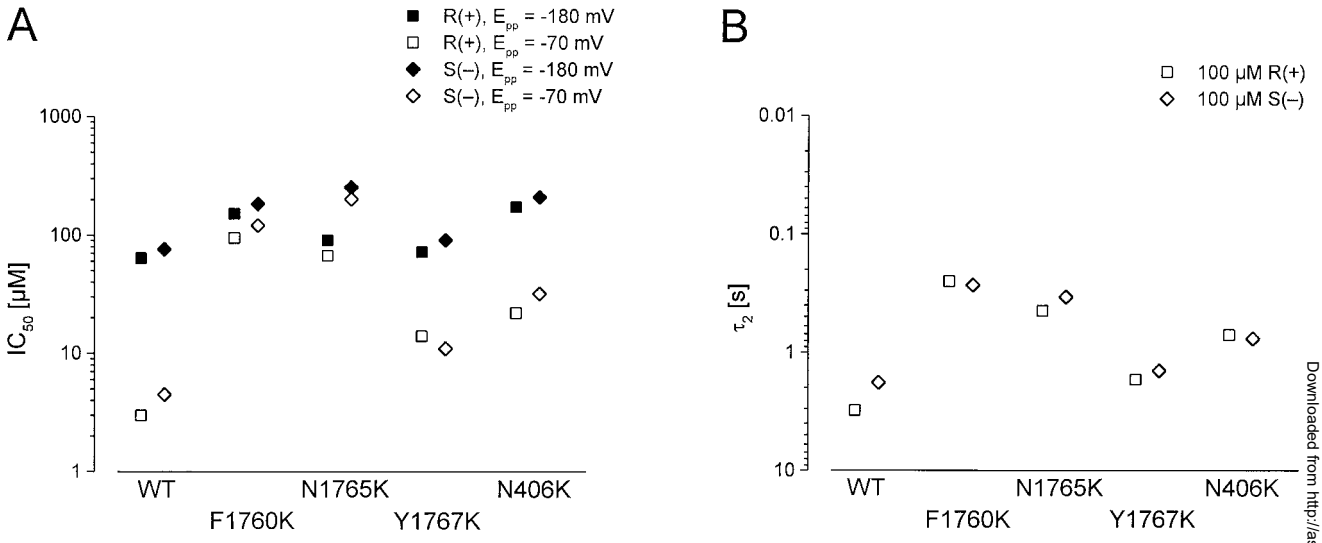


Fig. 6. Half-maximum inhibiting concentrations (IC_{50}) and recovery from block by bupivacaine enantiomers for human heart (hH1) wild-type and mutant channels. (*A*) The IC_{50} values for block of wild-type channels were derived from concentration-inhibition experiments as described in figure 1B. The IC_{50} values for block of mutant channels were estimated from fractions of block (f_B) at −180 and −70 mV as obtained in the experiments shown in figure 4B using the equation $f_B = [LA]/([LA] + IC_{50})$. IC_{50} values are also given in table 3. (*B*) Slow time constants of recovery T2 were obtained as described in figure 5. T2 values are also given in table 2.

abouts of the hydrophobic binding domain in the Na^+ channel for LAs remain unresolved.

Intrinsic Affinities of Human Heart and Rat Skeletal Muscle Na^+ Channels for Bupivacaine Enantiomers

There is a striking difference in clinically effective concentrations of lidocaine required for treatment of arrhythmias (10^{-5} M) and to achieve nerve impulse blockade (10^{-3} M). Unequivocally, one reason for this phenomenon is that lidocaine binds with high affinity to the inactivated states of Na^+ channels, a predominant state of cardiac Na^+ channels as the duration of ventricular action potential is long (hundreds of milliseconds) compared with action potentials in nerve and skeletal muscle (few milliseconds). Molecular cloning strategies and heterologous expression offer the chance to study the intrinsic LA affinities of recombinant Na^+ channel isoforms during identical conditions and to determine unambiguously whether a higher intrinsic affinity of cardiac Na^+ channels contributes to preferential block of cardiac Na^+ channels by lidocaine. However, the intrinsic affinity of hH1 Na^+ channels for lidocaine has been reported to be both greater[23–25] or similar[16] compared

