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Local Anesthetic Block of Batrachotoxin-Resistant Muscle Na^+ Channels

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ABSTRACT

Local anesthetics (LAs) are noncompetitive antagonists of batrachotoxin (BTX) in voltage-gated Na^+ channels. The putative LA receptor has been delineated within the transmembrane segment S6 in domain IV of voltage-gated Na^+ channels, whereas the putative BTX receptor is within segment S6 in domain I. In this study, we created BTX-resistant muscle Na^+ channels at segment I-S6 (μ1-N434K, μ1-L437K) to test whether these residues modulate LA binding. These mutant channels were expressed in transiently transfected human embryonic kidney 293T cells, and their sensitivity to lidocaine, QX-314, etidocaine, and benzocaine was assayed under whole-cell, voltage-clamp conditions. Our results show that LA binding in BTX-resistant μ1 Na^+ channels was reduced significantly. At −100 mV holding potential, the reduction in LA affinity was maximal for QX-314 (by 17-fold) and much less for neutral benzocaine (by 2-fold). Furthermore, this reduction was residue specific; substitution of positively charged lysine with negatively charged aspartic acid (μ1-N434D) restored or even enhanced the LA affinity. We conclude that μ1-N434K and μ1-L437K residues located near the middle of the I-S6 segment of Na^+ channels can reduce the LA binding affinity without BTX. Thus, this reduction of the LA affinity by point mutations at the BTX binding site is not caused by gating changes induced by BTX alone. We surmise that the BTX receptor and the LA receptor within segments I-S6 and IV-S6, respectively, may align near or within the Na^+ permeation pathway.

Local anesthetics (LAs) are drugs that block the propagation of action potentials in excitable membranes. The primary target of LAs is the voltage-gated Na^+ channel, which controls the permeability of Na^+ ions (for review, see Hille, 1992; Catterall and Mackie, 1996). Under voltage-clamp conditions, the LA block of the Na^+ currents is rather complicated. First, LAs elicit the tonic block of Na^+ currents at the resting potential when the membrane is stimulated infrequently. Second, with a prolonged conditioning pulse, LAs shift the steady state inactivation curve in the hyperpolarizing direction (Bean *et al.,* 1983). Third, most LAs produce additional use-dependent block during repetitive pulses at a frequency of 1–5 Hz (Strichartz, 1973). According to the Modulated Receptor Hypothesis (Hille, 1977), both the shift in steady state inactivation curve and the additional use-dependent block are attributed to the preferential binding of LAs to the inactivated state of Na^+ channels.

BTX is a steroidal alkaloid found in the skin of *Phyllobates terribilis* frogs (Daly *et al.*, 1980). This neurotoxin also targets voltage-gated Na^+ channels for its action. Classified as an Na^+ channel activator (Catterall, 1980), BTX binds preferentially to the open form of the α subunit Na^+ channel and, upon binding, nearly eliminates the fast and slow inactivation. BTX does not bind noticeably with inactivated Na^+ channels (Tanguy and Yeh, 1991). In addition, BTX shifts drastically the activation process of Na^+ channels in the hyperpolarizing direction by 30–50 mV (Khodorov, 1978). It is well recognized that the potency of some LAs is reduced drastically in BTX-modified Na^+ channels, yet the LA receptor and the BTX binding site do not overlap. In particular, lidocaine and its quaternary derivative QX-314 seem to be much less potent (by >100-fold) in BTX-modified Na^+ channels than in drug-free counterparts (Moczydlowski *et al.*, 1986; Zamponi and French, 1993). The reason for this reduction in binding of lidocaine and QX-314 to BTX-modified Na^+ channels is not clear.

The purified mammalian Na^+ channel protein comprises a large α subunit and one or two smaller β subunits. The α subunit of the voltage-gated Na^+ channel consists of four repeated homologous domains (Fig. 1, I-IV), each of which contains six transmembrane segments (S1-S6) (Catterall, 1995). The α subunit voltage-gated Na^+ channel clone alone expresses functional Na^+ currents in mammalian transfected cells that are comparable with currents found in native

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ABBREVIATIONS: BTX, batrachotoxin; HEK cells, human embryonic kidney cells; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMA-OH, tetramethylammonium hydroxide; QX-314 chloride, lidocaine *N*-ethyl chloride salt.

tissues (Trimmer *et al.*, 1989). The LA binding site has been mapped near the center of segment S6 at domain IV (Ragsdale, *et al*, 1994), whereas the BTX site is in segment S6 at domain I (Trainer *et al.*, 1996; Wang and Wang, 1998; Fig. 1, *arrows*). How these two distinct binding sites influence each other through distance is not known; therefore, we have begun to characterize the LA block in BTX-resistant mutant muscle μ1 (skm1) Na^+ channels, μ1-N434K and μ1-L437K. A lysine substitution at each of these two residues in segment I-S6 apparently eliminates BTX binding to the Na^+ channel. The lysine residue is normally protonated at physiological conditions. The purpose of this study was to demonstrate that residues at the BTX binding site can indeed affect LA binding at a separate site even without BTX present. Among the LAs used were lidocaine, QX-314, etidocaine, and benzocaine. Block of etidocaine and benzocaine on the wild-type and mutant channels of the LA binding site at segment IV-S6 has been described before (Ragsdale *et al.* 1994; Wang *et al.*, 1998) and can be used for comparison. Both lidocaine and etidocaine are tertiary amine compounds. Benzocaine is neutral in saline solution, whereas QX-314 is permanently charged.

