

Multiple Open Channel States Revealed by Lidocaine and QX-314 on Rat Brain Voltage-dependent Sodium Channels

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ABSTRACT We have recently reported that brain sodium channels display periods with high (low- K_d) and low (high- K_d) levels of lidocaine-induced open channel block (Salazar, B.C., D.O. Flash, J.L. Walewski, and E. Recio-Pinto. 1995. *Brain Res.* 699:305–314). In the present study, we further characterize this phenomenon by studying the effects of the permanently charged lidocaine analogue, QX-314. We found that the detection of high- and low- K_d periods does not require the presence of the uncharged form of lidocaine. The level of block, for either period, at various QX-314 concentrations indicated the presence of a single local anesthetic binding site. Increasing the concentration of QX-314 decreased the lifetime of the high- K_d periods while it increased the lifetime of the low- K_d periods. These results could be best fitted to a model with two open channel conformations that display different local anesthetic K_d values (low and high K_d), and in which the channel area defining the local anesthetic K_d consists of multiple interacting regions. Amplitude distribution analysis showed that changes in the K_d values reflected changes in the k_{on} rates, without changes in the k_{off} rates. Both lidocaine and QX-314 were found to be incapable of blocking small-channel subconductance states (5–6 pS). Changes in the local anesthetic k_{on} rates for blocking the fully open state and the lack of local anesthetic block of the small subconductance state are consistent with the presence of channel conformational changes involving the intracellular permeation pathway leading to the local anesthetic binding site. **Key words:** lidocaine • QX-314 • brain voltage-dependent sodium channels • open channel block

INTRODUCTION

One effect of local anesthetics is to block the open state of voltage-dependent sodium channels (Frazier et al., 1970; Strichartz, 1976). One approach that facilitates the study of this local anesthetic effect is incorporating batrachotoxin (BTX)-modified sodium channels into planar lipid bilayers (Krueger et al., 1983). Because BTX modifies the channel inactivation process in such a manner that the channel remains open almost continuously at potentials more positive than -50 mV (Moczydlowski et al., 1984) and because of the high stability of the planar lipid bilayers, one can observe the channels for long periods (>30 min) both during con-

trol and in the presence of the anesthetic (Salazar et al., 1995). With this method, it has been shown that local anesthetics block the open state of BTX-modified sodium channels in a way that is consistent with their binding to a single site (Moczydlowski et al., 1986b; Wang, 1988; Zamponi et al., 1993a). Moreover, functional studies indicated that channel structural differences between brain- and muscle-derived channels affect the apparent binding affinity of some local anesthetics such as lidocaine (Salazar et al., 1995) but not of other local anesthetics such as cocaine (Wang, 1990). On the other hand, the location, with respect to the intracellular channel aspect, of the binding site mediating the local anesthetic-induced open channel block seems to be the same in brain- and muscle-derived channels (Salazar et al., 1995; Wang, 1990).

We have previously reported that brain- but not muscle-derived sodium channels showed periods during which there was a relief in the level of the lidocaine-induced open channel block, as though the open chan-

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¹Abbreviation used in this paper: BTX, batrachotoxin.

nel state displayed changes in its lidocaine apparent binding affinity (K_d) (Salazar et al., 1995). In this report we further characterize the changes in K_d values to investigate whether the underlying mechanism could be the presence of spontaneously occurring conformational changes of the fully open channel state.

METHODS

Channel Incorporation into Planar Lipid Bilayers

Planar lipid bilayers were formed with a mixture of neutral phospholipids, and the channels were incorporated as previously described (Salazar et al., 1995). The compartments on either side of the Teflon partition (1 ml capacity) contained symmetrical 0.5 M or 0.2 M NaCl. Solutions were buffered to pH 7.4 with 10 mM HEPES. The *trans* compartment contained 1 μ M of the sodium channel activator BTX. Channel function was studied before and after the addition of lidocaine or lidocaine *N*-ethyl (QX-314) to the same channels. Lidocaine was added to both channel aspects; QX-314 was added to either the intracellular, the extracellular, or both channel aspects. Lidocaine was purchased from Sigma Chemical Co. (St. Louis, MO) and QX-314 from Research Biochemicals International (Natick, MA). Experiments were conducted at room temperature (22–25°C).

A two-electrode voltage clamp (Dagan 3900; Dagan Corp., Minneapolis, MN) or an EPC7 patch clamp (List Electronic, Darmstadt, Germany) was used. Ag/AgCl electrodes made direct contact with both aqueous solutions. Output from the voltage clamp was split between a DAS/VCR 900 digital recorder (filtered 2–5 kHz) to be used for later analysis and an eight-pole Bessel filter (50–100 Hz). Output from the Bessel filter was in turn split between a microcomputer data acquisition (12-bit) interface (Labmaster; Axon Instruments, Foster City, CA) and a strip chart recorder (100–150 Hz response). Data acquisition and analysis were done using PCLAMP programs (Axon Instruments).

Plasma membranes were isolated from 1–7-d-old rat brains as previously described (Villegas et al., 1994). In this study, we used seven preparations of rat brain sodium channels.

Data Analysis

The fast partially resolved blocking events were analyzed using the amplitude distribution analysis described by Yellen (1984). Current traces were obtained by holding each membrane potential for several (>3) minutes. The data were collected at various filtering frequencies (200, 50, 20, and 10 Hz) by using an eight-pole filter (Frequency Devices, Haverhill, MA) and digitized at 2 kHz. Current traces collected at 200 Hz and 50 Hz were inspected to select sections where there were no changes in the membrane background conductance nor in the level of the anesthetic-induced open channel block. Channel closures were used to determine the baseline but were not included in the construction of the current amplitude histograms. The selected sections were then localized in the current traces collected at 20 Hz (or 10 Hz), and the latter were used for constructing current amplitude distribution histograms. Current amplitude distributions were normalized by dividing the individual amplitude values by the single channel current value. The single channel current value

was calculated from the slope conductance of current–voltage relationships. Slope conductances were obtained by linear regression fits of the current–voltage relationships for each channel during control. The shape of the amplitude histogram was fitted to a density function by using a nonlinear least square fit (MathCAD software; MathSoft Inc., Cambridge, MA). The rate constants were determined from the fits as described by Yellen (1984). To use this analysis, the level of the drug-induced open channel block, in combination with the filter frequency, should provide amplitude histograms that, when fitted with the beta function, give a and b values >2. Such conditions were found for +40 mV and/or higher positive potentials when the channels displayed low- K_d periods. However, when the channels displayed high- K_d periods, such conditions were not possible since the level of drug-induced open channel block was too low. Fits that gave a or b values <2 were not used. $a = (1/t_{\text{close}})*\tau$; $b = (1/t_{\text{open}})*\tau$; t (in ms) = 22.8/filtering frequency (with an eight-pole filter) (see Yellen 1984).

