Bupivacaine effects on hKv1.5 channels are dependent on extracellular pH

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1 Bupivacaine-induced cardiotoxicity increases in hypoxic and acidotic conditions. We have analysed the effects of R(+)bupivacaine on hKv1.5 channels stably expressed in Ltk^- cells using the whole-cell patch-clamp technique, at three different extracellular pH (pH_o), 6.5, 7.4 and 10.0.

2 Acidification of the pH_o from 7.4 to 6.5 decreased 4 fold the potency of R(+)bupivacaine to block hKv1.5 channels. At pH_o 10.0, the potency of the drug increased ~ 2.5 fold.

3 Block induced by R(+)bupivacaine at pH_o 6.5, 7.4 and 10.0, was voltage- and time-dependent in a manner consistent with an open state block of hKv1.5 channels.

4 At pH_o 6.5, but not at pH_o 7.4 or 10.0, R(+)bupivacaine increased by 95 ± 3 % ($n=6$; $P<0.05$) the hKv1.5 current recorded at -10 mV, likely due to a drug-induced shift of the midpoint of activation ($\Delta V = -8.5 \pm 1.4$ mV; $n=7$).

5 R(+)bupivacaine development of block exhibited an 'instantaneous' component of block at the beginning of the depolarizing pulse, which averaged 12.5 ± 1.8% ($n=5$) and 4.6 ± 1.6% ($n=6$), at pH_o 6.5 and 7.4, respectively, and that was not observed at pH_o 10.0.

6 It is concluded that: (a) alkalinization of the pH_o increases the potency of block of R(+)bupivacaine, and (b) at pH_o 6.5, R(+)bupivacaine induces an 'agonist effect' of hKv1.5 current when recorded at negative membrane potentials.
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Abbreviations: δ, fractional electrical distance; E_h, midpoint of activation; τ_{Block}, time constant of block

Introduction

Bupivacaine is an amide type local anaesthetic widely used in regional anaesthesia. Unfortunately, bupivacaine is a very cardiotoxic local anaesthetic, likely due to its ability to decrease intracardiac conduction velocity, contractile force and sinoatrial activity (Strichartz, 1987) and to prolong QRS and QTc intervals of the ECG in anaesthetized dogs (Kasten & Martin, 1985; Wheeler et al., 1988) and human volunteers receiving high doses of bupivacaine (Scott et al., 1989). These cardiac effects are the consequence of its blocking effects of Na^+, Ca^{2+} and several K^+ channels (Kv1.5, Kv2.1, Kv1.4, Kv4.3, *HERG* and KvLQT1+minK) (Clarkson & Hondeghem, 1985; Graf et al., 1997; Lipka et al., 1998; González et al., 2001). Bupivacaine presents a chiral carbon and, thus, it can be separated into two enantiomers. It has been reported that bupivacaine-induced cardiotoxicity is mostly related to the effects of the R(+) enantiomer (Åberg, 1972; Luduena et al., 1972). Bupivacaine induced block of cardiac Na^+ and hKv1.5 channels is stereoselective, being R(+)bupivacaine 1.6- and 7 fold more potent than S(−)-bupivacaine, respectively, whereas block of Kv2.1 and Kv4.3 is not (Valenzuela et al., 1995a, b; Franqueza et al., 1997; 1999). It has been postulated that the cationic form of bupivacaine blocks hKv1.5 channels by binding to a receptor site located at the inner mouth of the channel involving, at least, the amino acids located at positions T505, L508 and V512 in the S6 segment of the channel (Valenzuela et al., 1995a; Franqueza et al., 1997).

Changes in pH like those occurring during myocardial ischaemia, diabetic ketoacidosis, and respiratory acidosis due to hypoventilation alter the degree of protonation of an anaesthetic molecule and modulate both the therapeutic and the toxic effects of the drug (Strichartz, 1987). In fact, bupivacaine-induced cardiotoxicity increased in hypoxic and acidotic conditions (Rosen et al., 1985; Nancarrow et al., 1987). Acidosis increased the action potential duration in isolated guinea-pig ventricular muscle and isolated dog Purkinje fibres, causing abnormal repolarization and early afterdepolarizations that were suggested to be due to a decrease in delayed rectifying potassium currents, supporting the idea that K^+ channel-mediated repolarization is impaired at low pH (Coraboeuf et al., 1976; Fry & Poole-Wilson, 1981; Bethell et al., 1998). Recently, it has been demonstrated that Kv1.5 channels are sensitive to low extracellular pH changes like those occurring during myocardial ischaemia (Steidl & Yool, 1999). Therefore, in this study we have analysed the possible differences among the effects induced by R(+)bupivacaine on hKv1.5 channels due to changes in the extracellular pH values (pH_o) (6.5 and 10.0). Preliminary results of the present study have been published in abstract form (Longobardo et al., 1998).

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Methods

Cell culture

Stably transfected Ltk^- cells with the gene encoding the expression of hKv1.5 channels were cultured in DMEM supplemented with 10% horse serum and 0.25 mg ml^{-1} G418 (a neomycin analogue) under a 5% CO_2 atmosphere as previously described (Tamkun *et al.*, 1991; Snyders *et al.*, 1993). Cultures were passaged every 3–5 days by use of a brief trypsin treatment. Before experimental, subconfluent cultures were incubated with 2 μM dexamethasone for 24 h to induce expression of hKv1.5 channels. The cells were removed from the dish with a rubber policeman, a procedure that left the vast majority of the cells intact. The cell suspension was stored at room temperature (21–23°C) and used within 12 h for all the experiments reported.

Electrophysiological recording

Experiments were performed in a small volume (0.5 ml) bath mounted on the stage of an inverted microscope (Nikon model TMS, Garden City, NY, U.S.A.) perfused continuously at a flow rate of 0.5–1.0 ml min^{-1}. hKv1.5 currents were recorded at room temperature (21–23°C) using the whole-cell voltage-clamp configuration of the patch-clamp technique (Hamill *et al.*, 1981) with an Axopatch 1C patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). Currents were filtered at 2 kHz (four-pole Bessel filter), sampled at 4 kHz, and stored on the hard disk of a Hewlett-Packard Vectra 486 computer for subsequent analysis. Data acquisition and command potentials were controlled by the PCLAMP 6.0.1 software (Axon Instruments). Micropipettes were pulled from borosilicate glass capillary tubes (Narishige, GD-1, Tokyo, Japan) on a programmable horizontal puller (Sutter Instrument Co., San Rafael, CA, U.S.A.) and heat-polished with a microforge (Narishige). 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 6.50 or 7.40 with NaOH. In the experiments performed at extracellular pH 10.0, we used 10 mM CAPS-Na as buffer. When filled with the intracellular solution and immersed into the bath (external solution), the pipette tip resistance ranged between 1 and 3 MΩ. The micropipettes were gently lowered onto the cells to obtain a gigaohm seal (17$\pm$3 GΩ) after applying suction. After seal formation, cells were lifted from the bottom of the perfusion bath and the membrane patch was ruptured with brief additional suction. The capacitive transients elicited by symmetrical 10-mV steps from -80 mV were recorded at 50 kHz (filtered at 10 kHz) for subsequent calculation of capacitive surface area and access resistance (10.8$\pm$0.7 pF and 4.0$\pm$0.3 MΩ; $n=10$). Thereafter, capacitance and series resistence compensation were optimized, and 80% compensation of the effective access resistance was usually obtained, achieving a mean value of uncompensated access resistance of 2.2$\pm$0.2 MΩ ($n=10$). Since the mean maximum current recorded was 1.5$\pm$0.4 nA, the voltage error was under 5 mV (2.9$\pm$0.5 mV; $n=10$).

Drugs

R($+$)bupivacaine (a gift from Astra, Södertälje, Sweden) was dissolved in distilled deionized water to yield stock solutions of 1 mM from which further dilutions were made to obtain the desired final concentration.

Pulse protocol and analysis

The holding potential was maintained at -80 mV unless indicated otherwise. The effect of drug infusion was monitored with test pulses to $+60$ mV, applied every 30 s until steady-state was obtained. The cycle time for other pulse protocols was 10 s. Steady-state current-voltage relationships (IV) were obtained by averaging the current over a small window (2–5 ms) at the end of 250 ms depolarizing pulses. Between -80 and -40 mV only passive linear leak was observed and least squares fits to these data were used for passive leak correction. Deactivating 'tail' currents were recorded either at -40 mV (experiments performed at pH$_o$ 7.4 and 10.0) or -30 mV (experiments at pH$_o$ 6.5), in order to record deactivating tail currents at similar potentials within the activation of the channel. The activation curve was obtained from the tail current amplitude immediately after the capacitive transient. Measurements were done using the CLAMPFIT program of PCLAMP 6.0.1, Origin 5.0 (Microcal Software, Northampton, MA, U.S.A.) and by a custom-made analysis program.

