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Mechanisms of block of a human cloned potassium channel by the enantiomers of a new bradycardic agent: S-16257-2 and S-16260-2

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1 The effects of S-16257-2 (S57) and S-16260-2 (R60), the two enantiomers of a new bradycardic agent, were studied on human cloned K^+ channels (hKv1.5) stably expressed in a mouse L cell line using the whole-cell configuration of the patch-clamp technique.

2 S57 and R60 did not modify the sigmoidal activation time course of the current but reduced the amplitude and increased the rate of the decay of the current during the application of depolarizing pulses. Both, S57 and R60 produced a concentration-dependent block of hKv1.5 channels with apparent K_D values of 29.0 ± 1.9 μM and 40.9 ± 4.0 μM, respectively. Thus, S57 was 1.4 fold more potent than R60 in blocking hKv1.5 channels.

3 The blockade produced by S57 and R60 was voltage-dependent and increased steeply between -30 and 0 mV, which corresponded with the voltage range for channel opening. This result indicated that both enantiomers block the hKv1.5 channels, preferentially, when they are in the open state. Between 0 and $+60$ mV the blockade exhibited a shallow voltage-dependence which was described by an electrical distance of 0.18 ± 0.002 and 0.19 ± 0.004 for S57 and R60, respectively.

4 S57 and R60 also increased the rate of decline of the current during the application of depolarizing pulses. The time constant of such decline (τ_{Block}) was faster in the presence of R60 than in the presence of S57 (16.2 ± 1.5 ms vs. 24.0 ± 2.6 ms; $P < 0.01$). The apparent association rate constants (k) were similar for S57 and R60 ($(0.52 \pm 0.13) \times 10^6$ M^{-1} s^{-1} and $(0.66 \pm 0.13) \times 10^6$ $M^{-1} s^{-1}$, respectively), whereas the dissociation rate constant (l) was faster for R60 than for S57 (25.8 ± 1.8 s^{-1} and 13.0 ± 2.4 s^{-1}, respectively).

5 Both enantiomers slowed the deactivation of the tail currents elicited upon repolarization to -40 mV, thus inducing a 'crossover' phenomenon. These results suggested that drug unbinding is required before hKv1.5 channels can close.

6 It is concluded that R60 and S57 produced a similar time- voltage- and state-dependent block of hKv1.5 channels that can be interpreted as open channel block by the charged form of each enantiomer. The main difference between R60 and S57 were linked to the apparent dissociation rate constants.

Keywords: hKv1.5 channels; stereoselectivity; bradycardic agents; S-16257-2; S-16260-2

Introduction

Selective bradycardic agents represent a new therapeutic approach in the treatment of effort-induced stable angina pectoris, particularly in patients with impaired ventricular function and may present an alternate to β-blocking agents and calcium channel blockers (Kobinger & Lille, 1987; Reiffen *et al.*, 1990; Hjalmarson, 1991). S-16257-2 (S57) and S-16260-2 (R60) are the (**S**)- and (**R**)-enantiomers of a new compound [7,8 dimethoxy 3{{[1-4,5-dimethoxybenzocyclobutan-1-yl) methyl] methylamino}propyl} 1,3,4,5-tetrahidro-2H-benzazepine 2-one] which in isolated cardiac preparations exhibited bradycardic effects (Thollon *et al.*, 1994; Pérez *et al.*, 1995). This compound is structurally related to zatebradine which, in turn, can be considered as a chemical congener of the calcium channel blocking agent, verapamil (Kobinger & Lille, 1984). In guinea-pig papillary muscles, both enantiomers inhibited the maximum upstroke velocity (V_{max}) of the action potentials at concentrations that had no effect on the resting membrane potential, which suggested that they exhibited Na^+ channel blocking properties. Based on the onset-offset kinetics of the frequency-dependent V_{max} block, S57 and R60 were classified as intermediate kinetics Na^+ channel blockers (Pérez *et al.*, 1995). Furthermore, S57 and R60 did not modify the action potential duration (APD) in guinea-pig papillary muscles. In contrast, a marked lengthening was observed when the effects of S57 were studied in sheep Purkinje fibres (Thollon *et al.*, 1994). This prolongation has been attributed to the possible inhibition of the outward K^+ currents responsible for the repolarization in cardiac Purkinje fibres (Thollon *et al.*, 1994). However, the effects of R60 and S57 on cardiac K^+ channels are as yet unknown.

Recently, a rapidly activating delayed rectifier K^+ channel (denoted hKv1.5) was cloned from human ventricle and stably expressed in a mouse *Ltk*$^-$ cell line (Tamkun *et al.*, 1991; Snyders *et al.*, 1993). The current through hKv1.5 channel is similar in voltage-dependence, kinetics and 4-aminopyridine sensitivity to the 'ultrarapid' delayed rectifier K^+ current recorded from human atrial myocytes (Wang *et al.*, 1993). However, the mere resemblence in kinetics and pharmacological properties is not sufficient to correlate the hKv1.5 clone to this endogenous current. Very recently, immunofluorescence data have demonstrated the localization of the hKv1.5 channel protein in human atrial and ventricular myocardium obtained from newborn and adult patients, which indicated that the human Kv1.5 channel is a true myocyte channel (Mays *et al.*,

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1995). The range of activation of hKv1.5 channels (midpoint at −14 mV) indicated that they may contribute to the initial fast repolarization and to the K^+ conductance during the plateau phase of the cardiac action potential (Snyders *et al.*, 1993; Wang *et al.*, 1993). Thus, this cell line provides a means for examining drug effects on a single human K^+ channel subtype with improved voltage clamp quality and avoids the problems derived from the presence of other contaminating currents (Snyders *et al.*, 1993).