RESULTS

QX-314-induced Open Channel Block Shows Apparent Affinity Changes

Fig. 1 shows current traces for three single channel experiments before (*left*) and after (*right*) the addition of either lidocaine (Fig. 1 A) or QX-314 (Fig. 1 B). During control, channels remained mainly open and underwent shortlived closures (*left*). Upon the addition of lidocaine or QX-314, the open channel noise level increased (*right*). QX-314 did not produce this effect when it was added to the extracellular channel aspect (five experiments, not shown). The increase in the open channel noise level has been shown to reflect partially resolved fully open channel blocking events and will be referred to as fast-open channel block (Strichartz, 1973; Moczydlowski et al., 1986*b*). As previously reported (Salazar et al., 1995) in brain channels, the level of the lidocaine-induced fast-open channel block was not constant; that is, there were periods during which the level of the lidocaine-induced open channel block was greatly reduced (Fig. 1 A, *right*). Such periods did not result from the presence of uncharged lidocaine molecules (at pH 7.4, ~14% of the lidocaine molecules are uncharged), since they were also observed when the open channel block was induced by the permanently charged lidocaine analogue QX-314 (Fig. 1 B, *right*, and see Fig. 3, *right*). During these periods, the anesthetic remains capable of blocking the open channel state, but the anesthetic apparent binding affinity has somehow been greatly reduced (see Fig. 3, *right*). Such apparent binding affinity changes were reversible. Periods in which the level of the local anesthetic-induced open channel block was low will be referred to as high- K_d periods, while those in which the level of the local anesthetic-induced open channel block was high will be referred to as low- K_d periods.

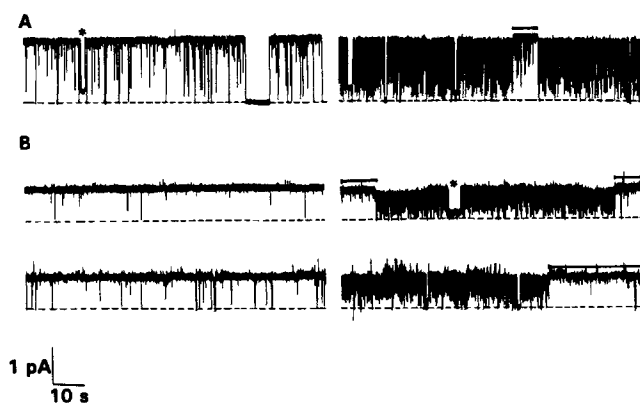


FIGURE 1. Fast-open channel block induced by lidocaine and QX-314 on rat brain sodium channels. Single channel current traces in the absence (*left*) and presence (*right*) of either lidocaine (A) or QX-314 (B). Lidocaine (1 mM) was added to both chamber compartments (+60 mV), whereas QX-314 (0.4 mM) was added only to the intracellular channel aspect (+30 mV). The dashed lines indicate the current level when the channel is closed and channel openings are upward (30 Hz). High- K_d periods for lidocaine and QX-314 are indicated with bars on top of the current traces (*right*). *Small subconductance states (~ 5 pS).

QX-314 Dose-Response Curves Indicate the Presence of a Single Local Anesthetic Binding Site during Both K_d Periods

To determine whether the QX-314-induced open channel block could be explained by binding to a single site, we measured the reduction of the channels' fractional open time by various QX-314 concentrations. Fig. 2 shows the dose-response curves of QX-314 on the fractional blocked time when the channels were in the low- K_d periods (*open circles*) and in the high- K_d periods (*filled circles*). A complete dose-response curve for the high- K_d period was hard to obtain because, as the QX-314 concentration was increased (>10 mM), the occurrence and lifetime of the high- K_d periods decreased and the membrane stability decreased (membranes broke rapidly). For both K_d periods, however, the fractional blocked time increased as the QX-314 concentration increased and could be well described by the Langmuir isotherm (Fig. 2), which is consistent with the idea that the QX-314-induced blocking events are due to the binding of one QX-314 molecule to one sodium channel during both K_d periods. This model has been previously validated for the action of various local anesthetics, including lidocaine, in muscle-derived BTX-modified channels where only one K_d period is observed (Moczydlowski et al., 1986*b*; Wang, 1988; Zamponi et al., 1993*b*).

Voltage Dependence of the Low- K_d and High- K_d Periods

Fig. 3 A shows current traces of a single channel at various membrane potentials in the absence (*left*) and pres-

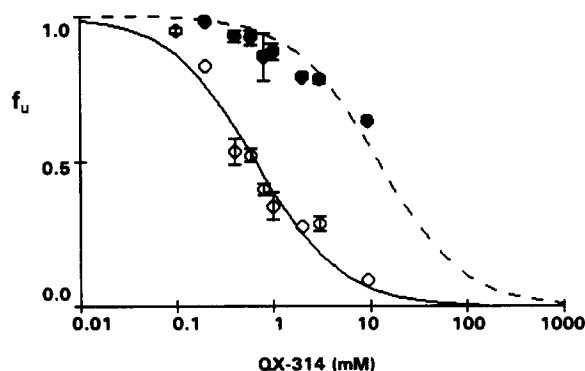


FIGURE 2. Open channel block induced by various QX-314 concentrations indicates the presence of a single local anesthetic (LA) binding site when the channel is in either the low- or high- K_d periods. Dose-response curves of QX-314 on fractional unblocked time (f_u) during low- K_d (*open circles*) and high- K_d periods (*filled circles*). For both K_d periods, the f_u decreased as the QX-314 concentration increased and could be fitted with a Langmuir isotherm ($f_u = 1 - [L]/(K_d + [L])$). From the fit of the data, the K_d at +60mV was 0.66 mM for the low- K_d period and 12.0 mM for the high- K_d period. The data points represent mean \pm SEM, $n = 3-5$, except for 0.2, 2.0, and 9.2 mM QX-314 where $n = 1$.

ence (*right*) of QX-314. The level of the QX-314-induced open channel block increased with depolarization both during the low- and high- K_d periods. Within the low- K_d period, changes in the time-averaged conductance were observed (Fig. 3, +30 mV, *arrows*). Under higher resolution (200 Hz, not shown), it became apparent that such changes did not reflect subconductance states but, rather, changes in the level of the QX-314-induced open channel block. Therefore, such events indicate the presence of more than one low- K_d level. This is more clearly seen in the highly filtered current trace shown at the bottom of Fig. 3 B, which indicates the levels of the time-averaged current when the channel was at the high- K_d (*upper dashed line*) and at two low- K_d periods (*two middle dashed lines*). There was also more than one high- K_d level (not shown).