Materials and Methods

Chemicals and reagents. Lidocaine base and benzocaine were purchased from Sigma Chemical Company (St. Louis, MO). Etidocaine-HCl and QX-314 chloride were gifts from Astra Pharmaceutical Products (Worcester, MA). A stock of human embryonic kidney cells (HEK 293t) and the plasmid CD8-pih3m were gifts from Dr. Stephen Cannon (Massachusetts General Hospital, Boston, MA). Plasmid μ1/skm1 was provided by Dr. James Trimmer (State University of New York at Stoneybrook).

Mutagenesis of μ1 channels and transient transfection of HEK 293T cells. The creation of μ1-N434K and μ1-L437K mutants was described previously (Wang and Wang, 1998). An additional mutant, μ1-N434D, was created in a similar manner on μ1-pcDNA1/amp by means of the Transformer Site-Directed Mutagenesis Kit (Clontech Lab, Palo Alto, CA). Two primers (a mutagenesis primer and a restriction primer) were synthesized and used to generate the desired mutants. *In vitro* synthesis was performed for a total of 4 hr, with one addition of NTPs and T4-DNA polymerase during the reaction. The potential mutants were identified by restriction digestion and confirmed by DNA sequencing.

The method of transient transfection of HEK 293T cells by the calcium phosphate precipitation was the same as described previously (Cannon and Strittmatter, 1993; Wright *et al.*, 1997). Transfected cells were used for experiments as late as 3–4 days after replating in 35-mm tissue culture dishes. Transfection-positive cells were identified by CD8-Dynabeads (Dynal, Lake Success, NY) before Na^+ current recording.

Electrophysiology and data acquisition. The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used to measure Na^+ currents. Data were recorded by an EPC-7 amplifier (List Electronic, Darmstadt/Eberstadt, Germany), filtered at 3 kHz, and collected with pCLAMP software (Axon Instruments, Burlingame, CA). The holding potential was −100 mV unless otherwise indicated. Patch electrodes contained 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES adjusted to pH 7.2 with CsOH. The electrodes had a tip resistance of 0.5–1.0 MΩ. The Na^+-free bath contained 150 mM choline chloride, 2 mM $CaCl_2$, and 10 mM HEPES adjusted to pH 7.4 with TMA-OH. This reversed Na^+ gradient was used to minimize the series resistance artifact near the activation threshold around −40 to −10 mV (Cota and Armstrong, 1987). The Na^+-containing bath with 130 mM NaCl, 20 mM choline chloride, 2 mM $CaCl_2$, and 10 mM HEPES, adjusted to pH 7.4 with TMA-OH, was applied to construct the relative conductance versus voltage relationship. For experiments described in Fig. 6, the bath solution contained 85 mM choline chloride, 65 mM NaCl, 10 mM HEPES and 2 mM CaCl2, titrated to pH 7.4 with TMA-OH. Liquid junction potentials were < 3 mV and series resistance errors < 4 mV after compensation. For internal perfusion of drug QX-314, perfusing patch pipettes were used as described by Tang *et al.* (1990). Experiments were performed at room temperature (23 ± 2°). Data are presented as mean ± standard error or fitted value ± standard error of the fit. An unpaired Student's *t* test (SigmaStat, Jandel Scientific Software, San Rafael, CA) was used to examine the significance of changes in mean values. *p* Values of < 0.05 were considered statistically significant.

Results

Potency of lidocaine and QX-314 in μ1-N434K Na^+ channels. To determine the LA concentration that inhibits 50% of peak Na^+ currents (IC_{50}) in wild-type channels and in BTX-resistant μ1-N434K mutant, we started to construct the dose-response curve. Fig. 2 shows the current traces of μ1 wild-type channels and μ1-N434K mutant before and after application of various concentrations of external lidocaine (Fig. 2, A and B) or after internal 30 μM QX-314 (Fig. 2, C and D). QX-314 was perfused intracellularly because of its permanent charge. For QX-314, only one concentration was applied in a given cell because of the slow rate of internal perfusion. It took about 15–25 min to reach steady state block by internal QX-314 application. The peak amplitudes of Na^+ currents were measured, normalized with respect to the peak current amplitude without drug, and plotted against drug concentration (Fig. 3). The values of IC_{50} for internal QX-314 were 8.9 ± 0.8 μM and 155 ± 11 μM for wild-type channels and μ1-N434K mutant, respectively, with a difference of ~17-fold (Table 1). In comparison, the values of IC_{50} for external lidocaine were 239 ± 6 μM and 696 ± 22 μM for wild-type channels and μ1-N434K mutant, respectively, or a difference of ~3-fold. Thus, the potency of charged QX-314 was reduced far more than that of tertiary amine lidocaine under our experimental conditions. The Hill coefficients for both lidocaine and QX-314 in wild-type and mutant channels

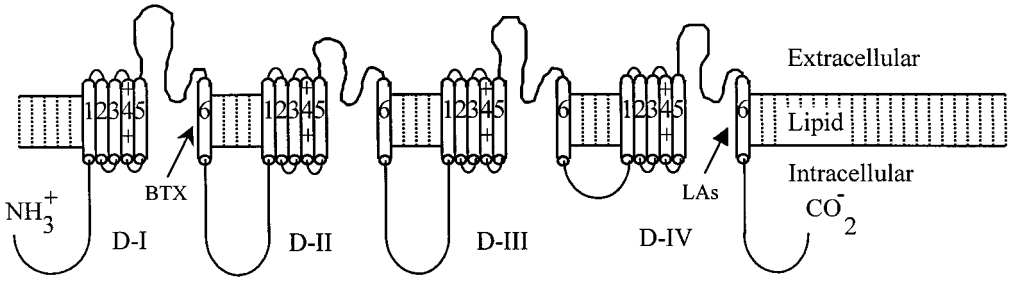

Fig. 1. The transmembrane organization of the Na^+ channel α subunit.

