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Enhancement of delayed-rectifier potassium conductance by low concentrations of local anaesthetics in spinal sensory neurones

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1 Combining the patch-clamp recordings in slice preparation with the 'entire soma isolation' method we studied action of several local anaesthetics on delayed-rectifier K^+ currents in spinal dorsal horn neurones.

2 Bupivacaine, lidocaine and mepivacaine at low concentrations (1–100 μM) enhanced delayed-rectifier K^+ current in intact neurones within the spinal cord slice, while exhibiting a partial blocking effect at higher concentrations (>100 μM). In isolated somata 0.1–10 μM bupivacaine enhanced delayed-rectifier K^+ current by shifting its steady-state activation characteristic and the voltage-dependence of the activation time constant to more negative potentials by 10–20 mV.

3 Detailed analysis has revealed that bupivacaine also increased the maximum delayed-rectifier K^+ conductance by changing the open probability, rather than the unitary conductance, of the channel.

4 It is concluded that local anaesthetics show a dual effect on delayed-rectifier K^+ currents by potentiating them at low concentrations and partially suppressing at high concentrations. The phenomenon observed demonstrated the complex action of local anaesthetics during spinal and epidural anaesthesia, which is not restricted to a suppression of Na^+ conductance only.
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Keywords: Patch-clamp; spinal cord; local anaesthetics; delayed rectifier potassium channel; single channel

Abbreviations: CNS, central nervous system; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; ESI, entire soma isolation; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); IC_{50}, half-maximal inhibiting concentration; TEA, tetraethylammoniumchloride; TTX, tetrodotoxin

Introduction

Different types of local anaesthetics are widely used in clinical practice for spinal, epidural and local anaesthesia. Although their principal action is usually explained through a suppression of voltage-gated Na^+ conductance, there is a continuously increasing number of reports showing that the block of some other systems of ionic channels (Ca^{2+}, K^+ or transmitter-activated) can be also involved (Aracava *et al*., 1984; Ikeda *et al*., 1984; Castle, 1990; Barann *et al*., 1993; Valenzuela *et al*., 1995; Hirota *et al*., 1997; Rossner & Freese, 1997; Nilsson *et al*., 1998; Bräu *et al*., 1995; 1998; Kindler *et al*., 1999; Nau *et al*., 1999). Among them, K^+ channels represent the most diverse family of ionic channels with multiple physiological functions. Since different types of K^+ channels influence the resting potential, firing threshold, spike depolarization and repetitive firing frequency (Connor & Stevens, 1971; Llinas, 1988; Koh *et al*., 1992; Safronov *et al*., 1996; Hille, 2001), their block or modulation by local anaesthetics may considerably affect the generation of simple spikes as well as complex patterns of activity in the neurone. As a consequence, this can modify the pattern of the entire afferent input and its processing in the spinal cord.

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Dorsal horn neurones from superficial laminae of the spinal cord are involved in processing of nociception (Light & Perl, 1979; Light *et al*., 1979). During epidural and spinal anaesthesia they are exposed to local anaesthetics, which directly diffuse into the spinal cord (Bromage *et al*., 1963). In our previous study performed on intact dorsal horn neurones in the spinal cord slice (Olschewski *et al*., 1998) it was shown that bupivacaine, lidocaine, and mepivacaine at concentrations between 1 μM and 1 mM suppress a fast inactivating A-type K^+ current, whereas delayed-rectifier currents were not remarkably affected. However, a more elaborate analysis of those data has unexpectedly revealed that low concentrations of local anaesthetics enhanced, rather than blocked, the delayed-rectifier K^+ currents, so that their activation was seen at more negative potentials. The present study was undertaken to describe this phenomenon in more detail. For this purpose, we used the 'entire soma isolation' (ESI) method (Safronov *et al*., 1997; Safronov, 1999) which enabled the study of channel pharmacology in isolated somata of visually identified dorsal horn neurones under conditions where diffusion of the blocker molecules toward the cell membrane was not impeded by connective tissue within the slice and the quality of current recording was not affected by insufficient space clamp (Olschewski *et al*., 2000).

This study shows that low concentrations of local anaesthetics indeed potentiate delayed-rectifier K^+ current

by shifting its activation to more negative potentials and increasing its maximum conductance. Local anaesthetics interact with the activation gates of the channel and increase its open probability without affecting the unitary conductance. At high concentrations, the local anaesthetics additionally blocked the current. It is concluded that bupivacaine, lidocaine and mepivacaine exert a dual effect on delayed-rectifier K^+ channels.

Methods

Preparation

Experiments were performed by means of the patch-clamp technique (Hamill *et al*., 1981) on 200 μm slices, cut from lumbar enlargements (L3–6) of the spinal cord of 4–16-day-old rats (Edwards *et al*., 1989; Takahashi, 1990; Olschewski *et al*., 1998). Animals were rapidly decapitated and the spinal cords were carefully cut out in ice-cold preparation solution bubbled with O_2–CO_2 (95%–5%). After removal of the pial membrane with fine forceps the spinal cord was embedded in a preparation solution containing 2% agar cooled down to 39°C. To accelerate solidification of the agar, the beaker with the preparation was placed in ice-cold water. The agar block containing the lumbar enlargement of the spinal cord was cut out and glued to a glass stage fixed in the chamber of the tissue slicer. The spinal cord was sliced in ice-cold preparation solution under continuous bubbling. The slices were thereafter incubated for 1 h at 32°C.

The procedures of animal decapitation have been reported to the local veterinarian authority and are in accordance with the German guidelines.

Solutions

Preparation solution contained (in mM): NaCl 115, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11, NaH_2PO_4 1, $NaHCO_3$ 25 (pH 7.4 when bubbled with 95% O_2–5% CO_2). In the experimental chamber, the slices were superfused by low-Ca^{2+} solution, in order to reduce spontaneous synaptic activity and to prevent activation of Ca^{2+} currents and Ca^{2+}-dependent K^+ currents. It was obtained from the preparation solution by setting the concentration of Ca^{2+} to 0.1 mM and increasing the concentration of Mg^{2+} to 5 mM. Tetraethylammonium-containing solution (TEA-solution) used for Na^+ current recordings in Figure 5 contained (in mM): NaCl 95, KCl 5.6, $CaCl_2$ 0.1, $MgCl_2$ 5, glucose 11, NaH_2PO_4 1, $NaHCO_3$ 25 and TEA-Cl 20 (pH 7.4 when bubbled with 95% O_2–5% CO_2). The study of K^+ currents was carried out in Na^+-free choline-Cl solution containing (in mM): choline-Cl 141, KCl 0.6, $CaCl_2$ 0.1, $MgCl_2$ 5, glucose 11, HEPES 10 (pH 7.4 adjusted with 5 mM KOH). TTX (0.2 μM) was added to this solution to block voltage-gated Na^+ channels.

Bupivacaine-HCl and lidocaine-HCl were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Mepivacaine was taken as Scandicaine from Astra Chemicals (Wedel, Germany). The S(−) and R(+) forms of bupivacaine were donated by Dr. Rune Sandberg (Astra-Pain Control, Södertälje, Sweden). The drugs were dissolved in distilled water to give 20 mM stock solutions. TTX was obtained from Latoxan (Rosans, France). The experimental chamber with a volume of 0.4 ml was continuously perfused by external solution at a rate of 2–3 ml min^{-1}.