Fig. 4 shows the estimated mean K_d values for lidocaine (*squares*) and for QX-314 (*circles*) at different membrane potentials. In this figure, the K_d values were obtained using time-averaged conductance measurements before (Ug) and after (Dg) the addition of the drug; Eqs. 1 and 2 were then applied. This method assumes that lidocaine- and QX-314-induced channel block results from a bimolecular reaction between lidocaine and the open sodium channel state ($O + D = OD$; O = open channel; D = drug (lidocaine or QX-314); OD = open blocked channel).

$$f_b = (Ug - Dg)/Ug \quad (1)$$

$$K_d = [D](1/f_b - 1) \quad (2)$$

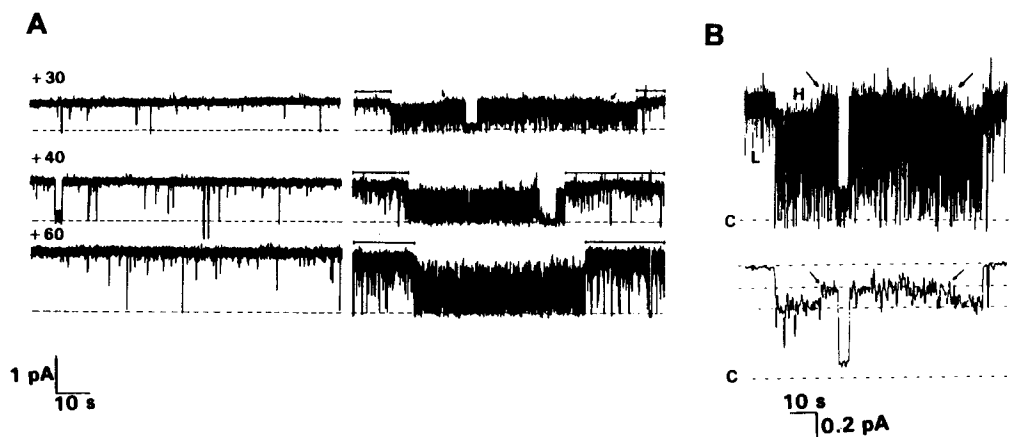


FIGURE 3. Voltage dependence of the low- and high- K_d periods. (A) Current traces for a single channel are shown at various positive membrane potentials in the absence (left) and presence of QX-314 (right) (0.4 mM). Dashed lines indicate the current level when the channel is closed and channel openings are upward (30 Hz). During control, the channel re-

mained mainly open, undergoing few brief closures and subconductance states (at +40 mV). In the presence of QX-314, the channel displayed periods of low level of channel block (high- K_d periods), indicated with the bars, and periods of high level of channel block (low- K_d periods). The level of open channel block increased with depolarization in both the high- and low- K_d periods. (B) Shown is the current trace at +30 mV at a different scale and at 30 Hz (top) and 1 Hz (bottom). The current level when the channel is fully closed is indicated by the dashed line labeled C. Periods in which the level of the open channel block during the low- K_d periods changed are indicated with arrows. In the highly filtered trace (bottom, 1 Hz), the averaged current level for the high- K_d period (top dashed line) as well as the current levels for the two low- K_d periods (two middle dashed lines) are indicated.

where f_b is the channel's fractional blocked time, and $[D]$ is the drug concentration. The voltage dependence of the lidocaine K_d was estimated by fitting the data to Eq. 3 (below), which was derived by Woodhull (1973).

$$K_d(V) = K_d(0) \exp(-\delta V e/kT), \quad (3)$$

where V is the applied potential, $K_d(0)$ is the K_d at 0 mV, e is the elementary charge, k is Boltzmann's constant, T is temperature in Kelvin, and δ is an equivalent valence that describes how the applied potential affects drug binding.

The QX-314 and lidocaine mean low- K_d values decreased with depolarization after a single exponential that had similar slopes. The mean low- K_d values were higher for QX-314 (Fig. 4, open circles) than for lidocaine (Fig. 4, filled squares). However, some of the individual K_d values for QX-314 were as low as those seen with lidocaine. The reason for the difference between mean low- K_d values is that, in the presence of lidocaine, the predominant low- K_d level had the highest affinity (lowest K_d), whereas, in the presence of QX-314, the predominant low- K_d level had a lower affinity value. The magnitude of the error bars reflects in part the presence of various and similar low- K_d periods.

The QX-314 mean high- K_d values (Fig. 4, filled circles) also decreased as an exponential function of voltage. The number of measured high- K_d values was low mostly because the level of the drug-induced open channel block was too low, especially at low positive potentials and low drug concentrations (Figs. 1 and 2); hence, accurate measurements were difficult. Moreover, increasing the drug concentration decreased the probability of detecting high- K_d periods (see next section); and in

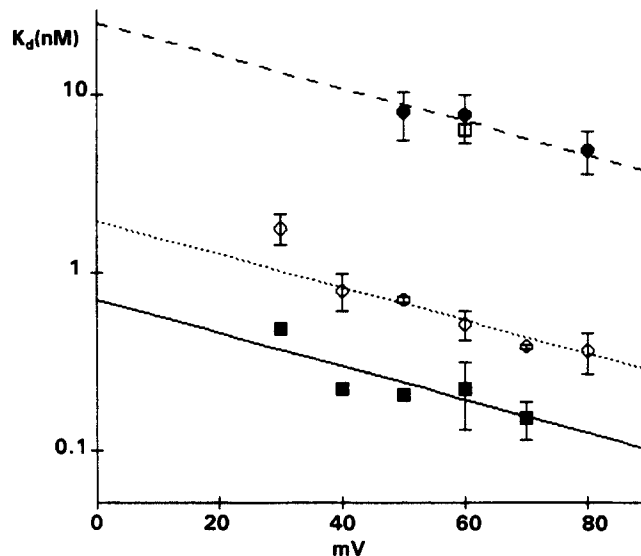


FIGURE 4. Voltage dependence of the high- and low- K_d periods for QX-314 and lidocaine. The K_d values were estimated using time-averaged current measurements and Eqs. 1 and 2 as described in the text. The various K_d values were plotted as a function of the membrane potential. Estimated mean low- K_d values in the presence of QX-314 (open circles) and lidocaine (filled squares), and the estimated mean high- K_d values in the presence of QX-314 (filled circles) as well as a value for the high- K_d period in the presence of lidocaine (open square) are shown. The data points represent mean \pm SEM, $n = 3-7$, except for the open square ($n = 2$). The lines represent visual fits to the data to Eq. 3. The fits give the following values: For the high- K_d period of QX314, $K_d(0) = 25.5$ mM, $\delta = 0.55$ (dashed line); for the low- K_d period of lidocaine, $K_d(0) = 0.70$ mM, $\delta = 0.55$ (continuous line); and for the low- K_d period of QX-314's, $K_d(0) = 1.95$ mM, $\delta = 0.55$ (dotted line). The mean low- K_d values were higher for QX-314 (open circles) than for lidocaine (filled squares). In all cases, the K_d values decreased with depolarization after a single and similar ($\sim\delta$) exponential.

