Local Anesthetics Potently Block a Potential Insensitive Potassium Channel in Myelinated Nerve

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ABSTRACT Effects of some local anesthetics were studied in patch clamp experiments on enzymatically demyelinated peripheral amphibian nerve fibers. Micromolar concentrations of external bupivacaine depolarized the excised membrane considerably. The flicker K^+ channel was found to be the most sensitive ion channel to local anesthetics in this preparation. Half-maximum inhibiting concentrations (IC_{50}) for extracellular application of bupivacaine, ropivacaine, etidocaine, mepivacaine, lidocaine, and QX-314 were 0.21, 4.2, 8.6, 56, 220, and >10,000 μM, respectively. The corresponding concentration-effect curves could be fitted under the assumption of a 1:1 reaction. Application from the axoplasmic side resulted in clearly lower potencies with IC_{50} values of 2.1, 6.6, 16, 300, 1,200, and 1,250 μM, respectively. The log(IC_{50})-values of the local anesthetics linearly depended on the logarithm of their octanol:buffer distribution coefficients with two regression lines for the piperidine derivatives and the standard amino-amides indicating an inherently higher potency of the cyclic piperidine series. Amide-linked local anesthetics did not impair the amplitude of the single-channel current but prolonged the time of the channel to be in the closed state derived as time constants τ_c from closed-time histograms. With etidocaine and lidocaine τ_c was 133 and 7.2 ms, and proved to be independent of concentration. With the most potent bupivacaine time constants of wash in and wash out were 1.8 and 5.2 s for 600 nM bupivacaine. After lowering the extracellular pH from 7.4 to 6.6, externally applied bupivacaine showed a reduced potency, whereas at higher pH of 8.2 the block was slightly enhanced. Intracellular pH of 6.4, 7.2, 8.0 had almost no effect on internal bupivacaine block.

It is concluded that local anesthetics block the flicker K^+ channel by impeding its gating but not its conductance. The slow blocker bupivacaine and the fast blocker lidocaine compete for the same receptor. Lipophilic interactions are of importance for blockade but besides a hydrophobic pathway, there exists also a hydrophilic pathway to the binding site which could only be reached from the cytoplasmic side of the membrane. Under physiological conditions, blockade of the flicker K^+ channel which is more sensitive to bupivacaine than the Na^+ channel might lead via

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membrane depolarization and the resulting sodium channel inactivation to a pronounced block of conduction in thin fibers.

INTRODUCTION

Local anesthesia in peripheral nerve is produced by the blockade of voltage-gated Na^+ currents as has been established in numerous voltage clamp studies (for reviews see Elliott and Haydon, 1989; Hille, 1992; Strichartz, 1987). The effect of local anesthetics upon the delayed rectifier K^+ current is of minor importance, because high concentrations of local anesthetics are needed to block this current (Århem and Frankenhaeuser, 1974). Furthermore, the contribution of potential-activated K^+ currents to the action potential at least in mammalian fibers is almost negligible (Vogel and Schwarz, 1995). Recently, single ionic channels responsible for the sodium current as well as for the delayed rectifier current of the peripheral nerve have been described (Jonas, Bräu, Hermsteiner, and Vogel, 1989). Further investigations revealed additional less voltage-sensitive K^+ channels in peripheral nerve axons, the functional roles of which are not yet well understood (Jonas, Koh, Kampe, Hermsteiner, and Vogel, 1991; Koh, Jonas, Bräu, and Vogel, 1992; Koh, Jonas, and Vogel, 1994). Among these background channels, the flicker K^+ channel is of special interest (Koh et al., 1992). This channel is mostly found in thin nerve fibers, and it may be a main candidate for generating the resting potential of these fibers. Furthermore, in our preparation, this channel is more sensitive than the Na^+ channel to lipophilic, amine-linked local anesthetics like bupivacaine and ropivacaine (Bräu, unpublished observations).

In this work, the basic properties of local anesthetic blockade of the flicker K^+ channel are studied. In current clamp experiments, depolarization of the membrane induced by local anesthetics could be observed. In thin nerve fibers, blockade of the flicker K^+ channel might lead via depolarization to an increased amount of inactivated Na^+ channels. Inactivated Na^+ channels cannot contribute to the action potential and are more sensitive to local anesthetics, thus, block of impulse propagation is enhanced. This mechanism may be a possible explanation for the clinically well-known differential block of motor and sensory nerve fibers, the so-called differential nerve block (Raymond and Gissen, 1987).

Parts of the results have been presented to the American and European Society of Regional Anesthesia (Bräu, Vogel, and Hempelmann, 1992) and to the German Physiological Society (Nau, Bräu, Hempelmann, and Vogel, 1993).

MATERIALS AND METHODS

Preparation and Recording

The patch clamp method (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was applied to enzymatically demyelinated nerve fibers of the clawed toad (*Xenopus laevis*) as described by Jonas et al. (1989). After dissecting and desheathing the tibial and peroneal nerve, the fibers were incubated with 3.5 mg/ml collagenase (Worthington type CLS II, Biochrom, Berlin, Germany) in Ringer solution for 135 min and subsequently with 1 mg/ml protease (type XXIV, Sigma Chemical Co., St. Louis, MO) in calcium-free Ringer solution for 35 min. During this procedure the nerve was kept in a chamber at a temperature of 23.5 ± 0.5°C, and mild shaking

was applied. The preparation was washed in calcium-free Ringer solution, cut into 1–3 mm long segments, transferred into culture dishes with the bottom coated with laboratory grease (Glisseal, Borer Chemie, Solothurn, Switzerland) and stored at 4°C for at least 30 min. These preparations have been used for up to 10 h.

Patch pipettes were pulled from borosilicate glass tubes (GC 150-7.5, Clark Electromedical Instruments, Pangbourne, England), coated with Sylgard 184 (Dow Corning, Seneffe, Belgium) and fire polished just before the experiment to give a resistance of 35 ± 10 MΩ. Currents were measured using an EPC-7 patch clamp amplifier (List, Darmstadt, Germany) and stored on video tape via a modified PCM-501ES pulse code modulation unit. For analysis data were filtered with a 4-pole low-pass Bessel filter and digitized with a Labmaster TM-40 AD/DA board (Scientific Solutions, Solon, OH). Recordings were made at 15.5 ± 2°C. Membrane potentials (E) are given for the inner side with respect to the outer side of the membrane, in all figures downward deflections indicate inward currents. Errors are given as standard errors of the mean (SEM). Differences in means were tested for significance using the unpaired t test.

Solutions

Experiments were performed with external solutions containing (in millimolar) either 105 KCl, 13 NaCl, 2 $CaCl_2$, 5 BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), and 100 nM tetrodotoxin (105 mM K_o) or 110 NaCl, 2.5 KCl, 2 $CaCl_2$, 5 BES, and 100 nM tetrodotoxin (Ringer-TTX) both adjusted to pH 7.4 with tris(hydroxymethyl)-aminomethane (TRIS) base and internal solution containing (in millimolar) 105 KCl, 13 NaCl, 5 BES, 3 EGTA (ethylenebis-[oxyethylenenitrilo]-tetraacetate) (105 mM K_i) adjusted to pH 7.2 with TRIS base. Local anesthetics were dissolved in distilled water to give 10 or 100 mM stock solution. Drugs were rapidly applied to excised patches by a multibarrel perfusion system (Yellen, 1982) starting with the lower concentrations when different concentrations were used in one experiment (cf. Figs. 4, 6, and 9). Moving the patch from bath into a test solution or back took less than a second. Bupivacaine-HCl, procaine-HCl, and tetracaine-HCl were purchased from Sigma Chemical Co. and QX314 from Alomone Labs (Jerusalem, Israel). Lidocaine-HCl was taken from different sources. Mepivacaine was purchased as Scandicaine 4% and etidocaine as Dur-Anest 1% (both from Astra Chemicals, Wedel, Germany).

