Fast Lidocaine Block of Cardiac and Skeletal Muscle Sodium Channels: One Site With Two Routes of Access

Gerald W. Zamponi,* Donald D. Doyle,‡ and Robert J. French*

*Department of Medical Physiology and Neuroscience Research Group, University of Calgary, Calgary, Alberta, T2N 4N1 Canada;
‡Department of Medicine, The University of Chicago, Chicago, Illinois 60637 USA

ABSTRACT   We have studied the block by lidocaine and its quaternary derivative, QX-314, of single, batrachotoxin (BTX)-activated cardiac and skeletal muscle sodium channels incorporated into planar lipid bilayers. Lidocaine and QX-314, applied to the intracellular side, appear to induce incompletely resolved, rapid transitions between the open and the blocked state of BTX-activated sodium channels from both heart and skeletal muscle. We used amplitude distribution analysis (Yellen, G. 1984. *J. Gen. Physiol.* 84:157–186.) to estimate the rate constants for block and unblock. Block by lidocaine and QX-314 from the cytoplasmic side exhibits rate constants with similar voltage dependence. The blocking rate increases with depolarization, and the unblocking rate increases with hyperpolarization. Fast lidocaine block was virtually identical for sodium channels from skeletal (rat, sheep) and cardiac (beef, sheep) muscle. Lidocaine block from the extracellular side occurred at similar concentrations. However, for externally applied lidocaine, the blocking rate was voltage-independent, and was proportional to concentration of the uncharged, rather than the charged, form of the drug. In contrast, unblocking rates for internally and externally applied lidocaine were identical in magnitude and voltage dependence. Our kinetic data suggest that lidocaine, coming from the aqueous phase on the cytoplasmic side in the charged form, associates and dissociates freely with the fast block effector site, whereas external lidocaine, in the uncharged form, approaches the same site via a direct, hydrophobic path.

INTRODUCTION

Class 1 antiarrhythmic agents, widely used for treatment of patients after cardiac infarction, have been known to block sodium channels for nearly four decades (1). Historically they have been divided, based on their blocking kinetics, into three subgroups, 1a, 1b, and 1c (for a review, see Ref. 2) with class 1b including the fastest blockers (Camm et al. (3) provide a more recent discussion of antiarrhythmic classification). The common class 1b drug, lidocaine, has been shown to block neuronal (4–7) and cardiac (8) sodium channels as well as batrachotoxin (BTX)-activated skeletal muscle (9, 10) sodium channels.

Single sodium channels, activated by BTX and incorporated into planar lipid bilayers, provide a convenient model system for studies of channel block. Even though quantitative data must be viewed with the knowledge that BTX modifies many channel properties, this system offers the particular advantage that drug interactions with different channel isoforms may be compared under identical conditions. We have observed two modes of lidocaine block at millimolar concentrations of the drug. Slow blocking fluctuations occur on a time scale of seconds and appear to be specific for the cardiac sodium channel isoform. This slow blocking mode is analyzed in the companion paper (11). Fast block, by either lidocaine or its quaternary derivative, QX-314, was seen as a noisy reduction in the open channel current.

In previous bilayer studies, lidocaine eluded a detailed kinetic analysis because of the difficulty of resolving the fast blocking fluctuations. Cocaine, another local anesthetic, shows a higher affinity for BTX-activated sodium channels, binding with 1:1 stoichiometry for long enough to produce discretely resolved blocking events (12). Depolarization enhances cocaine block, consistent with the idea that the charged form of the drug enters and leaves freely via the cytoplasmic mouth of the channel (12, 13). Since the permanently charged lidocaine derivative, QX-314, and cocaine compete for the same binding site, one might expect to find a similar mechanism and voltage dependence for lidocaine block. We estimated the rate constants for fast block using the amplitude distribution analysis developed by Yellen (14). The analysis revealed essentially identical fast block of cardiac and skeletal channels and provided evidence for two paths of access by lidocaine to the fast block effector site.

Applied internally, QX-314 exhibits blocking kinetics and voltage dependence similar to internally applied lidocaine, suggesting a common receptor and mechanism. Permanently charged QX-314 cannot diffuse easily across the membrane and is only effective when applied from the intracellular side of the channel. Lidocaine, however, in its uncharged form, is thought to be highly permeant through biological membranes, and hence is also effective when applied from the extracellular side. Based on a difference between the voltage-dependence of blocking rates for internally and externally applied lidocaine, and the dependence of the block on the charged and uncharged forms of the drug, we suggest that the receptor responsible for fast block can be reached via two independent pathways and is accessible directly from the extracellular side of the channel (cf. Ref. 7). The novel contribution of this study is to provide direct evidence, from

single-channel analysis, that a single, well-characterized mode of block is effected via those two pathways.

MATERIALS AND METHODS

Membrane preparations

Plasma membrane fractions from adult bovine heart, adult rat skeletal muscle, as well as lamb skeletal muscle from a 30-day-old animal were prepared as previously described by Guo et al. (15). Membrane fractions from sheep heart were prepared as described by Doyle and Winter (16). Membrane vesicles (protein concentrations: 2 mg/ml for rat skeletal muscle membranes from three preparations; 2–2.5 mg/ml for bovine heart membranes from two preparations; 3.5–18 mg/ml for sheep heart membranes from three preparations; 0.2 mg/ml for lamb skeletal muscle membranes from one preparation) were incubated with 6 μM BTX, stored at $-20°C$, removed from the freezer and stored on ice for 8 h on each of 4 days. The preparations were then successfully used to incorporate channels into bilayers for up to 1 week.

Bilayer methods

Unless otherwise stated, experiments were performed at room temperature ($\approx$22°C), under symmetric conditions in a chamber containing 1.5 ml of 200 mM NaCl and 20 mM 4-morpholinepropanesulfonic acid (MOPS) buffered to pH 7.0. Bilayers made of uncharged synthetic lipids (40 mg/ml 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine and 10 mg/ml 1-palmitoyl-2-oleoylphosphatidylcholine in decane) were formed on a Teflon partition with hole diameters between 120 and 250 μm. Between 5 and 20 μl of preparation were added to the cis side of the chamber which was continuously stirred. Voltages were alternated between +70 mV and −70 mV. Channel incorporation was observed as an increase in membrane conductance. Only single channel incorporations were used for experiments. The channel orientation was determined according to the gating behavior of the channel (17).

Single channel recordings

The bilayer was voltage-clamped, and the transmembrane current was measured using an Axopatch 200 amplifier (Axon Instruments Inc., Foster City, CA). All current traces were filtered at 1 KHz and stored on videotape, and they were later low-pass filtered during transcription via an Axolab-1 Interface (Axon Instruments Inc.) into a Compaq 386 personal computer for analysis (see Data Analysis).

Lidocaine was dissolved in the presence of MOPS to give a stock solution of 200 mM lidocaine/200 mM MOPS at pH 6.7. QX-314 was dissolved in water to make a stock solution of 200 mM QX-314. The drug solution was added to the chamber facing the extracellular or intracellular side of the channel. In general, in experiments involving lidocaine, the pH of the bath did not drop below 6.9, thus the associated error in lidocaine activity was less than 2%. In one experiment, the pH of the solution was deliberately allowed to vary in order to vary the ratio of charged to uncharged forms of lidocaine (see Fig. 7).

Data analysis

For analysis, data were acquired for up to 1 min at each voltage. Blocking events were analyzed using the amplitude distribution analysis described by Yellen (14). Data were filtered at 200 Hz using an 8-pole Bessel filter and sampled at 500 Hz during transcription from the videotape to the PC. pCLAMP software was used to generate amplitude histograms which were normalized, baseline-subtracted, and subsequently fitted with a filter output density function in MathCAD (MathSoft Inc., Cambridge, MA). The rate constants were determined from the best fit by eye. The procedure was checked with the use of CSIM software (Axon Instruments Inc.) by simulating three sets of data with rate constants similar to the ones determined for fast lidocaine block and with noise characteristics comparable with our

experiments. Rate constants obtained from blind fits to the simulated histograms fell within 20% of the simulation parameters. In some cases, the fractional block and the equilibrium dissociation constants were determined independently of the kinetic fits by subtracting the baseline and then calculating the ratio of the mean currents in presence and absence of the drug.