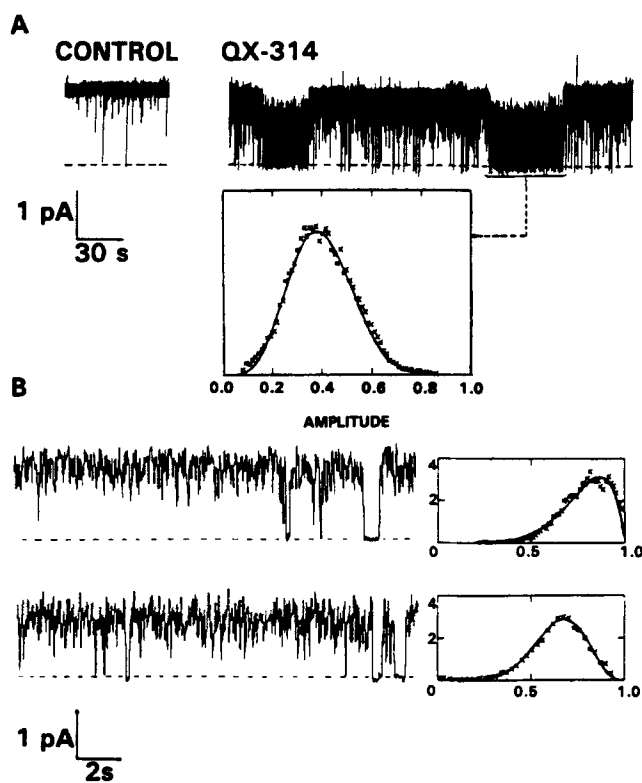


FIGURE 5. Various low- K_d periods reflect changes in the drug's k_{on} but not in the k_{off} rates. (A) Shown are current traces for a channel before (left) and in the presence of QX-314 (right) (30 Hz, +60 mV). The section indicated with the bar shows a constant level of QX-314-induced open channel block. This section was filtered at 10 Hz to obtain the amplitude distribution histogram shown below the current trace. Data points are indicated with x , and the continuous line is the fit of the data to a beta function from which a $k_{on} = 896 \text{ mM}^{-1}\text{s}^{-1}$ and a $k_{off} = 238 \text{ s}^{-1}$ were obtained. (B) Shown are two current traces for the same channel collected at +50 mV (10 Hz) during two different levels of channel block during the low- K_d period. To the right of each are their corresponding amplitude distribution histograms (x); the fit of the data for the top current trace gave a $k_{off} = 316 \text{ s}^{-1}$ and a $k_{on} = 219 \text{ mM}^{-1}\text{s}^{-1}$ and for the bottom current trace a $k_{off} = 339 \text{ s}^{-1}$ and a $k_{on} = 459 \text{ mM}^{-1}\text{s}^{-1}$.

the case of lidocaine, but not of QX-314, increasing the drug concentration (>1 mM) increased the rate of channel disappearance (not shown).

According to the model of Strichartz, the voltage dependence of the local anesthetic-induced open channel block gives the electrical distance from the intracellular channel entrance to the local anesthetic binding site (Strichartz, 1973). The resemblance in the voltage dependence between the high- and low- K_d periods (similar slopes in Fig. 4) suggests that the channel may display at least two open conformations in which the local anesthetic binding site seems to be located at the same electrical distance from the intracellular channel entrance, but the conformation of at least some of the

TABLE I
Changes in Low- K_d Values

A	K_d (mM) average	K_d (mM) amplitude	k_{off} (s^{-1})	k_{on} ($\text{mM}^{-1}\text{s}^{-1}$)
Channel a	0.24	0.27	238	896
Channel b	0.64	0.57	253	449
Channel c	0.57	0.59	291	494
Channel c	0.91	0.97	256	264

B	NaCl (M)	K_d (mM) amplitude	k_{off} (s^{-1})	k_{on} ($\text{mM}^{-1}\text{s}^{-1}$)
QX-314 ($n = 3$)	0.5	0.910 ± 0.27	304 ± 24	382 ± 81
Lidocaine ($n = 3$)	0.5	0.223 ± 0.007	477 ± 28	$2,124 \pm 78$
Lidocaine ($n = 4$)	0.2	0.101 ± 0.01	226 ± 30	$2,337 \pm 107$

(A) Changes in the QX-314's low- K_d values reflect changes in the binding (k_{on}) but not in the unbinding (k_{off}) rates. Data were collected in symmetrical 0.5 M NaCl at 160 mV. Data are shown for three channels (a, b, and c). (B) Apparent binding affinity (K_d), binding (k_{on}), and unbinding (k_{off}) rates of lidocaine at symmetrical 0.2 and 0.5 M NaCl and of QX-314 at symmetrical 0.5 M NaCl (at 150 mV). n = number of membranes containing single channels. Data = mean \pm SEM.

channel regions defining the local anesthetic binding affinity appear to be different.

Binding and Unbinding Rates for the Various K_d Periods in the Presence of QX-314

In an effort to understand the basis for the changes in the drug's K_d values, the drug's binding (k_{on}) and unbinding (k_{off}) rates were estimated by using the amplitude distribution analysis as described in Methods. Fig. 5 A shows current traces for a channel before (left) and after (right) the addition of QX-314. Amplitude histograms were constructed from sections of current traces that showed a constant level of drug-induced open channel block, as the one indicated with a bar in Fig. 5 A (right). The distribution of the data in the amplitude histograms was fitted to a beta function (continuous line), and from this fit the drug's k_{on} and k_{off} rates were obtained (see Methods). This method could be used when the channels displayed drug-induced low- K_d but not high- K_d periods (see Methods).

Table I shows some of the data obtained using the amplitude distribution method, and, for comparison, part A also shows the data obtained using the time-averaged current method. The estimated K_d values obtained with either method were equivalent.

In the presence of QX-314, a given channel could display more than one low- K_d level (Figs. 3 and 5 B). Fig. 5 B shows current traces for a single channel displaying two low- K_d levels while being held at +50 mV. To the right of these current traces, their corresponding amplitude distributions (x) and fits to the beta function (continuous lines) are shown. Changes in the QX-314's low- K_d values appear to reflect changes in the drug's k_{on}

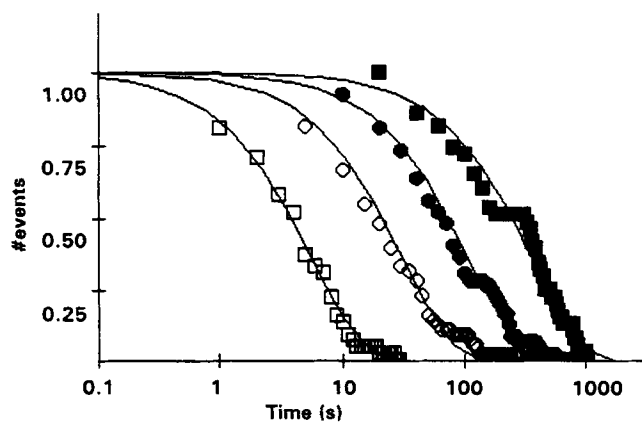


FIGURE 6. Stabilization of the low- K_d state with increasing concentrations of QX-314. Survival plots for the lifetimes of the high- (*open symbols*) and low- K_d (*filled symbols*) periods in the presence of 0.4 mM (*circles*) and 3.0 mM (*squares*) QX-314. (No. of events = normalized number of events with lifetime > time t). The membrane potential was held at +60 mV continuously, except for short periods when the membrane became unstable and the potential was lowered to prevent membrane rupture. When the QX-314 concentration was increased from 0.4 to 3.0 mM, the lifetime of the low- K_d period increased by 3.4 times (from 105 to 352 s), while the lifetime of the high- K_d period decreased by 5.4 times (from 30 to 5.5 s). The data for 0.4 mM came from six channels, and that for 3.0 mM came from two channels. The total number of events was 60 for the open circles, 52 for the filled circles, 48 for the open squares, and 43 for the filled squares.

rate but not in the drug's k_{off} rate. This was true for data collected from a given channel as well as for data collected from different channels (Fig. 5 B, Table I, part A). The absence of changes in the k_{off} rate suggests that the interactions between the drug and the binding region remain constant, while changes in the k_{on} rate suggest that channel regions defining the drug's access to the binding region undergo changes.