RESULTS

Inward currents through K^+ channels of a myelinated nerve fiber can be best observed if symmetrical potassium concentrations are used at both sides of the membrane (Dubois, 1981; Bräu, Dreyer, Repp, Jonas, and Vogel, 1990). In most of our experiments investigating flicker K^+ channels voltage was held at −90 mV, a potential at which this rather potential-insensitive channel is active and disturbances from voltage-activated channels are not to be expected. The first recording of Fig. 1 shows the typical fast gating kinetics of a single flicker K^+ channel, a feature which is probably due to a voltage-dependent block by external potassium ions themselves (Koh et al., 1992). Considering the flickering state as the open state of the channel, the open probability under control conditions was high, although it varied to a certain extent. For our purposes we preferred patches with channel open probabilities > 0.9 which facilitates studies of blocking kinetics.

Local Anesthetics Reduce Open Probability of the Flicker K^+ Channel

As shown in Fig. 1, external application of 300 μM lidocaine, 10 μM etidocaine, and 300 nM bupivacaine induce short closings in the range of milliseconds, tens of

milliseconds, and several seconds, respectively. Thus, lidocaine can be considered as a fast, etidocaine as an intermediate and bupivacaine as a very slow blocker of the flicker K^+ channel. The current recordings in Fig. 1 demonstrate that the single-channel current amplitude was not altered by the local anesthetics. In quantitative evaluations using amplitude histograms (Fig. 2) the single-channel current amplitude

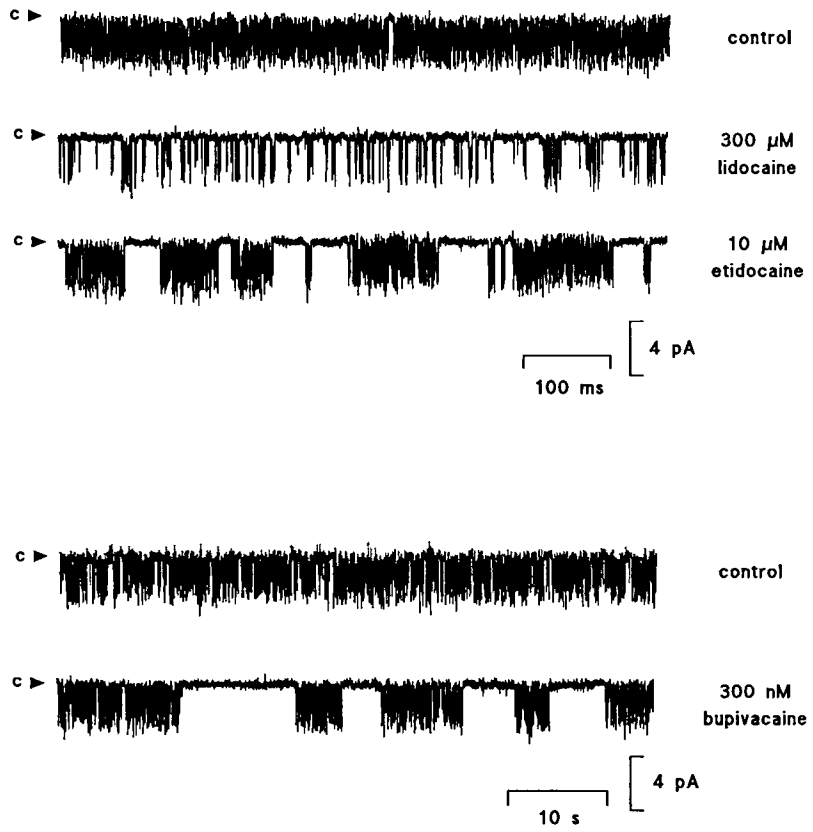

FIGURE 1. Local anesthetic induced closures of single flicker K^+ channels. Recordings from different single-channel outside-out patches at a holding potential of E_h = −90 mV in the absence of local anesthetics (*control*) and after addition of 300 μM lidocaine, 10 μM etidocaine and 300 nM bupivacaine to the external solution. Bath: 105 mM K_o, pipette: 105 mM K_i, filter frequency 3 kHz, temperature 15.5 ± 2.0°C. Current levels of closed channels are marked by arrow heads. Note the long lasting blocks shown at different time scale in the lower two recordings.

was measured to be 4.21 ± 0.20 pA (n = 4) at −90 mV in control solution and was not altered under lidocaine (4.21 ± 0.17 pA, n = 4) and bupivacaine (4.30 ± 0.23 pA, n = 4). The less potent blocker procaine, however, induces a significant reduction ($P < 0.005$) in single-channel amplitude (3.10 ± 0.14 pA, n = 4). This can be accounted to the typical fast blocking kinetic of a weak blocker, too fast to be resolved

by our measuring system. In conclusion, the effect of local anesthetics on the flicker K^+ channel is not due to a reduction of the channel conductance but to a reduction of the probability of the channel to be in the open state. From these experiments, it cannot be decided whether the bound drug molecule prevents channel opening or induces channel closing.

When returning with the outside-out patch into a drug-free control solution, flicker K^+ channels in most cases soon resumed their activity. Quantification of reversibility,

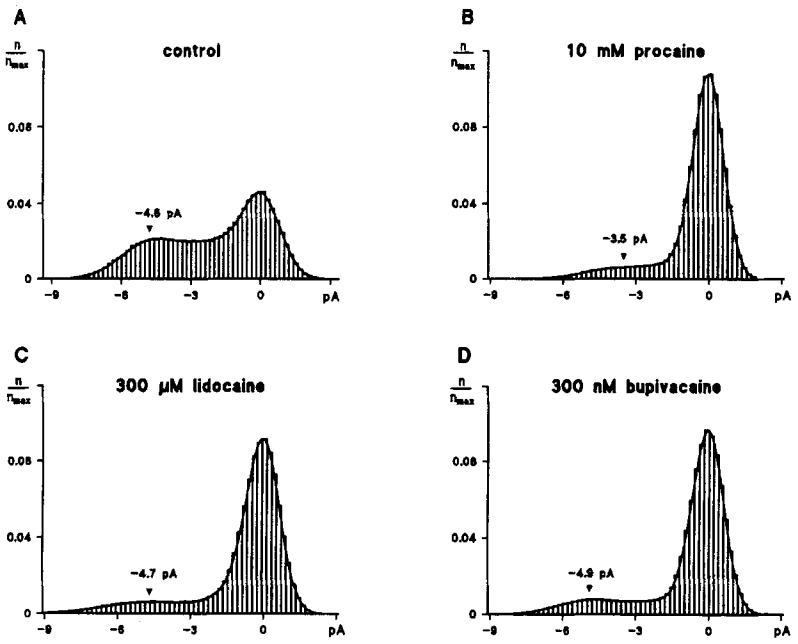

FIGURE 2. Estimation of single-channel amplitude by means of amplitude histograms. (*A*) Control solution; (*B*) external 10 mM procaine; (*C*) 300 µM lidocaine; and (*D*) 300 nM bupivacaine. 1-min records each, $E_h = -90$ mV, symmetrical 105 mM K_o, filter frequency 10 kHz, sampling rate 38.5 kHz, bin width 0.2 pA. Histograms are normalized as n/n_{max}, where n is the number of points in each interval and n_{max} is the total number of points. Histograms were fitted with the sum of three Gaussian curves to give a sufficient fit. The outer two Gaussians represent the channel open and closed state. The single-channel current is measured as the difference between these two peaks. The inner Gaussian curve is probably due to filtering of intrinsic channel flickering.

however, is difficult for three reasons: bupivacaine in particular requires long recording times, channel activity waxes and wanes to a certain degree and some run down exists.

