Stereoselective Block of a Human Cardiac Potassium Channel (Kv1.5) by Bupivacaine Enantiomers

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ABSTRACT Stereoselective drug-channel interactions may help to elucidate the molecular basis of voltage-gated potassium channel block by local anesthetic drugs. We studied the effects of the enantiomers of bupivacaine on a cloned human cardiac potassium channel (hKv1.5). This channel was stably expressed in a mouse Ltk^- cell line and studied using the whole-cell configuration of the patch-clamp technique. Both enantiomers modified the time course of this delayed rectifier current. Exposure to 20 μM of either S(−)-bupivacaine or R(+)-bupivacaine did not modify the activation time constant of the current, but reduced the peak outward current and induced a subsequent exponential decline of current with time constants of 18.7 ± 1.1 and 10.0 ± 0.9 ms, respectively. Steady-state levels of block (assessed with 250-ms depolarizing pulses to +60 mV) averaged 30.8 ± 2.5% (n = 6) and 79.5 ± 3.2% (n = 6) ($p < 0.001$), for S(−)- and R(+)-bupivacaine, respectively. The concentration dependence of hKv1.5 inhibition revealed apparent K_D values of 27.3 ± 2.8 and 4.1 ± 0.7 μM for S(−)-bupivacaine and R(+)-bupivacaine, respectively, with Hill coefficients close to unity, suggesting that binding of one enantiomer molecule per channel was sufficient to block potassium permeation. Analysis of the rate constants of association (k) and dissociation (l) yielded similar values for l (24.9 s^{-1} vs. 23.6 s^{-1} for S(−)- and R(+)-bupivacaine, respectively) but different association rate constants (1.0×10^6 vs. 4.7×10^6 M^{-1} s^{-1} for S(−)- and R(+)-bupivacaine, respectively). Block induced by either enantiomer displayed a shallow voltage dependence in the voltage range positive to 0 mV, i.e., where the channel is fully open, consistent with an equivalent electrical distance δ of 0.16 ± 0.01. This suggested that at the binding site, both enantiomers of bupivacaine experienced 16% of the applied transmembrane electrical field, referenced to the inner surface. Both bupivacaine enantiomers reduced the tail current amplitude recorded on return to −40 mV and slowed their time course relative to control, resulting in a "crossover" phenomenon. These data indicate 1) the charged form of both bupivacaine enantiomers block the hKv1.5 channel after it opens, 2) binding occurs within the transmembrane electrical field, 3) unbinding is required before the channel can close, 4) block of hKv1.5 channels by bupivacaine is markedly stereoselective, with the R(+)-enantiomer being the more potent one, 5) this stereoselective block was associated with a 1.11-kcal/mol difference in binding energy between both enantiomers, and 6) the stereoselectivity derives mainly from a difference in the association rate constants, suggesting that the S(−)-enantiomer is less likely to access the binding site in an optimal configuration.

INTRODUCTION

Bupivacaine is a long-acting and potent local anesthetic agent widely used for regional anesthesia (Strichartz and Ritchie, 1987) that exhibits a high affinity for the inactivated state of Na^+ channels (Clarkson and Hondeghem, 1985). Because it contains a chiral carbon atom, the clinically used form is a racemic mixture of S(−)-bupivacaine and R(+)-bupivacaine. We have observed that inactivated state block of cardiac Na^+ channels displays a moderate stereoselectivity, with R(+)-bupivacaine being 1.6 times more potent than S(−)-bupivacaine, whereas the interactions of bupivacaine enantiomers with the rested and the activated/open states were not stereoselective (Valenzuela et al., 1993).

Local anesthetics are useful probes of ion channel function and structure. Stereoselective interactions are especially of interest because they can reveal three-dimensional relationships between drug and channel with otherwise identical biophysical properties. In addition, identification of such stereospecific interactions may have practical consequences. Indeed, in vivo studies have demonstrated that the potency and duration of the anesthesia were equal or even larger for S(−)-bupivacaine than the R(+)-enantiomer (Luduena et al., 1972; Åberg, 1972; Aps and Reynolds, 1978). More important, the LD_{50} for R(+)-bupivacaine was ~30–40% lower than for S(−)-bupivacaine (Luduena et al., 1972; Åberg, 1972). Although the higher potency of R(+)-bupivacaine to inhibit I_{Na} (Valenzuela et al., 1993) could explain in part its higher cardiotoxicity over S(−)-bupivacaine, several studies have also shown a prolongation of the Q-Tc interval of the electrocardiogram (ECG) in anesthetized dogs (Avery et al., 1984; Wheeler et al., 1988; Solomon et al., 1990) and human volunteers (Scott et al., 1989) receiving high doses of bupivacaine. In some cases, this was accompanied by *torsades de pointes* (Kasten and Martin 1985). These results suggest that the cardiotoxicity of bupivacaine also involves block of K^+ channels.

As previously described for other local anesthetic agents (Strichartz and Ritchie, 1987), racemic bupivacaine blocks

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several cardiac K^+ currents, including the transient outward current (I_{TO}) and the delayed outward K^+ current (I_K) (Castle, 1990; Sánchez-Chapula, 1988; Courtney et al., 1988). Recently, a fast activating outward potassium current has been described in rat atrial myocytes (Boyle and Nerbonne, 1991), neonatal canine ventricular myocytes (Jeck and Boyden, 1992), and human atria (Fedida et al., 1993; Wang et al., 1993). The properties of this channel are most similar to those reported for the cloned cardiac hKv1.5 channel (Philipson et al., 1991; Tamkun et al., 1991; Snyders et al., 1993; Fedida et al., 1993). The hKv1.5-like current is involved in the control of the cardiac action potential duration (Wang et al., 1993) and, thus, represents a potential molecular target for drugs that prolong the cardiac action potential duration (Roberds et al., 1993).

Expression of this channel in a system without contaminating conductances allows for a detailed analysis of drug-channel interactions, which forms a necessary basis to elucidate structure-function relationships. The goals of the present study were 1) to characterize the voltage-dependent effects of bupivacaine enantiomers on hKv1.5, 2) to determine the kinetic state of the channel for which the enantiomers exhibit their highest affinity, and 3) to determine the basis for the possible stereoselectivity of the interaction between bupivacaine enantiomers and the hKv1.5 channel. A preliminary report of the present study has been published in abstract form (Valenzuela et al., 1994).