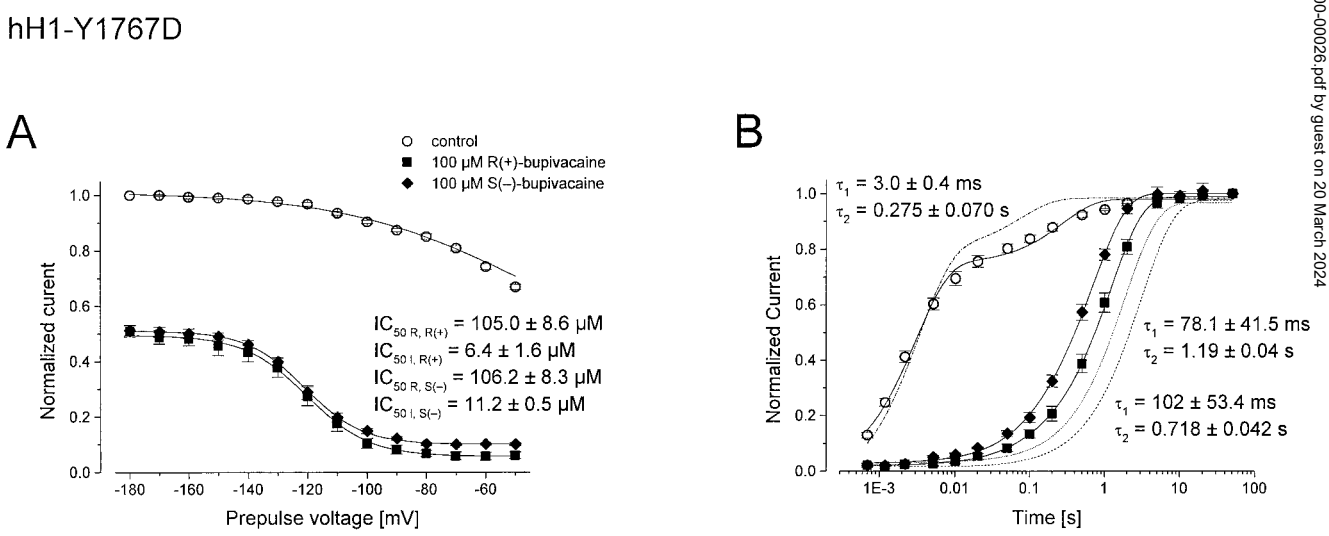


Fig. 7. State-dependent block and recovery from block of mutation hH1-Y1767D. (*A*) State-dependent block by 100 μM *R*(+)- and *S*(−)-bupivacaine. Data were obtained as described in figure 4B. (*B*) Recovery from block by 100 μM *R*(+)- and *S*(−)-bupivacaine. Data were obtained as described in figure 5. The data for recovery of human heart (hH1) wild-type currents from block by 100 μM *R*(+)- (dashed lines) and *S*(−)-bupivacaine (dotted lines) are given for comparison.

