



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

*¹T. González, ¹M. Longobardo, ¹R. Caballero, ¹E. Delpón, ²J.V. Sinisterra, ¹J. Tamargo & ¹C. Valenzuela

¹Institute of Pharmacology and Toxicology, CSIC/UCM. School of Medicine, Universidad Complutense. 28040 Madrid, Spain and ²Department of Organic and Pharmaceutical Chemistry. School of Pharmacy, Universidad Complutense. 28040 Madrid, Spain

1 The N-substituent of IQB-9302 has the same number of carbons as bupivacaine, but it exhibits a different spatial localization (n-butyl vs cyclopropylmethyl). Thus, the study of the effects of IQB-9302 enantiomers on hKv1.5 channels will lead to a better knowledge of the determinants of stereoselective block.

2 The effects of the IQB-9302 enantiomers were studied on hKv1.5 channels stably expressed in *Ltk*⁻ cells using the whole-cell configuration of the patch-clamp technique. Drug molecular modelling was performed using Hyperchem software.

3 Block induced by IQB-9302 was stereoselective with the R(+) enantiomer being 3.2-fold more potent than the S(-) one (K_D of $17.8 \pm 0.5 \mu\text{M}$ vs $58.6 \pm 4.0 \mu\text{M}$).

4 S(-)- and R(+)-IQB-9302 induced-block was time- and voltage-dependent consistent with an electrical distance from the cytoplasmic side of 0.173 ± 0.022 ($n=12$) and 0.181 ± 0.018 ($n=10$), respectively.

5 Potency of block of pipercoloxylidide local anaesthetics was linearly related to the length between the cationic tertiary amine and the end of the substituent.

6 Molecular modelling shows that only when S(-) and R(+) enantiomers are superimposed by their aromatic ring, their N-substituents are in opposite directions, which can explain the stereospecific block induced by bupivacaine and IQB-9302 with hKv1.5 channels.

7 These results suggest that: (a) IQB-9302 enantiomers block the open state of hKv1.5 channels, and (b) the length of the N-substituent in these local anaesthetics and not its volume determines the potency and degree of their stereoselective hKv1.5 channel block.

British Journal of Pharmacology (2001) **132**, 385–392

Keywords: IQB-9302; hKv1.5; bupivacaine; local anaesthetics; stereoselectivity

Abbreviations: δ , fractional electrical distance; TEA, tetraethylammonium; τ_B , time constant of block

Introduction

Local anaesthetics 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 & Katzung, 1977; Strichartz, 1987). However, voltage-gated Na^+ channels from other tissues when exposed to sufficient concentrations of these agents will be blocked. This can explain why the accidental intravascular injection or the use of high concentrations of local anaesthetics can produce profound systemic effects, especially in the central nervous and cardiovascular systems (Strichartz, 1987). Indeed, several local anaesthetics also exhibit class I antiarrhythmic actions on the myocardium at concentrations lower than those used for local anaesthesia (Hondeghem & Katzung, 1977; Strichartz, 1987).

Bupivacaine is a potent local anaesthetic widely used for long-lasting regional local anaesthesia (Strichartz, 1987). Bupivacaine decreases intracardiac conduction velocity,

contractile force, depresses spontaneous sinoatrial activity (Strichartz, 1987) and prolongs QRS and QTc intervals of the ECG in anaesthetized dogs (Kasten & Martin, 1985; Wheeler *et al.*, 1988) and human volunteers (Scott *et al.*, 1989) receiving high doses of bupivacaine. It has been reported that this cardiotoxicity is mostly related to the R(+) enantiomer (Åberg, 1972; Luduena *et al.*, 1972) likely due to its higher potency for blocking cardiac sodium and potassium channels (Valenzuela *et al.*, 1995a,b). In human cardiac myocytes, K^+ currents activated by depolarization have been identified as transient outward (I_{TO}) (Escande *et al.*, 1987; Shibata *et al.*, 1989; Beuckelmann *et al.*, 1993; Wettwer *et al.*, 1993) and delayed rectifier currents, I_{Ks} , I_{Kr} and I_{Kur} (Fedida *et al.*, 1993; Wang *et al.*, 1993a,b). The functional and pharmacologic characteristics of I_{Kur} are similar to those reported for the cloned cardiac human Kv1.5 channel, thus suggesting that it is the native counterpart of hKv1.5 channels cloned from human ventricle (Snyders *et al.*, 1992; 1993; Fedida *et al.*, 1993; Wang *et al.*, 1993a). Bupivacaine blocks several potassium channels, including hKv1.5, Kv2.1, Kv4.3,

*Author for correspondence; E-mail: carmenva@eucmax.sim.ucm.es

KvLQT1+minK and *HERG* channels, the K_D values being ~ 9 , 22, 24, 200 and 17 μM , respectively (Lipka *et al.*, 1998; Franqueza *et al.*, 1999; González *et al.*, 2000a). However, stereoselective bupivacaine block has only been demonstrated on hKv1.5 channels, but not on Kv2.1 or Kv4.3 (Franqueza *et al.*, 1997; 1999).

IQB-9302 is a new amide type local anaesthetic, chemically related to bupivacaine (Figure 1) (Gallego-Sandín *et al.*, 1999). Like bupivacaine, IQB-9302 blocks Kv2.1, Kv4.3 and *HERG* channels with similar potency than bupivacaine ($K_D \sim 20 \mu\text{M}$). IQB-9302 also inhibits hKv1.5 current, but it is 2.5 fold less potent than bupivacaine ($K_D = 22 \mu\text{M}$) (González *et al.*, 2000a). It contains a chiral carbon in its structure and therefore, it can be separated into two enantiomers: R(+) and S(-)IQB-9302. In this study we have analysed the effects of both enantiomers on hKv1.5 channels. It will permit us to better understand the structural determinants of pipercoloxylidide local anaesthetics induced-block of these channels, since the N-substituent of IQB-9302 has the same number of carbons than bupivacaine, but exhibits a different spatial localization (n-butyl *vs* cyclopropylmethyl). Moreover, the comparison of the results derived from this study with the effects on hKv1.5 channels of bupivacaine and ropivacaine enantiomers (Valenzuela *et al.*, 1995a; 1997) will permit us to hypothesize about the possible cardiotoxicity of this new local anaesthetic. Preliminary results of this study have been published in abstract form (González *et al.*, 1999; 2000b).

