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# Structural Determinants of Potency and Stereoselective Block of hKv1.5 Channels Induced by Local Anesthetics

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## ABSTRACT

Block of hKv1.5 channels by bupivacaine is stereoselective, with (*R*)-(+)-bupivacaine being 7-fold more potent than (*S*)-(−)-bupivacaine. The study of the effects of chemically related enantiomers on these channels may help to elucidate the structural determinants of stereoselective hKv1.5 channels block by local anesthetics. In this study, we analyzed the effects of (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine on hKv1.5 channels stably expressed in Ltk$^-$ cells. (*R*)-(+)-Ropivacaine inhibited hKv1.5 current and induced a fast initial decline superimposed to the slow inactivation during the application of depolarizing pulses, which reached steady state at the end of 250-msec depolarizing pulses. The concentration-dependence block induced by (*R*)-(+)-ropivacaine yielded a $K_D$ value of 32 ± 1 μM [i.e., 2.5-fold more potent than (*S*)-(−)-ropivacaine]. (*R*)-(+)-Ropivacaine block also was voltage dependent, with a fractional electrical distance (δ) of 0.156 ± 0.003 (n = 14) referred to the inner surface. Both (*S*)-(−)- and (*R*)-(+)-mepivacaine blocked hKv1.5 channels, with $K_D$ values of 286.8 ± 34.1 and 379.0 ± 56.0 μM, respectively [i.e., block was not stereoselective ($p > 0.05$)]. (*S*)-(−)-Mepivacaine and (*R*)-(+)-mepivacaine block displayed no apparent time-dependence due to a very fast dissociation rate constant. However, block by mepivacaine enantiomers was voltage dependent, with δ values of 0.154 ± 0.015 and 0.160 ± 0.008 for the (*S*)-(−)- and (*R*)-(+)-enantiomers, respectively. We conclude that (1) (*R*)-(+)-ropivacaine and mepivacaine enantiomers block the open state of hKv1.5 channels and (2) the length of their alkyl substituent at position 1 determines the potency and the degree of stereoselectivity.

TEA and its alkyl derivatives (QA) compounds are potent blockers of voltage-gated $Na^+$ and $K^+$ channels that represent useful probes of ion channel function and structure. Most voltage-gated $K^+$ channels have an external and an internal receptor site for QA (Hille, 1991; Yellen *et al*., 1991), which can be distinguished by their affinity for TEA. In squid axon and in *Shaker* $K^+$ channels, an increase of the length of one or more alkyl chains of QA favors the interaction with the TEA internal receptor, suggesting that this binding site contains a hydrophobic region (Armstrong, 1971; Armstrong and Hille, 1972; French and Shoukimas, 1981; Swenson, 1981; Villarroel *et al*., 1988; Choi *et al*., 1993). Local anesthetics are well known ion channel blockers, and most of them are tertiary amines, which are predominantly present in their charged (cationic) form at the physiological pH (Courtney and Strichartz, 1987; Strichartz and Ritchie, 1987; Hille, 1991). Therefore, these local anesthetics can be considered as highly hydrophobic QA analogs.

Bupivacaine, ropivacaine, and mepivacaine are local anesthetics that exhibit a common chemical structure; they differ only in the length of the alkyl substituent of the tertiary nitrogen (position 1), which is a butyl, propyl, or methyl group, respectively (Fig. 1A). These three chemically related drugs possess an asymmetrical carbon and thus exist as separate (*S*)-(−)- and (*R*)-(+)-enantiomers (Fig. 1A). We previously reported that bupivacaine block of cardiac $Na^+$ and hKv1.5 channels is stereoselective, with (*R*)-(+)-bupivacaine being 1.6- and 7-fold more potent than the (*S*)-(−)-enantiomer, respectively, which could explain the higher cardiotoxicity of (*R*)-(+)-bupivacaine versus (*S*)-(−)-bupivacaine (Åberg, 1972; Aps and Reynolds, 1978; Valenzuela *et al*., 1995a, 1995b). Ropivacaine is a pure (*S*)-(−)-enantiomer, developed as a less cardiotoxic alternative to the racemic bupivacaine used in the clinical practice (McClure, 1996), which blocks $Na^+$ and hKv1.5 channels with a lower potency than (*S*)-(−)-bupivacaine (Akerman *et al*., 1988; Valenzuela *et al*., 1995a, 1997; Moller and Covino, 1997).

The stereoselectivity of bupivacaine block of hKv1.5 channels seems to require both polar and hydrophobic interactions with presumably pore-lining residues in the S6-helix (Franqueza *et al*., 1997). Bupivacaine, ropivacaine, and mepivacaine contain two hydrophobic groups: the aromatic ring

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**ABBREVIATIONS:** TEA, tetraethylammonium; QA, quaternary ammonium derivatives; δ, fractional electrical distance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

and the alkyl-substituted saturated ring (Fig. 1A). Several studies have demonstrated that similar to the binding of QA to *Shaker* $K^+$ channels (Choi *et al*., 1993), the length of this side chain is a structural determinant of the potency of these local anesthetics to inhibit cardiac $I_{Na}$ and $I_{TO}$ (Courtney, 1980b, 1980a; Castle, 1990): the longer the side chain, the higher potency of the drug. However, there is no experimental evidence regarding to the possible role of this substituent in the degree of stereoselective $K^+$ channels block. Thus, to determine whether, specifically, the length of the side chain represents one of the structural requirements implicated in the potency and in the stereoselective block of hKv1.5 channels, we studied the effects of (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine on hKv1.5 channels stably expressed in $Ltk^-$ cells. The comparison of the current results with those previously reported with bupivacaine enantiomers (Valenzuela *et al*., 1995a) and (*S*)-(−)-ropivacaine (Valenzuela *et al*., 1997) may provide valuable clues about the nature of the local anesthetic/channel interactions. Preliminary results of the current study have been published in abstract form (Longobardo *et al*., 1997, 1998).

## Materials and Methods

**Cell culture and solutions.** We used a cell line stably expressing hKv1.5 as described previously (Snyders *et al*., 1992, 1993). Transfected cells were cultured in Dulbecco's modified Eagle medium 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 hr to induce the expression of hKv1.5 channels (which is driven by a dexamethasone inducible promoter). 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 (21–23°) and used within 12 hr for all the experiments reported here.

