Block of Inactivation-deficient Na+ Channels by Local Anesthetics in Stably Transfected Mammalian Cells: Evidence for Drug Binding Along the Activation Pathway

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ABSTRACT According to the classic modulated receptor hypothesis, local anesthetics (LAs) such as benzocaine and lidocaine bind preferentially to fast-inactivated Na+ channels with higher affinities. However, an alternative view suggests that activation of Na+ channels plays a crucial role in promoting high-affinity LA binding and that fast inactivation per se is not a prerequisite for LA preferential binding. We investigated the role of activation in LA action in inactivation-deficient rat muscle Na+ channels (rNav1.4-L435W/L437C/A438W) expressed in stably transfected Hek293 cells. The 50% inhibitory concentrations (IC$_{50}$) for the open-channel block at +30 mV by lidocaine and benzocaine were 20.9 ± 3.3 μM ($n = 5$) and 81.7 ± 10.6 μM ($n = 5$), respectively; both were comparable to inactivated-channel affinities. In comparison, IC$_{50}$ values for resting-channel block at −140 mV were >12-fold higher than those for open-channel block. With 300 μM benzocaine, rapid time-dependent block ($\tau \approx 0.8$ ms) of inactivation-deficient Na+ currents occurred at +30 mV, but such a rapid time-dependent block was not evident at −30 mV. The peak current at −30 mV, however, was reduced more severely than that at +30 mV. This phenomenon suggested that the LA block of intermediate closed states took place notably when channel activation was slow. Such closed-channel block also readily accounted for the LA-induced hyperpolarizing shift in the conventional steady-state inactivation measurement. Our data together illustrate that the Na+ channel activation pathway, including most, if not all, transient intermediate closed states and the final open state, promotes high-affinity LA binding.

KEY WORDS: sodium channel • fast inactivation • persistent sodium currents • local anesthetics • modulated receptor hypothesis

INTRODUCTION

The in vivo targets of local anesthetics (LAs) are voltage-gated Na+ channels responsible for the generation of action potentials in excitable membranes (Hille, 2001). Molecular cloning has thus far identified nine Na+ channel α-subunit isoforms in mammals. The α-subunit isoforms contain four repeated domains (D1–D4), each with six transmembrane domains (S1–S6) and can be functionally expressed in frog oocytes or in mammalian expression systems (Catterall, 2000). LAs interact with residues in the middle of D4S6, D3S6, and D1S6 segments, probably situated within the inner cavity of the Na+ permeation pathway (Ragsdale et al., 1994; Wang et al., 1998, 2000; Yarov-Yarovoy et al., 2001, 2002).

The block of Na+ currents by lidocaine and benzocaine has been studied extensively. Lidocaine contains a tertiary amine group, which can be protonated in the aqueous solution. In contrast, benzocaine is a neutral LA.

Lidocaine not only elicits a tonic block of Na+ currents when infrequently stimulated but also produces an additional use-dependent block during repetitive pulses (Hille, 2001). On the other hand, benzocaine fails to produce any use-dependent block. As a neutral drug, benzocaine may escape too rapidly during the interpulse to accumulate such a block. However, both drugs significantly shift the steady-state inactivation of Na+ channels to the hyperpolarizing direction as if LAs stabilize the inactivated state. Hille (1977) proposed a modulated receptor hypothesis to account for the complicated block of LAs. His hypothesis states that the LA receptor changes its conformation during state transitions and that the fast-inactivated state has a higher LA affinity than does the resting state. A similar hypothesis (Hondeghem and Katzung 1977, 1984) suggested that a class 1 antiarrhythmic agent, lidocaine, may additionally block open cardiac Na+ channels with a higher affinity.

To date, the proof of the high-affinity open-channel block by LAs remains controversial. Several pieces

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Abbreviation used in this paper: LA, local anesthetic.

of evidence indicate that the open-channel block by lidocaine plays a minimal role in the use-dependent block. For example, both Cahalan (1978) and Yeh (1978) reported that QX-314, a quaternary ammonium derivative of lidocaine, failed to block inactivation-deficient Na^+ currents in pronase-treated squid axons. Their results supported the conclusion that the inactivated state plays a dominant role in the use-dependent block of Na^+ currents by LAs. Bean et al. (1983) later found that lidocaine binds strongly to the inactivated state of cardiac Na^+ channels with an estimated dissociation constant of ~10 μM, a value within the therapeutic plasma concentration (6.4–21.3 μM). Recent experiments with inactivation-deficient mutant Na^+ channels (IFM/QQQ) also show that the inactivated state has a higher LA affinity than does the open state (Bennett et al., 1995; Grant et al., 2000; O'Leary and Chahine, 2002).

Wang et al. (1987), however, reported that QX-314 retains its potency on inactivation-deficient Na^+ currents in squid axons treated with chloramine-T and suggested that the open-channel block plays a significant role in the use-dependent block. Vedantham and Cannon (1999) found that the lidocaine-induced slowing of Na^+ channel repriming was not the result of a slowing of recovery of the fast-inactivation gate. Their finding indicates that use-dependent block does not involve an accumulation of fast-inactivated channels. Moreover, various class 1 antiarrhythmic agents, such as lidocaine, flecainide, amiodarone, and mexiletine, potently block persistent late Na^+ currents either derived from genetic diseases or under pathological conditions (Ju et al., 1992; An et al., 1996; Wang et al., 1997, 1999; Nagatomo et al., 2000; Maltsev et al., 2001).

To investigate the possible role of the activation pathway in LA block by benzocaine and lidocaine, we chose to study LA actions on stably transfected HEK293 cells expressing inactivation-deficient rat skeletal muscle Na^+ channels, rNav1.4-L435W/L437C/A438W (WCW mutant; Wang et al., 2003a). The point mutations of WCW mutant channels are at the positions 19, 21, and 22 of the D1S6 COOH terminus. The advantage of WCW mutant Na^+ channels is that, unlike the IFM/QQQ mutant cardiac Na^+ channels (Grant et al., 2000), they are well expressed in HEK293 cells. A large persistent late Na^+ current in HEK293 cells allows direct examinations of the open-channel block by LAs. The disadvantage of this approach is that any mutations or chemical/enzymatic treatments in theory could alter indirectly or directly the structure of the lidocaine/benzocaine receptor. Such receptor alteration could also occur in studies of late Na^+ currents under pathological conditions or by S6 mutations that cause genetic diseases (Takahashi and

Cannon, 2001). Despite this uncertainty, our result suggests an important therapeutic role of the open-channel block by lidocaine in vivo. To our surprise, we found that benzocaine blocks the open channel potently at +30 mV as well as the closed intermediate states at ≤-30 mV.

