Effects of the enantiomers of a new local anesthetic, IQB-9302, on a human cardiac potassium channel (Kv1.5)

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Introduction

Local anesthetics block the generation and conduction of nerve impulses by inhibiting the current through voltage-gated Na^+ channels in the nerve cell membrane (Hille, 1977; Hondeghem and Katzung, 1977; Strichartz and Ritchie, 1987). Nevertheless, all voltage-gated Na^+ channels exposed to sufficient concentrations of these agents will be affected. This can explain why the accidental intravascular injection or the use of high concentrations of local anesthetics can produce profound systemic effects, especially in the central nervous and cardiovascular systems (Covino, 1987). Indeed, several local anesthetics also exhibit class I antiarrhythmic actions on the myocardium at lower concentrations than those used for local anesthesia (Grant et al., 1984; Bennett et al., 1995).

Bupivacaine is a potent local anesthetic widely used for long-lasting regional local anesthesia. In isolated cardiac tissues, bupivacaine decreases intracardiac conduction velocity and contractile force and depresses spontaneous sinoatrial activity (Block and Covino, 1982; Moller and Covino, 1988). In anesthetized animals, bupivacaine decreases cardiac output, myocardial contractility, and intracardiac conduction velocity as evidenced by increased PR and QRS durations (Covino, 1987; Liu et al., 1982). Furthermore, several studies have shown a correlation between cardiac sodium channel (I_{Na}) inhibition and depression of the cardiovascular system (Covino, 1987; Block and Covino, 1988). Although the high potency of bupivacaine to inhibit I_{Na} (Clarkson and Hondeghem, 1985) can partially explain its high cardiotoxicity, it has also been shown that bupivacaine induces a prolongation of the QTc interval of the ECG in anesthetized dogs (Avery et al., 1984; Wheeler et al., 1988; Solomon et al., 1989) 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 both block of Na^+ and K^+ channels, as it has been demonstrated (Valenzuela et al., 1995a;b). Ropivacaine, the S(−) enantiomer of AL381, which is chemically related to bupivacaine was synthetized as a less toxic alternative to bupivacaine. Although cardiotoxicity of this compound is lower than that previously reported to bupivacaine (Scott et al., 1989; Akerman et al., 1988; Pitkanen et al., 1992), it has also been demonstrated that ropivacaine is able to induce the appearance of early-depolarizations in isolated preparations (Moller and Covino, 1990), probably secondary to a blockade of K^+ channels.

IQB-9302 is a new amide type local anesthetic, chemically related to bupivacaine and synthetized as a less toxic alternative to bupivacaine and ropivacaine. Preliminary experiments have demonstrated that it is less cardiotoxic than bupivacaine. In order to test this

hypothesis we will study the effects of IQB-9302 on hKv1.5 channels. It will permit us to know if this drug would inhibit the native counterpart of this current (I_{Kur}) (Wang et al., 1993), since selective block of this current affects action potential duration in human atria (Wang et al., 1993), thus indicating that hKv1.5 current represents one of the molecular targets for class III antiarrhythmic agents. Therefore, the purpose of this study is to determine the mechanism of action of IQB-9302 on hKv1.5 channels expressed in a mammalian cell line (*Ltk*-) without the complications of overlapping currents.

Materials and Methods

Electrophysiological recording

Use of the stable *Ltk*$^-$ cell line expressing Kv1.5 channels has been described previously in detail (Snyders et al., 1993, Valenzuela et al., 1995; Franqueza et al., 1997; Longobardo et al., 1998). The intracellular pipette filling solution contained (in mM): K-aspartate 80, KCl 50, phosphocreatine 3, KH_2 PO_4 10, MgATP 3, HEPES-K 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-Na 10, and glucose 10, and was adjusted to pH 7.40 with NaOH. IQB-9302 enantiomers were dissolved in distilled deionized water to yield a stock solution of 10 mM from which further dilutions were made to obtain the desired final concentration. 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. 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. Data acquisition and command potentials were controlled by the pCLAMP 6.0.1 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±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 resistence compensation were optimized, and 80% compensation of the effective access resistance was usually obtained.

Pulse protocol and analysis

The holding potential was maintained at –80 mV unless indicated otherwise. After control data were obtained, bath perfusion was switched to drug-containing solution. The

effects of drug infusion was monitored with test pulses to +60 mV, applied every 10 s until steady-state was obtained (after −12 s). Steady-state current-voltage relationships (IV) were obtained by averaging the current over a small window (2-5 ms) at the end of 250 ms depolarizing pulses. 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 either at +40 mV. The activation curve was obtained from the tail current amplitude immediately after the capacitive transient. Measurements were done using the CLAMPFIT program of PCLAMP 6.0.1 and by a custom-made analysis program.

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

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

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

$$k \cdot [D] + l = 1 / \tau_B = \lambda \quad (2a)$$

$$l / k = K_D \quad (2b)$$

in which τ_B represents the time constant of the fast initial drug-induced current decay after activation from the holding potential to +60 mV. The activation kinetics of hKv1.5 channels have been described as a sigmoidal process (Snyders et al., 1993). However, in the present study and in order to describe the dominant time constant of this process and the effects of drugs on it, the latter part of the current was fitted to a single exponential, following a procedure previously described and used for the same purpose (White and Bezanilla, 1975; Snyders et al., 1993; Valenzuela et al., 1995; Delpón et al., 1996). On the other hand, deactivation and inactivation were fitted to a biexponential process. Thus, both processes were fitted to an equation of the form:

$$y = C + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + ... + A_n \exp(-t/\tau_n) \quad (3)$$

where τ_1, τ_2 ... τ_n are the system time constants, A_1, A_2 ... A_n 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: $y = 1/[1 + \exp(!(E!E_h)/s)]$, 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 curve-fitting procedure used a non-linear 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.