Fractional Block Studied at Different Potentials

A fairly low voltage dependence of the flicker K^+ channel gating behavior has been demonstrated by Koh et al. (1992). The fractional block of the mean current by 300

nM bupivacaine, 10 μM etidocaine, and 300 μM lidocaine in the range of −120 to +60 mV is presented in Fig. 3. No clear voltage dependence of block by all three local anesthetics is seen. Other substances have not been tested for voltage dependence of block.

Different Kinetics of Flicker K^+ Channel Block

To evaluate more quantitatively the blocking kinetics, closed- and open-time histograms were derived from single-channel patches (Fig. 4). The mean closed and open

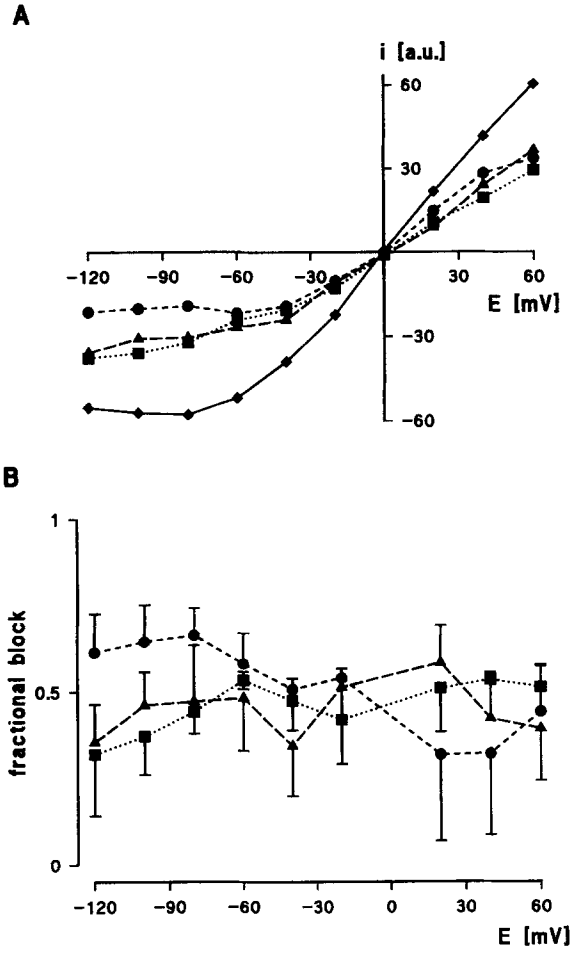

FIGURE 3. Dependence of flicker channel block by LA on membrane potential. (*A*) Normalized currents in control (*diamonds*) as well as in 300 nM bupivacaine (*triangles*), 10 μM etidocaine (*squares*), and 300 μM lidocaine (*circles*) solutions. In each experiment the currents in control solution were normalized so that the slope of the *i-E* curve between −40 and +60 mV was arbitrarily scaled to one. The same scaling factor was further used to normalize the currents in drug-containing solutions. (*B*) Fractional block of mean currents plotted versus membrane potential as derived from (*A*). Bath: 105 mM K_o; Pipette: 105 mM K_i; 11 outside-out patches.

times τ_c and τ_o were evaluated by fitting the closed- and open-time histograms with a single exponential which describes the distribution sufficiently. The calculated rate constants from values for τ_c and τ_o are listed in Table I. As already suggested by Fig. 1, lidocaine, etidocaine, and bupivacaine induce short, intermediate and long closings, respectively. For lidocaine and etidocaine they can be quantitized by time constants τ_c of 7.2 and 133 ms, respectively.

The short durations of the open states in lidocaine could not be distinguished from intrinsic flickering of the channel. Thus, open-time distributions and open-time constants could not be determined for lidocaine. On the other hand, studies of bupivacaine blocking kinetics make long registrations (>30 min to establish good results) necessary, too long for extended kinetic studies.

Because of its intermediate blocking kinetics, etidocaine was chosen for further kinetic studies. Evaluation of time constants from closed- and open-time histograms with different etidocaine concentrations revealed τ_c as concentration independent, whereas τ_o decreased with increasing etidocaine concentration (Fig. 4 *C*).

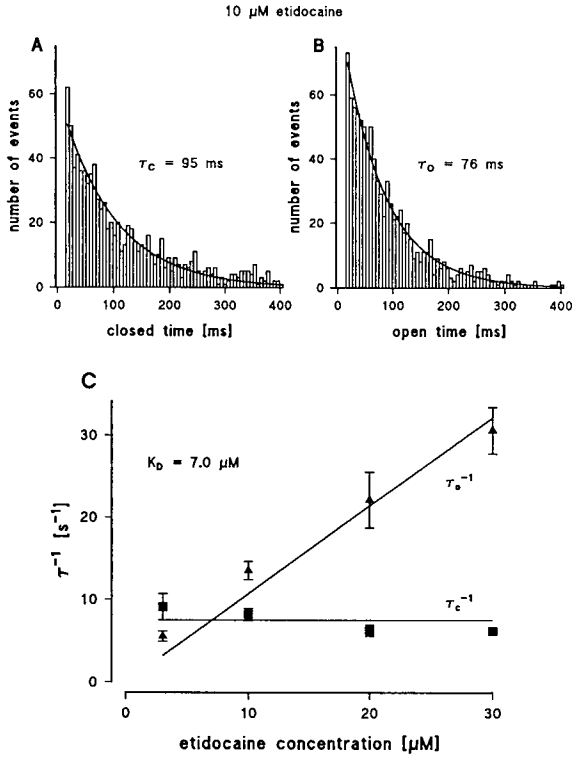

FIGURE 4. Kinetic properties of single flicker K^+ channel block induced by local anesthetics. Frequency density is plotted versus time. Closed and open time distributions are derived from the same recording. (*A* and *B*) Histograms of a 4-min record with extracellular 10 µM etidocaine containing 900 events were fitted with single exponentials, revealing a mean closed time τ_c = 95 ms and a mean open time τ_o = 76 ms. All currents were filtered at 60 Hz and sampled with 5 ms. Gaps <15 ms were ignored to eliminate the influence of the channel flickering upon closed and open time detection. (*C*) Dependence of reciprocal time constants upon external etidocaine concentrations. Mean open and closed time constants were obtained from four single-channel outside-out patches exposed to four different concentrations of etidocaine as described in *A* and *B*. The τ_o^{-1} values were fitted to the equation $\tau_o^{-1} = k_1 \cdot c$, where the steepness factor k_1 represents the association rate constant. The line for the τ_c^{-1}-values simply represents their mean value (k_{-1}). K_D was calculated as 7.0 µM from τ_c and τ_o at the point of intersection (see Table I).