(Table 1) were about unity, which suggests that a single LA binding site is present for these drugs.

Block of benzocaine and etidocaine on μ1-N434K Na^+ channels. For comparison, we determined the values of IC_{50} for neutral benzocaine and tertiary amine etidocaine under the same experimental conditions. The blocking effects of these two drugs on wild-type and mutant channels (μ1-I1575A, F1579A, and N1584A) have been reported in HEK 293t cells previously under identical conditions (Wang *et al.*, 1998). Fig. 4 shows the dose-response curve of benzocaine and etidocaine. The Hill coefficient value for benzocaine was estimated about 1.5 ± 0.1 and 1.9 ± 0.1 for wild-type and μ1-N434K mutant channels, respectively, a value significantly larger than that for other LAs, such as lidocaine, QX-314, and etidocaine ($p < 0.05$). The high Hill coefficient for benzocaine and its possible explanations have been reported previously in native Na^+ channels by Meeder and Ulbricht (1987). The IC_{50} values for benzocaine were 802 ± 16 μM and 1562 ± 48 μM for wild-type and μ1-N434K channels, respectively, or with a difference of ~2 fold. On the other hand, the IC_{50} values for etidocaine were 27 ± 2 μM and 194 ± 11 μM for wild-type and μ1-N434K channels, respectively, or a difference of ~7-fold. Thus, the reduction of LA potency in μ1-N434K mutant channels followed the order of QX-314 (17×) > etidocaine (7×) > lidocaine (3×) > benzocaine (2×), which paralleled the ranking potency of these drugs on μ1 wild-type Na^+ channels (Table 1). In other words, the more potent the drug, the greater reduction of its potency in μ1-N434K mutant channels.

Shifts in h_∞ by benzocaine, lidocaine, and etidocaine in μ1-N434K Na^+ channels. Benzocaine is known to shift the steady state inactivation (h_∞) drastically in the hyperpolarizing direction (Hille, 1977). This shift was taken as crucial evidence that benzocaine binds preferentially to the inactivated state and stabilizes such an inactivated form of Na^+ channels upon binding. Fig. 5 shows that benzocaine shifts the midpoint of the h_∞ curve by about 33 mV and 22 mV in the hyperpolarizing direction in wild-type and μ1-N434K mutant channels, respectively. Under identical pulse protocols, however, the shifts for lidocaine (Fig. 5) and etidocaine were minimal (Table 2), probably because the prepulse duration of 50 msec is too short to allow sufficient binding of lidocaine and etidocaine to the inactivated Na^+ channels. In previous studies, a prepulse duration of > 1 sec was required for these LAs to reach steady state binding (Bean *et al.*, 1983; Ragsdale *et al.*, 1994; Wright *et al.*, 1997).

Reduction of etidocaine potency in resting and inactivated μ1-N434K Na^+ channels. Recently, we applied a pulse protocol to assay directly the potency of LAs on the resting and inactivated states. A prepulse duration of 10 sec at various voltages was applied before an interpulse of 100 msec at −140 mV, followed by a brief test pulse to determine the drug-free Na^+ channels (Wright *et al.*, 1997). For these experiments, the holding potential of −140 mV was applied to remove all drug-free inactivated channels. With this protocol, we found that etidocaine at 30 μM blocked about 15% of peak μ1 current at voltage ≤ −130 mV and about 90% of peak current at voltage at −70 mV (Fig. 6, ●). In contrast, etidocaine at the same concentration blocked only about 4% of peak μ1-N434K current at voltage ≤ −120 mV and about 45% at −70 mV. Both resting (at ≤ −120 mV) and inactivated (at −70 mV) states of μ1-N434K mutant channels displayed a reduced binding affinity toward etidocaine. For reason of overall potency comparison, the IC_{50} values shown in Table 2 were determined with a holding potential of −100 mV, as described previously (Wang *et al.*, 1998). Under such a condition, there was a mixture of resting and inactivated states present. If we applied Langmüir isotherm to estimate