Activation curves were fitted with a Boltzmann equation:

$$y - 1/[1 + \exp - (E - E_h)/s], \qquad (1)$$

in which s represents the slope factor, E the membrane potential and E_h the voltage at which 50% of the channels are open. The activation kinetics of hKv1.5 have been described as a sigmoidal process (Snyders *et al.*, 1993). However, in the present study and in order to describe the dominant time constant of this process and the effects of drugs on it, the latter part of the current was fitted to a single exponential, following a procedure previously described and used for the same purpose (Snyders *et al.*, 1993; Valenzuela *et al.*, 1994; Delpón *et al.*, 1995). Deactivation was fitted to a biexponential process (Rich & Snyders, 1998). Thus, this process was fitted to an equation of the form:

$$y = C + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \qquad (2)$$

where τ_1, and τ_2 are the system time constants, A_1, and A_2 are the amplitudes of each component of the exponential, and C is the baseline value. The 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.

Drug-channel interactions were described by one or two binding curves. Apparent affinity constant, K_D, and Hill coefficient, n_H, for hKv1.5 channels at pH$_o$ 7.4 were obtained from fitting the fractional block, f, at various drug concentrations [D] to one Hill curve:

$$f = 1/[1 + (K_D/[D])_H^n] \qquad (3)$$

where [D] is the drug concentration. Experimental data from hKv1.5 channels at pH$_o$ 6.5 and 10.0 were fitted to the sum

of two Hill equations:

$$f = (I_1/(1 + K_{D1}/[D])) + (I_2/(1 + K_{D2}/[D])) \qquad (4)$$

where I_1 and I_2 are the fractional current of each component ($I_1 + I_2 = 1$), K_{D1} and K_{D2} are the apparent dissociation constants and [D] has the same meaning as above. Apparent rate constants for binding (k) and unbinding (l) were obtained from solving:

$$k \times [D] + l = \tau_{Block}^{-1} \qquad (5a)$$

$$l/k = K_D \qquad (5b)$$

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:

$$f = [D]/([D] + K_D^* \times exp(-\delta z F E / R T)), \qquad (6)$$

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

Statistical methods

Results are expressed as mean $\pm$ s.e.mean. Direct comparisons between mean values in control conditions and in the presence of drug for a single variable were performed by paired Student's t-test. Student's t-test was also used to compare two regression lines. Differences were considered significant if $P < 0.05$. To analyse drug or electrophysiological effects at multiple pH_o, two-way ANOVA was used (Wallenstein et al., 1980). Throughout the manuscript, results obtained at pH_o 6.5 or 10.0 were compared to those obtained at pH_o 7.4.

Results

Effects of R(+)bupivacaine on hKv1.5 channels at pH_o 6.5, 7.4 and 10.0

Figure 1A shows original records elicited at pH_o 6.5, 7.4 and 10.0 in the absence and in the presence of R(+)bupivacaine by depolarizing pulses from a holding potential of −80 mV to +60 mV in steps of 10 mV. In the absence of drug, current rapidly activated during depolarization at +60 mV and the dominant time constant of this process was faster at more alkaline pH_o [4.23 ± 0.30 ms ($n = 20$; $P < 0.05$), 2.10 ± 0.20 ms ($n = 21$) and 0.76 ± 0.04 ms ($n = 13$; $P < 0.05$) at pH_o 6.5, 7.4 and 10.0, respectively]. After reaching a maximum peak value, hKv1.5 current slowly and partially inactivated during the application of the depolarizing pulse. Tail currents were recorded upon repolarization to −40 mV (pH_o 7.4 and 10.0) or −30 mV (pH_o 6.5). The time course of the deactivation process was fitted to a biexponential equation (see equation 2 in Methods). The fast time constants (τ_f) averaged 21.4 ± 1.7 ($n = 24$; $P > 0.05$), 19.1 ± 1.1 ($n = 38$) and 20.5 ± 1.4 ms ($n = 13$; $P > 0.05$) at pH_o 6.5, 7.4 and 10.0, respectively. This component

equally contributed [$36.9 \pm 3.2\%$ ($n = 25$; $P > 0.05$), $49.8 \pm 3.3\%$ ($n = 38$) and $46.8 \pm 5.3\%$ ($n = 13$; $P > 0.05$) at pH_o 6.5, 7.4 and 10.0, respectively] to the total deactivation of the current. The slow time constants (τ_s) of this process were also similar at the three pH_o studied averaging 67.4 ± 5.6 ms ($n = 24$; $P > 0.05$), 60.0 ± 6.0 ms ($n = 38$) and 67.5 ± 5.8 ms ($n = 13$; $P > 0.05$), at pH_o 6.5, 7.4 and 10.0, respectively, this component contributing to a similar extent to the total deactivation process ($P > 0.05$) at the three pH_o studied, averaging values of $63.1 \pm 3.2\%$ ($n = 24$), $50.2 \pm 3.3\%$ ($n = 38$) and $53.2 \pm 5.3\%$ ($n = 13$), respectively.

Table 1 shows the theoretical intracellular and extracellular concentrations of charged and uncharged R(+)bupivacaine after adding 5 μM R(+)bupivacaine to the external solution at pH_o 6.5, 7.4 and 10.0. Whereas at pH_o 6.5, the drug will be mostly present in its extracellular charged form; at pH_o 10.0, the opposite will occur, so that the drug will accumulate inside the cell in its charged form (~ 39 μM). However, at pH_o 7.4, similar concentrations of the cationic form of R(+)bupivacaine are expected at both sides of the cell membrane. Figure 1A shows hKv1.5 current records obtained in the absence and in the presence of equipotent concentrations of R(+)bupivacaine at the three pH_o studied (20, 5 and 1 μM). It can be observed that the concentration required to induce a 50% block is reduced as the pH_o is higher, which indicates that most of the blocking properties of R(+)bupivacaine on hKv1.5 channels are the consequence of its binding to a receptor site located at the inner mouth of the ion pore of the channel. In all cases, block induced by R(+)bupivacaine was time-dependent, inducing a fast initial decline at the beginning of the depolarizing pulse, suggestive of open channel block (Armstrong, 1971). Therefore, steady-state block was measured at the end of 250 ms-depolarizing pulses from a holding potential of −80 mV to +60 mV. At pH_o 6.5, 20 μM R(+)bupivacaine decreased hKv1.5 current by $62 \pm 4\%$ ($n = 7$), at pH_o 7.4, 5 μM R(+)bupivacaine inhibited the current by $61 \pm 2\%$ ($n = 5$), and at pH_o 10.0, R(+)bupivacaine (1 μM) inhibited hKv1.5 current by $47 \pm 5\%$ ($n = 5$). Therefore, acidification of the pH_o decreased 4 fold the potency of R(+)bupivacaine to block hKv1.5 channels while at pH_o 10.0, the potency of the drug increased ~ 2.5-fold versus the potency that it exhibits at the physiological pH_o of 7.4 (Valenzuela et al., 1995a).

