Kvβ1.3 Reduces the Degree of Stereoselective Bupivacaine Block of Kv1.5 Channels

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Background: Kvβ1.3 subunit modifies the gating and the pharmacology of Kv1.5 channels, decreasing their sensitivity to block induced by drugs, suggesting that Kvβ3 competes with them for a binding site at Kv1.5 channels.

Methods: Currents generated by the activation of Kv1.5 and Kv1.5 + Kvβ1.3 channels expressed in HEK293 cells and Xenopus oocytes were recorded by using whole cell patch clamp and voltage clamp techniques.

Results: Block of Kv1.5, but not that produced on Kv1.5 + Kvβ1.3 channels, was voltage dependent. In both channels, bupivacaine block was time dependent. R(+) and S(−)-bupivacaine blocked Kv1.5 with IC50 4.4 ± 0.5 μM (n = 15) and 39.8 ± 8.2 μM (IC50 < 0.05), respectively (n = 5). These values increased twofold for R(+) -bupivacaine (17.2 ± 2.2 μM) and twofold for S(−)-bupivacaine (71.9 ± 11.5 μM) in Kv1.5 + Kvβ1.3 channels. Therefore, the degree of stereoselectivity (θ) decreased from 9 to 4 in the presence of Kvβ1.3. The decrease in potency to block Kv1.5 + Kvβ1.3 channels was the result of a less stable interaction between bupivacaine enantiomers and channels. Differences in stereoselectivity in each situation were due to a more favorable interaction between the channel and R(+) -bupivacaine. In the presence of Kvβ1.3, stereoselectivity was abolished for VS14A mutant channels (involved in bupivacaine binding but not in Kvβ1.3 binding) but not for L510A (part of Kvβ1.3 binding site).

Conclusions: The degree of stereoselective block of Kv1.5 decreases from 9 to 4 when Kvβ1.3 is present. L510 is determinant for the modulation of bupivacaine block, because it is the only residue of the S6 segment that binds to both bupivacaine and Kvβ1.3. These findings support an overlapping binding site for drugs and Kvβ1.3.

THE diversity of voltage-gated K+ (Kv) channels structure and function is enhanced by heteromultimerization of different α subunits and by their association with accessory Kvβ subunits,1 forming complexes of four α subunits and four β subunits that modify the electrophysiologic characteristics of the α subunit. Kv channels play critical roles in the electrical responses throughout the cardiovascular system, being responsible for establishing the resting membrane potential and cellular repolarization in heart and peripheral vascular beds.2-4 Kv1.5 channels are widely represented in the cardiovascular system.5 In the heart, the Kv1.5 protein has been located in human atrial and ventricular myocardium explaned from newborn and adult patients.6 However, electrophysiologic studies7,8 have shown the absence of Kv1.5-like current in human ventricular myocytes. These results suggest that Kv1.5 channels in human cardiac myocytes are likely due to its ability to depress the intracellular system.5 In the heart, the Kv1.5 protein has been located in human atrial and ventricular myocardium explaned from newborn and adult patients.6 However, electrophysiologic studies7,8 have shown the absence of Kv1.5-like current in human ventricular myocytes. These results suggest that Kv1.5 channels in human atria,9-12 and therefore, it can contribute to the repolarization process of the human atrial action potential.11,12 It has been reported that the expression of Kv1.5 protein is reduced in atrial appendages of chronic atrial fibrillation patients,13 and more recently, a familial form of atrial fibrillation has been attributed to a loss-of-function mutation in Kv1.5.14 In the pulmonary vasculature, Kv1.5 plays a critical role in the oxygen-sensitive regulation of arterial tone.15 Thus, there is significant interest in Kv1.5 as a potential pharmacologic target for diseases such as chronic atrial fibrillation and chronic hypoxic pulmonary arterial hypertension. In the human myocardium and in blood vessels, the expression of two β subunits (Kvβ1.3 and Kvβ2.1) that show overlapping expression patterns with Kv1.5 has been demonstrated. The Kvβ2.1 subunit increases the degree of slow inactivation of the current, whereas Kvβ1.3 induces a fast and incomplete inactivation of the current.16,17 Both, Kvβ2.1 and Kvβ1.3 shift the midpoint of the activation curve toward more negative voltages and slow the deactivation process.16,17 In addition, Kvβ1.3 subunits modify the pharmacology of the Kv1.5 subunits, decreasing their sensitivity to block induced by bupivacaine, quinidine, and S0100176.18,19