MATERIALS AND METHODS

Cell culture and solutions

The method to establish hKv1.5 expression in a clonal mouse *Ltk*$^-$-cell line has been described previously (Snyders et al., 1992, 1993). Transfected cells were cultured in DMEM supplemented with 10% horse serum and 0.25 mg/ml G418, under a 5% CO_2 atmosphere. The cultures were passed every 3–5 days, using a brief trypsin treatment. Before experimental use, subconfluent cultures were incubated with 2 μM dexamethasone for 24 h. The cells were removed from the dish with a rubber policeman, a procedure that left the vast majority of the cells intact. The cell suspension was stored at room temperature (20–22°C) and used within 12 h for all experiments reported here.

The intracellular pipette filling solution contained (in mM): K-aspartate 80, KCl 50, KH_2PO_4 10, MgATP 3, HEPES 10, EGTA 5, and was adjusted to pH 7.25 with KOH. The bath solution contained (in mM): NaCl 130, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, and glucose 10, and was adjusted to pH 7.35 with NaOH. Both bupivacaine enantiomers (a gift from Astra Hässle AB, Mölndal, Sweden) were dissolved in distilled deionized water to yield stock solutions of 10 mM from which further dilutions were made to obtain the desired final concentration.

Electrical recording

Experiments were performed in a small volume (0.5 ml) bath mounted on the stage of an inverted microscope (Nikon model TMS, Garden City, NY) perfused continuously at a flow rate of 0.5–1.0 ml/min. The hKv1.5 currents were recorded at room temperature (20–22°C) using the whole-cell voltage-clamp configuration of the patch-clamp technique (Hamill et al., 1981) with an Axopatch-1C patch-clamp amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 2 kHz (four-pole Bessel filter), sampled at 4 kHz, and stored on the hard disk of a Hewlett-Packard Vectra QS/16S computer for subsequent analysis. Data acquisition and command potentials were controlled by the pClamp 5.5.1. software (Axon Instruments).

Micropipettes were pulled from borosilicate glass capillary tubes (Narishige, GD-1, Tokyo, Japan) on a programmable horizontal puller (Sutter Instrument Co., San Rafael, CA) and heat-polished with a microforge (Narishige). When filled with the intracellular solution and immersed into the bath (external) solution, the pipette tip resistance ranged between 1 and 3 MΩ. The micropipettes were gently lowered onto the cells to obtain a gigaohm seal (16 $\pm$ 6 GΩ) after applying suction. After seal formation, cells were lifted from the bottom of the perfusion bath and the membrane patch was ruptured with brief additional suction. The capacitive transients elicited by symmetrical 10-mV steps from -80 mV were recorded at 50 kHz (filtered at 10 kHz) for subsequent calculation of capacitative surface area, access resistance, and input impedance. Thereafter, capacitance and series resistance compensation were optimized, and 80% compensation of the effective access resistance was usually obtained.

Pulse protocol and analysis

After control data were obtained, bath perfusion was switched to drug-containing solution. Drug infusion or removal was monitored with test pulses from -80 to $+30$ mV, applied every 30 s until steady-state was obtained (within 10–15 min). The holding potential was maintained at -80 mV. The cycle time for any protocol was 10 s to avoid accumulation of block or incomplete deactivation of the current.

The protocol to obtain current-voltage (I-V) relationships and activation curves consisted of 250-ms pulses that were imposed in 10-mV increments between -80 and $+60$ mV, with additional interpolated pulses to yield 5-mV increments between -30 and $+10$ mV, i.e., the activation range of hKv1.5 (Snyders et al., 1993). The "steady-state" I-V relationships were obtained by measuring the current at the end of the 250-ms depolarizations. Between -80 and -40 mV, only passive linear leak was observed and least-squares fits to these data were used for passive leak correction. Deactivating "tail" currents were recorded at -40 mV. The activation curve was obtained from the tail current amplitude immediately after the capacitive transient or from the amplitude of the exponential fit to its time course. Both procedures gave similar results in controls. Measurements were done using the clampfit program of pClamp 5.5.1. and by a custom-made analysis program.

Activation curves were fitted with a Boltzmann equation:

$$y = 1/[1 + \exp(-)(E - E_h)/s], \quad (1)$$

in which s represents the slope factor, E the membrane potential, and E_h the voltage at which 50% of the channels are open. The time course of tail currents and the slow inactivation were fitted with the sum of exponentials. The activation kinetics was determined with the dominant time constant of activation approach in which a single exponential was fitted to the latter 50% of activation (White and Bezanilla, 1985; Snyders et al., 1993; Valenzuela et al., 1994). The curve-fitting procedure used a nonlinear least-squares (Gauss-Newton) algorithm; results were displayed in linear and semilogarithmic format, together with the difference plot. Goodness of fit was judged by the χ^2 criterion and by inspection for systematic non-random trends in the difference plot.

A first-order blocking scheme was used to describe drug-channel interaction. The apparent affinity constant, K_D, and Hill coefficient, n_H, were obtained from fitting of the fractional block, f, at various drug concentrations [D]:

$$f = 1/\{1 + (K_D/[D])^{n_H}\}, \quad (2)$$

and apparent rate constants for binding (k) and unbinding (l) were obtained from solving

$$k \times [D] + l = 1/\tau_B \quad (3a)$$

$$l/k = K_D. \quad (3b)$$

Voltage dependence of block was determined as follows: leak-corrected current in the presence of drug was normalized to matching control to yield the fractional block at each voltage ($f = 1 - I_{drug}/I_{control}$). The voltage dependence of block was fitted to

$$f = [D]/\{[D] + K_D^* \times \exp(-\delta zFE/RT)\}, \qquad (4)$$

where z, F, R, and T have their usual meaning, δ represents the fractional electrical distance, i.e., the fraction of the transmembrane electrical field sensed by a single charge at the receptor site and K_D^* represents the apparent dissociation constant at the reference potential (0 mV).

Statistical Methods

Results are expressed as mean ± SEM. Direct comparisons between mean values in control conditions versus mean values in the presence of drug for a single variable were performed by a paired Student's t-test. ANOVA was used to compare more than two groups. Student's t-test was also used to compare two regression lines. Differences were considered significant if $p < 0.05$.

RESULTS

Dose-dependent and reversible block by R(+)- and S(−)-bupivacaine

The top panels of Fig. 1, *A* and *B* show superimposed tracings of potassium current through hKv1.5 channels expressed in mouse Ltk^- cells after the application of 250-ms depolarizing pulses from −80 mV to different test potentials between −60 and +60 mV. Under control conditions, the hKv1.5 current rose rapidly with a sigmoidal time course to a peak and then declined slowly (slow and partial inactivation). Outward tail currents were observed upon repolarization to −40 mV. Activation time constants ranged from 25.8 ± 1.8 ms at −10 mV ($n = 23$) to 2.1 ± 0.2 ms at +60 mV ($n = 23$), as described previously (Philipson et al., 1991; Snyders et al., 1992, 1993). Induction of block after switching of perfusion with each enantiomer progressed with a time constant of 3–4 min, which was about 5 times slower than the effect of changing extracellular K^+ concentration at similar flow rates. This delay suggested an intramembrane or intracellular site of action and, therefore, 10–15 min of equilibration were allowed before assessment of drug effects.