This analysis also shows that the difference between the low- K_d values of QX-314 and lidocaine resulted from QX-314 having a k_{on} rate that was about six times lower than that of lidocaine (Table I, part B). As expected, lowering the NaCl concentration from 0.5 to 0.2 M resulted in a decrease in lidocaine's low- K_d value. Such a decrease in the K_d value reflected mainly a decrease in the k_{off} rate (Table I, part B).

Stabilization of the Low- K_d State with Increasing Concentrations of QX-314 Is Consistent with the Presence of Two Open States with Different Drug Affinities

To investigate the underlying mechanism for the low- and high- K_d periods, we measured the drug concentration dependence of the lifetime of these periods. Fig. 6 shows survival plots for the lifetimes of the high- K_d periods (*open symbols*) and low- K_d periods (*filled symbols*) at

0.4 mM (*circles*) and 3.0 mM (*squares*) of QX-314. When the QX-314 concentration was increased from 0.4 to 3.0 mM, the lifetime of the low- K_d period increased by 3.4 times while the lifetime of the high- K_d period decreased by 5.5 times. This observation is consistent with a two-state model (Colquhoun, 1973); that is, the channel is able to exist in two open channel conformations even in the absence of drug, and the stabilization of one of the conformations (the low- K_d period) as the drug concentration is increased simply results from the drug having a higher affinity for that conformation (see Discussion).

Lidocaine and QX-314 Do Not Block Subconductance States

Rat brain sodium channels, in the absence of local anesthetics, displayed several levels of subconductance states; the predominant had a conductance value of ~ 4 –5 pS (Fig. 7, A and B, *top traces*), and it was observed in 63% of the experiments (in 26 out of 41 membranes; membranes contained one to two channels). Other less frequent subconductance states had a conductance value of ~ 16 pS observed in 24% of the experiments (in 10 out of 41 membranes). The noise level and the conductance value of the small subconductance state (~ 5 pS) was not changed by addition of lidocaine or QX-314 (Fig. 7, A and B, *bottom traces*). Fig. 7 C shows a histogram of the time-averaged conductance values for the small subconductance states observed before (*open bars*) and after (*filled bars*) the addition of lidocaine to a single channel. The time-averaged conductance value of the small subconductance state was not reduced by lidocaine, as would have been expected if lidocaine were blocking it with fast kinetics. This was observed in all of the channels undergoing small subconductance states. In fact, the mean conductance value for the small subconductance state in 0.5 M NaCl was the same during control (6.2 ± 0.5 pS, mean \pm SEM, $n =$ seven single channel membranes at 0.5 M NaCl) and in the presence of the local anesthetics (6.1 ± 0.4 , same seven single channel membranes). The same was true in 0.2 M NaCl (control: 5.2 ± 0.4 , lidocaine: 5.2 ± 0.4 , mean \pm SEM $n =$ four single channel membranes). This lack of drug effect on the small subconductance state was observed in all channels exposed to lidocaine and QX-314. On the other hand, the quiet large subconductance state (16 pS, same noise level as in control) was not observed in the presence of either local anesthetic. However, in the presence of local anesthetics in 2 out of 24 experiments, we observed a total of four noisy large subconductance states, suggesting the possibility that local anesthetics could block the large subconductance state. However, such events were so rare that we could not establish a clear correlation between them and the quiet large subconductance states observed

DISCUSSION

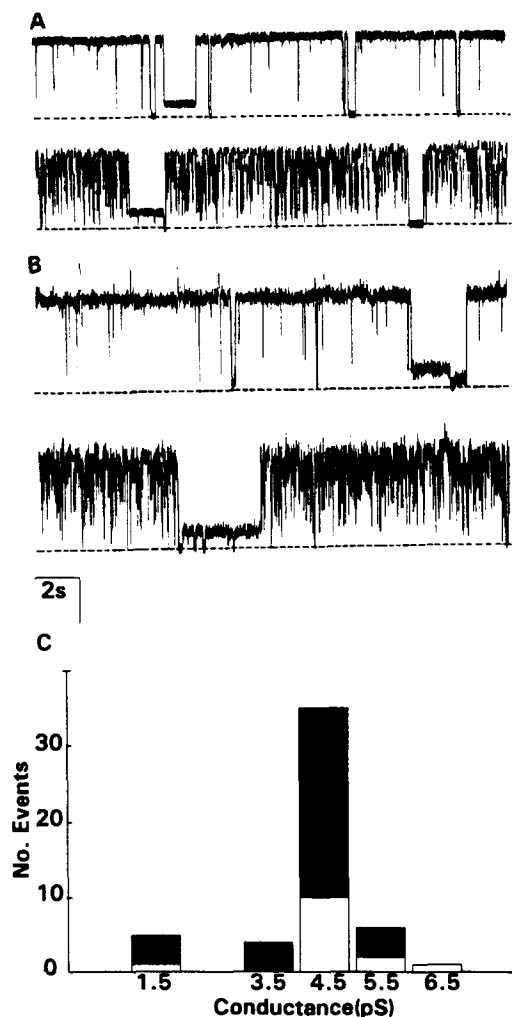


FIGURE 7. Lidocaine and QX-314 do not induce fast block of small subconductance states. (A) Current traces before (*top*) and in the presence of 30 mM lidocaine (*bottom*), (0.2 M NaCl, 50 Hz, +70 mV). (B) Current traces before (*top*) and in the presence of 0.4 mM QX-314 (*bottom*), (0.5 M NaCl, 50 Hz). Both during control and in the presence of local anesthetic, the channels displayed small subconductance states (~ 5 pS). Even though both local anesthetics produced high levels of block when the channel displayed its highest conductance (the fully open state became noisy), they did not change the noise level or time-averaged conductance level of the small subconductance state. (C) Cumulative histogram of small subconductance events before (*open bars*) and in the presence of 30 mM lidocaine (*filled bars*). Measurements were obtained within positive membrane potentials (+40 to +80 mV), in 0.2 M NaCl. At those potentials, the total observation time during control was 61.4 min, and in the presence of lidocaine it was 31.1 min.

during control. Local anesthetics induced fast block when the channels displayed their fully open state and probably large subconductance state but not when they displayed their low subconductance state.