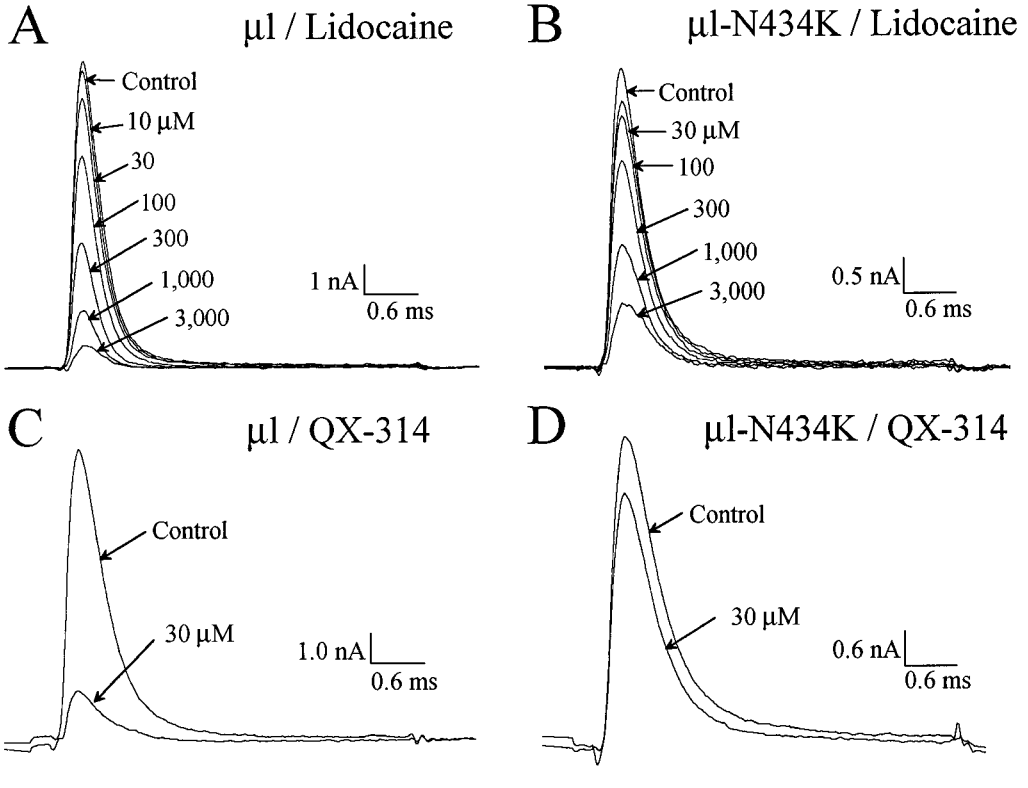

Fig. 2. Block of wild-type and μ1-N434K mutant currents by lidocaine and QX-314. A, Current traces before and after attaining the tonic block of wild-type Na^+ channels by external lidocaine are shown. The cell was first dialyzed and a test pulse of 4 msec at +30 mV (preceded by a prepulse of 100 msec at −130 mV and an interpulse of 0.2 msec at −100 mV) was applied to assay the tonic block of drugs. This pulse protocol was repeated at 30-sec intervals. B, Current traces of μ1-N434K are shown in the presence of various concentrations of lidocaine. The numbers near current traces indicate the concentration (in μM) applied. Traces of wild-type (C) and μ1-N434K (D) currents were recorded before and after attaining tonic block with intracellular perfusion of QX-314 at 30 μM. Holding potential was set at −100mV.

etidocaine affinity for the inactivated Na^+ channels alone, we obtained the IC_{50} values of 3.3 μM and 37 μM for the wild-type and μ1-N434K mutant channels, respectively, or an 11-fold difference. Unfortunately, this pulse protocol is not applicable for benzocaine and lidocaine because these drugs dissociate significantly from their receptors during the 100-msec interpulse (Wright *et al.*, 1997).

Use-dependent block of μ1-N434K Na^+ channels by etidocaine. During repetitive pulses, etidocaine at 30 μM produced significant 50–60% use-dependent block of wild-type Na^+ current (Fig. 7A; Wang *et al.* 1998). The time constant of this use-dependent block was measured about 2.4-pulse. To determine whether the ability of etidocaine to elicit use-dependent block was affected in μ1-N434K mutant, we applied an identical pulse protocol and assayed the use-dependent block on μ1-N434K currents in the presence of 200 μM etidocaine. At this etidocaine concentration, about 50% of μ1-N434K currents were tonically blocked at the holding potential of −100 mV. Fig. 7B shows the selected μ1-N434K current traces during repetitive pulses in the presence of 200 μM etidocaine. The peak currents were measured, normalized with respect to that of the first pulse, and plotted against the pulse number (Fig. 7D). The time constant of the use-dependent block (0.6-pulse) was faster than that of the wild-type channels, and the magnitude of use-dependent block by etidocaine in μ1-N434K currents was also severely reduced even at the equivalent concentration near the IC_{50} value.