The middle panels of Fig. 1, *A* and *B* illustrate the differential effectiveness of either enantiomer at an identical concentration (20 μM). R(+)-bupivacaine displayed a higher potency than S(−)-bupivacaine to inhibit the hKv1.5 current. Both enantiomers not only reduced the current amplitude but also altered the time course of the current during depolarization, without modifying the initial activation time course of the current. The drug-induced accelerated decline of hKv1.5 current to the reduced steady-state level proceeded with time constants of 18.7 ± 1.1 and 10.0 ± 0.9 ms for this concentration of the S(−)- and R(+)-enantiomers, respectively. At the end of a 250-ms step to +60 mV, this concentration of S(−)-bupivacaine and R(+)-bupivacaine reduced the hKv1.5 current by 31 ± 2% ($n = 5$) and 79 ± 3% ($n = 5$) ($p < 0.001$), respectively. The

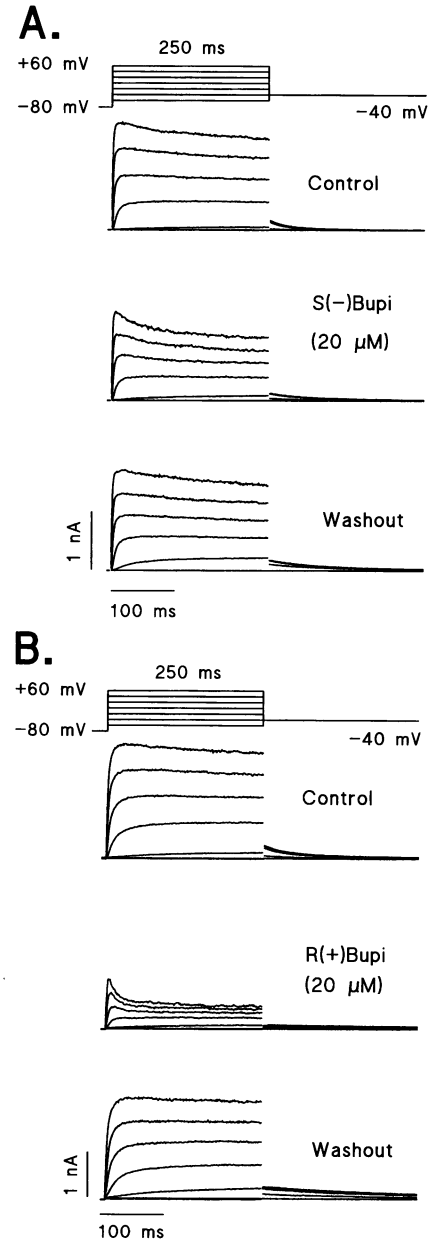

FIGURE 1 Effects of S(−)-bupivacaine and R(+)-bupivacaine on hKv1.5 currents. Currents are shown for depolarizations from −80 mV to voltages between −60 and +60 mV in steps of 20 mV. Tail currents were obtained on return to −40 mV. (*A*) Effects of S(−)-bupivacaine (20 μM) on hKv1.5. Traces were obtained in control conditions (*top*), in the presence of 20 μM S(−)-bupivacaine (*middle*), and after 20 min of wash out (*bottom*). Cell capacitance, 20 pF. Data filtered at 2 kHz (four-pole Bessel) and digitized at 10 kHz; additional digital filtering at 1 kHz. (*B*) Effects of R(+)-bupivacaine (20 μM) on hKv1.5. Traces were obtained in control conditions (*top*), in the presence of 20 μM R(+)-bupivacaine (*middle*), and after 20 min of wash out (*bottom*). Cell capacitance, 22 pF. Data filtered at 2 kHz (four-pole Bessel) and digitized at 10 kHz; additional digital filtering at 1 kHz.

reversibility of these effects are illustrated by the bottom panels of Fig. 1, *A* and *B*. The amplitude of the current was restored to 95 and 88%, respectively, after 20 min perfusion of the cells with drug-free solution. On average, the current

was restored to 88 ± 2.1% (n = 50) of its initial control value after a 20-min washout.

Fig. 2 shows the concentration dependence of S(−)-bupivacaine and R(+)-bupivacaine block of hKv1.5. To allow for the slower kinetics with lower concentrations (see below), we used suppression of current at the end of 250-ms depolarizations to +60 mV as an index of steady-state inhibition (*fractional block* = $1 - I_{drug}/I_{control}$). At all concentrations tested (0.3–100 μM), the degree of hKv1.5 block induced by R(+)-bupivacaine was significantly greater than that induced by S(−)-bupivacaine ($p < 0.001$). Thus, in the presence of 10 μM S(−)-bupivacaine and R(+)-bupivacaine, hKv1.5 inhibition averaged 29 ± 3% (n = 4) and 62 ± 6% (n = 9), respectively. A nonlinear least-squares fit of the concentration-response equation (Eq. 2, see Materials and Methods) to the individual data points yielded an apparent K_D of 27.3 ± 2.8 and 4.1 ± 0.7 μM ($p < 0.01$) for S(−)-bupivacaine and R(+)-bupivacaine, respectively. The Hill coefficients were 1.22 ± 0.22 and 0.85 ± 0.10 ($p > 0.05$) for S(−)-bupivacaine and R(+)-bupivacaine, respectively. The dashed line in Fig. 2 illustrates a fit of the same data with the Hill coefficient fixed at 1. The apparent K_D values obtained for both bupivacaine enantiomers were similar to those obtained without constraining the Hill coefficients. These results suggest that binding of a single bupivacaine molecule is sufficient to block the hKv1.5 channel.