The pipette solution used for K^+ current recordings from intact neurones contained (in mM): NaCl 5, KCl 144.4, $MgCl_2$ 1, EGTA 3, HEPES 10 (pH 7.3 by 10.6 mM KOH). Internal solution for K^+ current recording from isolated somata contained (in mM): NaCl 5, KCl 144.4, $MgCl_2$ 1, EGTA 3, HEPES 10 (pH 7.3 by 10 mM NaOH). This solution was also applied as bath solution for the single-channel recording from inside-out patches. The whole-cell Na^+ currents were measured in isolated somata using pipette solution (in mM): NaCl 5.8, CsCl 134, $MgCl_2$ 1, EGTA 3, HEPES 10 (pH 7.3 adjusted with 9.2 mM NaOH).

Current recording

Pipettes pulled from borosilicate glass tube (GC 150, Clark Electromedical Instruments, Pangbourne, U.K.) were fire-polished to give a final resistance of 3–4 MΩ for whole-cell recording and of 4–11 MΩ in the single-channel experiments. The pipettes used in the single-channel experiments were coated with Sylgard 184 (Dow Corning, Midland, U.S.A.). The patch-clamp amplifier was an EPC-7 (List, Darmstadt, Germany). The effective corner frequency of the low-pass filter was 3 kHz and the frequency of digitization was 10 kHz in all whole-cell experiments. For the single-channel recording the frequencies were 1 and 2 kHz, respectively. The data were stored and analysed using commercially available software (pCLAMP, Axon Instruments, Foster City, CA, U.S.A.). Transients and leakage currents were digitally subtracted in all experiments using records with hyperpolarizing pulses that activated no currents. Offset potentials were nulled directly before formation of the seal. Errors in the clamped potential evoked by the series resistance of the electrode were not corrected. The range of series resistances, estimated as a pipette resistance multiplied by a factor of 3, was 9–12 MΩ. For currents from the isolated somata used for analysis in this study, voltage errors due to resistance in series were smaller than 4 mV. All experiments were carried out at room temperature of 21–23°C.

Identification of dorsal horn neurones

The dorsal horn neurones were identified in spinal cord slices as multipolar cells with a soma (8–12 μm diameter) located in laminae I–III (Safronov *et al*., 1997). In voltage-clamp mode, neurones were distinguished from glial cells on the basis of the magnitude of Na^+ currents as described previously (Safronov *et al*., 1997). In low-Ca^{2+} solution, all neurones studied possessed a large Na^+ current exceeding 1 nA, were able to generate action potentials and showed spontaneous synaptic activity. The resting potentials measured in intact neurones were between −76 and −55 mV. In experiments with inside-out patches, the neurones were identified during establishing of the giga-seal contact, when the attached patch pipette could be also used for the extracellular recording of spontaneous action potentials invading the cell.

The ESI method

A detailed description of the ESI method has been given elsewhere (Safronov *et al*., 1997; Safronov, 1999; Olschewski *et al*., 2000). Briefly, in whole-cell recording mode, the entire soma of the neurone was isolated from the slice by slow withdrawal of the recording pipette. The isolated structure was classified as soma (*soma*) if it had lost all of its processes during isolation and preserved only 10–20% of original Na^+ current recorded from the neurone in the slice before its isolation. The good physiological state of the isolated structures was confirmed by a considerable increase in their input resistances (reflecting a decrease in membrane leakage conductance), by stable or even improved membrane resting potentials (Safronov *et al*., 1997; Safronov, 1999).

Separation of delayed-rectifier K^+ currents

Potassium currents were recorded in external choline-Cl solution. Delayed-rectifier K^+ currents were separated as described previously (Olschewski *et al*., 1998). Total K^+ currents activated by depolarizing steps following a 150 ms prepulse to −120 mV consisted of both rapidly inactivating A-type and delayed-rectifier components. A similar depolarization applied after 150 ms prepulse to −60 mV (at which A-type channels are almost completely inactivated) elicited only a non-inactivating component of K^+ current, which was considered in the present study as a delayed-rectifier current. The amplitudes of the delayed-rectifier currents were measured at the end of 200 ms depolarizing pulses.

Statistical analysis and fitting

The present study is based on recordings from 35 intact neurones in the spinal cord slice, 46 isolated *somata* and eight inside-out membrane patches. All numerical values are given as mean ± standard error of the mean (s.e.mean). The parameters obtained by fitting the data points using a non-linear least-squares procedure are given as mean ± standard error (s.e.).

The all point-amplitude histograms for the unitary currents see (Figure 6) were built for the patch containing two active channels. They were fitted using a sum of three Gauss functions given as (for the n^{th} peak):

$$A_n/[(2\pi)^{1/2}\sigma] \times \exp\,[-(i-i_n)^2/(2\sigma^2)], \qquad [1]$$

where n was 0, 1 or 2 for the peaks corresponding to the baseline, to the opening of one channel or to simultaneous opening of two channels, respectively. A_n and i_n were the histogram amplitude and the mean current for the n^{th} peak. Presented in this form, A_n was equal to the area under the n^{th} peak. Thus, the open probability (P_o) for the patch assumed to contain two active channels could be simply calculated from the fit to the all point-amplitude histogram as: $P_o = \frac{1}{2}(A_1 + 2A_2)/(A_0 + A_1 + A_2)$.

ANOVA was used to evaluate the significance of differences in the effects of bupivacaine and its enantiomers on the delayed-rectifier K^+ currents. Intergroup differences were assessed by a factorial analysis of variance with *post hoc* analysis with Fischer's least significant difference test. $P < 0.05$ was considered as significant.

Results

Local anaesthetics potentiate delayed-rectifier K^+ currents in intact neurones

Delayed-rectifier K^+ currents were first recorded from intact dorsal horn neurones in spinal cord slices in external choline-Cl solution. The currents were activated by a 200 ms voltage step to +20 mV following a 150 ms prepulse to −60 mV. Addition of 10 μM bupivacaine ($n = 5$), lidocaine ($n = 5$) or mepivacaine ($n = 5$) increased the amplitude of delayed-rectifier current at +20 mV by 15–35% (Figure 1A). At 100 μM of these local anaesthetics the current was still 5–11% larger than in control solution. Plotting the normalized amplitudes of the current at +20 mV as a function of local anaesthetics concentration demonstrated its enhancement by all three drugs in a range between 1 and 100 μM (Figure 1B). At 1 mM the local anaesthetics produced a slight reduction (3–7%) of the current (see also Olschewski *et al*., 1998).

These experiments have shown that local anaesthetics at low concentrations enhance delayed-rectifier K^+ currents while producing a slight block at higher concentrations. It