This kinetic experiment strongly suggests that the local anesthetic block of this channel is achieved by the binding of one local anesthetic molecule to a specific receptor at the channel molecule and is not due to nonspecific membrane effects. Further, the kinetic experiments performed with etidocaine can be described by a simple kinetic model (Scheme I) assuming a one-to-one reaction between the local

anesthetic molecule and the receptor of the channel protein.

$$O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C$$

SCHEME I

O stands for the open, C for the closed (local anesthetic bound) channel, k_1 and k_{-1} for the forward and backward rate constants. K_D can be calculated from the rate constants as

$$K_D = k_{-1}/k_1. \quad (1)$$

To derive kinetic data for bupivacaine block, τ_{on} and τ_{off} were estimated from the current recordings of a multichannel patch during wash in and wash out of the blocker (Fig. 5). The wash in and wash out records were fitted beginning at the

TABLE I

Kinetic and Steady State Constants

Substance	k_1 $M^{-1}\cdot s^{-1}$	k_{-1} s^{-1}	K_D μM	n
Lidocaine	$(0.63\cdot10^6)$	138 ± 9	(219)*	4
Etidocaine	$1.07 \pm 0.06\cdot10^6$	7.5 ± 0.55	7.0	4
Bupivacaine	$0.56 \pm 0.05\cdot10^6$	0.19 ± 0.01	0.34	11

Rate constants of local anesthetic block. Dissociation rate constants (k_{-1}) were derived from frequency density *vs* closed time histograms of lidocaine and etidocaine as $k_{-1} = \tau_c^{-1}$ (Fig. 8 *A*, Fig. 4 *C*). Association rate constants (k_1) were derived for etidocaine from the concentration dependence of the open time constants from Fig. 4 *C* as $k_1 = (\tau_o\cdot c)^{-1}$. For bupivacaine, rate constants were derived from on- and offset of block as $k_{-1} = \tau_c^{-1}$ and $k_1 = (\tau_{on}^{-1} - \tau_{off}^{-1})\cdot c^{-1}$; (Fig. 5 *C*). K_D values were calculated from rate constants with Eq. 1 for etidocaine and bupivacaine. For lidocaine, the association constant was calculated from the IC_{50} value of Table II and k_{-1}.

*IC_{50} value, derived from Table II.

moment when solution exchange had definitely taken place. With our method, we focus on the drug-receptor interaction and neglect possible drug-membrane diffusion effects. As shown by the upper recording in Fig. 5 *A*, duration of solution exchange was in a similar time range as the process of wash in. Thus, drug-receptor kinetics especially for the higher concentrations may be faster than it appears from the current decay. The process of wash out was slower than that of wash in and was therefore not influenced by the speed of solution exchange. Values for τ_{on} and τ_{off} were obtained by monoexponential fits. The rate constant for dissociation k_{-1} can be derived directly from wash out time constants ($k_{-1} = \tau_{off}^{-1}$). The association rate constant is calculated as $k_1 = (\tau_{on}^{-1} - \tau_{off}^{-1})\cdot c^{-1}$ were τ_{on} is the time constant for the wash in of the drug (Ulbricht, 1981) and c is the concentration of the drug. From these data, a remarkably low K_D of 0.3 μM was found for bupivacaine (Fig. 5 *B*).

Comparative Blocking Potency of Anesthetics

To compare typical local anesthetics with regard to their potency of blocking the flicker K^+ channel, concentration dependence was investigated by measuring the

mean current through multichannel patches under different externally and internally applied local anesthetic concentrations (Fig. 6). The slow blocker bupivacaine turned out to be the most potent one with a remarkable low half-maximum inhibition concentration of 0.21 μM when applied externally. The other amide local anesthetics tested were less potent in the sequence ropivacaine, etidocaine, mepivacaine, and lidocaine, together spanning a concentration range over three orders of magnitude (Table II). When applied intracellularly, the sequence of potency is unchanged, but the substances are less potent by factors of 1.6 to 10. The data points of externally

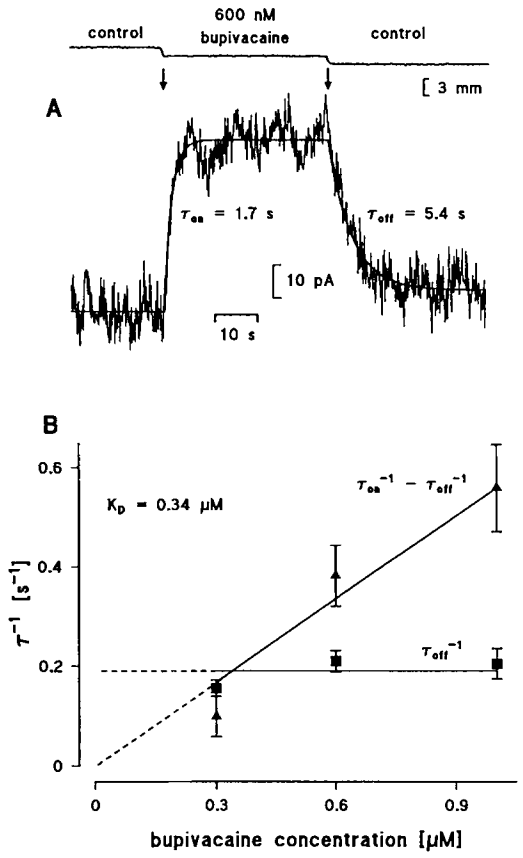

FIGURE 5. Kinetics of wash in and wash out of 600 nM bupivacaine. Outside-out patch containing many flicker K^+-channels. Bath: 105 mM K_o, pipette: 105 mM K_i. (*A*) Top line shows mechanical movement of the multibarrel pipette indicating time course of solution exchange. Recording shows inward current at −90 mV and its change during wash in and wash out of the drug. Filter frequency 60 Hz. Single exponentials were used for fitting wash in and wash out kinetics neglecting an apparent irreversibility (cf. Results). (*B*) Dependence of reciprocal time constants upon external bupivacaine concentrations. Mean time constants of onset and offset of bupivacaine block were obtained with 60 solution changes from 11 patches as shown in *A*. Rate constants were evaluated as given in legend of Fig. 4. K_D was calculated as 0.34 μM from the rate constants (see Table I).

applied drugs can be fitted under the assumption of a one-to-one reaction with a certain deviation in the low concentration range. Surprisingly, for internal application best least square fits have been obtained with variable steepness coefficients between 0.63 for bupivacaine and 1.03 for lidocaine. The positively charged quarternary lidocaine derivative QX-314 has almost no effect when applied externally, but its internal application revealed the same IC_{50} value as lidocaine. The ester-bound local anesthetics procaine and tetracaine applied from the external side are nearly ineffective even in millimolar concentrations. The IC_{50} values of external bupivacaine

and etidocaine obtained from these experiments are in good agreement with the K_D values from kinetic experiments (see Table I).