Bupivacaine is an amide-type local anesthetic widely used for regional anesthesia20 that exhibits a high cardiotoxicity, likely due to its ability to depress the intracardiac conduction velocity and cardiac contractility.21 These effects have been related to its blocking properties of Na+ and Ca2+ channels.22,23 Moreover, several cases of long QT (QT interval of the electrocardiogram) syndrome have been reported in animal models and in humans.24-26 These cardiotoxic effects have been related to its blocking properties of K+ channels.27-31 Bupivacaine is a racemic drug, and its cardiotoxicity has been mostly related to the effects of its R(+) enantiomer.32,33 R(+) -bupivacaine exhibits a higher potency for
blocking cardiac Na\textsuperscript+ channels and Kv1.5 channels,\textsuperscript{28,34,35} whereas its potency to block human ether-a-go-go-related gene (HERG) channels is lower.\textsuperscript{29,31} Stereoselective bupivacaine interactions with Kv1.5 channels has been shown to involve at least three amino acids located at the S6 segment (T507, L510, and V514).\textsuperscript{30} Mutation of L510 was previously reported to decrease channel block induced by quinidine, bupivacaine, and tetracetylimmonium.\textsuperscript{36–38} Although leucine at position 510 is predicted to face away from the central cavity, it has been recently demonstrated that mutation of this residue to alanine prevents the interaction with Kv\textbeta1.3 and, thus, its ability to induce N-type inactivation and to alter Kv1.5 channel gating.\textsuperscript{18} If L510 determines bupivacaine stereoselectivity and it is also required for Kv\textbeta1.3-induced inactivation, it could be hypothesized that the inactivating ball of the \beta subunit and bupivacaine may compete for the same internal receptor site, as it has been suggested for quaternary ammonium derivatives.\textsuperscript{39} Stereoselective interactions are very specific and suggest direct and specific three-dimensional relations in the receptor site. Therefore, possible changes in the degree of bupivacaine stereoselectivity on Kv1.5 and Kv1.5 + Kv\textbeta1.3 channels may help us to test this hypothesis. Preliminary results have been published in abstract form.\textsuperscript{40}

**Materials and Methods**

The surgery of *Xenopus laevis* for the isolation of oocytes was approved by the Regierungspräsidium in Giessen, Germany.

**Cell Transfection**

Human Kv1.5 (−22 to 1,894 nt) and Kv\textbeta1.3 (−53 to 1,500 nt) were inserted in tandem into the same pBK vector with the Kv1.5 subunit placed 3’ to the Kv\textbeta1.3 subunit and behind an internal ribosome entry sequence, thus generating a dual cistronic messenger RNA as previously described.\textsuperscript{41} The pBK construct used for Kv1.5 alone has been described.\textsuperscript{42} HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine fetal serum, penicillin-streptomycin (Sigma-Aldrich, Alcobendas, Spain), and 1% non-essential amino acids. Transfection of Kv1.5 (0.3 μg) or Kv1.5 + Kv\textbeta1.3 channel (0.3 μg) and reporter plasmid CD8 (1.6 μg) was performed by use of lipofectamine (10 μl). Before experimental use, cells were incubated with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450; Dynal, Oslo, Norway), as previously described.\textsuperscript{19,43} Because HEK293 cells express endogenous Kv currents, the effects of R(+) and S(−)-bupivacaine on untransfected HEK293 cells and on HEK293 cells transfected only with Kv\textbeta1.3 were analyzed. In these experiments, we observed that both bupivacaine enantiomers stereoselectively blocked the endogenous Kv channels. However, the degree of this stereoselective block was not modified by Kv\textbeta1.3. Therefore, in these experiments, we observed that S(−)-bupivacaine (50 μM) blocked the Kv current of untransfected and of Kv\textbeta1.3-transfected HEK293 cells by 69.2 ± 5.1 and 67.2 ± 0.9, respectively (n = 3; P > 0.05). R(+)bupivacaine (10 μM) blocked the Kv current present in untransfected and in Kv\textbeta1.3-transfected HEK293 cells by 26.5 ± 6.5 and 26.9 ± 6.2, respectively (n = 3; P > 0.05). These results indicate that the possible contribution of the endogenous Kv current on the effects of bupivacaine enantiomers is negligible.

**Electrophysiologic Technique and Data Acquisition from HEK293 Cells**

The intracellular pipette filling solution contained 80 mM K-aspartate, 50 mM KCl, 3 mM phosphocreatine, 10 mM KH\textsubscript{2}PO\textsubscript{4}, 3 mM MgATP, 10 mM HEPES-K, and 5 mM EGTA and was adjusted to pH 7.25 with KOH. The bath solution contained 130 mM NaCl, 4 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES-Na, and 10 mM glucose and was adjusted to pH 7.40 with NaOH. Bupivacaine enantiomers (Astra, Södertälje, Sweden) were dissolved in distilled deionized water to yield stock solutions of 10 mM.