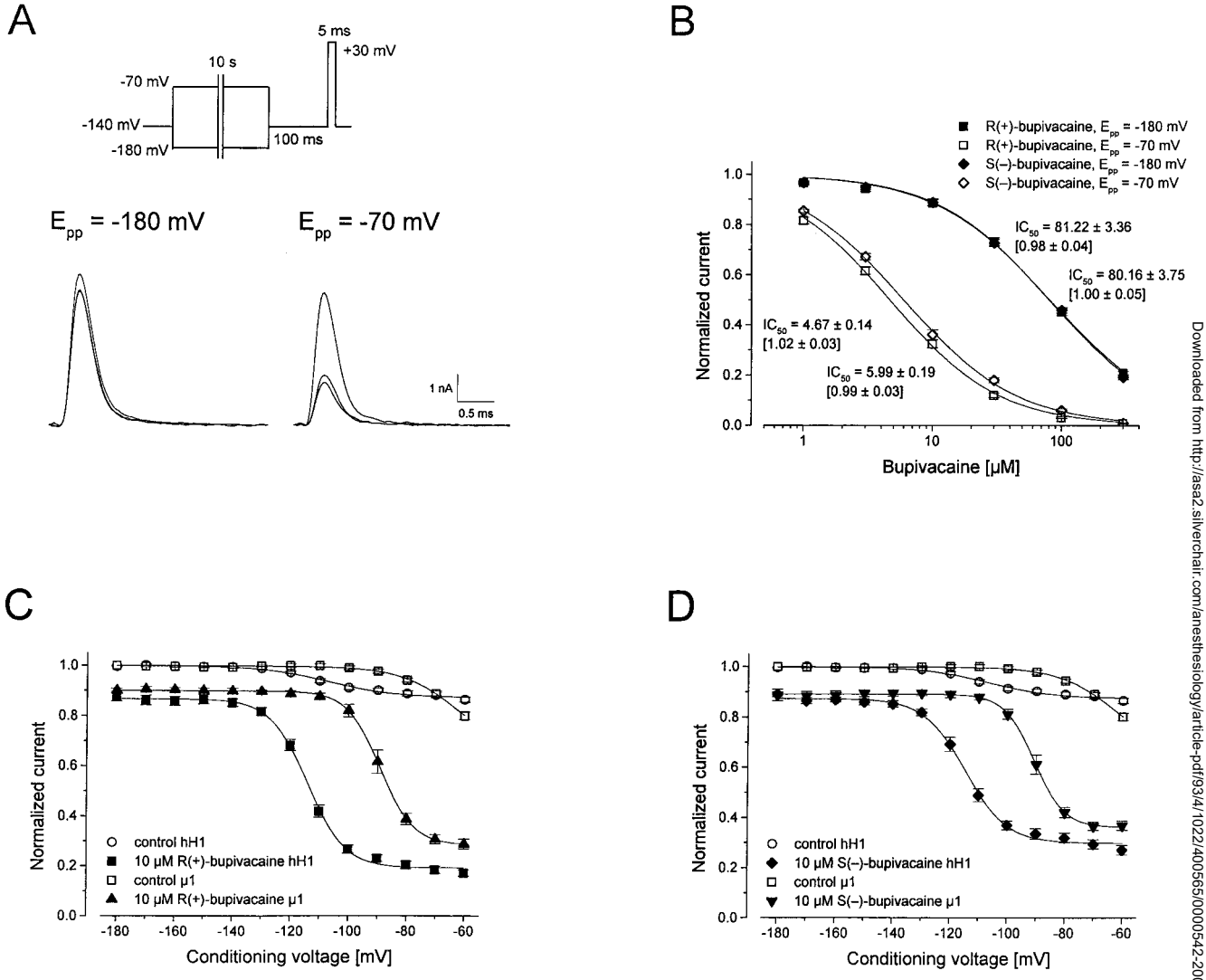


Fig. 8. State-dependent block of rat skeletal muscle (μ1) Na^+ channels by bupivacaine enantiomers. (*A*) μ1 wild-type Na^+ currents in control and in the presence of 10 μM *R*(+)- or *S*(−)-bupivacaine. Data were obtained as described in figure 1A. (*B*) Concentration dependence of block of resting (solid symbols) and inactivated (open symbols) μ1 wild-type Na^+ channels by *R*(+)-bupivacaine (squares) and *S*(−)-bupivacaine (diamonds). Data were obtained as described in figure 1B. (*C* and *D*) Comparison of state-dependent block of human heart (hH1) and μ1 wild-type Na^+ channels by 10 μM *R*(+)- (*C*) and *S*(−)-bupivacaine (*D*). The pulse protocol as described in figure 4B was used.

with skeletal muscle isoforms. On the other hand, differences found were consistently less than an order of magnitude, and thus clinical consequences are likely to be minor.

Regarding bupivacaine, a higher intrinsic affinity of cardiac Na^+ channels, especially for *R*(+)-bupivacaine compared with *S*(−)-bupivacaine, could help to explain the higher cardiotoxicity of this enantiomer. Similarly to results with lidocaine, the differences in affinities between hH1 and μ1 Na^+ channels found in this study for block of resting channels and for block of inactivated channels by *R*(+)-and *S*(−)-bupivacaine are small, with affinity ratios (hH1/μ1) of 1.3, 1.0, 1.5, and 1.3, respectively. However, for the inactivated states they are in a direction that might help to explain a preferential block of cardiac Na^+ channels by *R*(+)-bupivacaine.

On the other hand, the small differences in LA affinities among different Na^+ channel isoforms might well have biophysical significance. As residues putatively contributing to LA binding are conserved in cardiac, neuronal, and skeletal muscle voltage-gated Na^+ channel isoforms, differences in intrinsic LA affinities must result from interaction with other structures and might point to a direct or indirect involvement of the channels' gating movement in LA binding.

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