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:

$$f = [D]/([D] + K_D^* \cdot \exp(-\delta z FE/RT)), \quad (4)$$

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 and in the presence of drug for a single variable were performed by paired Student's *t*-test. Student's *t*-test was also used to compare two regression lines. Differences were considered significant if $p<0.05$.

Results

Figure 1 shows original hKv1.5 current records obtained in the absence and in the presence of 50 μM S(−)IQB-9302 or R(+)IQB-9302. The holding potential was maintained at -80 mV and 250 ms depolarizing pulses in duration to membrane potentials between -80 mV and +60 mV were applied. Tail currents were recorded upon repolarization to -40 mV. Under control conditions hKv1.5 current activates rapidly, reaches a maximum peak current and slowly inactivates, as previously described (Snyders et al., 1993). At 50 μM, both enantiomers inhibited hKv1.5 current, and R(+)IQB-9302 was 4-fold more potent than the S(−) enantiomer (70.4 ± 3.0%, n=4, *vs* 44.8 ± 3.6%, n=8, $p<0.01$, respectively). The effects of both enantiomers were reversible upon perfusion of the cells with drug-free external solution (94 ± 1% of the control values, n=21). S(−)IQB-9302 or R(+)IQB-9302 did not modify the activation kinetics of the current [1.33 ± 0.14 ms *vs* 1.16 ± 0.12 ms, n=6, $p>0.05$; and 1.73 ± 0.20 ms *vs* 1.27 ± 0.04, n=6, $p>0.05$; respectively]. Their more prominent effect was the induction of a fast decline of the current at the beginning of the depolarizing pulse, which was faster at higher drug concentrations, thus suggesting an open channel block mechanism.

Block induced by S(−)IQB-9302 and R(+)IQB-9302 was concentration dependent. Figure 2 shows the concentration response curve when using as an index of block the suppression of the current induced by both enantiomers at +60 mV at the end of 250 ms depolarizing pulses. A non-linear least-squares fit of the concentration-response equation (see eq. 1 in Materials and Methods section) yielded, for S(−)IQB-9302, a K_D and the n_H values of 65.3 ± 5.0 μM and 0.879 ± 0.061, respectively. For R(+)IQB-9302, these values averaged 18.6 ± 1.5 μM and 0.806 ± 0.055. When n_H was fixed to unity, the K_D values for S(−)IQB-9302 and R(+)IQB-9302 were 64.4 ± 5.2 μM ($p>0.05$) and 18.7 ± 1.9 μM ($p>0.05$), suggesting that the binding of only one molecule of the drug was enough to block potassium permeation through the ion pore.

Voltage dependence of IQB-9302 induced block of hKv1.5 channels

Figure 3 shows the IV relationships obtained in the absence and in the presence of either S(−)IQB-9302 (50 μM) or R(+)IQB-9302 (10 μM). Both enantiomers decreased the amplitude of the current at all membrane potentials tested, although, the inhibition of the current was more pronounced at more positive than at more negative membrane potentials, thus suggesting an open channel block mechanism similar to that described for bupivacaine, ropivacaine and mepivacaine enantiomers (Valenzuela et al., 1995, 1997; Longobardo et al.,

1998). In fact, both enantiomers induced a downward curvature of the IV relationship, indicating an open channel block mechanism. In order to better quantitate this voltage dependence we represented the relative current in the presence of either 50 μM S(−)IQB-9302 or 10 μM R(+)IQB-9302 *versus* membrane potential. As it can be observed, block steeply increased in the range of membrane potentials of the activation of the channels, which indicates that the drug needs that the channel will open before it can bind. At membrane potentials positive to 0 mV a shallower but significant increase in block was observed. Both enantiomers are weak bases (pKa=7.99 ± 0.006) and thus, they are predominantly charged at the physiological pH. Thus, this increase in block can be attributed to the effect of the transmembrane electrical field on the interaction between the cationic form of the drug and the receptor at the channel. This effect can be explained following a Woodhull formalism (Woodhull, 1973). By using this model, a non linear curve fitting of the data (see eq. 4 in the Materials and methods section) yielded apparent dissociation constant at the reference potential (0 mV) (K_D^*) and the fractional electrical distance from the inner side of the membrane (δ). In the presence of S(−)IQB-9302 and R(+)IQB-9302 the δ values averaged 0.192 ± 0.026 and 0.181 ± .018, respectively. This indicates that they have to cross ~18% of the transmembrane electrical field to bind their receptor site in the hKv1.5 channel. The K_D^* averaged 103.5 μM and 28.7 μM for S(-)IQB-9302 and R(+)IQB-9302 , respectively.

Time dependent block

One of the more prominent effects observed in the presence of IQB-9302 enantiomers was the appearance of a fast initial decline of the current which was superimposed to the slow inactivation (Figure 4a). In the presence of S(-)IQB-9302 and R(+)IQB-9302 this fast decline appeared at concentrations higher than 100 μM and 10 μM, respectively. The time constant of this fast decline of the current was lower at higher concentrations and thus, it was considered to be a good index of the kinetics of binding of the drug (τ_B). From the values of τ_B we calculated the apparent drug association (*k*) and dissociation (*l*) kinetics constants (see eq. 2 in Materials and Methods). In the case of S(-)IQB-9302, these kinetic constants reached mean values of 0.59 ± 0.09 μM^{-1} s^{-1} and 39.0 ± 6.1 s^{-1} (n=11), respectively. For R(+)IQB-9302, *k* was 4.6-fold higher than that obtained for S(-)IQB-9302 (2.90 ± 0.63 μM^{-1} s^{-1}, n=7, $p<0.01$) whereas *l* was similar than that obtained with S(-!)IQB-9302 (52.2 ± 11.4 s^{-1}, n=7, $p>0.05$). Time dependent block was also observed in the deactivating process. Figure 4b shows superimposed current traces obtained under control conditions and in the presence of

S(-)IQB-9302 (20 μM) or R(+)IQB-9302 (10 μM). Under control conditions, the deactivating process was fitted following a biexponential process with an slow (τ_s) and a fast (τ_f) time constants. S(-)IQB-9302 increased the two time constants from 17.9 ± 3.3 ms and 58.6 ± 12.3 ms to 31.2 ± 4.0 ms (n=5, $p<0.05$) and 167.4 ± 30.4 ms (n=5, $p<0.05$). R(+)IQB-9302 increased the two process of deactivation from 18.7 ± 2.9 ms and 48.4 ± 6.5 ms to 28.0 ± 5.0 ms (n=5, $p<0.05$) and 73.0 ± 8.6 ms (n=5, $p<0.05$). This slowing of the deactivating process induced by IQB-9302 enantiomers produced a "crossover" phenomenon when the tail currents obtained under control conditions and in the presence of the drug are superimposed and this phenomenon is an indicator of open channel block (Armstrong, 1971).