MATERIALS AND METHODS

HEK293 Cells with Stably Transfected rNav1.4-L435W/L437C/A438W cDNA

Mutagenesis of rNav1.4-L435W/L437C/A438W cDNA in the pcDNA3 vector was performed as described previously (Wang et al., 2003a). Cultured HEK293 cells were maintained at 37°C in a 5% CO_2 incubator in DMEM (Life Technologies) containing 10% FBS (HyClone), and 1% penicillin and streptomycin solution (Sigma-Aldrich). The HEK293 cells were transfected with the mutant clone in pcDNA3 vector (10 μg) by a calcium phosphate precipitation method. Transfected HEK293 cells were treated with 1 mg/ml G-418 (Invitrogen) in 100-mm culture dishes, and individual G-418 resistant colonies were isolated using glass cylinders (i.d. = 6 mm) ~2 wk after transfection as described previously (Wang et al., 2004a). One colony was frozen, later reestablished, and maintained in Ti-25 flasks without the addition of G-418 for all studies described here.

Electrophysiology and Data Acquisition

The whole-cell configuration of a patch-clamp technique (Hamill et al., 1981) was used to study Na^+ currents in HEK293 cells at room temperature (22 ± 2°C). Electrode resistance ranged from 0.5 to 1.0 MΩ. Command voltages were elicited with pCLAMP8 software and delivered by Axopatch 200B (Axon Instrument) or by EPC-7 (List Electronics). Cells were held at -140 mV and dialyzed for 10–15 min before current recording. Most of the capacitance and leak currents were cancelled with a patch-clamp device and by P/-4 subtraction. Liquid junction potential was not corrected. Peak currents at +30 mV were 2–20 nA for the majority of cells. Access resistance was 1–2 MΩ under the whole-cell configuration; series resistance compensation of >85% typically resulted in voltage errors of ≤4 mV at +50 mV. Most dose–response studies were performed at +30 mV for the outward Na^+ currents. Such recordings allowed us to avoid the complication of series resistance artifacts and to minimize inward Na^+ ion loading (Cota and Armstrong, 1989). Curve fitting was performed by Microcal Origin. An unpaired Student's t test was used to evaluate estimated parameters (mean ± SEM or fitted value ± SE of the fit); P values of <0.05 were considered statistically significant.

Solutions and Chemicals

Lidocaine-HCl and benzocaine were purchased from Sigma-Aldrich. Drugs were dissolved in DMSO at 100 mM as stock solutions and stored at 4°C. Final drug concentrations were made by serial dilution. The highest DMSO concentration in the bath solution (1%) had little effect on Na^+ currents. Cells were perfused with an extracellular solution containing (in mM) 65 NaCl, 85 choline-Cl, 2 $CaCl_2$, and 10 HEPES (titrated with tetramethylammonium-OH to pH 7.4). In some experiments, we used the Na^+-free extracellular solution by replacing 65 mM NaCl with 65 mM choline-Cl. The pipette (intracellular) solution consisted of (in mM) 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES (titrated with cesium-OH to pH 7.2).
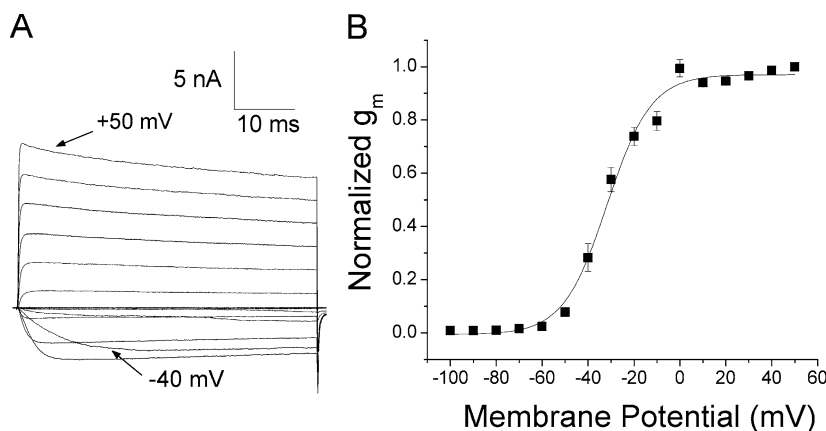
A

5 nA
10 ms

+50 mV

−40 mV

B

FIGURE 1. Activation of rNav1.4-WCW mutant Na$^+$ channels. (A) Currents were evoked by 50-ms test pulses from −100 to +50 mV in 10-mV increments. (B) Normalized membrane conductance (g$_m$) was plotted against the voltage. g$_m$ was calculated from the equation g$_m$ = I$_{Na}$/(E$_m$ − E$_{Na}$), where I$_{Na}$ is the peak current, E$_m$ is the amplitude of the voltage step, and E$_{Na}$ is the estimated reversal potential of the Na$^+$ current. Plots were fitted with a Boltzmann function (1/[1 + exp((V$_{0.5}$ − V)/k)]). The average midpoint voltage (V$_{0.5}$) and slope (k) were −31.7 ± 1.2 mV and 9.6 ± 1.0 mV, respectively (n = 6). Holding potential was set at −140 mV.

R E S U L T S

A Hek293 Cell Line Expressing Robust rNav1.4-WCW Inactivation-deficient Na$^+$ Currents

Fig. 1 A shows the Na$^+$ currents at various membrane potentials from a cell stably transfected with rNav1.4-WCW mutant channels. The inactivation-deficient Na$^+$ currents were activated at −50 or −60 mV. The peak currents were measured and the peak conductance/voltage relationship was then plotted (Fig. 1 B). The result shows that the activation parameters (midpoint voltage, V$_{0.5}$ = −31.7 ± 1.2 mV, and slope factor, k = 9.6 ± 1.0 mV, n = 6) for the WCW mutant channels are similar to those of wild-type counterparts (P > 0.05; V$_{0.5}$ = −32.0 ± 0.9 mV; k = 8.7 ± 0.8 mV; Wang et al., 2003a). The level of expression of this WCW-transfected cell line was relatively high with 407 ± 32 pA/pF at +50 mV (n = 31), compared with ∼250 pA/pF in transiently transfected cells expressing the wild-type rNav1.4 channels (Nau et al., 2003). The high level of Na$^+$ channel expression in this cell line remained stable for several months.