Voltage-dependent block by bupivacaine of hKv1.5 channels

Fig. 3 *A* shows the effects of 50 μM S(−)-bupivacaine on the steady-state current-voltage (I-V) relationship for the hKv1.5 channel. The I-V relationship under control conditions was quasi-linear for depolarizations positive to +10 mV, whereas the sigmoidicity between −30 and +10 mV reflects the voltage-dependence of channel gating (Snyders et al., 1993). In the presence of 50 μM S(−)-bupivacaine, the curve displayed a downward curvature at test potentials positive to 0 mV, which suggested that this enantiomer produced more extensive block at very positive depolarizations. To quantitate the voltage dependence of hKv1.5 block, the relative current $I_{drug}/I_{control}$ was plotted as a function of membrane potential (Fig. 3 *B*). Human Kv1.5 block increased steeply between −30 and 0 mV (*open triangles*), which corresponded to the voltage range of channel opening (*dashed line*). Between 0 and +60 mV (*closed circles*), block continued to increase but with a shallower voltage dependence, although all channels were open over this voltage range. These results suggested that S(−)-bupivacaine preferentially bound to the open state of the hKv1.5 channel. Fig. 4 illustrates that R(+)-bupivacaine induced a similar downward curvature of the steady-state I-V relationship at positive potentials, as well as a biphasic voltage-dependence of hKv1.5 block. The total voltage-dependency of block induced by both bupivacaine enantiomers appeared to be composed of a steep phase coinciding with channel activation (Figs. 3 *B* and 4 *B*, *dashed lines*) and a shallower phase at membrane potentials positive to 0 mV. Thus, the overall mechanism appears similar, but the effects occurred at lower concentrations compared to S(−)-bupivacaine.

It is unlikely that the shallow voltage dependence of block observed at membrane potentials positive to 0 mV was due to channel gating, because hKv1.5 activation had reached saturation over this voltage range with time constants of 10 ms or less. Bupivacaine is a weak base and has a tertiary amine group with pK_a = 8.1, so that at the

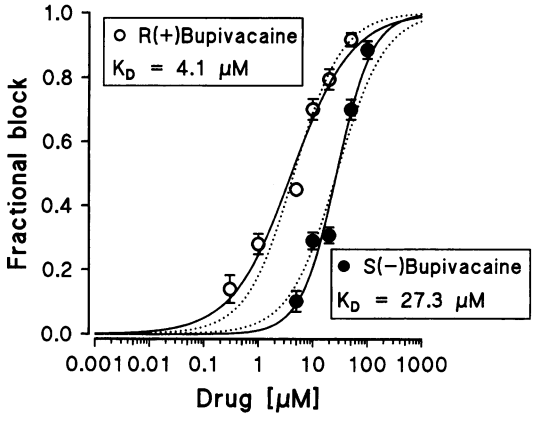

FIGURE 2 Concentration dependence of S(−)-bupivacaine- and R(+)-bupivacaine-induced block of hKv1.5. Reduction of current (relative to control) at the end of depolarizing steps from −80 mV to +60 mV was used as index of block. Data are mean ± SEM of a total of 51 experiments. The solid line represents the fit of the experimental data to the equation: $1/\{1 + (K_D/[D])^nH\}$. For comparison, the dashed line represents the fit for a Hill coefficient (n_H) of 1.

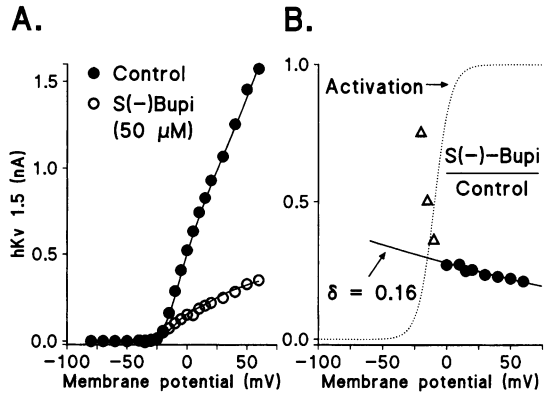

FIGURE 3 Voltage dependence of hKv1.5 block by S(−)-bupivacaine (50 μM). (*A*) Current-voltage relationship (250 ms isochronal) in control conditions (●) and in the presence of 50 μM S(−)-bupivacaine (○). (*B*) Relative current expressed as $I_{S(-)\text{-bupivacaine}}/I_{control}$ from data shown in *A*. The dashed line represents the activation curve for this experiment. Block increased steeply between −20 and 0 mV (Δ), which corresponds to the voltage range of activation of hKv1.5. For membrane potentials positive to 0 mV, a continued but more shallow voltage dependence was observed (●) that represents the effect of the membrane electrical field on the interaction between S(−)-bupivacaine and the hKv1.5 channel. This voltage dependence was fitted (——) with Eq. 4 (see Materials and Methods) and yielded $\delta = 0.16$.

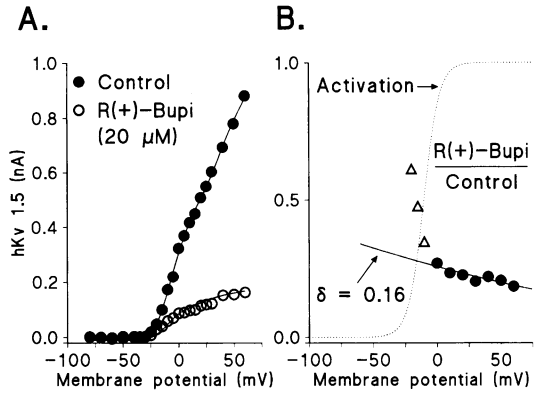

FIGURE 4 Voltage dependence of hKv1.5 block by R(+)-bupivacaine (20 μM). (*A*) Current-voltage relationship (250 ms isochronal) in control conditions (●) and in the presence of 20 μM R(+)-bupivacaine (○). (*B*) Relative current expressed as $I_{R(+)\text{-bupivacaine}}/I_{control}$ from data shown in *A*. The dashed line represents the activation curve for this experiment. Block increased steeply between −20 and 0 mV (Δ), which corresponds to the voltage range of activation of hKv1.5. For membrane potentials positive to 0 mV, a continued but more shallow voltage dependence was observed (●) that represents the effect of the membrane electrical field on the interaction between R(+)-bupivacaine and the hKv1.5 channel. This voltage dependence was fitted (——) with Eq. 4 (see Materials and Methods) and yielded $\delta = 0.16$.