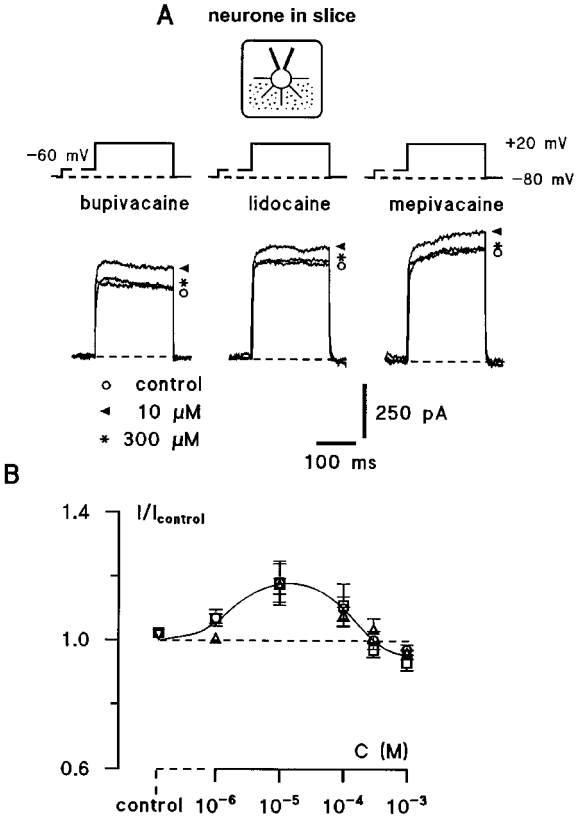

Figure 1 Potentiation of delayed-rectifier K^+ current by local anaesthetics in intact spinal sensory neurones. (A) Delayed-rectifier K^+ currents activated by a voltage pulse to +20 mV following a 150 ms prepulse to −60 mV in control solution and in the presence of 10 and 300 μM bupivacaine, lidocaine and mepivacaine. Holding potential −80 mV. (B) Relative amplitudes of the currents at +20 mV as a function of bupivacaine (circles), lidocaine (triangles) and mepivacaine (squares) concentration. The currents were normalized by the amplitude of the corresponding current recorded in control solution. Each data point is the mean of measurements from five cells. Connecting line was drawn by eye.

could be reasonably assumed that such an enhancement resulted either from an increase in total K^+ conductance or from a shift in the steady-state activation characteristic to more negative potentials. In order better to understand this phenomenon, we performed further experiments using the ESI technique. This has enabled current recordings under conditions of sufficient space clamp and small voltage errors due to resistance in series (remaining currents were much smaller than those in the whole neurone) as well as pharmacological investigation with well-controlled drug concentrations facing the cell membrane. The following study of local anaesthetic action on delayed-rectifier currents in isolated *somata* has been performed using bupivacaine.

Studies in isolated somata

For the intact neurones in the spinal cord slice, the concentration range of current potentiation was between 1 and 100 μM. According to our previous data (Olschewski *et al*., 2000) the effective drug concentrations are several times lower when studied in isolated *somata*, due probably to better diffusion of the blocker molecules to the cell membrane. Therefore, the concentration range of bupivacaine tested on *somata* was increased (30 nM to 1 mM).

The currents elicited by different depolarizing pulses were recorded in the presence of increasing concentrations of bupivacaine (Figure 2A, shown for +20 mV). The amplitudes of delayed-rectifier K^+ currents normalized to that measured in control solution at +40 mV ($I_{control}$(+40 mV)) are presented as a function of bupivacaine concentration in Figure 2B. At all potentials tested the currents were enhanced at low concentrations (100 nM to 10 μM) of local anaesthetic. The maximal enhancement of K^+ conductance was observed at 1 μM. The current was increased by 21±5% at +40 mV, by 39±3% at 0 mV and by 105±2% at −40 mV (n=5). However, the most striking effect occurred at −50 mV where the current has not been seen under control conditions but appeared after addition of 0.3–10 μM bupivacaine. At 10–30 μM bupivacaine, the current amplitude was very close to the control value and beginning with 100 μM a clear reduction of the current amplitude was observed. At 1 mM bupivacaine the current was reduced by 39±3% (at +40 mV) when recorded in isolated *somata*. This reduction was much larger than that of less than 7% seen in experiments with 1 mM bupivacaine on intact neurones (Figure 1, see also Olschewski *et al*., 1998).

To analyse the changes in delayed-rectifier K^+ conductance, the steady-state activation characteristics were constructed for currents recorded in control solution and in the presence of 1 μM, 10 μM and 1 mM bupivacaine. The data were fitted by the Boltzmann equation and then normalized by the maximum conductance (G_M) obtained for control solution (Figure 3A). The fitting parameters are given in Table 1. After addition of 1 μM bupivacaine, the activation

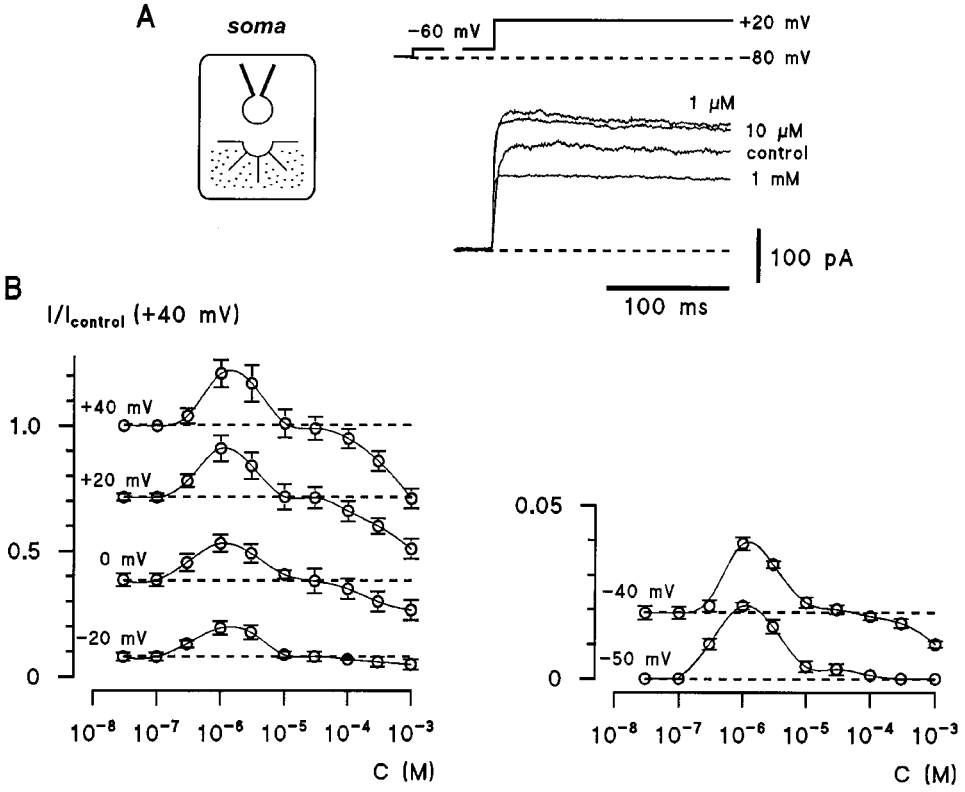

Figure 2 Effect of bupivacaine on the amplitude of the delayed-rectifier K^+ current in isolated *somata*. (A) Delayed-rectifier currents activated by depolarization to +20 mV following a 150 ms prepulse to −60 mV in the presence of different concentrations of bupivacaine (indicated near the corresponding trace). Holding potential, −80 mV. (B) Amplitudes of delayed-rectifier K^+ currents activated by different depolarizing potentials as a function of bupivacaine concentration. Each data point represents the mean of five measurements in different *somata*. All amplitudes were normalized by the amplitude of the control current recorded at +40 mV ($I_{control}$(+40 mV)). The data for −50 and −40 mV are shown at higher resolution. Data points are connected by eye.