The conventional steady-state inactivation curve (h$_∞$) showed incomplete inactivation using a two-pulse pro-

tocol. Fig. 2 A showed current traces at the test pulse of +30 mV for 5 ms (V$_{test}$; solid arrow) with various conditioning pulses (V$_{con}$; dashed arrow) for 100 ms. Peak currents at the test pulse were measured and plotted against the corresponding conditioning voltage (Fig. 2 B). The voltage dependence shown in this graph suggests that the decline in relative peak currents begins at the conditioning pulse near −50 mV, where the Na$^+$ channels are activated. One possibility is that the decline of peak currents after a conditioning pulse is due to the enhanced slow inactivation of the open Na$^+$ channel during depolarization, which was accelerated significantly in WCW mutant channels (Wang et al., 2003a).

Concentration- and Time-dependent Block of Inactivation-deficient Na$^+$ Currents by Lidocaine

Block of the open state of Na$^+$ channels by lidocaine was conspicuous in inactivation-deficient rNav1.4-WCW mutant channels. Such open-channel block was concentration and time dependent at +30 mV (Fig. 3 A). In general, the larger the lidocaine concentration the faster the blocking time course and the greater the steady-state block of the late Na$^+$ currents (Fig. 3 A, 1–100 μM

A

+30 mV

−140 mV

+30 mV
(V$_{test}$)

5 nA
20 ms

−30 mV (V$_{con}$)

B
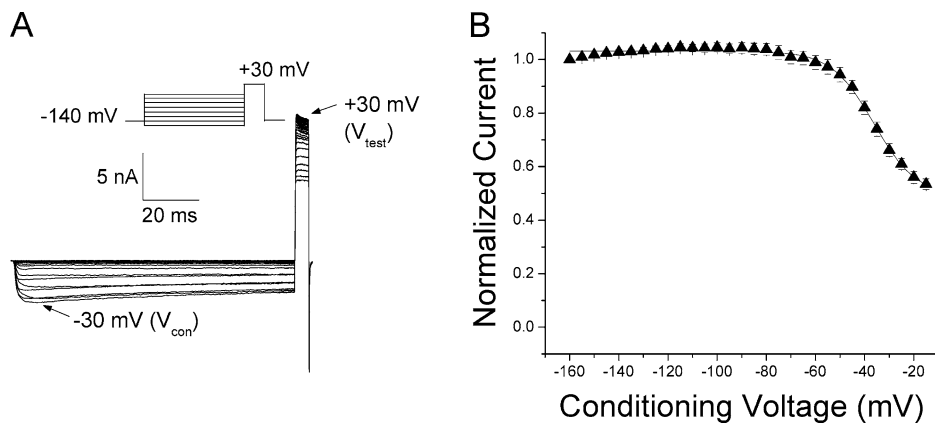

FIGURE 2. Conventional steady-state inactivation measurement (h$_∞$) of rNav1.4-WCW mutant Na$^+$ channels. (A) Currents were evoked by a 5-ms test pulse (V$_{test}$) at +30 mV. Test pulses were preceded by 100-ms conditioning pulses (V$_{con}$), increasing in 5-mV increments from −160 to −15 mV (inset). (B) Normalized availability of rNav1.4-WCW mutant channels plotted as a function of the conditioning pulse voltage. The plot was fitted with a Boltzmann function. The average midpoint (V$_{0.5}$) and slope (k) were −36.2 ± 0.9 mV and 8.7 ± 0.6 mV, respectively (n = 7). Holding potential was set at −140 mV.
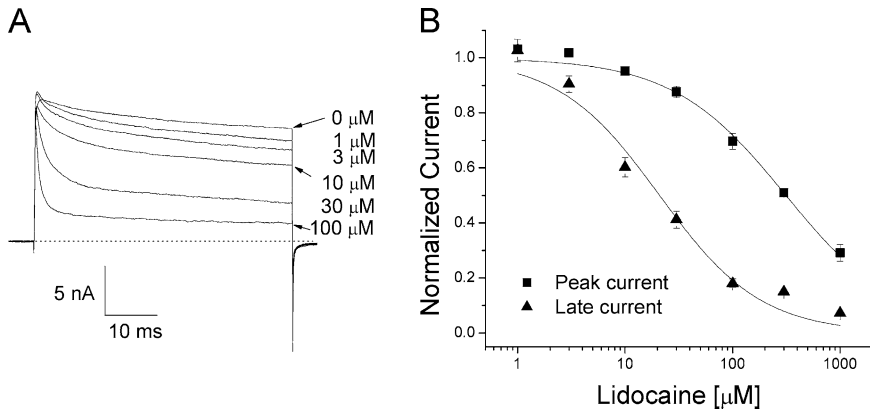
693   WANG ET AL.

FIGURE 3. Block of rNav1.4-WCW mutant currents by lidocaine. (A) Representative current traces are shown at various lidocaine concentrations. Cells were depolarized by a 50-ms test pulse at +30 mV. Pulses were delivered at 60-s intervals. (B) Peak and maintained late currents at the end of the test pulse as shown in A were measured at various lidocaine concentrations. Data were normalized to the control saline response and fitted with the Hill equation. The IC$_{50}$ value for the peak current was 314 ± 25 μM (Hill coefficient, 0.8 ± 0.1) (■, $n = 5$). For the maintained late current the IC$_{50}$ was 20.9 ± 3.3 μM (0.9 ± 0.1) (▲, $n = 5$).

lidocaine). This behavior is customarily found in the block of voltage-gated K$^+$ channels by quaternary ammonium ions such as nonyltriethyammonium and tetrapentylammonium ions (Armstrong, 1971; Hille, 2001).

Dose–Response Curve of Lidocaine

The peak currents at the beginning of the pulse and the maintained late Na$^+$ currents at the end of the pulse (Fig. 3 A) were measured at various lidocaine concentrations, normalized with the amplitude without drug and plotted against lidocaine concentrations (Fig. 3 B). The 50% inhibitory concentration (IC$_{50}$) for the late Na$^+$ currents is 20.9 ± 3.3 μM with a Hill coefficient of 0.9 ± 0.1 ($n = 5$; triangle), whereas the IC$_{50}$ for the peak Na$^+$ currents is 314 ± 25 μM with a Hill coefficient of 0.8 ± 0.1 ($n = 5$; square). Our interpretation of these data is that the IC$_{50}$ for the late currents reflects the lidocaine affinity of the open channel during depolarization, whereas the IC$_{50}$ for the peak currents is related to the lidocaine affinity of the resting channel before depolarization. The difference in IC$_{50}$ values between the resting- and the open-channel block by lidocaine is 15-fold (314 μM vs. 20.9 μM). The Hill coefficient was near unity, suggesting that one lidocaine molecule blocks one channel.

