Stereoselectivity of Bupivacaine in Local Anesthetic–sensitive Ion Channels of Peripheral Nerve

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Background: The local anesthetic bupivacaine exists in two stereoisomeric forms, R(+)- and S(−)-bupivacaine. Because of its lower cardiac and central nervous system toxicity, attempts were made recently to introduce S(−)-bupivacaine into clinical anesthesia. We investigated stereoselective actions of R(+)- and S(−)-bupivacaine toward two local anesthetic-sensitive ion channels in peripheral nerve, the Na^+ and the flicker K^+ channel.

Methods: In patch-clamp experiments on enzymatically demyelinated peripheral amphibian nerve fibers, Na^+ and flicker K^+ channels were investigated in outside-out patches. Half-maximum inhibiting concentrations (IC_{50}) were determined. For the flicker K^+ channel, simultaneous block by R(+)-bupivacaine and S(−)-bupivacaine was analyzed for competition and association (k_1) and dissociation rate constants (k_{-1}) were determined.

Results: Both channels were reversibly blocked by R(+)- and S(−)-bupivacaine. The IC_{50} values (±SEM) for tonic Na^+ channel block were 29 ± 3 μM and 44 ± 3 μM, respectively. IC_{50} values for flicker K^+ channel block were 0.15 ± 0.02 μM and 11 ± 1 μM, respectively, resulting in a high stereopotency ratio (±) of 73. Simultaneously applied enantiomers competed for a single binding site. Rate constants k_1 and k_{-1} were $0.83 \pm 0.13 \times 10^6$ $M^{-1} \cdot s^{-1}$ and 0.13 ± 0.03 s^{-1}, respectively, for R(+)-bupivacaine and $1.90 \pm 0.20 \times 10^6$ $M^{-1} \cdot s^{-1}$ and 8.3 ± 1.0 s^{-1}, respectively, for S(−)-bupivacaine.

Conclusions: Bupivacaine block of Na^+ channels shows no salient stereoselectivity. Block of flicker K^+ channels has the highest stereoselectivity ratio of bupivacaine action known so far. This stereoselectivity derives predominantly from a difference in k_{-1}, suggesting a tight fit between R(+)-bupivacaine and the binding site. The flicker K^+ channel may play an important role in yet unknown toxic mechanisms of R(+)-bupivacaine. (Key words: Enantiomers; patch-clamp method; piperidine derivatives; rate constants.)

STEREOSELECTIVITY is a recognized criterion in support of the existence of specific drug–receptor interactions. In addition to this scientific aspect of stereoselectivity, there is increasing awareness of its clinical importance because, although physicochemically identical in an achiral environment, stereoisomers can exhibit quantitative and qualitative differences in their pharmacologic actions. Thus, stereoisomers of a drug may show differences in potencies for the main effect or for unwanted side effects or for both. For that reason, stereoisomers similar to S(+)-ketamine or S(−)-ropivacaine have already been introduced into clinical practice.[1]

Because of a chiral carbon in the piperidine ring, the local anesthetic bupivacaine exists in two stereoisomeric forms: S(−)-bupivacaine and R(+)-bupivacaine. The clinically used form of bupivacaine is the racemic mixture. However, it is known that the enantiomers show differences in both their local anesthetic actions and their cardiac and cerebral effects. *In vivo* studies have shown that duration of local anesthesia is similar for the enantiomers,[2,3] or even longer for S(−)-bupivacaine than for R(+)-bupivacaine.[2,4,5]

In a previous work, no difference in nerve blocking potencies *in vitro* between R(+)- and S(−)-bupivacaine was observed.[2] More recently, R(+)-bupivacaine was shown to be 1.6-fold more potent in inhibition of compound action potentials in frog peripheral nerve.[6]

Cardiac and cerebral actions determine the most important side effects of bupivacaine. *In vivo* animal studies have shown a 1.4- to 1.8-fold higher systemic toxicity for R(+)-bupivacaine, measured as LD_{50} in mice and rats and as LD_{mean} in rabbits.[2] The major cause of death is cardiotoxicity caused by direct effects on the myocardium and indirect centrally mediated effects.[7,8] In iso-

lated hearts, QRS widening and the occurrence of severe arrhythmias were more pronounced[9] and atrioventricular conduction delay was more prolonged by R(+)-bupivacaine.[10]

Regarding the ion channel level, apparent affinity of bupivacaine to the inactivated state of cardiac sodium (Na$^+$) channels displayed a moderate stereoselectivity, with R(+)-bupivacaine being 1.7 times more potent than S(−)-bupivacaine. Interactions of bupivacaine enantiomers with the resting and the activated states were not significantly stereoselective.[11]

Blockade of Na$^+$ channels is of paramount importance for peripheral nerve block, although a variety of systems is discussed to be involved in mechanisms of local anesthesia.[12] Recent patch-clamp investigations of peripheral amphibian nerve axons revealed one type of Na$^+$ channel but a diversity of potassium (K$^+$) channels.[13] Among these channels, a voltage-insensitive flickering K$^+$ channel[14] has been found to be more sensitive than the Na$^+$ channel to lipophilic, amide-linked local anesthetics, and especially to the piperidine derivatives bupivacaine and ropivacaine.[15] It was shown that local anesthetics did not impair the amplitude of the single-channel current, but prolonged the time of the channel in the closed state. This block was voltage-independent and was described under the assumption that one local anesthetic molecule interacts with one specific binding site at the channel. The channel was insensitive to the ester-linked local anesthetics procaine and tetracaine. The flicker K$^+$ channel was mostly found in thin, myelinated nerve fibers and is a possible candidate for generating the resting potential of these fibers.[14] The high sensitivity of this channel toward racemic bupivacaine suggested a high stereoselectivity ratio for bupivacaine enantiomers.[16]