To clarify whether the artificial condition of high potassium solution has an effect upon local anesthetic block as has been shown for the highly potent K^+ channel

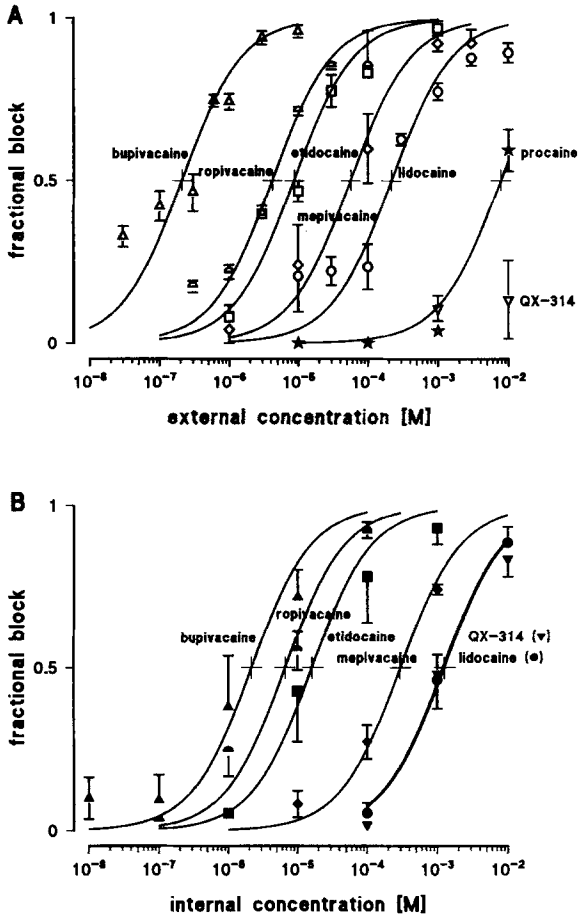

FIGURE 6. Concentration dependence of flicker K^+-channel block by (*A*) externally and (*B*) internally applied local anesthetics. Fractional block (f_B) of mean current is plotted versus concentration (c) of local anesthetics. Symbols denote bupivacaine (*triangles,* peak up), ropivacaine (*upper semicircles*), etidocaine (*squares*), mepivacaine (*diamonds*), lidocaine (*circles*), procaine (*stars*), and QX314 (*triangles,* peak down). Mean values ±SEM from (*A*) outside-out (n = 64) and (*B*) inside-out patches (n = 20) at −90 mV. Symmetrical 105 mM K. Curves represent nonlinear least-squares fits of the equation $f_B = c/(c + IC_{50})$ to the data points assuming a one-to-one reaction of the local anesthetic molecule with the flicker K^+-channel (Hill coefficient = 1). Half-maximum inhibiting concentrations (IC_{50}) are denoted by crosses (data listed in Table II).

blockers dendrotoxin and mast-cell-degranulating peptide (Bräu et al., 1990), a concentration-effect curve for externally applied bupivacaine was also obtained in Ringer solution at E = +40 mV, which revealed an IC_{50} value of 0.18 μM (n = 4, not shown) similar to the value in 105 mM K_o (0.21 μM).

External and Internal Access to the Binding Site

The low potency of externally applied QX-314 suggests a binding site which is accessible for charged molecules from the axoplasmic side only. Because the charge of the local anesthetic molecule depends on the pH-value of the solution, block of flicker K^+ channels should also depend on extracellular pH. At low pH-values more

local anesthetic molecules will be charged and therefore can block the channel to a lower extent than at higher pH values.

Under control conditions, the channel itself showed a sensitivity to external alterations of pH (Fig. 7 *A*). The mean current flowing through multiflicker K^+ channel patches is clearly reduced when the pH is lowered in both the extracellular and the intracellular solution. External bupivacaine, which was elected because of its high blocking potency at this channel, has a much lower blocking effect at acidic pH values than at alkaline pH. The fractional block induced by 300 nM bupivacaine is 0.19 at pH 6.6, 0.71 at pH 7.4, and 0.74 at pH 8.2 (Fig. 7 *B*). Internal alteration of pH has almost no effect upon internal bupivacaine block (Fig. 7, *C* and *D*). Both results support the idea of a hydrophobic pathway to the binding site for externally applied local anesthetics. Internally applied drugs can reach the receptor in either the charged or uncharged form of the molecule.

TABLE II

Half Maximum Inhibiting Concentrations, IC_{50}

Substance	External μM	Internal μM
Bupivacaine	0.21	2.1
Ropivacaine	4.2	6.6
Etidocaine	8.6	16.1
Mepivacaine	56.4	297
Lidocaine	219	1,198
QX-314	>10,000	1,250
Procaine	7,793	—
Tetracaine	>1,000	—

Data from nonlinear least-squares fits as shown in Fig. 6.

Furthermore, the question arises whether the blocking effect of externally and internally applied local anesthetics is due to the same binding site at the channel molecule or whether different mechanisms are responsible for block from either side. If only one receptor is present which can be reached from either side, kinetics and especially unbinding of the molecule from the channel should not depend upon the side of application, therefore revealing equal closed time constants (τ_c) for external and internal application. Closed time constants (τ_c) as derived from closed time histograms of single-channel patches (Fig. 8) are 7.2 ± 0.5 ms (n = 4) for externally and 6.9 ± 0.3 ms (n = 3) for internally applied lidocaine, thus being not significantly different. Comparable closed time constants support, although not unequivocally, the idea of only one binding site for lidocaine.

Competitive Antagonism

The big differences in affinity of amide-type local anesthetics to this channel, three orders of magnitude between lidocaine and bupivacaine, may be due to specific receptors of the channel for the different local anesthetics. To rule out this possibility, simultaneous block by lidocaine and bupivacaine was analyzed for competition. Fixed concentrations of bupivacaine elicit a decrease of mean current in