Currents were recorded using the whole cell configuration of the patch clamp technique with a patch clamp amplifier (Axopatch 200C patch clamp amplifier, Axon Instruments, Foster City, CA) and stored on a personal computer (TD Systems, Madrid, Spain) with an analog-to-digital converter DigiData 1322A (Axon Instruments). pClamp version 9 software (Axon Instruments) was used for data acquisition and analysis. Currents were recorded at room temperature (21°–23° C) at a stimulation frequency of 0.1 Hz and were sampled at 4 kHz after antialias filtering at 2 kHz. The average pipette resistance ranged between 1 and 3 MΩ (n = 12). Gigaohm seal formation was achieved by suction (2–5 GΩ, n = 12). After seal formation, cells were lifted from the bath, and the membrane patch was ruptured with a brief additional suction. The capacitive transients elicited by symmetrical 10-mV steps from −80 mV were recorded at 50 kHz and filtered at 10 kHz for subsequent calculations of capacitative surface area, access resistance, and input impedance. Thereafter, capacitance and series resistance compensation were optimized and, usually, 80% compensation of the effective access resistance was obtained. Microlab Origin 7.0 (Microlab Software, Northampton, MA) and custom-made programs were used to perform least-squares fitting and for data presentation.

Deactivation and inactivation were fitted to a biexponential process. Therefore, both processes were fitted to an equation of the form

\[
y = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + C,
\]

where \(\tau_1\) and \(\tau_2\) are the system time constants, \(A_1\) and \(A_2\) are the amplitudes of each component of the exponential, and \(C\) is the baseline value. The voltage dependence
of activation curves were fitted with a Boltzmann equation:
\[ y = \frac{1}{1 + \text{exp}(-\frac{E - E_{\text{h}}}{s})} \]
in which \( s \) represents the slope factor, \( E \) represents the membrane potential, and \( E_{\text{h}} \) represents the voltage at which 50% of the channels are open. Drug-induced block was measured at the end of 250-ms depolarizing pulses from -80 to +60 mV. The degree of inhibition obtained for each drug concentration was used to calculate the IC\(_{50}\) and \( n_{\text{H}} \) values from the fitting of these values to a Hill equation of the form \( 1/[1 + (\text{IC}_{50}/[D])^{n_{\text{H}}}] \).28

**Two-electrode Voltage Clamp Recordings in Xenopus Oocytes**

Polymerase chain reaction–based site directed mutagenesis and the human Kv1.5 (\( \text{KCNA5} \)) constructs for expression in \( \text{Xenopus} \) oocytes were previously described.44 Complementary RNA (cRNA) for injection into oocytes was prepared with T7 Capscribe (Roche Diagnostics GmbH, Mannheim, Germany) after linearization with \( \text{Nhe} \). Estimates of cRNA quality and quantity were determined by gel electrophoresis and ultraviolet spectroscopy. Ovarian lobes were dissected from mature \( \text{Xenopus laevis} \) anesthetized with tricaine and treated with collagenase (1 mg/ml, Worthington, type II) in OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), and 5 mM HEPES, pH 7.4) for 120 min. Isolated oocytes were stored at 18°C in ND96 recording solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 5 mM HEPES, pH 7.4) plus Na-pyruvate (275 mg/l), theophylline (90 mg/l), and gentamicin (50 mg/l). Microelectrodes were fabricated from glass pipettes filled with 3M KCl and had a resistance of 0.2–1.0 MΩ. Two to four days after injection of oocytes with cRNA, currents were recorded at room temperature (21°–23°C) with standard two-electrode voltage clamp techniques.

**Statistical Analysis**

Data are presented as mean ± SEM. Comparisons between mean values in control conditions and mean values in the presence of drug for a single variable were performed by use of the paired Student \( t \) test. One-way analysis of variance was used to compare more than two groups. Statistical significance was set at \( P < 0.05 \). 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 chi-square criterion and by inspection for systematic nonrandom trends in the difference plot.