The purpose of this study was to determine and compare the actions of R(+)-bupivacaine and S(−)-bupivacaine on Na$^+$ and flicker K$^+$ channels, two local anesthetic–sensitive ion channels in membranes of peripheral nerve fibers.

Materials and Methods

Preparation and Electrophysiologic Technique

Sciatic nerves of freshly killed *Xenopus laevis* were dissected. The decapitation procedures were reported to the local veterinarian authority and are in accordance with German guidelines. Nerve fibers were enzymatically prepared as described by Jonas *et al.*,[17] leading to dissociation and retraction of their myelin sheath. Na$^+$

and flicker K$^+$ currents were recorded using the outside-out configuration of the patch-clamp method.[18]

Micropipettes were pulled from borosilicate glass tubes (GC150; Clark Electromedical Instruments, Pangbourne, UK), coated with sylgard 184 (Dow Corning, Seneffe, Belgium), and fire-polished to give a resistance of 5–10 MΩ for Na$^+$ channel and 15–30 MΩ for flicker K$^+$ channel recording.

Solutions

External solution contained 110 mM NaCl, 2.5 mM KCl, 2 mM CaCl$_2$, 5 mM N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic-acid (BES) and 10 mM tetraethylammonium chloride (TEA) (Ri-TEA) for Na$^+$ channel recording and 105 mM KCl, 13 mM NaCl, 2 mM CaCl$_2$, 5 mM BES, and 0.1 μM tetrodotoxin (high-K$_o$) for flicker K$^+$ channel recording; both solutions were adjusted to pH 7.4 with tris[hydroxymethyl]-aminomethane (TRIS).

Pipette solution for Na$^+$ channel recording (CsCl$_i$) contained 110 mM CsCl, 13 mM NaCl, 3 mM ethylene glycol-bis[β-aminoethyl ether] N,N,N′,N′-tetraacetic acid (EGTA), 5 mM BES, adjusted to pH 7.2 with TRIS. For flicker K$^+$ channel recording CsCl was replaced by 105 mM KCl (KCl$_i$). All chemicals were obtained from Sigma, Deisenhofen, Germany. Temperature was kept constant at 14 ± 1°C.

Control and test solutions were rapidly applied to outside-out patches with a multiple-barrel perfusion system. Bupivacaine enantiomers were gifts from ASTRA Pain Control, Södertälje, Sweden, and were dissolved in distilled water to give 10-mM stocks.

Data Acquisition

Currents were recorded using an EPC 7 patch-clamp amplifier (List, Darmstadt, Germany), filtered with a four-pole low-pass Bessel filter at 1 kHz, digitized with a Labmaster TM-40 AD/DA board (Scientific Solutions, Solon, OH), and stored on a personal computer. PClamp 5.5.1 software (Axon Instruments, Burlingame, CA) was used for data acquisition and evaluation.

For Na$^+$ channel recordings, outside-out patches were formed from nodal membrane areas. The membrane potential was held at −90 mV in the voltage-clamp mode of the amplifier. Inward Na$^+$ currents were evoked at 10-s intervals by applying a 50-ms prepulse to −130 mV to remove fast inactivation, followed by a 50-ms test pulse to −40 mV. Noise of recordings was reduced by averaging 20 successive traces before Na$^+$ current measurement. Fractional inhibition (f$_I$) of averaged peak Na$^+$ current was calculated measuring the peak current am-

plitude in different drug concentrations (c) with respect to control to give concentration–inhibition curves.

Flicker K^+ channels were continuously recorded in outside-out patches formed from thin fibers (about 5 μm). Membrane potential was held at -90 mV and no test pulse was applied. Fractional inhibition was calculated measuring the mean current through multiple-channel patches from a 30-s recording under different drug concentrations with respect to control.

Statistical Analysis

Concentration–inhibition curves were constructed by plotting the fractional current inhibition against blocker concentration. Half-maximal blocking concentrations (IC_{50}) were derived from nonlinear least-squares fits of the Hill function,

$$f_I = c^n / (c^n + IC_{50}^n), \qquad (1)$$

to the data points of the curves. The Hill coefficient (n) was set to 1, assuming a one-to-one reaction of the local anesthetic molecule with the channel, unless mentioned otherwise.

Data for the concentration-dependent block of the flicker K^+ channel by R(+)-bupivacaine were additionally fitted with the sum of two Hill functions,

$$f_I = F_1 \cdot c / (c + IC_{50,1}) + F_2 \cdot c / (c + IC_{50,2}), \qquad (2)$$

where F_1 and F_2 ($F_1 + F_2 = 1$) are the fractions of current with the half-maximal blocking concentrations $IC_{50,1}$ and $IC_{50,2}$, respectively.