Discussion

The main findings of the present study are: 1) IQB-9302 induced block of hKv1.5 channels is stereoselective, 2) both enantiomers of this new local anesthetic block the open state of hKv1.5 channels and 3) the stereoselectivity observed for the IQB-9302 enantiomers is due to the spatial localization of the N-substituent of the molecule, as well as that observed for ropivacaine and bupivacaine (Valenzuela et al., 1997; Longobardo et al., 1998).

IQB-9302 enantiomers block the open state of hKv1.5 channels

There are several pieces of evidence suggesting that IQB-9302 enantiomers block hKv1.5 channels by binding to the open state. As it is observed in Fig. 1 and 4, at high drug concentrations, both enantiomers induce a fast initial decline of the maximal activated current, that superimposes to the intrinsic C-type inactivation of these channels. Block steeply increased in the range of activation of the hKv1.5 channels activation, indicating that the drug need that the channel opens to bind to its receptor site and block the K^+ efflux. IQB-9302 enantiomers induced-block was also voltage dependent at membrane potentials positive to 0 mV and this voltage dependence was shallower than that observed in the activation range of hKv1.5 channels. This effect was explained following a Woodhull model (Woodhull, 1973), which led us to calculate the fractional electrical distance from the inside of the membrane (δ–0.19 for both enantiomers). Finally, when the tail currents obtained in the absence and in the presence of each enantiomer were superimposed, we observed a tail current "crossover" as a consequence of an slowing of the time course of deactivation of the current and which is a reflect of an open channel block mechanism (Armstrong, 1971; Follmer et al., 1990; Snyders et al., 1992; Valenzuela et al., 1995; 1997; Longobardo et al., 1998).

Affinity related to the length between the tertiary amine and the end of the N-substituent

Bupivacaine, ropivacaine, IQB-9302 and mepivacaine are local anesthetics which only differ in the N-substituent which is a butyl, propyl, methyl-ciclopropyl and methyl, respectively. In order to quantitate the relationship between the affinity and the length of the N-substituent we have measured the maximal length between the tertiary amine and the end of the substituent and we have represented the logK_D *vs* the maximal length (Figure 5).For each pair of enantiomers, we could observe a good linear relationship between the potency and the length of the N-substituent, thus suggesting that the part of this substituent plays an important role determining the potency and the degree of stereoselective block induced by the drug.

Stereoselective block of hKv1.5 channels induced by IQB-9302

Block induced by IQB-9302 was stereoselective like in the case of bupivacaine (Valenzuela et al., 1995a). However, although similarly to bupivacaine, the more potent enantiomer was the R(+), the degree of stereoselective block was lower, being the R(+) enantiomer 4.6-fold more potent than S(-)IQB-9302. Figure 6 shows the two enantiomers of bupivacaine superimposed by the pipecoloxilidide ring. As we can observe, under these circumstances, both enantiomers almost fit completely, which does not explain the experimental results that reveal a 7-fold difference in potency between the S(-) and R(+)-bupivacaine (Valenzuela et al., 1995a). Therefore, we suggest that the channel recognizes first the aromatic ring of the local anesthetic and afterwards it fits the N-substituent. In Figure 7 we show the superimposed S(-) and R(+) enantiomers and we can observe that the structure that differs in both is the N-substituent, which are in opposite directions for each enantiomer, which agree with our experimental results for this type of local anesthetics (bupivacaine, ropivacaine, IQB-9302 and mepivacaine) (Valenzuela et al., 1995a; 1997; Longobardo et al., 1998). Figure 8 shows a proposed type of interaction between the channel protein and this type of local anesthetics. First, the aromatic ring would interact with an amino acid containing an aromatic ring (Phe, Tyr or Trp) establishing a π interaction between both bencene rings and then, the N-substituent would interact with some of the two amino acids previously identified as part of the receptor site for bupivacaine in hKv1.5 channels: Leu or Val at positions 508 and 512, respectively (Franqueza et al., 1997).