The intracellular pipette filling solution contained 80 mM K-aspartate, 3 mM phosphocreatine, 50 mM KCl, 10 mM $KH_2PO_4$, 5 mM MgATP, 10 mM HEPES, and 5 mM EGTA, adjusted to pH 7.25 with KOH. The bath solution contained 130 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.35 with NaOH. (*R*)-(+)-Ropivacaine (a gift from Chiroscience, Cambridge, UK) and (*R*)-(+)-mepivacaine and (*S*)-(−)-mepivacaine (gifts from Astra Pain Control, Södertälje, Sweden) were dissolved in distilled deionized water to yield stock solutions of 10 mM. Further dilutions in external solution 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 (model TMS; Nikon, Garden City, NY) continuously perfused at a flow rate of 0.5–1.0 ml/min. The hKv1.5 currents were recorded at room temperature (21–23°) using the whole-cell voltage-clamp configuration of the patch-clamp technique with an Axopatch-1C patch-clamp amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 2 kHz (four-pole Bessel filter) and sampled at 4 kHz. Data

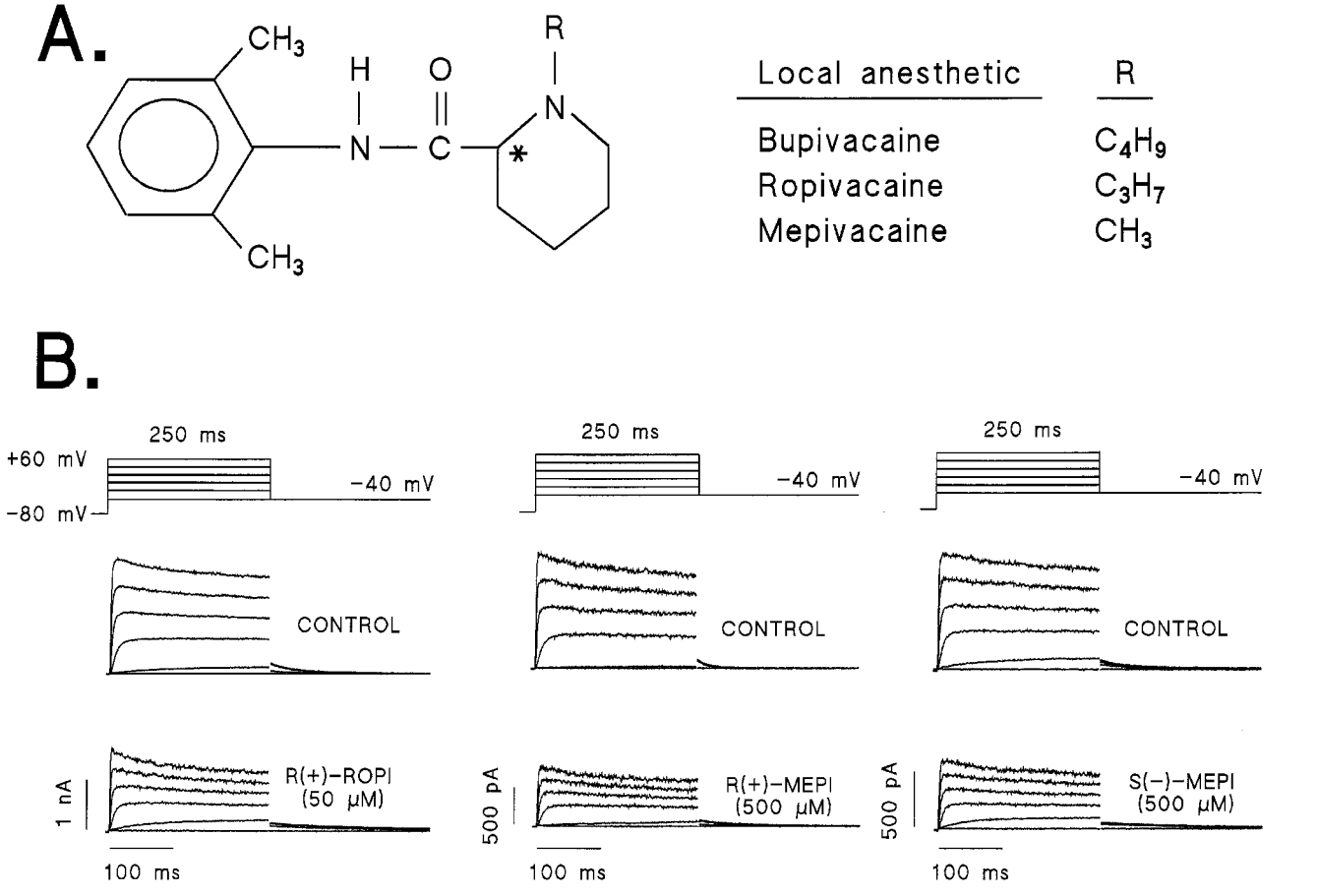


**Fig. 1.** A, Chemical structures of bupivacaine, ropivacaine, and mepivacaine. *, Asymmetrical carbon of the molecule. B, Current traces obtained in the absence and presence of (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine. Cells were held at −80 mV, and 250-msec depolarizing steps between −40 and +60 mV in increments of 20 mV were applied. Tail currents were recorded at −40 mV. Data were filtered at 2 kHz (four-pole Bessel) and digitized at 10 kHz; additional digital filtering occurred at 1 kHz.

acquisition and command potentials were controlled by the pClamp 5.5.1. software (Axon Instruments).

Micropipettes were pulled from borosilicate glass capillary tubes (GD-1; Narishige, Tokyo, Japan) on a programmable horizontal puller (Sutter Instrument, San Rafael, CA, USA) and heat polished with a microforge (Narishige, Tokyo, Japan). When filled with the intracellular solution and immersed into the bath (external) solution, the pipette resistance ranged between 1 and 3 MΩ. The micropipettes were gently lowered onto the cells to obtain a gigaohm seal (17 ± 2 GΩ) after application of 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 usually was 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 mV to +30 mV, applied every 30 sec until steady state was obtained (within 10–15 min). The holding potential was maintained at −80 mV. The cycle time within each protocol was 10 sec to avoid accumulation of block or incomplete deactivation of the current.

The protocol to obtain current-voltage relationships and activation curves consisted of 250-msec 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 the hKv1.5 channels) (Snyders *et al*., 1993). The "steady state" current-voltage relationships were obtained by measuring the current at the end of the 250-msec 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 maximum value of the tail current amplitude after the capacitative transient. Measurements were performed using the ClampFit program of pClamp 6.0.1. and by a custom-made analysis program.

Activation curves were fitted with a Boltzmann equation

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

where s is the slope factor, E is the membrane potential, and $E_h$ is 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; 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 the fit was judged by the $\chi^2$ criterion and by inspection for systematic nonrandom trends in the difference plot.

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

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

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

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

$$l/k = K_D \quad (4)$$

where $\tau_B$ is the time constant of the fast initial drug-induced current decay after activation from the holding potential to +60 mV.