Competition experiments were performed to rule out the existence of two different binding sites for R(+)-bupivacaine and S(−)-bupivacaine at the flicker K^+ channel. When two drugs (A and B) compete for the same receptor, with both drugs reacting in a one-to-one fashion, the presence of drug B reduces the receptor occupation by drug A so that its apparent dissociation constant is enlarged. In the presence of increasing concentrations of the competitive agonist B, the concentration-inhibition curve for A is shifted along the concentration axis to higher concentrations, without a change in shape and height of the curve. The apparent IC_{50} of drug A can be calculated as follows:

$$IC_{50,A,app} = IC_{50,A}(1 + [B]/IC_{50,B}). \qquad (3)$$

Assuming two different binding sites to which drugs A and B bind independently in a one-to-one reaction and assuming the channel is blocked if either one, the other, or both sites are occupied, a simplified model predicts

no change in the dissociation constant of drug A in the presence of increasing concentrations of drug B.

Kinetic data for S(−)-bupivacaine were derived from closed- and open-time histograms. To generate closed- and open-time histograms, outside-out patches with only one active flicker K^+ channel were analyzed. Currents were filtered at 200 Hz and recorded with a sample rate of 1 ms to eliminate the flickering in the open state of the channel. Then, a semiautomatic procedure was used to detect the idealized channel events. Closings less than 30 ms and openings less than 15 ms were ignored. Open- and closed-time histograms were constructed with a bin width of 5 ms and fitted with single exponentials using unweighted, nonlinear least-squares fitting to give open-(τ_o) and closed-time constants (τ_c). From the time constants, the rate constants can be easily calculated with

$$k_{-1} = \tau_c^{-1} \qquad (4)$$

and

$$k_1 = (\tau_o c)^{-1}. \qquad (5)$$

Kinetic data for R(+)-bupivacaine were estimated from current recordings of multiple-channel patches during wash-in and wash-out of the local anesthetics. These recordings were filtered at 60 Hz and fitted with a single exponential using unweighted, nonlinear least-squares fitting to give wash-in (τ_{on}) and wash-out time constants (τ_{off}). The rate constants for dissociation k_{-1} can be derived directly from wash-out time constants with

$$k_{-1} = \tau_{off}^{-1}. \qquad (6)$$

The association rate constant is calculated as a function of τ_{on} and τ_{off} as

$$k_1 = (\tau_{on}^{-1} - \tau_{off}^{-1})c^{-1}. \qquad (7)$$

Data points are given as mean $\pm$ SEM, IC_{50} values are given as fitted values $\pm$ standard error of the fit. Fig.P 5.0 software (Biosoft, Cambridge, UK) was used for creating figures and performing fitting procedures.

Results

Na$^+$ channels

Average Na^+ currents in outside-out patches, evoked as described, had a peak amplitude of 20–150 pA. Assuming a single-channel current of 1 pA per channel at -40 mV,[17] this corresponds to 20–150 channels simultaneously open at peak. Because of this small number of

channels present in the outside-out patches, amplitude fluctuations originating from random channel openings in subsequent traces were considerable and had to be smoothed by averaging procedures before peak current measurement. These procedures, however, impeded investigation of phasic block, which is measured as the increase of Na^+ current inhibition during successive membrane depolarizations at high frequency. Therefore, only tonic block measured at low depolarization frequencies was investigated.

The enantiomers $R(+)$-bupivacaine and $S(-)$-bupivacaine reversibly blocked the peak amplitude of axonal Na^+ currents (fig. 1) in a concentration-dependent manner. Concentration–inhibition experiments gave IC_{50} values for tonic block of 29 ± 3 μM ($n = 10$) and 44 ± 3 μM ($n = 14$) for $R(+)$-bupivacaine and $S(-)$-bupivacaine, respectively, revealing a weak stereoselectivity with a stereopotency ratio $(+/-)$ of 1.5.

Flicker K^+ channels

Flicker K^+ channels were easily identified by their flickery appearance in high-K_o solution at a holding potential of -90 mV. At that potential, this voltage-insensitive channel is active, and disturbances from voltage-activated channels are not to be expected. It was shown previously that high-K_o, in comparison to physiologic Ringer's solution, had no influence on the potency of externally applied bupivacaine.[15]
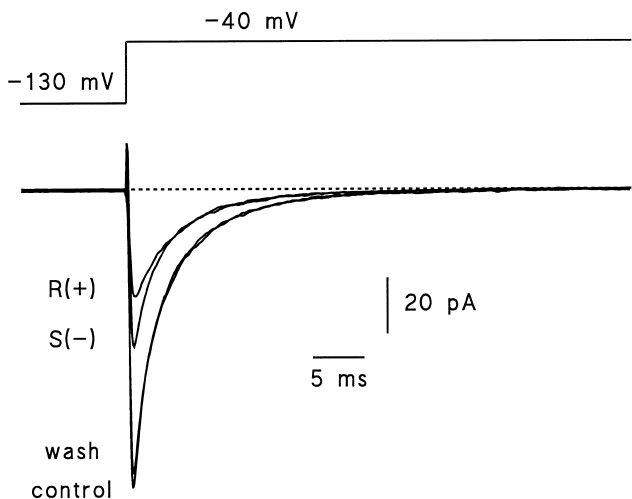