Figure 1B shows the activation curves of hKv1.5 channels obtained at the three pH_o tested in the absence and in the presence of R(+)bupivacaine. Under control conditions, the midpoint of activation (E_h) of hKv1.5 channels was shifted to more positive membrane potentials as the pH_o decreased [$+3.1 \pm 0.7$ mV ($n = 27$; $P < 0.05$), -14.2 ± 2.1 mV ($n = 38$) and -23.1 ± 1.3 mV ($n = 13$; $P < 0.05$) at pH_o 6.5, 7.4 and 10.0, respectively], without modifications in the slope factor [5.2 ± 0.1 mV ($n = 27$), 4.9 ± 0.2 mV ($n = 38$) and 4.6 ± 0.2 mV ($n = 13$) at pH_o 6.5, 7.4 and 10.0]. In order to study the effects of the pH_o on hKv1.5 channels, we represented the shift of the E_h values against the pH_o (Figure 2). Considering that ΔE_h saturates at pH_o 10.0, its value would be zero at this pH_o. As it has been previously described for other ionic channels (Prod'hom et al., 1989; Ito et al., 1992; Yamane et al., 1993), the E_h shift of the activation of hKv1.5 channels induced by extracellular protons is consistent with a pKa value of 6.4, which is very close to the pKa of histidine (6.0). Therefore, these results suggest that titration of some histidine within the outer pore of hKv1.5 channels might be
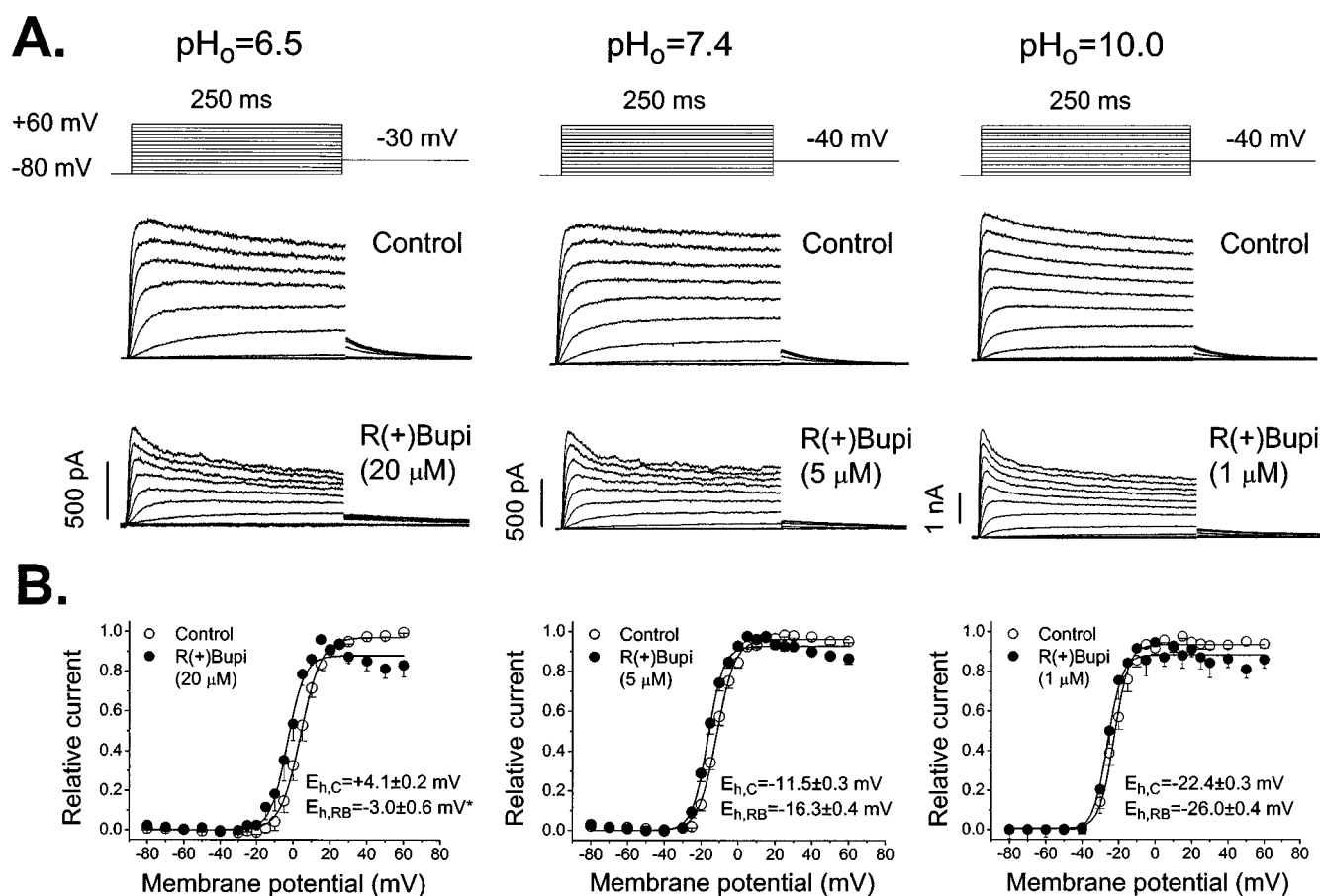
Figure 1 Effects of R(+)bupivacaine on hKv1.5 channels at different pH_o values. Original records obtained in the absence and in the presence of equipotent drug concentrations after applying depolarizing pulses from -80 to $+60$ mV in 10 mV steps of 250 ms duration. Tail currents were recorded upon repolarization to -40 mV (pH_o 7.5 and 10.0) or -30 mV (pH_o 6.5). (A) Shows original records obtained at pH_o 6.5, 7.4 and 10.0 in the absence and in the presence of equipotent concentrations of R(+)bupivacaine. R(+)bupivacaine 20, 5 and 1 μM induced a time-dependent block which yields $62\pm4\%$ ($n=6$), $61\pm2\%$ ($n=5$) and $47\pm5\%$ ($n=5$) at pH_o 6.5, 7.4 and 10.0, respectively. (B) Shows the activation curves at pH_o 6.5, 7.4 and 10.0 in the absence and in the presence of equipotent R(+)bupivacaine concentrations. The midpoint of activation (E_h) was shifted to more negative potentials as the pH_o value was more alkaline. Data points represent the mean$\pm$s.e.mean of a $6-7$ experiments. $*P<0.01$.

Table 1 Left: concentration of neutral or charged R-bupivacaine inside and outside the cell after adding 5 μM R-bupivacaine to the external solution at different pH_0. Right: concentration of charged R-bupivacaine (RB^+) inside and outside the cell membrane at the K_D values obtained at pH_0 6.5, 7.4 and 10.0

| pH_0 | *Intracellular concentrations* | | *Extracellular concentrations* | | K_D | $[RB^+]_{in}$ | $[RB^+]_{out}$ |
	$[RB^+]_{in}$ (μM)	$[RB]_{in}$ (μM)	$[RB^+]_{out}$ (μM)	$[RB]_{out}$ (μM)	(μM)	(μM)	(μM)
6.5	0.9732	0.1225	4.8775	0.1225	19	3.5	18
7.4	6.6063	0.8317	4.1683	0.8317	4.7	4.5	5.4
10.0	39.2226	4.9378	0.0622	4.9378	1.7	38	0.0622

$[RB^+]_{in}$: Intracellular concentration of the cationic form of bupivacaine. $[RB]_{in}$: Intracellular concentration of the neutral form of bupivacaine. $[RB^+]_{out}$: Extracellular concentration of the cationic form of bupivacaine. $[RB]_{out}$: Extracellular concentration of the neutral form of bupivacaine. These values were calculated applying the Henderson-Hasselbalch equation $\{pH = pKa + \log([RB^+]/[RB])\}$, where pKa represents the dissociation constant of the drug (8.1 for R(+)bupivacaine).

involved in this effect. As it is shown in Figure 1B, R(+)bupivacaine shifted the activation curve of hKv1.5 channels at the three pH_o studied. However, this shift was only statistically significant at pH_o 6.5 ($\Delta V = -8.5\pm1.4$ mV, $n=7$; $P<0.01$), whereas at pH_o 7.4 and 10.0, R(+)bupivacaine at 5 and 1 μM only shifted the activation curves by -4.5 ± 0.5 ($n=6$; $P>0.05$) and -3.6 ± 0.2 mV ($n=7$; $P>0.05$), respectively. R(+)bupivacaine did not modify the

slope factor of the activation curve under any experimental condition studied.

Figure 3 shows the concentration-response curves obtained at pH_o 6.5, 7.4 and 10.0. Although the concentration dependence for block of hKv1.5 channels at pH_o 7.4 was adequately described by a single binding site model (Valenzuela *et al.*, 1995a), block induced by R(+)bupivacaine of hKv1.5 channels at pH_o 6.5 or 10.0 was better fit assuming two binding

sites, with the fraction of channels blocked with high affinity being ~30%, similarly to that previously described for R(+)- and S(−)-bupivacaine-block of some pore-mutant hKv1.5 channels (Franqueza *et al.*, 1997). A nonlinear least-squares fit of the concentration-response equation (see Methods) to the individual data points yielded apparent $K_{D's}$ of 0.066 ± 0.040 and 19.8 ± 2.7 μM ($n=39$) for R(+)bupivacaine hKv1.5 block at pH_o 6.5, and 0.027 ± 0.007 and 1.7 ± 0.2 μM ($n=23$) for R(+)bupivacaine block at pH_o 10.0.