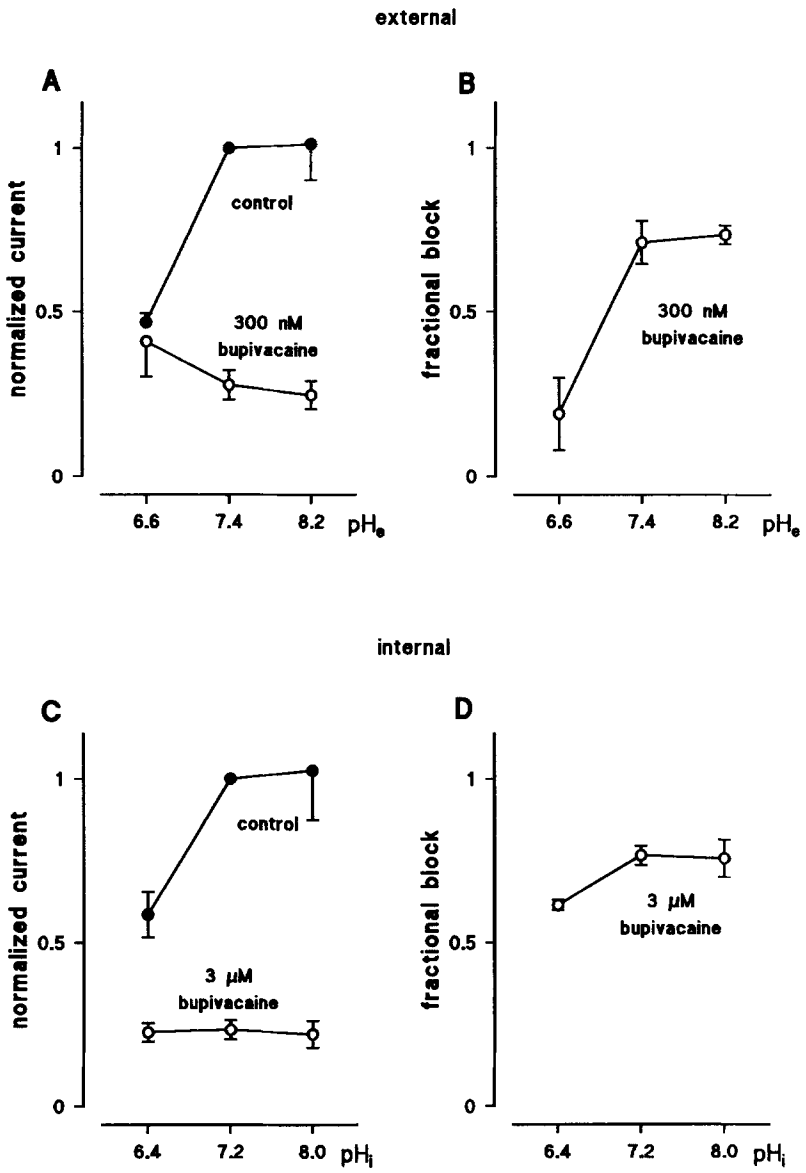

FIGURE 7. pH-dependence of the external and internal bupivacaine block. (*A*) Mean currents at different pH values of extracellular solution before (*filled circles*) and after external application of 300 nM bupivacaine (*open circles*). Currents were normalized to the mean current at pH 7.4. (*B*) pH dependence of fractional block as obtained from data in *A*. Seven outside-out patches containing several flicker K^+-channels, $E = -90$ mV, symmetrical 105 mM K. (*C* and *D*) Corresponding experiments with alteration of pH and application of 3 μM bupivacaine from the axoplasmic side, four inside-out patches.

multiflicker K^+ channel patches. Concentration-effect curves obtained from addition of increasing lidocaine concentrations to the partially bupivacaine-blocked channels would reveal equal IC_{50} values if two different receptors would exist for lidocaine and bupivacaine. In the case of one receptor, competition between lidocaine and bupivacaine would shift the apparent concentration-effect curve of lidocaine to higher values with increasing previously applied bupivacaine concentrations. In competition experiments we started with constant bupivacaine concentrations and measured the concentration-effect curves for lidocaine. With 300, 600, and 1,000 nM

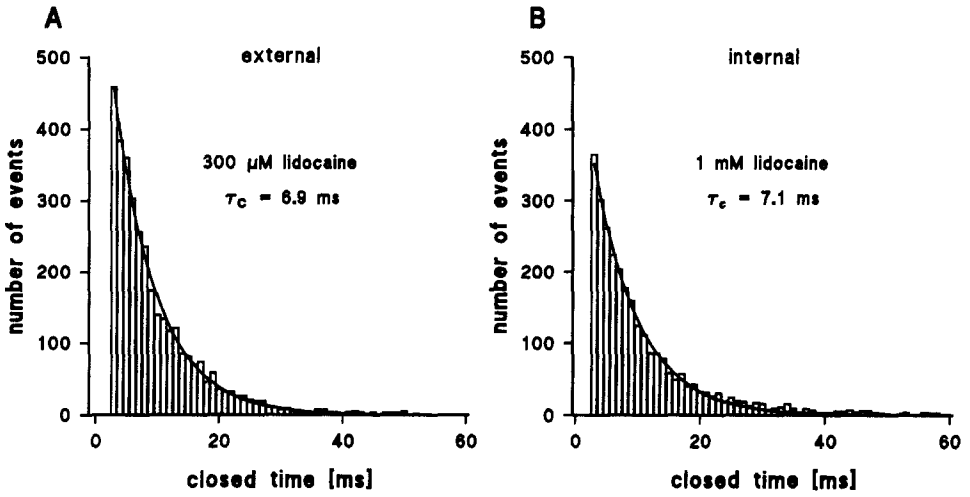

FIGURE 8. Closed time histograms of (*A*) externally and (*B*) internally applied lidocaine. Histograms fitted with single exponentials revealing mean closed time constants τ_c = 6.9 ms and 7.1 ms for external 300 µM and internal 1 mM lidocaine, respectively. 26-s records containing 7,345 and 6,095 events. Filter frequency 10 kHz, sampling rate 38.5 kHz. A relative large number of closed events shorter than 2 ms has been omitted to exclude the fast gating component of the flicker K^+ channel seen also under control conditions from the data fit.

bupivacaine apparent IC_{50} values of 354, 868, and 1,570 µM lidocaine, respectively, were found as shown in Fig. 9. These data rule out the possibility of two independent receptors for each lidocaine and bupivacaine and are clearly in favor of the one-receptor hypothesis. A simple scheme of competition between the two different substances lidocaine (*L*) and bupivacaine (*B*) for one receptor can be established:

$$\begin{array}{ccccc} & & & & B \\ & & & & \downarrow \\ O{\cdot}L & \rightleftharpoons & O & \rightleftharpoons & O{\cdot}B \\ & & \downarrow & & \\ & & L & & \end{array}$$

SCHEME II

where *O* is the open channel, *O·L* or *O·B* is the channel blocked by lidocaine or bupivacaine, respectively. Bupivacaine can only bind if lidocaine is not bound to the channel and vice versa. From Scheme II, the following equation can be derived to

calculate the apparent half-maximum blocking concentration value ($IC_{50,app}$):

$$IC_{50,lido,app} = IC_{50,lido}\,[1 + [bupi]/([bupi] + IC_{50,bupi})] \quad (2)$$

where $IC_{50,lido}$ and $IC_{50,bupi}$ are the half-maximum blocking concentration of lidocaine and bupivacaine as evaluated from concentration-effect curves and [bupi] is the concentration of bupivacaine applied first.

Dependence of IC_{50} upon Lipid Distribution Coefficient

From our data, a correlation of the potency of local anesthetics with their hydrophobic characteristics appears. This can be demonstrated when IC_{50} values as derived from our concentration-effect studies (Fig. 6, *A* and *B;* Table II) are plotted on a logarithmic scale versus the logarithm of their distribution coefficients log Q using the spectrophotometrically determined data for octanol:buffer distribution coefficients at pH 7.4 published by Strichartz, Sanchez, Arthur, Chafetz, and Martin (1990).