Channel blocking activity of cardioactive drugs is influenced not only by physicochemical properties (i.e., molecular weight, p*K*a or lipid solubility), but also by the stereochemistry of the molecule (Campbell, 1983; Courtney, 1987). Many cardioactive drugs are racemic mixtures of enantiomers with different pharmacological characteristics (Scott, 1991; Ariëns, 1993). It is, therefore, of interest to analyze and compare the properties of individual enantiomers in order to select those with better pharmacological profiles. Very recently, it has been reported that block of hKv1.5 channels induced by the local anaesthetic, bupivacaine, displays a marked stereoselectivity: the **R**-(+)-enantiomer was 7 fold more potent than S-(-)-bupivacaine in inhibiting the hKv1.5 current (Valenzuela *et al.*, 1995b).

Therefore, the present study was undertaken to study the concentration-, voltage- and state-dependent effects of R60 and S57 on hKv1.5 channels expressed in a stable murine L cell line and to determine if the blockade displayed stereoselectivity. Our results indicated that both enantiomers produced a similar concentration-, voltage- and state-dependent inhibition of hKv1.5 channels, with moderate stereoselectivity. A preliminary report of this study has been published in abstract form (Delpón *et al.*, 1994).

Methods

Transfection and cell culture

We used the stable cell line expressing hKv1.5 that has been described in detail elsewhere (Snyders *et al.*, 1993). Cells were cultured in DMEM supplemented with 10% horse serum and 0.25 mg ml^{-1} G418 (a neomycin analogue). The cultures were passed every 4–5 days using a brief trypsin treatment. Prior to experimental use, subconfluent cultures were incubated with 2 μM dexamethasone for 24 h to induce efficient channel expression. The cells are removed from the dish with a rubber policeman, a procedure that leaves the vast majority of the cells intact. The cell suspension was stored at room temperature and used within 12 h for electrophysiological experiments.

Electrical recording

A small aliquot of cell suspension was transferred to a 0.5 ml chamber placed on the stage of an inverted microscope (Nikon TMS, Nikon Co. Tokyo, Japan). After settling to the bottom of the chamber, cells were superfused with standard saline solution at 1 ml min^{-1}. This saline solution contained (in mM): NaCl 130, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10 and glucose 10 and was adjusted to pH 7.4 with NaOH. Pipettes were filled with an 'internal' solution containing (in mM): K-aspartate 80, KCl 50, KH_2PO_4 10, MgATP 3, HEPES 10 and EGTA 5 (adjusted to pH 7.2 with KOH). Measurements in *Ltk*$^-$ cells dialyzed with internal solutions containing low concentrations of EGTA (2.1 mM) yielded internal calcium concentrations below 10^{-9} M (pCa>9) (Hosoi & Slayman, 1985). All the experiments were performed at 24–25°C.

Recording techniques

HKv1.5 currents were measured by the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) using an Axopatch-1D clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). Micropipettes were pulled from Narishige (GD1) (Narishige Co., Ltd, Tokyo, Japan) borosilicate capillary tubes using a programmable patch micropipette puller (Model P-87 Brown-Flaming, Sutter Instruments Co., Novato, CA, U.S.A.) and were heat polished with a microforge (Model MF-83, Narishige). To ensure voltage-clamp quality, micropipette resistance was kept below 3.5 MΩ when filled with the internal solution and immersed in the external solution. The micropipettes were gently lowered onto the cells to get a gigaohm seal after applying suction. After seal formation, cells were lifted from the bottom of the perfusion bath and the membrane patch was ruptured with brief additional suction. The capacitive transients elicited by symmetrical 10 mV steps from −80 mV were recorded at 50 kHz (filtered at 10 kHz) for subsequent calculation of capacitative surface area, access resistance, and input impedance. Thereafter, capacitance and series resistance compensation were optimized and ≈80% compensation was usually obtained. Maximum outward current amplitudes at +60 mV averaged 2.0±0.1 nA. Thus, no significant voltage errors (<5 mV) due to series resistance were expected with the electrodes used and this was confirmed by the calculated access resistance (R_a). Moreover the low capacitance (26.7±2.8 pF) enabled fast clamp control. Voltage-clamp command pulses were generated by a 12-bit digital-to-analog converter. The current records were sampled at 3–10 times the antialias filter setting and stored on the hard disk of a Tandon 386/25 for subsequent analysis. Data acquisition and command potentials were controlled by pCLAMP 5.5.1. software (Axon Instruments, Foster City, CA, U.S.A.).

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 s until steady-state was obtained. Following exposure to S57 and R60 induction of block appeared within 3–4 min and, therefore, an equilibration period of 10 min was allowed before the drug effects were measured.

The holding potential was maintained at −80 mV and the cycle time for any protocol was 10 s in order to avoid accumulation of block. The protocol to obtain current-voltage (*I-V*) relationships and activation curves consisted of 500 ms pulses that were imposed in 10 mV increments between −80 mV and +70 mV, with additional interpolated pulses to yield 5 mV increments between −30 and +10 mV (activation range of hKv1.5) (Snyders *et al.*, 1993). The 'steady-state' *I-V* relationships were obtained by plotting the current level after 250 ms as a function of the membrane potential. 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 on return to −40 mV. The activation curve was obtained from the tail current amplitude immediately after the capacitive transient.

Activation curves have been fitted with a Boltzmann equation:

$$y = 1/\{1 + \exp[(V_h - V_m)/k]\} \quad (1)$$

where V_h is the half-point of activation (in mV), V_m is the test potential and k represents the slope factor for the activation curve (in mV). In order to describe the time course of currents during depolarizing pulses, tail currents upon repolarization and activation kinetics, exponential analysis was used as an operational approach, fitting these processes to an equation of the form:

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

where τ_1, τ_2 and τ_n are the system time constants, A_1, A_2 and A_n are the amplitudes of each component of the exponential, and C is the baseline value. The curve fitting procedure used a

nonlinear least-squares (Gauss-Newton) algorithm; results were displayed in linear and semilogarithmic format, together with the difference plot. Goodness of fit was judged by the χ^2 criterion and by inspection for systematic nonrandom trends in the difference plot.

Fractional block was defined as:

$$f = 1 - I_{Drug}/I_{Control}$$

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 the fractional block, f, at various drug concentrations:

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

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

$$\tau_{Block} = 1/(k \times [D] + l) \quad (3)$$

in which τ_{Block} is the time constant of development of block.