intracellular pH of 7.2, it is present predominantly in its charged form. Thus, the shallow component in the voltage dependence of block that appears at membrane potentials positive to 0 mV could result from the influence of the transmembrane electrical field on the interaction between cationic bupivacaine and the channel receptor. If bupivacaine reaches the receptor from the inside, block is expected to increase in a voltage-dependent manner. Indeed, the percentage of block induced by 50 μM S(−)-bupivacaine significantly increased from 59.0 ± 5.6% at 0 mV to 69.5 ± 3.7% at +60 mV ($n = 5$; $p < 0.01$). With 20 μM R(+)-bupivacaine, block increased from 72.4 ± 3.6% at 0 mV to 79.5 ± 3.2% at +60 mV ($n = 5$; $p < 0.01$). In both cases, the voltage dependence of block observed in this range of membrane potentials (between 0 and +60 mV) was described by a Boltzmann relationship based on the Woodhull model (Eq. 4 in Materials and Methods). The parameter δ in this equation represents the fractional electrical distance, i.e., the fraction of the membrane electrical field sensed by a single charge at the receptor site (Woodhull, 1973). The solid lines in Figs. 3 *B* and 4 *B* represent the fits of this Boltzmann equation to the data points positive to 0 mV (*solid symbols*). The latter restriction was required to quantify this effect independent from the voltage dependence of channel opening. Using this analysis, we obtained a value of 0.16 for the fractional electrical distance (δ) for the two experiments shown. In the presence of 50 μM S(−)-bupivacaine, the δ value averaged 0.164 ± 0.002 ($n = 6$). Similarly, the averaged δ value obtained for R(+)-bupivacaine (20 μM) was 0.168 ± 0.007 ($n = 6$), a value that was not significantly different from δ obtained for S(−)-bupivacaine ($p > 0.05$).

Concentration dependence of time course of channel block

If both bupivacaine enantiomers can only access to their binding site when the channel is open, then the inhibition of the potassium current would only develop after the channels start to open, and development of block should be conspicuous if the blocking rate is slower than the opening rate. On the other hand, if the blocking rate exceeded that of channel opening, or if bupivacaine enantiomers blocked other states of the channel, then the current recorded in the presence of drug would be expected to be scaled down. Fig. 5, *A* and *B* show superimposed recordings of 250-ms depolarizations from −80 to +60 mV under control conditions and in the presence of three different concentrations of S(−)-bupivacaine and R(+)-bupivacaine, respectively. Under control conditions, the potassium current reached its maximum level within 10 ms and then declined slowly, with a time constant of 160 ms. In the presence of S(−)-bupivacaine and R(+)-bupivacaine, the peak was decreased in a concentration-dependent manner. In both cases, the subsequent time course displayed an additional rapid exponential component superimposed on the slow inactivation. As illustrated in Fig. 5, *A* and *B*, the time course of this decline was concentration-dependent. The time constants of the fast component in the presence of 20, 50, and 100 μM of S(−)-bupivacaine were 20, 17, and 7 ms, respectively. These time constants (τ_B) were at least 8 times faster than those of the (partial) slow inactivation observed under control conditions. Therefore, they were taken as an approximation of bimolecular drug-channel interaction kinetics, similar to the approach used previously for the interaction of quinidine with this channel (Snyders et al., 1992). Fig. 5 *C* shows the plot of $1/\tau_B$ vs. S(−)- and R(+)-bupivacaine concentration for data obtained in 40 experiments. The straight lines are the least-squares fit to the relation $1/\tau_B = k \times [D] + l$. Slope and intercept for the fitted relation yielded apparent association rate constants (k) of (1.03 ± 0.09) × 10^6 M^{-1} s^{-1} and (4.71 ± 0.45) × 10^6 M^{-1} s^{-1} for S(−)-bupivacaine and R(+)-bupivacaine, respectively ($p < 0.01$). The dissociation rate constants (l) resulted to be similar for both bupivacaine enantiomers, being 24.3 ± 6.4 and 23.6 ± 13.6 s^{-1} for S(−)-bupivacaine and R(+)-bupivacaine, respectively ($p > 0.05$). Thus, the dissociation rate constants were similar for both enantiomers, and the data suggest that the stereoselectivity derives from the differences in the binding rate constants.

Time course of tail currents

After a depolarizing step from −80 to +60 mV, the potassium currents deactivated completely on return to −40 mV with a time constant of 46 ± 4 ms ($n = 10$). This time constant reflects mainly the virtually irreversible closing of the channel. If both S(−)- and R(+)-bupivacaine bind only to the open state of hKv1.5 channels, then the dissociation of the enantiomers from the blocked channel should result

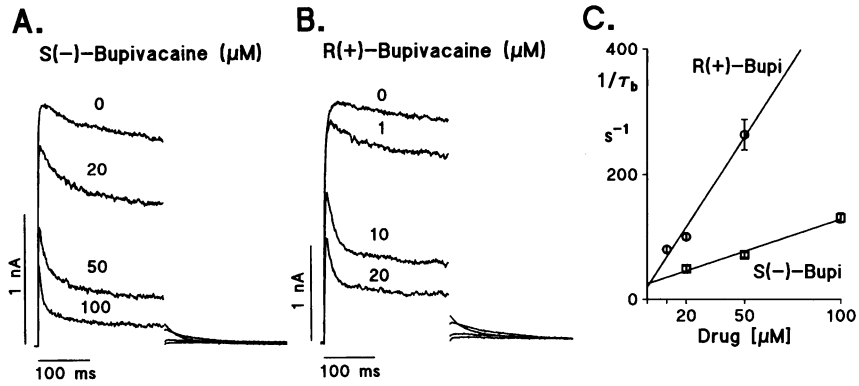

FIGURE 5 Kinetics of block induction by S(−)- and R(+)-bupivacaine. (*A*) Superimposed traces for steps from −80 to +60 mV and tail currents recorded on return to −40 mV under control conditions (0) and in the presence of 20, 50, and 100 μM S(−)-bupivacaine. (*B*) Superimposed traces for steps from −80 to +60 mV and tail currents recorded on return to −40 mV under control conditions (0) and in the presence of 1, 10, and 20 μM R(+)-bupivacaine. In the presence of both S(−)- and R(+)-bupivacaine, the current activated initially as under control conditions but reached a lower peak and subsequently declined more quickly. (*C*) Rate of block as a function of drug concentration. The time constant of S(−)- and R(+)-bupivacaine-induced fast component (τ_B) was obtained from biexponential fits to the falling phase of the tracings shown in *A* and *B*. The inverse of τ_B was plotted versus drug concentration. For a first-order blocking scheme, a linear relation is expected: $1/\tau_B = k\times[D] + l$. The solid lines represent the linear fits for τ_B of the fast component observed in the presence of S(−)- and R(+)-bupivacaine, from which the apparent binding and unbinding rate constants were obtained. Note that the unbinding rate constants (l) obtained from the *y*-intercept were similar for both enantiomers (24 s^{-1}), whereas the slope of the line (binding rate constant, k) were different, being faster in the presence of R(+)-bupivacaine (4.7×10^6 vs. 1.0×10^6 M^{-1} s^{-1}).