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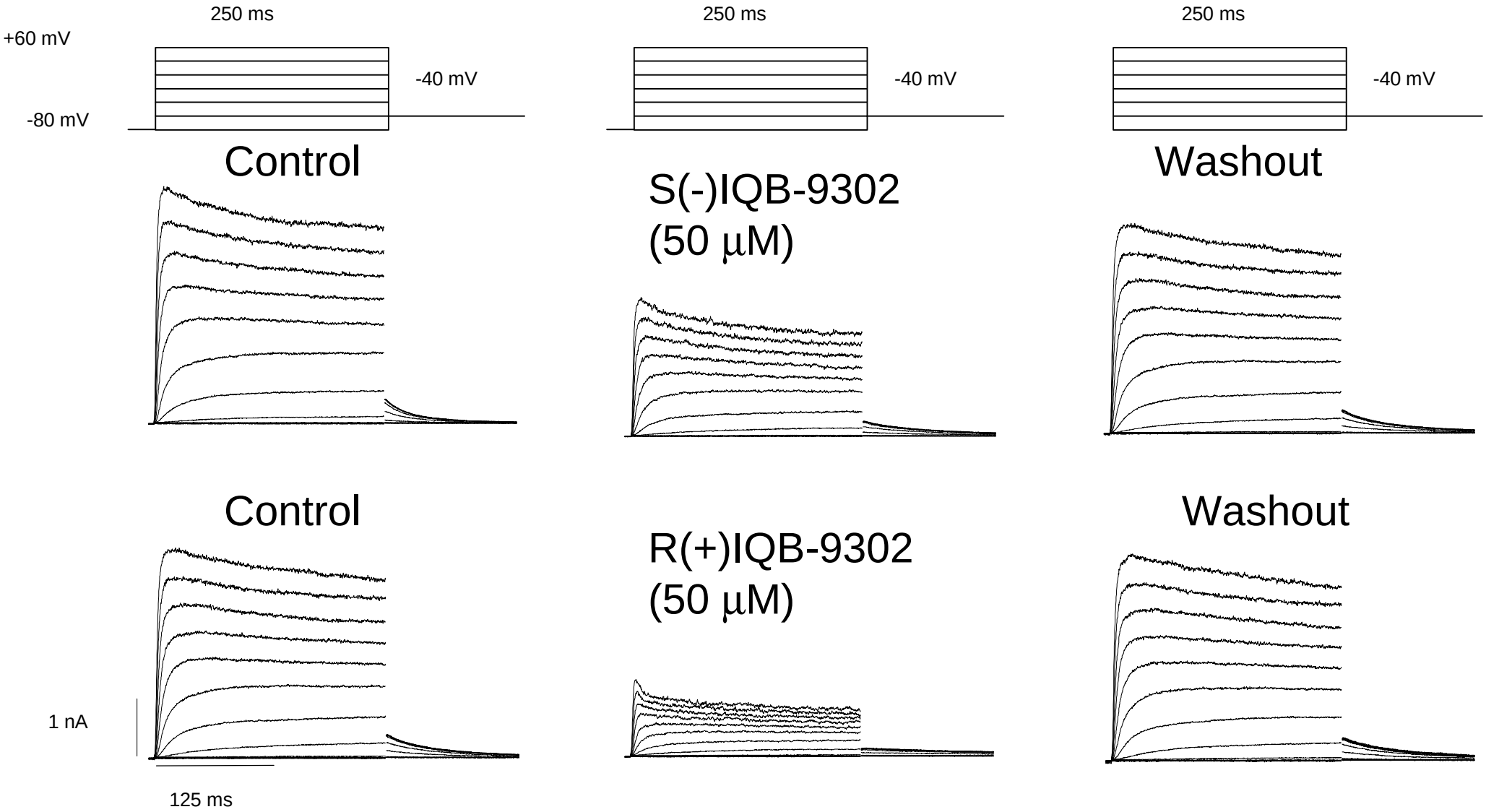

Figure 1. Efecto of S(-)IQB-9302 and R(+)IQB-9302 (50 μM) on hKv1.5 current. Holding potential was maintained at -80 mV and depolarizing pulses between -80 and +60 mV in 10 mv steps were applied.

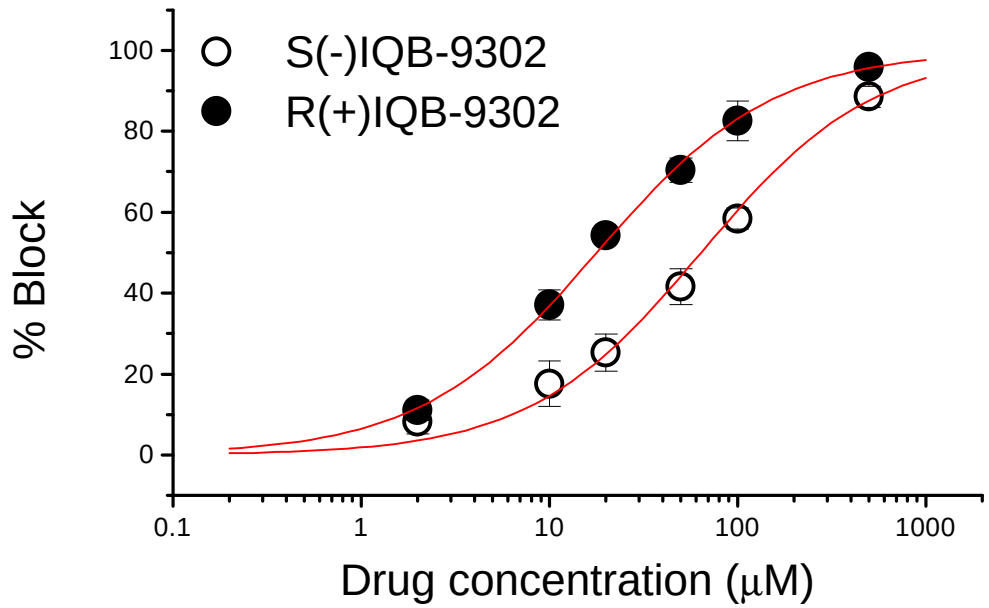

Figure 2. Concentration dependence of S(-)IQB-9302 ( ) and R(+)IQB-9302, ( ),block of hKv1.5. Reduction of current (relative to control) at the end of depolarizing steps from -80 mV to +60 mV was used as index of block. Data are mean±S.E.M. of a total of 48 experiments.