Methods

Electrophysiological recording

Use of the stable *Ltk*⁻ cell line expressing hKv1.5 channels has been described previously in detail (Valenzuela *et al.*, 1995a; Franqueza *et al.*, 1997; Longbardo *et al.*, 1998). The intracellular pipette filling solution contained (in mM): K-aspartate 80, KCl 50, phosphocreatine 3, KH_2PO_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.

hKv1.5 currents were recorded at room temperature (20–22°C) using the whole-cell patch-clamp technique (Hamill *et al.*, 1981) with an Axopatch 1C patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). Currents were filtered at 2 kHz (four-pole Bessel filter) and sampled at 4 kHz. Micropipettes were pulled from borosilicate glass capillary

tubes (Narishige, GD-1, Tokyo, Japan) on a programmable horizontal puller (Sutter Instrument Co., San Rafael, CA, U.S.A.) and heat-polished with a microforge (Narishige). Micropipettes resistance were 1–3 M Ω . Capacitance and series resistance compensation were optimized, and 80% compensation of the effective access resistance was usually obtained.

Drugs

IQB-9302 [1-(cyclopropylmethyl-2',6'-pipercoloxylidide) enantiomers (gift from Dr A. Galiano, IQB-Inibsa S.A., Spain) were dissolved in distilled deionized water to yield stock solutions of 10 mM from which further dilutions were made to obtain the desired final concentration in each experiment.

Pulse protocol and analysis

Cells were held at -80 mV . After control data were obtained, bath perfusion was switched to drug-containing solution. The effects of drug infusion was monitored with test pulses to $+60 \text{ mV}$, applied every 10 s until steady-state was obtained (after $\sim 12 \text{ min}$). 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 at -40 mV . The activation curve was obtained from the tail current amplitude measured just after the capacitive transient. Command potentials, data acquisition and measurements were done using PCLAMP 6.0.1., Origin 5.0 (Microcal Software, Northampton, MA, U.S.A.) and by a custom-made analysis program.

Apparent affinity constants, K_D , and Hill coefficients, n_H , were obtained from fitting of the fractional block, f , at various drug concentrations [D]:

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

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

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

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

where τ_B represents the time constant of the drug-induced fast initial decline during depolarization to $+60 \text{ mV}$.

The dominant time constant of the activation process was analysed fitting it to a single exponential, following a procedure previously described and used for the same purpose (Valenzuela *et al.*, 1995a). Deactivation and inactivation were fitted to a biexponential process:

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

where $\tau_1, \tau_2 \dots \tau_n$ are the system time constants, $A_1, A_2 \dots A_n$ are the amplitudes of each component of the exponential, and C is the baseline value. Half-maximal voltages (E_h) and slope factors (s) of activation were determined by fitting data with a Boltzmann equation: $y = 1/[1 + \exp(-(E - E_h)/s)]$. The

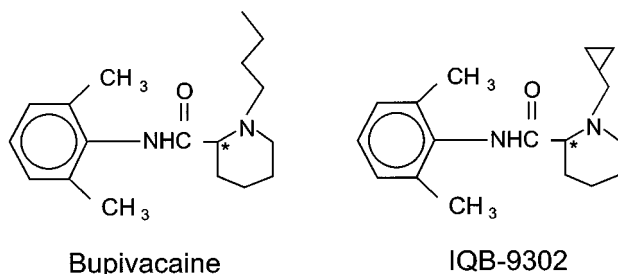


Figure 1 Chemical structure of bupivacaine and IQB-9302. The asterisk indicates the asymmetric carbon in the molecule.

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:

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

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).

Molecular modelling

Models of bupivacaine analogues with different N-substituents (n-butyl, cyclopropylmethyl, n-propyl and methyl) in their cationic forms were constructed and energy minimized with the Hyperchem software 5.1 (Molecular modelling system, Hypercube, Inc. and Autodesk Inc., 1998) following procedures previously reported (López-Belmonte *et al.*, 1997). MM⁺ force field and the conjugated algorithm Fletcher-Reeves were used to minimize the structures. MNDO semiempirical method was used to calculate the charges and molecular orbitals.

Statistical methods

Results are expressed as mean \pm s.e.mean. 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. Differences were considered significant if $P < 0.05$.

Results

Figure 2 shows original hKv1.5 current records obtained in control conditions, in the presence of 50 μ M S(-)IQB-9302 or R(+)IQB-9302, and after washout with drug-free external solution. Under control conditions, hKv1.5 current activates rapidly, reaches a maximum peak current and slowly inactivates, as previously described (Valenzuela *et al.*, 1995a). At 50 μ M, both enantiomers inhibited hKv1.5 current, being R(+)IQB-9302 3.2 fold more potent than S(-)IQB-9302, (70.4 \pm 3.0%, $n = 4$, vs 46.5 \pm 3.1%, measured at +60 mV, $n = 10$, $P < 0.01$, respectively). The effects of both enantiomers were reversible upon perfusion of the cells with drug-free external solution (93 \pm 1% of the control values, $n = 23$). S(-)IQB-9302 or R(+)IQB-9302 did not modify the activation kinetics of the current (1.28 \pm 0.11 ms vs 1.06 \pm 0.11 ms, $n = 8$, $P > 0.05$; and 1.73 \pm 0.20 ms vs 1.27 \pm 0.04, $n = 6$, $P > 0.05$; respectively), but induced a fast decline of the current at the beginning of the depolarizing pulse, faster at higher drug concentrations, suggesting an open channel block mechanism.