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:

$$f = [D]/([D] + K_D^* \times \exp(-\delta zFE/RT)) \quad (5)$$

where $z$, $F$, $R$, and $T$ have their usual thermodynamic meaning, $\delta$ is the fractional electrical distance (Woodhull, 1973) (i.e., the fraction of the transmembrane electrical field sensed by a single charge at the receptor site), and $K_D^*$ is the apparent dissociation constant at the reference potential (0 mV).

**Statistical methods.** Results are expressed as mean ± standard error. Paired Student's $t$ test was used to compare the effects of (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine with the control values. Statistical significance was taken as $p < 0.05$.

## Results

**Effects of (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine on hKv1.5 channels.** Fig. 1B (*top*) shows the original hKv1.5 current records obtained under control conditions. The holding potential was maintained at −80 mV, and pulses of 250 msec in duration to membrane potentials between −60 and +60 mV (in steps of 20 mV) were applied. Tail currents were recorded on return to −40 mV. The current activates with a fast time course, reaches a maximum peak, and slowly inactivates during the application of depolarizing pulses as described previously (Snyders *et al*., 1993). On repolarization to −40 mV, current deactivates with a dominant time constant of 31 ± 3 msec (17 experiments).

Fig. 1B (*left bottom*) shows hKv1.5 current records obtained in the presence of 50 μM (*R*)-(+)-ropivacaine. In the presence of this drug, the activation of the current was not modified, but the amplitude of the current measured at the end of the 250-msec depolarizing pulse to +60 mV decreased by 63 ± 1% (four experiments). The tail currents recorded at −40 mV also decreased, and their time course was slower than that under control conditions (see below). Fig. 2 shows the concentration dependence of (*R*)-(+)-ropivacaine block of hKv1.5 using suppression of current at the end of 250-msec depolarizations to +60 mV as an index of steady state inhibition. A nonlinear least-squares fit of the concentration-

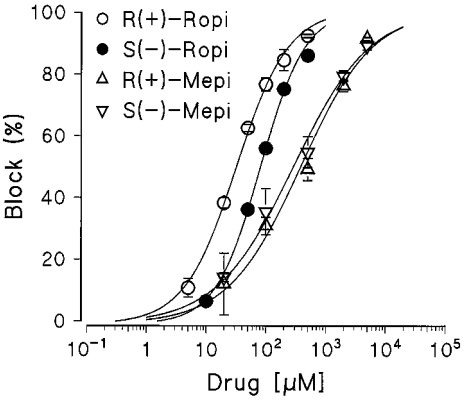


**Fig. 2.** Concentration dependence of (*R*)-(+)-ropivacaine (*Ropi*), (*S*)-(−)-ropivacaine, (*R*)-(+)-mepivacaine (*Mepi*), and (*S*)-(−)-mepivacaine block of hKv1.5. Reduction of current (relative to control) at the end of depolarizing steps from −80 mV to +60 mV was used as index of block. Data are mean ± standard error of a total of 60 experiments. Data for (*S*)-(−)-ropivacaine are from Valenzuela *et al*. (1997).

response equation (eq. 2; see Materials and Methods) yielded an apparent $K_D$ value of 32 ± 1 μM and a Hill coefficient of 1.003 ± 0.044 (22 experiments), suggesting that binding of a single (*R*)-(+)-ropivacaine molecule is sufficient to block the hKv1.5 channel. In a previous study, we demonstrated that the $K_D$ value of (*S*)-(−)-ropivacaine was 81 μM to block hKv1.5 channels [i.e., (*R*)-(+)-ropivacaine is 2.5-fold more potent than (*S*)-(−)-ropivacaine] (Valenzuela *et al*., 1997) (Fig. 2).

Fig. 1B (*middle bottom*) shows that 500 μM (*R*)-(+)-mepivacaine also inhibited hKv1.5 current and, like (*R*)-(+)-ropivacaine, did not modify the activation time course of the current (Fig. 1B). At this concentration, (*R*)-(+)-mepivacaine blocked hKv1.5 channels by 49 ± 4% (five experiments) when measured at the end of a 250-msec depolarizing pulse from −80 to +60 mV. A nonlinear least-squares fit of the concentration-response equation yielded $K_D$ and $n_H$ values of 379.0 ± 56.0 μM and 0.72 ± 0.07 (18 experiments), respectively (Fig. 2). Therefore, the presence of a methyl substituent instead a propyl one at position 1 of the molecule resulted in a 12-fold decrease the affinity, suggesting an important role of the length of the alkyl chain in determining the potency of the local anesthetic. More important, the effects of (*S*)-(−)-mepivacaine were quantitatively similar to those described with the (*R*)-(+)-enantiomer [i.e., block was not stereoselective (Fig. 1B, *right bottom*, and Fig. 2)]. (*S*)-(−)-Mepivacaine (500 μM) induced the same percentage of block as that produced by (*R*)-(+)-mepivacaine (54.8 ± 5.1%, six experiments). A nonlinear least-squares fit of the concentration-response equation (Fig. 2) yielded an apparent $K_D$ value of 286.8 ± 34.1 μM ($p > 0.05$, with respect to that obtained for (*R*)-(+)-mepivacaine), and a Hill coefficient of 0.68 ± 0.05 (23 experiments).

**Voltage-dependent block of hKv1.5 by (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine.** Block of hKv1.5 channels by either (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, or (*S*)-(−)-mepivacaine was voltage dependent (Fig. 3). The Fig. 3 (top) shows that the three enantiomers induced a downward curvature of the IV relationship, indicating a higher current inhibition at more positive potentials. To quantify this voltage dependence, we plotted the relative current in the presence of 50 μM (*R*)-(+)-ropivacaine, 500 μM (*R*)-(+)-mepivacaine, or 500 μM (*S*)-(−)-mepivacaine versus membrane potential (Fig. 3, *bottom*). As it can be observed, block induced by all three enantiomers steeply increased in the range of activation of the channel (between −30 and 0 mV), which strongly suggest that the channels must open before drug can bind and block permeation. For depolarizations positive to 0 mV, block continued increasing with a shallower voltage dependence. Because in this range of membrane potentials, activation curve is saturated, this increase in block cannot be attributed to channel

