

Lysine Point Mutations in Na⁺ Channel D4-S6 Reduce Inactivated Channel Block by Local Anesthetics

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ABSTRACT

Voltage-gated Na⁺ channels are a primary target for local anesthetics (LAs). Open or inactivated Na⁺ channels usually have a severalfold higher affinity for LAs than do resting channels. Hille's modulated receptor hypothesis attributed the changes in LA affinity to state-dependent alterations in the conformation of the LA receptor. We expressed wild-type and mutant rat skeletal muscle (μ 1) Na⁺ channels in human embryonic kidney cells to investigate the state-dependent modulation of LA receptor affinity. As an alternative approach to using alanine for point mutation, we substituted lysine (a hydrophilic residue) for native residues in the putative LA receptor located in D4-S6 of the μ 1 Na⁺ channel. Lysine mutation at Y1586 did not alter resting channel affinity for cocaine but did reduce resting affinity at F1579K and N1584K by 2- and 3-fold, respectively. Compared

with μ 1, resting benzocaine block did not change at F1579K, decreased at N1584K, and increased at Y1586K. These effects on resting block could largely be accounted for by either steric/charge interference or cation- π electron interactions between particular moieties on the LA and lysine. Surprisingly, lysine substitution at these residues allowed the channels to undergo steady state fast inactivation yet reduced inactivated channel block by cocaine by up to 27-fold and reduced the benzocaine-induced leftward shift in the h_{∞} curve by up to 22 mV. Our data suggest that transitions in channel state indeed invoke conformational changes in the LA receptor and that lysine mutations in the LA receptor region alter such conformational changes during the transition to the inactivated state.

Voltage-gated Na⁺ channels are membrane proteins that produce action potentials in excitable tissues. The α subunit of Na⁺ channels consists of four homologous domains, each of which contains six transmembrane segments that supposedly have an α -helical secondary structure (Numa and Noda, 1986). LA agents block the transmission of action potentials by binding to a receptor site on the Na⁺ channel α subunit. According to the modulated receptor model for LAs (Hille, 1977), channel state governs the conformation of the LA receptor. The receptor has a weak affinity for LA in the resting state but a strong affinity for LA in the activated or inactivated state. Ragsdale *et al.* (1994) first showed that point mutation of specific residues to alanine in Domain 4-S6 of the rat brain Na⁺ channel (NaIIa) strongly affected LA block of Na⁺ currents when the channels were expressed in *Xenopus laevis* oocytes. Recently, Wang *et al.* (1998) reported similar findings for comparable mutations of the rat skeletal muscle Na⁺ channel (μ 1; Trimmer *et al.*, 1989) when expressed in mammalian cells (HEK 293T). The study by Wang

et al. (1998) also confirmed that etidocaine, a tertiary amine LA, and neutral benzocaine (ethyl *p*-aminobenzoate) bind to a common receptor within Domain 4-S6.

In the present study, we substituted a charged amino acid (lysine) for single point mutations of the native residues at μ 1-F1579, N1584, and Y1586 and expressed the channels in HEK cells. Point mutation of comparable residues in NaIIa channels to alanine (NaIIa-F1764A, N1769A, and Y1771A) markedly altered receptor affinity for etidocaine (Ragsdale *et al.*, 1994). Our aim was to determine how incorporation of a charged residue at these positions might affect state-dependent LA affinity. We examined block of Na⁺ current by two LAs that are structurally very different. Cocaine (a tertiary amine) has a large rigid structure and the charged species, presumed to be active at the receptor (Narahashi *et al.*, 1970; Nettleton and Wang, 1990), is predominant at neutral pH. Benzocaine, on the other hand, is a more flexible compound and is uncharged. Our results indicated that lysine mutation at these three residues had little effect on the level of current expression and nearly eliminated high affinity, inactivated channel binding of both cocaine and benzocaine. The reduction in inactivated channel block could not be attributed to changes in channel gating because the mutations did not

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ABBREVIATIONS: LA, local anesthetic; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; h_{∞} , steady state availability function.

prevent the mutant channels from entering the fast inactivated state. Our data suggest that lysine mutation of residues within the LA receptor region prevents the receptor from occupying the high affinity conformation without drastically altering the kinetic transition from resting to inactivated channels.

Materials and Methods

Solutions and chemicals. Cocaine hydrochloride was purchased from Mallinckrodt (St. Louis, MO), and benzocaine was purchased from Sigma Chemical (St. Louis, MO). LAs were applied externally at the appropriate concentration. The extracellular solution used to perfuse HEK cells contained: 65 mM NaCl, 85 mM choline Cl, 2 mM CaCl_2 , and 10 mM HEPES (titrated with tetramethylammonium hydroxide to pH 7.4). The pipette solution contained 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES (titrated with cesium hydroxide to pH 7.2).

Mutagenesis of $\mu 1$ channels and transient transfection of HEK 293T cells. Site-directed mutagenesis was used to create lysine point mutations of the $\mu 1$ -pcDNA/amp vector at residues $\mu 1$ -F1579, $\mu 1$ -N1584, and $\mu 1$ -Y1586 as described previously (Wang and Wang, 1998; Wang et al., 1998). One additional application of nucleoside triphosphates and T4-DNA polymerase was given during the 4-hr *in vitro* synthesis. Potential mutants were identified by restriction mapping and confirmed by DNA sequencing.