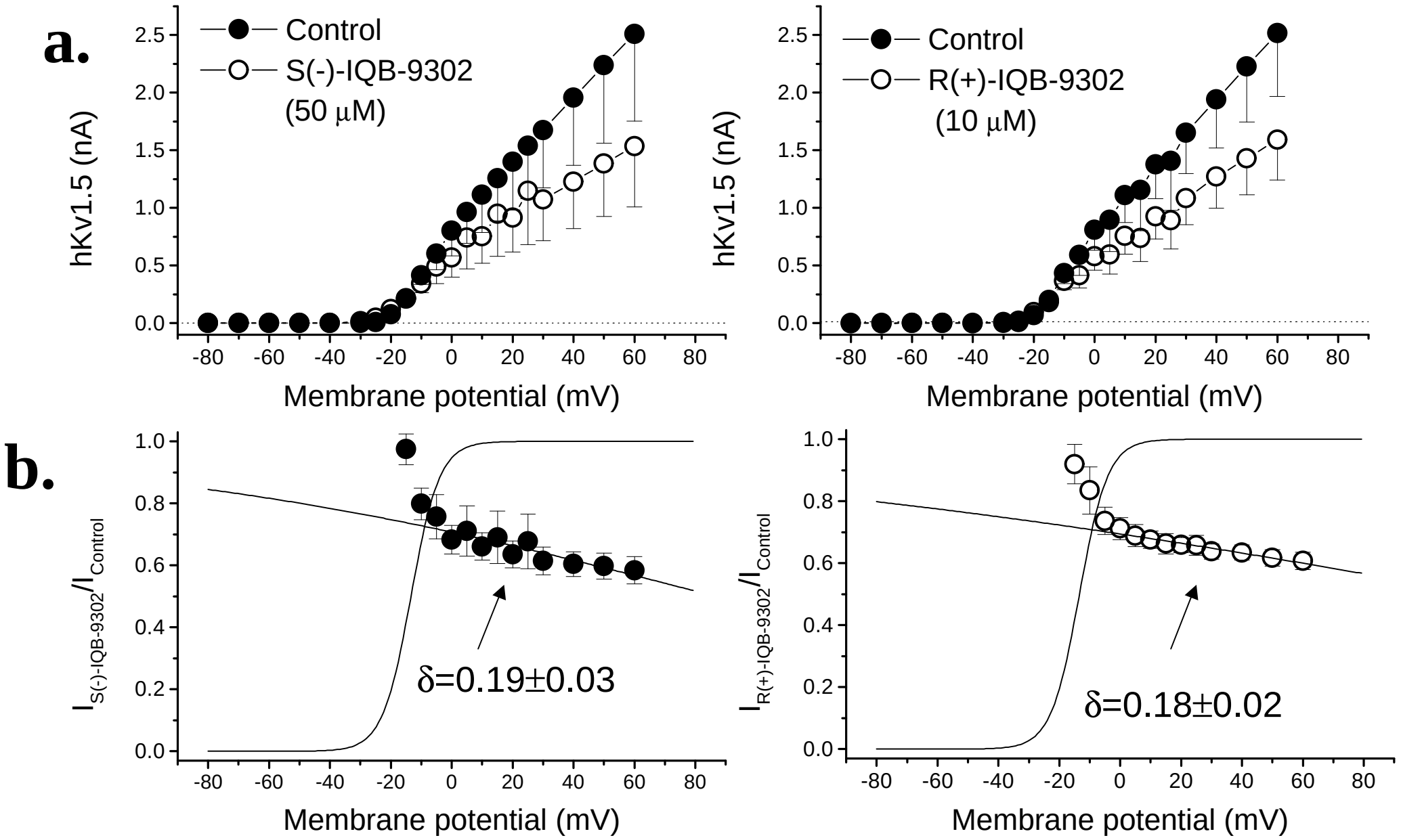

Figure 3. Voltage dependence of block of hKv1.5 channels induced by IQB-9302 enantiomers. Panel a: IV relationships in the absence and in the presence of each enantiomer. Panel b: Relative current in the presence of drug at each membrane potential.