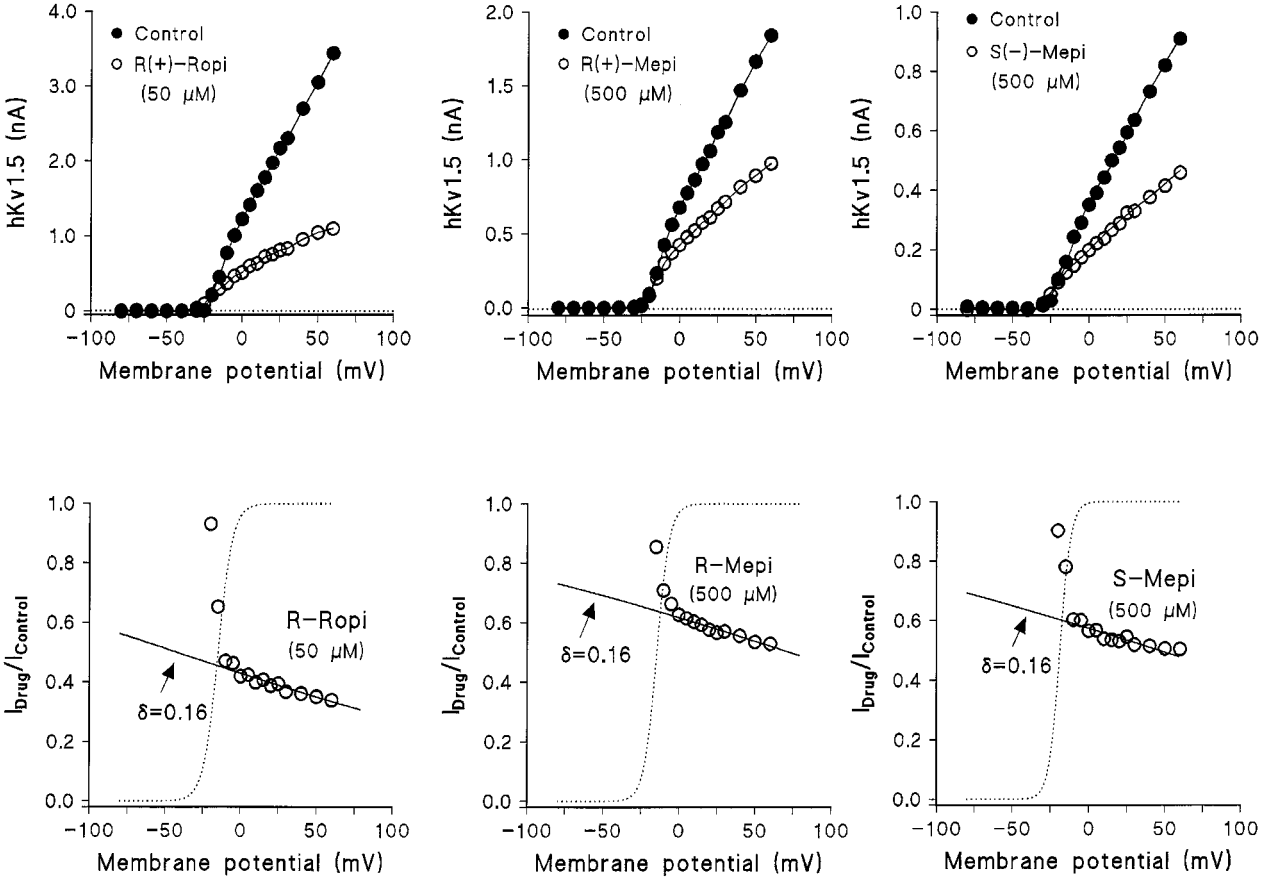


**Fig. 3.** Voltage-dependent block of hKv1.5 channels by (*R*)-(+)-ropivacaine (*Ropi*), (*R*)-(+)-mepivacaine (*Mepi*), and (*S*)-(−)-mepivacaine. *Top*, current-voltage relationship under control conditions (●) and in the presence of each drug (○). *Bottom*, relative current in the presence of the drugs at each membrane potential. *Dotted lines*, activation curves for each particular experiment. Block steeply increases in the range of membrane potential of activation of the channel (*dotted line*). For potentials positive to 0 mV, block continued to increase but with a shallower voltage dependence. *Solid line*, fit using a Woodhull model, which yielded an equivalent electrical distance of 0.16 for each of the drugs.

opening. Both drugs are weak bases [p$K_a$ = 8.1 and 7.8 for (*R*)-(+)-ropivacaine and mepivacaine enantiomers, respectively], and therefore, we attributed the increase in block in this voltage range to the effect of the transmembrane electrical field on the drug/channel interaction. This effect can be explained by a simple Woodhull model (Woodhull, 1973). Therefore, a nonlinear curve fitting of the data to eq. 5 (see Materials and Methods) yielded apparent dissociation constant at the reference potential (0 mV) ($K_D$*) and fractional electrical distance ($\delta$) values of 41 ± 2 $\mu$M and 0.156 ± 0.003 (14 experiments) for (*R*)-(+)-ropivacaine, 549 ± 121 $\mu$M and 0.160 ± 0.008 (10 experiments) for (*R*)-(+)-mepivacaine, and 538 ± 97 $\mu$M and 0.154 ± 0.016 (six experiments) for (*S*)-(−)-mepivacaine. These $K_D$* values were higher than those determined at +60 mV ($p < 0.01$), as would be expected for an open channel blocker.

**Kinetics of block of hKv1.5 channels induced by (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine.** (*R*)-(+)-Ropivacaine block of hKv1.5 channels was time dependent at concentrations of ≥100 $\mu$M (i.e., the drug induced a concentration-dependent fast decline of the current during the application of a depolarizing pulse that superimposed to the slow inactivation of the current) (Fig. 4). This fast decline was more evident and faster in the presence of higher concentrations of drug, and thus, the time constant of this process was taken as an approximation of the time constant of block ($\tau_B$), from which the association ($k$) and the dissociation rate constants ($l$) were derived. After this approach (see eq. 3), the $k$ and $l$ values were (1.37 ± 0.14) × $10^6$ $M^{-1}$ $sec^{-1}$ and 44.0 ± 4.4 $sec^{-1}$, respectively (11 experiments). Time-dependent block induced by 100 $\mu$M (*R*)-(+)-ropivacaine also was evident in the tail currents, which were slower in the presence of (*R*)-(+)-ropivacaine increasing the time constant of deactivation from 44.1 ± 5.9 to 221.5 ± 68.0 msec (six experiments; $p <$ 0.05) (Fig. 4). In the presence of (*R*)-(+)-ropivacaine, the tail currents displayed a rising initial phase that reflects the dissociation process of (*R*)-(+)-ropivacaine from drug-bound (not conducting) open hKv1.5 channels (OD → O). Subsequently, the tail current displayed a slower decline because some fraction of the open channels become blocked again rather than closing irreversibly (OD ⇄ OC →). Therefore, a "crossover" phenomenon between the tail current obtained in the presence of drug and that recorded under control conditions was observed, which is indicative of open channel block (Armstrong, 1971; Snyders *et al.*, 1992; Choi *et al.*, 1993; Valenzuela *et al.*, 1995a). Moreover, the experimental results obtained with (*R*)-(+)-ropivacaine were fairly well simulated using a kinetic scheme that assumes it only binds to the open state of hKv1.5 channels (Valenzuela *et al.*, 1995a, 1997; Delpón *et al.*, 1996) (Fig. 5A). This represents further evidence supporting that (*R*)-(+)-ropivacaine blocks hKv1.5 channels by binding to the open state of the channels.