**Results**

Figure 1 shows original records of \( K^+ \) current through Kv1.5 channels expressed in HEK293 cells in the absence and in the presence of \( S(-) \) or \( R(+) \)-bupivacaine (50 \( \mu \)M). Cells were held at -80 mV, and 250-ms depolarizing pulses to +60 mV in 10-mV steps were applied at a frequency of 0.1 Hz. Tail currents were recorded at -40 mV. \( S(-) \) and \( R(+) \)-bupivacaine blocked Kv1.5 channels by 48 ± 6% (\( n = 4 \)) and 87 ± 3% (\( n = 5 \), \( P < 0.05 \)), respectively, when measured at the end of the 250-ms depolarizing step to +60 mV. Figure 2 shows current records through Kv1.5 + \( \text{Kv1.3} \) channels expressed in HEK293 cells obtained in the absence and in the presence of \( S(-) \) or \( R(+) \)-bupivacaine (50 \( \mu \)M) after applying the same pulse protocol. \( \text{Kv1.3} \) produced an initial fast and incomplete inactivation of the current that exhibited a time constant of 3.8 ± 0.4 ms (\( n = 12 \)) as

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**Fig. 1. Effects of bupivacaine enantiomers on Kv1.5 currents.**

Currents are shown for depolarizations from -80 mV to voltages between -80 and +60 mV in steps of 10 mV. Tail currents were obtained on return to -40 mV. Data filtered at 2 kHz (four-pole Bessel) and digitized at 10 kHz.

**Fig. 2. Effects of bupivacaine enantiomers on Kv1.5 + \( \text{Kv1.3} \) currents.**

Currents are shown for depolarizations from -80 mV to voltages between -80 and +60 mV in steps of 10 mV. Tail currents were obtained on return to -40 mV. Data filtered at 2 kHz (four-pole Bessel) and digitized at 10 kHz.
Figure 3. Current–voltage relations of Kv1.5 (A) and Kv1.5 + Kvβ1.3 (B) obtained in control conditions (●) and in the presence of 50 μM S(−)-bupivacaine or R(+)bupivacaine (○).

Figure 4 shows the concentration–response curves when using as an index of block the suppression of the current induced by bupivacaine enantiomers at the end of 250-ms depolarizing pulses to +60 mV. For R(+) and S(−)-bupivacaine, nonlinear least-square fits yielded IC50 values of 4.4 ± 0.5 μM (n = 15) and 39.8 ± 8.2 μM (n = 16; P < 0.05), respectively. Therefore, the degree of stereoselectivity for bupivacaine block of Kv1.5 channels (θ) (obtained from the ratio IC50,S(−)-Bupi/IC50,R(+)Bupi) was 9. The potency of bupivacaine enantiomers to block Kv1.5 + Kvβ1.3 channels decreased with respect to that observed in the absence of Kvβ1.3, this decrease in potency being greater for R(+)bupivacaine (fourfold, 17.2 ± 2.2 μM, n = 25) than for S(−)-bupivacaine (twofold, 71.9 ± 11.5 μM, n = 31). Therefore, the θ for bupivacaine block of Kv1.5 + Kvβ1.3 channels achieved a value of 4 (ns. 9 in the absence of Kvβ1.3) in the presence of Kvβ1.3.

Figure 5A (top) shows normalized Kv1.5 current traces obtained in the absence and in the presence of 10 μM R(+) or S(−)-bupivacaine after applying 250-ms depolarizing pulses to +60 mV from a holding potential of −80 mV. Concentrations higher than 1 μM R(+)bupivacaine induced a fast initial decline of the current that was superimposed onto the slow inactivation (figs. 1 and 5). The time constant of this drug-induced initial current decline was faster at higher concentrations, and therefore, it was considered as a good index of the kinetics of block (τb). Figure 5A (bottom) shows graphs in which 1/τb was plotted versus different R(+) and S(−)-bupivacaine concentrations. The straight line represents the least-squares fit to the relation 1/τb = k × [Bupi] + 1, which yielded apparent association (k) and dissociation (l) rate constants of 5.7 ± 0.8 μM⁻¹·s⁻¹ and 22.8 ± 3.1

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s⁻¹ (n = 14), respectively, for R(+)-bupivacaine. For S(−)-bupivacaine, the fast initial decline was observed at higher concentrations (>10 μM), and the k and l reached mean values of 0.8 ± 0.2 μM⁻¹ · s⁻¹ (P < 0.05) and 33.0 ± 6.5 s⁻¹ (n = 13; P > 0.05), respectively. Therefore, the dissociation rate constants were similar for both bupivacaine enantiomers (22.8 ± 3.1 vs. 33.0 ± 6.5 s⁻¹, P > 0.05, respectively), suggesting that the stereoselectivity derives from the difference in the association rate constants. R(+) and S(−)-bupivacaine induced block of Kv1.5 + Kvβ1.3 was also time dependent (fig. 5B). To quantify the kinetics of Kv1.5 + Kvβ1.3 block for R(+) and S(−)-bupivacaine, we represented the ratio between the drug-sensitive current and the current in control conditions [(I_{Control} − I_{Drug})/I_{Control}] during the first 12 ms in the presence of different drug concentrations (fig. 5B, bottom). The time constant of this process was considered a good index of development of block (τ₀). Similarly to that described in Kv1.5 channels, we represented 1/τ₀ against different R(+) and S(−)-bupivacaine concentrations and fitted these values to a straight line. From these fits, k and l values were derived. For R(+)bupivacaine, k and l values averaged 6.7 ± 1.3 μM⁻¹ · s⁻¹ (n = 25) and 145.7 ± 33.2 s⁻¹ (n = 25), respectively. Values of k and l averaged 2.6 ± 0.5 μM⁻¹ · s⁻¹ (n = 18) and 182.8 ± 34.0 s⁻¹ (n = 18) for S(−)-bupivacaine. Similarly to what happened in Kv1.5 channels, the dissociation rate constants resulted to be similar for both enantiomers, and therefore, stereoselectivity may derive from differences in the association rate constants. Figure 6 summarizes the degree of stereoselectivity observed in Kv1.5 and Kv1.5 + Kvβ1.3 channels. As can be observed, the degree of stereoselectivity decreases in a parallel manner to the ratio of the association rate constants and independently from the dissociation rate constants, which are similar for both enantiomers either in the absence or in the presence of the Kvβ1.3 subunit.