in an open channel (which subsequently could close). Blocked channels are not conducting, and the conversion to open channels, therefore, should result initially in a rising phase of the tail current. Subsequently, the tail current should display a slower decline because some fraction of the open channels become blocked again, rather than closing irreversibly (Armstrong 1971; Choi et al., 1993; Snyders et al., 1992). Fig. 6 *A* shows the superposition of the tail currents obtained at −40 mV after 250-ms depolarization to +60 mV under control conditions and in the presence of 50 μM S(−)-bupivacaine. After exposure to this enantiomer, the initial amplitude of the tail current was reduced and the tail current displayed a rising phase reaching a peak after 10.0 ± 5.0 ms. The subsequent decline of the tail current was slower than in control conditions (44 ± 2 vs. 223 ± 45 ms, in control and in the presence of 50 μM S(−)-bupivacaine, respectively; $n = 5$), resulting in a "crossover" phenomenon. Similar modifications of the time course of the tail currents were observed with 10 μM R(+)-bupivacaine (Fig. 6 *B*). The peak tail current was obtained after 14 ± 4 ms, and the decay time constants increased from 45 ± 3 ms in control to 260 ± 73 ms in the presence of R(+)-bupivacaine, which again resulted in a "crossover" phenomenon. These results supports an open-channel interaction between both bupivacaine enantiomers and hKv1.5 channels.

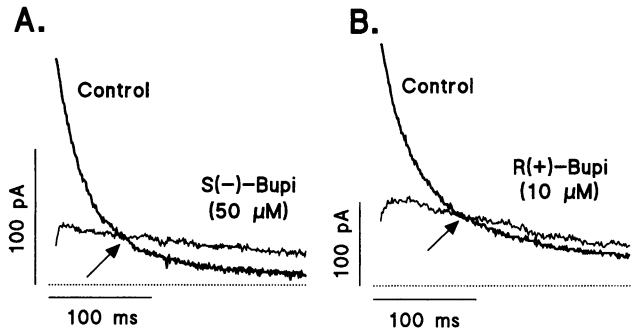

FIGURE 6 Tail current crossover. (*A*) Currents recorded in control conditions and in the presence of 50 μM S(−)-bupivacaine. (*B*) Currents recorded in control conditions and in the presence of 10 μM R(+)-bupivacaine. Tail currents were obtained at −40 mV after a 250-ms depolarizing pulse to +60 mV. Arrow shows the crossover of tracings recorded in the presence of S(−)-bupivacaine or R(+)-bupivacaine with those recorded under control conditions.

Open channel block model

The experimental results obtained in this study were interpreted and simulated using a simplified kinetic state diagram similar to that used for the related *Shaker* and RCK1 channels (Koren et al., 1990; Zagotta and Aldrich 1990) and was used previously to describe block of the hKv1.5 channel by quinidine (Snyders et al., 1992):

$$\begin{array}{ccccccccc} & 4\alpha & 3\alpha & 2\alpha & \alpha & k\times[\mathrm{D}] & & \\ \mathrm{C} \rightleftharpoons & \mathrm{C} & \rightleftharpoons \mathrm{C} & \rightleftharpoons \mathrm{C} & \rightleftharpoons \mathrm{O} & \rightleftharpoons & \mathrm{OB} \\ & \beta & 2\beta & 3\beta & 4\beta \updownarrow & l & & \\ & & & & \mathrm{I} & & & \end{array}$$

To incorporate the interaction of S(−)-bupivacaine and R(+)-bupivacaine into this diagram, we assumed that either enantiomer binds only to the open state of the channel. Because the activation process is fast at potentials positive to 0 mV, this system is functionally reduced to a three-state model (I ⇄ O ⇄ OB). In the presence of S(−)-bupivacaine and R(+)-bupivacaine, the inactivation became biexponen-

tial, as expected for this model. The drug-induced extra component of inactivation had a time constant (τ) that was much faster than the slow inactivation of the current under control conditions. Thus, this fast τ can be considered to represent the interaction of bupivacaine enantiomers (B) with the open state (O) of hKv1.5 channel (O $\rightleftarrows$ OB), $\tau_B = 1/(k\times[D] + l)$. Fig. 7 shows the mathematical simulation of the effects of 50 μM S(−)- and 10 μM R(+)-bupivacaine,

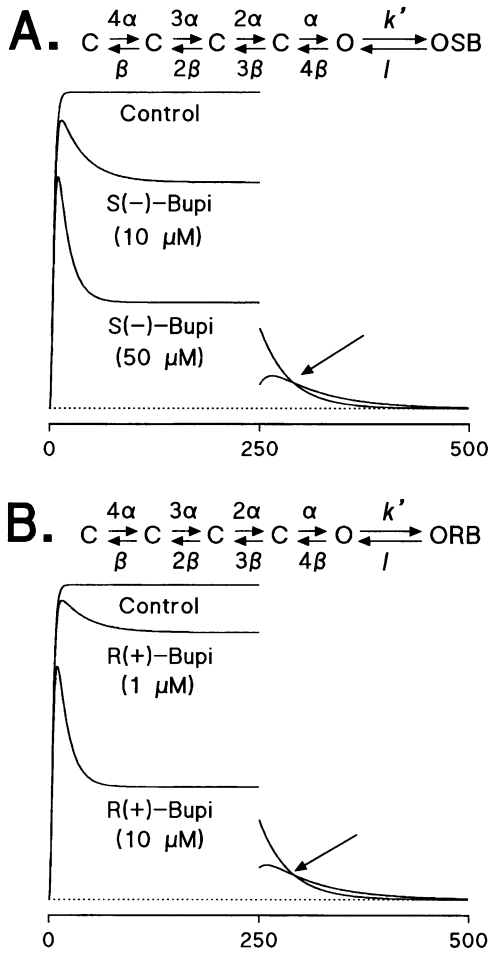

FIGURE 7 Mathematical modeling of S(−)-bupivacaine and R(+)-bupivacaine interactions with hKv1.5 channels. (*A*) Simulation of S(−)-bupivacaine-hKv1.5 interaction. The open-channel block model was used with the following rate constants: at +60 mV, α = 400 s^{-1}, β = 1 s^{-1}, k = 1.0 μM^{-1} s^{-1}, and l = 24 s^{-1}; at −40 mV, α = 0.1 s^{-1}, β = 7 s^{-1}, k = 0.5 μM^{-1} s^{-1} and l = 45 s^{-1}. For depolarization, simulations for control and for 10 and 50 μM S(−)-bupivacaine are displayed; for the tails, control and 50 μM S(−)-bupivacaine are shown. (*arrow*) Crossover. Currents for step and tail were scaled to reflect the difference in driving force. (*B*) Simulation of R(+)-bupivacaine-hKv1.5 interaction. The open-channel block model was used with the following rate constants: at +60 mV, α = 400 s^{-1}, β = 1 s^{-1}, k = 4.7 μM^{-1} s^{-1}, and l = 24 s^{-1}; at −40 mV, α = 0.1 s^{-1}, β = 7 s^{-1}, k = 3.2 μM^{-1} s^{-1} and l = 45 s^{-1}. For depolarization, simulations for control and for 1 and 10 μM R(+)-bupivacaine are displayed; for the tails, control and 10 μM R(+)-bupivacaine are shown. (*arrow*) Crossover. Currents for step and tail were scaled to reflect the difference in driving force. In this model, we omitted for simplicity the slow and partial inactivation and therefore this process was not reproduced by the mathematical simulation shown in this figure.