As described previously (Wright et al., 1997), HEK 293T cells were transfected with channel plasmid (2–10 μg) and reporter plasmid CD8-pih3m (1 μg) by the calcium phosphate precipitation method (Cannon and Strittmatter, 1993). The transfected cells were replated onto 35-mm culture dishes and used for experiments for up to 3 days. Transfection-positive cells, as identified by CD8 Dynabeads (Dyna, Lake Success, NY), were selected for whole-cell patch recording. Expression levels of the lysine mutants were generally > 1 nA, which were equal to or greater than the expression levels of corresponding alanine-substituted mutants (Wang et al., 1998).

Electrophysiology and data analysis. Whole-cell voltage clamp (Hamill et al., 1981) of HEK cells was used to study macroscopic Na^+ currents at room temperature ($23 \pm 2^\circ$). Electrode resistances ranged from 0.4 to 1.0 M Ω . Command voltages were programmed by pCLAMP software (Axon Instruments, Burlingame, CA) and delivered by a List EPC7 voltage clamp. Data were sampled at 50 kHz and filtered at 5 kHz. After establishment of whole-cell voltage clamp, the cells were dialyzed for 25–30 min before data were acquired (Wright et al., 1997). The holding potential for all experiments was -140 mV. Most of the capacitive current was canceled by the EPC7 circuitry; any remaining capacitive artifact and the leakage current were subtracted by the P/4 method. The voltage error at $+30$ mV was ≤ 5 mV. Pulses were separated by 5 sec when acquiring steady state activation and inactivation data (including steady state inactivation in benzocaine). For all other experiments, pulses were separated by 30 sec intervals at the holding potential. Least-squares curve-fitting was performed with Origin software (Microcal, Northampton, MA). Statistical analyses (Student's *t* test) were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA) to determine the significance of changes in mean values. *p* values of < 0.05 were considered statistically significant; *n* values indicate the number of cells examined. Data are presented as mean \pm standard error.

Results

Effects of lysine point mutations on $\mu 1$ channel kinetics. To determine the voltage dependence of activation of $\mu 1$ and mutant channels, we measured the peak Na^+ current during 5-msec depolarizations to test potentials ranging from -100 mV to $+50$ mV (Fig. 1A). Activation of N1584K chan-

nels closely resembled the activation of $\mu 1$ channels, whereas the midpoint voltages ($V_{0.5}$) of activation for F1579K and Y1586K were 11 mV and 12 mV, respectively, more positive than the $V_{0.5}$ value of activation for $\mu 1$. To determine the voltage dependence of steady state inactivation (h_∞) of the channels, we delivered 100-msec conditioning pulses ranging from -160 mV to -35 mV and measured the peak Na^+ current during a 5-msec test pulse to $+30$ mV (Fig. 1B). The $V_{0.5}$ value of steady state inactivation for Y1586K channels was similar to that of $\mu 1$ channels, whereas $V_{0.5}$ value of steady state inactivation for F1579K and N1584K channels were 6 mV and 12 mV, respectively, more positive than that of $\mu 1$. In addition, approximately 10% of the N1584K channels did not inactivate after conditioning pulses ranging from -50 mV to -35 mV.

Lysine point mutations in Domain IV-S6 affect state-dependent modulation of cocaine sensitivity. To obtain an initial assessment of cocaine sensitivity after lysine point