The voltage-dependence of block was fitted to:

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

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

Drugs

S-16257-2 (S57) and S-16260-2 (R60) were synthesized and kindly provided by IRIS (Courbevoie, Paris, France). Drugs as a powder were initially dissolved in distilled deionized water to make 10 mM stock solutions. Further dilutions were carried out in external solution to obtain final concentrations between 1 and 100 μM.

Statistical methods

Data obtained under control conditions were compared with those obtained after drug exposure in a paired manner. For comparisons at a single voltage or drug concentration differences were analyzed by Student's t test. To analyze block at multiple voltages or drug concentrations, two-way analysis of variance was used (Wallestein *et al.*, 1980). Results are expressed as mean ± s.e.mean. A P value of less than 0.05 was considered as significant. More details on each procedure are given under Results.

Results

Concentration-dependent hKv1.5 block by S57 and R60

Figure 1 shows superimposed potassium currents recordings from a mouse L cell expressing hKv1.5 channels in control conditions and in the presence of (a) R60 and (b) S57. The cell was held at −80 mV and subjected to 500 ms depolarizing pulses from −60 mV to +60 mV in 20 mV steps applied every 10 s. Outward currents were followed by decaying outward tail currents upon repolarization to −40 mV. Under control conditions, (the upper panels), depolarizations positive to −40 mV elicited outward currents that progressively increased with further depolarizations. The activation proceeded with a sigmoidal time course, the rate of activation being faster at more depolarized levels. At the most positive voltages, the current declined slowly during the maintained depolarization (slow and partial inactivation). Thus, the voltage- and time-dependent characteristics of this current correspond to those previously described for hKv1.5 currents (Snyders *et al.*, 1992; 1993).

In this typical experiment, exposure to 100 μM R60 (the middle panel in Figure 1a) did not modify the sigmoidal activation time course of the current but reduced the peak outward current elicited by pulses to +60 mV by 27% (from 2103 pA to 1533 pA). Moreover, R60 increased the rate of decay of the current during the application of the depolarizing pulse, which reached a pseudo steady-state level within 250 ms. Thus, the reduction of the hKv1.5 current measured after 250 ms depolarization to +60 mV was 73% (from 1563 pA to 420 pA). The bottom panel of Figure 1a shows current traces obtained after 15 min perfusion with drug-free solution. The currents were restored to 98% of control value, indicating that the effects of R60 were reversible upon washout. The middle panel in Figure 1b shows current traces obtained in the same cell after perfusion with 100 μM S57. The effects were similar to those observed with the R-enantiomer. The peak current was reduced by 33% (from 1915 pA to 1280 pA), but the most marked effect was an acceleration in the rate of decay of the current during the depolarizing pulses. In this experiment, S57 reduced by 84% the current measured after 250 ms at +60 mV (from 1529 pA to 250 pA). The bottom panel of Figure 1b shows that the effects of S57 were also largely reversible after 15 min perfusion with drug-free solution.

The concentration-dependence of the block of the hKv1.5 channel induced by R60 and S57, in a range of concentrations between 1 and 100 μM, is presented in Figure 2. The inhibition of the current was determined from the reduction in current amplitudes after 250 ms depolarizing pulses from −80 mV to +60 mV. As is indicated by the asterisks, only at 50 and 100 μM was the blockade induced by S57 significantly greater ($P<0.05$) than that induced by R60. A nonlinear least-squares fit of the concentration-response equation (eq. 2, see Methods) to the individual data points yielded an apparent K_D of 40.9 ± 4.0 μM and 29.0 ± 1.9 μM for R60 and

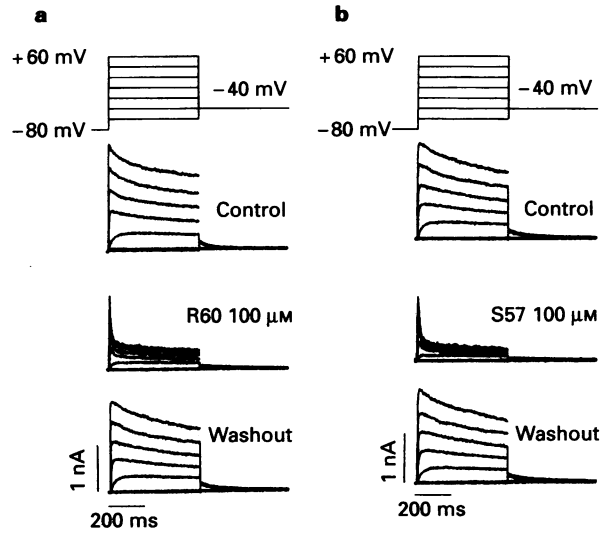


Figure 1 Effects of (a) R60 and (b) S57 on hKv1.5 channels expressed in a mouse L cell. Results for both enantiomers were obtained in the same cell. Superimposed current traces are shown for 500 ms depolarizing pulses from −80 mV to voltages between −60 and +60 mV in steps of 20 mV in control conditions (the upper panels), in the presence of 100 μM R60 and 100 μM S57 (the middle panels) and after washout of the drugs (the bottom panels). Data filtered at 1 kHz (four-pole Bessel) and digitized at 5 kHz. Cell capacitance, 28 pF.

S57, respectively. The Hill coefficients obtained by this fitting procedure in the presence of R60 and S57 were 0.89 and 1.13, respectively. A fit of the same data with the Hill coefficient fixed at 1, led to apparent K_D values for both enantiomers similar to those obtained when the coefficients were not constrained to unity (40.9±3.8 μM and 28.3±2.1 μM for R60 and S57, respectively). These results suggest that the blockade of hKv1.5 channels displayed a moderate stereoselectivity. Moreover, the Hill coefficients close to unity suggests that binding of one drug molecule/channel is sufficient to block potassium permeation.