Voltage-dependence of block of hKv1.5 channels by R(+)bupivacaine at different pH_o

Figure 4 shows the relative current obtained in the presence of equipotent concentrations of R(+)bupivacaine at the three pH_o studied *versus* membrane potential. In all cases, block
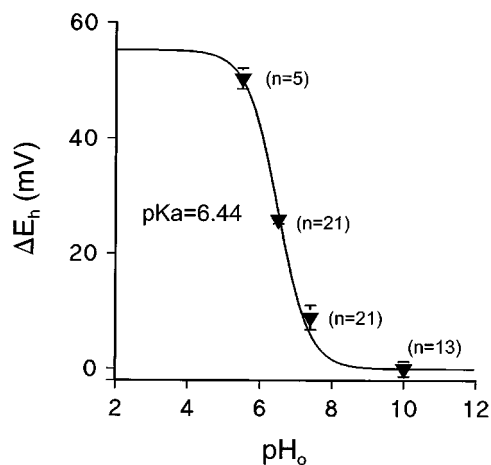
induced by R(+)bupivacaine steeply increased at the membrane potentials that coincided with the activation range of the channel, which strongly suggests that the drug binds to the open state of the channel. At membrane potentials positive to $+10$ mV ($pH_o=6.5$), 0 mV ($pH_o=7.4$) and -5 mV ($pH_o=10.0$), block still increased but with a shallower voltage dependence. At this range of voltages the activation curve of the channel has reached saturation and, therefore, an increase of the degree of block cannot be attributed to an opening of the channel. Since R(+)bupivacaine is a weak base ($pKa=8.1$) this shallow voltage dependence of block was interpreted as the effect of the transmembrane electrical field on the interaction between the cationic form of the drug and its receptor in the channel, according to a Woodhull model (Woodhull, 1973). Therefore,
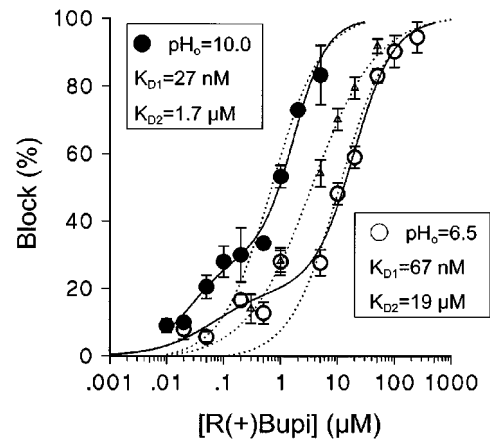
Figure 2 Effects of changes in extracellular pH (pH_o) on hKv1.5 channels. Considering the equation $\Delta E_h = A/(1 + 10^{(pH-pKa)})$; where A represents the maximum amplitude, and assuming that E_h saturates at pH_o 10.0 (so $\Delta E_h = 0$ at $pH_o=10.0$), we calculated a pKa value of 6.44. This is in close agreement with the pKa value of protonation for an histidine (6.0) and suggests that the titration of a histidine present within the pore region of hKv1.5 channels could be responsible for the observed proton-induced shift towards more positive membrane potentials as the pH_o is reduced.

Figure 3 Concentration-response curves for R(+)bupivacaine-induced block at all the pH_o studied. In this plot we represented the R(+)bupivacaine induced block at each concentration and under each experimental conditions. At pH_o 6.5 and 10.0 the concentration-response curves were better fit assuming a Hill equation of two components, i.e. biphasic concentration-response curves, from which two K_D values were obtained (K_{D1} and K_{D2}). Dotted lines represent the concentration-response curves when the fit assumed a Hill equation of one component. The concentration-response curve for R(+)bupivacaine at pH_o 7.4 (Δ) was taken from (Valenzuela *et al.*, 1995a) with permission.
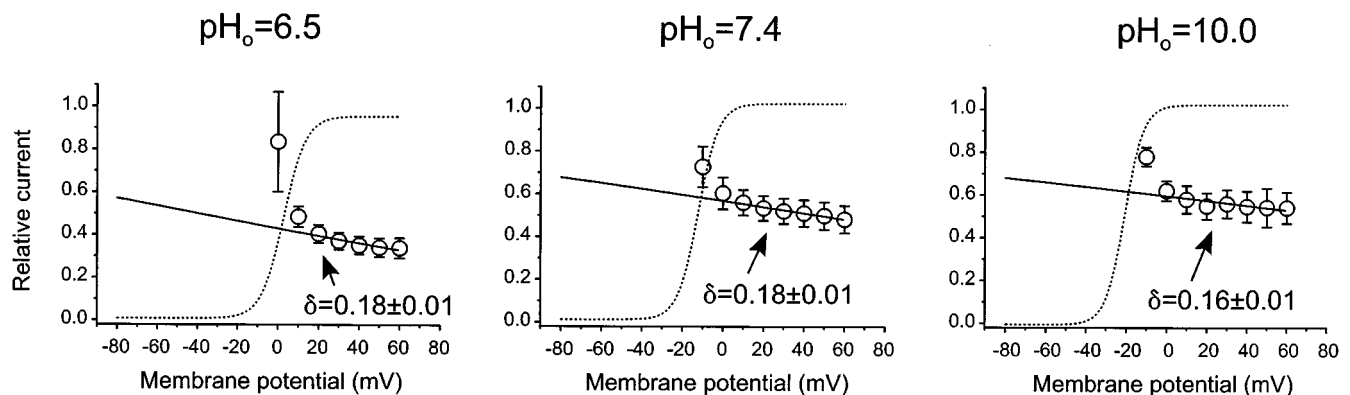
Figure 4 Voltage-dependent effects of equipotent R(+)bupivacaine concentrations on hKv1.5 channels at different pH_o values. Relationship between the relative current ($I_{Drug}/I_{Control}$) and membrane potential at pH_o 6.5, 7.4 and 10.0. At the three pH_o studied, block induced by R(+)bupivacaine steeply increased in the range of activation of the channel. At voltages positive to $+10$ mV (pH_o 6.5), 0 mV (pH_o 7.4) and -5 mV (pH_o 10.0) block increased with a shallower voltage-dependence consistent with δ values of 0.18 ± 0.01 ($n=14$), 0.18 ± 0.01 ($n=5$) and 0.16 ± 0.01 ($n=7$), respectively ($P>0.05$).

a nonlinear curve fitting of the data to equation 6 (see Methods) yielded the fractional electrical distance (δ) values that were 0.18 ± 0.01 ($n = 14$), 0.18 ± 0.01 ($n = 5$) and 0.16 ± 0.01 ($n = 7$) at pH_o 6.5, 7.4 and 10.0, respectively.

Figure 5 shows original hKv1.5 current traces recorded at very positive ($+60$ mV) and at the threshold for activation (-10 mV) at pH_o 6.5 in the absence and in the presence of R($+$)bupivacaine. At this pH_o value, R($+$)bupivacaine (20 μM) decreased hKv1.5 current recorded at $+60$ mV by $62 \pm 4\%$ ($n = 7$; $P < 0.01$), but increased the hKv1.5 current amplitude at -10 mV, by $95 \pm 3\%$ ($n = 6$; $P < 0.01$). This increase was not significant at pH_o 7.4 ($10 \pm 2\%$; $n = 6$, $P > 0.05$, measured at -20 mV) and at pH_o 10.0 only blocking effects could be observed ($12 \pm 1\%$; $n = 5$ $P < 0.05$, measured at -30 mV). The increase of the hKv1.5 current observed at negative membrane potentials at pH_o 6.5 can be the consequence of a drug-induced negative voltage shift of the midpoint of the activation curve from $+4.1 \pm 0.2$ to -3.0 ± 0.6 mV ($n = 7$; $P < 0.01$). We will use the term 'agonist effect' to describe the R($+$)bupivacaine-induced increase of hKv1.5 current amplitude.

Time dependence of block of hKv1.5 channels by R($+$)bupivacaine at different pH_o

As it has been mentioned above, one of the most prominent effects of R($+$)bupivacaine on hKv1.5 current at any pH_o tested was the development of a fast initial decline at the beginning of the depolarizing pulse that was superimposed on the slow inactivation characteristic of the current (Figure 6A). The time constant of this initial decline was faster at higher drug concentrations and, therefore, it was taken as a good index of the time constant of the drug-channel interaction (τ_{Block}). Figure 6B shows the apparent rate of block (τ_{Block}^{-1}) *versus* R($+$)bupivacaine concentrations at each pH_o value. The straight lines are the least squares fits to the equation $\tau_{Block}^{-1} = k \times [D] + l$. Slope and intercept for fitted relations yielded the apparent association (k) and dissociation (l) rates for R($+$)bupivacaine at the three pH_o studied. At pH_o 6.5 the k value for R($+$)bupivacaine was 3.7 fold smaller than that obtained at pH_o 7.4. However, l values were similar at both pH_o. The lower k value together with a

similar l value calculated at pH_o 6.5 can explain the lower potency of block induced by R($+$)bupivacaine at pH_o 6.5 compared to its potency at pH_o 7.4 ($K_D = l/k$). At pH_o 10.0, both rate constants, k and l, were modified compared to those calculated at pH_o 7.4. Thus, while the k value was 9.1 fold times faster, the l value was only 3.1 fold times faster than values described at pH_o 7.4.