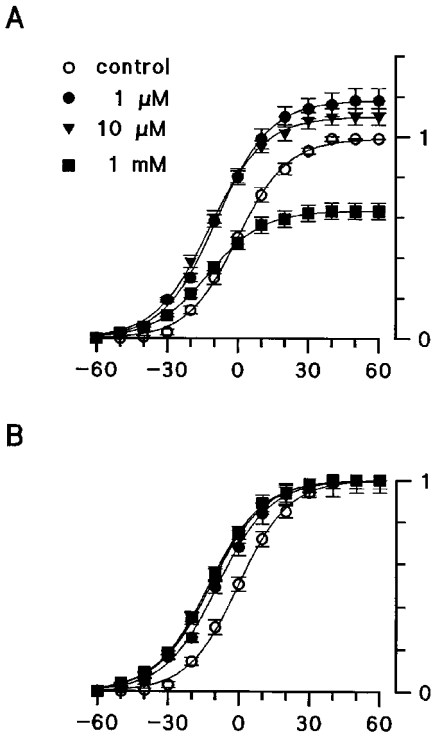

Figure 3 Effect of bupivacaine on delayed-rectifier K^+ conductance in isolated *somata*. (A) Voltage-dependence of delayed-rectifier K^+ conductance studied at different bupivacaine concentrations. Reversal potential for K^+ ions was assumed to be −84 mV. Data from five *somata*. Data points were fitted with a standard Boltzmann function and normalized by the maximum conductance measured in control solution:

$$G = G_M/G_M(0) \times 1/(1+\exp(-(E-E_{50})/k)), \qquad [2]$$

where G is conductance, G_M is the maximal conductance at a given concentration of blocker, $G_M(0)$ is maximal conductance in control solution, E is membrane potential, E_{50} is a potential at which a half-maximal conductance is activated and k is steepness factor. The fitting parameters are given in Table 1. (B) The same characteristics as in A normalized to 1.

Table 1 Changes in delayed-rectifier K^+ conductance evoked by application of different concentrations of bupivacaine

	E_{50} (mV)	k (mV)	G_M	P	n
Control	−0.2±0.4	10.8±0.3	1.00		5
1 μM	−8.9±0.3	11.3±0.3	1.18±0.01	<0.001	5
10 μM	−11.9±0.3	11.4±0.3	1.10±0.01	<0.001	5
1 mM	−12.6±0.2	11.4±0.2	0.63±0.01	0.1329	5

The numbers were obtained as a result of fitting of the data points in Figure 3A using the Boltzmann equation, where E_{50} is a potential of half-maximal activation, k is a steepness factor and (G_M) is the maximum delayed-rectifier conductance. The G_M value for each drug concentration was divided by that for control solution. Given P values describe the significance of the E_{50} value changes with increasing bupivacaine concentration: 1 μM bupivacaine *versus* control solution, 10 μM *versus* 1 μM bupivacaine and 1 mM *versus* 10 μM bupivacaine.

curve shifted to more negative potentials from −0.2±0.4 mV (control) to −8.9±0.3 mV ($P<0.001$) and the G_M value increased by 18±1% ($n=5$). In 10 μM bupivacaine, a small further shift of activation characteristic to −11.9±0.3 mV ($P<0.001$) was seen, while an increase in G_M by 10±1% ($n=5$) was smaller than in 1 μM. No further appreciable shift of activation was revealed in 1 mM bupivacaine, but G_M was reduced to 63±1% ($n=5$). Saturation of the shift of the activation curve became obvious after all G_M values were normalized to 1 (Figure 3B).

Effect of bupivacaine stereoisomers

The stereoselectivity of bupivacaine enantiomers was studied by comparing the effects of S(−) and R(+) forms on the macroscopic delayed-rectifier currents in isolated *somata*. The data given in Table 2 indicate that these isomers tested at concentrations from 1 μM to 1 mM did not show statistically significant differences either for enhancement or suppression of the current.

Modifications of the activation kinetics

In order to test whether the bupivacaine-induced shift of the steady-state activation characteristic is accompanied by the corresponding changes in the time course of current activation, the kinetics of the currents in control and drug-containing solutions were compared. Indeed, 1 μM, 10 μM and 1 mM bupivacaine accelerated the activation of the current elicited by a voltage step to +20 mV (Figure 4A). The effect became more evident after normalizing the maximal amplitudes of these four currents (Figure 4B). The largest acceleration of the activation kinetics took place after change from control solution to 1 μM bupivacaine. Increase in the drug concentration to 10 μM produced only a slight further acceleration that almost saturated at 1 mM.

We described the activation kinetics by measuring the time of half-maximum activation ($\tau_{0.5}$). In control solution the data points were satisfactorily fitted ($r=0.999$) with a mono-exponential function with an e-fold reduction per 31.8±0.8 mV (Figure 4C, six *somata*). This curve was then fixed and shifted along the voltage axis, in order to test whether it can be also used for fitting the $\tau_{0.5}$ values obtained in 1 μM and 1 mM bupivacaine. It can be seen that just a parallel shift of the control fitting curve by 13.1 and 21.3 mV to more negative potentials was sufficient to give good fits for the $\tau_{0.5}$ values in 1 μM ($r=0.999$) and 1 mM bupivacaine ($r=0.997$), respectively. The voltage-dependence of $\tau_{0.5}$ for 10 μM bupivacaine (shift of 19.4±0.6 mV from control curve; not shown) was very close to that for 1 mM. Thus, it could be concluded that the voltage-dependence of $\tau_{0.5}$ was shifted to more negative potentials and that the saturation of this shift was reached at about 10 μM bupivacaine.

Control experiments with Na^+ currents

Although the present data have indicated that the local anaesthetics specifically interact with an activation gate of the delayed-rectifier K^+ channel, it could not be excluded that the drugs can additionally produce a screening effect on stationary membrane charges resulting in a change of the voltage profile within the membrane (Hille, 2001). To test this hypothesis, recordings of voltage-gated Na^+ currents in control solution and in the presence of 1 μM bupivacaine were made (Figure 5A, upper part). Application of the drug

reduced the amplitude of Na^+ current at all potentials investigated to about a half, but no shift in voltage-current relationships was revealed (Figure 5A, bottom). For five isolated *somata*, the Na^+ conductances as a function of membrane potential in both solutions were plotted (Figure 5B). No apparent shift in steady-state activation curve was revealed. The fitting of the data points with the Boltzmann function gave $E_{50} = -40.4 \pm 0.2$ mV and $k = 5.6 \pm 0.3$ mV for control solution and $E_{50} = -40.3 \pm 0.3$ mV and $k = 5.4 \pm 0.5$ mV for 1 μM bupivacaine.

Table 2 No difference between effects of S(−) and R(+) bupivacaine

	$G_{M,\ S(-)bupivacaine} / G_{M,\ control}$	$G_{M,\ R(+)bupivacaine} / G_{M,\ control}$	$G_{M,\ S(-)bupivacaine} / G_{M,\ R(-)bupivacaine}$	P	n
1 μM	1.24±0.02	1.20±0.03	1.03	0.26	7
100 μM	0.94±0.02	0.91±0.03	1.03	0.38	6
1 mM	0.68±0.04	0.72±0.05	0.94	0.52	6

The ratios between the maximum delayed-rectifier K^+ conductances in drug-containing and control solutions were compared for S(−) and R(+) bupivacaine. A significant level (P) shows that there is no significance difference in effects of both isomers.

