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


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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 receiving high doses of bupivacaine has been reported that it is the native counterpart of hKv1.5 channels cloned from human ventricle (Snyders et al., 1994a,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_{kr}, I_{kr} and I_{khr} (Fedida et al., 1993; Wang et al., 1993a,b). The functional and pharmacologic characteristics of I_{khr} 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,b). Bupivacaine blocks several potassium channels, including hKv1.5, Kv2.1, and Kv4.3.
KvLQT1+minK and HERG channels, the $K_D$ values being $\sim 9, 22, 24, 200$ and $17 \mu M$, 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 to bupivacaine ($K_D \sim 20 \mu M$). IQB-9302 also inhibits hKv1.5 current, but it is 2.5 fold less potent than bupivacaine ($K_D = 22 \mu M$) (González et al., 2000a). It contains a chiral carbon in its structure and therefore can be separated into two enantiomers: $R(\pm)$ 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 pipecoloxylidide 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$_2$PO$_4$ 10, MgATP 3, HEPES-K 10, EGTA 5 and was adjusted to pH 7.40 with NaOH. 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 MΩ. 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'-pipecoloxylidide] 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 $+60$ mV, applied every $10$ s until steady-state was obtained (after $\sim 12$ 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 $[D]$:

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

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

$$k \times [D] + l = 1/\tau_B = \lambda$$

$$l/k = K_D$$

where $\tau_B$ represents the time constant of the drug-induced fast initial decline during depolarization to $+60$ 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:

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

where $\tau_1, \tau_2, \ldots, \tau_n$ are the system time constants, $A_1, A_2, \ldots, A_n$ are the amplitudes of each component of the exponential, and $C$ is the baseline value. Half-maximal voltages ($E_{50}$) and slope factors ($s$) of activation were determined by fitting data with a Boltzmann equation: $y = 1/[1 + \exp(-(E - E_{50})/s)]$. 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 $\chi^2$ 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 ($f = 1 - I_{\text{drug}}/I_{\text{control}}$). The voltage dependence of block was fitted to:

$$f = |D|/|D| + K_D^* \times \exp(-\delta zFE/RT),$$

where $z$, $F$, $R$, and $T$ have their usual meaning, $\delta$ 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; Longobardo et al., 1998). 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 ± 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 $\mu$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 $\mu$M, both enantiomers inhibited hKv1.5 current, being R(+)IQB-9302 3.2 fold more potent than S(-)IQB-9302, (70.4 ± 3.0%, $n=4$, vs 46.5 ± 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 ± 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 ± 0.11 ms vs 1.06 ± 0.11 ms, $n=8$, $P>0.05$; and 1.73 ± 0.20 ms vs 1.27 ± 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 $t_{1/2}$ values of 58.6 ± 4.0 $\mu$M and 0.874 ± 0.055, respectively. For R(+IQB-9302, these values averaged 17.8 ± 0.5 $\mu$M and 0.912 ± 0.025. When $t_{1/2}$ was fixed to unity, the $K_D$ values for S(-)IQB-9302 and R(+IQB-9302 were 58.1 ± 5.1 $\mu$M ($P>0.05$) and 18.1 ± 0.9 $\mu$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 $\mu$M) or R(+IQB-9302 (10 $\mu$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 block of the concentration-response equation yielded, for S(-)IQB-9302 or 10 $\mu$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 ± 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 (\(\delta\)). In the presence of S(-)IQB-9302 and R(+IQB-9302 the \(\delta\) values averaged 0.173 ± 0.022 (n = 12) and 0.181 ± 0.018 (n = 10), respectively, indicating that they have to cross ~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 ± 26.3 $\mu$M (n = 9) and 29.7 ± 2.2 $\mu$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 $\mu$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 (\(\tau_B\)). From the $\tau_B$ values, the apparent drug association (k) and dissociation (l) constants were calculated. For S(-)IQB-9302, k and l were 0.60 ± 0.08 $\mu$M$^{-1}$ s$^{-1}$ and 39.7 ± 5.2 s$^{-1}$ (n = 13), respectively. For R(+IQB-9302, k...
was 4.6 fold higher than that obtained for S(−)IQB-9302 (2.90 ± 0.63 μM s⁻¹, n = 7, P < 0.01) whereas I remained similar to S(−)IQB-9302 (52.2 ± 11.4 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 μM) or R(+)-IQB-9302 (10 μM). Under control conditions, the deactivating process was fitted following a biexponential process with a fast (τf) and a slow (τs) time constants. S(−)IQB-9302 increased the two time constants from 17.9 ± 3.3 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 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 (I) is fast enough, then the tail current may display a rising phase reflecting the dissociation of the drug from the blocked open channels (OD → 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).
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), logKD was plotted against several molecular parameters as logP, 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 (logP) 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 sterical 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 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 δ ~ 0.18.
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 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 μM S(−)IQB-9302 and 50 μM R(+)IQB-9302. (B) Tail current crossover. Currents recorded in control conditions and in the presence of 20 μM S(−)IQB-9302 or 10 μM 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.
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 stereo-chemistry 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 pipoocoloxilidide 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 modeling 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).

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References


Figure 7 Docking of minimum energy conformers of the two enantiomers of bupivacaine and IQB-9302 superimposed by the pipoocoloxilidide 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 pipoocoloxilidide 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 Hyperchem program.