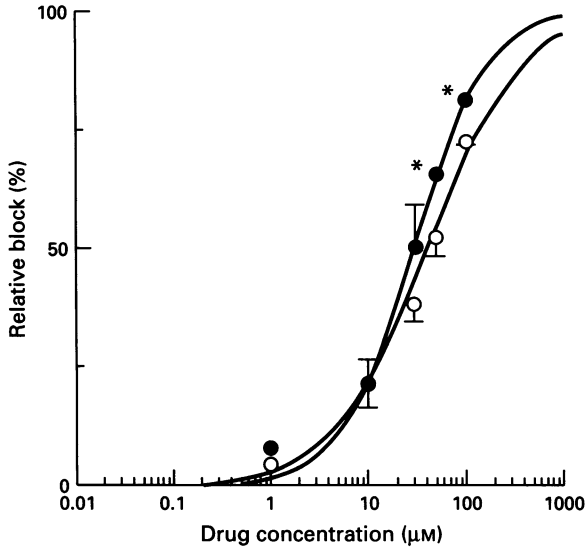


Figure 2 Concentration-dependence of R60 (○)- and S57 (●)-induced block of hKv1.5. Reduction of current (relative to control) after 250 ms depolarizations to +60 mV was used as index of block. K_D S57 = 29 μM; K_D R60 = 41 μM. In both cases, the continuous line represents the fit of the experimental data to eq. 2 (see methods). Each point represents the mean±s.e.mean of 3–8 experiments. *$P<0.05$ between R60 and S57.

Voltage-dependent block by R60 and S57 of hKv1.5 channels

Figure 3a shows the effects of 100 μM R60 and S57 on the steady state current-voltage (*I-V*) relationship for the hKv1.5 channel constructed by plotting the current amplitudes after 250 ms depolarization as a function of the test pulse voltage. The control *I-V* relationship was almost linear for depolarizations positive to + 10 mV. The sigmoidicity observed between −30 and +10 mV reflected the voltage-dependence of channel opening. R60 (○) and S57 (□) induced a voltage-dependent inhibition of the hKv1.5 current: the *I-V* curves tended to curve downward with a larger amount of block at stronger depolarizations (> −10 mV) than at smaller ones (−30 to −10 mV). In fact, the percentage of block induced by R60 increased from 56% at −20 mV to 74% at +70 mV, while that produced by S57 increased from 65% to 83%, respectively.

To quantify the voltage-dependence of hKv1.5 block, the relative current ($I_{drug}/I_{control}$) was plotted as a function of the membrane potential (Figure 3b). The voltage-dependence of activation of the hKv1.5 current was obtained from the deactivating tail current amplitude recorded at −40 mV following 500 ms depolarizing pulses from −80 mV to potentials between −80 and +70 mV. The dotted line shows the activation curve of the hKv1.5 channels in this particular experiment. The current activates at −30 mV and the conductance of the channel is fully saturated at 0 mV. The midpoint and slope factor from Boltzmann equation (eq. 1, see methods) yield values of −13 mV and 5.3 mV, respectively. In the presence of both R60 and S57 the blockade increased steeply between −30 and 0 mV, which corresponded with the voltage range for channel opening (Snyders *et al.*, 1993). These results suggested that both enantiomers bind preferentially to the open state of the hKv1.5 channels. Between +10 mV and +70 mV, block continued to increase with a more shallow voltage-dependence, despite the fact that all channels are open over this voltage-range. It is unlikely that this shallow voltage-dependence observed in the presence of either enantiomer was due to channel gating, because hKv1.5 activation had reached saturation over this voltage range (Snyders *et al.*, 1992; 1993). R60 and S57 are weak bases with $pK_a = 8.5$ and, therefore, at the intracellular pH of 7.2 both enantiomers are present predominantly in the charged form. The voltage-dependence of block could be due to the effect of the transmembrane elec-

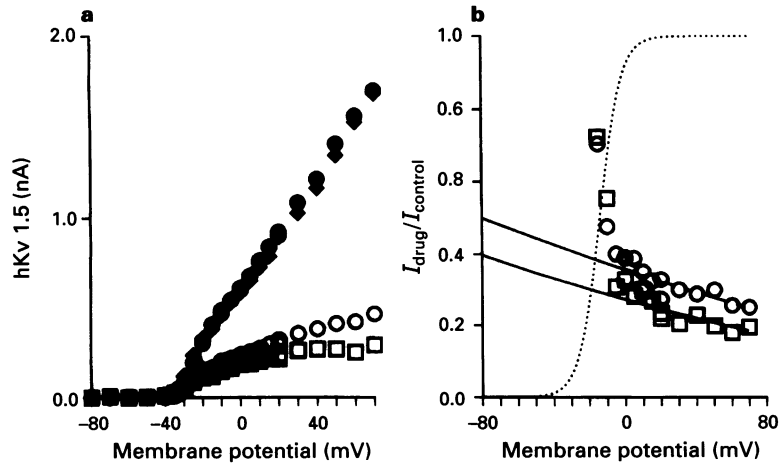


Figure 3 (a) Effect of 100 μM R60 and 100 μM S57 on the steady-state current-voltage (*I-V*) relationships (250 ms isochronal). Data were obtained from the same cell as in Figure 1. (●) Control; (○) R60; (◆) Wash; (□) S57. (b) Relative current expressed as $I_{drug}/I_{control}$ from data obtained in the presence of 100 μM R60 (○) or S57 (□). The dotted line shows the activation curve of the hKv1.5 channel. Block increased steeply between −30 mV and 0 mV. For membrane potentials more depolarized than 0 mV a more shallow voltage-dependence was observed for both enantiomers. This voltage-dependence was fitted (continuous line) to eq. 4 (see Methods) and yielded a δ value of 0.18.