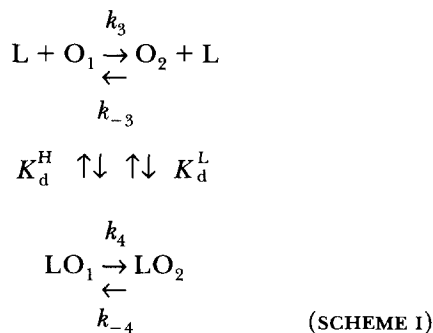
In this study, it was found that lidocaine and QX-314 detected various open channel conformations in brain sodium channels. Such open channel conformations became perceptible through reversible changes in the drugs' apparent binding affinity (K_d) for inducing their open channel block. It was also found that, while lidocaine and QX-314 blocked the fully open channel state(s) (~ 25 pS), they did not block the small subconductance channel state (~ 6 pS). We discuss how the presence of the low- and high- K_d periods is best explained by a two-open channel state model, and that the area defining the local anesthetic K_d consists of at least four interacting regions. In addition, changes in the apparent binding affinity values and absence of local anesthetic-induced block of the small subconductance state could be explained by the presence of channel conformational changes at the intracellular hydrophilic pathway leading to the anesthetic binding region.

Lidocaine and QX-314 have been reported to produce a constant level of fast-open channel block in various muscle-derived sodium channels (Moczydlowski et al., 1986a; Zamponi et al., 1993a; Salazar et al., 1995). However, we recently reported that lidocaine produced two levels of fast open channel block in brain channels (Salazar et al., 1995). In this study, we further characterize this phenomenon by using QX-314. We found that the various levels of lidocaine-induced open channel block do not require the presence of the uncharged form of lidocaine, since they were observed when the channels were exposed to the permanently charged lidocaine analogue, QX-314. Moreover, the presence of two affinity conformations does not reflect the presence of two channel types since they were observed in single channel-containing membranes.

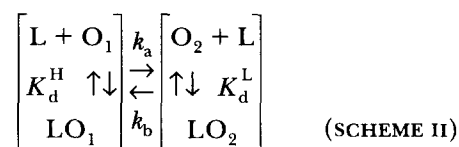
The behavior of the low- and high- K_d periods is consistent with the presence of two spontaneously occurring open channel conformations and with the local anesthetic binding affinity being defined by the interaction of various channel regions.

The rates of transition between both affinity periods (0.003 – 0.178 s^{-1}) were significantly slower than the local anesthetic k_{off} rates (200 – 500 s^{-1} range). Therefore, transitions between K_d periods are unlikely to reflect positional changes of the local anesthetic molecule within the binding region because such positional changes should be faster than the anesthetic's k_{off} rates (Gingrich et al., 1993). Even if present, such fast drug positional changes would not have been detected with the resolution of the bilayer system. It is also unlikely that the two K_d periods reflect the presence of two separate binding sites. In such a model ($L + O \leftrightarrow LO + L \leftrightarrow L_2O$; $L =$ QX-314, $O =$ open channel), the binding of the first ligand to form LO is characterized by a high affinity equilibrium constant, while the binding of the

second ligand to form L_2O is characterized by a lower affinity equilibrium constant. This model would not account for the detection of low affinity binding in the absence of high affinity binding; that is, it does not account for the presence of the high- K_d periods. A more probable model is one that assumes that the channel spontaneously exists in two freely interconvertible open channel conformations (O_1 and O_2):



In a two-open channel state model, when the binding steps ($O_1 + L \leftrightarrow LO_1$; and $O_2 + L \leftrightarrow LO_2$) are faster than the transition rates between the two open conformations ($O_1 \leftrightarrow O_2$, and $LO_1 \leftrightarrow LO_2$), a ligand is expected to increase the amount of time the channel spends in one of the open conformations only by having a higher affinity for that open conformation (e.g., O_2). With this model, low- K_d periods would reflect QX-314 binding and unbinding when the channel is in its high affinity open conformation ($O_2 + L \leftrightarrow LO_2$), whereas high- K_d periods would reflect QX-314 binding and unbinding when the channel is in its low affinity open conformation ($O_1 + L \leftrightarrow LO_1$). This model is also consistent with the observation that the time the channel spends on the low- K_d period ($[O_2] + [LO_2]$) increases, whereas the time the channel spends in the high- K_d period ($[O_1] + [LO_1]$) decreases as the concentration of QX-314 is increased. In the simplest two-state model, the channel area defining the local anesthetic K_d consists of only one region (Model 1). To determine whether the data could be explained by the simplest two-state model or whether a more complex two-state model would be required, we estimated the equilibrium constants between O_1 and O_2 ($K_3 = k_{-3}/k_3$) and between LO_1 and LO_2 ($K_4 = k_{-4}/k_4$) at +60mV by using Scheme II and Eqs. 4 and 5. The binding affinities (at +60 mV) when the channel was in its high affinity conformation (O_2 , $K_d^L = 0.58$ mM) and when the channel was in its low affinity conformation (O_1 , $K_d^H = 7.54$ mM) were obtained from the data shown in Figs. 2 and 5 C. These values were used to calculate the fractional time a given open state (O_1 or O_2) was blocked (f_b) and unblocked ($f_u = 1 - f_b$) in the presence of two concentrations of QX-314 (0.4 and 3.0 mM) by using Eq. 2 (in Results).

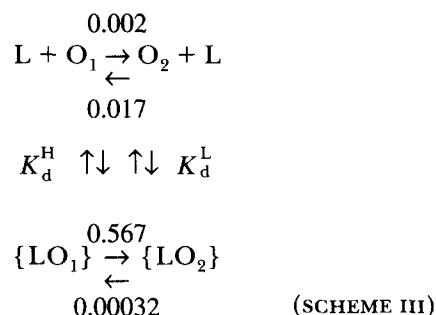


$$1/(\text{lifetime high-}K_d \text{ period}) = k_a = [O_1] * k_3 + [LO_1] * k_4$$

$$1/(\text{lifetime low-}K_d \text{ period}) = k_b = [O_2] * k_{-3} + [LO_2] * k_{-4}, \quad (4)$$

$$\begin{array}{l}
 \text{where } [O_1] = f_u \text{ during the } K_d^H \text{ period,} \\
 [LO_1] = f_b \text{ during the } K_d^H \text{ period,} \\
 [O_2] = f_u \text{ during the } K_d^L \text{ period,} \\
 [LO_2] = f_b \text{ during the } K_d^L \text{ period.}
 \end{array}
 \quad (5)$$

Solving Eqs. 4 and 5 gave the following equilibrium constant values for $K_3 = 8.5$ and $K_4 = 0.00056$. These values are inconsistent with the simplest two-state model (Model 1), since such a model predicts that $K_d^H * K_4 = K_d^L * K_3$, and that was not the case ($K_d^H * K_4 = 0.0042$; $K_d^L * K_3 = 4.93$) (Colquhoun, 1973). To indicate that a more complex two-state model is required to explain the data, we placed within braces the drug-bound open conformations in Scheme III ($\{LO_1\}$, $\{LO_2\}$):