The lack of a time-dependent component of block after channel opening might suggest a different mechanism of block for mepivacaine enantiomers. However, the voltage dependence of block and the tail crossover observed in the presence of both enantiomers suggest an interaction with the open state of the channel. In fact, in the presence of (*R*)-(+)-mepivacaine, tail currents decreased and became slower than in control conditions [27.4 ± 2.3 versus 62.0 ± 4.2 msec in the absence and in the presence of 500 $\mu$M (*R*)-(+)-mepivacaine, respectively; six experiments; $p < 0.01$]. The effects of 500 $\mu$M (*S*)-(−)-mepivacaine were very similar to those observed in the presence of (*R*)-(+)-mepivacaine: tail currents became slower [34.2 ± 4.3 versus 71.2 ± 3.2 msec in the absence and in the presence of (*S*)-(−)-mepivacaine, respectively; four experiments; $p < 0.01$]. In the presence of either enantiomer, the tail currents exhibit a fast initial rising phase, which suggests that the dissociation rate constants for mepivacaine enantiomers were very fast. Therefore, we tested if a kinetic change could explain the interaction between mepivacaine enantiomers and hKv1.5 channels. If we assume that the abrupt transition (compared with control) from the rising phase of channel opening into the reduced steady state level represents fast block (i.e., block occurring on the time scale of channel opening), then the time constant for block at the $K_D$ concentration of (*S*)-(−)- or (*R*)-(+)-mepivacaine should be <4 msec, and the binding rate ($\lambda = k \times [D] + l$) would equal 2$l$. With the constraint that the time constant should be <4 msec, we would expect $l$ to be >125 $sec^{-1}$. Fig. 5B shows the model of the experimental results using a kinetic scheme that assumes that the drug binds to the open state of the channel (Valenzuela *et al.*, 1995a, 1997; Delpón *et al.*, 1996) for two different $l$ values: the lower limit (125 $sec^{-1}$) and 2-fold the lower limit (250 $sec^{-1}$). Assuming an $l$ value of 125

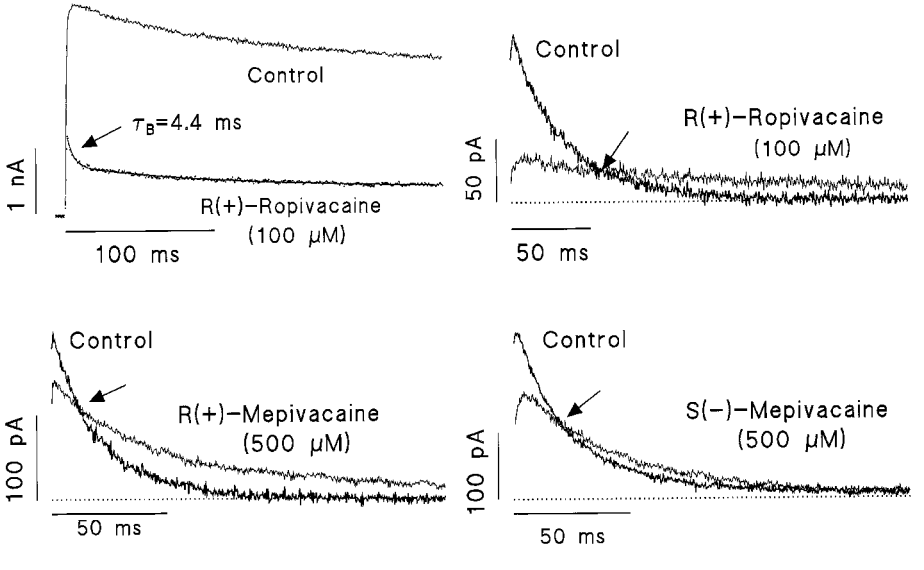


**Fig. 4.** Time dependence of block of hKv1.5 induced by (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine. *Top*, time-dependent block induced by 100 $\mu$M (*R*)-(+)-ropivacaine. *Left*, current records obtained in the absence and presence of 100 $\mu$M (*R*)-(+)-ropivacaine during the application of a depolarizing pulse from −80 to +60 mV. (*R*)-(+)-Ropivacaine induced a fast initial decline of the current, which superimposes to the slow inactivation of the current. *Right*, tail currents obtained under control conditions and in the presence of 100 $\mu$M (*R*)-(+)-ropivacaine. *Bottom*, tail current "crossover" of tails obtained in control conditions and in the presence of either (*R*)-(+)-mepivacaine or (*S*)-(−)-mepivacaine. *Arrow*, the "crossover" between the two traces.

$sec^{-1}$, the association rate constant would be 0.33 $\mu M^{-1}$ $sec^{-1}$, and for this case, the model predicts a time-dependent block of the current and a rising phase in the tail currents, effects we do not observe in the experiments presented in this study. Only the model in which an $l$ value of 250 $sec^{-1}$ [6-fold higher than the $l$ value obtained for (*R*)-(+)-ropivacaine] and a $k$ value of 0.66 $\mu M^{-1}$ $sec^{-1}$ [2-fold lower than the $k$ value for (*R*)-(+)-ropivacaine] were included could reproduce the experimental results obtained with (*R*)-(+)-mepivacaine. The experimental results obtained with (*S*)-(−)-mepivacaine also were well simulated with these kinetic values. These findings confirm that differences in potency between ropivacaine and mepivacaine enantiomers are mainly due to a less stable drug/channel complex in the case of mepivacaine.

## Discussion

We analyzed and compared the effects of (*R*)-(+)-ropivacaine, (*R*)-(+)-mepivacaine, and (*S*)-(−)-mepivacaine on hKv1.5 channels cloned from the human ventricle. The main findings of this study are that (1) (*R*)-(+)-ropivacaine and both mepivacaine enantiomers block the open state of hKv1.5 channels, (2) (*R*)-(+)-ropivacaine is ~10-fold more potent than (*R*)-(+)-mepivacaine and (*S*)-(−)-mepivacaine, and (3) mepivacaine block of hKv1.5 channels is not stereoselective.