Concentration- and Time-dependent Block of Inactivation-deficient Na$^+$ Currents by Benzocaine

Previous reports all indicate that benzocaine effectively stabilizes the inactivated sodium channels (Hille, 2001). To investigate the possible open-channel block by this drug, we applied benzocaine to inactivation-deficient mutant channels. The superimposed WCW mutant Na$^+$ currents are shown without and with various concentrations of benzocaine (Fig. 4 A; 10 μM to 1 mM). Beside concentration-dependent block, we unexpectedly found that benzocaine also elicited a time-dependent block of mutant currents similar to the phenotype produced by lidocaine (Fig. 3 A). Benzocaine was evidently capable of blocking the open state of mutant channels.

Dose–Response Curve of Benzocaine

To determine the resting- and the open-channel block by benzocaine we measured the peak and the late mutant currents at various benzocaine concentrations (Fig. 4 A), normalized with the control currents in the absence of drug, and plotted against concentrations. The IC$_{50}$ for the open-channel block is 81.7 ± 10.6 μM with a Hill coefficient of 0.9 ± 0.1 ($n = 5$; triangle), whereas
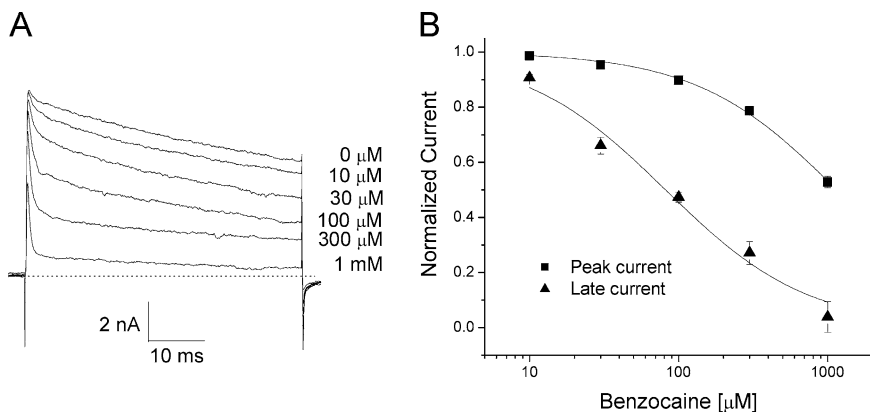

FIGURE 4. Block of rNav1.4-WCW Na$^+$ currents by benzocaine. (A) Representative current traces are shown at various benzocaine concentrations. Cells were depolarized by a 50-ms test pulse at +30 mV. Pulses were delivered at 60-s intervals. (B) Peak currents and maintained late currents at the end of the test pulse as shown in A were measured at various benzocaine concentrations. Data were normalized to the control saline response and fitted with the Hill equation. The IC$_{50}$ value for the peak current was 1.16 ± 0.05 mM (Hill coefficient, 0.9 ± 0.1) (■, $n = 5$). For the maintained current, the IC$_{50}$ was 81.7 ± 10.7 μM (0.9 ± 0.1) (▲, $n = 5$).

A

Normalized Peak Current (y-axis, values 0.3–1.0)

Δt (s) (x-axis)

+30 mV, 50 ms
−140 mV
Δt
+30 mV
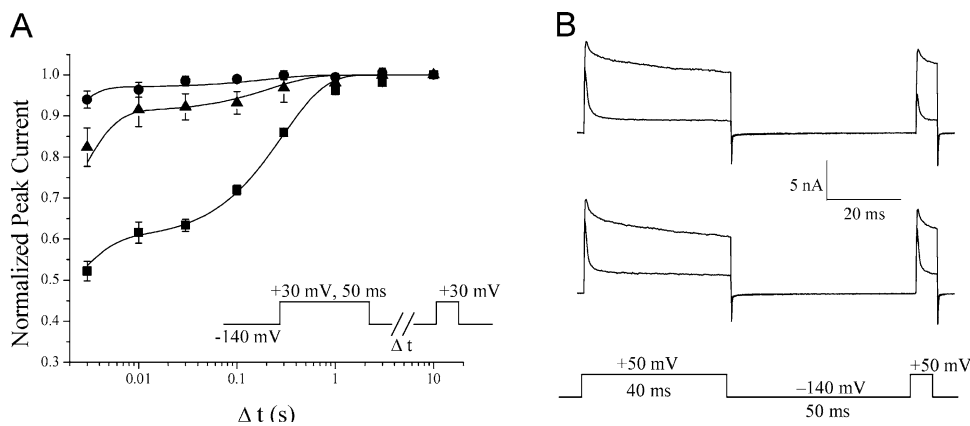
B

5 nA

20 ms

+50 mV
40 ms
−140 mV
50 ms
+50 mV

FIGURE 5. Recovery time courses from the open-channel block and additional use-dependent block by lidocaine and benzocaine. (A) Recovery time courses without (●) and with the open-channel block by lidocaine (■) and benzocaine (▲) at the holding potential are plotted against normalized currents. The pulse protocol is shown in inset. The fitted values and number of experiments are given in the text. (B) Additional use-dependent block by lidocaine (top) but not by benzocaine (middle) is found in the second pulse. The pulse protocol is shown at the bottom. Peak currents before and after LAs are normalized and the mean values are given in the text.

the IC$_{50}$ for the resting-channel block is 1.16 ± 0.05 mM with a Hill coefficient of 0.9 ± 0.1 (n = 5; square) (Fig. 4 B). It is unclear why the Hill coefficient is near unity in benzocaine block as previous reports indicate that the Hill coefficient deviates from unity in neuronal Na$^+$ channels, ranging from 0.5 to 2.4 (Meeder and Ulbricht, 1987). The difference between the IC$_{50}$ values of the resting- and the open-channel block by benzocaine was ~13-fold. Such a high ratio indicates a significant change in the binding affinity of benzocaine during activation. Previous results showed that the affinities for the resting and inactivated channels in myelinated nerve fibers were 0.7 mM (K$_R$) and 0.038 mM (K$_I$), respectively (Meeder and Ulbricht, 1987). However, these values were calculated from shifts in h$_\infty$ curve using a modulated receptor model. It was not possible to measure the inactivated block by benzocaine directly because of its rapid recovery at −140 mV. The ratio of K$_R$/K$_I$ was 17.5, which is comparable to the ratio we obtained for the resting and open-channel block in inactivation-deficient WCW mutant channels.