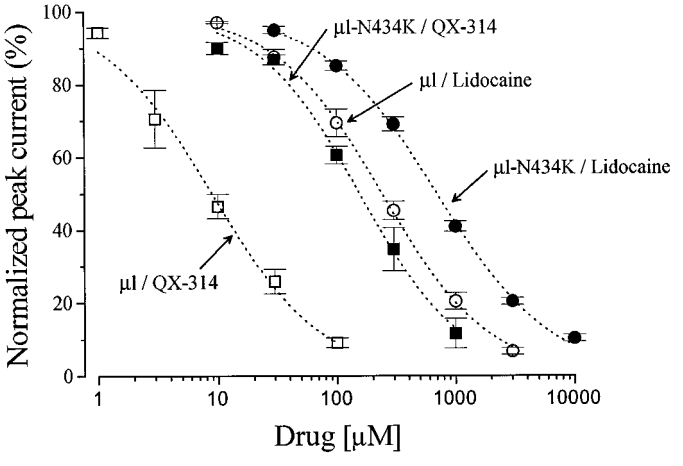

Fig. 3. Dose-response curves of wild-type and μl-N434K mutant channels with external lidocaine and internal QX-314. Experiments were performed according to the pulse protocol described in Fig. 2. Various concentrations of lidocaine were applied to each cell via external superfusion, whereas only one concentration of QX-314 was applied to each cell. The peak amplitudes of Na^+ currents were measured, normalized with respect to the peak amplitude without drug, and plotted against drug concentration. The Hill coefficient for lidocaine was 0.97 ± 0.02 for the wild-type and 0.91 ± 0.02 for μl-N434K channels. With internal QX-314, the Hill coefficients were 0.96 ± 0.08 and 1.02 ± 0.07, respectively, for the wild-type and μl-N434K mutant channels. The IC_{50} values for lidocaine were 239 ± 7 μM for the wild-type channels and 696 ± 22 μM for μl-N434K. With internal QX-314, the IC_{50} values were 8.9 ± 0.8 μM and 155 ± 11 μM, respectively. The IC_{50} values and the number of experiments are listed in Table 1.

LA block in μ1-L437K Na^+ channels. As with μ1-N434K channels, μ1-L437K channels were insensitive to BTX. We therefore examined the block of QX-314, lidocaine, etidocaine, and benzocaine in this mutant channel. Fig. 8 shows the μ1-L437K current traces before and after application of QX-314, etidocaine, lidocaine, and benzocaine, respectively. All drugs except QX-314 were applied externally. The peak currents were measured, normalized with respect to the control peak current without drug, and plotted against the concentration (as shown in Fig. 3). The estimated IC_{50} values from these plots, listed in Table 1, indicate that the mutation at μ1-L437K position also reduced the LA affinity toward the receptor. However, this reduction in affinity was less profound than the mutation at μ1-N434K position. Similarly, the h_∞ curve of μ1-L437K mutant is shifted less in the hyperpolarizing direction by benzocaine than is that of the μ1-N434K mutant (Table 2).

To ascertain that the resting and inactivated μ1-L437K Na^+ channels bind to etidocaine with diminished affinity, we used the protocol described in Fig. 6. The etidocaine block fell between the wild-type and μ1-N434K values, as expected (Fig. 6; ♦). Hence, the affinity of etidocaine for both resting and inactivated μ1-L437K channels is reduced, but to a lesser extent than for μ1-N434K channels.

LA block in μ1-N434D Na^+ channels. To determine whether the positive charge on the lysine residue is critical for the reduction in LA binding, we mutated the μ1-N434 position with a negatively charged residue, aspartic acid. In contrast to mutant μ1-N434K, mutant μ1-N434D channels displayed an increase in their binding affinity toward etidocaine. Fig. 9B shows μ1-N434D current traces before and after external etidocaine application with a holding potential of −100 mV. The IC_{50} value was 19 ± 1 μM, lower than that of the wild-type channel (27 ± 2 μM, $p < 0.05$). Similarly, we found higher etidocaine inhibitions for the inactivated Na^+ currents of μ1-N434D than for those of the wild-type channel, as shown in Fig. 6 (■). However, this was not true for the resting μ1-N434D channels, which were blocked less than

TABLE 1

Values of IC_{50} and the Hill coefficient of local anesthetics in μ1 wild-type and mutant channels.

The values of IC_{50} (mean ± standard error, n = number of experiments) were determined as described in Figure 3. The differences in LA potency between wild-type and mutant channels were all statistically significant. The Hill coefficient is listed in brackets. If estimated by a single concentration of 30 μM QX-314, the IC_{50} value was 8.8 μM for μ1-N434D, no difference from the wild-type channels.

LA drugs	μ1 wild-type	μ1-N434K[a]	μ1-L437K[a]	μ1-N434D[a]
QX-314	8.9 ± 0.8 (n = 5)	155 ± 11 (n = 5)	74 ± 13 (n = 5)	N.D.
$[\mu M]_{internal}$	[0.96 ± 0.08]	[1.02 ± 0.07]	[1.13 ± 0.21]	
Etidocaine	27 ± 2 (n = 14)	194 ± 11 (n = 9)	85 ± 6 (n = 5)	19 ± 1 (n = 6)
$[\mu M]_{external}$	[1.30 ± 0.11]	[1.11 ± 0.07]	[1.12 ± 0.08]	[1.13 ± 0.07]
Lidocaine	239 ± 7 (n = 8)	696 ± 22 (n = 7)	453 ± 15 (n = 5)	108 ± 3 (n = 6)
$[\mu M]_{external}$	[0.97 ± 0.02]	[0.91 ± 0.02]	[1.12 ± 0.04]	[0.96 ± 0.02]
Benzocaine	802 ± 16 (n = 8)	1562 ± 48 (n = 8)	960 ± 54 (n = 6)	437 ± 14 (n = 5)
$[\mu M]_{external}$	[1.47 ± 0.05]	[1.88 ± 0.10]	[1.53 ± 0.11]	[1.49 ± 0.06]

[a] *, $p < 0.05$.
N.D., not determined.

the wild-type channel. Note in Fig. 9 that the time constant for the fast inactivation of μ1-N434D current was only slightly slower than that for the wild-type channel (τ = 0.30 ± 0.05 msec, n = 11 versus 0.26 ± 0.01 msec, n = 8; p = 0.51), but the presence of a maintained current was evident. The results for this phenotype were opposite those of the mutant μ1-N434A, which displayed a time constant faster than that of the wild-type channel (Wang and Wang, 1997). Hence, depending on the substituted residues, the decaying phase of Na^+ currents could be accelerated (N434A), slightly slowed (N434D), or minimally affected (N434K). For comparison, the blocking effects of QX-314, lidocaine, and benzocaine are also shown in Fig. 9, and their IC_{50} values are listed in Table 1. Evidently, these LAs have higher affinities for μ1-N434D mutant channels than for the wild-type counterparts at the holding potential of −100 mV, probably because of an increase in affinity for inactivated Na^+ channels.