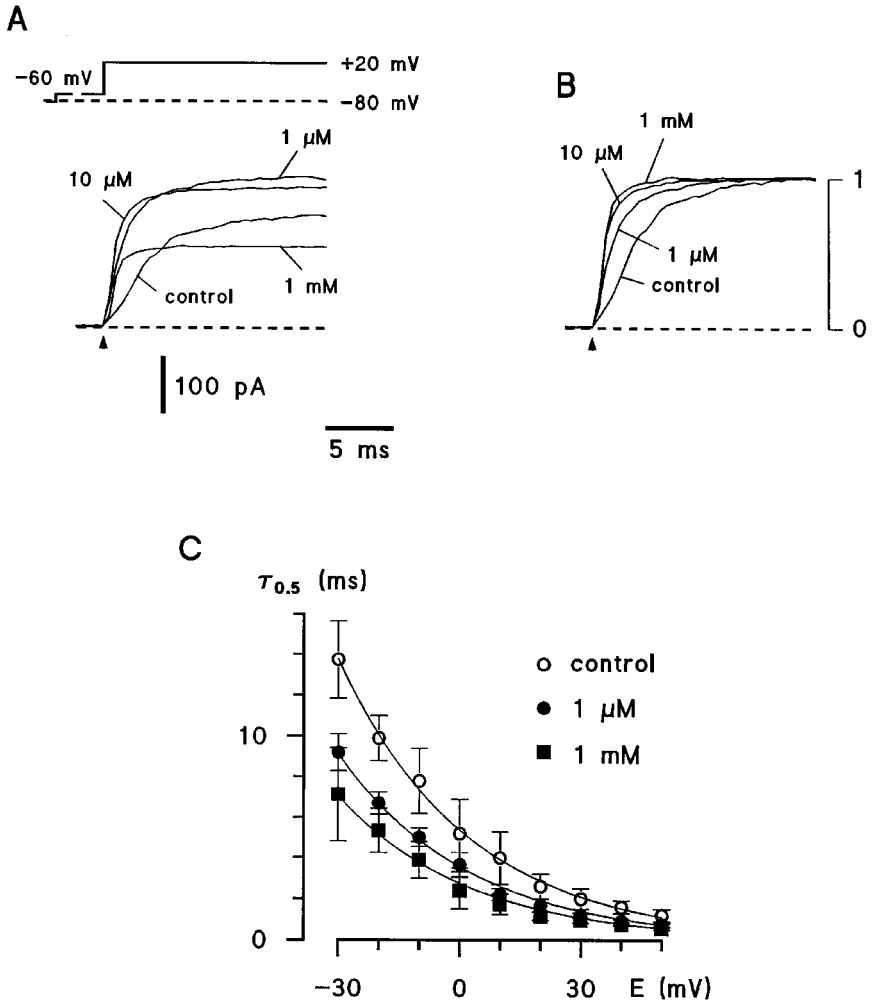

Figure 4 Acceleration of the current activation by bupivacaine. (A) delayed-rectifier K^+ currents in the presence of different bupivacaine concentrations. The currents were activated using the same pulse protocol as in Figure 2A. Beginning of the depolarizing pulse is indicated by an arrowhead. (B) Current traces normalized to 1. (C) Time constant of half-maximal activation ($\tau_{0.5}$) as a function of membrane potential in control solution and in the presence of 1 μM and 1 mM bupivacaine. Data from six *somata*. The data points were fitted with a mono-exponential function: $A \times \exp[-(E-\Delta E)/k]$. For the data in control solution ΔE was assumed to be zero and the values of $A = 5.4 \pm 0.1$ ms and $k = 31.8 \pm 0.8$ mV were obtained by optimal fitting ($r = 0.999$). For 1 μM and 1 mM bupivacaine, the values of A and k were fixed at 5.4 ms and 31.8 mV, respectively, and the ΔE parameter was varied to give best fit -13.1 ± 0.5 mV ($r = 0.999$) for 1 μM bupivacaine and -21.3 ± 0.7 mV ($r = 0.997$) for 1 mM bupivacaine.

Thus, the shift in the steady-state activation curve of delayed-rectifier K^+ current was not a property shared by Na^+ current.

Single-channel analysis

The dual effect of bupivacaine on delayed-rectifier K^+ currents was further investigated at the single-channel level. Recordings were done in inside-out patches originating from the soma of intact neurones in the spinal cord slices. These patches usually contained only a few delayed-rectifier K^+ channels, which were identified according to a procedure described previously (Wolff *et al*., 1998). The drug was applied with bath solution to the cytoplasmic side of the membrane, while the pipettes were filled with external Na^+-free choline-Cl solution. The channels were activated by a depolarization to +40 mV at which the steady-state activation characteristic reached its saturation.

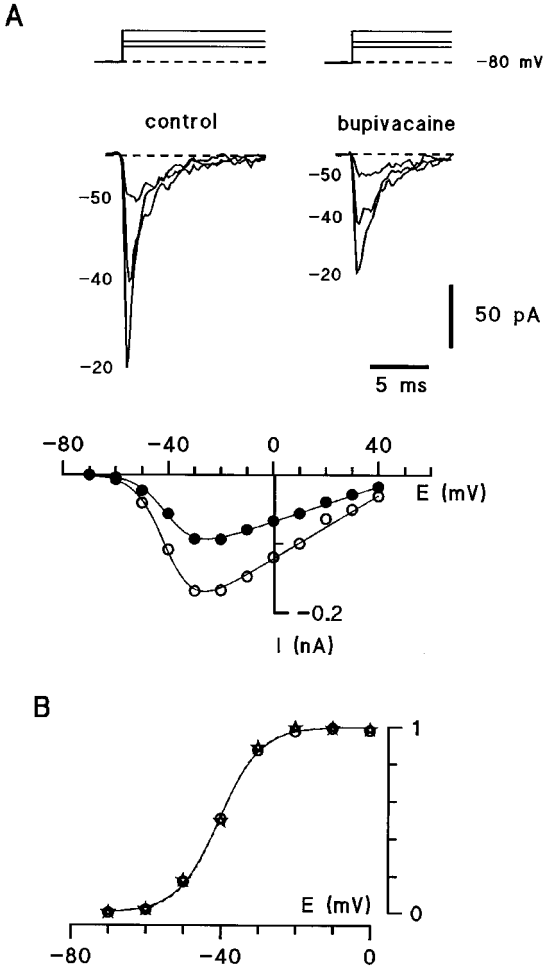

Figure 5 Control experiments with Na^+ currents. (A) Na^+ currents activated in an isolated *soma* by different depolarizing pulses (indicated near the corresponding trace) in control solution and in 1 μM bupivacaine. Holding potential, −80 mV. Below, the current-voltage relationships for Na^+ currents in control solution (open symbols) and in the presence of 1 μM bupivacaine (filled symbols). Connection lines were drawn by eye. (B) Voltage-dependence of Na^+ conductance in control solution (circles) and in the presence of 1 μM bupivacaine (stars). The reversal potential for Na^+ ions was assumed to be +53 mV. The data points (five *somata*) were fitted using the Boltzmann function and normalized to 1. Fitting parameters are given in the text.

Effects of low (1 μM) bupivacaine concentration on the gating and conductance of delayed-rectifier K^+ channels are shown in Figure 6A. In spite of complete activation of the current at +40 mV, the channels are not permanently open but rather show regular transitions between open and closed states. Addition of 1 μM bupivacaine increased both the frequency of openings and the relative time spent by the channel in the open state. As a result, the averaged single-channel currents (based on total of 70 episodes each) became clearly larger in the presence of the drug. The same effect was observed in five patches which were subjected to a detailed analysis using all point-amplitude histograms.

Figure 6B and C show the data from one of these patches (the same as in Figure 6A). For estimation of the change in the channel open probability (P_o) induced by bupivacaine, the all point-amplitude histograms based on all 70 episodes for each solution were analysed (Figure 6B). The P_o values calculated from the peak amplitudes of histogram fitting according to a description given in method section were 0.075 for control solution and 0.14 for 1 μM bupivacaine.