Recovery from the Open-channel Block

We measured the recovery time course from the open-channel block by lidocaine and benzocaine. Most of mutant channels are noninactivating after a 30-mV pulse for 50 ms (Fig. 5 A, circle) and can be reactivated rapidly. With 100 μM lidocaine, the recovery time course at holding potential (−140 mV) followed a two-exponential function (square). The slow component (40.2%) had a time constant of 292 ± 19 ms (n = 5), whereas the fast component had a time constant of <5 ms. This slow recovery time constant for lidocaine block is slower than that found for the inactivated rNav1.4 wild-type Na$^+$ channels (179 ± 17 ms; Wright

et al., 1997; P < 0.05). With 300 μM benzocaine, most of mutant channels recovered quickly with a fast time constant of <5 ms (triangle) while a small component (9%) recovered with a time constant of 220 ms.

The presence of a major slow component in the recovery from lidocaine block predicted that repetitive pulses at a high frequency would result in additional use-dependent block of mutant currents. To illustrate this phenotype we applied a two-pulse protocol shown in Fig. 5 B (bottom). The peak currents before and after 100 μM lidocaine (Fig. 5 B, top) were measured, normalized, and compared. For the first pulse, there were 73.1 ± 0.7% peak currents remained but only 52.2 ± 1.3% in the second pulse (P < 0.001, n = 9). Thus, lidocaine produced considerable use-dependent block of mutant channels during the second pulse. In comparison with 300 μM benzocaine, there were 74.2 ± 0.8% peak currents in the first pulse and 72.5 ± 1.6% in the second pulse (P = 0.358, n = 8) (Fig. 5 B, middle). Benzocaine therefore failed to elicit additional use-dependent block in mutant channels.

Minimal Effects of External Na$^+$ Ions on Lidocaine or Benzocaine Block

Barber et al. (1992) reported that lidocaine block was not affected by external Na$^+$ ion concentrations ranging from 20 to 150 mM. In our experiments, the concentration of Na$^+$ ions in the external solution was 65 mM. We therefore tested whether complete removal of Na$^+$ ions from the external solution has any effects on lidocaine and benzocaine block of mutant currents. Without external Na$^+$ ions, the IC$_{50}$ values for the resting- and open-channel block are 324 ± 26 μM (with Hill coefficient of 0.97 ± 0.07, n = 5; Fig. 6 A, square) and 17.6 ± 3.2 μM (0.63 ± 0.07, n = 5; triangle) for
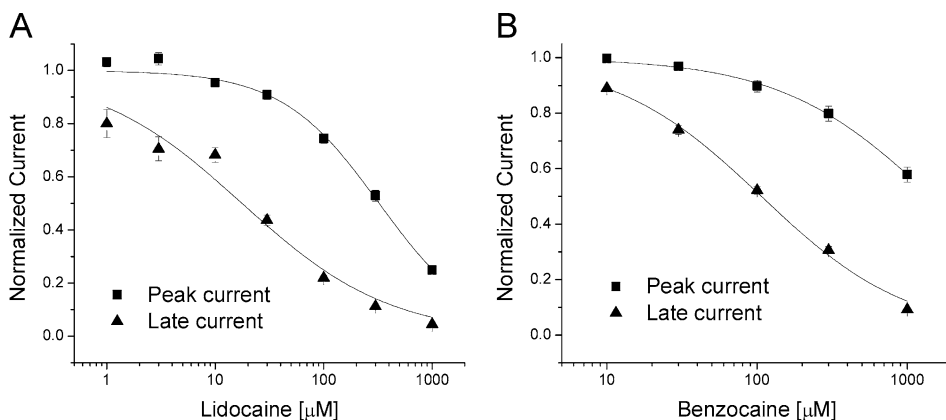
lidocaine and 1.15 ± 0.07 mM (0.92 ± 0.04, n = 5; Fig. 6 B, square) and 107 ± 6 μM (0.88 ± 0.04, n = 5; triangle) for benzocaine. These IC$_{50}$ values are not significantly different from those obtained in the presence of external Na⁺ ions at 65 mM (Figs. 3 and 4; P > 0.05). We conclude that external Na⁺ ions have minimal effects on the block of mutant currents by benzocaine or by lidocaine.

Current–Voltage Relationship in the Presence of 300 μM Benzocaine

Since benzocaine is a neutral compound, its binding should not be influenced directly by voltage within the membrane electric field. Fig. 7 A shows the current traces at various membrane voltages in the presence of 300 μM benzocaine. There is strong time-dependent benzocaine block at voltages higher than +10 mV (vs. Fig. 1 A for control without drug). The time constant for open-channel block by 300 μM benzocaine was 0.82 ± 0.08 ms ($n = 5$) at +30 mV. However, such rapid time-dependent block is minimal at voltages near −30 mV. This lack of time-dependent block could be due to the slower time course of channel activation at −30 mV, which overlap with the fast time course of benzocaine block. In fact, the extent of the block of peak currents at the lower voltages is actually larger (>60% is blocked at −30 mV) than that at the high voltages (<30% is blocked at +30 mV) (Fig. 7 B). These results indicate that channel activation is important for benzocaine block but not the channel opening per se. Also, our data suggest that the resting block by benzocaine is better determined at +30 mV than at −30 mV (Fig. 7 B) because the activation time course is more rapid at +30 mV. Otherwise, the IC$_{50}$ value of the resting-channel block will be <300 μM for benzocaine

if measured at −30 mV. It is interesting to note that the late currents at the end of 50 ms pulse were blocked almost constantly by benzocaine from −40 to +50 mV (∼65%; Fig. 7 C, triangle, $n = 4$) unlike the large variation in block of peak currents (Fig. 7 C, square).