Discussion

Reduced etidocaine affinity in BTX-resistant Na^+ channels. A substitution of μ1-N434 and μ1-L437 with a K residue clearly reduces the potency of etidocaine in these voltage-gated mutant Na^+ channels, which have been reported to be completely BTX resistant (Wang and Wang, 1998). The reduction in etidocaine potency occurs in both the resting and the inactivated states of mutant channels. The degree of use-dependent block by etidocaine is also substantially reduced in mutants. These blocking phenomena are surprisingly similar to those found in mutant μ1-F1579A located at IV-S6 segment (Wang *et al.*, 1998). These results together demonstrate for the first time that residues at the BTX binding site can alone influence the LA binding affinity without BTX and provide evidence that these residues are not only critical for BTX binding, but also important for LA binding at a separate site. Changes in specific residues within the BTX binding domain, therefore, can mimic the effect of BTX binding on the LA affinity, although the effect may not be as drastic as it is when BTX is present at the site.

Relative potency of LA drugs in μ1 wild-type channels and in BTX-resistant mutants. The quaternary ammonium derivative of lidocaine, QX-314, is the most potent drug among LA drugs tested in this study (IC_{50} ~10 μM for

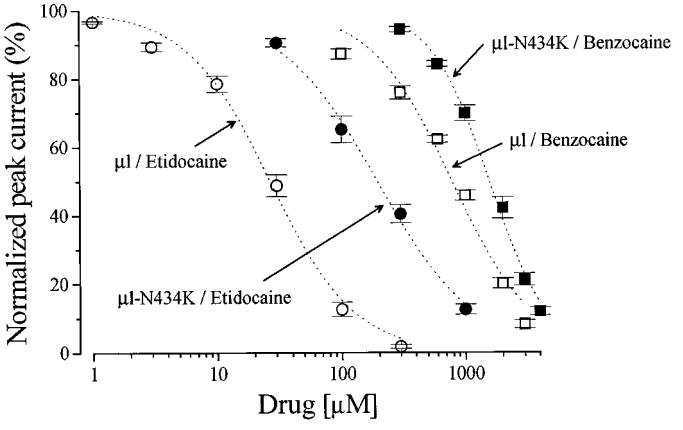

Fig. 4. Dose-response curves of wild-type and μl-N434K channels with external benzocaine and etidocaine. Dose response curves of benzocaine and etidocaine were constructed as described in Fig. 3. The Hill coefficients for external etidocaine were 1.30 ± 0.11 for the wild-type channels and 1.11 ± 0.07 for μl-N434K channels. For external benzocaine, the values were 1.47 ± 0.05 and 1.88 ± 0.10 for the wild-type and μl-N434K channels, respectively. The IC_{50} values for benzocaine were 0.80 ± 0.02 mM and 1.56 ± 0.05 mM for the wild-type and μl-N434K channels, respectively. For etidocaine, the values were 27 ± 2 μM and 194 ± 11 μM, respectively. The IC_{50} values and the number of experiments are listed in Table 1.