Fig. 1. Axonal Na^+ currents in one outside-out patch in the absence of (control) and after addition of 30 μM $S(-)$-bupivacaine and 30 μM $R(+)$-bupivacaine. Traces are plotted as averages of 20 successive recordings. The impulse protocol to elicit Na^+ currents is given above the traces. Holding potential: -90 mV; bath: tetraethylammonium chloride; pipette: $CsCl_i$.
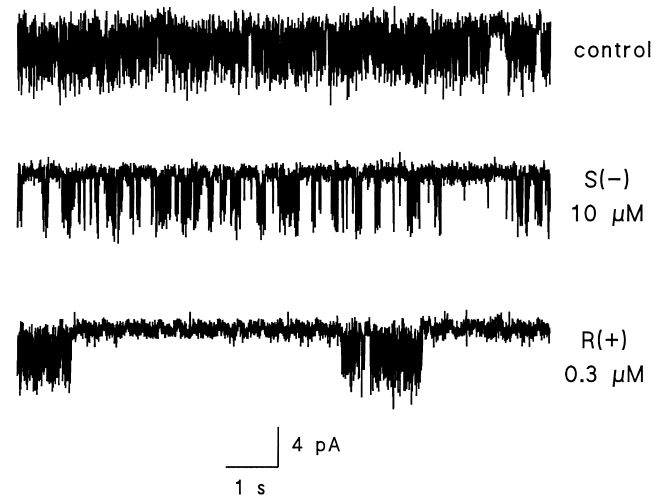

Fig. 2. Recordings of a single flicker K^+ channel in different single-channel outside-out patches at a holding potential of -90 mV in the absence of bupivacaine (control) and after addition of 10 μM $S(-)$-bupivacaine and 0.3 μM $R(+)$-bupivacaine. Bath: high-K_o, pipette: KCl_i.

Externally applied bupivacaine enantiomers reversibly reduced the open probability of the channel in a concentration-dependent manner. $R(+)$-bupivacaine induced long closings in the range of seconds, and $S(-)$-bupivacaine induced short closings in the range of milliseconds (fig. 2).

Concentration–inhibition experiments gave IC_{50} values of 0.15 ± 0.02 μM ($n = 8$) and 11 ± 1 μM ($n = 7$) for $R(+)$-bupivacaine and $S(-)$-bupivacaine, respectively, revealing a stereopotency ratio $(+/-)$ of 73 (fig. 3).

The data points from concentration–inhibition experiments were all fitted with a constrained Hill coefficient of 1 under the assumption of a single binding site (equation 1). Fitting of the data with a variable Hill coefficient revealed values close to unity for block of the Na^+ channel by $R(+)$-bupivacaine and $S(-)$-bupivacaine (0.97 ± 0.13 and 0.90 ± 0.10, respectively) and for block of the flicker K^+ channel by $S(-)$-bupivacaine (0.97 ± 0.05, fits not shown). Best fits for the concentration-dependent block of the flicker K^+ channel by $R(+)$-bupivacaine, however, have been obtained under the assumption of two independent binding processes (equation 2), revealing a fraction F_1 of 0.73 exhibiting high affinity binding ($IC_{50,1} = 0.06 \pm 0.01$ μM) and a fraction F_2 of 0.27 exhibiting binding with a lower affinity ($IC_{50,2} = 1.9 \pm 0.9$ μM).

To rule out the existence of two different binding sites for $(+)$-enantiomers and $(-)$-enantiomers, simultaneous block by $R(+)$-bupivacaine and $S(-)$-bupivacaine was

analyzed for competition. For reasons of simplicity, we conducted and analyzed these experiments under the assumption of one binding site for R(+)-bupivacaine, with an apparent IC_{50} of 0.15 μM. Concentration–inhibition experiments for S(−)-bupivacaine in the presence of 0.3, 0.6, and 1 μM R(+)-bupivacaine revealed apparent IC_{50} values for S(−)-bupivacaine of 33 ± 3, 66 ± 7, and 76 ± 18 μM, respectively. Predicted values obtained from equation 3, assuming a competitive interaction between R(+)-bupivacaine and S(−)-bupivacaine are 30, 50, and 77 μM, respectively (fig. 4). The experimental data clearly support competition of the enantiomers for a single binding site.

To characterize the blocking kinetics that underlie stereoselective action, we investigated the association and dissociation rate constants k_1 and k_{-1} of block for R(+)-bupivacaine and S(−)-bupivacaine. As during control conditions, the flicker K^+ channel had an open probability of more than 0.95; the kinetics of the induced block by S(−)-bupivacaine could be conveniently characterized. Closed- and open-time histograms were derived from single-channel patches (figs. 5A and B, respectively). The mean closed and open times τ_c and τ_o, respectively, were evaluated by fitting the closed- and open-time histograms with single exponentials (see Materials and Methods). From the time constants, the rate constants can be easily calculated (equations 4 and 5).
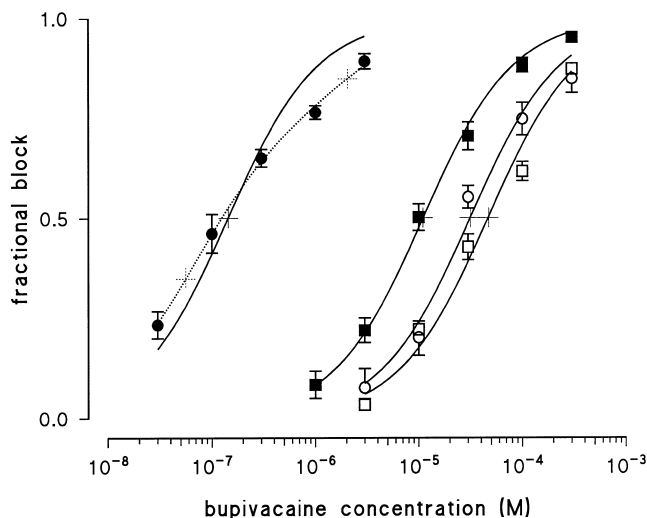