R($+$)bupivacaine (1 μM) did not modify the activation time constant at pH_o 10.0 [0.85 ± 0.10 *versus* 0.72 ± 0.08 ms ($n = 5$; $P > 0.05$)] at $+60$ mV. However, at pH_o 6.5 and 7.4 R($+$)bupivacaine (10 and 5 μM; respectively) accelerated the activation time course of the current [2.59 ± 0.17 ms *versus* 4.12 ± 0.53 ms ($n = 5$; $P < 0.05$) at pH_o 6.5, and 1.45 ± 0.11 ms *versus* 2.00 ± 0.11 ($n = 6$; $P < 0.01$) at pH_o 7.4]. These results can suggest that, at pH_o 6.5 and 7.4, R($+$)bupivacaine may block some state of the hKv1.5 channel previous to the open one. In order to analyse this hypothesis we plotted the ratio between the drug-sensitive current and that recorded under control conditions [$(I_{Control} - I_{Drug})/I_{Control}$] at $+60$ mV *versus* time of depolarization (inset of Figure 6B). As it can be observed, at pH_o 6.5, R($+$)bupivacaine development of block exhibited an 'instantaneous' component of block at the beginning of the depolarizing pulse ($t = 0$ ms), which averaged $12.5 \pm 1.8\%$ ($n = 5$). At pH_o 7.4, a lower degree of 'instantaneous' block was observed ($4.6 \pm 1.6\%$; $n = 6$). At these two different pH_o, block increased further during the application of the depolarizing pulses, indicating that R($+$)bupivacaine binds also to the open state of the channel. At pH_o 10.0 development of block began during the depolarization without an 'instantaneous' component of block ($n = 5$; $P < 0.05$ *versus* results obtained at pH_o 6.5).

Time dependent block of hKv1.5 channels was also observed in the tail currents, which represent the transition from the open to the closed state of the channel. As it has been previously described, under control conditions, the deactivation of hKv1.5 channels recorded at -40 mV follows a biexponential function with fast and slow time constants that were similar at the three pH_o values studied. If R($+$)bupivacaine blocks hKv1.5 channels in the open state, as suggested by the time- and voltage-dependence block observed, then dissociation of R($+$)bupivacaine from the blocked channels results in an open channel (which
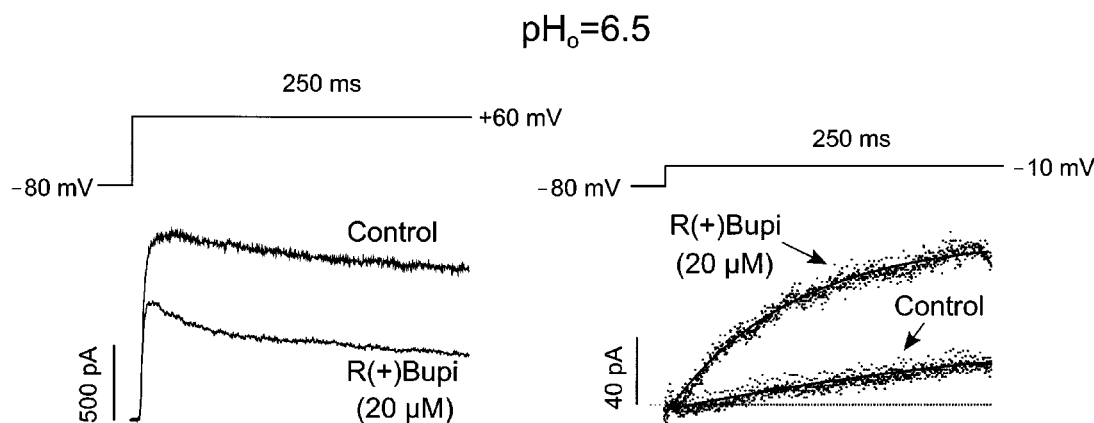
$pH_o = 6.5$

Figure 5 Effects of R($+$)bupivacaine on hKv1.5 channels at pH_o 6.5. Left panel shows original records obtained after depolarizing the cell from -80 to $+60$ mV. Right panel shows the drug effects after depolarizing the cell membrane to -10 mV. Note that, under these circumstances, the drug increased the amplitude of the hKv1.5 current by $95 \pm 3\%$ ($n = 6$; $P < 0.05$), whereas after strong depolarizations (left panel), R($+$)bupivacaine inhibited the hKv1.5 current.
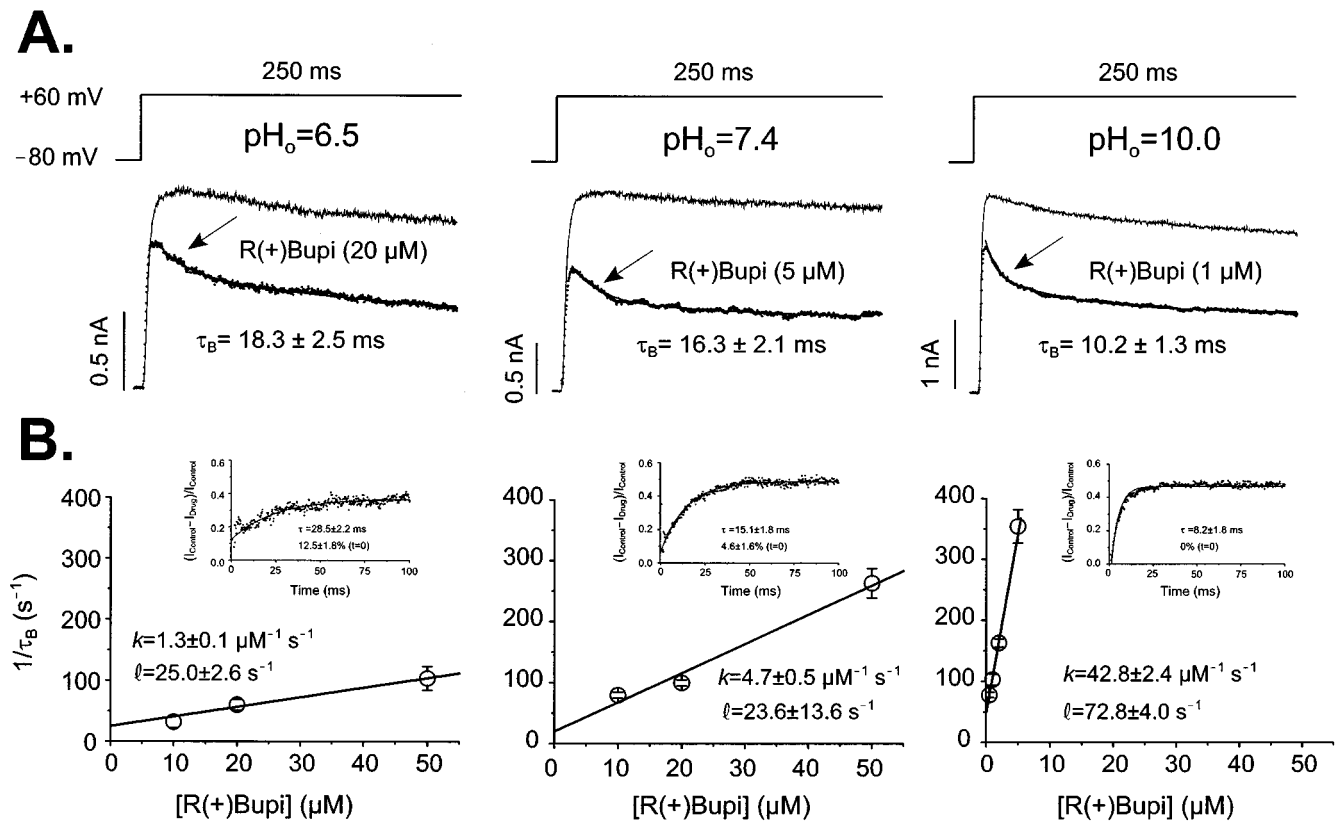
Figure 6 Time-dependent block of hKv1.5 channels induced by R(+)bupivacaine at pH_o 6.5, 7.5 and 10.0. (A) Shows original records obtained after applying a depolarizing pulse from -80 mV to $+60$ mV for 250 ms in duration in the absence and in the presence of R(+)bupivacaine (20, 5 and 1 μM) at the different pH_o values. R(+)bupivacaine induced a fast initial decline of the current which was faster at higher drug concentrations and thus, it was considered as a good index of the time constant of block (τ_{Block}). (B) The inverse of τ_{Block} was plotted *versus* bupivacaine concentration. For a first-order blocking scheme, a linear relation is expected: $\tau_{Block}^{-1} = k \times [D] + l$ (see Methods section). The solid line represents the linear fit, from which the apparent binding and unbinding rate constants were obtained. Inset: Representative traces of time course of the development of block of hKv1.5 after a depolarizing pulse to $+60$ mV from a holding potential of -80 mV at pH_o 6.5, 7.4 and 10.0 induced by R(+)bupivacaine at concentrations of 10, 5 and 1 μM, respectively. The reduction of hKv1.5 current in the presence of the drug is expressed as a proportion of the control current at any given time after the beginning of the depolarizing pulse. In the presence of R(+)bupivacaine at all the pH_o studied inhibition of the current increases exponentially during depolarization. Note that at pH_o 6.5 and 7.4, R(+)bupivacaine (10 μM) induced an instantaneous block at $t = 0$ ms.

subsequently could close). Blocked channels are not conducting, and the conversion to the open state should, therefore, result initially in a rising phase of the tail current; subsequently, the tail current should display a slower decline because some fraction of the open channels become blocked again, rather than closing irreversibly. Figure 7 shows the superposition of the tail currents obtained at -40 mV (pH_o 7.4 and 10.0) or -30 mV (pH_o 6.5) after a 250 ms depolarization to $+60$ mV under control conditions and in the presence of equipotent concentrations of R(+)bupivacaine at the three pH_o. In all cases, R(+)bupivacaine slowed the time course of deactivation resulting in the 'crossover' phenomenon (Figure 7), which represents another piece of evidence supporting an open channel block mechanism (Armstrong, 1971). At the three pH_o studied, equipotent concentrations of R(+)bupivacaine (20, 5 and 1 μM) eliminated τ_f and therefore, the time course of deactivation followed a monoexponential function with time constants of 114.2 ± 26.2 ms ($n = 4$), 93.8 ± 11.7 ms ($n = 6$) and 95.1 ± 13.6 ms ($n = 5$), at pH_o 6.5, 7.4 and 10.0, respectively.