To measure the single-channel currents, 10 episodes with clear and frequent openings of the channels were selected (from total 70 episodes) for each solution. Such a histogram had a relatively large peak corresponding to channel opening (Figure 6C) and allowed precise estimation of the current amplitude. The fitting of this histogram has shown that the single-channel current was not changed: 1.08 ± 0.01 pA in control solution *versus* 1.11 ± 0.01 pA in 1 μM bupivacaine.

In all five patches subjected to detailed analysis with all point-amplitude histograms the P_o values changed from 0.187 ± 0.047 to 0.242 ± 0.041 (by $42.3 \pm 15.8\%$, $n=5$), whereas the single-channel currents did not change significantly (0.99 ± 0.04 *versus* 1.00 ± 0.05, $n=5$).

Thus, an increase in P_o rather than in single-channel current was responsible for enhancement of delayed-rectifier K^+ current by low concentrations of local anaesthetics.

The mechanisms of partial K^+ current block by high concentrations of the drug were also studied at the single-channel level (Figure 7). As seen 100 μM bupivacaine produced a flickery block of the currents reducing their apparent amplitude within the bursts (three inside-out patches). This pattern was typical for a fast block of ion channels (Hille, 2001).

Discussion

The present results have shown that low concentrations of the local anaesthetics bupivacaine, lidocaine, and mepivacaine enhance the delayed-rectifier K^+ currents in spinal sensory neurones. Detailed analysis of this phenomenon performed on isolated *somata* has revealed that the enhancement occurs at drug concentrations of 0.1–10 μM, which are much below those sufficient for suppression of Na^+ conductance (Olschewski *et al*., 1998). Besides, starting from 30 μM the drug partially blocked delayed-rectifier currents. Similar enhancement of currents through cloned and native cardiac delayed-rectifier K^+ channels was also observed with anti-arrhytmic agents almokalant (Carmeliet, 1993) and quinidine (Tseng *et al*., 1996), class III antiarrthymic drug

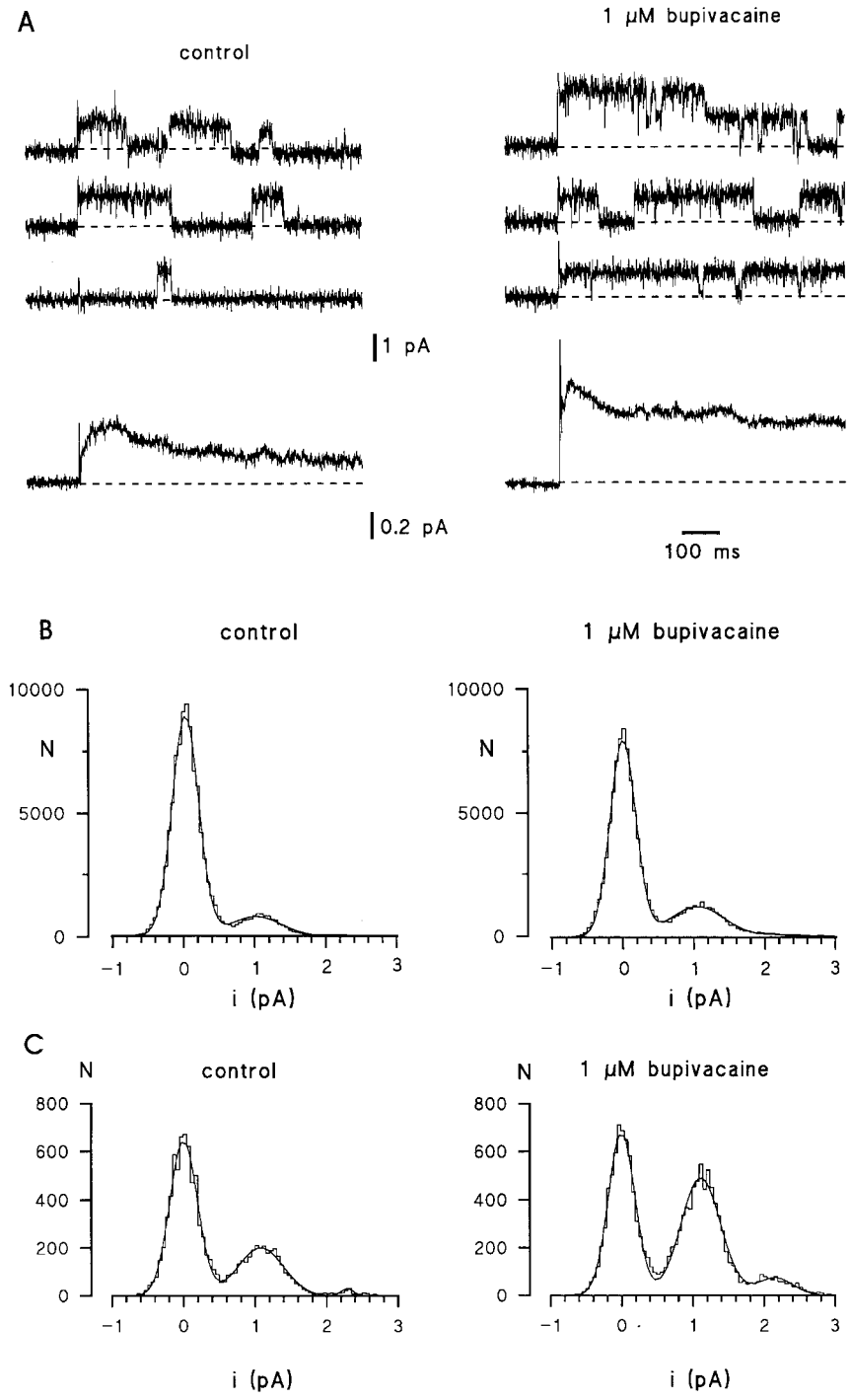

Figure 6 Bupivacaine increases the P_o but does not change the amplitude of the single-channel current. (A) Recordings from an inside-out patch containing only delayed-rectifier K^+ channels (at least two active) before and after addition of 1 μM bupivacaine to bath solution. Holding potential, −80 mV. The channels were activated by a depolarization to +40 mV following a 250 ms prepulse to −60 mV. The lowermost traces are averages of total 70 episodes each. Note that the averaged traces are given at higher resolution. The averaged currents were smaller than the single-channel currents because some episodes either were empty or had only a few channel openings. (B) All point-amplitude histograms, each based on all 70 episodes. The histograms are fitted using three Gauss functions with the amplitudes $A_0=4171$ ($\sigma=0.187$ pA), $A_1=698$ ($\sigma=0.356$ pA; $i_1=1.01$ pA) and $A_2=19$ ($\sigma=0.167$ pA; $i_2=2.20$ pA) for control solution and $A_0=3620$ ($\sigma=0.182$ pA), $A_1=1172$ ($\sigma=0.383$ pA; $i_1=1.05$ pA) and $A_2=89$ ($\sigma=0.361$ pA; $i_2=2.31$ pA) for 1 μM bupivacaine. Using the equation given in the methods section, the P_o was calculated to be 0.075 for control solution and 0.14 for 1 μM bupivacaine. (C) All point-amplitude histogram for delayed-rectifier channels in the presence and absence of 1 μM bupivacaine. For these histograms, only the episodes with very clear openings were selected (10 of total 70 for each solution). In addition, some parts of the recording at the level of the base line were digitally 'cut out', to increase the relative amplitude of the peak corresponding to the channel opening. The data were fitted using the sum of three Gauss functions as described in the method section. The single-channel currents determined from the fitting were $i_1=1.08\pm0.01$ pA in control solution and $i_1=1.11\pm0.01$ pA in 1 μM bupivacaine.