Figure 3 shows the concentration response curve when using as an index of block the suppression of the current induced by both enantiomers at the end of 250 ms depolarizing pulses to +60 mV. A non-linear least-squares fit of the concentration-response equation yielded, for S(-)IQB-9302, a K_D and the n_H values of 58.6 \pm 4.0 μ M and 0.874 \pm 0.055, respectively. For R(+)IQB-9302, these values averaged 17.8 \pm 0.5 μ M and 0.912 \pm 0.025. When n_H was fixed to unity, the K_D values for S(-)IQB-9302 and R(+)IQB-9302 were 58.1 \pm 5.1 μ M ($P > 0.05$) and 18.1 \pm 0.9 μ M ($P > 0.05$).

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

Figure 4A shows the IV relationships obtained in the absence and in the presence of either S(-)IQB-9302 (50 μ M) or R(+)IQB-9302 (10 μ M). Both enantiomers decreased the amplitude of the current at membrane potentials positive to -10 mV, 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.*, 1995a; 1997; Longobardo *et al.*, 1998). In order to quantitate this voltage dependence we represented the relative current in the presence of either 50 μ M S(-)IQB-9302 or 10 μ M R(+)IQB-9302 vs membrane potential (Figure 4B). Block steeply increased in the range of membrane potentials of the activation of the channels, which indicates that the drug needs the channel to 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 \pm 0.006) and thus, they are predominantly charged at the physiological pH. Therefore, this increase in block can be attributed to the effect of the transmembrane electrical field on the interaction between the cationic drug and the channel. Following a Woodhull formalism (Woodhull, 1973), a non linear curve fitting of the data 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 0.173 \pm 0.022 ($n = 12$) and 0.181 \pm 0.018 ($n = 10$), respectively, indicating that they have to cross \sim 18% of the transmembrane electrical field to bind their receptor site in the hKv1.5 channel. In addition, the calculated K_D^* averaged 78.8 \pm 26.3 μ M ($n = 9$) and 29.7 \pm 2.2 μ M ($n = 10$) for S(-)IQB-9302 and R(+)IQB-9302, respectively.

Time-dependent block

S(-) and R(+)IQB-9302 induced a fast initial decline of the current which was superimposed to the slow inactivation at concentrations higher than 100 and 10 μ M, respectively (Figure 5A). The time constant of this decline of the current was lower at higher drug concentrations and thus, it was considered to be a good index of the kinetics of binding of the drug (τ_B). From the τ_B values, the apparent drug association (k) and dissociation (l) constants were calculated. For S(-)IQB-9302, k and l were 0.60 \pm 0.08 μ M⁻¹ s⁻¹ and 39.7 \pm 5.2 s⁻¹ ($n = 13$), respectively. For R(+)IQB-9302, k

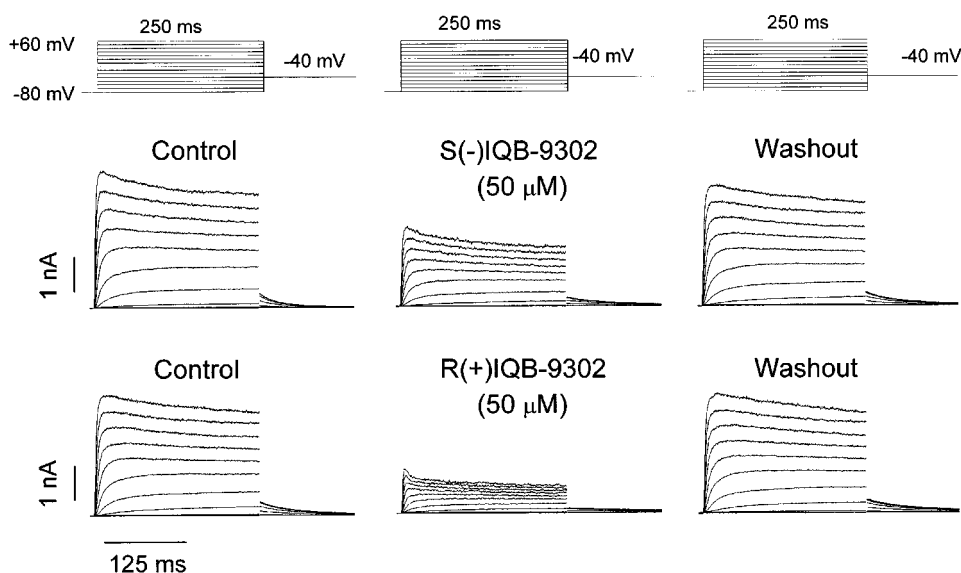


Figure 2 Effects of IQB-9302 enantiomers on hKv1.5 currents. Currents are shown for depolarization from -80 mV to voltages between -80 and $+60$ mV in steps of 10 mV. Tail currents were obtained on return to -40 mV. Traces were obtained in the absence (left panel), in the presence of $50 \mu\text{M}$ of each enantiomer of IQB-9302 (middle panel) and after washout with drug-free external solution (right panel). All records shown in this Figure were recorded from the same cell, which was first perfused with S(-)IQB-9302 and afterwards with R(+)-IQB-9302. The control for R(+)-IQB-9302 is the washout of S(-)IQB-9302. Cell capacitance, 23 pF.