Table 1 Percentage of inhibition induced by S57 and R60 on hKv1.5 currents measured by applying 250 ms pulses to +60 mV from a holding potential of −80 mV

Drug	*1 μM*	*10 μM*	*30 μM*	*50 μM*	*100 μM*
S57	7.7±0.2 (3)	21.3±5.0 (4)	51.5±5.3 (4)	66.3±1.2* (5)	81.6±1.4** (5)
R60	4.2±0.2 (3)	21.4±5.4 (8)	35.5±3.1 (4)	52.4±3.9 (8)	72.7±1.0 (8)

Values are presented as the mean±s.e.mean and were compared by Student's unpaired *t* test. *$P<0.05$ and **$P<0.01$ compared with percentage of block in the presence of R60. Numbers in parentheses indicate the number of experiments.

trical field on the interaction between the charged form of both enantiomers and the channel receptor. If these compounds reached the receptor from the inside, then the channel block is expected to increase in a voltage-dependent manner, according to equation (4) (Woodhull, 1973; Snyders *et al.*, 1992). The solid lines in Figure 3b represent the fits of this equation to the data points for membrane potentials positive to 0 mV for S57 and R60. In both cases the electrical distance δ was 0.18. In 15 experiments the average values for δ were 0.18±0.002 ($n=6$) and 0.19±0.004 ($n=9$) in the presence of S57 and R60, respectively ($P>0.05$).

Time course of channel block and its concentration-dependence

Figure 4a shows superimposed outward current recordings elicited by applying 500 ms depolarizing pulses from −80 mV to +60 mV in the absence and in the presence of S57 (the upper panel) or R60 (the bottom panel). At 50 μM, S57 and R60 reduced the peak current by 21.6±5.7% ($n=5$) and 28.8±2.6% ($n=6$, $P>0.05$), respectively. Moreover, both enantiomers accelerated the time to peak current but did not significantly modify the activation kinetics of hKv1.5 current. Under control conditions, the activation time constant of the currents elicited by test pulses to +60 mV averaged 1.7±0.1 ms, whereas in the presence of 30 μM of both S57 and R60 were 1.6±0.1 ms ($n=4$, $P>0.05$) and 1.5±0.2 ms ($n=4$, $P>0.05$), respectively. However, the most noticeable feature of the inhibition of hKv1.5 currents induced by S57 and R60 was a concentration-dependent increase in the rate of decline of the currents during the depolarizing pulse. The increased rate of decay was well fitted by a double exponential function (continuous line in both panels of Figure 4a), comprised of a slow component and a fast one which superimposed on the slow inactivation. At drug concentrations higher than 30 μM, this extra component was at least 10 times faster than the slow inactivation (time constant of about 150 ms). Visual inspection of Figures 1 and 4a revealed that the decline of the current was somewhat faster in the presence of R60 than in the presence of S57. Thus, in the presence of 50 μM of R60 or S57, the time constants of the fast component were 16.2±1.5 ms ($n=21$) and 24.0±2.6 ms ($n=13$, $P<0.01$), respectively. The time constant of this fast decline (τ_{Block}) decreased monotonically when the concentration of each enantiomer increased and thus,

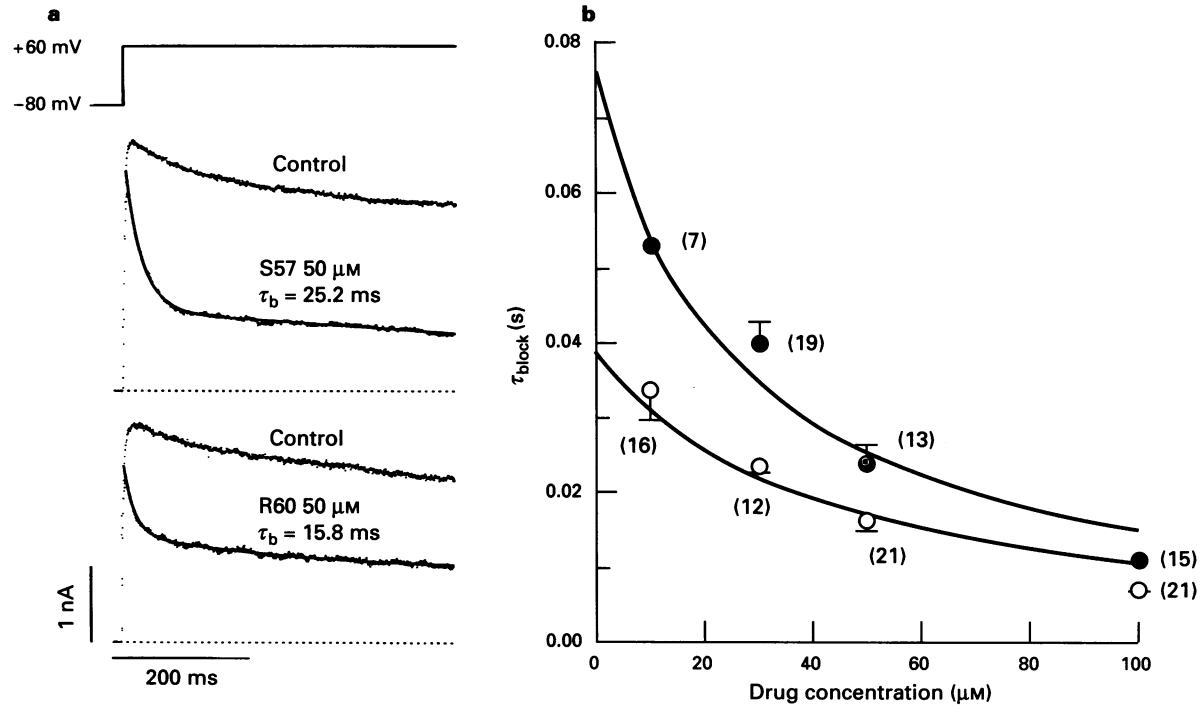


Figure 4 (a) Human Kv1.5 current traces obtained by applying 500 ms depolarizing pulses from −80 mV to +60 mV in control and in the presence of 50 μM S57 (upper panel) or 50 μM R60 (lower panel) in two different cells. Dotted line represents the zero current level. Data filtered at 1 kHz (four-pole Bessel) and digited at 5 kHz. Cell capacitance 20.4 and 37 pF, respectively. (b) Time constant of block as a function of drug concentration. The time constant of the fast component of decline of hKv1.5 current (τ_{Block}) obtained from the biexponential fits of the falling phase of the current traces was plotted versus the concentration of S57(●) or R60(○). The solid line represents the fit to eq.3 (see Methods) from which the apparent association (k) and dissociation rate constants (l) were calculated.