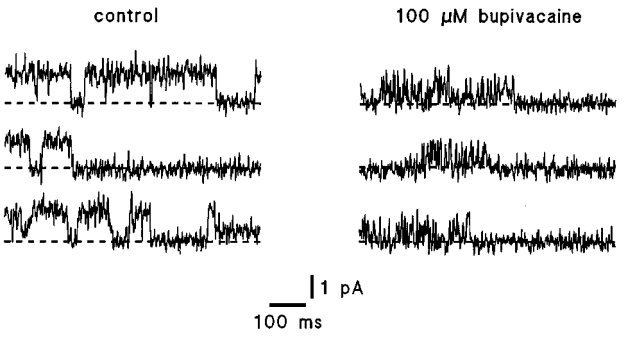

Figure 7 Block of K^+ channels by high concentrations of bupivacaine. Single K^+ channel currents in control solution and in the presence of 100 μM bupivacaine. The experimental protocol was the same as in Figure 6A. Current traces were digitally filtered at 200 Hz.

NE-10064 (Davies *et al*., 1996) as well as local anaesthetics benzocaine (Delpón *et al*., 1999) and bupivacaine (at pH 6.5, Longobardo *et al*., 2001). The present data provide evidence that a dual effect also occurs when standard local anaesthetics are applied to native delayed-rectifier K^+ channels expressed in neuronal membrane.

The molecular mechanism of this dual effect is not yet clear. The simplest explanation would be an existence of two independent processes, enhancement and blockade, each associated with a specific binding site on the channel molecule (Delpón *et al*., 1999; Longobardo *et al*., 2001). According to our measurements, these binding sites have different affinities to local anaesthetics, the site for an enhancement being by two orders of magnitude more sensitive.

The enhancement of the current resulted from a parallel shift of the steady-state activation curve (Carmeliet, 1993; Tseng *et al*., 1996; Delpón *et al*., 1999) and an additional increase in the maximum conductance. Potentiation of the current was especially pronounced at threshold potentials of -50 to -40 mV, where the current magnitude became several times larger than in control solution. At saturating potentials of $+40$ to $+60$ mV enhancement of the maximum conductance was considerably smaller (18% at 1 μM). As the single-channel recording at saturating potentials showed, an enhancement resulted not from the change of the unitary conductance but from an increase in the open probability or, in other words, from modification of the channel gating.

It should be noted that the shift of the activation characteristic is unlikely to be due to a screening effect of local anaesthetics on the membrane charges (Hille, 2001), since it was not observed for Na^+ currents. Thus, it can be assumed that the phenomenon of enhancement resulted from a direct interaction of the local anaesthetic with the activation gate of the channel.

A partial block of the K^+ current occurring at bupivacaine concentrations above 100 μM was accompanied neither by an additional shift of the activation curve nor by the acceleration of the activation kinetics. As the single-channel recordings have shown, this suppression resulted from the fast type block of the channels leading to an apparent reduction of the single-channel current. All these observations indicate that this partial block was not associated with the interaction between the local anaesthetic and the channel gate.

The present data allow us to assume that the local anaesthetics act on two independent sites of delayed-rectifier K^+ channels in spinal dorsal horn neurones of rat. The interaction with the activation gate and resulting modification of the channel gating seem to be specific features of the delayed-rectifier K^+ channels, since no shift of the steady-state activation curve has been seen for the voltage-gated Na^+ current. The blocking effect of local anaesthetics at high concentrations is a well-known phenomenon reported for other types of ionic channels (Butterworth & Strichartz, 1990; Hille, 2001).

A number of reports have shown that local anaesthetics and some other clinically used drugs are able to block different types of K^+ currents (Carmeliet *et al*., 1976; Castle, 1990; Olschewski *et al*., 1996; 1998; 1999; 2001; Bräu *et al*., 1995; 1997; 1998; 2000; Nilsson *et al*., 1998; Kindler *et al*., 1999; Nau *et al*., 1999). Until now, the meaning of this K^+ channel block is much less understood than that for the voltage-gated Na^+ channels. As the Na^+ channels are mostly involved in the spike generation and propagation in peripheral axons and central neurones, their suppression by local anaesthetics directly results in a conduction block. Unfortunately, the role of K^+ conductance in cell excitability is less straightforward than that of Na^+ channels. The functions of K^+ channels are much more diverse and include setting and/or modulation of the resting potential, depolarization during the action potential and regulation of the interspike intervals during repetitive firing (Connor & Stevens, 1971; Llinas, 1988; Koh *et al*., 1992; Safronov *et al*., 1996; Hille, 2001). In other words, K^+ conductances are involved in the modulation of complex firing patterns in peripheral axons and spinal neurones and therefore their block or potentiation by local anaesthetics can influence the sensory, i.e. nociceptive, inputs or their processing in the spinal cord. A considerable increase in K^+ conductance at threshold potentials may also affect the initiation of action potential. Suppression of inactivating A-type current reported previously (Olschewski *et al*., 1998) and the enhancement of delayed-rectifier K^+ currents by low concentrations of local anaesthetics may change the firing rates of spinal sensory neurones during onset of or recovery from anaesthesia.

Clinically bupivacaine is used as the racemic mixture of R(+) and S(−) enantiomers. Recently, the S(−) enantiomer known as levobupivacaine is introduced into clinical practice because of its smaller adverse effects compared to the R(+) form. However, the local anaesthetic activity is similar for both enantiomers. Our study demonstrates that bupivacaine shows stereoselectivity neither for the high affinity activation site nor for the low affinity blocking site. The lack of stereoselectivity in the action of low bupivacaine concentration on delayed rectifier K^+ channels thus is in agreement with clinical observations.

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References

ARACAVA, Y., IKEDA, S.R., DALY, J.W., BROOKES, N. & ALBUQUERQUE, E.X. (1984). Interactions of bupivacaine with ionic channels of the nicotinic receptor. Analysis of single-channel currents. *Mol. Pharmacol.,* **26,** 304–313.

BARANN, M., GOTHERT, M., FINK, K. & BONISCH, H. (1993). Inhibition by anaesthetics of 14C-guanidinium flux through the voltage-gated sodium channel and the cation channel of the 5-HT3 receptor of N1E-115 neuroblastoma cells. *Naunyn. Schmiedeberg's Arch. Pharmacol.,* **347,** 125–132.

BRÄU, M.E., KOCH, E.D., VOGEL, W. & HEMPELMANN, G. (2000). Tonic blocking action of meperidine on Na^+ and K^+ channels in amphibian peripheral nerves. *Anesthesiology,* **92,** 147–155.

BRÄU, M.E., NAU, C., HEMPELMANN, G. & VOGEL, W. (1995). Local anesthetics potently block a potential insensitive potassium channel in myelinated nerve. *J. Gen. Physiol.,* **105,** 485–505.

BRÄU, M.E., SANDER, F., VOGEL, W. & HEMPELMANN, G. (1997). Blocking mechanisms of ketamine and its enantiomers in enzymatically demyelinated peripheral nerve as revealed by single-channel experiments. *Anesthesiology,* **86,** 394–404.