Fitting of data points and preparation of figures was conducted using Sigmaplot (Jandel Scientific, Corte Madera, CA).

RESULTS

Internal lidocaine causes fast block

In the absence of lidocaine and in the voltage range used for this study (−60 to +60 mV), BTX-activated sodium channels are usually on the plateau of their activation curve and show an approximately constant open probability, P_{open}, as observed in earlier studies (e.g., Refs. 18 and 19). Skeletal muscle channels were generally open most of the time ($P_{open} > 0.9$), whereas the maximal open probability for cardiac channels was generally somewhat lower (e.g., we observed $P_{open} = 0.62 \pm 0.15$, mean $\pm$ SD for five bovine cardiac channels at −40 mV). The skeletal muscle sodium channels show closures due to gating which are usually briefer than 20 ms. Cardiac sodium channels exhibit an additional slower gating component with a dwell time constant of up to several hundred milliseconds, the closed time distribution generally requires an additional time constant for fitting (Ref. 20, and manuscript in preparation) compared to the skeletal muscle channels. The spontaneous gating behavior is illustrated in the control traces in Figs. 1 and 9. Internally applied lidocaine induces fast blocking which is favored by depolarizing voltages (Fig. 1, A and B). Blocking events were not resolved as discrete steps, but rather as a broadening of the current amplitude distribution and a reduction in the time-averaged, apparent single channel conductance. Hence, it was not possible to directly determine the individual rate constants for block and unblock. However, the rate constants could be estimated from the shape of the amplitude distribution in presence of the blocker. The theoretical shape of this distribution is mathematically described by a filter output density function with two parameters which are directly proportional to the blocking and unblocking rate constants (14). Hence, a fit of the amplitude distribution with the theoretical function (see Fig. 2) provides estimates of the association and dissociation rate constants for fast block.

Fast block by internal lidocaine is voltage-dependent and not tissue-specific

Both bovine cardiac and rat skeletal muscle channels exhibit a similar fast block by internal lidocaine. The voltage dependence of the rate constants for each channel type is displayed in Fig. 3. Block is enhanced at depolarizing potentials (Fig. 3 A), unblock is favored at hyperpolarizing voltages (Fig. 3 B). This is consistent with a positively charged blocker entering the transmembrane electric field to reach the blocking site. The voltage sensitivity of the K_d values (which have been calculated from the individual rate constants) reflects apparent electrical distances ($z\delta$) of the binding sites,
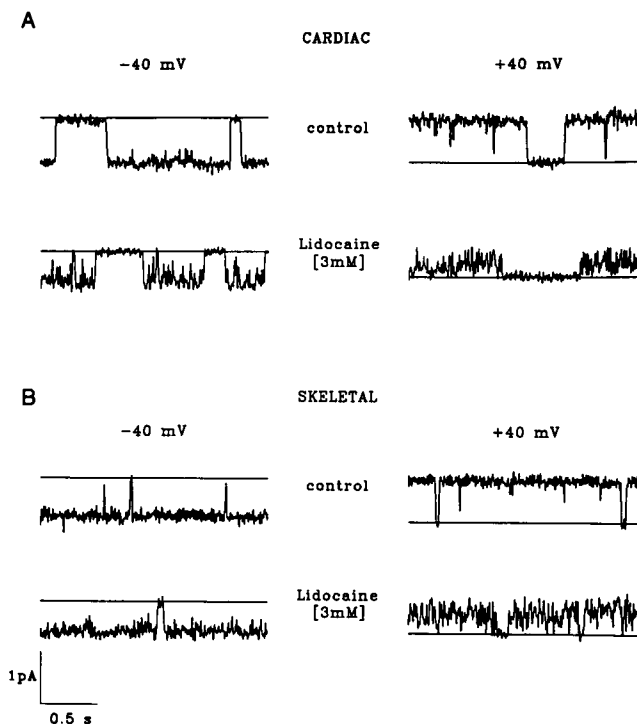
FIGURE 1   Traces recorded from BTX-activated sodium channels of (A) bovine heart, or (B) rat skeletal muscle in absence and presence of lidocaine. In the absence of the drug both channel types are open most of the time at voltages more positive than −60 mV. As seen above, internally applied lidocaine acts on both channel types in a similar manner by inducing rapid blocking events. At this bandwidth (50 Hz) the fast blocking events cannot be resolved as rectangular steps, but rather appear as a flickery open state with a reduced single channel amplitude. The solid lines indicate the closed level.

from the cytoplasmic side, of 0.33 and 0.28 for the cardiac and skeletal muscle sodium channels, respectively (Fig. 3 C). The mean values of rate and equilibrium constants for the two channel types at $E = 0$ mV are presented in Table 1.

Fig. 4 A depicts dose-response curves for fast lidocaine block of sodium channels from rat skeletal muscle, bovine heart, lamb skeletal muscle, and sheep heart. The open probability was calculated from the rate constants, plotted as a function of the logarithm of lidocaine concentration and fitted with a simple hyperbola (see legend to Fig. 4) which implies a 1:1 interaction between lidocaine and the binding

TABLE 1   Fast block from the intracellular side

	K_d	k_{on}	k_{off}	$z\delta$	Number of points
	[mM]	[1/mM^{-s}]	[1/s]		(Number of exp.)
Lidocaine					
Heart	4.1	1,985	8,185	0.38	34(3)
Skeletal muscle	4.2	2,356	9,941	0.33	27(4)
QX-314					
Heart	6.4	1,762	10,641	0.32	22(3)
Skeletal muscle	5.1	1,665	8,511	0.25	36(5)

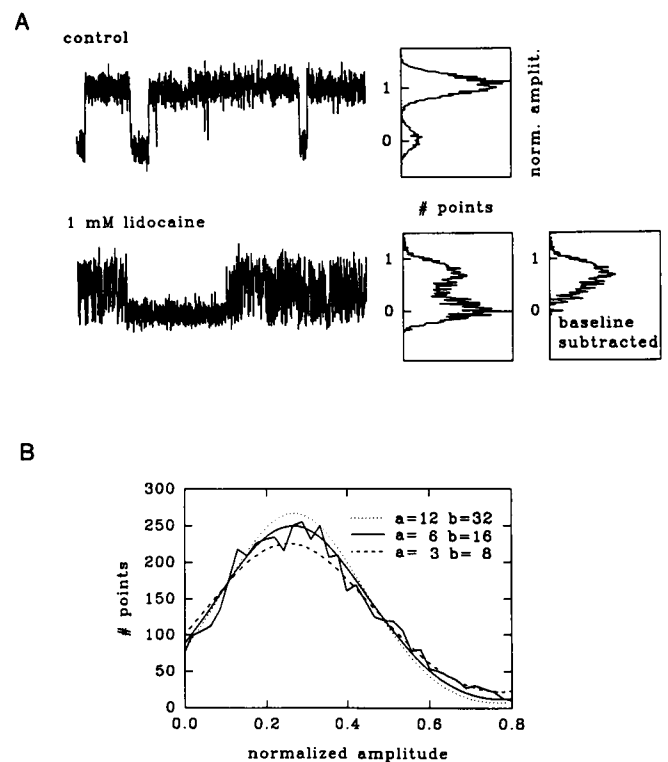Equilibrium and kinetic parameters were measured at 0 mV.