Time dependency was also observed in the deactivation process of the current (fig. 7). Under control conditions, the deactivating process of Kv1.5 channels was best fitted to a biexponential process with slow (τ₁) and fast (τ₀) time constants. On the contrary, the deactivating process of Kv1.5 + Kvβ1.3 channels was monoexponential, as previously reported.19,42 In both experimental conditions, the deactivation became slower in the presence of either bupivacaine enantiomer, as can be observed in figure 7 and in table 1. Moreover, in all cases, a “crossover” phenomenon indicative of an open channel block interaction was also observed.28,45

All these results could be explained if both, the inactivating Kvβ1.3 particle and bupivacaine compete for a receptor site, in which case, bupivacaine enantiomers would be able to slow down the N-type inactivation induced by the Kvβ1.3 subunit. In fact, as it is shown in figure 8, this is what happened in the presence of 1 μM of either R(+) or S(−)-bupivacaine. The time constant of inactivation (τ_{inact}) in the absence and in the presence of R(+)bupivacaine averaged 3.2 ± 0.4 and 5.0 ± 0.9 ms (n = 5; P < 0.05), respectively. The τ_{inact} values also increased from 3.8 ± 0.8 to 4.3 ± 0.7 ms (n = 5; P < 0.05) in the absence and in the presence of S(−), respectively.

**Effects of Bupivacaine Enantiomers on S6 Mutant Kv1.5 Channels with or without Kvβ1.3**

To directly analyze the possible competition between bupivacaine and the Kvβ1.3 subunit, we studied the inhibition induced by bupivacaine enantiomers of wild-type Kv1.5 and mutant Kv1.5 channels in the ab-
Fig. 5. Time-dependent block of Kv1.5 and Kv1.5 + Kvβ1.3 channels. (A) Time-dependent block of Kv1.5 channels induced by S(-)- and R(+)-bupivacaine. Top: Superimposed traces for steps from -80 to +60 mV and normalized to match control values. Bottom: Relation between $1/\tau_B$ values at different S(-)- and R(+)-bupivacaine (from 10 to 300 μM). Each point represents the mean ± SEM of three to seven experiments. For a first-order blocking scheme, a relation is expected: $1/\tau_B = k \times [\text{Drug}] + l$. The solid line represents the fit from which the apparent binding (k) and unbinding (l) rate constants were obtained. (B) Time-dependent block of Kv1.5 + Kvβ1.3 channels induced by S(-)- and R(+)-bupivacaine. Top: Relation between the sensitive current and depolarizing time at three different bupivacaine enantiomers concentrations. Bottom: Relation between $1/\tau_B$ values at different S(-)- and R(+)-bupivacaine (from 10 to 1,000 μM). Each point represents the mean ± SEM of three to seven experiments. The solid line represents the fit from which the apparent binding and unbinding rate constants were obtained.
sence and presence of Kvβ1.3, using two electrode voltage clamp recordings in Xenopus oocytes. We focused on two residues at the S6 level previously described as part of the bupivacaine binding site, L510 and V514.36 To test whether these residues also mediate the loss of stereoselectivity induced by Kvβ1.3, we studied the inhibition potencies of the bupivacaine enantiomers for these mutants alone or in presence of Kvβ1.3. Overall bupivacaine affinity was reduced compared with recordings in HEK293 cells, a phenomenon previously described for Xenopus oocytes and other drugs. Similar as in HEK293 cells, the R(+)-bupivacaine was more potent blocking wild-type Kv1.5 channels, and stereoselectivity was reduced in the presence of Kvβ1.3 (fig. 9A). The mutations L510A and V514A preserved the stereoselectivity in the absence of Kvβ1.3 (figs. 9B and C), as previously described.36 In the presence of Kvβ1.3, stereoselectivity was abolished for V514A mutant channels but not for L510A. The residue L510 is, in contrast to V514, a part of the Kvβ1.3 binding site18 and the bupivacaine binding site.36 Consistently, the Kvβ1.3 mediated reduction in stereoselectivity was not present for the L510A mutation (fig. 9B), which lacks inhibition by Kvβ1.3.