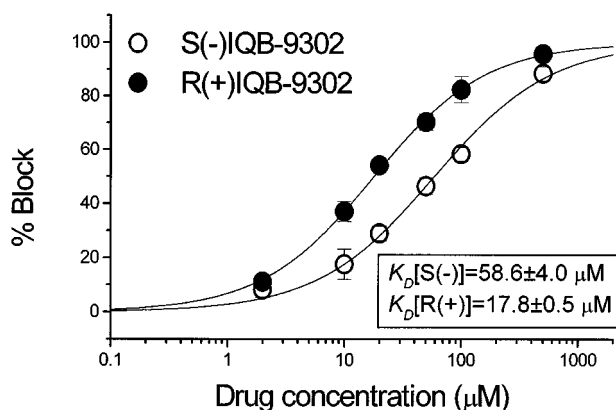


Figure 3 Concentration dependence of IQB-9302 enantiomers-induced block of hKv1.5 channels. Reduction of current (relative to control) at the end of depolarizing steps from -80 mV to $+60$ mV was used as index of block. Data are mean \pm s.e. mean of a total of 51 experiments. The continuous line represents the fit of the experimental data to the equation: $1/[1 + (K_D/[D])^n]$.

was 4.6 fold higher than that obtained for S(-)IQB-9302 ($2.90 \pm 0.63 \mu\text{M}^{-1} \text{s}^{-1}$, $n=7$, $P < 0.01$) whereas l remained similar to S(-)IQB-9302 ($52.2 \pm 11.4 \text{s}^{-1}$, $n=7$, $P > 0.05$).

Figure 5B shows superimposed current traces obtained on return to -40 mV under control conditions and in the presence of S(-)IQB-9302 ($20 \mu\text{M}$) or R(+)-IQB-9302 ($10 \mu\text{M}$). Under control conditions, the deactivating process was fitted following a biexponential process with a fast (τ_f) and a slow (τ_s) time constants. S(-)IQB-9302 increased the two time constants from 17.9 ± 3.3 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 and 48.4 ± 6.5 ms to 28.0 ± 5.0 ms ($n=5$, $P < 0.05$) and 73.0 ± 8.6 ms ($n=5$, $P < 0.05$). Upon repolarization, channel deactivation is fast and virtually irreversible. If a large fraction of channels are blocked (OD) and the unbinding rate (l) is fast enough, then the tail current may display a rising phase reflecting the dissociation of the drug from the blocked open channels (OD \rightarrow O). Subsequently, channels reach the open state and may be blocked again before they deactivate. This process will be reflected by a slower time course of the tail current and, thus, a tail 'crossover' suggestive of open channel block will be observed (Armstrong, 1971).

Discussion

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. First, at high concentrations, both enantiomers induced a fast initial decline of the maximal activated current, that superimposes to the intrinsic C-type inactivation of these channels. Second, block steeply increased in the range of activation of the hKv1.5 channels activation, indicating that the drug needs the channel to open to bind to its receptor site and block the K^+ efflux. Finally, when the tail currents obtained in the absence and in the presence of each enantiomer were superimposed, we observed a tail current 'crossover', indicating fast recovery from block during deactivation, consistent with an open channel block mechanism (Armstrong, 1971; Valenzuela *et al.*, 1995a).