Discussion

The main findings of the present study are: (1) alkalinization of the pH_o increases the potency of R(+)bupivacaine to block hKv1.5 channels at positive membrane potentials, consistent with the existence of an internal receptor bupivacaine site responsible of most of the blocking effects of R(+)bupivacaine. (2) R(+)bupivacaine at pH_o 6.5 induces an 'agonist effect' on hKv1.5 channels when recorded at negative membrane potentials (physiological membrane potentials).

Effects of pH_o on hKv1.5 channels

As it has been previously described for other ion channels, changes in pH_o modify the gating of hKv1.5 channels in a manner consistent with a surface screen of negative charges (Busch *et al.*, 1991; Coulter *et al.*, 1995; Fakler *et al.*, 1996; Hoth *et al.*, 1997). In fact, a decrease or an increase of the pH_o induced a positive or a negative shift in the E_h of the activation curve, respectively. This suggests that protons
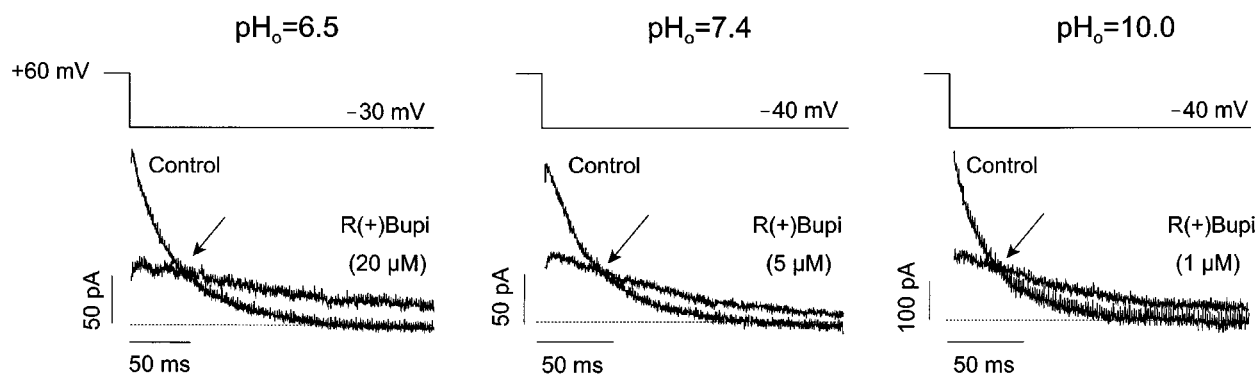
Figure 7 Time-dependent block of hKv1.5 channels induced by R(+)bupivacaine at different pH_o values observed during deactivation at -40 mV (at pH_o 7.4 and 10.0) and -30 mV (at pH_o 6.5). R(+)bupivacaine induced an initial rising phase due to the unblocking of R(+)bupivacaine from open channels and slowed the deactivation time course at all the pH_o studied which resulted in a crossover phenomenon indicative of an open-channel block mechanism. The arrows indicate the 'crossover' between the tail currents recorded in the presence of drug and those recorded under control conditions.

decrease the negative charge at the external surface of the membrane, in the vicinity of the hKv1.5 channel gate and thus, alter the voltage field sensed by the channel mechanism. The activation kinetics was also modified by the pH_o, being faster as the pH_o increased, indicating that changes in the surface charges modify the kinetics of this channel, maybe due to a modification of the channel gating. The pH_o-dependent properties of different inwardly rectifying potassium channels and Kv1.3 channels have been attributed to the protonation of titrable amino acids such as histidine (Busch *et al.*, 1991; Coulter *et al.*, 1995; Hoth *et al.*, 1997), cysteine (Coulter *et al.*, 1995) or lysine (Fakler *et al.*, 1996). Most of these amino acids are located near the outer part of the pore region. Recently, it has been demonstrated that the rat isoform of Kv1.5 channels (rKv1.5) is extremely sensitive to pH_o (Steidl & Yool, 1999). Protonation of a histidine located at position 452 in the pore region causes channels to accumulate in the C-type inactivated state inducing a reduction of Kv1.5 current amplitude (Steidl & Yool, 1999). In the present study, the observed E_h shift induced by extracellular protons is consistent with a pK_a value of 6.4, which is very close to pK_a of histidine (6.0). Since the human isoform of Kv1.5 channels (hKv1.5) exhibits an histidine at equivalent position to 452 in rKv1.5 channels, this amino acid might be responsible of the changes in E_h.

R(+)bupivacaine blocks the open state of hKv1.5 channels at the three pH_o studied

Block induced by R(+)bupivacaine at the three pH_o studied was voltage dependent, consistent with a δ value of ~ 0.17 when measured from the inside of the membrane (Figure 4). Also, at the three pH_o studied, R(+)bupivacaine induced a fast initial decline of the maximum outward current upon depolarization from -80 to $+60$ mV, indicative of an open channel mechanism (Figure 6). Furthermore, R(+)bupivacaine slowed the deactivation process inducing a tail 'crossover' phenomenon at all pH_o studied (Figure 7). All these results are consistent with an open channel block mechanism in which the cationic form of R(+)bupivacaine (RB^+) binds to a receptor located at the inner mouth of the ion pore of hKv1.5 channels blocking them when they achieve their open state (Valenzuela *et al.*, 1995a; Franqueza *et al.*, 1997).

At pH_o 6.5 and 7.4, R(+)bupivacaine induced an instantaneous block at $t = 0$ ms that was not observed at pH_o 10.0. This initial block, which appears before channel opening could be attributed to the drug interaction with a non conducting state of the channel. Consistent with this hypothesis, at pH_o 6.5 and 7.4, R(+)bupivacaine accelerated the activation time constant at $+60$ mV *vs* that recorded in the absence of the drug in the same conditions.

As it can be observed in Figures 1, 3 and 6, acidification of the pH_o produced a 4 fold decrease in the potency of R(+)bupivacaine to block hKv1.5 channels when compared to data obtained at pH_o 7.4. This lower potency could be attributed to a slower association time constant (k) for R(+)bupivacaine (the k value at pH_o 7.4 was 3.7 times faster than at pH_o 6.5), since the dissociation time constants (l) at the two pH_o were similar. However, at pH_o 10.0 the potency of R(+)bupivacaine to block hKv1.5 channels was 2.5 fold times higher than that described at pH_o 7.4. This higher potency could be explained by the acceleration observed in the k value (9.1 fold times faster than that calculated at pH_o 7.4) together with a 2.8 fold times faster l value.

Similarly to that reported for R(+)bupivacaine in blocking the flicker K^+ channel present in thin myelinated nerve fibres and in hKv1.5 mutated channels (T505I, T505S and T477S), at pH_o 6.5 and 10.0, concentration-dependent block of hKv1.5 channels by R(+)bupivacaine best fits have been obtained under the assumption of two independent binding processes (Franqueza *et al.*, 1997; Nau *et al.*, 1999). These findings could be explained on the basis of the existence of two different populations of channels with different R(+)bupivacaine affinity. However, this seems unlikely to occur with a cloned channel that forms homomultimers. Heteromultimer formation with endogenous subunits is also unlikely, since the Ltk^- cells used do not contain endogenous voltage gated ion currents or detectable K^+ channel mRNA (Snyders *et al.*, 1993). A second possible explanation is that changes observed in channel gating observed in mutant channels or following changes in pH_o may introduce a high-affinity binding site for bupivacaine in hKv1.5 channels. However, if this would be the case, then all the current would be inhibited by the high-affinity binding site before the low-affinity site could be occupied, thus masking low-affinity binding. The hypothesis we favour is that even in wild type hKv1.5 channels multiple

open states exist with different bupivacaine affinities. Bupivacaine binding to the high-affinity open state could represent an intermediate transition state in wild type hKv1.5 channels under physiological conditions (pH$_o$ = 7.4), as has been proposed previously to explain the blockade of cardiac Na$^+$ channels by QX-314 (Gingrich *et al.*, 1993). Under this framework, hKv1.5 channels with a modified gating induced by changes in pH$_o$ would be stabilizing an ultrafast (higher affinity) interaction between bupivacaine and hKv1.5 channels. In fact, there is evidence that multiple open states exist in hKv1.5 channels and that conversion between them can be influenced by drug concentration (Rich, 1996). Thus, as bupivacaine concentration increases, an open state with low affinity is favoured. This hypothesis requires a drug-induced shift in gating that is independent of open-channel block, such as that observed at pH$_o$ 6.5. Thus, at pH$_o$ 6.5 and 10.0, conformational changes induced by a higher or a lower proton concentration would promote a drug-induced shift in channel gating that reveal an open state with lower affinity for bupivacaine. However, the mechanism responsible for the biphasic dose-response curves requires further investigation.