FIGURE 2   (A) Extraction of histograms for the amplitude distribution analysis as introduced by Yellen (14). The current amplitude distributions of the channels in the absence and presence of the drug are determined. The mean amplitude of the open channel under drug-free conditions is defined as 1, the closed level as 0. Lidocaine reduces the apparent current amplitude, and the position of the peak of the amplitude density shifts to values smaller than one. The extent of the shift reflects the equilibrium dissociation constant of the drug binding. The peak representing full channel closures due to gating is digitally subtracted. (B) A normalized, baseline-subtracted amplitude histogram is fitted with a filter output density function. The fitting parameters b and a are related to the rate constants for block and unblock by lidocaine ($k_{on} = f_c b/0.228$ and $k_{off} = f_c a/0.228$ with $f_c = 200$ Hz being the corner frequency for 3-dB attenuation). The solid line represents the best fit by eye, the dashed lines demonstrate the deviation from the best fit upon variation of the fitting parameters, while their ratio (and hence the equilibrium dissociation constant) is held constant. Sheep heart, + 40 mV.

site. The concentrations at $P_{open} = 0.5$ are close to the K_d values directly obtained from the ratios of the rate constants. The K_d values for the four curves span a range of less than 1.5 mM, hence the potency of internally applied lidocaine is virtually identical for channels from these four preparations. This suggests that the binding site is conserved.

The concentration- and voltage-dependent reduction in apparent single channel amplitude can be used to confirm K_d values and electrical distances independent of the assumptions of the beta distribution analysis. An example for the bovine cardiac sodium channel is shown in Fig. 4 B. The curve was fitted with a Boltzmann relation (see legend to Fig. 4), the $z\delta$ value (0.33) estimated from the fit is close to the value obtained from the regression in Fig. 3 C. We have also applied this method to determine open probabilities and construct dose-response curves. The results obtained were similar to those in Fig. 4 A (data not shown).
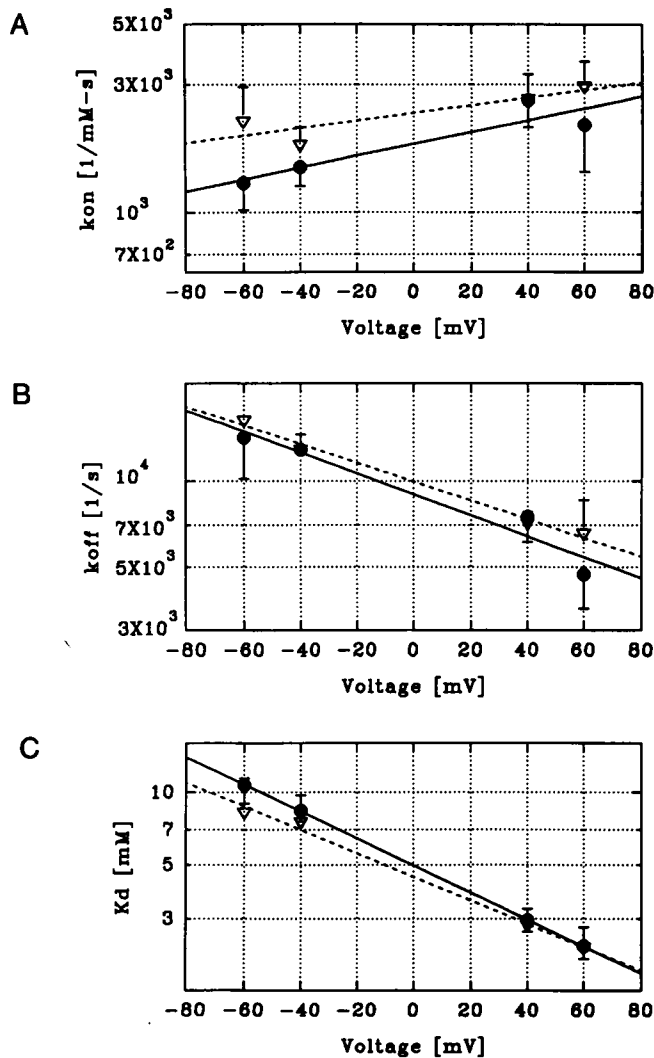
FIGURE 3 Voltage dependence of the rate constants and equilibrium dissociation constant for fast lidocaine block of bovine cardiac (*circles, solid lines*) and rat skeletal muscle sodium channels (*triangles, dashed lines*). Rate constants were obtained by fitting normalized amplitude histograms as described in the legend to Fig. 2. Solid lines are least square fits of the data, error bars (skeletal muscle upward, cardiac downward) indicate standard deviations. (*A*) The block rates are increased by depolarization as expected for an internally applied, positively charged blocker that enters the transmembrane electric field. (*B*) The unblock rates are increased by hyperpolarization. (*C*) The equilibrium dissociation constants, K_d, were calculated from the ratio of unblock to block rate. The K_d changes e-fold over a voltage range of 77 mV. for the cardiac channels and 90 mV for the skeletal muscle channels which places the binding sites 33 and 28% of the way through the transmembrane voltage (29) from the cytoplasmic end of the channel.

Fast block by external lidocaine shows weaker voltage dependence

Application of lidocaine to the external side of the channel results in fast blocking fluctuations similar to those observed for internal lidocaine. It has been shown that the permanently charged lidocaine derivative QX-314, which induces fast block similar to the one exhibited by lidocaine, is only effective when applied to the intracellular side of the channel

(12). These results suggested that fast block by lidocaine, when applied to the extracellular side of the channel, would require the drug to pass through the membrane and then act as an internal blocker. Hille (6) suggested a diffusion coefficient for lidocaine in lipid bilayers in the order of 0.5 cm²/s, implying that the drug would reach the internal end of the channel almost as rapidly as if the bilayer were not present. Thus, one would expect rate constants and voltage dependencies almost identical to the ones for internally applied lidocaine.

Our results, however, contradict this prediction. Fig. 5 compares the rate constants for fast block by internally versus externally applied lidocaine for bovine cardiac sodium channels from five experiments. The unblocking rates are virtually identical, but the blocking rate appears to show little or no voltage dependence. The lack of voltage dependence of the blocking rate after external application suggests that the drug does not rapidly cross the membrane and then act in its charged form from the intracellular side of the channel.

This is supported by the observation that the effect of lidocaine was reversed only when the drug was washed out from the side to which it was applied. Perfusing the opposite side of the chamber had little or no effect (data not shown). Thus, there was no significant accumulation of lidocaine in the internal chamber during the time course of the experiment.

Fig. 6 shows current traces from a single experiment in which lidocaine was applied to each side of a skeletal muscle channel. Block from the extracellular side is significantly weaker at positive potentials. Note the substantial difference between mean currents in the presence of internally and externally applied drug at +35 mV. At −45 mV, the difference between internal and external application is less obvious. This is further reflected in K_d values determined, at +60 mV and −60 mV, from our collected data (see Table 2). At −60 mV, internally applied lidocaine is only marginally more potent than externally applied lidocaine. When the voltage is changed to +60 mV, potency increases about 5 × for internal lidocaine, but only about 3 × for external lidocaine. Consistent with Figs. 5 and 7, this represents a weaker voltage dependence of equilibrium block from the extracellular side.