**Discussion**

In the current study, we have analyzed and compared the interactions between bupivacaine enantiomers and Kv1.5 or Kv1.5 + Kvβ1.3 channels transiently expressed in HEK293 cells. The main findings of the current study are as follows: (1) the potency of both bupivacaine enantiomers to block these currents decreases when Kvβ1.3 is present; (2) the decrease in potency is higher for R(+)-bupivacaine than for S(-)-bupivacaine; (3) the degree of stereoselective block of Kv1.5 channels decreases from 9 to 4 when the Kv1.5 subunit is assembled with Kvβ1.3; (4) bupivacaine enantiomer slow the time

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**Table 1. Time Constants of the Deactivation Process of Kv1.5 and Kv1.5 + Kvβ1.3 Channels in the Absence (Control) and in the Presence of Either Bupivacaine Enantiomer**

<table>
<thead>
<tr>
<th></th>
<th>Kv1.5</th>
<th>Kv1.5 + Kvβ1.3</th>
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<tbody>
<tr>
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<tr>
<td>$\tau_i$, ms</td>
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<tr>
<td>$\tau_d$, ms</td>
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<td>$\tau_d$, ms</td>
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<td>83.1 ± 16.0*</td>
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<tr>
<td>$\tau_d$, ms</td>
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<td>92.5 ± 3.8*</td>
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<td>S(-)-bupivacaine (10 $\mu$M)</td>
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<tr>
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<td>19.7 ± 6.2*</td>
<td>86.8 ± 34.9*</td>
</tr>
<tr>
<td>$\tau_d$, ms</td>
<td>86.8 ± 34.9*</td>
<td>83.2 ± 7.2*</td>
</tr>
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* $P < 0.05$ compared with control values.

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**Fig. 6. Relation between the degree of stereoselective bupivacaine block ($\theta$), the association rate constants ratio for bupivacaine enantiomers, and the dissociation rate constants ratio for both bupivacaine enantiomers. Note that the decrease of the degree of stereoselectivity is accompanied by a parallel decrease in the association rate constants ratio and that is independent of the dissociation rate constant ratio.**

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**Fig. 7. Tail current crossover of Kv1.5 and Kv1.5 + Kvβ1.3 channels. (A) Superimposed tail Kv1.5 currents recorded on return to $-40$ mV, under control conditions and with 10 $\mu$M $S(-)$- and $R(+)$-bupivacaine. (B) Superimposed tail Kv1.5 + Kvβ1.3 currents recorded on return to $-40$ mV, under control conditions and with 10 $\mu$M $S(-)$- and $R(+)$-bupivacaine. Arrows show the “crossover” of traces recorded in the presence of drug with that recorded under control conditions.**
course of the N-type inactivation induced by Kvβ1.3; and (5) Kvβ1.3 subunit abolishes stereoselective bupivacaine block only when it binds properly to the inner cavity of Kv1.5 channels.