based on this open-channel block model, reproducing the experimental observed changes in time course during depolarization and deactivating tails (Figs. 1, 5, and 6). As it can be observed, a model that only assumes an open channel interaction reproduces the experimental data reasonably well.

DISCUSSION

In the present study, we have analyzed the interaction between S(−)- and R(+)-bupivacaine with human cardiac Kv1.5 channels expressed in a stable cell line (*Ltk*$^-$). The main conclusions of the present paper are as follows: 1) the charged form of both bupivacaine enantiomers blocks the hKv1.5 channel after it opens; 2) binding occurs within the transmembrane electrical field; 3) unbinding is required before the channel can close; 4) bupivacaine-induced block of hKv1.5 channels is markedly stereoselective, with R(+)-bupivacaine being sevenfold more potent than S(−)-bupivacaine; 5) this stereoselective block was associated with a 1.11-kcal/mole difference in binding energy between both enantiomers; and 6) the stereoselectivity derives mainly from a difference in the association rate constants, which suggests that S(−)-bupivacaine is less likely to access the binding site in an optimal configuration.

Time-dependent interactions between bupivacaine enantiomers and hKv1.5 channels

S(−)-bupivacaine and R(+)-bupivacaine did not modify the time course of activation, but induced an initial fast decline of the hKv1.5 current during depolarization (see Fig. 1). The time course of this fast decline (τ_B) was concentration-dependent and was taken as an index of the binding process of the drug. In the present experiments, R(+)-bupivacaine was sevenfold more potent to block hKv1.5 channels than S(−)-bupivacaine, which indicated that the blockade produced by bupivacaine was stereoselective. These data are consistent with an open-channel block mechanism in which both bupivacaine enantiomers only access their binding site when the channel is opened and with a slower blocking rate than the opening rate of the channel. Moreover, mathematical modeling assuming the interaction of each enantiomer only with the open state of the channel, reproduced fairly well the experimental data (see Fig. 7). At lower depolarizations, activation of hKv1.5 is much slower. The rate of activation at 0 mV is comparable with the blocking rate that would preclude to observe a distinct drug-induced decline of current. This was indeed the case for the tracings at −20 and 0 mV in Fig. 1. Similarly, it was not possible to extract reliable rate constants at low drug concentrations because the time course of block became similar to that of slow inactivation. Therefore, we did not include data obtained in the presence of low concentrations of S(−)-bupivacaine (below 20 μM) and R(+)-bupivacaine (below 10 μM) in Fig. 5. The apparent binding and unbinding rate constants

for S(−)-bupivacaine were calculated to be $k = 1.0\times10^6$ M^{-1} s^{-1} and $l = 24.9$ s^{-1}, whereas for R(+)-bupivacaine these rate constants were $k = 4.7\times10^6$ M^{-1} s^{-1} and $l = 23.6$ s^{-1}, respectively. From these rate constants the apparent affinity ($K_D = l/k$) is estimated to be 5.0 and 24.9 μM for (R+) and S(−)-bupivacaine, respectively. These estimates are in good agreement with the values obtained from steady-state suppression of the hKv1.5 current (4.1 and 27.3 μM). This agreement between both independent methods to estimate the affinity further supports the open-channel block model used to derive the rate constants. It is of interest to note that bupivacaine-induced block of nerve and cardiac Na^+ channels is also stereoselective. In cardiac Na^+ channels, the higher potency of the R(+)-enantiomer was also related to a faster association rate constant compared to the S(−)-bupivacaine (Wang, 1990; Valenzuela et al., 1993).

Open channel block not only modifies the time course of the current during depolarization, but can also influence the time course of the deactivating currents (Follmer et al., 1992; Snyders et al., 1992; Choi et al., 1993, Carmeliet, 1993). Under control conditions, channel deactivation upon repolarization is fast and virtually irreversible ($4\beta \gg \alpha$). However, if a large fraction of channels is blocked by either bupivacaine enantiomer during the preceding depolarization and if the unbinding rate (l) is fast enough, then the tail current may display a rising phase that will reflect the unblocking process. Subsequently, channels reach the open state and may be blocked again before they eventually deactivate. This process will be reflected by a slower time course of the tail current (Fig. 6). The mathematical model based on the experimentally derived rate constants predicted both effects: 1) a rising phase, and 2) a slower time course of deactivation, resulting in the "crossover" phenomenon (Fig. 7), as observed in the records shown in Figs. 5 and 6. These data further support the proposed interaction of both enantiomers with the open state of the hKv1.5 channel.

Voltage dependence of bupivacaine block

Figs. 3 *B* and 4 *B* show that block of hKv1.5 channels induced by S(−)-bupivacaine and R(+)-bupivacaine was voltage-dependent, reaching a higher degree of block at more positive than at more negative membrane potentials. These results are consistent with the proposed open channel block mechanism, because the probability of opening increases at more positive membrane potentials. The voltage dependence of block produced by both bupivacaine enantiomers consisted of two different phases: a steep one, which coincides with the range of membrane potentials of channel activation (−30 and 0 mV) and a shallower one, observed at membrane potentials positive to 0 mV. Bupivacaine is a weak base with a pK_a of 8.1, which indicates that at the intracellular pH of 7.2, most bupivacaine is present in its charged form (88.8%). The access of the hydrophilic form of the drug to the receptor requires a hydrophilic pathway (Hille, 1977), as has been proposed for quaternary ammonium block of various potassium channels (Armstrong, 1971; French and Shoukimas, 1981; Yellen et al., 1991) as well as for quinidine block of these hKv1.5 channels (Snyders et al., 1992). This mode of access requires that the charged drug moves into the electrical membrane field from the inside, so that membrane depolarization will promote entry of the cationic form into the channel. If a drug has a high affinity for activated (open) channels, the voltage dependence of channel activation superimposes on the intrinsic voltage dependence of drug binding because channel activation makes the receptor available. Therefore, the total voltage dependency of open channel block is composed of a steep phase over the range of membrane potentials at which the channel activates and a shallower phase over the voltage range where all channels are open. The latter reflects the additional effect of the electrical field on the interaction between the charged drug and the channel, as is the case of bupivacaine enantiomers. From the shallow voltage dependence, we obtained a fractional electrical distance (δ) of 0.16 for both enantiomers. Not only is this result consistent with binding in the open ion channel, but the identical value also supports the assumption that both enantiomer bind at the same site in the channel.