Another indication that the simplest two-state model (Model 1) did not explain the data was that the measured fractional time the channel expended in the high affinity conformation [$FO_2 = ([O_2] + [LO_2]) / ([O_1] + [LO_1] + [O_2] + [LO_2])$] was significantly different from that predicted for Model 1 (Table II) which is given by Eq. 6 (Colquhoun, 1973) (Table II):

$$FO_2 = \frac{1}{1 + K_3 [(1 + A) / (1 + B)]},$$

$$\text{where } A = [L] / K_d^H ; B = [L] / K_d^L. \quad (6)$$

We considered other variations of two-state models (Colquhoun, 1973), again by assuming that the only effect of QX-314 was to bind to the two open channel conformations with different binding affinities. One of these models, Model 2, assumes that the channel area defining the local anesthetic binding affinity consists of more than one region ($n > 1$). Each region can exist in two conformations (x or x'), and all regions must be in the x' conformation for the channel to enter the O_2 state. Moreover, the conformation of each region is in-

TABLE II
Measured and Expected FO₂ Values Using the Two-State Model 1

[QX-314]	Model 1	
	Measured FO ₂	Expected FO ₂
<i>mM</i>		
0.4	0.655	0.158
3.0	0.983	0.341

The measured FO₂ was obtained by measuring all the time the channel expended in either state while being held almost continuously at +60 mV (see Fig. 6 legend). For 0.4 mM QX-314, the data were collected from six channels and the total observation time was 3.66 h. For 3.0 mM QX-314, the data were collected from two channels and the total observation time was 4.32 h.

dependent of all the other regions. Model 2 is described by Eq. 7 (Colquhoun, 1973). The measured FO₂ values were used to estimate the number of *n* channel regions. Model 2 does not fit the data, since the estimated *n* was <1 and significantly different (~10 times) when estimated at 0.4 and 3.0 mM QX-314 (Table III):

$$FO_2 = (1/\{1 + K_3[(1 + A)/(1 + B)]\})^n \quad (7)$$

The last model that was considered (Model 3) was as Model 2, but instead of being independent, the *n* regions are assumed to be linked in such a way that all *n* regions are constrained to adopt the same conformation. This model is described by Eq. 8 (Colquhoun, 1973):

$$FO_2 = \frac{1}{1 + K_3 \{ [(1 + A)/(1 + B)] \}^n} \quad (8)$$

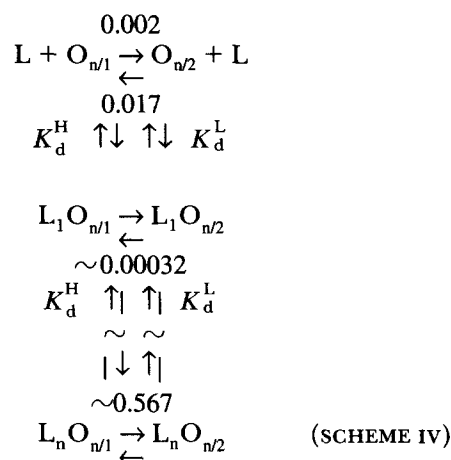
Model 3 gives a number of channel regions between 4 and 6 (Table III). The fact that the value varies between 4 and 6 may in part reflect that the data were collected from different channels (channel variability), that there may be more than two open channel states (as suggested by the detection of various low- and high-*K_d* periods), or that the FO₂ in 0.4 mM QX-314 was overestimated. In spite of these limitations, the data could be

TABLE III
Estimated Number of *n* Regions with the Two-State Models 2 and 3

[QX-314]	Model 2: estimated <i>n</i> = number of independent regions	Model 3: estimated <i>n</i> = number of interacting regions
<i>mM</i>		
0.4	0.23	5.9
3.0	0.02	4.1

The estimated number of regions defining the local anesthetic binding affinity (*n*) was done with Eqs. 5 and 6 for Models 2 and 3, respectively.

better fitted with Model 3 than with Models 1 and 2. Based on this finding, the overall equilibrium between {LO₁} ↔ {LO₂} (Scheme III) was expanded in Scheme IV. In Scheme IV, the subscript *n* is the number of regions that are linked. Since the *n* regions are constrained to adopt the same conformation, there are still only two open states, O_{*n*1} (low affinity state) and O_{*n*2} (high affinity state). The subscript *i* on L (L_{*i*}) indicates the number of *n* regions with which L is interacting. The equilibrium constants between L_{*i*}O_{*n*1} ↔ L_{*i*}O_{*n*2} (*i* > 0) are defined by the other equilibrium constants in the following way: *K₃*(*K_d^L*/*K_d^H*)^{*i*} (Colquhoun, 1973). Estimation of the expected equilibrium constants for *i* between 1 and 6 (Table IV) indicates that the equilibrium constant between the low and high affinity conformations becomes smaller as the number of *n* regions increases. The lifetime of the high-*K_d* period would then be dominated by the transition rate from L_{*n*}O_{*n*1} → L_{*n*}O_{*n*2} and the lifetime of the low-*K_d* period would be dominated by the transition rate from L₁O_{*n*2} → L₁O_{*n*1}.



Our findings indicate that the low- and high-*K_d* periods reflect the presence of at least two open channel conformations with the same conductance but differ-

TABLE IV
Estimated Equilibrium Constants for Transitions between QX-314-bound Open States for Model 3

Model 3	Estimated equilibrium constants
L ₁ O _{<i>n</i>1} ↔ L ₁ O _{<i>n</i>2}	0.657
L ₂ O _{<i>n</i>1} ↔ L ₂ O _{<i>n</i>2}	0.050834
L ₃ O _{<i>n</i>1} ↔ L ₃ O _{<i>n</i>2}	0.003931
L ₄ O _{<i>n</i>1} ↔ L ₄ O _{<i>n</i>2}	0.000304
L ₅ O _{<i>n</i>1} ↔ L ₅ O _{<i>n</i>2}	0.000024
L ₆ O _{<i>n</i>1} ↔ L ₆ O _{<i>n</i>2}	0.0000018

The equilibrium constants between L_{*i*}O_{*n*1} → L_{*i*}O_{*n*2} (*i* > 0) were estimated using the following relation: *K₃*(*K_d^L*/*K_d^H*)^{*i*}, and the following measured values: *K_d^L* = 0.58 mM, *K_d^H* = 7.5 mM, and *K₃* = 8.5.

ent local anesthetic binding affinities. In addition, it indicates that the local anesthetic binding area is defined by at least four interacting channel regions. One such region has already been identified as segment 6 of domain IV in rat brain sodium channels (Ragsdale et al., 1994). The other regions may involve segment 6 in the other three channel domains; however, this remains to be determined.