Fig. 7 shows the voltage dependence of rate constants (Fig. 7, *A* and *B*) and of steady state fractional block (Fig. 7 *C*) for fast block by external and internal lidocaine of the skeletal muscle subtype. In this case, block from either side was studied on the same channel. Fig. 7 *C* shows the voltage dependencies for the block from both sides determined independently of the beta distribution analysis. The value of $z\delta$ estimated from the degree of block is reduced from 0.3 for internal application to 0.25 for external application. This reflects the loss of voltage dependence of the blocking rate, but not of the unblocking rate (see Fig. 7, *A* and *B*). In contrast with lidocaine, QX-314 showed no effect when it was applied to the extracellular side of either channel subtype. Overall, these data suggest the possibility of two independent
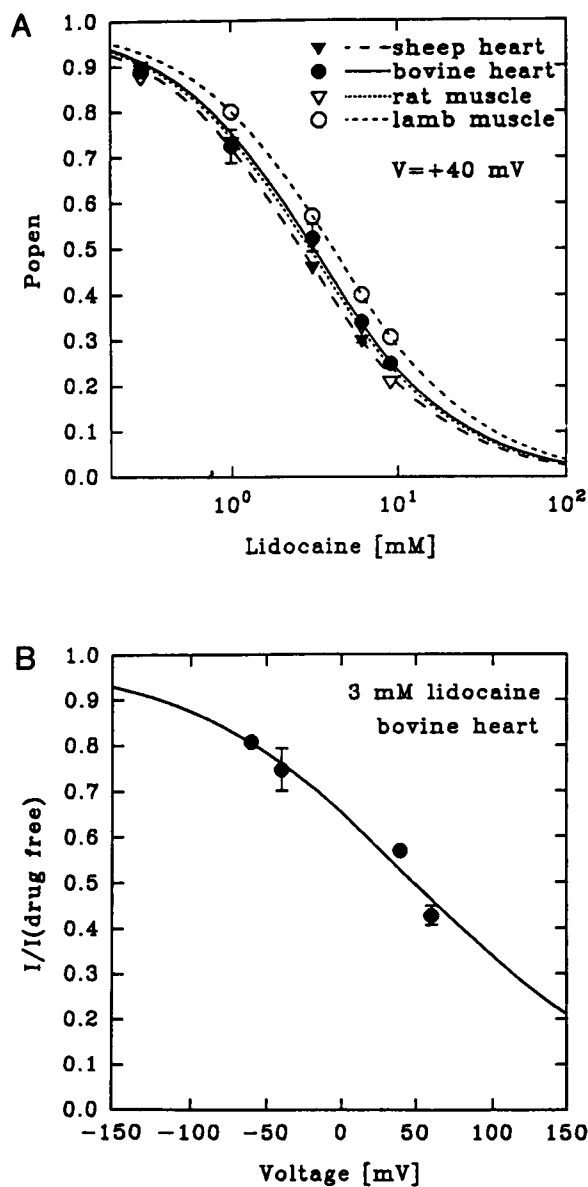
FIGURE 4 (A) Dose-response curves for fast internal lidocaine block of sodium channels from various tissues. The open probability was calculated from the rate constants [$P_{open} = k_{off}/(k_{on} + k_{off})$]. The data were fitted with a single hyperbola $P_{open} = 1/(1+[L]/K_d)$, where [L] is the drug concentration and K_d is the equilibrium dissociation constant. The K_d values at this potential (+40 mV) for lamb skeletal muscle, rat skeletal muscle, bovine heart, and sheep heart are 4.0, 2.9, 3.1, and 2.6 mM, respectively. Upward and downward error bars indicate standard deviations for the bovine cardiac and the rat skeletal muscle channels respectively. (B) The voltage dependence of fast block of the bovine cardiac sodium channel by 3 mM internal lidocaine. The data points represent the mean, baseline-subtracted current in the presence of 3 mM internal lidocaine and are obtained without the use of the beta distribution analysis. The data points were fitted with a Boltzmann relation ($P_{open} = 1/\{1 + \exp[z\delta(V - V_H)/25.4]\}$), where V is the membrane potential, V_H is the potential at half block, and $z\delta$ is the proportional to the slope of the fit. The value of $z\delta$ indicates that the binding site is located 33% across the transmembrane voltage from the cytoplasmic end of the channel. This is consistent with the results obtained in Fig. 3.
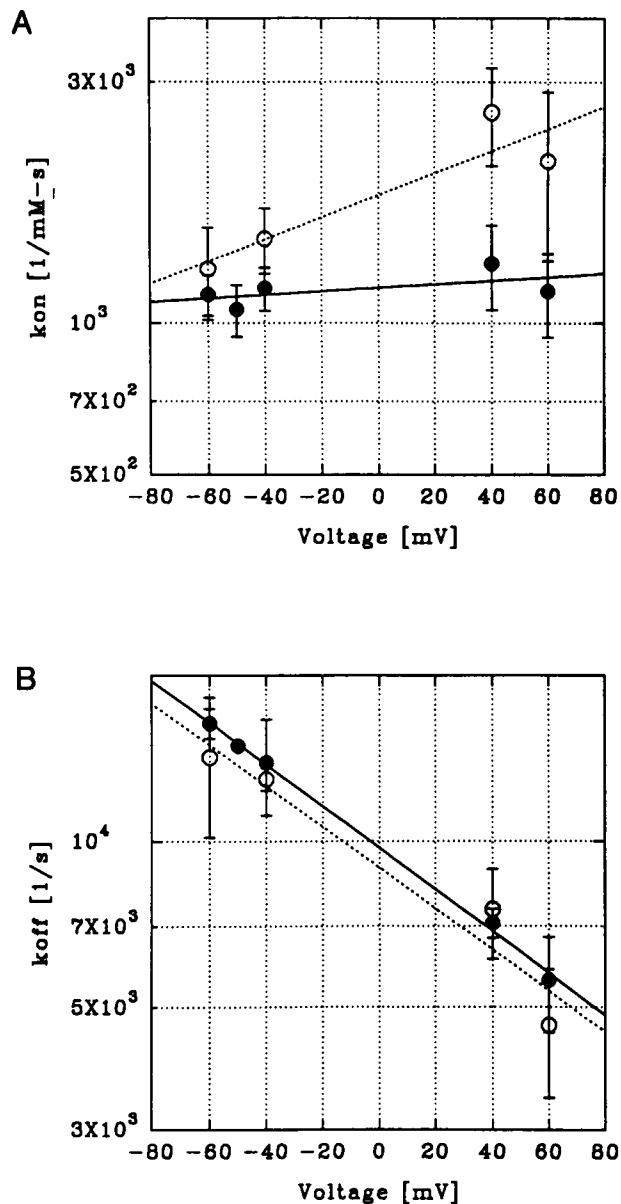
FIGURE 5 Comparison between the voltage dependence of fast block of internally applied (open circles, dashed lines) versus externally applied (filled circles, solid lines) lidocaine for the bovine cardiac sodium channel. The rate constants were obtained as described in Fig. 3. Solid lines are least square fits of the data, error bars indicate standard deviations. (A) Externally and internally applied lidocaine differ in their blocking kinetics. The block rate for internal application is voltage-dependent. In contrast, the block rate for externally applied lidocaine exhibits little or no voltage dependence. (B) The unblock rates for both internal and external application are virtually identical in magnitude and voltage dependence.

pathways for internal and external lidocaine to one common receptor responsible for fast block (cf. Refs. 6, 7, and 21).

Fast block by external lidocaine requires the uncharged form of the drug

We used lidocaine, which was dissolved in H_2O and HCl to give a stock solution of 300 mM lidocaine-HCl at a pH of

control   lidocaine [3 mM]

                internal   external

                                              +35 mV

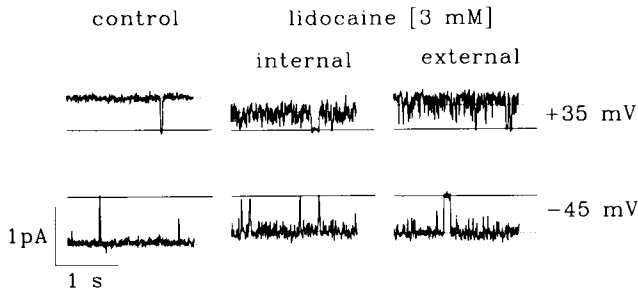1 pA   -45 mV

1 s

FIGURE 6 Traces recorded from the same rat skeletal muscle sodium channel for internally versus externally applied lidocaine. All records were filtered at 50 Hz. The solid lines indicate the closed level, and the dashed lines indicate the mean, baseline-subtracted current. At +35 mV, intracellular application of the drug causes an almost twofold reduction in mean current, while block from the extracellular side is weaker. At -45 mV, block from the intracellular and the extracellular side is similar in potency. These data suggest that internally applied lidocaine causes a more voltage-dependent block.