BRÄU, M.E., VOGEL, W. & HEMPELMANN, G. (1998). Fundamental properties of local anesthetics: Half-maximal blocking concentrations for tonic block of Na^+ and K^+ channels in peripheral nerve. *Anesth. Analg.,* **87,** 885–889.

BROMAGE, P.R., JOYAL, A.C. & BINNEY, J.C. (1963). Local anesthetic drugs: Penetration from the spinal extradural space into the neuroaxis. *Science,* **140,** 392–394.

BUTTERWORTH, IV J.F. & STRICHARTZ, G.R. (1990). Molecular mechanisms of local anesthesia: A review. *Anesthesiology,* **72,** 711–734.

CARMELIET, E. (1993). Use-dependent block and use-dependent unblock of the delayed-rectifier K^+ current by almokalant in rabbit ventricular myocytes. *Circ. Res.,* **492,** 857–868.

CARMELIET, E., XHONNEUX, R., VAN GLABBEEK, A. & RENEMAN, R. (1976). Electrophysiological effects of droperidol in different cardiac tissues. *Naunyn. Schmiedeberg's Arch. Pharmacol.,* **293,** 57–66.

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.

CONNOR, J.A. & STEVENS, C.F. (1971). Inward and delayed outward membrane currents in isolated neural somata under voltage clamp. *J. Physiol.,* **213,** 1–19.

DAVIES, M.P., FREEMAN, L.C. & KASS, R.S. (1996). Dual action of the novel class III antiarrhytmic drug NE-10064 on delayed potassium channel currents in guinea pig ventricular and sinoatrial node cells. *J. Pharmacol. Exp. Ther.,* **490,** 1149–1154.

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

EDWARDS, F.A., KONNERTH, A., SAKMANN, B. & TAKAHASHI, T. (1989). A thin slice preparation for patch clamp recordings from neurones of the mammalian central nervous system. *Pflügers. Arch.,* **414,** 600–612.

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. (2001). In *Ionic Channels of Excitable Membrane*. 3rd edn. ed. Hille, B., Sunderland, Massachusetts: Sinauer Associates Inc. Publishers.

HIROTA, K., BROWNE, T., APPADU, B.L. & LAMBERT, D.G. (1997). Do local anaesthetics interact with dihydropyridine binding sites on neuronal L-type Ca^{2+} channels? *Br. J. Anaesth.,* **78,** 185–188.

IKEDA, S.R., ARONSTAM, R.S., DALY, J.W., ARACAVA, Y. & ALBUQUERQUE, E.X. (1984). Interactions of bupivacaine with ionic channels of the nicotinic receptor. Electrophysical and biochemical studies. *Mol. Pharmacol.,* **26,** 293–303.

KINDLER, C.H., YOST, C.S. & GRAY, A.T. (1999). Local anesthetic inhibition of baseline potassium channels with two pore domains in tandem. *Anesthesiology,* **90,** 1092–1102.

KOH, D.S., JONAS, P., BRÄU, M.E. & VOGEL, W. (1992). A TEA-insensitive flickering potassium channel active around resting potential in myelinated nerve. *J. Membr. Biol.,* **130,** 149–162.

LIGHT, A.R. & PERL, E.R. (1979). Reexamination of the dorsal root projection to the spinal dorsal horn including observations on the differential termination of coarse and fine fibers. *J. Comp. Neurol.,* **186,** 117–132.

LIGHT, A.R., TREVINO, D.L. & PERL, E.R. (1979). Morphological features of functionally defined neurones in the marginal zone and substantia gelatinosa of the spinal dorsal horn. *J. Comp. Neurol.,* **186,** 151–172.

LLINAS, R.R. (1988). The intrinsic electrophysiological properties of mammalian neurons: Insights into central nervous system function. *Science,* **242,** 1654–1664.

LONGOBARDO, M., GONZALEZ, T., CABALLERO, R., DELPON, E., TAMARGO, J. & VALENZUELA, C. (2001). Bupivacaine effects on hKv1.5 channels are dependent on extracellular pH.. *Br. J. Pharmacol.,* **491,** 359–369.

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

NILSSON, J., ELINDER, F. & ARHEM, P. (1998). Mechanisms of bupivacaine action on Na^+ and K^+ channels in myelinated axons of Xenopus laevis. *Eur. J. Pharmacol.,* **360,** 21–29.

OLSCHEWSKI, A., BRÄU, M.E., HEMPELMANN, G., VOGEL, W. & SAFRONOV, B.V. (2000). Differential block of fast and slow inactivating tetrodotoxin-sensitive sodium channels by droperidol in spinal dorsal horn neurons. *Anesthesiology,* **92,** 1667–1676.

OLSCHEWSKI, A., BRÄU, M.E., OLSCHEWSKI, H., HEMPELMANN, G. & VOGEL, W. (1996). ATP-dependent potassium channel in rat cardiomyocytes is blocked by lidocaine. *Circulation,* **93,** 656–659.

OLSCHEWSKI, A., HEMPELMANN, H., VOGEL, W. & SAFRONOV, B.V. (1998). Blockade of Na^+ and K^+ currents by local anesthetics in the dorsal horn neurons of the spinal cord. *Anesthesiology,* **88,** 172–179.

OLSCHEWSKI, A., HEMPELMANN, H., VOGEL, W. & SAFRONOV, B.V. (2001). Suppression of potassium conductance by droperidol has influence on excitability of spinal sensory neurons. *Anesthesiology,* **94,** 280–289.

OLSCHEWSKI, A., OLSCHEWSKI, H., BRÄU, M.E., HEMPELMANN, G., VOGEL, W. & SAFRONOV, B.V. (1999). Effect of bupivacaine on ATP-dependent potassium channels in rat cardiomyocytes. *Br. J. Anaesth.,* **82,** 435–438.

ROSSNER, K.L. & FREESE, K.J. (1997). Bupivacaine inhibition of L-type calcium current in ventricular cardiomyocytes of hamster. *Anesthesiology,* **87,** 926–934.

SAFRONOV, B.V. (1999). Spatial distribution of Na^+ and K^+ channels in spinal dorsal horn neurones: Role of the soma, axon and dendrites in spike generation. *Prog. Neurobiol.,* **59,** 217–241.

SAFRONOV, B.V., BISCHOFF, U. & VOGEL, W. (1996). Single voltage-gated K^+ channels and their functions in small dorsal root ganglion neurones of rat. *J. Physiol.,* **432,** 393–408.

SAFRONOV, B.V., WOLFF, M. & VOGEL, W. (1997). Functional distribution of three types of Na^+ channel on soma and processes of dorsal horn neurones of spinal cord. *J. Physiol.,* **503,** 371–385.

TAKAHASHI, T. (1990). Membrane currents in visually identified motoneurones of neonatal rat spinal cord. *J. Physiol.,* **423,** 27–46.

TSENG, G.N., ZHU, B., LING, S. & YAO, J.A. (1996). Quinidine enhances and suppresses Kv1.2 from outside and inside the cells, respectively. *J. Pharmacol. Exp. Ther.,* **489,** 844–855.

VALENZUELA, C., DELPON, E., TAMKUN, M.M., TAMARGO, J. & SNYDERS, D.J. (1995). Stereoseletive block of a human cardiac potassium channel (Kv1.5) by bupivacaine enantiomers. *Biophys. J.,* **69,** 18–427.

WOLFF, M., VOGEL, W. & SAFRONOV, B.V. (1998). Uneven distribution of K^+ channels in soma, axon and dendrites of rat spinal neurones: Functional role of the soma in generation of action potentials. *J. Physiol.,* **509,** 767–776.

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