Fig. 4. Simultaneous application of bupivacaine enantiomers. Ordinate gives apparent IC_{50} values of S(−)-bupivacaine, as found in concentration–effect experiments for S(−)-bupivacaine in the presence of various R(+)-bupivacaine-concentrations (abscissa). Data points represent mean of IC_{50} values ± SEM. Numbers of experiments are given next to each data point. Dashed lines represent theoretical changes of the apparent S(−)-bupivacaine IC_{50}, assuming models of either competitive interaction (one site) or noncompetitive, noninteracting agonism (two sites) of bupivacaine enantiomers. Dotted lines give 95% confidence interval to the linear regression (not shown) of the data.

The long closed times induced by R(+)-bupivacaine would necessitate analysis of single-channel recordings, with a duration of up to 60 min, a duration that exceeds the usual lifetime of an outside-out patch in this preparation. To obtain kinetic data for R(+)-bupivacaine, the time constants τ_{on} and τ_{off} were estimated from the current recordings of a multiple-channel patch during wash-in and wash-out of the drugs (fig. 6). This method is not applicable for S(−)-bupivacaine because its offset kinetics are too fast to be resolved by the system of solution exchange used in this study. The wash-in and wash-out records were fitted beginning at the moment when solution exchange was completed. Values for τ_{on} and τ_{off} were obtained by monoexponential fits of the current traces. The rate constants for dissociation k_{-1} can be derived directly from wash-out time constants (equation 6); the association rate constant is calculated as a function of τ_{on} and τ_{off} (equation 7). As shown in figure 6, duration of solution exchange was in a time range close to the process of wash-in of R(+)-bupiva-

Fig. 3. Concentration dependence of axonal Na^+ channel (*open symbols*) and flicker K^+ channel block (*filled symbols*) by R(+)-bupivacaine (*circles*) and S(−)-bupivacaine (*squares*). Solid lines represent fits to the data points under the assumption of a single binding site (equation 1), the dotted line represents a fit under the assumption of two independent binding processes (equation 2). Crosses mark IC_{50} values.
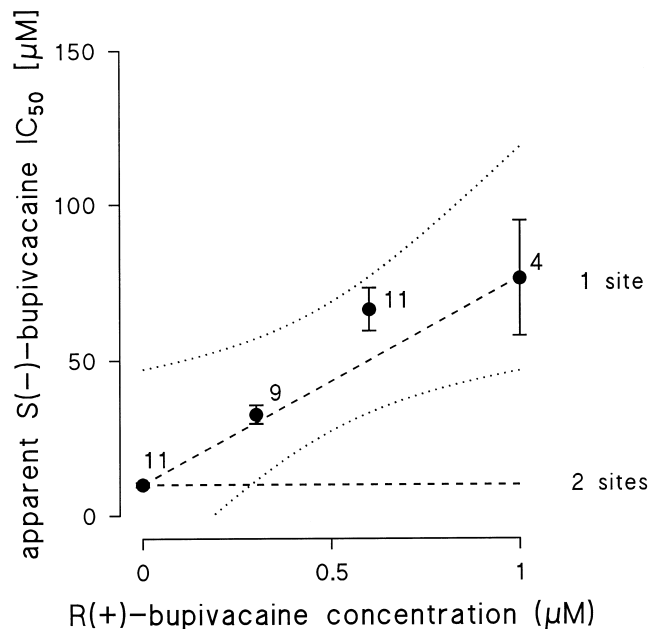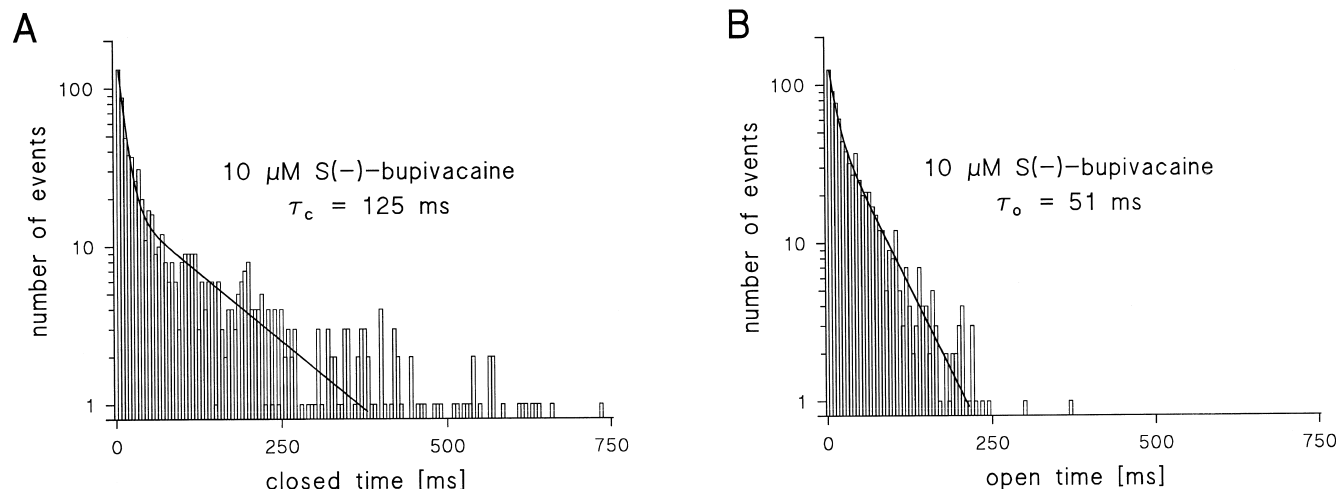
A