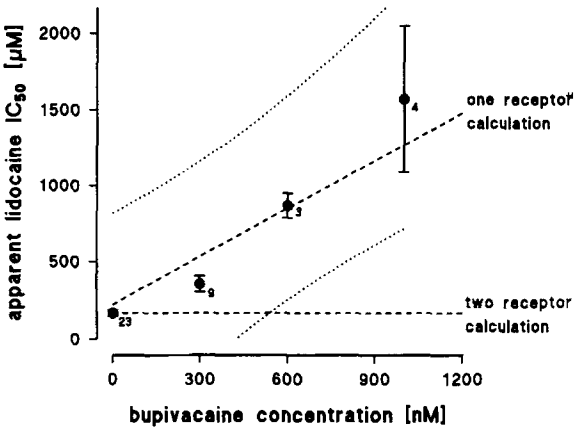

FIGURE 9. Simultaneous application of bupivacaine and lidocaine. Ordinate gives apparent IC_{50} values of lidocaine as found in separate concentration-effect experiments for lidocaine, where various concentrations of bupivacaine (abscissa) were present. (*Dashed lines*) Theoretical change of the apparent lidocaine IC_{50} in dependence of the simultaneously present concentrations of bupivacaine assuming models of either competition of both drugs for one receptor (according to Eq. 2) or of two independent receptors. Dotted lines give 95% confidence interval to the data. Numbers of outside-out patches are given next to each measuring point.

Distribution coefficients for intracellularly applied drugs were calculated as established by Sanchez, Arthur, and Strichartz (1987) with $Q = (10^{(pka\text{-}pH)}P^{+} + P^{0})/(1 + 10^{(pka\text{-}pH)})$ due to our more acid internal solution with a pH of 7.2. (P^0, P^+: ratio of the relative concentrations of neutral and protonated drugs, respectively, between octanol and buffer).

Fig. 10 clearly shows that the potencies of the substances increase with higher distribution coefficients in octanol. Surprisingly, two regression lines between potency and lipid solubility for the three piperidine derivatives bupivacaine, ropivacaine and mepivacaine and the two standard amino-amides etidocaine and lidocaine have been obtained indicating an inherently higher potency of the cyclic piperidine series. Of internally applied piperidine derivatives the regression lines of the IC_{50} values are shifted 2.1-fold to higher IC_{50} values indicating that externally applied they are

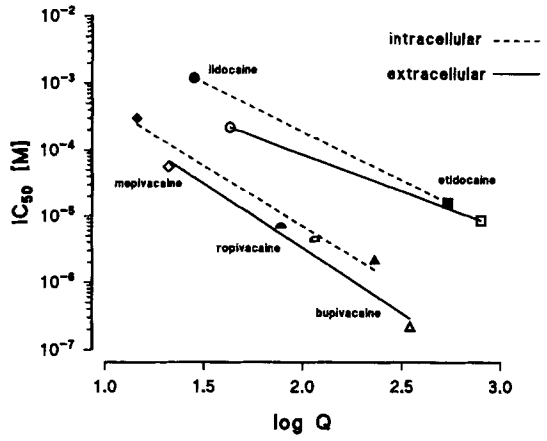

FIGURE 10. Dependence of blocking potency on hydrophobicity. Half-maximum inhibiting concentrations, IC_{50}, of extracellular (*open symbols*) and intracellular (*filled symbols*) application (see Table II) are plotted versus log Q, the logarithm of octanol:buffer distribution coefficients, as found in Strichartz et al. (1990). Regression lines for extra- and intracellularly applied piperidine derivatives bupivacaine, ropivacaine, and mepivacaine differ by a mean factor of 2.1, their respective correlation coefficients are −0.986 and −0.984.

approximately two times more potent. A similar shift can be seen for the standard amino-amides etidocaine and lidocaine.

Depolarization of the Membrane

Under current clamp conditions we were able to measure a membrane potential of −80 to −50 mV from axon-free outside-out patches containing several flicker K^+ channels. In the experiment of Fig. 11, a patch was investigated which previously had shown I channel (delayed rectifier potassium channels, Jonas et al., 1989) and flicker K^+ channel currents. When the external Ringer solution was changed to Ringer's plus 10 mM tetraethylammonium, TEA, only a small depolarization was detected because classical delayed rectifier channels were not active at this potential and flicker K^+ channels are fairly insensitive to TEA (Koh et al., 1992). 3 μM bupivacaine, however, reversibly induced a quick depolarization by ~15 mV.

This experiment may show that the local anesthetic bupivacaine is able to depolarize dramatically the nerve membrane and thus will have strong impact on potential sensitive ion channels in general and predominantly Na^+ channels and thus on excitability of the nerve. However, in our preparation measurements of the resting

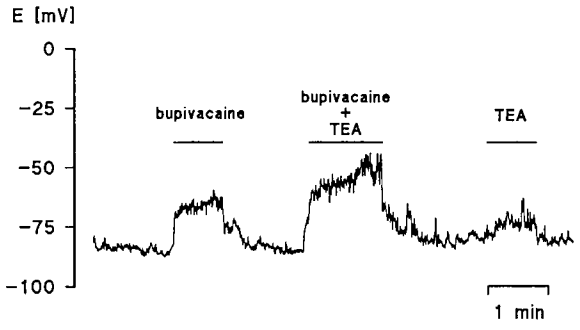

FIGURE 11. Membrane potential as affected by 3 μM bupivacaine as well as 10 mM TEA. Outside-out patch in current-clamp mode. Bath: Ringer's solution, pipette: 105 mM K_i.

potential are restricted to axon-free membrane patches and can not be conducted on intact axons.

DISCUSSION

Blockade of the fast sodium inward current seems to be the main mechanism of local anesthesia in peripheral nerve. Besides sodium current, also neuronal calcium currents (Guo, Castle, Chernoff, and Strichartz, 1991) and delayed rectifier potassium currents are affected by local anesthetics (Taylor, 1959; Hille, 1966; Århem and Frankenhaeuser, 1974). The contribution of fast K^+ channel block to conduction block of the fiber is small because these channels are mainly responsible for the fast repolarization of the action potential, as can be shown in computer simulations (Ritchie and Stagg, 1982; Vogel and Schwarz, 1995). The resting potential which to some extent may be generated by voltage-independent potassium permeability is slightly depolarized by (general) anesthetics in the squid axon (Urban and Haydon, 1987; Haydon, Requena, and Simon, 1988; Elliott, Elliott, and Haydon, 1989) whereas hippocampal CA1 neurones are hyperpolarized (MacIver and Kendig, 1991).

Our investigations describe the blockade of voltage-independent K^+ channels in peripheral nerve membrane by several local anesthetics. The recently detected flicker K^+ channel is rather specifically blocked leading to a depolarization of the membrane potential which inactivates the Na^+ channels and thus impedes conduction of the nervous impulse. After binding in a one-to-one reaction between the drug molecule and the channel receptor, the open probability, but not the conductance of this channel, is affected. Bupivacaine with the highest distribution coefficient in octanol is the most potent of the local anesthetics tested. It is more effective when applied as an uncharged molecule from the external side of the membrane. With a potency of IC_{50} = 200 nM, bupivacaine has a three times higher affinity to the flicker K^+ channel than it has to the α_1-acid glycoprotein in human serum, the protein with the highest affinity to local anesthetics known so far (Denson, Coyle, Thompson, and Myers, 1984).