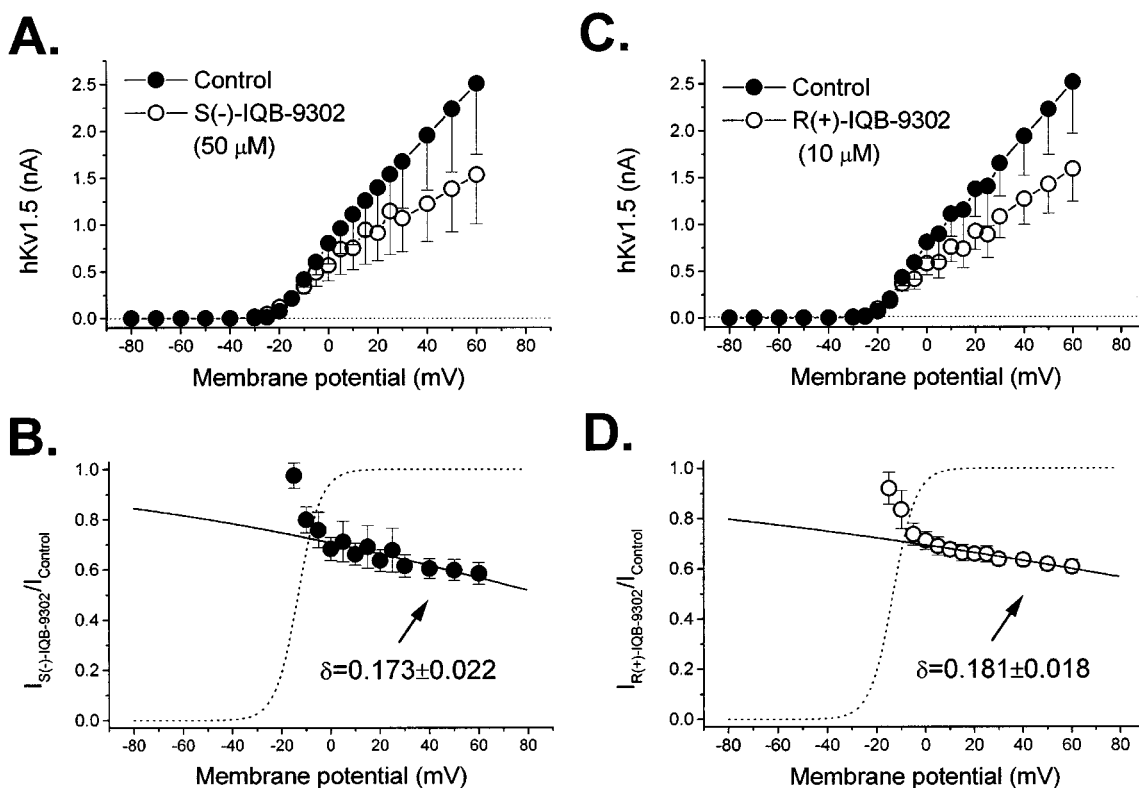


Figure 4 Voltage dependence of hKv1.5 block by IQB-9302 enantiomers. (A) Current-voltage relationship (250 ms isochronal) in control conditions and in the presence of 50 μM S(-)-IQB-9302. (B) Relative current expressed as $I_{S(-)IQB-9302}/I_{Control}$ from data shown in (A). (C) Current-voltage relationship (250 ms isochronal) in control conditions and in the presence of 10 μM R(+)-IQB-9302. (D) Relative current expressed as $I_{R(+)-IQB-9302}/I_{Control}$ from data shown in (C). The dashed lines in (B) and (D) represent the activation curve of the hKv1.5 channel for each experiment. In both cases, block increased steeply between -20 and 0 mV, which corresponds to the voltage range of activation of hKv1.5. For membrane potentials positive to 0 mV, a continued but more shallow voltage dependence was observed. This voltage dependence was fitted (continuous line) with eq. 4 (see Methods) and yielded $\delta \sim 0.18$.

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

Bupivacaine, ropivacaine, IQB-9302 and mepivacaine only differ in the N-substituent which is a n-butyl, n-propyl, cyclopropylmethyl and methyl, respectively. In order to quantitate the relationship between the affinity and the structure of these compounds (as cations), $\log K_D$ was plotted against several molecular parameters as $\log P$, van der Waals volume, atomic charges or energy of HOMO or LUMO, but no significant interactions were obtained. Nevertheless, the relationship between the affinity constant and the length of the alkyl chain bond to the tertiary nitrogen (in Å) revealed a good correlation coefficient (Figure 6) (Valenzuela *et al.*, 1995a; 1997; Longobardo *et al.*, 1998). For each pair of enantiomers, we could observe a good linear relationship between the potency and the length of the N-substituent group, suggesting that this region of the molecule determines the potency of the drug.

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

Block induced by IQB-9302 was stereoselective. As observed with bupivacaine (Valenzuela *et al.*, 1995a), the more potent enantiomer was the R(+) one, although for IQB-9302, the

degree of stereoselective block was lower than that observed for bupivacaine. The difference in potency between both enantiomers was related to a faster association rate constant, like it occurs with bupivacaine and ropivacaine (Valenzuela *et al.*, 1995a; 1997; Longobardo *et al.*, 1998). Stereoselective interactions suggest that unidirectional structural properties of the chain such as hydrophobicity ($\log P$) or van der Waals volume (Vw) cannot be the factor responsible for differences in potency (Figure 6). In contrast, it seems to be the consequence of some stereochemical property of the drug, such as the length of the N-substituent, which would adopt opposite spatial localization for each pair of enantiomers.

To further analyse this issue, the minimum energy conformers of the N-protonated bupivacaine (N-n-butyl), ropivacaine (N-n-propyl), IQB-9302 (N-cyclopropylmethyl) and mepivacaine (N-methyl) were modelled using the Hyperchem program. The cationic forms were selected because all these drugs are weak bases (with pKa values ~ 8) and, therefore, they will be mostly protonated at the physiological pH. This analysis was carried out both for S(-) and R(+) enantiomers and docking of the conformers of minimum energies were performed in order to explore steric differences. Figure 7A,B show the minimum energy conformers of the two enantiomers of bupivacaine and IQB-9302 superimposed by the piperidine ring. Under these circumstances, each pair of enantiomers fit almost comple-

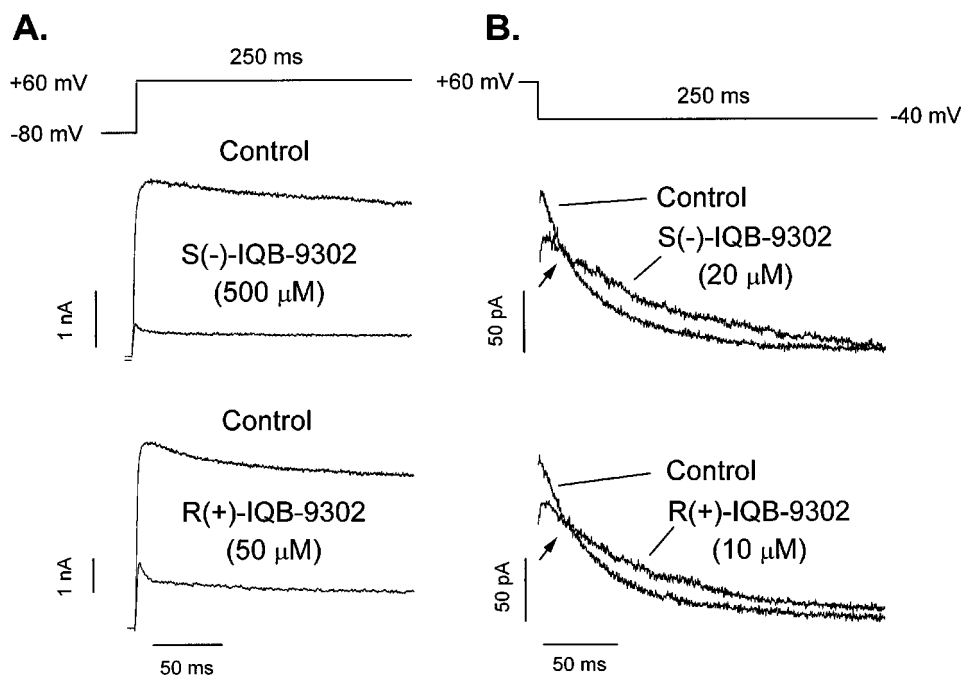


Figure 5 Time-dependent block induced by IQB-9302 enantiomers. (A) Superimposed traces for steps from -80 mV to $+60$ mV under control conditions and in the presence of $500 \mu\text{M}$ S(-)-IQB-9302 and $50 \mu\text{M}$ R(+)-IQB-9302. (B) Tail current crossover. Currents recorded in control conditions and in the presence of $20 \mu\text{M}$ S(-)-IQB-9302 or $10 \mu\text{M}$ R(+)-IQB-9302 were superimposed. Tail currents were obtained at -40 mV after a 250 ms-depolarizing pulse to $+60$ mV. Arrow shows the crossover of tracings recorded in the presence of IQB-9302 enantiomers with those recorded under control conditions.