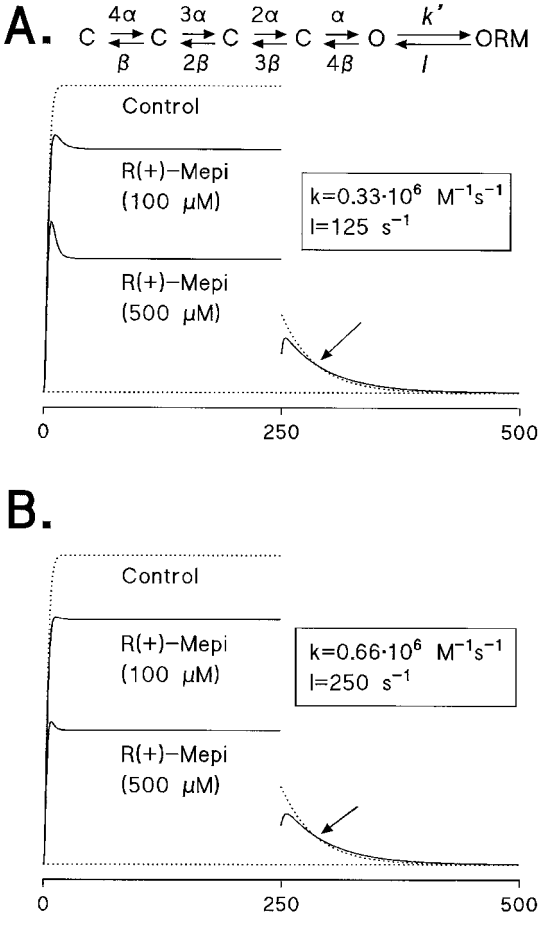


**Fig. 5.** Mathematical modeling of the effects of (*R*)-(+)-mepivacaine on hKv1.5 channels assuming an open-channel block mechanism. Note that only a model in which the dissociation rate constant is increased can reproduce the experimental data.

**(*R*)-(+)-Ropivacaine and (*R*)-(+)- and (*S*)-(−)-mepivacaine block open hKv1.5 channels.** Block of hKv1.5 channels induced by (*R*)-(+)-ropivacaine was concentration, time, and voltage dependent. At concentrations of ≥100 $\mu M$, the most prominent effect of this enantiomer was the induction of a fast decline of the current at the beginning of depolarizing pulses positive to +50 mV, which was faster at higher drug concentrations. This effect suggests an open-channel block mechanism. Moreover, block was voltage dependent, being higher at more positive step potentials, which can be explained by the effect of the transmembrane electrical field on the interaction between the cationic drug and the receptor in the channel. This voltage dependence was consistent with a $\delta$ value of 0.156 ± 0.003. Finally, tail currents exhibited a "crossover" phenomenon, which is typical of open-channel block mechanism (Armstrong, 1971; Snyders *et al.*, 1992; Choi *et al.*, 1993; Valenzuela *et al.*, 1995a). Block induced by either (*R*)-(+)-mepivacaine or (*S*)-(−)-mepivacaine did not exhibit an identifiable time dependency, but block induced by both enantiomers was voltage dependent, which is consistent with a fractional electrical distance similar than that observed with (*R*)-(+)-ropivacaine. Furthermore, the tail currents also exhibited a "crossover" phenomenon. The lack of time-dependent block can be attributed to the very fast dissociation rate constant, which also explains the low affinity of these enantiomers to block hKv1.5 channels. Indeed, a kinetic scheme assuming open-channel block and a very fast dissociation rate constant reproduces fairly well the experimental results (Fig. 5). This finding represents an additional evidence supporting the open-channel block induced by mepivacaine enantiomers. In fact, a similar approach was used to explain differences in potency and time dependence between quinidine and quinine on hKv1.5 channels (Snyders and Yeola, 1995).

**Relationship between the affinity and the alkyl chain length of the *N*-substituent.** Bupivacaine, ropivacaine, and mepivacaine differ only in the length of the *N*-substituent, which is a butyl, propyl, or methyl group, respectively. In an attempt of establish a relation among the potency of block of each enantiomer ($K_D$), the dissociation rate constants ($l$), and the number of methyl groups of the *N*-substituent of these drugs, we plotted $l$ and $K_D$ values versus the number of —$CH_2$ groups (Fig. 6A). For both (*S*)-(−) and (*R*)-(+) enantiomers, the $K_D$ values decreased as the number of —$CH_2$ groups increased, which suggests that the length of the side chain determines the potency of the local anesthetic. The decrease in the $K_D$ values was parallel to the decrease of the $l$ values, which seems to indicate that the lower potency to block hKv1.5 channels by local anesthetics with shorter side chains is derived from a more unstable drug/channel interaction.

**Relationship between the *N*-alkyl chain length and stereoselectivity.** In the current study, we show that block of hKv1.5 channels induced by (*R*)-(+)-ropivacaine exhibited a $K_D$ value of 32 $\mu M$ [i.e., it was 2.5-fold more potent than (*S*)-(−)-ropivacaine] (Valenzuela *et al.*, 1997). It is interesting to note that the stereoselectivity previously described for bupivacaine enantiomers was much higher, with (*R*)-(+)-bupivacaine being 7-fold more potent than (*S*)-(−)-bupivacaine (Valenzuela *et al.*, 1995a; Franqueza *et al.*, 1997). Moreover, in the current study, we also found that mepivacaine block of hKv1.5 channels is not stereoselective. All

these results demonstrate that the length of the *N*-substituent is a key structural determinant of stereoselective block of bupivacaine-related local anesthetics, which suggests that stereoselective block of hKv1.5 channels by this type of local anesthetics involves a hydrophobic interaction between the side chain of the local anesthetic at position 1 and the receptor in the channel. Because the dissociation rate constants are similar for each pair of enantiomers studied and most of their physicochemical properties are identical, stereoselectivity requires a more favored conformation of one of the enantiomers of each drug. This would lead to a faster association rate constant for the more potent enantiomers (*R*) versus the less potent ones (*S*), which would increase as the length of the side chain becomes longer and therefore more hydrophobic. To analyze this hypothesis, we plotted the relationship among the degree of stereoselectivity [$\theta = K_D$ (*R*)-(+)/$K_D$ (*S*)-(−)] (Courtney and Strichartz, 1987), the association rate constants, and the number of —$CH_2$ groups at the N-position (Fig. 6B). As can be observed, the $\theta$ values increased with the number of —$CH_2$ groups. This increase in stereoselectivity (from 1.3 to 7) was accompanied by a parallel increase in the $k$ values obtained for (*R*)-(+)-enantiomers, whereas the $k$ values for the (*S*)-(−)-enantiomers remained constant for the three drugs studied, suggesting that (*S*)-(−) enantiomers need to adopt an energetically less favored conformation than (*R*)-(+) enantiomers to block hKv1.5 channels.