The results of identical analyses for lidocaine block at 100 μM (Fig. 8, A–C) unfortunately are not as explicit as those shown for benzocaine (Fig. 7, A–C) but the trend is similar. For example, although the time-dependent block by lidocaine is present at −30 mV (Fig. 8 A), it is less profound than that at +50 mV. The block of late mutant currents increases progressively from −40 to 0 mV (Fig. 8 C). This overlaps with the voltage where channels open (Fig. 1 B) and is indicative for drugs that preferentially block open channels. Also, the reduction in the peak current is greater at −30 mV than that at +50 mV for lidocaine, but the difference is not very large (40% vs. 30%; Fig. 8 C, square, $n = 5$). Nonetheless, the largest deviation in the block of peak vs. late currents is at +50 mV (30% vs. 80%), as for benzocaine (20% vs. 65%; Fig. 7 C). Possible explanations for these differences are addressed in DISCUSSION.

Apparent Shift in the Steady-state Inactivation Curve by Benzocaine and Lidocaine

The results shown in Figs. 7 and 8 suggest that LAs may block the mutant channels during activation even when the channel is not yet open. To test this possibility, we applied a conventional two-pulse protocol with various conditioning voltages (Fig. 2). The superimposed current traces using this pulse protocol are shown in Fig. 9 A after the application of 300 μM benzocaine. The peak currents were measured in the absence of external Na⁺ ions, normalized with respect to the value with a conditioning pulse at −160 mV and plotted against
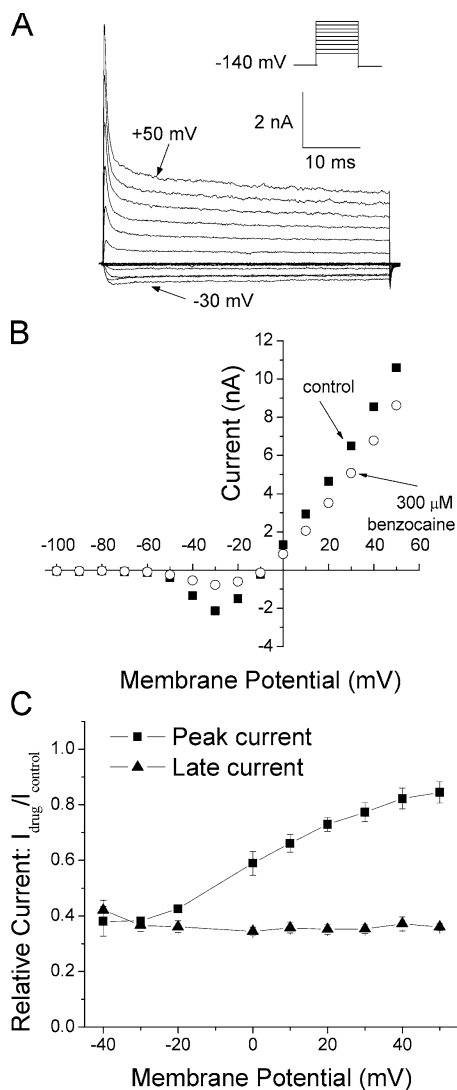
FIGURE 7. Current–voltage relationship in the presence of 300 μM benzocaine. (A) A representative family of Na$^+$ currents were evoked by 50-ms test pulses increasing in 10-mV increments from −100 to +50 mV (pulse protocol shown in inset) in the presence of 300 μM benzocaine. Holding potential was set at −140 mV. Control data similar to those illustrated in Fig. 1 A in the absence of drug were not shown. (B) Peak currents for control (■) and 300 μM benzocaine (○) were plotted against membrane voltage. (C) Both peak (■, $n = 4$) and persistent (▲, $n = 4$) late currents at the end of the test pulse as shown in A were measured, normalized to peak currents measured from the same cell in control saline ($I_{drug}/I_{control}$), and plotted against membrane voltage.

FIGURE 8. Current–voltage relationship in the presence of 100 μM lidocaine. (A) A representative family of Na$^+$ currents were evoked by 50-ms test pulses increasing in 10-mV increments from −100 to +50 mV (inset) in the presence of 100 μM lidocaine. Control data similar to those illustrated in Fig. 1 A without drug were not shown. (B) Peak currents for control (■) and 100 μM lidocaine (□) were plotted against membrane voltage. (C) Both peak (■, $n = 5$) and persistent (▲, $n = 5$) late currents as shown in A were measured, normalized to peak currents measured from the same cell in control saline ($I_{drug}/I_{control}$), and plotted against membrane voltage.

the conditioning pulse (h$_\infty$ curve). At −60 mV, where few channels were activated or inactivated (Fig. 9 B, square), ~60% of channels were inhibited by benzocaine at 300 μM (Fig. 9 B, triangle). Even at −80 mV, a significant fraction of mutant Na$^+$ channels were blocked by benzocaine without channel opening. The shift in the midpoint voltage by benzocaine was −34 mV, providing additional evidence that benzocaine also blocks most, if not all, of the intermediate closed states
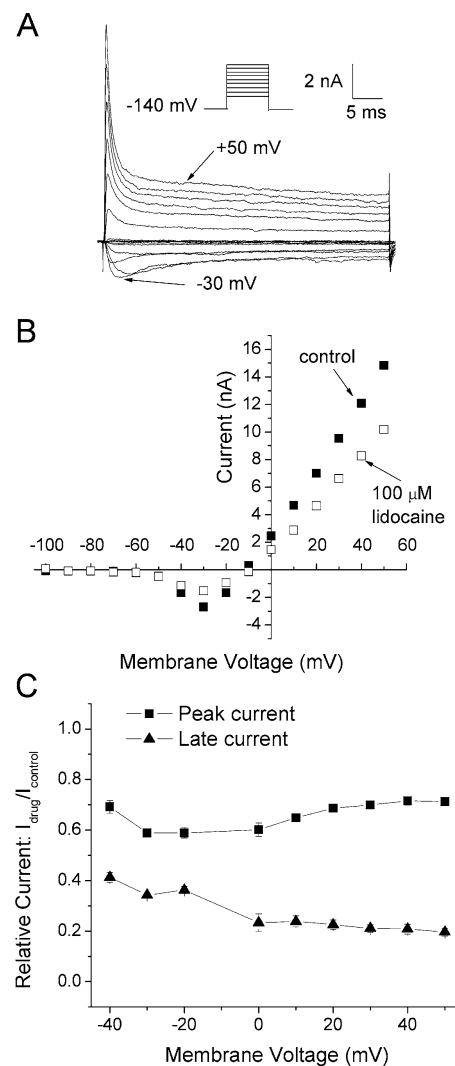
during channel activation. Similar results were obtained for lidocaine at 300 μM (Fig. 9, C and D). The shift in the midpoint voltage by lidocaine was −27 mV. Evidently, a significant intermediate closed-channel block by benzocaine and lidocaine can occur in inactivation-deficient Na$^+$ channels.