TABLE 2 Apparent dissociation constants (K_d) for fast block of cardiac channels by internally or externally applied lidocaine

	K_d	
E	-60	+60
mV	mM	
External	14.7	4.7
Internal	11.3	2.1

The voltage dependence of block is weaker for external application. K_d values are derived from the regression lines in Fig. 5, A and B.

1.7, to drop the pH of the external bath in a concentration-dependent manner. This procedure allowed us to study the extent of lidocaine block at several pH values without having to perfuse the chamber facing the extracellular end of the channel and thus risk breakage of the bilayer. We varied the total lidocaine concentration, measured the pH of the bath at each of these concentrations, and determined the concentration of the charged and the uncharged species according to the Henderson-Hasselbalch equation. Finally, we estimated the blocking rate constant at each of the concentrations using the beta distribution analysis. Fig. 8 shows the result of this experiment. Variation in the block rate clearly parallels the concentration of the uncharged species rather than that of the charged species which is consistent with the lack of block by external QX-314.

This result cannot be due to the direct effect of low pH on the channel. At pH < 6 block of the sodium channel by hydrogen ions becomes significant (13). However, we did not drop the pH of our bath below 6.5. Furthermore, the effect of hydrogen ions would appear as an increase in block and not as the reduction shown in Fig. 8. The correlation between blocking rate and the concentration of the uncharged form, and the voltage-independent blocking rate described in the previous section, are consistent with the involvement of a direct, hydrophobic path of access to the receptor.
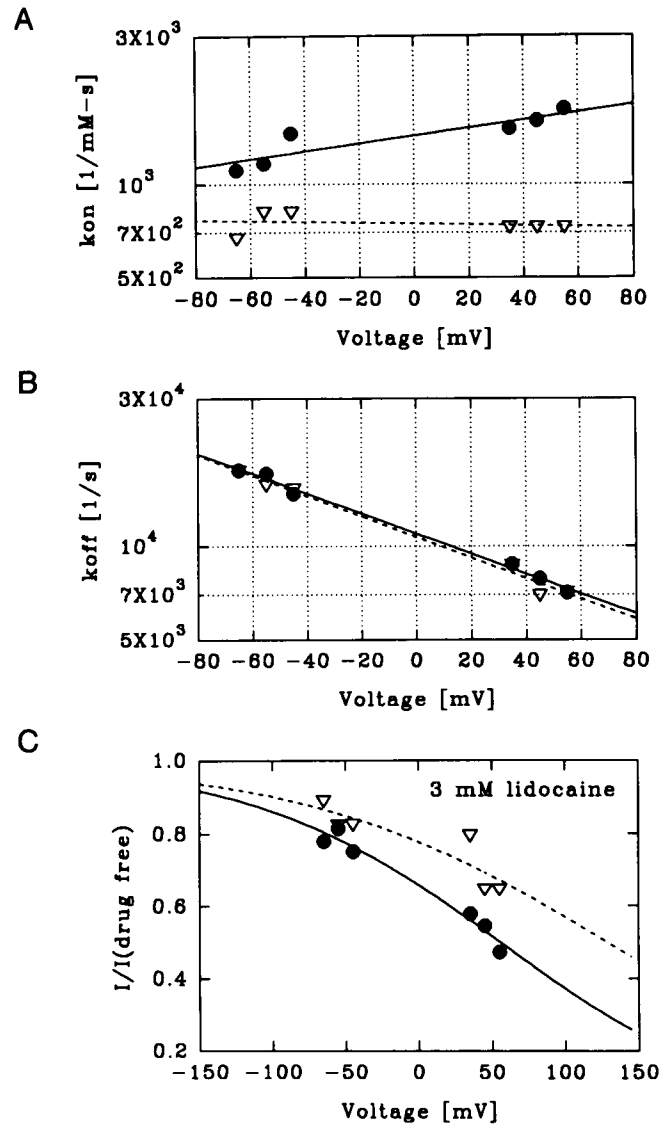
A

B

C

FIGURE 7 Comparison between the voltage dependence of fast block of internally applied (circles, solid lines) versus externally applied (triangles, dashed lines) lidocaine for a single rat skeletal muscle sodium channel. Lidocaine was first applied to the intracellular side, then both chambers were perfused and lidocaine was added to the extracellular side. The rate constants were obtained as described in Fig. 3. (A) The blocking rate for extracellularly applied lidocaine is voltage-independent in contrast with the voltage-dependent blocking rate for internal application. (B) The unblocking rates are identical for internal and external application of the drug. (C) Fractional block from the external side is less voltage-dependent; the value of $z\delta$ decreases from 0.3 to 0.25 when the drug is applied from the extracellular side, as compared with internal application. The data points represent the mean, baseline-subtracted current in the presence of 3 mM internal or external lidocaine. This method does not rely on the beta distribution analysis. The data points were fit as described for Fig. 4.

Lidocaine and QX-314 act similarly from the internal side

Fig. 9 shows examples of traces recorded in the absence and presence of QX-314 for the bovine cardiac (Fig. 9 A) and the rat skeletal muscle (Fig. 9 B) subtype. Similar to lidocaine, QX-314 appears to induce rapid transitions between open
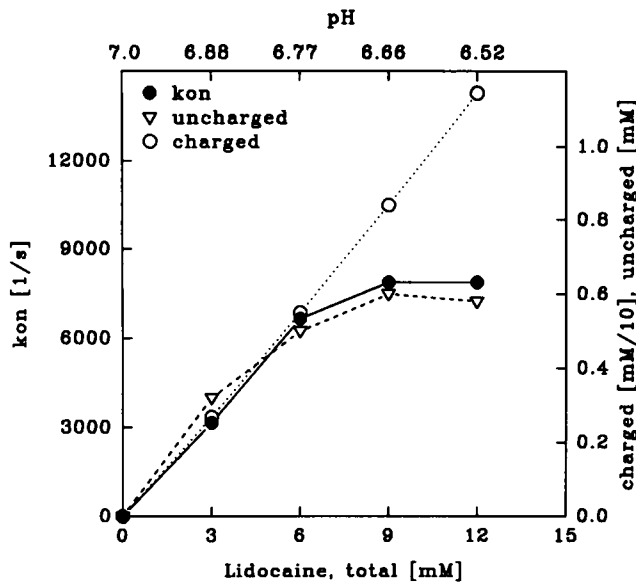
FIGURE 8 Fast external block is correlated with the concentration of the uncharged form of lidocaine. The pH of the bath was allowed to vary from 7 to 6.5 using a stock solution of 300 mM lidocaine-HCl at a pH of 1.7, added to give final bath concentrations of total lidocaine from 0–12 mM. Hence, the concentration of uncharged drug does not increase linearly with the total drug concentration. The pH of the bath was measured at each concentration, and the ratio of charged to uncharged form of the drug was determined according to the Henderson-Hasselbalch equation. The blocking rate parallels the concentration of the uncharged form, but diverges from the concentration of the charged form of the drug.
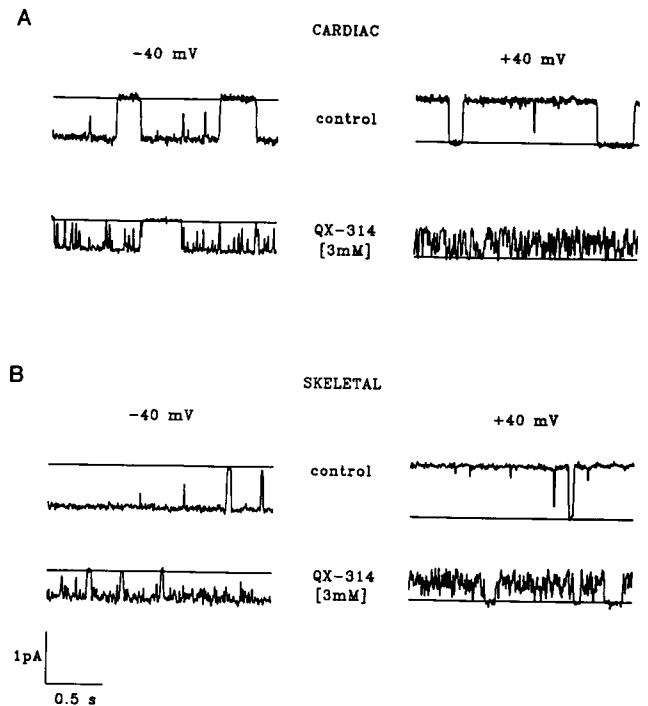