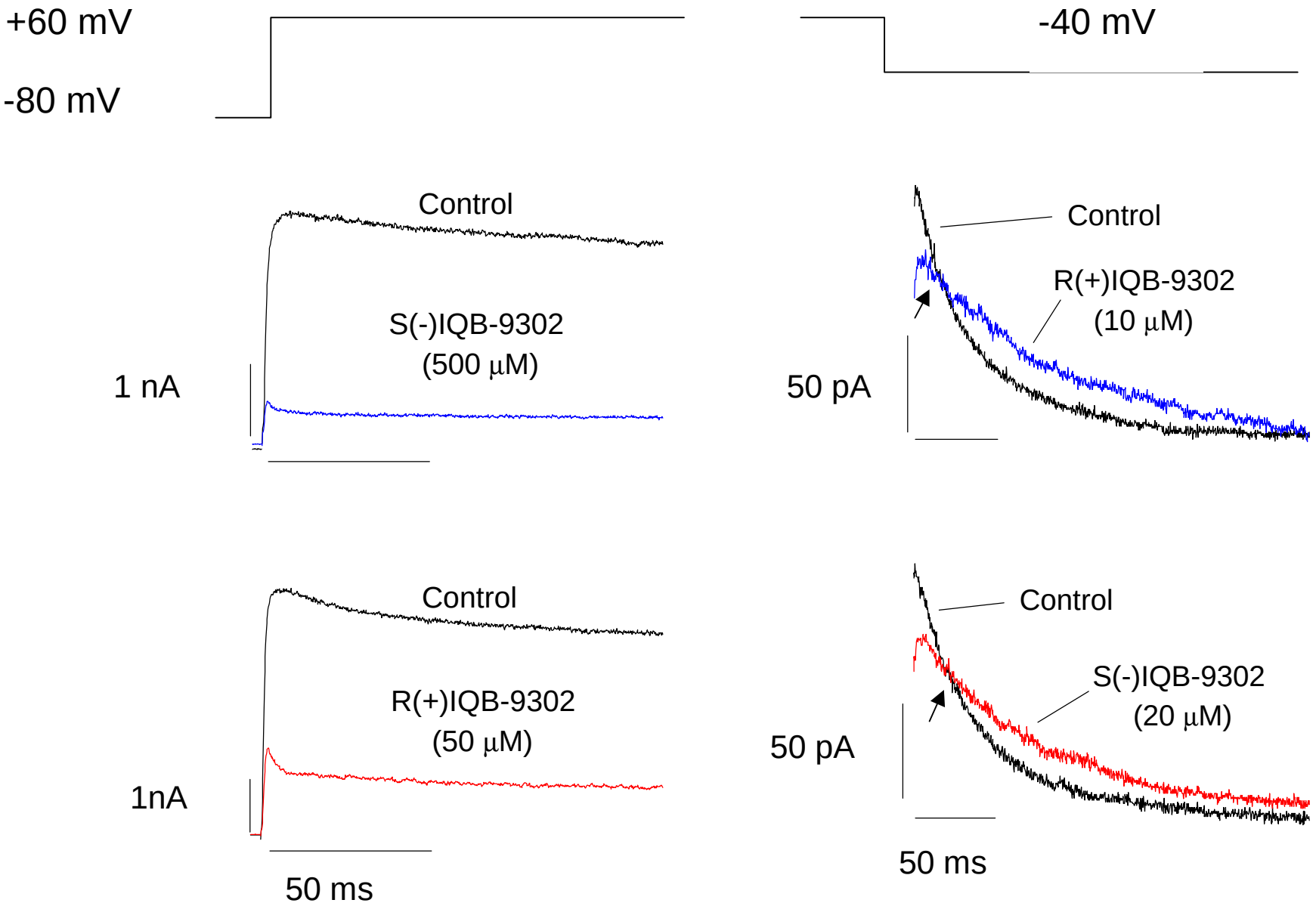

Figure 4. Time dependent effects of the IQB-9302 enantiomers measured at the maximum activated current (Panel a) and at the tail currents recorded at -40 mv after a test pulse to +60 mV (Panel b).

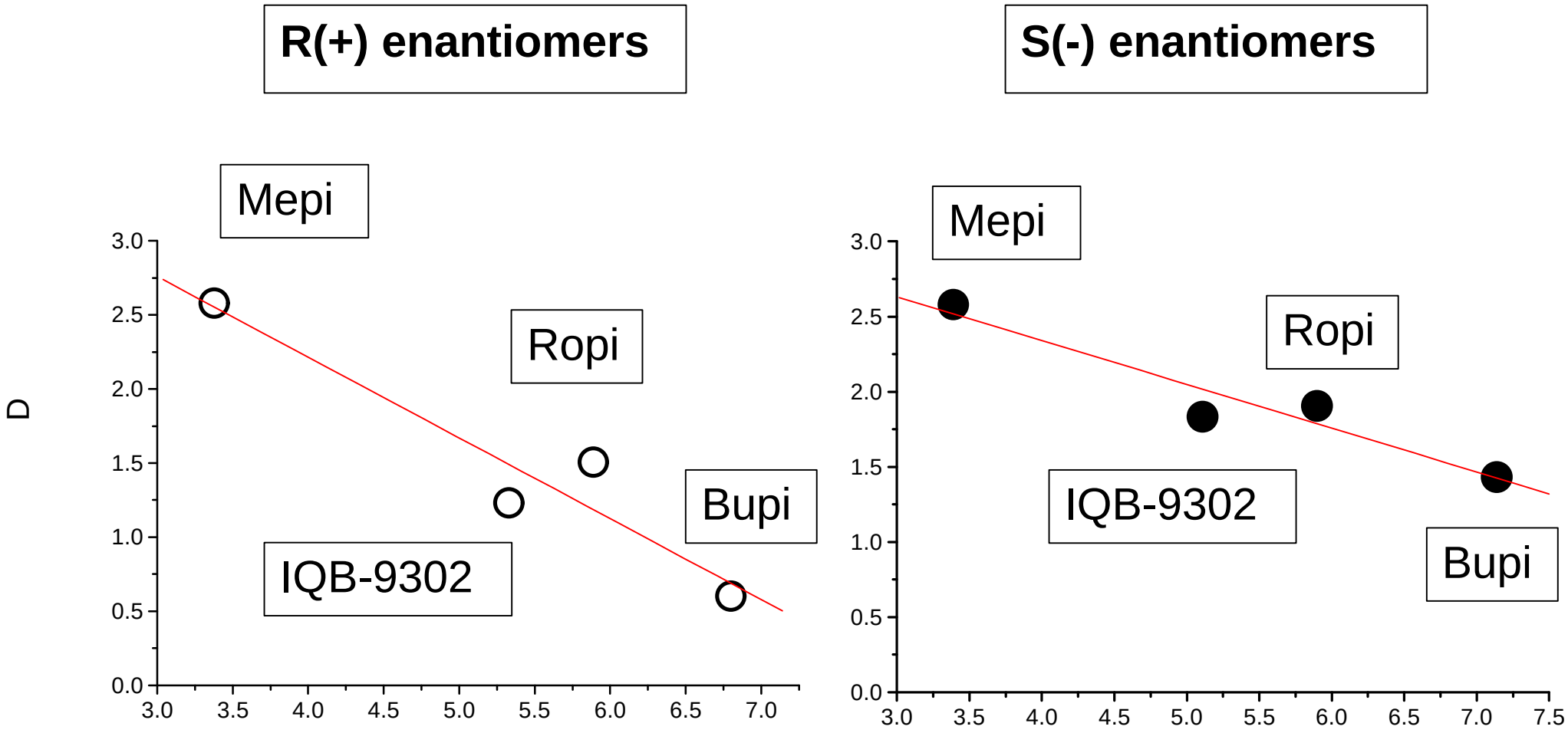

Figure 5. Relationship between the potency and the maximal distance between the tertiary amine and the end of the N-substituent for the enantiomers of bupivacaine (Bupi), IQB-9302, ropivacaine (Ropi) and mepivacaine (Mepi).