Possible contribution of R(+)bupivacaine-binding to the external binding site to the total block induced on hKv1.5 channels

It has been generally accepted that the blocking effects of R(+)bupivacaine were mostly due to the interaction between the intracellular cationic form of the drug with the inner mouth of the ion pore (Valenzuela *et al.*, 1995a; Franqueza *et al.*, 1997). If this hypothesis is correct, we would expect to find K_D values at different pH$_o$ that correspond to similar intracellular cationic concentration of R(+)bupivacaine ([RB$^+$]$_{in}$). However, as it can be observed in Table 1, at the three K_D values obtained in this study, the theoretical [RB$^+$]$_{in}$ were different. In fact, if we consider only the [RB$^+$]$_{in}$ at the K_D value calculated at each pH$_o$ studied, the concentration required to induce a similar degree of block increases as the pH$_o$ is more alkaline, indicating that R(+)bupivacaine potency decreases as the pH$_o$ increases. At the three pH$_o$ tested, the concentrations of RB$^+$ at the external side of the membrane ([RB$^+$]$_{out}$) are very different. Recently, we have demonstrated the existence of an external binding site for R(+)bupivacaine in hKv1.5 channels (Longobardo *et al.*, 2000). Therefore, these results may suggest that the relative contribution of R(+)bupivacaine-binding to the external receptor site to the total block observed is prominent at pH$_o$ 6.5, less important at pH$_o$ 7.4 and almost negligible at pH$_o$ 10.0. As mentioned above, it has been described that at pH 6.2, the protonation of a histidine at position 452 of Kv1.5 channels, induces the channels to accumulate in the inactivated state (Steidl & Yool, 1999). In addition, we have described that binding of a permanently charged bupivacaine analog to the internal bupivacaine receptor site modifies the effects induced by the permanently charged analogue extracellularly applied (RB$^+$1C$_{out}$) when the experimental conditions promote the inactivated state of the channel (long depolarizing pulses) (Longobardo *et al.*, 2000). In fact, the degree of block induced by RB$^+$1C$_{out}$ was higher in the presence of RB$^+$1C at the internal side of the membrane at the end of a 4 s depolarizing pulse than in the absence of drug in the intracellular media. Therefore, a possible

explanation for the observed increased block at lower [RB$^+$]$_{in}$ (observed at pH$_o$ 6.5) could be the functional coupling between both receptor sites.

Agonist effects of R(+)bupivacaine at pH$_o$ 6.5

During the application of 250 ms depolarizing pulses from −80 mV to +60 mV, R(+)bupivacaine blocked hKv1.5 channels at the three pH$_o$ studied. However, at membrane potentials close to the threshold for channel activation, R(+)bupivacaine effects were different depending on the pH$_o$ value. At low pH$_o$, at which the concentration of the extracellular cationic form of the drug is much higher, we observed an agonist effect at negative potentials (−10 mV) that was not observed neither at pH$_o$ 7.4 nor at pH$_o$ 10.0. This effect was likely due to the drug-induced shift of the activation curve to more negative potentials, as it has been described for other potassium channels and drugs (Carmeliet, 1993; Tseng *et al.*, 1996; Davies *et al.*, 1996; Delpón *et al.*, 1999). At pH$_o$ 6.5, drug is mostly present in its cationic form at the external side of the membrane, which may suggest that it is the charged form of bupivacaine the one which exherts the 'agonist effect' by binding to the receptor site located at the external side of the membrane. On the other hand, bupivacaine is a very hydrophobic drug with a lipid phase/aqueous phase partition coefficient (logP) of 4.0. Given its pKa value (8.1), the lipid/buffer distribution coefficient (logQ) is 2.4, 3.2 and 4.0 at pH$_o$ 6.5, 7.4 and 10.0, respectively. These values predict that drug concentration at the cell membrane will increase 240, 320 and 400 fold at pH$_o$ 6.5, 7.4 and 10.0, respectively. Thus, at the K_D values calculated for these pH$_o$ values, the theoretical expected drug concentration at the cell membrane will be 4560, 1504 and 6783 μM, respectively. Therefore, we cannot rule out the possibility that the uncharged form of the drug would be responsible of the observed shift in the activation curve. In fact, it has been described that neutral local anaesthetics, such as benzocaine, induce, at low concentrations, similar effects on these potassium channels (Delpón *et al.*, 1999)

Possible clinical consequences of the present study

It has been described that bupivacaine cardiotoxicity increases in acidosis and hypoxia (Rosen *et al.*, 1985; Nancarrow *et al.*, 1987). During acidosis, most ionic currents, including hKv1.5, I_{Na}, I_{Ca}, I_{TO}, I_{Kr} and I_{Ks}, are inhibited by extracellular protons (Prod'hom *et al.*, 1989; Zhang & Siegelbaum, 1991; Yamane *et al.*, 1993; Stengl *et al.*, 1998; Anumonwo *et al.*, 1999; Steidl & Yool, 1999). These effects on ion channels are translated, at the multicellular level, into a depolarization of the membrane potential, a reduction of conduction velocity, and a prolongation of the action potential duration, with eventual oscillations at the plateau level and occurrence of early afterdepolarizations (Coraboeuf *et al.*, 1980; Cordeiro *et al.*, 1994). Bupivacaine not only blocks hKv1.5 channels, but also those responsible of the activation of I_{Kr}, I_{Ks} and I_{TO} (Castle, 1990; Lipka *et al.*, 1998; Franqueza *et al.*, 1999; González *et al.*, 2001). Therefore, a further decrease of hKv1.5 current, would be expected to be accompanied by a prolongation of the atrial action potential, which could be, together with its effects on other K$^+$ currents, responsible of the higher bupivacaine cardiotoxicity.

However, the increased bupivacaine cardiotoxicity at low pH_o would also involve its blocking effects on cardiac Na^+ channels. In fact, under these conditions, the resting membrane potential is depolarized to levels which promote the transition of Na^+ channels from the rested to the inactivated state, which makes them more susceptible to be blocked by bupivacaine (Clarkson & Hondeghem, 1985; Valenzuela et al., 1995b), and, as a consequence, the conduction velocity will decrease to a higher extent, contributing to the observed higher cardiotoxicity.

Conclusions

In this study, we demonstrated that R(+)bupivacaine potency to block hKv1.5 channels increases in parallel with the increase in the pH_o and, thus, with the increase in the intracellular concentration of the cationic form of the drug.

Also, at low pH_o (6.5), R(+)bupivacaine increases hKv1.5 current at negative membrane potentials, likely due to the drug induced shift of the activation curve. At the K_D values, intracellular concentrations of the cationic form of R(+)bupivacaine do not match at the different pH_o, suggesting that either: (a) binding to the extracellular bupivacaine binding site, or (b) the influence of hKv1.5 channel inactivation, that predominates at low pH_o, might determine the blocking effects of R(+)bupivacaine on hKv1.5 channels.

References

ANUMONWO, J.M., HORTA, J., DELMAR, M., TAFFET, S.M. & JALIFE, J. (1999). Proton and zinc effects on HERG currents. Biophys. J., **77,** 282–298.

ARMSTRONG, C.M. (1971). Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. J. Gen. Physiol., **58,** 413–437.

ÅBERG, G. (1972). Toxicological and local anaesthetic effects of optically active isomers of two local anaesthetic compounds. Acta Pharmacol. Toxicol., **31,** 273–286.

BETHELL, H.W., VANDENBERG, J.I., SMITH, G.A. & GRACE, A.A. (1998). Changes in ventricular repolarization during acidosis and low- flow ischemia. Am. J. Physiol., **275,** H551–H561.

BUSCH, A.E., HURST, R.S., NORTH, R.A., ADELMAN, J.P. & KAVANAUGH, M.P. (1991). Current inactivation involves a histidine residue in the pore of the rat lymphocyte potassium channel RGK5. Biochem. Biophys. Res. Comm., **179,** 1384–1390.

CARMELIET, E. (1993). Use-dependent block and use-dependent unblock of the delayed rectifier K^+ current by almokalant in rabbit ventricular myocytes. Circ. Res., **73,** 857–868.