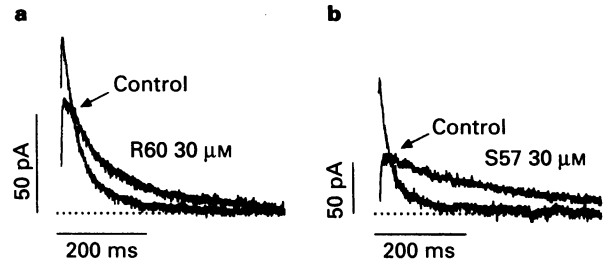


Figure 5 Tail current crossover in the presence of (a) R60 and (b) S57. Tail currents elicited on return to −40 mV after 500 ms duration depolarizing pulses from a holding potential of −80 mV to +60 mV in control conditions and in the presence of 30 μM R60 or S57. In both panels the *arrow* indicates the tail crossover phenomenon.

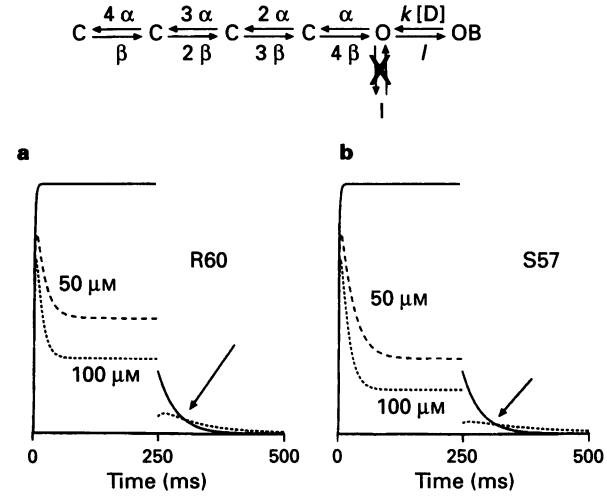


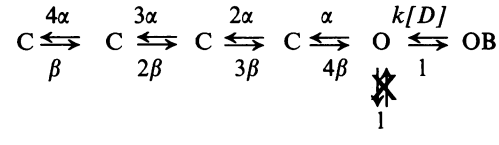
Figure 6 Mathematical modelling of (a) R60 and (b) S57 interactions with hKv1.5 channels. Channel opening was modelled using four independent activating units (see upper diagram) reflecting the fourfold symmetry of the channel. For the simulation of the channel opening at +60 mV in the absence of drug the rate constants were: $\alpha = 400\,s^{-1}$, $\beta = 1\,s^{-1}$. In both panels currents for step and tail were scaled to reflect the difference in driving force, α and β at −40 mV were $0.1\,s^{-1}$ and $7\,s^{-1}$, respectively. For depolarization, simulations for control and for 50 and 100 μM R60 (a) or S57 (b) are displayed; for the tails, control and 100 μM R60 or S57 are shown. (a) Simulation of the time course of block for R60 with the following time constants $k = 0.66\,\mu M^{-1}s^{-1}$, and $l = 25.8\,s^{-1}$ at +60 mV; at −40 mV, $k = 0.44\,\mu M^{-1}s^{-1}$ and $l = 38.7\,s^{-1}$. (b) The open-channel block model for S57 was used with the following rate constants: at +60 mV, $k = 0.52\,\mu M^{-1}s^{-1}$, and $l = 13.0\,s^{-1}$; at −40 mV, $k = 0.35\,\mu M^{-1}s^{-1}$ and $l = 19.5\,s^{-1}$. The *arrow* indicates the crossover.

it was considered to be a reasonable approximation of the drug-channel interaction kinetics. In Figure 4b the τ_{Block} was plotted as a function of the concentration of each enantiomer. The relationship between τ_{Block} and drug concentration (continuous line in Figure 4b) was well described by equation (3). Slope and intercept with the ordinate axis for the fitted relation yielded an apparent association rate constant (k) of $(0.52 \pm 0.13) \times 10^6\ M^{-1}\ s^{-1}$ and a dissociation rate constant (l) of $13.0 \pm 2.4\ s^{-1}$ in the presence of S57, while in the presence of R60 the k and l values were $(0.66 \pm 0.13) \times 10^6\ M^{-1}\ s^{-1}$ and $25.8 \pm 1.8\ s^{-1}$ respectively. These results indicated that the onset rate of block was very similar and that the differences in the kinetics of block between both enantiomers were linked to differences in the dissociation rate constant, which was faster in the presence of R60. Furthermore, the K_D ($= l/k$) values derived from this relationship for hKv1.5 block by S57 and R60 were 24.8 μM and 39.1 μM, respectively. These values were in reasonable agreement with those obtained from the concentration-response curves presented in Figure 2. Therefore, the effects of S57 and R60 on the time course of outward currents during the depolarizing pulse suggested that both enantiomers access to their receptor only if the channel is in the open state, and that block was visible because the blocking rate was slower than the opening rate.

To characterize further the time-dependent interactions of S57 and R60 on hKv1.5 channels, their effects on the time course of tail currents deactivation were studied. Drugs which block the open state of the K^+ channels and exhibit a fast kinetics of open-channel unblocking would be expected to induce an initial rising phase of the tail current, because the open channels that become unblocked during the tail will generate outward current, followed by slower decline which leads to larger currents at later times ('crossover'). This can be seen in Figure 5, where tail currents recorded in two different cells on return to −40 mV after a 500 ms depolarization from −80 mV to +60 mV have been superimposed to compare the time course of decay of the current, in control conditions and in the presence of 30 μM R60 or S57. In control conditions, the tail deactivated with a time constant of 51.8 ± 5.7 ms ($n = 8$), while in the presence of both enantiomers the tail current amplitude was reduced and the subsequent decline of the current was slower than in control conditions, which resulted in a 'crossover' phenomenon. In the presence of 30 μM S57 and R60 the time constant of decline of the tail currents was significantly increased, but the effect was more marked in the presence of S57 (163.3 ± 15.4, $n = 4$, $P < 0.01$) than in the presence of R60 (120.5 ± 29.3 ms, $n = 4$, $P < 0.05$). These results supported an open-channel interaction between both enantiomers and the hKv1.5 channels.