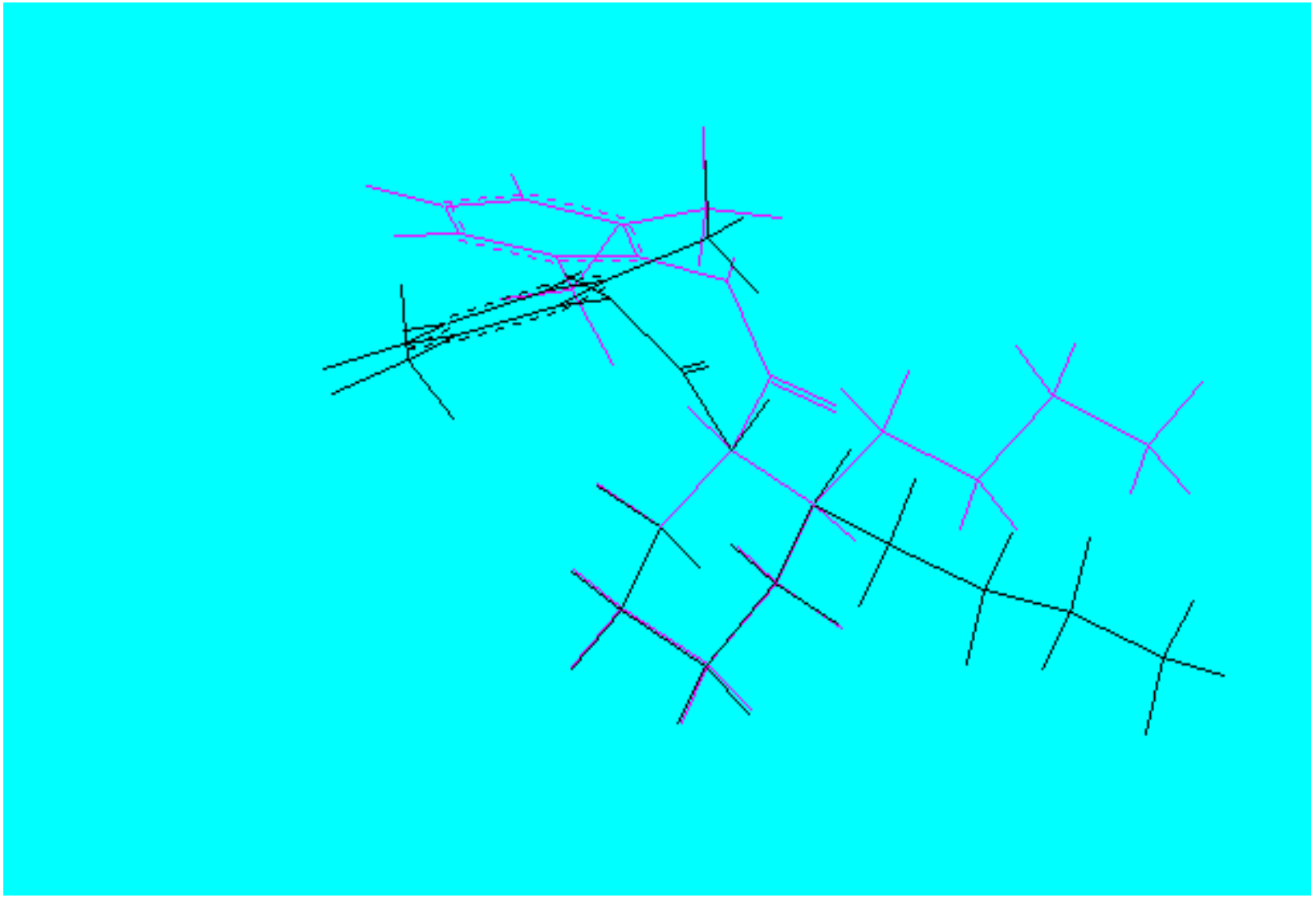

Figure 6. Superimposed S(-) and R(+)bupivacaine when the pipecoloxilidide are fixed between them.