Putative receptor site for bupivacaine on hKv1.5 channels

In *Shaker* channels, residues at the innermost turn of the P-loop (connecting S5 and S6) as well as those located at the putative S6 have been implicated in the binding of quaternary ammonium derivatives (Yellen et al., 1991). The electrical distance observed for bupivacaine enantiomers in this study is very similar to the values obtained for internal tetraethylammonium (TEA) block ($\delta = 0.16$) of *Shaker* channels (Yellen et al., 1991; Choi et al., 1993) and for quinidine block of hKv1.5 channels ($\delta = 0.19$) (Snyders et al., 1992). Therefore, these results are consistent with a common binding site for TEA, antiarrhythmic drugs and local anesthetics in the internal mouth of the channel. However, because both bupivacaine and quinidine are very hydrophobic molecules ($\log P \sim 4.0$ and 2.4, respectively) (Snyders et al., 1992), it is likely that the binding of these drugs to the hKv1.5 channel is influenced by hydrophobic residues in the channel, as was described recently for alkyl-TEA derivatives in *Shaker* channels (Choi et al., 1993). Hydrophobic interaction have also been suggested to explain the differences in hKv1.5 block between clofilium, tetrapentylammonium, quinidine, and quinine (Snyders et al., 1992).

Although the results for bupivacaine indicate a stereoselective interaction with the hKv1.5 pore, open channel block of a cardiac transient outward current was not stereoselective (Castle, 1990). The molecular identity of this channel has not yet been established, but Kv1.4 and Kv4.2 are potential candidates. The high degree of sequence conservation in the pore regions of *Shaker*-related potassium

channels (Pongs, 1992) can explain the similar mechanism of block. However, more subtle differences may be responsible for isoform-specific differences in drug binding.

The values for the apparent dissociation constant (K_D) can be converted into apparent binding energies (ΔG) relative to a 1 M standard concentration using the relation:

$$\Delta G = -RT \times \ln[1M/K_D],$$

where R is the universal gas constant and T is the absolute temperature. The free energy indicates the relative stability of the drug-bound channel in the presence of each enantiomer. The values for ΔG were -6.16 and -7.27 kcal/mol for S(−)-bupivacaine and R(+)-bupivacaine, respectively. Thus, the stereoselectivity in hKv1.5 block corresponds to a difference in free energy of 1.11 kcal/mol between R(+)-bupivacaine and S(−)-bupivacaine blocked channels. This could reflect a more stable interaction of the R(+)-enantiomer with the receptor. However, differences in potency between both enantiomers was explained largely by their different association rate constants, which were faster for R(+)-bupivacaine. Because these drugs are enantiomers, most properties are identical, including the free energy associated with similar mirror image conformations. Therefore, the difference in association rates suggests that the S(−)-enantiomer needs to adopt a less favored conformation, i.e., 1.11 kcal/mol above that of the antipode of the conformation in which R(+)-bupivacaine binds. Additional studies, probably including molecular modeling, will be needed to elucidate the detailed structural requirements for stereoselectivity and binding of local anesthetic and antiarrhythmic drugs.

Practical implications regarding toxicity

Bupivacaine is a potent local anesthetic widely used for long lasting regional local anesthesia (Strichartz and Ritchie, 1987). In isolated cardiac tissues, racemic bupivacaine decreased intracardiac conduction velocity and widened the QRS complex of the electrocardiogram by inhibiting I_{Na} (Clarkson and Hondeghem, 1985; Wheeler et al., 1988). Both in animal models and in humans, bupivacaine prolonged the duration of the cardiac action potential (Avery et al., 1984; Kasten and Martin, 1985; Scott et al., 1989; Solomon et al., 1990) and the Q-Tc interval of the ECG (Scott et al., 1989). This prolongation of the Q-Tc interval occasionally can result in the development of a polymorphic ventricular tachycardia similar to that seen in *torsades de pointes* (Kasten and Martin, 1985). Therefore, the electrical aspects of bupivacaine-induced cardiotoxicity could be explained by its inhibition of both cardiac Na^+ and K^+ channels. Moreover, the prolongation of the cardiac action potential induced by their K^+ channel block would further enhance block of the inactivated state of the cardiac Na^+ channel. Because the R(+)-enantiomer has a higher affinity for both the inactivated state of the sodium channel and for hKv1.5 channels than S(−)-bupivacaine, the synergistic interaction could explain the higher toxicity of the R(+) enantiomer. Although mechanical failure may only be apparent with very high plasma concentrations (Tucker and Mather, 1975), a major cause of death with bupivacaine appears to be ventricular fibrillation (Albright, 1979). Adverse effects on both conductivity and contractility of myocardium can be detected at much lower plasma bupivacaine concentrations (1.22 μg/ml = 4.1 μM) than those reported at cardiotoxic levels, when more sensitive methods are used (Scott et al., 1989). In fact, these authors showed that cardiovascular changes can be detected before the clinical symptoms will appear. Comparison of the cardiovascular effects of S(−)-bupivacaine with those of the racemate in male volunteers demonstrated a reduced cardiotoxicity of S(−)-bupivacaine: no significant electrocardiographic effects were detected with S(−)-bupivacaine, whereas racemic bupivacaine induced both mechanical depression and small but significant ECG alterations (Gristwood et al., 1994). Moreover, the systemic disposition of racemic bupivacaine is stereoselective in humans, resulting in a slower elimination of the unbound R(+)-enantiomer (Burm et al., 1994). Because the R(+)-enantiomer has a higher affinity for both the inactivated state of the sodium channel and for this cardiac potassium channel, the synergistic interaction could explain the higher toxicity of the R(+) enantiomer. Thus, in clinical practice S(−)-bupivacaine, which exhibits a lower affinity for both Na^+ and hKv1.5 channels, could be a less cardiotoxic alternative to racemic bupivacaine. Obviously, further clinical studies will be needed to evaluate whether this difference in biophysical properties between both enantiomers has practical implications regarding safety and toxicity.

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