Mathematical simulation of the effects of R60 and S57

To interpret our results, we have used a partial kinetic state model derived from a more complex one developed for *Shaker* (Koren *et al.*, 1990; Zagotta & Aldrich, 1990; Bezanilla *et al.*, 1994) and hKv1.5 channels (Snyders *et al.*, 1992):



where C, O and I are the closed, the open and the inactivated states of the channel, respectively. The voltage-dependence of the drug channel interaction was incorporated, but slow inactivation was omitted for simplicity. Channel activation was modelled with four independent gating units (corresponding with the tetramer structure of the channel) governed by voltage-dependent forward (α) and reverse (β) activation rate constants. This results in four closed (C) states which must be transversed before opening (O) which results in the sigmoidal activation time course. Upon depolarization the channel opens rapidly and then slowly inactivates (O→I). The scheme predicts that the decay of the current will be accelerated by R60 and S57 because the open channels can close by slow inactivation and by moving to a non-conducting drug-blocker state (O→OB).

Figure 6 shows the results of a mathematical stimulation of the effects of both R60 and S57 at two different concentrations (50 and 100 μM), based on this open-channel block model. Incorporation of the experimentally obtained values for k and l for each enantiomer reproduced fairly well the main effects of the enantiomers: (a) the reduction of peak current without altering the initial sigmoidal activation time course of the current (Figures 1 and 4a); (b) the fast decline of hKv1.5 current during the depolarizing step (Figures 1 and 4a); and (c) the crossover phenomenon of the tail currents (Figure 5). Since the model assumes that R60 and S57 bind only to the open

channel, the concordance with the results obtained further suggested that the effects of both enantiomers can be explained considering that they preferentially bind to the open state of the hKv1.5 channels.

Discussion

We have shown that the two enantiomers, R60 and S57, of a new bradycardic agent block the delayed rectifier K^+ (hKv1.5) channel cloned from human ventricle and stably expressed in mouse L cell line. This cell line system allows study of the effects of drugs on a single human K^+ channel subtype in the absence of other contaminating currents to be studied, eliminating the need for various ionic substitutions and improving voltage clamp quality and speed due to the small cell size (Tamkun *et al.*, 1995). The current through hKv1.5 channels is similar in voltage-dependence and kinetics to the delayed rectifier current recorded in adult rat atrial myocytes (Boyle & Nerbonne, 1991), neonatal canine epicardial myocytes (Jeck & Boyden, 1992) and human atrial myocytes (Fedida *et al.*, 1993; Wang *et al.*, 1993). Moreover, the sensitivity of cardiac hKv1.5 channels to quinidine and other antiarrhythmics (Snyders *et al.*, 1992; Tamkun *et al.*, 1995) suggests that this channel can be a good model to study the effects of antiarrhythmic drugs.

The present results indicated that R60 and S57 produced a concentration-dependent and reversible block of hKv1.5 channels. Furthermore, the time course of current activation was unaffected, which indicated that block did not occur until the channel opens. From the analysis of the concentration-dependence of block, it was evident that the potency of R60 and S57 in blocking hKv1.5 channels was very similar. In fact, the apparent K_D values calculated from the fit of the experimental values to the concentration-response equation were 29 μM and 41 μM for S57 and R60, respectively. Therefore, S57 was only 1.4 fold more potent than R60 in blocking hKv1.5 channels, which indicated that the channel block displays a moderate stereoselectivity. The lack of stereoselective block suggested that the chiral centre must be located in a structurally highly tolerant region of the molecule, irrelevant for their interaction with the receptor site, i.e., 'silent chirality' (Ariëns, 1993). At present the effects of the enantiomers of bupivacaine (Valenzuela *et al.*, 1995b), terfenadine (Yang *et al.*, 1995) and quinidine (Snyders *et al.*, 1992) and its diastereomer, quinine (Snyders & Yeola, 1995) on hKv1.5 channels have been compared. The blockade produced by bupivacaine enantiomers was highly stereoselective, **R**-(+)-bupivacaine being 7 fold more potent than S-(-)-bupivacaine in blocking hKv1.5 currents. Stereoselective differences also exist between quinidine and quinine in affinity and kinetics of hKv1.5 block, quinidine being almost 3 fold more potent than quinine. In contrast, as reported in the present study with R60 and S57, the terfenadine enantiomers were approximately equipotent open state blockers of this human channel (K_D for **R**- and S-terfenadine were 1.19 μM and 1.16 μM, respectively) (Yang *et al.*, 1995). Therefore, further studies are needed to elucidate if the stereoselective block of the hKv1.5 channels is linked to differences in the 'size' (molecular weights of bupivacaine and quinidine are 325 as compared to 501 for R60 and S57 or to 472 for terfenadine) or in lipid solubility of these compounds (logP = 4 for bupivacaine, 3.6 for quinidine and 0.9 for R60 and S57) or spatial arrangements. When the K_D values obtained in the present study for R60 and S57 are compared with those previously reported for other cardioactive drugs, it was evident that these bradycardic agents are less potent than quinidine ($K_D = 6.2$ μM, Snyders *et al.*, 1992), terfenadine racemate ($K_D = 0.367$ μM, Rampe *et al.*, 1993a; $K_D = 0.88$ μM, Yang *et al.*, 1995), **R**-(+)-bupivacaine ($K_D = 4$ μM, Valenzuela *et al.*, 1995b) and zatebradine ($K_D = 1.8$ μM, Valenzuela *et al.*, 1995a), but as potent as verapamil ($K_D = 45$ μM, Rampe *et al.*, 1993b).