B

Fig. 5. Kinetics of closures of single flicker K^+ channels induced by S(−)-bupivacaine. Frequency density is plotted *versus* time. Closed- (*A*) and open-time distributions (*B*) are derived from single-channel recordings of 129 s containing 1,565 events. Currents were filtered at 200 Hz and sampled at 1 kHz. The histograms were generated with a bin width of 5 ms. Events less than 30 ms for the closed-time histogram and events less than 15 ms for the open-time histogram were ignored. Histograms were fitted with single exponential functions, revealing mean closed (τ_c) and open times (τ_o).

caine. Thus, onset kinetics of R(+)-bupivacaine might be faster than it appears from the current decay, and we might have underestimated association rate constants for R(+)-bupivacaine. The kinetic constants are given in table 1. The equilibrium constants (K_D) calculated from corresponding rate constants are comparable with the IC_{50} values obtained in concentration–inhibition experiments and therefore justify the different methods that were applied to obtain kinetic data for R(+)-bupivacaine and S(−)-bupivacaine.

As already suggested by figure 2, the closings induced by S(−)-bupivacaine are much shorter (k_{-1} = 8.3 s^{-1}) than those induced by R(+)-bupivacaine (k_{-1} = 0.13 s^{-1}). In other words, unbinding of S(−)-bupivacaine

from the binding site is much faster compared to R(+)-bupivacaine. The corresponding ratio $k_{-1,S(-)\text{-bupivacaine}}$ / $k_{-1,R(+)\text{-bupivacaine}}$ is 64, which is close to the stereopotency ratio obtained in concentration–inhibition experiments. Association rate constants for S(−)-bupivacaine and R(+)-bupivacaine differ by 2.3-fold.

Discussion

The Na$^+$ channel investigated in this work displays a weak stereoselectivity for tonic block by bupivacaine enantiomers with a stereopotency ratio (+/−) of 1.5. This is in accordance with results from peripheral nerve
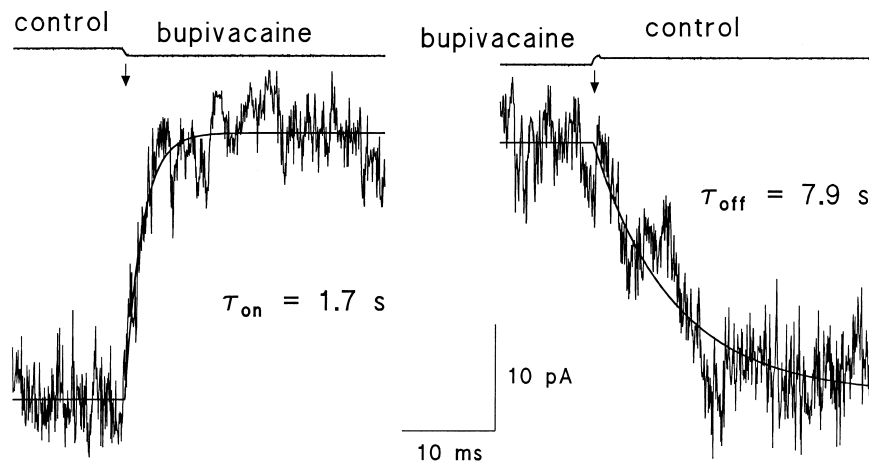
Fig. 6. Kinetics of wash-in and wash-out of 0.6 μM R(+)-bupivacaine. Inward current trace of an outside-out patch containing a large number of flicker K^+ channels, filtered with 60 Hz. The top trace indicates mechanical movement of the multiple-barrel perfusion system, giving an estimate of time course of solution exchange. Values for τ_{on} and τ_{off} were obtained by monoexponential fits of the current traces.