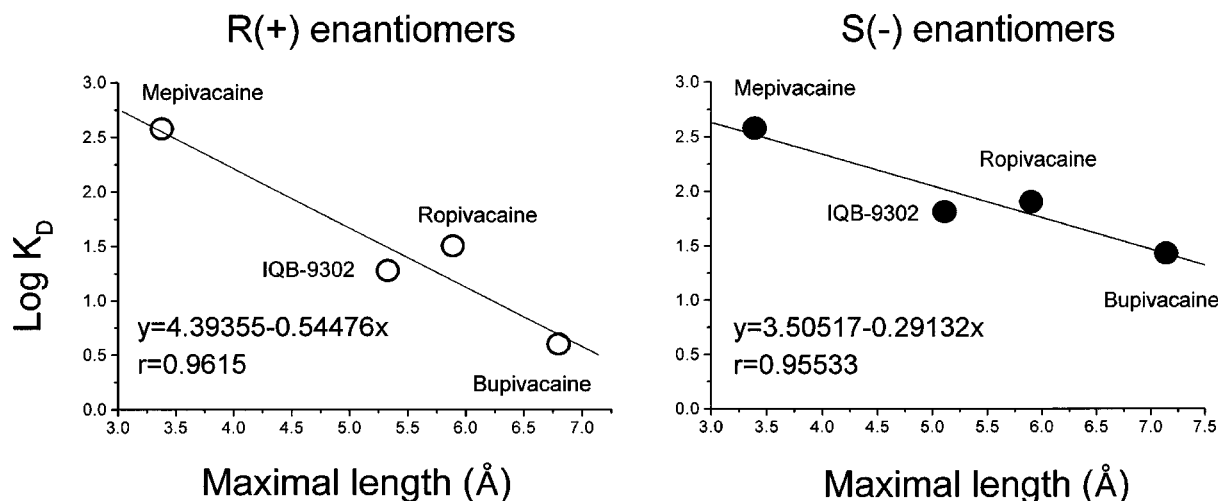


Figure 6 Relationship between the potency of block of hKv1.5 channels and the maximal length between the tertiary amine and the end of the N-substituent in mepivacaine, IQB-9302, ropivacaine and bupivacaine.

tely, which does not explain the experimental results that revealed the 7- and 3.2 fold differences in potency for bupivacaine and IQB-9302, respectively (Valenzuela *et al.*, 1995a). Figure 7C,D show the superimposed S(-) and R(+) enantiomers of bupivacaine and IQB-9302, in this case by their aromatic rings. It is observed that in both cases S(-) and R(+) enantiomers differ in the position of the N-substituents, which are in opposite directions for each enantiomer. According to Figure 6, R(+) enantiomers adopt a more favourable conformation in energetic terms than the S(-) enantiomers, that explain their higher potency. This simple approach led us to postulate a simple recognition

model for this type of local anaesthetics (Valenzuela *et al.*, 1995a; 1997; Longobardo *et al.*, 1998), suggesting a restrictive site for the recognition of the aromatic ring. Under this hypothesis, first, the aromatic ring of the drug would interact with an amino acid containing an aromatic ring (Phe, Tyr or Trp) establishing a π - π interaction between both aromatic rings, which are more stable, in energetic terms, than the hydrophobic interaction between alkyl chains. Therefore, they have been described as crucial for this kind of compound when they interact with other proteins such as pancreatic porcine lipase (Borreguero *et al.*, 1999). Afterwards, the N-substituent would interact with some of the two