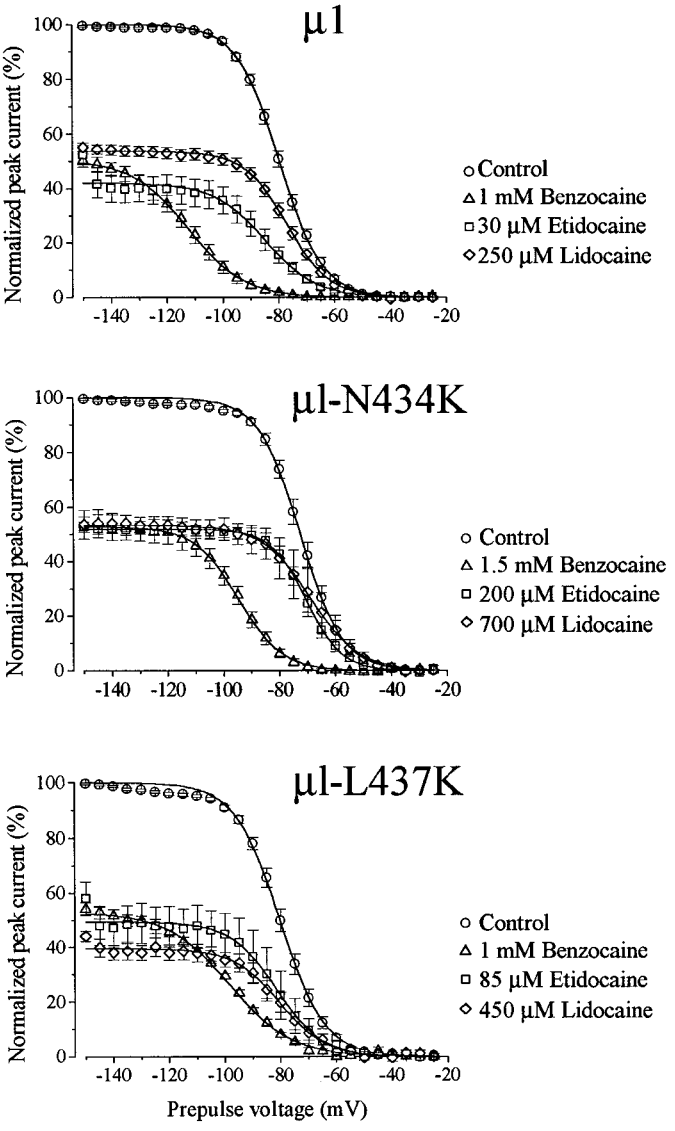

Fig. 5. Shifts in h_∞ inactivation by benzocaine and lidocaine in μl wild-type, μl-N434K, and μl-L437K Na^+ channels. Peak Na^+ currents were measured by a test pulse of +50 mV for 3.6 msec with various prepulse voltages (E_{pp}) ranging from −150 to −25 mV for 50 ms at 10-sec intervals. Holding potential was set at −100 mV. After a complete set of control traces was recorded, a second set was taken when the steady state tonic block was reached by drug. Each family of peak amplitudes was normalized with respect to the value of the prepulse voltage at −150 mV without drug, plotted against the prepulse voltage, and fitted by a Boltzmann equation, $y = \{1 + \exp[(E_{pp} - E_{0.5})/k_h]\}$, where k_h is the slope factor and $E_{0.5}$ is the voltage at which 50% of peak Na^+ currents were inactivated. These estimated values are listed in Table 2. The value of $h_{0.5}$ for the wild-type channel was shifted by −33 mV in the presence of 1 mM benzocaine. A shift of −22 mV was observed for μl-N434K in the presence of 1.5 mM benzocaine. A similar shift of −18.2 mV was also seen for μl-L437K in the presence of 1 mM benzocaine. Such large shifts were not seen in the presence of lidocaine. The value of $h_{0.5}$ in the wild-type channel shifted little by 250 μM lidocaine. For μl-N434K, the shift was also minimal by 700 μM lidocaine. For μl-L437K, the shift was again minimal by 450 μM lidocaine.

wild-type channels), whereas neutral benzocaine is the least potent (IC_{50} ~1 mM). It is interesting that the reduction in LA potency in BTX-resistant μ1-N434K mutant channels is the highest for charged QX-314 (~17-fold), but minimal for neutral benzocaine (~2-fold). Etidocaine and lidocaine display intermediate potency in blocking wild-type Na^+ channels and a potency reduction of ~7-fold and ~3- fold, respectively, in μ1-N434K BTX -resistant mutant channels. Why the ranking order of LA potency in μ1 wild-type channels parallels the ranking order of reduction in LA binding in BTX-resistant channels remains unclear. One possible explanation for this phenomenon is that a high affinity binding component is affected more than other binding components within the LA binding domain. This component may be involved in binding with the protonated amine component of LAs because the binding of benzocaine (lacking the amine component) is affected the least, whereas the permanently charged QX-314 is affected the most.

Differential effects on LA affinity by positive and negative charges at the N434 position. Because the lysine residue at μ1–434 and μ1–437 positions reduces the LA affinity drastically, could the charge alone explain this reduction? In theory, a positively charged residue at the I-S6 transmembrane segment could induce extensive conformational changes in Na^+ channels. An aspartic acid residue, however, does not cause a reduction in etidocaine binding

TABLE 2

Gating parameters of wild type versus BTX-resistant channels and $h_{0.5}$ values with and without local anesthetics.

The gating parameters were estimated as described in Wang and Wang (1998). The number of experiments per value for activation was 6–11 and for inactivation was 4–9. The differences in $h_{0.5}$ with and without drug are listed in brackets.

Gating parameters	μ1 wild-type	μ1-N434K	μ1-L437K
		mV	
Activation			
$E_{0.5}$	−37.8 ± 2.7	−35.0 ± 2.5	−29.7 ± 2.7
k_a	6.8 ± 0.9	8.7 ± 0.8	7.6 ± 0.6
Inactivation (h_∞)			
$h_{0.5}$ (control)	−79.6 ± 0.9	−72.3 ± 1.1	−80.1 ± 1.2
k_h (control)	7.1 ± 0.2	6.3 ± 0.2	7.4 ± 0.2
$h_{0.5}$ (benzocaine)	−112.6 ± 1.8 [−33]^a	−94.4 ± 2.2 [−22.1]^a	−98.3 ± 1.3 [−18.2]^a
k_h (benzocaine)	9.3 ± 0.4^a	7.1 ± 0.4	11.0 ± 0.5^a
$h_{0.5}$ (lidocaine)	−77.1 ± 1.2 [2.5]	−69.9 ± 4.5 [2.4]	−80.6 ± 2.3 [−0.5]
k_h (lidocaine)	7.5 ± 0.3	6.6 ± 0.5	7.7 ± 0.4
$h_{0.5}$ (etidocaine)	−87.5 ± 2.1 [−7.9]^a	−70.6 ± 1.7 [1.7]	−83.0 ± 3.8 [−2.9]
k_h (etidocaine)	10.1 ± 1.3^a	6.0 ± 0.3	7.3 ± 0.3

^{a} $p < 0.05$.