**Hydrophobic interactions determine potency and degree of stereoselectivity.** Several important molecular determinants for block of hKv1.5 channels and *Shaker* $K^+$ channels by alkyl-TEA derivatives, as well as quinidine and bupivacaine, are located in the S6 segment of the $\alpha$ subunit (Yellen *et al.*, 1991; Choi *et al.*, 1993; Yeola *et al.*, 1996; Franqueza *et al.*, 1997). In a previous study, we reported that threonine at position 477 (i.e., hKv1.5 internal TEA binding site) also is involved in the binding of bupivacaine (Franqueza *et al.*, 1997), thus suggesting that bupivacaine binds to a receptor site in the S6 segment, which overlaps the internal TEA binding site, as also was proposed by Baukrowitz and Yellen (1996) for alkyl-TEA derivatives and local anesthetics in *Shaker* $K^+$ channels. The potency of block of hKv1.5 channels induced by alkyl-TEA derivatives in *Shaker* channels is related to the length of the side chain (Villarroel *et al.*, 1988; Choi *et al.*, 1993). Our results demonstrate that the length of the *N*-substituent at position 1 in bupivacaine-related local anesthetics determines the potency of these drugs to block hKv1.5 channels. The observed decrease in the dissociation rate constants with the number of —$CH_2$ groups in the side chain could be explained if the stability of the drug/channel complex is mainly due to a hydrophobic interaction between the alkyl chain of the local anesthetic and some amino acid residue of the channel protein.

Stereoselective bupivacaine block of hKv1.5 channels is determined by a polar and two hydrophobic interactions at positions Thr505, Leu508, and Val512, respectively (Franqueza *et al.*, 1997). The current results indicate that the length of the alkyl substituent at position 1 in the molecule of these drugs also is a chemical determinant of the degree of stereoselectivity. The faster association rate constants observed for (*R*)-(+)-enantiomers suggests that stereoselectivity is mainly determined by a conformation of these enantiomers that

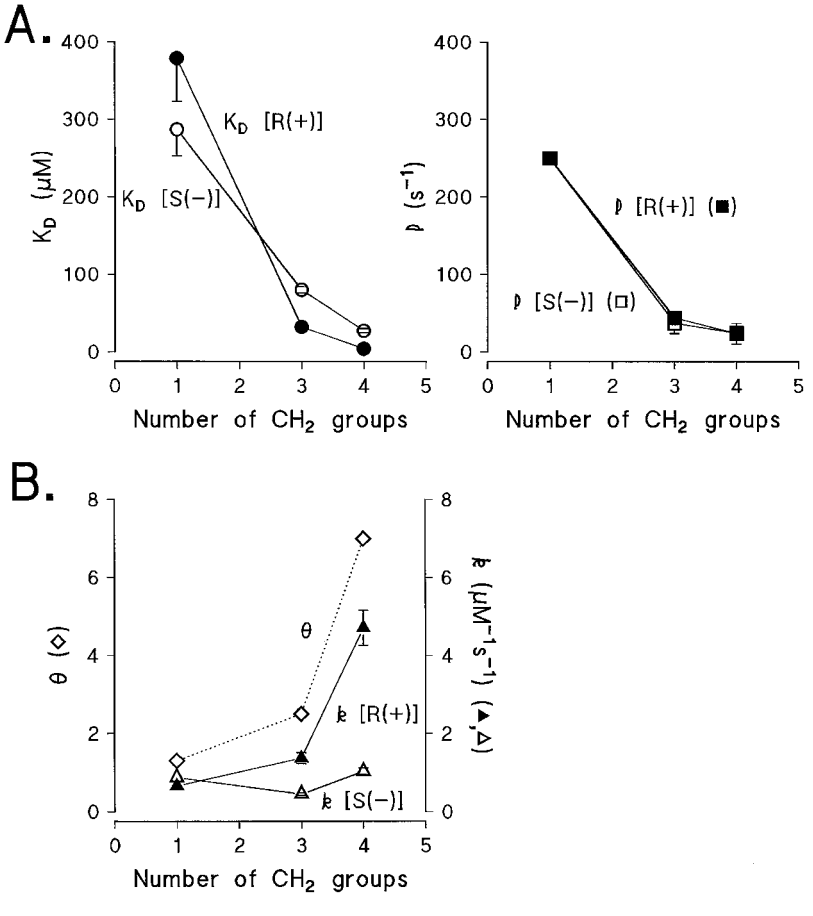


**Fig. 6.** A, Relation between $K_D$ (left panel) and dissociation rate constants ($l$) (*right*) and number of —$CH_2$ groups in the molecule for the two enantiomers of bupivacaine, ropivacaine, and mepivacaine. Note that $K_D$ and $l$ decrease when the number of —$CH_2$ groups increase in the molecule, indicating that the higher potency observed in the presence of bupivacaine enantiomers than in the presence of mepivacaine enantiomers is due to a more stable drug/channel interaction. B, Relation among the degree of stereoselective block ($\theta$), the association rate constant ($k$), and the number of —$CH_2$ groups present in the (*R*)-(+)- and the (*S*)-(−)-enantiomers. The degree of stereoselective block increases when the number of —$CH_2$ groups is higher, as do the $k$ values for these enantiomers, whereas the $k$ values for the (*S*)-(−)-enantiomers did not change with the number of —$CH_2$ groups, suggesting that the degree of stereoselective block is determined by a more favored conformation, which is reflected by a faster $k$ value. Data for bupivacaine enantiomers and (*S*)-(−)-ropivacaine were taken from previous reports from our laboratory (Valenzuela *et al.*, 1995a, 1997).

better allows the hydrophobic interaction between the *N*-substituent and the channel at the S6 segment level. Further studies, using site-directed hKv1.5 mutant channels, are needed to determine the amino acids involved in this interaction.

**Conclusions.** The length of the *N*-substituent of bupivacaine-related local anesthetics ropivacaine and mepivacaine determines the potency of block, being higher with longer chains. Moreover, stereoselectivity also is determined by the length of this side chain, in such a way that only bupivacaine-related anesthetics with alkyl substitutions of at least more than one —$CH_2$ group block hKv1.5 channels in a stereoselective manner.

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**References**

Åberg G (1972) Toxicological and local anaesthetic effects of optically active isomers of two local anaesthetic compounds. *Acta Pharmacol Toxicol* **31:**273–286.