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Table 1. Kinetic and Steady-State Constants of Flicker K$^+$ Channel Block

Local Anesthetic	IC$_{50}$ (μM)	n	K$_1$ (M^{-1}s^{-1})	n	k$_{-1}$ (s^{-1})	n	K$_D$ (μM)
R(+)-bupivacaine	0.15 ± 0.02	8	0.83 ± 0.13 · 10^6	6	0.13 ± 0.03	9	0.16
S(−)-bupivacaine	11 ± 1	7	1.90 ± 0.20 · 10^6	4	8.3 ± 1.0	4	4.4

IC$_{50}$ values as obtained from concentration–effect experiments (fig. 3). Dissociation rate constants (k$_1$, k$_{-1}$) for S(−)-bupivacaine were derived from closed- and open-time histograms (fig. 5, equations 4 and 5). For R(+)-bupivacaine rate constants were derived from on- and offset of block (fig. 6, equations 6 and 7). K$_D$ values were calculated from rate constants as K$_D$ = k$_{-1}$ k$_1$$^{-1}$.

conduction block measured as tonic block of compound action potentials revealing a stereopotency ratio (+/−) of [6] 1.6. Na$^+$ currents in cells of a rat pituitary cell line (GH$_3$ cells) have a similar sensitivity and stereoselectivity to bupivacaine enantiomers, with a ratio of 1.6 for R(+)-bupivacaine over S(−)-bupivacaine at a holding potential of −70 mV.[19]

Two different modes account for Na$^+$ current inhibition by local anesthetics: Tonic inhibition is measured as the decrease in Na$^+$ current at low-depolarization frequencies and is thought to be accomplished by binding of local anesthetics to channels in the resting state and inactive states at rest. Use-dependent, or phasic, inhibition is measured as the further decrease in Na$^+$ current during brief membrane depolarizations repeated at high frequency. This phenomenon is thought to result from increased binding of local anesthetics to open and inactivated states of the channel. Resting channels have a low affinity for local anesthetics, whereas open or inactivated channels have a higher affinity. The changes between low- and high-affinity channels have been explained with voltage-dependent conformational changes that modulate the affinity of the local anesthetic binding site ("modulated receptor hypothesis"[20,21]) or with voltage-dependent gates that govern local anesthetic access to a binding site with constant affinity ("guarded receptor hypothesis"[22]).

In this work, interpretations concerning Na$^+$ channel block are limited insofar as only tonic block could be investigated. However, stereoselectivity of bupivacaine to decrease compound action potentials in isolated frog peripheral nerve is only slightly enhanced for phasic over tonic block with the (+)-isomer being more potent than the (−)-isomer.[6]

Interestingly, block of the inactivated state of the Na$^+$ channel in guinea pig ventricular myocytes displayed a stereoselectivity with a ratio of 1.7 for R(+)-bupivacaine over S(−)-bupivacaine that is comparable to neuronal Na$^+$ channels. Tonic block was not stereoselective.[11] It seems to be a common feature of Na$^+$ channels not to

show more than a twofold stereoselectivity for the enantiomers of bupivacaine.

The most striking result of this work is the high stereoselectivity of the flicker K$^+$ channel block, with a stereopotency ratio of 73 for R(+)-bupivacaine over S(−)-bupivacaine. The high stereoselectivity underlies the extremely high and specific affinity of bupivacaine to this channel.

The existence of two different binding sites for (+)-enantiomers and (−)-enantiomers could be clearly ruled out by competition experiments. The theoretical IC$_{50}$ value for the racemic mixture of bupivacaine, assuming equal presence of enantiomers and competition for one binding site, is calculated to be 0.3 μM and is in good agreement with previously reported experimental values for the racemic mixture (0.21 μM[15]).

Comparing the potencies of R(+)-bupivacaine and S(−)-bupivacaine is complicated because, for concentration-dependent block of the flicker K$^+$ channel by R(+)-bupivacaine, best fits have been obtained under the assumption of two independent binding processes, whereas concentration-dependent block by S(−)-bupivacaine was adequately described under the assumption of one binding site. Interestingly, a similar phenomenon was seen in a study that investigated the molecular basis for stereoselective bupivacaine block of the human cardiac K$^+$ channel hKv1.5,[23] which is preferentially blocked by R(+)-bupivacaine, with a stereoselectivity ratio of 7 over S(−)-bupivacaine.[24] Concentration-dependent block of mutations T505I, T505S, and T477S by either enantiomer revealed biphasic concentration–inhibition curves that were not apparent in wild-type channels. This observation was not dependent on stereoselective properties because these were preserved for mutations T477S and T505S, but not for T505I. The favored hypothesis to explain this phenomenon was the existence of multiple open states with different bupivacaine affinities. Mutations T505I, T505S, and T477S would enhance a drug-induced shift in gating to reveal an open state with lower affinity at higher bupivacaine concentrations.

However, based on kinetic and pharmacologic properties, the flicker K^+ channel does not seem to be related to hKv1.5. Thus, different mechanisms are likely to underlie stereoselective properties and biphasic concentration–effect curves of these two K^+ channels. Further conclusions regarding the molecular structure of the local anesthetic binding site at the flicker K^+ channel cannot be drawn.

Unlike in a study that investigates heterologously expressed channels, in our preparation, we cannot exclude the existence of two different channel populations with different affinities for R(+)-bupivacaine. Unfortunately, we were not able to obtain adequate closed- and open-time histograms for R(+)-bupivacaine to answer the question of the existence of either two channel populations or different channel conformations with different affinities for R(+)-bupivacaine. However, both binding processes apparent for R(+)-bupivacaine show higher affinities (183-fold and 5.8-fold) compared to binding of S(−)-bupivacaine.