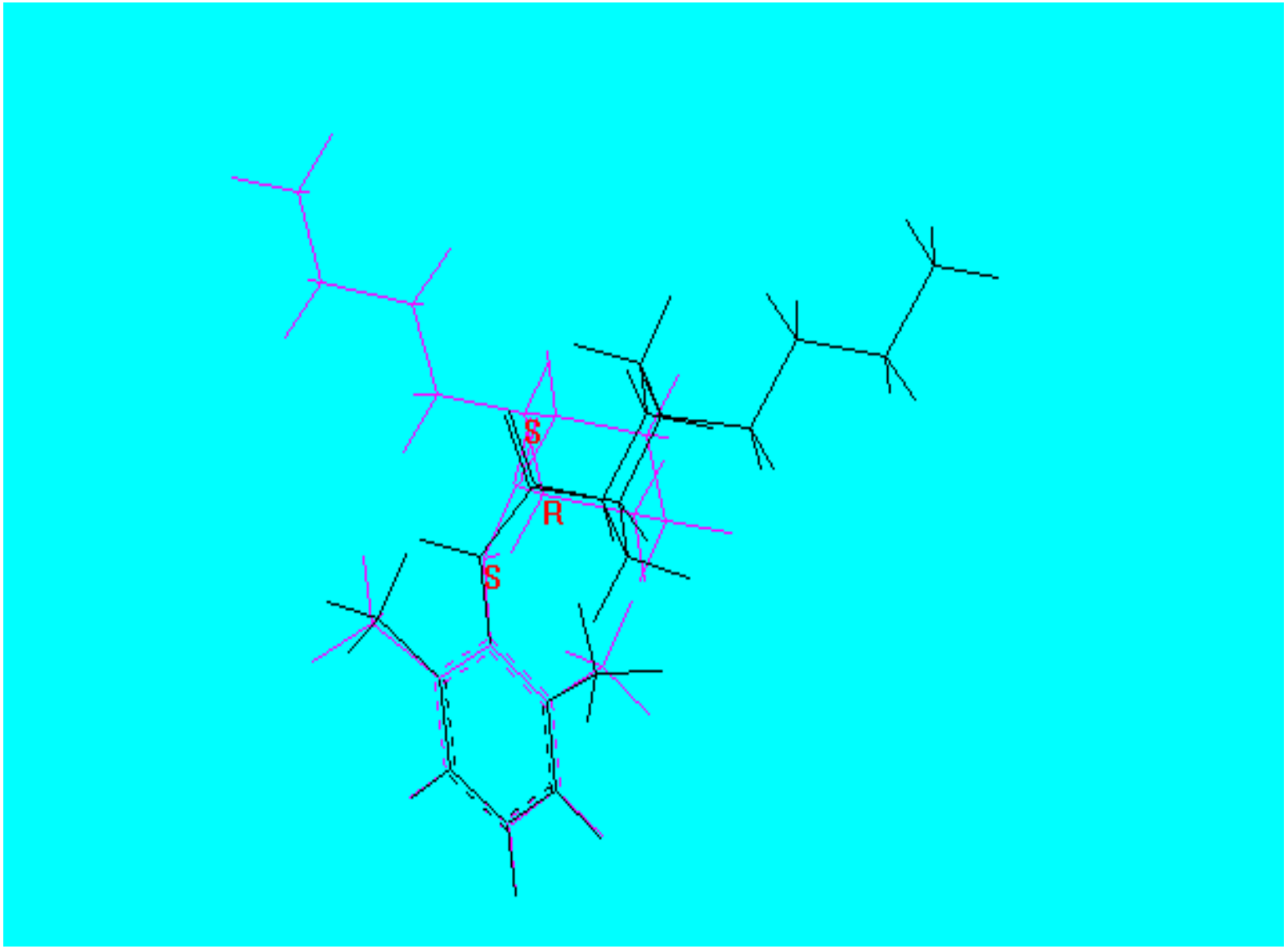

Figure 7. Superimposed structures of S(-) and R(+)bupivacaine when the aromatic rings are fixed between them.

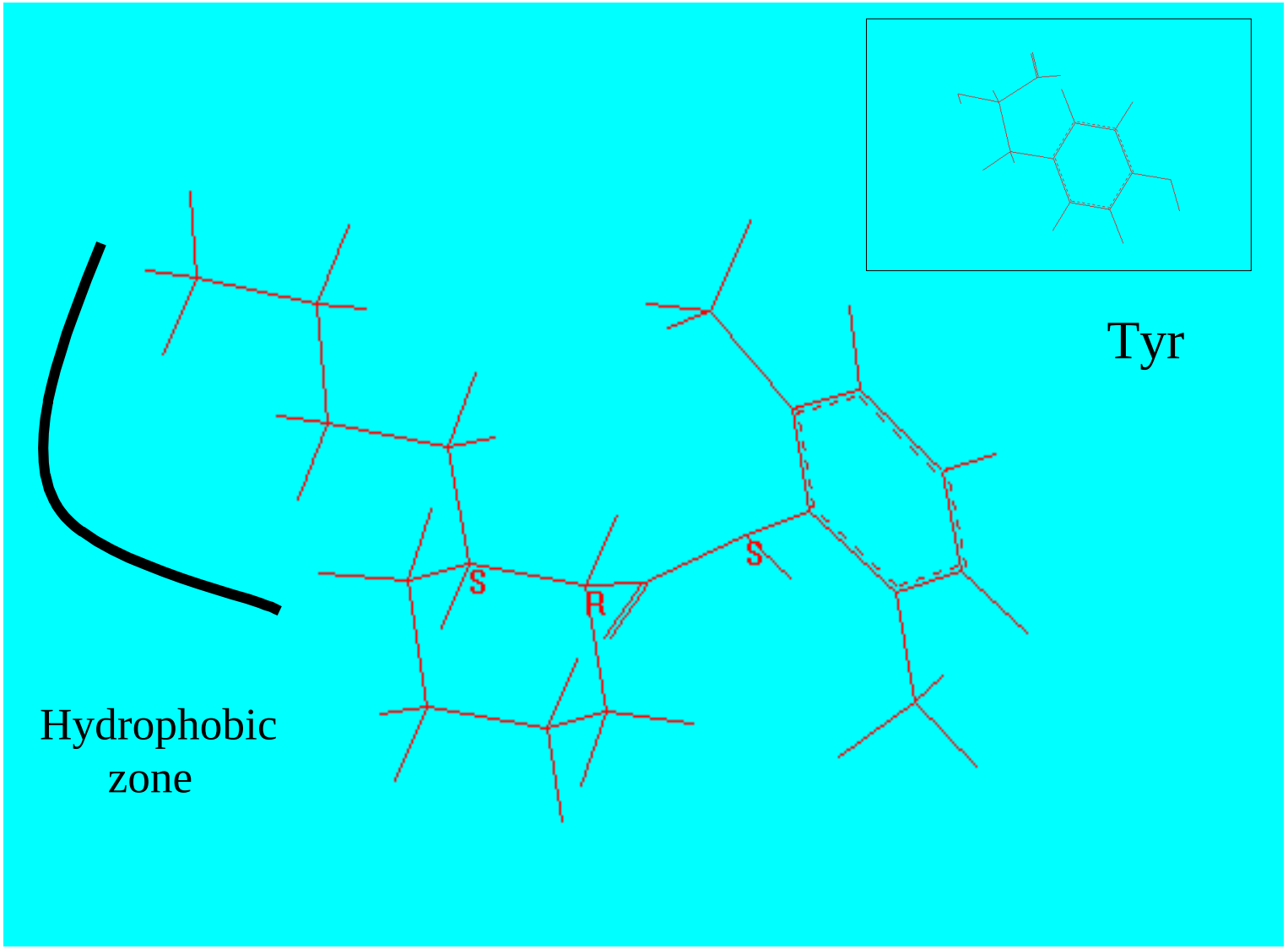

Figure 8. Proposed interaction between the hKv1.5 channel protein and bupivacaine-like local anesthetics: the aromatic ring fits withTyr, Phe or Trp and the N-sunstituent will do with L508 and/or V512 (hydrophobic zone).