A Potassium Channel Highly Sensitive to Local Anesthetics

Kinetic studies of the flicker K^+ channel block reveal different dwell times for local anesthetics: substances with low IC_{50} values exert much longer closings than substances with higher half-maximum inhibiting concentrations. The block of the flicker K^+ channel is furthermore specific for amide local anesthetics. Clearly, higher concentrations of ester-bound drugs like procaine and tetracaine (> 1 mM) are needed to block this channel. This specifity of amide-type local anesthetics compared to ester-bound drugs has also been found for the delayed rectifier K^+ current. Århem and Frankenhaeuser (1974) observed a nine times higher IC_{50} value for K^+ current than for Na^+ current as blocked by the amide local anesthetic lidocaine, whereas for the ester procaine this ratio was 47.

A correlation between local anesthetic potency and their distribution coefficient was observed in our experiments. In other structure-activity studies on local anesthetics and Na^+ channel block, however, some deviations from this relationship have been

found as more compounds were studied (Ehring, Moyer, and Hondeghem, 1988). Our observations could suggest that nonspecific membrane effects rather than receptor guided mechanisms are responsible for local anesthetic action or that lipophilic drugs are favored penetrating diffusion barriers to reach their site of action. Since in our experiments, epi- and perineurium had been removed, the membrane remains as a hydrophobic barrier to reach a specific receptor (Hille, 1977*a*) and the receptor itself may be of lipid structure.

The higher potencies of the cyclic piperidine series compared to the standard amino-amides with similar hydrophobicities demonstrate that besides hydrophobic properties, structural elements are important for the blocking effect of local anesthetics. Further, the higher affinities of amide- over ester-bound drugs suggests a specific interaction of these drugs with a membrane receptor.

More evidence for a drug-receptor interaction comes from kinetic data, derived from single, partially blocked flicker K^+ channels. The channel block can readily be described by a first-order reaction between one molecule and one receptor. Its predictions, monoexponential closed- and open-time histograms with concentration dependence of the open-time constants and concentration independence of the closed-time constants, as well as a Hill coefficient of one in the block effect vs concentration curve, are all matched by our observations. We therefore conclude that block of flicker K^+ channels is established by a one-to-one reaction between the local anesthetic molecule and the channel protein. Interestingly, for internal application fits of the block effect vs concentration curve with variable steepness factors resulted in Hill coefficients between 0.63 for the most potent bupivacaine and 1.03 for the least potent lidocaine as opposed to fits for externally applied drugs where the Hill coefficient was constantly 1.0.

Local anesthetics produce, in addition to tonic block, also phasic (use-dependent) block of Na^+ channels. According to the modulated receptor hypothesis (Hille, 1977*b;* Hodeghem and Katzung, 1977) this is due to different affinities of local anesthetics to different conformational states of the Na^+ channel. Independently of the modulated receptor hypothesis, Na^+ channel block by local anesthetics is voltage dependent, even if the channel is in the same conformational state (open) over a wide voltage range, as it can be studied in batrachotoxin-activated channels (Wang, 1988). In contrast to the Na^+ channel, no voltage dependence of the flicker K^+ channel block has been observed in this study. Because the flicker K^+ channel shows no voltage-dependent gating (Koh et al., 1992) no different voltage-dependent conformation states exist and therefore a phasic block is not to be expected for this channel.

Pathways to the Receptor

When given from the extracellular side of the membrane, local anesthetics can block the flicker K^+ channel more potently when they are in their uncharged form, whereas a block from the inner side does not depend on the dissociation state of the molecule. This conclusion is drawn from two observations: first, the permanently charged lidocaine derivative QX-314 blocks the flicker K^+ channel from the inner side of the membrane with a comparable affinity to lidocaine but shows no effect when being given from the outer side. Similar observations exist for Na^+ channels in the squid axon (Frazier, Narahashi, and Yamada, 1970). Second, external bupivacaine block is

strongly dependent on extracellular pH. At lower pH values, where bupivacaine (pK = 8.21, Strichartz et al., 1990) exists predominantly in its charged form, the block is weaker than at higher pH values. Internal alteration of pH has little effect upon internal bupivacaine block. With Na^+ channels, however, internally applied local anesthetics have a higher blocking potency at low pH when they are in their charged forms (Narahashi, Frazier, and Yamada, 1970).

It is likely that local anesthetics, when given extracellularly, must penetrate into the membrane and reach the receptor of the channel molecule directly from the membrane or gain access to the receptor from the internal side, a requirement which only uncharged molecules can fulfill.

Allowing for the pH dependence of lipid distribution coefficients, the differences of apparent affinities for extra- and intracellularly applied local anesthetics decrease to a mean factor of 2.1 and 1.9 for the piperidine derivatives and standard amino-amides, respectively (cf. Figs. 6 and 10).

Additionally, the difference in internal and external pH values (7.2 and 7.4, respectively) induces an asymmetric partitioning of local anesthetics between outer and inner solution with an internal accumulation of the substance when applied externally. This effect can raise the inner concentration of local anesthetics by a factor of 1.6 at most as calculated from the Henderson-Hasselbalch equation. To achieve the same fractional block, concentrations of externally applied substances can therefore be nominally lower than the concentrations of internally applied drugs when an internal access to the binding site is assumed.

Both effects together may explain the apparently higher affinity of externally applied local anesthetics. Further the sidedness suggested by the apparent affinity does not seem to be true because the dwell times (an expression of true affinity) of extra- and intracellularly applied lidocaine and etidocaine are similar.

Also, at the axoplasmic side, no diffusion barrier for charged substances like QX-314 or protonated bupivacaine exists, and most likely, the receptor can be reached through the aqueous pore of the channel. Corresponding findings of Hille (1977*b*) for the Na^+ channel suggest similar mechanisms of local anesthetic block at flicker K^+ and Na^+ channels although the latter are more sensitive to internal application.

A New Mode of Nerve Block

Three of the characteristic features of the flicker K^+ channel, K^+ selectivity, voltage independence, and TEA insensitivity may indicate its main physiological function, to generate the resting potential or at least to give a certain contribution to it (Koh et al., 1992). Blockade of this channel by bupivacaine indeed depolarizes the membrane. This effect inevitably will reduce excitability of nervous conduction by increasing the portion of Na^+ channels in the inactivated state. Inactivated Na^+ channels have a much higher affinity to local anesthetics (modulated receptor hypothesis: Hille 1977*b;* Hondeghem and Katzung, 1977). According to Drachman and Strichartz (1991), conduction block is facilitated under depolarization. Both depolarizing and hyperpolarizing changes of the membrane potential as induced by anesthetics have been reported earlier (Frazier et al., 1970; Haydon et al., 1988; Elliott et al., 1989; MacIver and Kendig, 1991). In our study with amphibian

myelinated nerve, flicker K^+ channels are more frequently found in thin nerve fibers (< 5 μm) which in general conduct pain. Therefore, a specific blockade of these fibers may lead to a predominant block of pain, a so-called differential nerve block (Rosenberg and Heinonen, 1983; Raymond and Gissen, 1987). In fact, bupivacaine and ropivacaine in low concentrations are clinically used for this purpose. Among the local anesthetics we studied so far, only bupivacaine and ropivacaine have a higher affinity to flicker K^+ channels than to Na^+ channels of the peripheral nerve (Bräu, unpublished results) which thus explains their differential block effect.

In conclusion, mechanisms of conduction block in peripheral nerve may not only consist of the specific blockade of the voltage-gated Na^+ channel but also other and more complex functional mechanisms are conceivable (Frenkel, Duch, and Urban, 1993) including a depolarizing nerve block.

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