In addition to their concentration-dependent effects, the interaction of both enantiomers with the hKv1.5 channels was also voltage-dependent. The blockade increased steeply in the voltage range of channel activation (between −30 and 0 mV), thus suggesting that both enantiomers bind preferentially to the open state of the channel (i.e., O↔OB transition). Moreover, the block produced by S57 and R60 continues to increase at potentials positive to 0 mV, when the channel activation reached saturation. This shallow voltage-dependence cannot be attributed to channel opening, but is similar to that previously described for quinidine (Snyders *et al.*, 1992) and bupivacaine enantiomers (Valenzuela *et al.*, 1995b). Because R60 and S57 are weak bases (p*Ka* = 8.5. Joulin, Y., personal communication), it is expected that they will predominate in the charged form at the intracellular pH (7.2). Therefore, if it is assumed that both enantiomers block hKv1.5 channels by interacting with the open state of the channel and access their binding site from the intracellular side of the membrane, the extra voltage-dependence of block can be attributed to the effects of the transmembrane electrical field on the charged form at the receptor site. The δ value for the voltage-dependence of the apparent K_D calculated by fitting our experimental results to the Boltzmann relationship (eq.4) yielded a value of about 0.18 which was interpreted to indicate that S57 and R60 moved about 18% into the membrane electrical field to reach its receptor on the channel and block the hKv1.5 current. This δ value was also very similar to that previously described for quinidine (Snyders *et al.*, 1992), bupivacaine (Valenzuela *et al.*, 1995b), terfenadine (Rampe *et al.*, 1993a; Yang *et al.*, 1995) or verapamil (Rampe *et al.*, 1993b) in hKv1.5 channels and for internal tetraethylammonium block in *Shaker* K^+ channels (Yellen *et al.*, 1991; Choi *et al.*, 1993), which probably reflects that R60 and S57 could bind to the same receptor site as these compounds.

As was evident from inspection of Figures 1 and 4a, the most noticeable effect observed in the presence of R60 or S57, was the induction of a fast decline of the current during the depolarizing pulses which superimposed on the slow inactivation. At concentrations equal to or higher than 30 μM this initial decline was 10 times faster than the inactivation process of the hKv1.5 current. This fast phase of decline can be considered to represent the interaction of both enantiomers to the open state of the channel (O↔OB) and its time constant (τ_B) an approximation of the drug-channel interaction kinetics. Based on this assumption, from the plot of τ_B as a function of the concentration of each enantiomer (Figure 4b), we were able to calculate the apparent association (k) and the dissociation (l) rate constants. The experimental results indicated that the apparent association rate constants were very similar in the presence of both enantiomers, whereas the l values were faster for R60 (25.8 s^{-1}) than for S57 (12.6 s^{-1}). Binding rates for S57 and R60 can also be derived from the apparent K_D (29 μM and 41 μM, respectively) and the average value of the time constants of block at 50 μM. Using this procedure, the calculated k values for S57 and R60 were 0.52×10^6 M^{-1} s^{-1} and 0.68×10^6 M^{-1} s^{-1} and the l values 15.2 s^{-1} and 27.8 s^{-1}, respectively. As can be observed, these values are close to those obtained when the τ_{Block} was plotted as a function of the concentration of each enantiomer. The apparent association rate constants of R60 and S57 are 5–8 times slower than those obtained for quinidine (4.5×10^6 M^{-1} s^{-1}; Snyders *et al.*, 1992), **R**-bupivacaine (4.7×10^6 M^{-1} s^{-1}; Valenzuela *et al.*, 1995b) or terfenadine (3.5×10^6 M^{-1} s^{-1}; Rampe *et al.*, 1993a; Yang *et al.*, 1995). This may be due to the higher molecular weight of R60 and S57. On the other hand, it is also important to note that the main difference in the effects of R60 and S57 on hKv1.5 channels was linked to the apparent dissociation rate constant. A similar finding was observed in the presence of quinidine and quinine, whereas for the enantiomers of bupivacaine, the derived dissociation rate constant was similar and the main differences in potency were related to the apparent association rate constant which was 5 fold faster for **R**-(+)-bupivacaine.

Open channel block not only can affect the time course of

the currents during the depolarizing pulse but also the tail currents. This is actually a direct prediction of the model shown in Figure 6. Under control conditions, tail currents reflected the rapid and irreversible closing of the channels upon repolarization. If a large fraction of channels are blocked (OB) and the unbinding kinetics are fast enough, then the tail current may initially display a rising phase reflecting the OB→O unblocking. Subsequently, the tail current should decline more slowly than in control conditions, because a fraction of unblocked channels become blocked again (O→OB) rather than closing irreversibly, thus resulting in the 'crossover phenomenon'. The present data demonstrated that both enantiomers induced a crossover phenomenon, which indicated that unbinding is required before channels can close and provided further evidence for the proposed open channel interaction. This phenomenon has been also reported with other open state cardiac potassium channel blockers like encainide, flecainide, almokalant and quinidine (Follmer *et al.*, 1992; Snyders *et al.*, 1992; Carmeliet, 1993). In contrast, propafenone which also acts as open state blocker of the rapid and slow components of the delayed rectifier current did not induce a tail crossover in guinea-pig ventricular myocytes, probably because the drug exhibits a slow unblocking kinetics (Delpón *et al.*, 1995).

All these results indicated that R60 and S57 exhibited a similar concentration-, voltage- and time-dependent block of hKv1.5 channels, i.e., the blockade displays a moderate stereoselectivity. Since hKv1.5 current is similar to the ultrarapid delayed rectifier observed in human atrial myocytes (Wang *et al.*, 1993), the present results may suggest that both enantiomers would tend to lengthen the cardiac action potential. Final effect of R60 and S57 on action potential duration would be the result of their effects on the multiple ionic currents involved in control of cardiac repolarization.

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