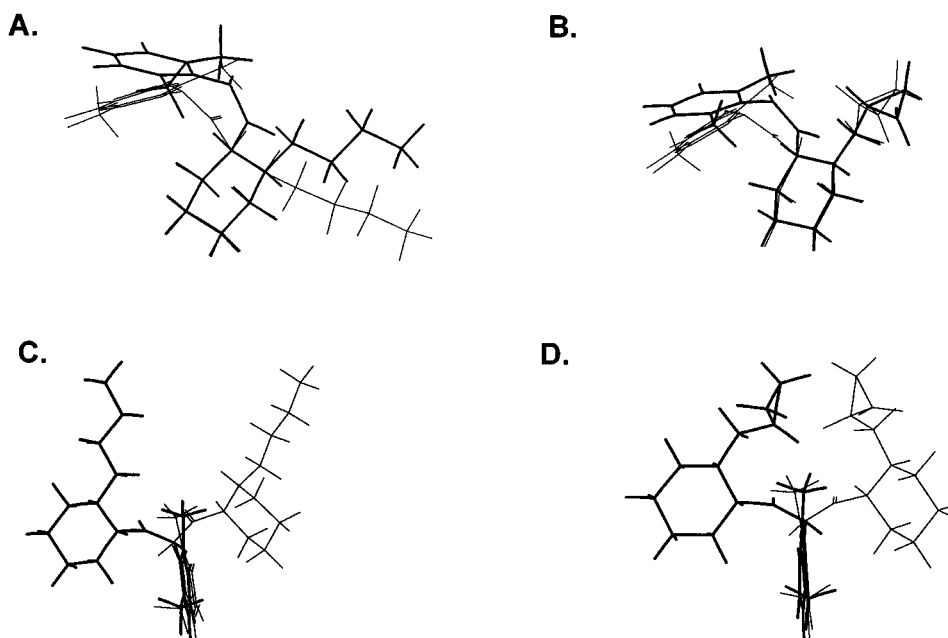


Figure 7 Docking of minimum energy conformers of the two enantiomers of bupivacaine and IQB-9302 superimposed by the pipercoloxylidide ring (A,B) or by the aromatic ring (C,D). In both cases, the R(+) enantiomers are shown with thicker lines than the S(-) ones. Note that only when each pair of enantiomers are superimposed by the pipercoloxylidide a clear difference in the spatial location of the drugs is observed. This finding could explain the experimental differences in potency observed for R(+) and S(-) enantiomers. Both models were constructed by using the Hiperchem program.

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). Under this approach, this interaction is stereocontrolled by the stereochemistry of the chiral carbon of the local anaesthetic, as we can observe in Figure 7C,D. Possible candidates for the anchoring of the aromatic ring of the pipercoloxylidide type local anaesthetics in hKv1.5 channels would be F437 and F438, located at the S5 segment, although we cannot exclude other aromatic amino acids present at the inner part of the ion pore region. Indeed, following the recent proposed structural 'bent S6' model for Kv channels by Del Camino *et al.* (2000), some of the aromatic amino acids of the F(N)YFY amino acid sequence at the inner part of the ion pore would be responsible for the anchoring of this kind of drug in hKv1.5 channels. However, a more detailed Molecular Dynamic study is needed to verify this theoretical hypothesis.

Conclusions

The results obtained in the present study show that both potency of block of hKv1.5 channels and the degree of stereoselective block is lower in the presence of IQB-9302

than in the presence of bupivacaine (Valenzuela *et al.*, 1995a), which could be related to lesser cardiotoxic effects than those previously reported for bupivacaine. Therefore, IQB-9302 could represent a less cardiotoxic alternative to bupivacaine. Moreover, the molecular modelling of these results suggest that potency and stereoselective hKv1.5 block induced by bupivacaine-like local anaesthetics are related to the length of their N-substituent and not to its hydrophobicity or van der Waals volume. As a qualitative binding model, we propose that these drugs may first bind to the hKv1.5 channels by anchoring the aromatic ring onto some aromatic amino acid and this interaction could redirect the entire molecule determining the spatial localization of the N-substituent, that will interact with L508 and/or V512 at the S6 level (Franqueza *et al.*, 1997).

The authors want to express their thanks to Drs D.J. Snyders and M.M. Tamkun for providing us with the cell line expressing the gene encoding hKv1.5 channels. We also thank Ms Guadalupe Pablo and Mr Jose Luis Llorente for their excellent technical assistance. This work was supported by CICYT SAF98-0058 (C. Valenzuela), CICYT SAF99-0069 (J. Tamargo), CAM 08.4/0016198 (E. Delpón), IQB-Inibsa S.A. and U.S.-Spain Science and Technology Program 98131 (C. Valenzuela) Grants.

References

- ÅBERG, G. (1972). Toxicological and local anaesthetic effects of optically active isomers of two local anaesthetic compounds. *Acta Pharmacol. Toxicol.*, **31**, 273–286.
- ARMSTRONG, C.M. (1971). Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. Gen. Physiol.*, **58**, 413–437.
- BEUCKELMANN, D.J., NÄBAUER, M. & ERDMANN, E. (1993). Alterations of K^+ currents in isolated human ventricular myocytes from patients with terminal heart failure. *Circ. Res.*, **73**, 379–385.