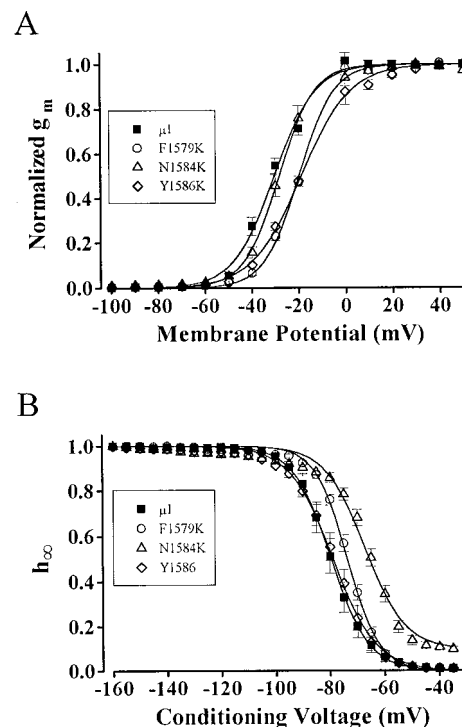


Fig. 1. Activation and steady state inactivation of $\mu 1$ channels after lysine point mutation. A, Normalized membrane conductance (g_m) plotted versus the amplitude of the 10-msec voltage step. g_m was determined from the equation $g_m = I_{\text{Na}} / (E_m - E_{\text{Na}})$. The data were fitted with an empirical Boltzmann function $\{1/[1 + \exp((V_{0.5} - V)/k)]\}$ to determine the midpoint voltage ($V_{0.5}$) of activation and slope (k). For $\mu 1$ ($n = 6$), the average $V_{0.5}$ and k values were -30.4 ± 1.4 mV and 8.5 ± 0.2 mV, respectively. For F1579K ($n = 7$), $V_{0.5}$ and k values were -19.7 ± 0.7 mV ($p < 0.05$ compared with $\mu 1$) and 7.8 ± 0.6 mV, respectively. For N1584K ($n = 8$), $V_{0.5}$ and k values were -28.0 ± 1.6 mV and 7.3 ± 0.5 mV ($p = 0.04$ compared with $\mu 1$), respectively. For Y1586K ($n = 7$), $V_{0.5}$ and k values were -18.4 ± 0.7 mV ($p < 0.05$ compared with $\mu 1$) and 10.9 ± 0.9 mV ($p < 0.05$ compared with $\mu 1$), respectively. B, Normalized Na^+ current availability function (h_∞) for the four channels. Cells were held at -140 mV and received a 100 ms conditioning pulse ranging in amplitude from -160 mV to -35 mV followed by a test pulse to $+30$ mV. The average $V_{0.5}$ values (50% availability) and k values for the fitted Boltzmann functions were: -79.5 ± 2.1 mV and 6.2 ± 0.4 mV, respectively for $\mu 1$ ($n = 7$); -73.6 ± 0.8 mV ($p < 0.05$ compared with $\mu 1$) and 5.5 ± 0.1 mV ($p = 0.04$ compared with $\mu 1$), respectively, for F1579K ($n = 11$); -67.2 ± 1.2 mV ($p < 0.05$ compared with $\mu 1$) and 7.1 ± 0.4 mV, respectively, for N1584K ($n = 14$); and -78.8 ± 1.9 mV and 7.3 ± 0.2 mV ($p < 0.05$ compared with $\mu 1$), respectively, for Y1586K ($n = 7$).

mutation, we examined cocaine block of the channels using a pulse protocol that consisted of 10-sec conditioning pulses to various voltages followed by a 100-msec interval at the holding potential and a subsequent test pulse to +30 mV (Fig. 2). The 10-sec conditioning pulses allowed cocaine binding to reach steady state, and the 100-msec interval at the holding potential permitted inactivated but drug-free channels to recover from fast inactivation (Wright *et al.*, 1997). The percentage of block after strongly negative conditioning pulses provided an estimate of resting channel affinity for cocaine whereas the percentage of block after the least negative conditioning pulses provided an estimate of the inactivated channel affinity.

Fig. 2A shows the currents evoked during test pulses to +30 mV after the cells had received conditioning pulses to -140 mV (Fig. 2A, *left traces*) and to -70 mV (Fig. 2A, *right traces*). After a conditioning pulse to -140 mV, 50 μ M cocaine blocked similar percentages of current at resting μ 1 and Y1586K channels but blocked a smaller percentage of current at resting F1579K and N1584K channels. After inactivating the channels with a 10-sec conditioning pulse to -70 mV, 50 μ M cocaine blocked a much larger percentage of μ 1 current than at any of the three mutant channels. Although the conditioning pulse to -70 mV in control saline elicited large amounts of slow inactivation at Y1586K channels (Fig. 2A, *dashed line*), the cocaine-induced reduction in test-current amplitude was much less at Y1586K channels than at μ 1 channels.

Fig. 2B shows the effect of the pulse protocol on normalized test currents evoked in control saline. We normalized the control data by dividing the peak amplitude of the test current at each conditioning voltage by the peak amplitude of

the test current evoked after the conditioning pulse to -160 mV. In control saline, 10-sec conditioning pulses more negative than -80 mV had little effect on the test current amplitude of μ 1, F1579K, or N1584K channels. Conditioning pulses more positive than -80 mV, which began to elicit slow inactivation, had similar effects on test current amplitude at μ 1 and F1579K channels but had little effect on the current amplitude at N1584K channels. In contrast, conditioning pulses more positive than -120 mV elicited increasing amounts of slow inactivation at Y1586K channels.

We delivered the pulse protocol in the presence of cocaine (Fig. 2C) to determine the cocaine sensitivities of resting and inactivated channels. To obtain the percentage of cocaine block at each conditioning voltage, we divided the peak amplitude of the test currents evoked in 50 μ M cocaine by the peak amplitude of the test currents evoked in control saline. Cocaine block of the resting channels (-160 mV to -120 mV) was weak, although both F1579K and N1584K channels seemed less sensitive than either μ 1 or Y1586K. As the conditioning voltage became less negative, an increasing percentage of μ 1 channels became fast inactivated and subsequently blocked by cocaine during the 10-sec conditioning pulse. The percentage of block of inactivated μ 1 channels reached steady state at -70 mV. In contrast, even the most positive conditioning pulses induced very little block of inactivated F1579K and N1584K channels. Cocaine block of Y1586K channels resembled the block of μ 1 channels up to -100 mV, and conditioning pulses to between -90 mV and -70 mV produced modest increases in the block of Y1586K channels. Note that after a conditioning pulse to -60 mV (Fig. 2C), block of Y1586 decreased by 20% ($p < 0.05$; $n = 6$) compared with block after a conditioning pulse to -70 mV.