FIGURE 9 Traces recorded from BTX-activated bovine cardiac (A) and rat skeletal muscle (B) sodium channels in absence and presence of QX-314. Similar to lidocaine, QX-314 induces rapid transitions between the open and the blocked state which, at a bandwidth of 50 Hz, cannot be resolved as discrete steps. The solid lines indicate the closed level.

and blocked states. Depolarizing voltages increase the degree of block, and both channel subtypes are similarly affected. The voltage dependencies of the individual rate constants and the equilibrium dissociation constant for both channel subtypes are similar and are displayed in Fig. 10. The electrical distances ($z\delta$) determined from the voltage sensitivity of the K_d are 0.33 and 0.24 for the cardiac and the skeletal muscle subtype, respectively. Fig. 11 A displays dose-response curves for the two channel subtypes. To check the voltage dependence of QX-314 block independently of the kinetic analysis, we fitted the voltage-dependent reduction in apparent single channel current amplitude with a Boltzmann function and obtained $z\delta$ values of 0.27 and 0.28 for the cardiac and the skeletal muscle subtypes (Fig. 11 B, note that the data points for the cardiac channel are from the same channel). The data in Fig. 11 A were well-fit by a first order hyperbola, thus suggesting a bimolecular reaction. The dose-response curves, as well as the magnitude and voltage dependencies of the individual rate constants for both channel subtypes, are similar. Hence, block by QX-314 does not appear to be tissue-specific. The block by QX-314 is very similar to that for internally applied lidocaine. This is consistent with QX-314 and lidocaine acting at the same site.

External sodium inhibits block by QX-314

Wang (12) demonstrated that external sodium ions reduced the affinity of cocaine for sodium channels from skeletal

muscle, and suggested that external sodium ions were physically expelling the drug from its receptor. Since QX-314 and cocaine compete for a common binding site (12), external sodium ions should also affect the blocking kinetics of QX-314 and lidocaine. We chose QX-314 to test this hypothesis, because of its one-sided blocking action. Fig. 12 shows the effect of replacing external sodium with the channel-impermeant N-methyl-D-glucamine (NMG). In one experiment (circles), after incorporation of a skeletal muscle sodium channel into the bilayer and determination of its orientation with symmetric 200 mM NaCl, 20 mM MOPS (pH 7.0), the extracellular side was perfused with 200 mM NMG (pH 7.0) and 6 mM QX-314 was added to the chamber facing the intracellular side of the channel. Then both chambers were perfused with 200 mM NaCl, 20 mM MOPS (pH 7.0) to restore symmetric conditions, and 6 mM QX-314 was added to the intracellular side. In two experiments (triangles) the experimental protocol was reversed. Fig 12 C shows records in the presence and absence of external sodium. As can be clearly seen, removal of external sodium ions results in increased block by QX-314. Our data demonstrate that removal of external sodium increases the blocking rate and decreases the unblocking rate while leaving the voltage dependence of the rates virtually unaffected. Hence, it appears that entry of sodium ions from the extracellular solution decreases the affinity of the receptor for QX-314. These data also suggest that penetration of sodium ions into the transmembrane electric field does not contribute to the voltage dependence of the QX-314 block.
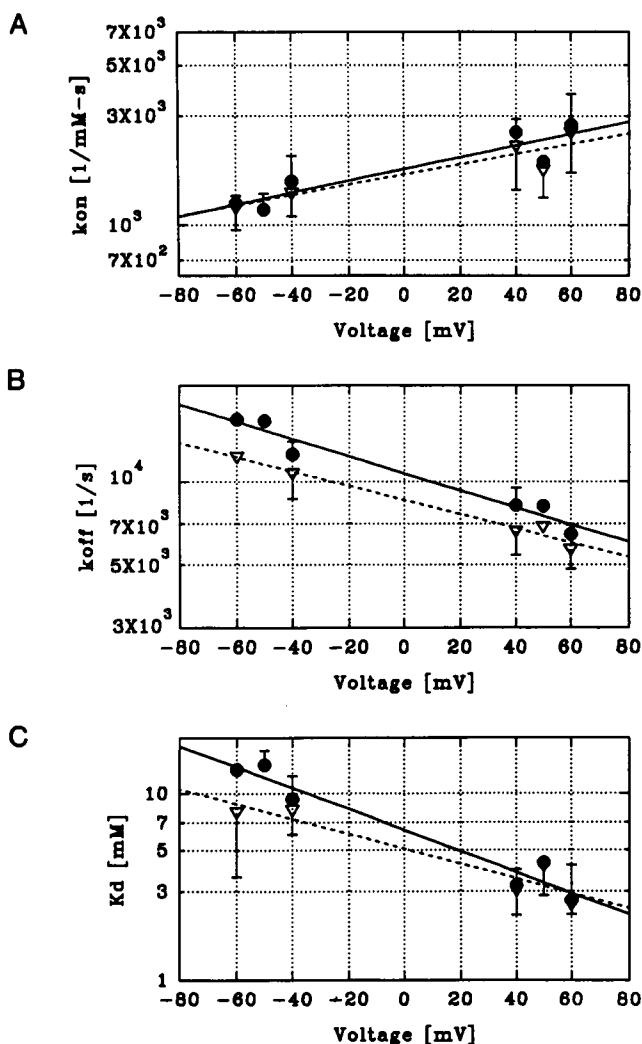
FIGURE 10   Voltage dependence of the rate constants and equilibrium dissociation constant for fast block of bovine cardiac (*circles, solid lines*) and rat skeletal muscle (*triangles, dashed lines*) sodium channels by internal QX-314. Data points were obtained as described in Fig. 3. The association rate constants (*A*), the dissociation rate constants (*B*), and the equilibrium dissociation constants (*C*) show a voltage dependence similar to lidocaine block. The voltage sensitivity of the equilibrium dissociation constants reflects apparent electrical distances ($z\delta$) of 0.33 and 0.24 for the cardiac and the skeletal muscle subtypes. Error bars (cardiac upward, skeletal muscle downward) indicate standard deviations.