In addition to the overall equilibrium constants, rate constants of association and dissociation give additional information about the process of drug–receptor interaction, although great care has to be taken in comparing affinities and rate constants of drugs for a receptor and deriving general features concerning the structure of the receptor and the forces that constitute the interaction.[25]

According to our data, the potencies of the enantiomers are predominantly dependent on the dwell times of the molecules. This is a phenomenon suggested in the rate theory of drug action.[26] It could account for the fact that the binding site is easily and equally accessible for both molecules, whereas the more potent molecule has a more stable interaction with the binding site and therefore stays bound longer at the site. In other words, the stereoselective difference between the bupivacaine enantiomers to block the flicker K^+ channel derives from the difference in the rate constants of dissociation, implying that there is equal access to the binding site for both enantiomeric forms.

For the open-channel block of the human cardiac K^+ channel Kv1.5, it was shown, in contrast, that the difference in potencies of R(+)- over S(−)-bupivacaine derived from a difference in the association rate constants, suggesting that the less potent molecule is less likely to access the binding site in an optimal configuration.[24] One possible explanation was that the S(−)-enantiomer needs to adopt a less favored conformation with a higher free energy profile to bind at the site compared to the R(+)-enantiomer.

Likewise, the calculated dissociation rate constants for the slightly stereoselective block of the inactivated state of the cardiac Na^+ channel were shown to be similar, whereas the association rate constants seemed to determine the stereoselective difference between the twofold more potent R(+)-bupivacaine over S(−)-bupivacaine.[11]

In our experiments, we found a 2.3-fold difference in association rate constants for S(−)-bupivacaine over R(+)-bupivacaine. In contrast to the discussed data for cardiac K^+ channels (Kv1.5) and cardiac Na^+ channels, in our investigation, the association rate constant for the less potent S(−)-bupivacaine was faster compared to the association rate constant for R(+)-bupivacaine. This again indicates that the association process does not account for the stereoselective difference in potency between R(+)-bupivacaine and S(−)-bupivacaine and may even attenuate it.

In conclusion, our kinetic data show that, not only quantitatively, but also qualitatively, block of the flicker K^+ channel by bupivacaine enantiomers represents a unique example for stereoselectivity with a tight fit between R(+)-bupivacaine and the binding site.

However, the pharmacologic relevance of the flicker K^+ channel block remains elusive. The channel has only been described in *Xenopus laevis* so far, and a putative human correlate might be less sensitive to bupivacaine. Furthermore, the role of the flicker K^+ channel in neuronal excitability is unclear. Because the channel is mainly found in thin, myelinated fibers, it was suggested that block of this channel might lead *via* membrane depolarization to an enhanced conduction block of thin fibers, giving a possible explanation for differential nerve block.[15] Recently, the effect of bupivacaine was analyzed on voltage-clamped intact *Xenopus laevis* nodes of Ranvier,[27] in which no effect of bupivacaine on the voltage-independent leak current or the resting potential was found. However, the axons that were studied ranged in diameter from 10 μm to 20 μm and thus were too large to contain flicker K^+ channels.

Given a conduction block of thin fibers by bupivacaine, differential nerve block should exhibit a high stereoselectivity, favoring R(+)-bupivacaine. However, clinical studies do not support this theory. The 0.75% S(−)-bupivacaine applied for extradural anesthesia gave a significantly longer sensory block compared to racemic bupivacaine and no difference in onset time, maximum spread of sensory block, or intensity of motor block between the groups.[5] Another investigation found a

shorter onset of pain relief after the main dose of extra-durally applied 0.5% racemic bupivacaine compared to 0.5% ropivacaine (the S(−)-enantiomer of 1-propyl-2′,6′-pipecoloxylidide, a structural homologue of bupivacaine, 1-butyl-2′,6′-pipecoloxylidide) but no significant differences in duration, onset of pain relief with the subsequent dose, quality of analgesia, or spread of sensory and motor block.[28] The majority of studies reported that ropivacaine produced a less intense motor block than racemic bupivacaine.[29,30]

Recently, a new family of K^+ channels has been described. Their molecular specialties are two-pore domains and four-transmembrane segments. Members of this family have been cloned from mammalian and human tissue and are abundant, particularly in the brain, heart, and lungs.[31–34] Similar to the flicker K^+ channel, these channels are K^+-selective background channels and most likely play a pivotal role in the control of the resting membrane potential and thus in the modulation of electrical activity of excitable cells. Among these channels, electrophysiologic and pharmacologic properties of the TWIK (tandom pore domain weak inwardly rectifying K^+ channel)-related acid-sensitive K^+ channel (rTASK)[34] best resemble those of the flicker K^+ channel.[14,15] However, the IC_{50} for racemic bupivacaine to block rTASK is, at 68 μM, remarkably higher than the IC_{50} for racemic bupivacaine to block the flicker K^+ channel.[34]

Further investigation will show whether the flicker K^+ channel belongs to this specific family of K^+ channels or whether other channels that share structural features with the flicker K^+ channels are targets for highly stereoselective side effects in local anesthesia.

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