Open Channel Block of Kv1.5 and Kv1.5 + Kvβ1.3 Channels

Similarly to racemic bupivacaine and to bupivacaine enantiomers on Kv1.5 expressed in Ltk" cells (that endogenously express Kvβ2.1 subunits), bupivacaine enantiomers exert their actions on Kv1.5 by interacting with the open state of the channel. There are several pieces of evidence that support this open channel mechanism: (1) Bupivacaine enantiomers induce an initial decline of the maximum outward current that exhibited a time course faster as the concentration of either bupivacaine enantiomer increased. (2) Block increased as the depolarizing step was more positive, i.e., when the open channel probability increases. Indeed, the IC_50 for R(+) - and S(-)-bupivacaine when measured at the end of 250-ms depolarizing pulses from −80 to 0 mV were 21.3 ± 1.2 μM (vs. 4.4 ± 0.5 μM when measured at +60 mV; P < 0.05) and 71.5 ± 19.7 μM (vs. 39.8 ± 8.2 μM when measured at +60 mV; P < 0.05), respectively. (3) Finally, the time course of deactivation became slower in the presence of either bupivacaine enantiomer and, after superimposing control and bupivacaine tail currents, a “crossover” of the currents, indicative of an open channel block mechanism, was observed. Bupivacaine enantiomer-induced block of Kv1.5 + Kvβ1.3 channels seems also to be the consequence of an open-channel interaction. Similarly to that observed in Kv1.5 channels, (1) bupivacaine enantiomers produced an initial current decline that was faster as the drug concentration was increased; and (2) the time course of deactivation became slower in the presence of either bupivacaine enantiomer, and after superimposing control and bupivacaine tail currents, a “crossover” of the currents was observed. However, block was not voltage dependent. In fact, the IC_50 for R(+) - and S(-)-bupivacaine when
measured at the end of 250-ms depolarizing pulses from −80 to 0 mV were 17.0 ± 3.4 μm (rs. 17.2 ± 2.2 μm when measured at +60 mV; P > 0.05) and 81.7 ± 30.1 μm (rs. 71.9 ± 11.5 μm when measured at +60 mV; P > 0.05). This lack of voltage-dependent block of Kv1.5 + Kvβ1.3 channels induced by bupivacaine enantiomers may be due to the competition between the bupivacaine molecule and Kvβ1.3. In fact, it has been described that the inactivation of Kv1.5 induced by Kvβ1.3 exhibits a voltage dependency similar to that induced by bupivacaine enantiomers in Kv1.5.32,47 which supports the hypothesis of a competition between bupivacaine and Kvβ1.3.

**Stereoselective Block of Kv1.5 and Kv1.5 + Kvβ1.3 Channels Induced by Bupivacaine**

The results presented in this study demonstrate that bupivacaine enantiomers block Kv1.5 and Kv1.5 + Kvβ1.3 channels in a stereoselective manner. The degree of stereoselectivity decreases from a value of 9 to 4, when examined in Kv1.5 and Kv1.5 + Kvβ1.3 channels, respectively. The IC50 values obtained from the block induced by bupivacaine enantiomers of Kv1.5 and Kv1.5 + Kvβ1.3 channels can be assumed as a good approximation of the apparent dissociation constant (K_D) values, which can be converted into apparent binding energies (∆G) relative to a 1 m standard concentration using the relation

\[ \Delta G = -RT \times \ln[1 \text{ m/K}_D], \]

where R is the universal gas constant and T is the absolute temperature. The free energy indicates the relative stability of the drug–channel complex. The values for ∆G were −7.20 and −5.91 kcal/mol for Kv1.5 block induced by R(+)- and S(−)-bupivacaine, respectively, and −6.40 and −5.57 kcal/mol for Kv1.5 + Kvβ1.3 block induced by R(+)- and S(−)-bupivacaine, respectively. Therefore, the difference in the degree of stereoselectivity between Kv1.5 and Kv1.5 + Kvβ1.3 channels corresponds to a difference in free energy of 1.31 and 0.83 kcal/mol between R(+)- and S(−)-bupivacaine blocked channels, respectively. This may reflect a more stable interaction of the R(+)bupivacaine with the channel. However, differences in potency between both enantiomers were largely explained by their different association rate constants, which were faster for R(+)bupivacaine in both experimental conditions. Because these drugs are enantiomers, most of their properties are identical, including the free energy associated with similar mirror image conformations. Therefore, differences in association rate constants suggest that the S(−) enantiomer needs to adopt a less favorable conformation, i.e., 1.31 and 0.83 kcal/mol above the conformation in which R(+)-bupivacaine binds to Kv1.5 and Kv1.5 + Kvβ1.3 channels, respectively. All these results, together with the fact that both enantiomers, at low concentrations, slow down the fast inactivation induced by Kvβ1.3, can be explained by a competition mechanism between bupivacaine enantiomers and Kvβ1.3.

**Partial Overlap between Kvβ1.3 and Bupivacaine Binding Site in Kv1.5**

From the results obtained in mutant Kv1.5 channels alone or assembled with Kvβ1.3 subunit, we conclude that Kvβ1.3 reduces the stereoselectivity of bupivacaine block, by a direct binding to the inner cavity of the Kv1.5 channels. The L510A mutant (in contrast to L510M) preserves stereoselectivity of bupivacaine block36 while inhibition by Kvβ1.3 is diminished.18 As L510A is a part of the Kvβ1.3 binding site,18 the mutant L510A channels are not blocked by Kvβ1.3. Accordingly, Kvβ1.3 is not able to eliminate the stereoselectivity of bupivacaine block. In contrast, the stereoselectivity of mutant V514A channels, which have an intact Kvβ1.3 binding ability,18 remains modulated by the inhibition of Kvβ1.3. The residue L510 is particularly important for the modulation of bupivacaine block, because from our current knowledge L510 is the only residue of the S6 segment that is binding to both bupivacaine36,37 and Kvβ1.3.18 These findings support our previous reports of an overlapping binding site for drugs and Kvβ1.318,19 and pinpoint the stereoselective effects of Kvβ1.3 to the S6 segment residue L510.