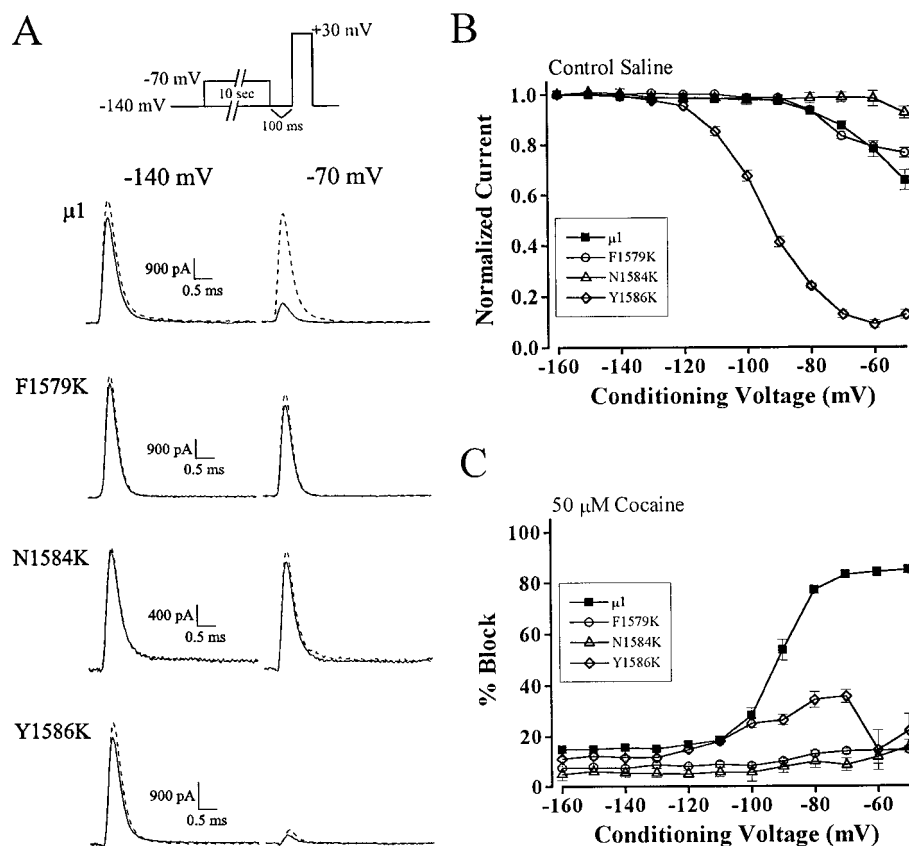


Fig. 2. Steady state block of wild-type and mutant μ 1 Na^+ channels by 50 μ M cocaine. **A**, The pulse protocol consisted of a 10-sec conditioning pulse to -140 mV or to -70 mV, followed by a 100-msec interval at -140 mV, and a subsequent test pulse to +30 mV. *Traces*, effects of the pulse protocol on test current amplitude when delivered in control saline (*dashed traces*) and in 50 μ M cocaine (*solid traces*). **B**, Conditioning pulses (as in part A) ranging from -160 mV to -50 mV were delivered in control saline. The test current amplitude after each conditioning pulse was normalized according to the amplitude of the test pulse after the conditioning pulse to -160 mV and plotted versus the conditioning voltage. **C**, Normalized percentage of 50 μ M cocaine block at each conditioning voltage. At each conditioning voltage, test current amplitude in 50 μ M cocaine was normalized according to the amplitude of the test current evoked in control saline. In B and C, $n = 6$ (μ 1), 6 (F1579K), 5 (N1584K) and 6 (Y1586K).

The decrease in cocaine block of Y1586K channels at -60 mV most likely resulted from modest amounts of channel activation and knockout of the drug by external Na^+ ions (Wang, 1988). These data indicated that introduction of a lysine residue in the region of the LA receptor markedly reduced the affinity of inactivated channels for cocaine.

To more accurately determine the affinities of resting and inactivated channels for cocaine, we performed dose-response experiments and fitted the data with the Hill equation to estimate the dissociation constants of resting (K_R) and inactivated (K_I) channels (Fig. 3). In all cases, the fitted curves had a Hill coefficient of 1, which indicates that one cocaine molecule binds to each channel. We determined the resting

channel affinity by performing the experiments at a holding potential of -140 mV and measuring the percentage of cocaine block at $+30$ mV (Fig. 3A). Cocaine had a similar affinity for resting $\mu 1$ and Y1586K channels with K_R values of $222.5 \pm 15.7 \mu\text{M}$ ($n = 4$) and $226.4 \pm 8.6 \mu\text{M}$ ($n = 6$; $p > 0.05$), respectively. Compared with $\mu 1$, resting F1579K and N1584K channels had a 2- to 3-fold ($p < 0.05$) lower affinity for cocaine with K_R values of $455.5 \pm 23.4 \mu\text{M}$ ($n = 4$) and $631.1 \pm 33.1 \mu\text{M}$ ($n = 7$), respectively (Table 1).