Akerman B, Hellberg I, and Trossvik C (1988) Primary evaluation of the local anaesthetic properties of the amino amide agent ropivacaine (LEA 103). *Acta Anaesthesiol Scand* **32:**571–578.

Aps C and Reynolds F (1978) An intradermal study of the local anaesthetic and vascular effects of the isomers of bupivacaine. *Br J Clin Pharmacol* **6:**63–68.

Armstrong CM (1971) Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J Gen Physiol* **58:**413–437.

Armstrong CM and Hille B (1972) The inner quaternary ammonium ion receptor in potassium channels of the node of Ranvier. *J Gen Physiol* **59:**388–400.

Baukrowitz T and Yellen G (1996) Two functionally subsites for the binding of internal blockers to the pore of voltage-activated $K^+$ channels. *Proc Natl Acad Sci USA* **93:**13357–13361.

Castle NA (1990) Bupivacaine inhibits the transient outward $K^+$ current but not the inward rectifier in rat ventricular myocytes. *J Pharmacol Exp Ther* **255:**1038–1046.

Choi KL, Mossman C, Aube J, and Yellen G (1993) The internal quaternary ammonium receptor site of *Shaker* potassium channels. *Neuron* **10:**533–541.

Courtney KR (1980a) Structure-activity relations for frequency-dependent sodium channel block in nerve by local anesthetics. *J Pharmacol Exp Ther* **213:**114–119.

Courtney KR (1980b) Interval-dependent effects of small antiarrhythmic drugs on excitability of guinea-pig myocardium. *J Mol Cell Cardiol* **12:**1273–1286.

Courtney KR and Strichartz GR (1987) Structural elements which determine local anesthetic activity, in *Local Anesthetics* (Strichartz GR, ed), pp 53–89, Springer-Verlag, Berlin, Germany.

Delpón E, Valenzuela C, Perez O, Franqueza L, Gay P, Snyders DJ, and Tamargo J (1996) Mechanisms of block of a human cloned potassium channel by the enantiomers of a new bradycardic agent: S-16257–2 and S-16260–2. *Br J Pharmacol* **117:**1293–1301.

Franqueza L, Longobardo M, Vicente J, Delpón E, Tamkun MM, Tamargo J, Snyders DJ, and Valenzuela C (1997) Molecular determinants of stereoselective bupivacaine block of hKv1.5 channels. *Circ Res* **81:**1053–1064.

French RJ and Shoukimas JJ (1981) Blockage of squid axon potassium conductance by internal tetra-n-alkylammonium ions of various sizes. *Biophys J* **34:**271–291.

Hille B (1991) *Ionic Channels of Excitable Membranes*. Sinauer Associates, Sunderland, MA.

Longobardo M, Franqueza L, Caballero R, Delpón E, Tamargo J, and Valenzuela C (1997) Block of hKv1.5 channels by R(+)-ropivacaine and R(+)-mepivacaine: structure-activity relationship. *Br J Pharmacol* **122:**88P.

Longobardo M, Caballero R, Delpón E, Tamargo J, and Valenzuela C (1998) Chemical determinants of stereoselective local anesthetics block of hKv1.5 channels. *Biophys J* **74:**A211.

McClure JH (1996) Ropivacaine. *Br J Anaesth* **76:**300–307.

Moller R and Covino BG (1997) Cardiac electrophysiologic properties of bupivacaine and lidocaine compared with those of ropivacaine, a new amide local anesthetic. *Anesthesiology* **72:**322–329.

Snyders DJ, Knoth KM, Roberds SL, and Tamkun MM (1992) Time-, voltage-, and state-dependent block by quinidine of a cloned human cardiac potassium channel. *Mol Pharmacol* **41:**322–330.

Snyders DJ, Tamkun MM, and Bennett PB (1993) A rapidly activating and slowly inactivating potassium channel cloned from human heart: functional analysis after stable mammalian cell culture expression. *J Gen Physiol* **101:**513–543.

Snyders DJ and Yeola SW (1995) Determinants of antiarrhythmic drug action. Electrostatic and hydrophobic components of block of the human cardiac hKv1.5 channel. *Circ Res* **77:**575–583.

Strichartz GR and Ritchie JM (1987) The action of local anesthetics on ion channels of excitable tissues, in *Local Anesthetics* (Strichartz GR, ed), pp 21–52, Springer-Verlag, Berlin, Germany.

Swenson RP Jr (1981) Inactivation of potassium current in squid axon by a variety of quaternary ammonium ions. *J Gen Physiol* **77:**255–271.

Valenzuela C, Sanchez-Chapula J, Delpón E, Elizalde A, Perez O, and Tamargo J (1994) Imipramine blocks rapidly activating and delays slowly activating $K^+$ current activation in guinea pig ventricular myocytes. *Circ Res* **74:**687–699.

Valenzuela C, Delpón E, Tamkun MM, Tamargo J, and Snyders DJ (1995a) Stereoselective block of a human cardiac potassium channel (Kv1.5) by bupivacaine enantiomers. *Biophys J* **69:**418–427.

Valenzuela C, Snyders DJ, Bennett PB, Tamargo J, and Hondeghem LM (1995b) Stereoselective block of cardiac sodium channels by bupivacaine in guinea pig ventricular myocytes. *Circulation* **92:**3014–3024.

Valenzuela C, Delpón E, Franqueza L, Gay P, Snyders DJ, and Tamargo J (1997) Effects of ropivacaine on a potassium channel (hKv1.5) cloned from human ventricle. *Anesthesiology* **86:**718–728.

Villarroel A, Alvarez O, Oberhauser A, and Latorre R (1988) Probing a $Ca^{2+}$-activated $K^+$ channel with quaternary ammonium ions. *Pflueg Arch Eur J Physiol* **413:**118–126.

White M and Bezanilla F (1985) Activation of squid axon $K^+$ channels: ionic and gating current studies. *J Gen Physiol* **85:**539–554.

Woodhull AM (1973) Ionic blockade of sodium channels in nerve. *J Gen Physiol* **61:**687–708.

Yellen G, Jurman ME, Abramson T, and MacKinnon R (1991) Mutations affecting internal TEA blockade identify the probable pore-forming region of a $K^+$ channel. *Science (Washington DC)* **251:**939–942.

Yeola SW, Rich TC, Uebele VN, Tamkun MM, and Snyders DJ (1996) Molecular analysis of a binding site for quinidine in a human cardiac delayed rectifier $K^+$ channel: role of S6 in antiarrhythmic drug binding. *Circ Res* **78:**1105–1114.

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