DISCUSSION

Lidocaine affinity for BTX-activated sodium channels

Sheldon et al. (22) reported a remarkable correlation, now extending over more than three orders of magnitude, between the concentrations of type I antiarrhythmic drugs required to inhibit the binding of [^{3}H]batrachotoxinin A 20α-benzoate to rat cardiac myocytes, and the clinically effective doses of the drugs. This result motivated us to examine the electrical consequences of antiarrhythmic drug interaction with single, BTX-activated cardiac and skeletal muscle sodium channels, beginning with the archetypal local anesthetic/antiarrhythmic, lidocaine. Apparent dissociation constants,
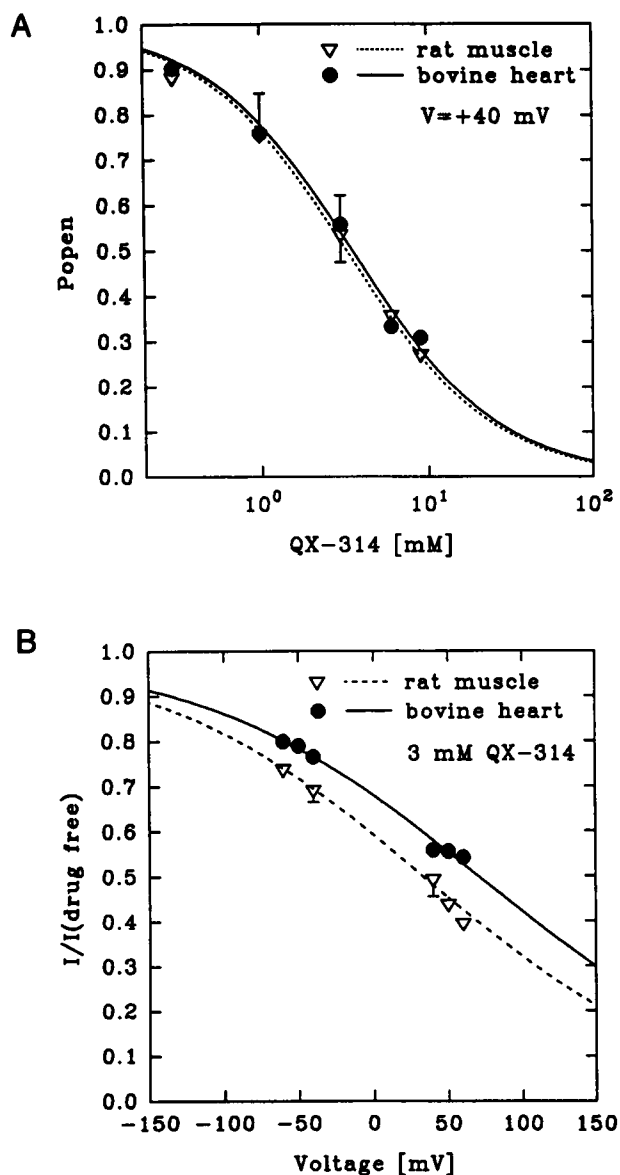

FIGURE 11   (*A*) Dose response curves for the block of the bovine cardiac and the rat skeletal muscle sodium channel by QX-314 at +40 mV. The data points were fitted as described in Fig. 4. Upward and downward error bars indicate standard deviations for the cardiac and the skeletal muscle subtypes, respectively. The K_d values obtained from the fit are 3.3 mM for the skeletal muscle and 3.5 mM for the cardiac subtype. (*B*) The voltage dependence of QX-314 block was also determined directly from the reduction in apparent single channel amplitude. The data were fitted as described in Fig. 4; the $z\delta$ values obtained from the slopes of the fits are 0.27 and 0.28 for the cardiac and the skeletal muscle subtypes, respectively. The data points for the cardiac channel were taken from one experiment, the downward error bars indicate standard deviations for the skeletal muscle data.

K_d, for lidocaine block of single skeletal or cardiac sodium channels at 0 mV are ≃5 mM (Fig. 3). The estimated K_d for lidocaine binding is about 100-fold lower (22). A substantial part of the discrepancy between these values appears to arise from the fact that the single channel experiments reflect a drug interaction with a single, open channel to which BTX is continuously bound throughout the experiment, whereas the binding data primarily reflect lidocaine interaction
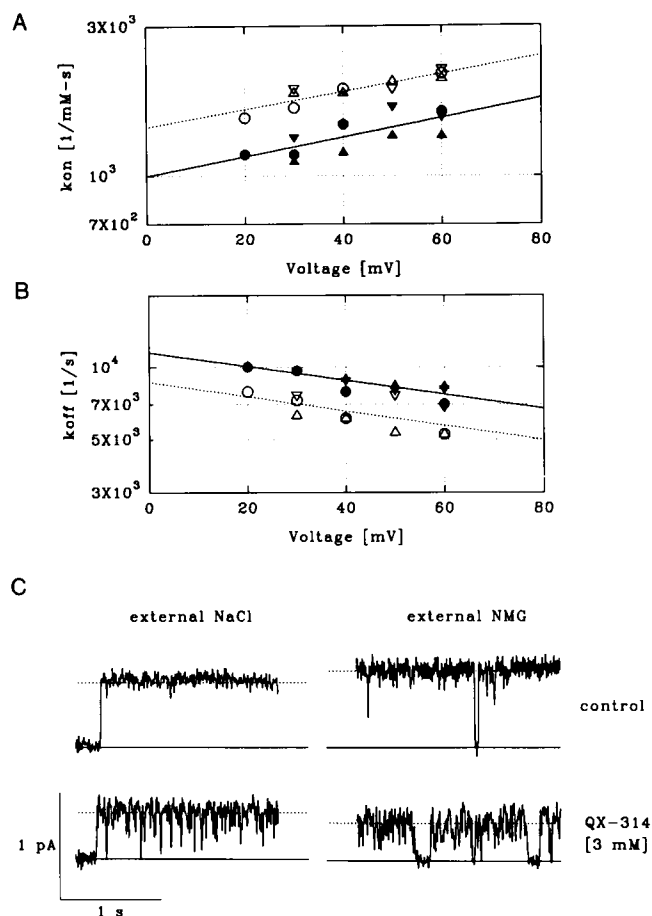
FIGURE 12   Effect of external sodium ions on the blocking and unblock-ing rate of QX-314 for the rat skeletal muscle sodium channel. To remove external sodium ions, the external bath was replaced with 200 mM NMG. The data in the presence (*filled symbols, solid lines*) and absence (*open symbols, dashed lines*) of external sodium were taken from the same channel in three separate experiments. Each of the symbols used (*upward triangles, downward triangles, circles*) represents data from one channel. (*A*) The blocking rate is reduced in the presence of external sodium ions; the voltage dependence is not affected. (*B*) The unblocking rate is enhanced in the presence of external sodium ions; the voltage dependence is not altered. (*C*) Traces from one rat skeletal muscle channel to illustrate the effect of external sodium ions. The traces (+40 mV) were filtered at 50 Hz, solid lines indicate the closed level, dashed lines indicate the mean baseline subtracted current. In the absence of the drug, replacement of external sodium by NMG results in an increase in apparent single channel amplitude. In the presence of QX-314, the apparent single channel amplitude is decreased due to fast block. The current decreases to a lower level when external sodium ions are removed despite the increased control current.

with the nonactivated (i.e., BTX-free) channel. A later study by the same group (23) showed that lidocaine binding is state-dependent, and it is now known that the K_d for bind-ing to the activated ([^{3}H]batrachotoxinin A 20α-benzoate-bound) channel is more than 10-fold higher than for the nonactivated state. Antiarrhythmic binding by BTX-bound channels appears to be relatively nondiscriminating among different drugs, as well as showing low affinity, based on both the binding studies of Hill et al., (23) and single channel experiments with drugs having widely differing clinically effective doses (G. W. Zamponi, unpublished data).

The site for fast lidocaine block is highly conserved

Our study is the first to estimate the individual rate constants for block and unblock of lidocaine on BTX-activated sodium channels. Fast block is clearly block of the open channel, however, we cannot rule out the possibility that the drug also binds rapidly to a closed state of the channel. Fast block of internally applied lidocaine and QX-314 has been previously described for BTX-activated rat skeletal muscle sodium channels (9, 24). Moczydlowski and coworkers reported a similar electrical distance as well as a K_d (0 mV) for lidocaine of the same order of magnitude as in the present study. How-ever, their study found QX-314 to be only half as potent as lidocaine. We have found block by lidocaine and QX-314 to be similar in potency. Also the similar voltage dependence supports the idea of a common binding site. Our data indicate that this site is conserved in sodium channel isoforms from at least three species and two different tissues.