We determined the cocaine affinity at inactivated channels by delivering a 10-sec conditioning pulse followed by a 100-msec interval at -140 mV and a subsequent test pulse to $+30$ mV (Fig. 3B). We used a conditioning pulse to -70 mV for determining the inactivated channel affinities of $\mu 1$, F1579K, and Y1586K. Because the $V_{0.5}$ value of steady state inactivation for N1584K channels was about 10 mV more positive than the $V_{0.5}$ values for the other channels (Fig. 1B), we determined the inactivated channel affinity of N1584K channels using a conditioning pulse to -60 mV. Lysine point mutation substantially reduced the relative increase in cocaine affinity at inactivated channels (K_R/K_I ratio; Table 1). At $\mu 1$ channels, the K_R value for cocaine was 18 times larger than the K_I value. In contrast, the K_R/K_I ratios at F1579K, N1584K, and Y1586K channels were 1.8, 1.9, and 3.1, respectively. Furthermore, lysine point mutation resulted in a markedly reduced inactivated channel affinity compared with the inactivated channel affinity of $\mu 1$ channels. The reduction in inactivated channel affinity was smallest at Y1586K channels (~ 6 -fold) and was largest at N1584K channels (~ 27 -fold). Note that inactivated F1579K channels had a similar affinity for cocaine, and inactivated N1584K channels had a weaker affinity for cocaine compared with $\mu 1$ resting affinity at -140 mV.

Effect of lysine point mutation on block by neutral benzocaine. To estimate the benzocaine affinity of resting channels, we measured the percentage of current blocked by 1 mM benzocaine during a step from the holding potential to $+30$ mV (Fig. 4, A and B). Interestingly, benzocaine block of resting channels differed at each of the three mutant channels. On average, 1 mM benzocaine blocked a similar amount of current at $\mu 1$ channels (40%) and F1579K channels (41%; $p > 0.05$). Compared with block of $\mu 1$ channels, 1 mM benzocaine blocked a significantly smaller ($p < 0.05$) percentage of current at N1584K channels (35%) but a significantly larger ($p < 0.05$) percentage of current at Y1586K channels (55%). We did not perform dose-response experiments with benzocaine because the Hill coefficient varies from < 1 to > 1 depending on the concentration range (Meeder and Ulbricht, 1987; Wang et al., 1998) and because the solubility of benzocaine is less than 4 mM.

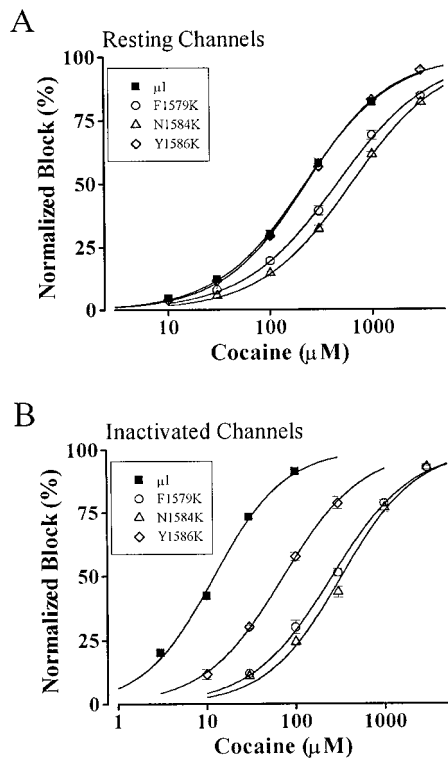


Fig. 3. Cocaine dose-response curves for wild-type and mutant $\mu 1$ Na^+ channels. A, The resting channel affinity (K_R) was determined by holding the cells at -140 mV and measuring the percentage of blocked current during a test pulse to $+30$ mV. B, The inactivated channel affinity (K_I) of $\mu 1$, F1579K, and Y1586K channels was determined by delivering a 10-sec conditioning pulse to -70 mV, followed by a 100-msec interval at -140 mV and a test pulse to $+30$ mV; for N1584K channels, a 10-sec conditioning pulse to -60 mV was used. The percentage of block was determined by comparing the peak amplitude of the test current at each cocaine concentration with the peak amplitude of the test current in control saline. The slopes (h) of the fitted Hill equation ranged from 0.94 to 1.08 and thus were indicative of a 1:1 stoichiometry. The average K_R and K_I values for each channel are given in Table 1.

TABLE 1
 K_D values of cocaine at resting and inactivated channels

The K_D values were obtained from dose-response experiments. K_R values for all channels were determined at -140 mV; K_I values for $\mu 1$, F1579K, and Y1586K were determined at -70 mV and the K_I value for N1584K was determined at -60 mV.

Channel	K_R	K_I	K_R/K_I	$K_R/K_{R(\mu 1)}$	$K_I/K_{I(\mu 1)}$
	μM				
$\mu 1$	222.5 ± 15.7 (4)	12.2 ± 0.5 (5)	18.2	—	—
F1579K	$455.5 \pm 23.4^*$ (4)	$260.1 \pm 16.5^*$ (4)	1.8	2.1	21.3
N1584K	$631.1 \pm 33.1^*$ (7)	$334.5 \pm 23.5^*$ (7)	1.9	2.8	27.4
Y1586K	226.4 ± 8.6 (6)	$74.3 \pm 4.2^*$ (4)	3.1	1.0	6.1

Numbers in parentheses indicate the number of cells; *, $p < 0.05$ compared with $\mu 1$. The values in the last two columns were determined by dividing the mean mutant K_D values by the mean $\mu 1$ K_D values.