Changes in the Low- K_d Values and the Lack of Local Anesthetic Block of the Small Subconductance State Suggest Channel Conformational Changes at the Intracellular Permeation Pathway

When exposed to QX-314, the channels displayed more than one low- K_d period. Most of the QX-314's low- K_d values were larger than the one observed with lidocaine. However, some of the QX-314's low- K_d values were as low as the one observed with lidocaine. The open conformation with the highest local anesthetic affinity was then detected by both of these local anesthetics. However, even when the low- K_d value was the same, the binding and unbinding rates were about two times lower for QX-314 than for lidocaine (not shown). This may reflect differences in the interactions between the molecules and the binding region, as well as differences in the number of routes the anesthetic molecule uses to access its binding region. Lidocaine can access the binding region through a hydrophilic and a hydrophobic route, whereas QX-314 can access the binding region only through the hydrophilic route (Hille, 1977). For a given time period, fewer molecules will enter and leave the channel; hence, the binding and unbinding rates will be lower for QX-314 than for lidocaine.

At a given membrane potential, the various low- K_d values detected with QX-314 result from changes in the QX-314's k_{on} rate without changes in its k_{off} rate. This observation is consistent with the idea that the QX-314 molecule senses changes in the conformation of the hydrophilic path leading to the binding region from the intracellular channel aspect. At a given membrane potential in the presence of lidocaine mainly one low- K_d value was detected. The conformational changes at the hydrophilic path seem to be detected more easily by QX-314 than by lidocaine, probably because the QX-314 molecule is slightly larger and is permanently charged. The various low- K_d periods probably reflect intermediate channel open conformations between those displaying the highest (lowest- K_d) and lowest (high- K_d) drugs' affinities. We were unable to obtain accurate binding and unbinding rates during the high- K_d periods because of the low level of drug-induced open channel block during those periods. However, based on the kinetic analysis from the various low- K_d periods, we favor the idea that the high- K_d period reflects an

open conformation in which the hydrophilic path leading to the local anesthetic binding region has been significantly changed (narrowed) to a level that still allows but decreases the rate of entry of the large anesthetic molecules without affecting the rate of entry of the small sodium ions (same maximal conductance). We propose as the underlying mechanism for the various K_d periods the presence of various fully open channel conformations whose hydrophilic path leading to the binding site is different in conformation (different k_{on} rates), whereas the binding site mediating the drug's open channel block has the same conformation (same k_{off} rates) and electrical location (same voltage dependence).

The small subconductance state (~ 6 pS) was not blocked by lidocaine or QX-314. It has been proposed that the hydrophilic pathway used by QX-314 and by the charged form of lidocaine is the intracellular aspect of the channel pore (Hille, 1977). The lack of drug-induced block suggests that the underlying mechanism of the small subconductance state may involve a significant narrowing of the intracellular pore entrance. Such narrowing will not only block the entry of larger ions such as QX-314 and lidocaine, resulting in the elimination of their capacity to block the channel, but it will also slow down the entry and passage of smaller ions such as sodium, resulting in the decrease of the channel's conductance (from ~ 26 to 6 pS). Since lidocaine can access the binding site through both a hydrophilic and a hydrophobic path, the narrowing of the hydrophilic path should lead to a decrease but not an elimination of the lidocaine-induced block. Therefore, the lack of detectable lidocaine-induced block when the channel enters the small subconductance state most likely reflects the small level of uncharged molecules. However, we cannot rule out the presence of additional conformational changes either at the hydrophobic path or at the binding region.

Increasing the concentration of NaCl decreased lidocaine's K_d mainly through a reduction (50%) in the k_{off} rate and partly through a small increase in the k_{on} rate (Table I, part B). This is consistent with previous observations in which it was found that increasing the external Na^+ concentration reduced the K_d of internally applied QX-314 (Cahalan and Almers, 1979) and of cocaine (Wang, 1988). The decrease in the local anesthetic's K_d cannot be explained by a strict competition where Na^+ should only reduce the anesthetic k_{on} rate without affecting the anesthetic k_{off} rate (Wang, 1988). The increase in the k_{off} rate could reflect electrostatic repulsion within the channel pore; however, indirect interactions between Na^+ and the local anesthetic binding site cannot be ruled out (Wang, 1988).

In the presence of either lidocaine or QX-314, the quiet large subconductance state (~ 16 pS) was not ob-

served. This could mean that local anesthetics decreased the rate of transition from the fully open to the large subconductance state or, as suggested by some of the current traces, that local anesthetics induced block of the large subconductance state and consequently would appear as noisy rather than quiet large subconductance states. In either case, these results indicate that the underlying mechanism for the large subconductance state must be different from the one proposed for the small subconductance state.

The drug's low- and high- K_d periods were long-lived (seconds to minutes), and such long lifetimes resemble those displayed by the various channel activation-gating modes (Chabala et al., 1991). In fact, all the studied channels showed activation-gating modes (not shown). Based on this resemblance, one could postulate the presence of various open channel states with different equilibrium rates with the closed (resting) state as the underlying mechanism for the activation-gating modes. Brain- (Chabala et al., 1991) and muscle-derived (French et al., 1986; Moczydlowski et al., 1984; Recio-Pinto et al., 1987) sodium channels displayed activation-gating modes, whereas only brain channels displayed various anesthetics' K_d periods. This suggests that, in muscle-derived sodium channels, the various open channel states have different equilibrium with the resting state but the same affinity for local anesthetics, whereas in brain channels these open states have different local anesthetic affinity and different equilibrium with the resting state. Further studies are required to establish whether there is a correlation between the presently observed local anesthetic's K_d periods and the previously observed channel activation modes.

In the rat brain sodium channel II, eight amino acids

have been identified at the transmembrane segment S6 of domain IV to be involved in defining the action of local anesthetics (Ragsdale et al., 1994). Such amino acids are identical in brain- and muscle (mammalian skeletal and cardiac muscle, and eel electroplax)-derived sodium channels. Therefore, these amino acids by themselves do not account for the presence of various levels of lidocaine- and QX-314-induced open channel block in brain- but not in muscle-derived channels (Salazar et al., 1995, and this study). Other amino acid residues or channel structures (β -subunits, post-translational modifications) must be involved in defining the number of the drug's K_d periods.

In summary, the data in this study suggest that the various levels of lidocaine (or QX-314)-induced open channel block may reflect the presence of at least two open channel conformations that display the same conductance but different anesthetic binding affinities, and that the local anesthetic binding area is defined by at least four interacting channel regions. Kinetic analysis indicates that the various proposed open channel states appear to display conformational differences at the hydrophilic intracellular pathway that lead to the local anesthetic binding region (changes in k_{on} rates), whereas the conformation of the binding region (same k_{off} rates) and its location (same voltage dependence) seem to remain constant. Finally, a narrowing of the intracellular pore entrance could explain the decrease in channel conductance and lack of drug-induced block when the channel displays the small subconductance state. The latter supports Hille's model (1977) that the intracellular hydrophilic pathway used by local anesthetics is the channel pore itself.

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