DISCUSSION

We created a HEK293 cell line expressing robust inactivation-deficient rNav1.4-WCW mutant Na$^+$ currents

A

-140 mV

+ 30 mV

2 nA

20 ms

B

C

-140 mV

+ 30 mV

2 nA

20 ms

D
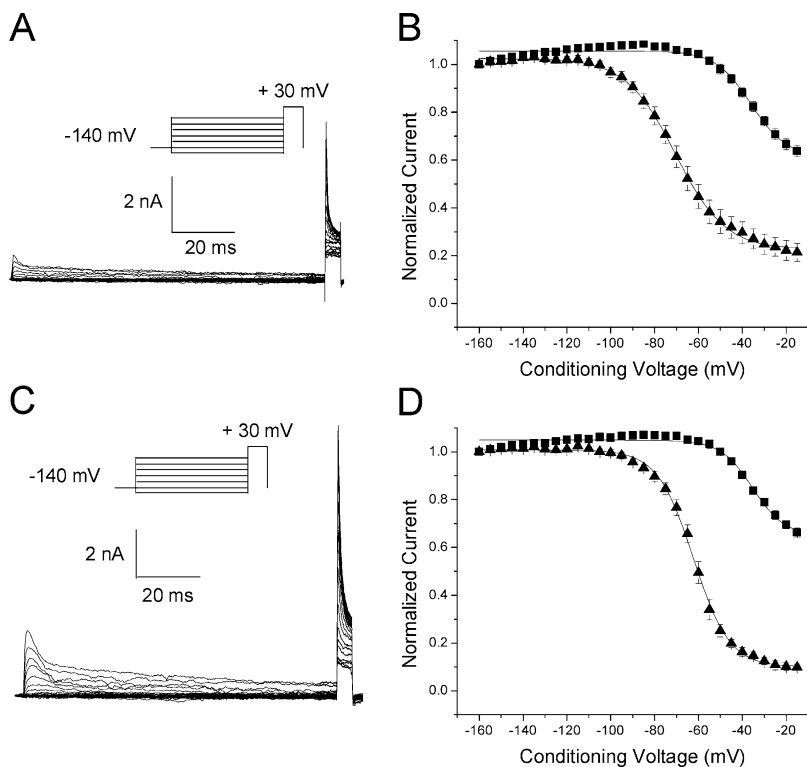
FIGURE 9. Conventional h_∞ measurement of rNav1.4-WCW Na$^+$ channels with LAs presence. (A) Superimposed current traces were evoked by a 5-ms test pulse to +30 mV in the presence 300 μM benzocaine. Test pulses were preceded by 100-ms conditioning pulses, ranging from −160 to −15 mV in 5-mV increments (inset). Notice that Na$^+$ currents were activated at the conditioning pulse >-50 mV. (B) Peak currents at the test pulse of +30 mV were measured, normalized, and plotted as a function of the conditioning voltage. The plot was fitted with a Boltzmann function $(1/[1 + \exp((V_{0.5} - V)/k)])$. The average midpoint ($V_{0.5}$) and slope ($k$) for the control were −36.0 ± 1.77 mV and 8.2 ± 1.2 mV, respectively (■, $n = 5$), and for the cells treated with benzocaine were −70.5 ± 0.4 mV and 11.3 ± 0.3 mV, respectively (▲, $n = 5$). (C) Currents were evoked as described (A) in the presence of 300 μM lidocaine. (D) Normalized Na$^+$ current availability, plotted as in B, and fitted with a Boltzmann function. The average midpoint ($V_{0.5}$) and slope (k) for the control were −35.3 ± 1.5 mV and 7.3 ± 1.0 mV, respectively (■, $n = 6$), and for cells treated with lidocaine were −62.3 ± 0.3 mV and 8.2 ± 0.3 mV, respectively (▲, $n = 6$). All cells were perfused with external solution containing no Na$^+$ ions.

and demonstrated that both lidocaine and benzocaine blocked the open state of WCW mutant channels with high affinities. The IC$_{50}$ values for such open-channel block were comparable to those for the inactivated-channel block. The external Na$^+$ ions did not significantly affect the open-channel block by benzocaine or lidocaine. In addition, we provided evidence that benzocaine and lidocaine blocked intermediate closed states during channel activation, probably via their hydrophobic pathway. The term "high-affinity" in LA binding is only a relative term for closed intermediate and open states with respect to the "low-affinity" LA binding for the resting state. The significance of these findings is discussed below.

Robust Expression of WCW Mutant Na$^+$ Channels in HEK293 Cells

The high expression of rNav1.4-WCW mutant muscle Na$^+$ channels in stably transfected HEK293 cells is unusual, since a previous report showed rather poor expression of inactivation-deficient hNav1.5-IFM/QQQ mutant cardiac Na$^+$ channels in HEK293 cells (Grant et al., 2000). Evidently, the inactivation-deficient phenotype alone cannot explain the limited expression of IFM/QQQ cardiac mutant channels in HEK293 cells. Regardless the reasons for this robust expression, our cell line will be useful for the study of the open-channel block by LAs and by class 1 antiarrhythmic agents. In addition, this cell line can be used for the investiga-

tion of the possible involvement of intermediate closed states in drug binding during channel activation as shown in this report. Such a cell line should also be suitable for the high throughput screening of novel open-channel blockers (Sanguinetti and Bennett, 2003; Wang et al., 2004a).

Open-channel Block of WCW Mutant Channels by Lidocaine and Benzocaine

The time-dependent inhibition of WCW mutant currents by lidocaine, benzocaine, and class 1 antiarrhythmic agents (Wang et al., 2003b, 2004b) was taken as evidence for the open-channel block. This phenotype is consistent with the location of the LA receptor within the pore-forming S6 segments. Such a phenotype was found in the block of K$^+$ channels by internal TEA ions and various homologues (Armstrong, 1971). The dose–response curves show that this open-channel block by benzocaine and lidocaine is nearly as potent as the inactivated-channel block (Meeder and Ulbricht, 1987; Wright et al., 1997). In addition, lidocaine, but not benzocaine, elicited an additional use-dependent block (Fig. 5 B) because of the slow recovery of the open-channel block by lidocaine. Our findings imply that the open-channel block by lidocaine may also play an important role in vivo, particularly during ectopic high-frequency firings when Na$^+$ channels open repetitively (>20 Hz; Devor et al., 1992). Furthermore, the IC$_{50}$ value for the open-channel block by lidocaine is near

the therapeutic plasma concentration range for its anti-arrhythmic action (Roden, 2001) and for its treatment of neuropathic pain (Boas et al., 1982), implying that ectopic hyperexcitability and/or persistent late Na^+ currents found under pathological conditions are susceptible for LA open-channel block, as also recently demonstrated for LQT-3 syndromes (An et al., 1996; Wang et al., 1997; Nagatomo et al., 2000).