To compare the inactivated channel affinities for benzocaine, we measured the leftward shift in the h_{∞} curves (Fig. 4C) induced by 1 mM benzocaine. We assumed that the magnitude of the leftward shift provided relative information about the affinity of benzocaine at inactivated channels. Benzocaine shifted the h_{∞} curve of $\mu 1$ channels by 26 mV in the negative direction, whereas the negative shifts at F1579K, N1584K, and Y1586K channels were 7 mV, 4 mV, and 18 mV, respectively. For each channel, the effect of benzocaine on the Boltzmann function slope factor (k) reflected the magnitude of the leftward shift, with $\mu 1$ having the largest increase in k value (see legend, Fig. 4C). Thus, the reduction in leftward shift in h_{∞} at the three mutant channels was generally consistent with the reduction in cocaine affinity for the inactivated state. For both cocaine and benzocaine, the affinity of inactivated N1584K channels was most reduced and the affinity of inactivated Y1586K channels was least reduced.

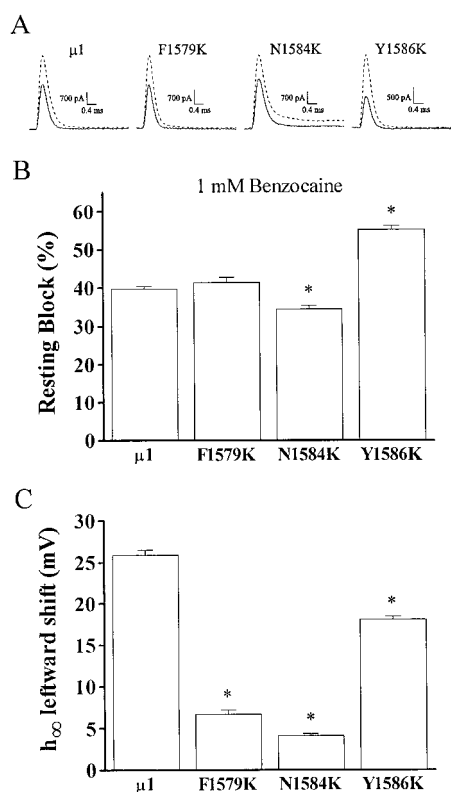


Fig. 4. Block of wild-type and mutant $\mu 1$ Na^+ channels by 1 mM benzocaine. A, Representative current traces of resting channel block by 1 mM benzocaine at -140 mV measured during a test pulse to $+30$ mV. B, Averaged percentages of resting channels blocked by 1 mM benzocaine [$n = 10$ ($\mu 1$), 9 (F1579K), 8 (N1584K) and 8 (Y1586K)]. C, Average leftward (negative) shift in the $V_{0.5}$ value of the steady state inactivation curve (h_{∞}) produced by 1 mM benzocaine [$n = 7$ for each channel]. For each cell, the h_{∞} pulse protocol (Fig. 1B) was delivered after 25–30 min of dialysis and superfusion with control saline. After resting block by 1 mM benzocaine reached steady state (~ 2 min), the h_{∞} pulse protocol was repeated. $V_{0.5}$ values in control saline and in benzocaine were determined by fitting the data with a Boltzmann function. The mean slope factors (k) of the fitted Boltzmann function were (control k value; benzocaine k value): $\mu 1$ ($6.2 \text{ mV} \pm 0.4$; $8.9 \pm 0.3 \text{ mV}^*$), F1579K ($5.5 \pm 0.2 \text{ mV}$; $5.8 \pm 0.1 \text{ mV}$), N1584K ($6.9 \pm 0.4 \text{ mV}$; $7.0 \pm 0.6 \text{ mV}$), Y1586K ($7.3 \pm 0.2 \text{ mV}$; $9.5 \pm 0.3 \text{ mV}^*$), where * indicates a significant ($p < 0.05$) increase in the slope factor in the presence of benzocaine. On the figure: *, $p < 0.05$ compared with $\mu 1$.

Discussion

The idea that channel state modulates the conformation and affinity of the LA receptor (Hille, 1977) has been well supported (for review, see Butterworth and Strichartz, 1990). The inherent complexity of a state-dependent modulated receptor and the lack of information about the receptor's conformational changes that accompany channel state transition have thus far hampered the study of LA action. Recent studies have shown that residues within Domain 4-S6 of rat brain IIa Na^+ channels are important determinants for binding etidocaine (Ragsdale *et al.*, 1994) as well as Class I antiarrhythmic drugs and anticonvulsant agents (Ragsdale *et al.*, 1996). In this study, we explored the mechanism of state-dependent receptor modulation using lysine point mutations of critical residues within Domain 4-S6 of $\mu 1$ Na^+ channels. Our data generally support the LA binding model described by Ragsdale *et al.* (1994) and offer further explanation into the interaction between LA agents and voltage-gated Na^+ channels. The major finding in this report is that lysine mutation of the native residues at $\mu 1$ -F1579, N1584, and Y1586 nearly eliminates high affinity LA binding with the inactivated state. The implications of our findings are that 1) these residues are critical for LA binding and 2) lysine mutation of these residues prevents the LA receptor from occupying the high affinity conformation through charge interference or some unidentified allosteric mechanism.