External sodium ions do not contribute to the voltage dependence of fast block

We have shown that replacing extracellular sodium ions with NMG results in an increased blocking rate and a decreased unblocking rate for QX-314 without changing their voltage dependence. Similar observations have been previously re-ported by Cahalan and Almers (25) for QX-314 and Wang (12) for cocaine. Wang suggested that external sodium ions were able to physically expel the cocaine molecule from its binding site rather than act in a strict competition with co-caine for the same binding site. In case of strict competition, the unblocking rate of cocaine or QX-314 should not be af-fected by the presence of sodium ions. The observation that the voltage dependencies of the rate constants are not af-fected by external sodium suggests that external sodium ions do not contribute to the voltage dependence of fast block. Hence, the observed voltage dependence of QX-314 block may arise solely from the charge on the local anesthetic mol-ecule traversing a part of the transmembrane potential.

Sidedness of fast block suggests two pathways to the local anesthetic receptor

Block of sodium channels by local anesthetics has been de-scribed as block from the internal side of the channel (5, 7, 9, 12). The drugs were thought to cross the membrane in their uncharged form, become protonated on the inside, and then bind to the local anesthetic receptor in the charged form (6, 13). We believe that the drugs do not have to accumulate internally in order to bind to the receptor, but are able to directly block the channel from the extracellular side in their uncharged form. If an externally applied drug were to cross the membrane, accumulate in the cytoplasmic bathing so-lution, and subsequently bind to an internal receptor, the con-centration at the cytoplasmic end of the channel would be less

than or equal to that in the extracellular bath. One would expect the unblocking rates to be the same for external and internal application, while the blocking rates might differ in absolute magnitude, but not in voltage dependence. We have, however, observed a drastic change in voltage dependence for the block rate when the drug was applied extracellularly. The identical magnitude and voltage dependence of the un-block rates suggest that the drug is acting on only one site, regardless of whether it is applied from the internal or the external side of the channel. A similar argument has been recently made for the *l-cis*-diltiazem block of cyclic GMP-gated channels of photoreceptors (26). Hence, fast block from the external side must involve a direct voltage-independent access to the receptor. The observations that external block is reduced by lowering the external pH (see Fig. 7) and that external application of the permanently charged lidocaine derivative QX-314 was without effect (data not shown) also suggest the involvement of the un-charged species in block from the external side.

This leaves two possibilities: either the externally applied drug finds its way to the receptor via a hydrophobic pathway, or the drug is able to reach the receptor directly by entering the channel mouth from the outside.

The following consideration speaks against the latter case. No ion containing a methyl group is able to pass through the sodium channel (27) and, thus, neither would the lidocaine tail. Therefore, the site would have to be located exactly at the selectivity filter in order to be reached from either side. The drug, in its uncharged form, could directly reach the binding site through the external mouth of the channel (hence accounting for the lack of voltage dependence of the blocking rate) and become protonated in the vicinity of, or while binding to, the site. Unblock should then be favored by hyper-polarizing voltages, because the drug would be expected to exit the channel mouth toward the extracellular side. Thus, the observation that the voltage dependence of unblocking kinetics is identical for external and internal application argues against this mechanism.

The second possibility, that external lidocaine reaches a common site via a hydrophobic route, appears to be more likely. Lidocaine, in its uncharged form, could reach the hydrophilic receptor environment via a hydrophobic pathway, then become protonated and bind to the site. Unblock then would occur as if the drug had reached the site directly from the inside. The lack of voltage dependence of the blocking rate is consistent with this model. The protonation rate for lidocaine at pH 7 lies between 200 and 2500/s (28) which is the diffusion-limited maximum in free solution. At lidocaine concentrations up to about 1 mM, lidocaine binding to the receptor would be the rate-limiting step. At high lidocaine concentrations (9 mM), the predicted protonation rate is slightly less than the block rate, but both rates are of the same order of magnitude. Preliminary data suggest that the block-ing rate may saturate at high external lidocaine concentra-tions. The protonation rate at the local environment near the binding site, however, might be higher than in free solution

(e.g., influence of an acidic sidegroup at the selectivity filter as suggested by Woodhull (29)). Thus, even at higher drug concentrations, binding to the receptor rather than protona-tion might be the rate-limiting step.

We cannot completely exclude the possibility that inter-nally applied lidocaine acts, in part, in the uncharged form. It proved difficult to test this possibility rigorously, because our bilayers became less stable at very high pH. A complete study would require variation of pH over a wide range on both sides of the bilayer and is beyond the scope of this paper. However, in one experiment we observed a decrease in po-tency of internal lidocaine, with no change in voltage de-pendence of block, when internal pH was raised from 6.9 to 7.6. This result is consistent with the charged form being the predominant blocking species from the inside, as is the sim-ilarity of actions of internally applied lidocaine and perma-nently charged QX-314.

Our results support the following mechanism: 1) The drug can only find its way into the pore from the outside in its uncharged form, and, once it is close to the receptor, the drug becomes protonated and interacts with the binding site. 2) Unblock follows the same kinetics as if the drug had reached the site from the inside. Our model is consistent with the observation that raising the external pH increases the block rate of cocaine, whereas increasing the internal pH has the opposite effect (13). Nettleton and Wang (13) had suggested that cocaine would be prevented from crossing the membrane to the internal side by low external pH and thus show a reduced block rate from the inside, however, this result could be interpreted according to our model if cocaine in its neutral form were able to reach the binding site directly from the channel outside.

Taken together, the evidence supports direct fast block of the channel from the outside via a hydrophobic pathway. This might be part of the mechanism underlying the in vivo action of class I antiarrhythmics.

Is lidocaine accumulation across the membrane significant?

We have clearly demonstrated differential effects depending on the side of lidocaine application. We have also observed that the effect of the drug can be readily reversed by perfusing the chamber to which the drug had initially been added, but not by perfusion of the opposite chamber. Furthermore, block from the external or internal side reaches its steady state within seconds after application and stirring. However, it has been suggested (6) that bilayers exhibit a high permeability for lidocaine ($D = 0.5$ cm^2/s) so that the drug would virtually not recognize the bilayer as a barrier. Given the small thick-ness of the bilayer and the proposed high rate of lidocaine diffusion, the drug would equilibrate rapidly across the bi-layer resulting in identical concentrations in the immediate vicinity of both sides of the membrane. Gutknecht and Toste-son (30) suggested that the size of the unstirred layers on either side of the bilayer was rate-limiting for the diffusion

of hydrophobic drugs across the bilayer. Hence, a difference in thickness of the unstirred layers on either side of the membrane could result in differential blocking effects of the drug depending on the side of application. However, even if unstirred layers limited the diffusion of the drug across the bilayer, and hence, reduced the apparent affinity of the drug for its receptor, the voltage dependence of the steady state block should not be affected. In addition, the potency of internal lidocaine and QX-314 was not affected by the orientation of the channel in bilayers formed on asymetrically constructed holes in the partition of the bilayer chamber (31). Thus, the unstirred layers on both sides of the bilayer appear to have similar effects on fast lidocaine block under our experimental conditions. The observation that fast block by external lidocaine is essentially voltage-independent suggests that little of the drug actually reaches the internal mouth of the channel to block from the inside. A possible explanation is that the bilayers have a significantly lower permeability to lidocaine than is commonly believed. We know of no reports of direct measurement of the permeability of phospholipid bilayers for lidocaine. Consequently we suggest that, in our experiments, lidocaine mainly acts directly from the side on which it is applied. In small cells, with a much higher surface-to-volume ratio, bulk accumulation in the cytoplasm after external application would be much greater.

CONCLUSIONS

We have investigated fast block of the BTX-activated cardiac and skeletal muscle sodium channel by lidocaine and its quaternary derivative QX-314. Fast block by lidocaine and QX-314 applied to the intracellular side of the channel is not tissue-specific, is antagonized by external sodium ions, and both blocking and unblocking rates are voltage-dependent. Lidocaine, applied to the extracellular side of the channel, accesses the same binding site principally via a direct, hydrophobic route rather than by passing through the bilayer to act from the intracellular side.

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