- BORREGUERO, I., SINISTERRA, J.V., RUMBERO, A., HERMOSO, J.A., MARTINEZ-RIPOLL, M. & ALCANTARA, A.R. (1999). Acyclic phenylalkanedioles as substrates for the study of enzyme recognition. Regioselective acylation by porcine pancreatic lipase: A structural hypothesis for the enzymatic selectivity. *Tetrahedron*, **55**, 14961–14974.
- DEL CAMINO, D., HOLMGREN, M., LIU, Y. & YELLEN, G. (2000). Blocker protection in the pore of a voltage-gated K⁺ channel and its structural implications. *Nature*, **403**, 321–325.
- ESCANDE, D., COULOMBE, A., FAIVRE, J., DEROUBAIX, E. & CORABOEUF, E. (1987). Two types of transient outward currents in adult human atrial cells. *Am. J. Physiol.*, **252**, H142–H148.
- FEDIDA, D., WIBLE, B., WANG, Z., FERMINI, B., FAUST, F., NATTEL, S. & BROWN, A.M. (1993). Identity of a novel delayed rectifier current from human heart with a cloned K⁺ channel current. *Circ. Res.*, **73**, 210–216.
- FRANQUEZA, L., LONGOBARDO, M., VICENTE, J., DELPON, E., TAMKUN, M.M., TAMARGO, J., SNYDERS, D.J. & VALENZUELA, C. (1997). Molecular determinants of stereoselective bupivacaine block of hKv1.5 channels. *Circ. Res.*, **81**, 1053–1064.
- FRANQUEZA, L., VALENZUELA, C., ECK, J., TAMKUN, M.M., TAMARGO, J. & SNYDERS, D.J. (1999). Functional expression of an inactivating potassium channel (Kv4.3) in a mammalian cell line. *Cardiovasc. Res.*, **41**, 212–219.
- GALLEGO-SANDIN, S., NOVALBOS, J., GALVEZ-MUGICA, M.A., GARCIA, A.G. & ABAD, F. (1999). Comparison of hemodynamic effects of IQB-9302 and bupivacaine in anaesthetized rats. *Meth. Find. Exp. Clin. Pharmacol.*, **21**, 158.
- GONZALEZ, T., LONGOBARDO, M., CABALLERO, R., DELPON, E., TAMARGO, J. & VALENZUELA, C. (1999). Effects of IQB-9302 enantiomers on a human cardiac cloned K⁺ channel (Kv1.5). *Meth. Find. Exp. Clin. Pharmacol.*, **21**, 152.
- GONZALEZ, T., LONGOBARDO, M., CABALLERO, R., DELPON, E., TAMARGO, J. & VALENZUELA, C. (2000a). Effects of bupivacaine and a novel local anesthetic, IQB-9302, on human cardiac K⁺ channels. *J. Pharmacol. Exp. Ther.* (in press).
- GONZALEZ, T., LONGOBARDO, M., CABALLERO, R., DELPON, E., TAMARGO, J. & VALENZUELA, C. (2000b). Effects of a new local anesthetic, IQB-9302, and its enantiomers on hKv1.5 channels. *Biophys. J.*, **78**, 212A.
- HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.
- HILLE, B. (1977). Local anesthetics: Hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.*, **69**, 497–515.
- HONDEGHEM, L.M. & KATZUNG, B.G. (1977). Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochim. Biophys. Acta.*, **472**, 373–398.
- KASTEN, G.W. & MARTIN, S.T. (1985). Bupivacaine cardiovascular toxicity: comparison of treatment with bretylium and lidocaine. *Anesth. Analg.*, **64**, 911–916.
- LIPKA, L.J., JIANG, M. & TSENG, G.N. (1998). Differential effects of bupivacaine on cardiac K channels: role of channel inactivation and subunit composition in drug-channel interaction. *J. Cardiovasc. Electrophysiol.*, **9**, 727–742.
- LONGOBARDO, M., DELPON, E., CABALLERO, R., TAMARGO, J. & VALENZUELA, C. (1998). Structural determinants of potency and stereoselective block of hKv1.5 channels induced by local anesthetics. *Mol. Pharmacol.*, **54**, 162–169.
- LOPEZ-BELMONTE, M.T., ALCANTARA, A.R. & SINISTERRA, J.V. (1997). Enantioselective esterification of 2-arylpropionic acids catalyzed by immobilized *Rhizomucor miehei* lipase. *J. Org. Chemistry*, **62**, 1831–1840.
- LUDUENA, F., BOGADO, E. & TULLAR, B. (1972). Optical isomers of mepivacaine and bupivacaine. *Arch. Int. Pharmacodyn. Ther.*, **200**, 359–369.
- SCOTT, D.B., LEE, A., FAGAN, D., BOWLER, G.M., BLOOMFIELD, P. & LUNDH, R. (1989). Acute toxicity of ropivacaine compared with that of bupivacaine. *Anesth. Analg.*, **69**, 563–569.
- SHIBATA, E.F., DRURY, T., REFSUM, H., ALDRERTE, V. & GILES, W. (1989). Contributions of transient outward currents to repolarization in human atrium. *Am. J. Physiol.*, **257**, H1773–H1781.
- SNYDERS, D.J., KNOTH, K.M., ROBERDS, S.L. & TAMKUN, M.M. (1992). Time-, voltage-, and state-dependent block by quinidine of a cloned human cardiac potassium channel. *Mol. Pharmacol.*, **41**, 322–330.
- SNYDERS, D.J., TAMKUN, M.M. & BENNETT, P.B. (1993). A rapidly activating and slowly inactivating potassium channel cloned from human heart. Functional analysis after stable mammalian cell culture expression. *J. Gen. Physiol.*, **101**, 513–543.
- STRICHARTZ, G.R. (1987). *Local Anesthetics*, Berlin, Springer-Verlag.
- VALENZUELA, C., DELPON, E., FRANQUEZA, L., GAY, P., SNYDERS, D.J. & TAMARGO, J. (1997). Effects of ropivacaine on a potassium channel (hKv1.5) cloned from human ventricle. *Anesthesiology*, **86**, 718–728.
- VALENZUELA, C., DELPON, E., TAMKUN, M.M., TAMARGO, J. & SNYDERS, D.J. (1995a). Stereoselective block of a human cardiac potassium channel (Kv1.5) by bupivacaine enantiomers. *Biophys. J.*, **69**, 418–427.
- VALENZUELA, C., SNYDERS, D.J., BENNETT, P.B., TAMARGO, J. & HONDEGHEM, L.M. (1995b). Stereoselective block of cardiac sodium channels by bupivacaine in guinea pig ventricular myocytes. *Circulation*, **92**, 3014–3024.
- WANG, Z., FERMINI, B. & NATTEL, S. (1993a). Sustained depolarization-induced outward current in human atrial myocytes. Evidence for a novel delayed rectifier K⁺ current similar to Kv1.5 cloned channel currents. *Circ. Res.*, **73**, 1061–1076.
- WANG, Z., FERMINI, B. & NATTEL, S. (1993b). Delayed rectifier outward current and repolarization in human atrial myocytes. *Circ. Res.*, **73**, 276–285.
- WETTWER, E., AMOS, G., GATH, J., ZERKOWSKI, H.R., REIDEMISTER, J.G. & RAVENS, U. (1993). Transient outward current in human and rat ventricular myocytes. *Cardiovasc. Res.*, **27**, 1662–1669.
- WHEELER, D.M., BRADLEY, E.L. & WOODS, JR. W.T. (1988). The electrophysiologic actions of lidocaine and bupivacaine in the isolated, perfused canine heart. *Anesthesiology*, **68**, 201–212.
- WOODHULL, A.M. (1973). Ionic blockade of sodium channels in nerve. *J. Gen. Physiol.*, **61**, 687–708.

(Received May 23, 2000

Revised October 30, 2000

Accepted November 14, 2000)