With respect to the resting channel affinity for LA, most of our results can be accounted for by the Ragsdale *et al.* (1994) model for LA binding to Na^+ channels (Fig. 5A). The model contends that the contact point for the charged region of a tertiary amine LA lies deep within the channel pore at $\mu 1$ -F1579, whereas the contact point for the phenyl group lies closer to the cytoplasmic mouth of the pore at $\mu 1$ -Y1586. The attraction for LA is thought to occur through cation- π electron interaction (Heginbotham and MacKinnon, 1992) between the aromatic moiety on the F1579 residue and the tertiary amine moiety on the LA, and through hydrophobic interaction (Butterworth and Strichartz, 1990) between the aromatic moieties on the Y1586 residue and on the LA. Our data show that substitution of lysine at $\mu 1$ -F1579 reduced resting affinity for cocaine by 2-fold (Fig. 3A and Table 1), which suggests that the charged lysine inhibited cocaine binding with the tertiary amine moiety, perhaps by a charge-charge interaction. On the other hand, lysine substitution at residue $\mu 1$ -Y1586 had no effect on the resting affinity for cocaine. Replacement of the hydrophobic Y residue with the basic lysine residue could invoke a cation- π electron interaction between the aromatic ring on cocaine and the charged amine moiety on lysine, resulting in no net change in resting affinity.

We can draw similar conclusions about the effects of lysine substitution on resting benzocaine affinity. Compared with the wild-type channels, mutation of the F1579 residue to lysine had no detectable effect on benzocaine block of resting channels, whereas lysine substitution at Y1586 increased block of resting channels (Fig. 4B). These results are also consistent with the Ragsdale *et al.* model, because there is no tertiary amine moiety on benzocaine to interact with residue F1579. Furthermore, the increase in benzocaine affinity at Y1586K can be explained if a cation- π electron interaction between the aromatic ring on benzocaine and the amine

moiety on Y1586K is stronger than the hydrophobic interaction between the aromatic ring on benzocaine and the aromatic ring on tyrosine at Y1586. Note also that benzocaine contains a 4-amino group on its phenyl ring that is not present on cocaine.

One significant difference between our data and those of Ragsdale *et al.* (1994) was the effect on LA affinity after point mutation at residue $\mu 1$ -N1584 (NaIIa-N1769). Both Ragsdale *et al.* (1994) and Wang *et al.* (1998) showed that alanine substitution at this residue increased the resting affinity for etidocaine by severalfold. In contrast, substitution of lysine for $\mu 1$ -N1584 reduced receptor affinity for cocaine and benzocaine at both resting and inactivated channels more so than did the mutations at $\mu 1$ -F1579 and Y1586. Indeed, mutation $\mu 1$ -N1584K reduced cocaine affinity at resting channels by 3-fold and reduced the inactivated affinity by almost 30-fold. Ragsdale *et al.* (1994) attributed the increase in resting etidocaine affinity at mutant NaIIa-N1769A ($\mu 1$ -N1584) to indirect effects because the model has the residue facing away from the channel pore as shown in the helical wheel plot of the D4-S6 segment in Fig. 5B, where the *dashed line* indicates the pore lining. If alanine point mutation at $\mu 1$ -N1584 increases LA affinity through indirect effects, then it is conceivable that lysine point mutation could have vastly different indirect effects than those of alanine. The decreases in cocaine affinity at $\mu 1$ -N1584K can not be attributed to changes in gating because we determined K_R at -140 mV where steady state inactivation is completely removed, and we determined K_I at -60 mV where steady state inactivation

of $\mu 1$ -N1584K was comparable with that of $\mu 1$ at -70 mV. Note that although approximately 10% of the N1584K channels did not fast inactivate, this small component could not be responsible for the cocaine resistance of the inactivated state in N1584K channels because both F1579K and Y1586K were cocaine-resistant, even though their steady state inactivation reached completion. These data imply that residue N1584 and/or the surrounding microenvironment have a substantial role in determining LA affinity. Further study of N1584 with hydrophilic and hydrophobic residues as substitutes may help clarify the role of this residue in LA binding.

Our most striking observation was that lysine substitution virtually eliminated inactivated channel block at F1579 and N1584 and markedly reduced inactivated channel block at Y1586. In addition to the severalfold reduction in the inactivated channel affinity for cocaine, lysine mutation at these residues significantly decreased the benzocaine-induced left shift in the h_∞ curve despite having variable effects on resting benzocaine affinity. One interpretation of our data is that lysine point mutation of the D4-S6 segment could, in theory, alter LA access to the receptor when the channel occupies the inactivated conformation. Although we can not completely rule out this possibility, benzocaine is a small, flexible, hydrophobic LA whose access to the receptor would not likely be affected by lysine point mutation. Furthermore, lysine substitution reduced inactivated channel block by cocaine in a manner consistent with the reduction in benzocaine block, suggesting that the mechanism for the reduction in inacti-