The results described above, however, remain incompatible with the observations that show (a) the lack of open-channel block by QX-314 in pronase-treated squid axon and (b) the absence of the high-affinity block by LAs in IFM/QQQ mutant Na^+ channels (see INTRODUCTION). One interpretation is that WCW mutations at D1-S6 fortuitously modify the LA receptor, which is different from that in normal wild-type Na^+ channels. The argument against this view is that LAs also potently block the open state of normal Na^+ channels treated with chloramine-T (Ulbricht and Stoye-Herzog, 1984; Wang et al., 1987, 2004b). Alternatively, the discrepancy may be due to differences in expression systems, mutation sites, and/or pulse protocols. This discrepancy is yet to be resolved.

Lack of Na$^+$ Ion Effects in Lidocaine and Benzocaine Block

External Na^+ ions significantly antagonize binding of QX-314, a quaternary ammonium derivative of lidocaine (Cahalan and Almers, 1979). This antagonism was due to repulsion between the Na^+ ion and the positive charge carried by QX-314 within the pore region. As anticipated, we did not observe antagonism between external Na^+ ions and bound neutral benzocaine (Fig. 6 B). However, since lidocaine can also carry a positive charge when protonated, the lack of antagonism between the external Na^+ ions and lidocaine block is unexpected (Fig. 6 A). Barber et al. (1992) reported a similar negative result by external Na^+ ions for lidocaine block of normal cardiac Na^+ channels. The reason for this phenomenon is unclear. One possibility is that H^+ dissociated from lidocaine when Na^+ ions are approaching the LA receptor. This could not occur for QX-314 because it carries a permanent charge. Yeh and Tanguy (1985) showed that unlike QX-314, lidocaine in its neutral form leaves the closed channel rapidly through the hydrophobic pathway. This hydrophobic pathway could also in part provide unprotonated lidocaine with an LA block that is not antagonized by external Na^+ ions. This possibility is discussed next.

Direct Intermediate Closed-channel Block of WCW Mutant Channels by LAs

At +30 mV, the time-dependent block by 300 μM benzocaine is rapid, with a time constant of ∼0.8 ms, whereas at −30 mV, there is no evidence of a time-dependent benzocaine block. Closer examinations of benzocaine block revealed a slow activation time course at −30 mV as compared with the rapid time course of benzocaine block at +30 mV (Fig. 7). Could block of Na^+ channels by benzocaine occur during transitions to the intermediate closed states before Na^+ currents reach their peak (i.e., first latency distribution; Aldrich et al., 1983)? If so, the peak current at −30 mV should be reduced more than that at +30 mV. A much greater reduction in peak currents by benzocaine was indeed observed at −30 mV (Fig. 7, B and C). By the same token, this result suggests that the rapid open-channel block by neutral benzocaine at +30 mV does not require that benzocaine come exclusively via the hydrophilic pathway.

The results for lidocaine were less clear-cut than those for benzocaine but the trend with respect to intermediate closed-channel block appears the same (Fig. 8). This ambiguity could be due to (a) differences in size and hydrophobicity of lidocaine and benzocaine, (b) the protonation/deprotonation of lidocaine within the aqueous pore, or (c) differences in open times of mutant channels at various voltages. These factors will likely affect the kinetics of the access/exit of the drug via hydrophobic and hydrophilic pathways (Hille, 1977).

Vedantham and Cannon (1999) recently hypothesized that transitions to intermediate closed states, rather than transitions to inactivated states as stated in the classic modulated receptor hypothesis, play a crucial role in the shift of the h_∞ curve by lidocaine. Assuming that intermediate closed state interacts with benzocaine or lidocaine, a significant block by LAs should develop during the 100-ms conditioning pulse because of the significant increase in the availability of intermediate closed states along the activation pathway (e.g., at −70 mV conditioning pulse). Our experimental data from inactivation-deficient Na^+ channels (Fig. 9) strongly support such a hypothesis for LA block by benzocaine and lidocaine.

It is worth noting that we make no distinction between the high-affinity LA binding among closed or closed/inactivated channels. Perhaps fast inactivation in normal Na^+ channels masks the high-affinity LA interactions with intermediate closed and the final open states. Slow-inactivated Na^+ channels may also display high-affinity binding with LAs as residual slow inactivation is evident for the open and intermediate preopen mutant Na^+ channels (Figs. 1 and 2). Transient intermediate "activated" states will in time enter the absorbing inactivated state (Aldrich et al., 1983). Inactivated Na^+ channels are likely to bind to lidocaine with a high affinity albeit more slowly (Wright et al., 1997) than open Na^+ channels. A similarly high-affinity LA receptor should be present in the intermediate closed, in-

termediate closed/inactivated, open, and open/inactivated channels. If so, the use-dependent action of LAs during depolarization does not require inactivated states but may simply be explained by higher affinity for intermediate closed and open states (Vedantham and Cannon, 1999).

In molecular terms, the formation of a high-affinity LA receptor during activation gating could well be due to the rearrangement of S6 segments. This is feasible considering that S6 segments are structurally analogous to the pore-forming inner helices of K^+ channels (Yarov-Yarovoy et al., 2002). These inner helices contain a gating hinge (glycine) that bends $\sim30°$ in K^+ channels upon activation (Jiang et al., 2002). In the straight configuration, four inner helices form a bundle, closing the channel near its intracellular surface, whereas in the bent configuration, the inner helices splay open, creating a wide pathway. Thus, Na^+ channel activation could easily modulate the configuration of the LA receptor situated at pore-forming S6 segments. In contrast, the movement of the intracellular fast-inactivation gate (IFM motif) is not involved in the formation of a high-affinity lidocaine receptor as inferred by Vedantham and Cannon (1999). Nonetheless, fast inactivation may play an important but indirect role for the LA binding since (a) the activation and inactivation gating processes are coupled and (b) the inactivation gate, if docked within the pore region near the LA receptor (Yarov-Yarovoy et al., 2002; Wang et al., 2003b), will likely limit the LA access/exit via the hydrophilic pathway.

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