CASTLE, N.A. (1990). Bupivacaine inhibits the transient outward K^+ current but not the inward rectifier in rat ventricular myocytes. J. Pharmacol. Exp. Ther., **255,** 1038–1046.

CLARKSON, C.W. & HONDEGHEM, L.M. (1985). Mechanism for bupivacaine depression of cardiac conduction: fast block of sodium channels during the action potential with slow recovery from block during diastole. Anesthesiology, **62,** 396–405.

CORABOEUF, E., DEROUBAIX, E. & COULOMBE, A. (1980). Acidosis-induced abnormal repolarization and repetitive activity in isolated dog Purkinje fibers. J. Physiol. (Paris), **76,** 97–106.

CORABOEUF, E., DEROUBAIX, E. & HOERTER, J. (1976). Control of ionic permeabilities in normal and ischemic heart. Circ. Res., **38,** I92–I98.

CORDEIRO, J.M., HOWLETT, S.E. & FERRIER, G.R. (1994). Simulated ischaemia and reperfusion in isolated guinea pig ventricular myocytes. Cardiovasc. Res., **28,** 1794–1802.

COULTER, K.L., PERIER, F., RADEKE, C.M. & VANDENBERG, C.A. (1995). Identification and molecular localization of a pH-sensing domain for the inward rectifier potassium channel HIR. Neuron, **15,** 1157–1168.

DAVIES, M.P., FREEMAN, L.C. & KASS, R.S. (1996). Dual actions of the novel class III antiarrhythmic drug NE-10064 on delayed potassium channel currents in guinea pig ventricular and sinoatrial node cells. J. Pharmacol. Exp. Ther., **276,** 1149–1154.

DELPÓN, E., CABALLERO, R., VALENZUELA, C., LONGOBARDO, M., SNYDERS, D. & TAMARGO, J. (1999). Benzocaine enhances and inhibits the K^+ current through a human cardiac cloned channel (Kv1.5). Cardiovasc. Res., **42,** 510–520.

DELPÓN, E., VALENZUELA, C., PEREZ, O., CASIS, O. & TAMARGO, J. (1995). Propafenone preferentially blocks the rapidly activating component of delayed rectifier K^+ current in guinea pig ventricular myocytes. Voltage-independent and time-dependent block of the slowly activating component. Circ. Res., **76,** 223–235.

FAKLER, B., SCHULTZ, J.H., YANG, J., SCHULTE, U., BRANDLE, U., ZENNER, H.P., JAN, L.Y. & RUPPERSBERG, J.P. (1996). Identification of a titratable lysine residue that determines sensitivity of kidney potassium channels (ROMK) to intracellular pH. EMBO J., **15,** 4093–4099.

FRANQUEZA, L., LONGOBARDO, M., VICENTE, J., DELPÓN, 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.

FRY, C. & POOLE-WILSON, P. (1981). Effects of acid-base changes on excitation-contraction coupling in guinea-pig and rabbit cardiac ventricular muscle. J. Physiol. (Lond)., **313,** 141–160.

GINGRICH, K.J., BEARDSLEY, D. & YUE, D.T. (1993). Ultra-deep blockade of Na^+ channels by a quaternary ammonium ion: catalysis by a transition-intermediate state? J. Physiol. (Lond.), **471,** 319–341.

GONZÁLEZ, T., LONGOBARDO, M., CABALLERO, R., DELPÓN, E., TAMARGO, J. & VALENZUELA, C. (2001). Effects of bupivacaine and a novel local anesthetic, IQB-9302, on human cardiac K^+ channels. J. Pharmacol. Exp. Ther., **296,** 573–583.

GRAF, B.M., MARTIN, E., BOSNJAK, Z.J. & STOWE, D.F. (1997). Stereospecific effect of bupivacaine isomers on atrioventricular conduction in the isolated perfused guinea pig heart. Anesthesiology, **86,** 410–419.

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.

HOTH, S., DREYER, I., DIETRICH, P., BECKER, D., MULLER ROBER, B. & HEDRICH, R. (1997). Molecular basis of plant-specific acid activation of K^+ uptake channels. Proc. Natl. Acad. Sci. U.S.A., **94,** 4806–4810.

ITO, H., VEREECKE, J. & CARMELIET, E. (1992). Intracellular protons inhibit inward rectifier K^+ channel of guinea-pig ventricular cell membrane. Pflügers Arch., **422,** 280–286.

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., DELPÓN, E., CABALLERO, R., TAMARGO, J. & VALENZUELA, C. (1998). Effects of changes in extracellular pH on R(+)-bupivacaine-induced block of hKv1.5 channels. *Br. J Pharmacol.,* **124,** 110P.

LONGOBARDO, M., GONZÁLEZ, T., NAVARRO-POLANCO, R., CABALLERO, R., DELPÓN, E., TAMARGO, J., SNYDERS, D.J., TAMKUN, M.M. & VALENZUELA, C. (2000). Effects of a quaternary bupivacaine derivative on delayed rectifier K^+ currents. *Br. J. Pharmacol.,* **130,** 391–401.

LUDUENA, F., BOGADO, E. & TULLAR, B. (1972). Optical isomers of mepivacaine and bupivacaine. *Arch. Int. Pharmacodyn. Ther.,* **200,** 359–369.

NANCARROW, C., RUNCIMAN, W.B., MATHER, L.E., UPTON, R.N. & PLUMMER, J.L. (1987). The influence of acidosis on the distribution of lidocaine and bupivacaine into the myocardium and brain of the sheep. *Anesth. Analg.,* **66,** 925–935.

NAU, C., VOGEL, W., HEMPELMANN, G. & BRAU, M.E. (1999). Stereoselectivity of bupivacaine in local anesthetic-sensitive ion channels of peripheral nerve. *Anesthesiology,* **91,** 786–795.

PROD'HOM, B., PIETROBON, D. & HESS, P. (1989). Interactions of protons with single open L-type calcium channels. Location of protonation site and dependence of proton-induced current fluctuations on concentration and species of permeant ion. *J. Gen. Physiol.,* **94,** 23–42.

RICH, T.C. (1996). Gating of the hKv1.5 delayed rectifier: the implications of multiple conducting states. UMI, Ann Arbor, Mich (Microfilm #9714424). PhD Thesis.

RICH, T.C. & SNYDERS, D.J. (1998). Evidence for multiple open and inactivated states of the hKv1.5 delayed rectifier. *Biophys. J.,* **75,** 183–195.

ROSEN, M., THIGPEN, J., SHNIDER, S., FOUTZ, S., LEVINSON, G. & KOIKE, M. (1985). Bupivacaine-induced cardiotoxicity in hypoxic and acidotic sheep. *Anesth. Analg.,* **64,** 1089–1096.

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.

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.

STEIDL, J.V. & YOOL, A.J. (1999). Differential sensitivity of voltage-gated potassium channels Kv1.5 and Kv1.2 to acidic pH and molecular identification of pH sensor. *Mol. Pharmacol.,* **55,** 812–820.

STENGL, M., CARMELIET, E., MUBAGWA, K. & FLAMENG, W. (1998). Modulation of transient outward current by extracellular protons and Cd^{2+} in rat and human ventricular myocytes. *J Physiol. (Lond.)* **511,** 827–836.

STRICHARTZ, G.R. (1987). *Local Anesthetics,* 1 Ed., Berlin, Springer-Verlag.

TAMKUN, M.M., KNOTH, K.M., WALBRIDGE, J.A., KROEMER, H., RODEN, D.M. & GLOVER, D.M. (1991). Molecular cloning and characterization of two voltage-gated K^+ channel cDNAs from human ventricle. *FASEB J.,* **5,** 331–337.

TSENG, G.N., ZHU, B., LING, S. & YAO, J.A. (1996). Quinidine enhances and suppresses Kv1.2 from outside and inside the cell, respectively. *J. Pharmacol. Exp. Ther.,* **279,** 844–855.

VALENZUELA, C., DELPÓN, 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., SANCHEZ CHAPULA, J., DELPÓN, E., ELIZALDE, A., PEREZ, O. & TAMARGO, J. (1994). Imipramine blocks rapidly activating and delays slowly activating K^+ current activation in guinea pig ventricular myocytes. *Circ. Res.,* **74,** 687–699.

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.

WALLENSTEIN, S., ZYCKER, C. & FLEISS, J. (1980). Some statistical methods useful in circulation research. *Circ. Res.,* **47,** 1–8.

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.

YAMANE, T., FURUKAWA, T., HORIKAWA, S. & HIRAOKA, M. (1993). External pH regulates the slowly activating potassium current IsK expressed in Xenopus oocytes. *FEBS Lett.,* **319,** 229–232.

ZHANG, J.F. & SIEGELBAUM, S.A. (1991). Effects of external protons on single cardiac sodium channels from guinea pig ventricular myocytes. *J Gen. Physiol.,* **98,** 1065–1083.