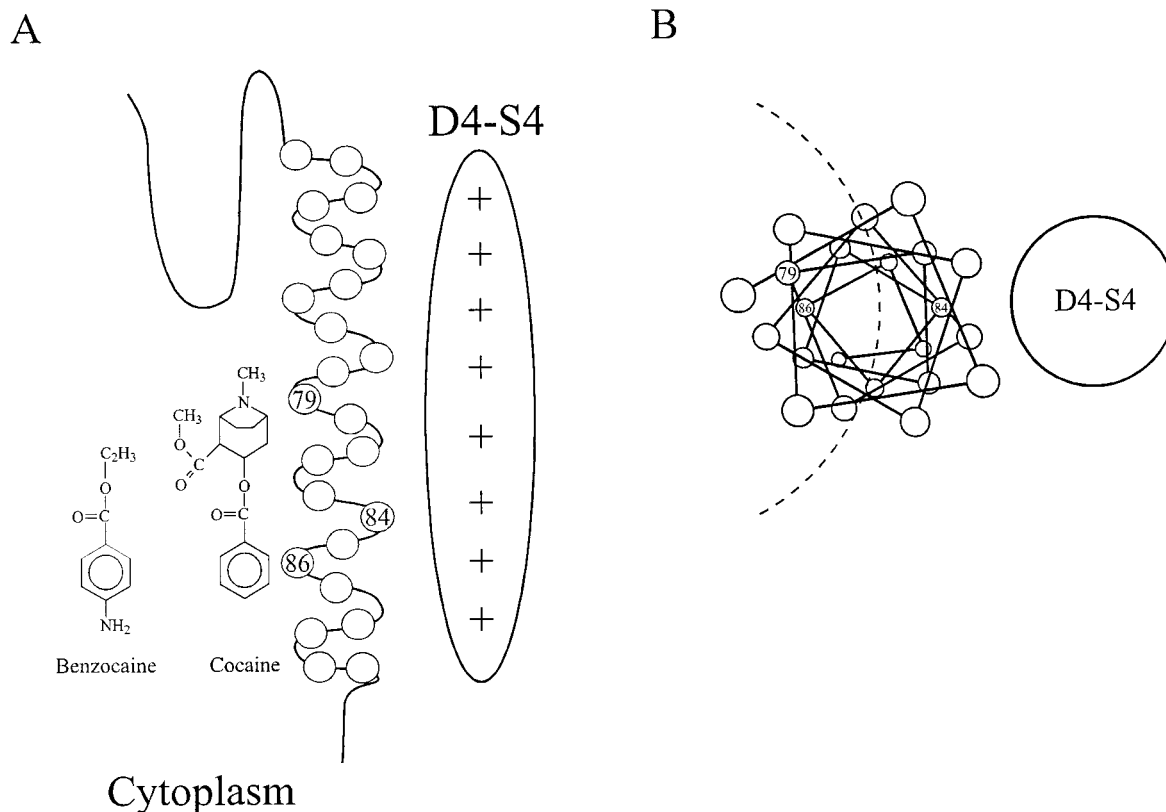


Fig. 5. The LA receptor on $\mu 1$ channels. A, Putative LA contact points on the D4-S6 segment of $\mu 1$ channels. The model by Ragsdale *et al.* (1994) was adapted to show the appropriate $\mu 1$ residue numbers. The tertiary amine moiety (on cocaine or other tertiary amine LAs) is believed to bind at residue F1579 and the phenyl moiety (on tertiary amine LAs and neutral benzocaine) is believed to bind at Y1586. The adjacent D4-S4 segment (Yang *et al.*, 1996) is also shown. B, Extracellular view of a helical wheel plot of the D4-S6 segment and the adjacent D4-S4 segment. The plot shows residues F1579 and Y1586 inside the pore region; *dashed half circle*, the lining of the pore region.

vated channel block by these two very different LAs is at the level of the receptor and not a change in LA access.

Lysine substitution at F1579 had no detectable effect on resting benzocaine affinity but reduced the magnitude of the leftward shift in the h_{∞} curve by 20 mV. According to the Ragsdale *et al.* model, F1579 should not interact directly with benzocaine because benzocaine lacks the tertiary amine, yet mutation of F1579 to lysine reduced the inactivated channel affinity for benzocaine. The reduction in inactivated channel binding of benzocaine at F1579K may be caused by charge interference or steric interaction between the amino group of lysine and the amino group of benzocaine when the channel occupies the high affinity conformation. Our findings thus suggest that conformational changes in the LA receptor indeed accompany changes in kinetic state. Furthermore, lysine mutation in the LA receptor region affected the local conformational transition of the receptor from the low affinity state to the high affinity state without markedly affecting the global transition from the resting state to the inactivated state. In normal channels, the transition from the resting state to the inactivated state could increase the affinity of the LA receptor by shifting the orientation of the S6 segment residues. The entire α helical structure of the S6 segment could twist or become tilted in response to outward movement of the S4 segment during depolarization (Yang and Horn, 1995; Yang *et al.*, 1996). A more drastic alteration in receptor configuration could occur if depolarization induces changes in the secondary structure of the S6 segment as has been suggested for the S4 segment of K channels (Aggarwal and MacKinnon, 1996). Because the Na^+ channel selectivity filter also strongly influences LA binding (Sunami *et al.*, 1997), further examination of the pore region using charged residue substitutions should extend our present understanding of LA interactions and could also prove to be a valuable tool for investigating the structural determinants of other transmembrane segments of ion channels.

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