Previous studies have analyzed the pharmacologic consequences of the assembly of HERG + MiRP1 and Kv4.3 + KChIP2.2,30,48,49 Whereas MiRP1 did not modify the degree of block produced by bupivacaine in HERG channels, bupivacaine interfered with the effects of KChIP2.2 on Kv4.3. The results obtained in the current study demonstrate that bupivacaine and Kvβ1.3 compete for a binding site that overlaps for bupivacaine and Kvβ1.3 at the inner cavity of the Kv1.5 channel. This unique feature observed in Kv1.5 + Kvβ1.3 seems to be the consequence of their interaction, because the N-terminus of the β subunit (like bupivacaine and other antiarrhythmic drugs) blocks the open state of Kv1.5 channels, whereas KChIP2.2 assembly to the N-terminal tetramerization (T1) domain of Kv4.3, it does not interact with the ion pore of the channel.50

**Clinical Consequences of This Study**

The degree of stereoselective bupivacaine block of Kv1.5 + Kvβ1.3 channels strongly decreases with respect to the stereoselective block of Kv1.5 channels (from 9 to 4). The differences in free energy of bupivacaine enantiomers for blocking Kv1.5 and Kv1.5 + Kvβ1.3 channels correspond with 0.80 and 0.54 kcal/mol for R(+) and S(−)-bupivacaine, respectively. These differences reflect a more stable interaction between bupivacaine enantiomers with Kv1.5 than with Kv1.5 + Kvβ1.3 channels, as observed by the higher dissociation rate constants obtained for the interaction with Kv1.5 +

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Kvβ1.3 channels. However, the difference in free energy mostly affects to the more potent blocker, R(+)-bupivacaine. It has been described that the levels of Kvβ1.3 expression vary in the different heart regions and also in different smooth muscle cells.\(^{16,51}\) Kvβ subunits can modulate the pharmacology of Kv1 channels in addition to their well-characterized ability to alter channel gating and expression. For example, Kvβ1.3 subunits reduce the block of Kv1.5 by racemic bupivacaine, quinidine, and S0100176.\(^{18,19}\) Previous findings suggested that S0100176 and the Kvβ1.3 subunit compete for an overlapping but not identical binding site located in the inner cavity of Kv1.5.\(^{18}\) Moreover, the Kv1.5 pore-blocker AVEO1118 interacts with a similar subset of residues at the S6 than S0100176 including residues L510 and V516, similar to the binding of Kvβ1.3.\(^{52}\) The current study demonstrates that the assembly of Kv1.5 and Kvβ1.3 subunits decreases the block induced by bupivacaine enantiomers and also the degree of stereoselectivity. Therefore, the sensitivity to Kv1.5 channel blocking drugs will vary depending on the regional distribution of β regulatory subunits. The expression of Kvβ1.3 subunits in the myocardium is not homogeneous, for this subunit is expressed to a higher degree in the ventricle than in atria.\(^{53}\) Similarly to Kvβ1.3, KChIP2.2 subunits show a gradient of expression in cardiac ventricle, and it has been suggested that this ancillary subunit influences the effects of local anesthetics on Kv channels.\(^{49}\) In accordance, Kvβ1.3 might affect bupivacaine block of Kv1.5 channels in a transmural fashion. Moreover, a Kvβ1.3 gradient might be important for the transmural distribution of K\(^+-\) channels, especially because Kvβ1.3 might be influencing the expression of a variety of Kv channels.\(^{54}\) Within various vascular beds, there are marked differences in β subunit expression, whereas Kv1.5 levels change little.\(^{51}\) Therefore, the differential assembly between the Kvα and Kvβ subunits present in the cardiovascular system is a variable to be accounted for the development of new ion channel modifying agents and can account, at least partially, for the less cardiotoxicity observed with S(−)-bupivacaine.

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References

31. Delpo E, Snyders DJ, Bennett PB, Tamargo J, Hondeghem LM: Stere-
selective block of cardiac sodium channels by bupivacaine in guinea pig ventricular myocytes. Circulation 1995; 92:3014–24
40. Arias C, David M, Guizy M, Valenzuela C: Kvβ1.3 reduces the degree of stereoselective bupivacaine block of Kv1.5 channels (abstract). Biophys J 2006; 97A
49. Solth A, Siebrands CG, Friederich P: Inhibition of Kv4.3/KChIP2.2 channels by bupivacaine and its modulation by the pore mutation Kv4.3V401I. Anesthesiology 2005; 103:796–804

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