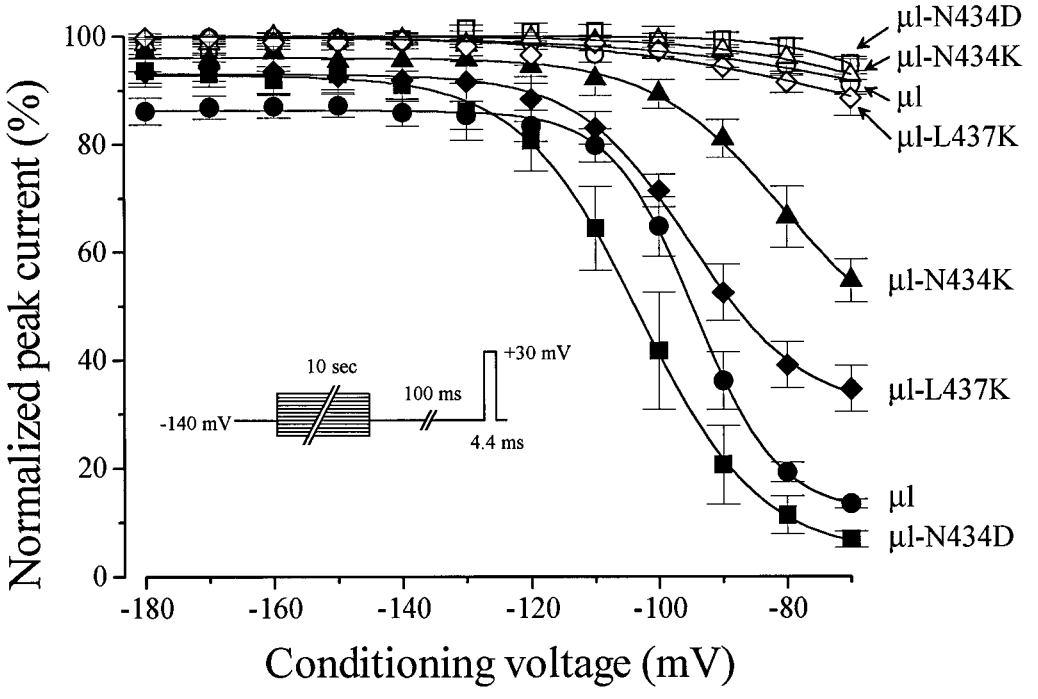

Fig. 6. Steady state block of wild-type and mutant Na^+ channels by 30 μM etidocaine at various voltages. The pulse protocol is shown in the inset. Cells were given a 10-sec conditioning voltage ranging from −180 mV to −70 mV, followed by a 100-msec interpulse at −140 mV, then a test pulse of 4.4 msec at +50 mV (*open symbols*). Holding potential was set at −140 mV. A second set of traces was taken after attaining tonic inhibition by 30 μM etidocaine (*solid symbols*). Each family of peak amplitudes was normalized with respect to the control peak current value with the conditioning voltage at −180 mV, plotted against conditioning voltage, and fitted with a Boltzmann equation. The average midpoint in the wild-type was −94.5 ± 0.3 mV (n = 5) and the average slope factor was 6.5 ± 0.3 mV. In μl-N434D, the average midpoint was −103.1 ± 0.4 mV (n = 6) and the slope was 9.4 ± 0.4. In μl-N434K, the average midpoint was −80.6 ± 2.1 mV (n = 5) and the slope factor 10.1 ± 1.0 mV. In μl-L437K, the average midpoint was −95.2 ± 0.6 mV (n = 5) and the slope factor was 8.7 ± 0.5.

affinity (Fig. 9), nor does the mutant channel become BTX resistant (Wang S-Y, unpublished observations). This result suggests that a positive charge is essential for the BTX-resistant phenotype and for the reduction of LA binding but a negative one is not. It is possible that the positive charge at segment I-S6 may repel the positive charge of QX-314 during binding, provided that they are in close proximity. For example, during binding external and internal tetraethyl ammonium ions were shown to repel each other within the permeation pathway of cloned potassium channels (Newland *et al.*, 1992). According to Coulomb's law, these two positive charges have to be relatively close to experience the charge repulsion; residue lysine, with its long side chain, may be particularly effective for such an effect. If so, then our results imply that the I-S6 segment and the IV-S6 segment align adjacently along the Na^+ permeation pathway. The fact that both the LA receptor (Ragsdale *et al.*, 1994) and the BTX receptor (Wang and Wang, 1998) are located at residues near the middle of the S6 segment is consistent with this notion. Alternatively, it is also possible that a positively charged residue at I-S6 segment induces conformational changes that have an indirect effect at the LA receptor. It is interesting that μ1-N434D mutant currents contain a maintained steady state current component, which suggests that fast inactivation is hampered by this mutation. This phenotypic effect is not completely unexpected, because upon binding, BTX inhibits fast inactivation gating. The BTX binding site must somehow couple to the fast inactivation gating process when BTX is present.

Regions that modulate LA affinity. Multiple regions in α subunit Na^+ channels are known to modulate the LA affinity. One region that modulates the LA binding is at or near the putative LA binding site. Different residues within the IV-S6 segment are critical for LA binding (Ragsdale *et al.*, 1994). Besides the LA receptor site, Sunami *et al.* (1997) recently reported that the Na^+ channel selectivity filter also regulates LA binding. They concluded that the selectivity ring participates in LA binding and is adjacent to the S6 segment.

A second mechanism to modulate the LA receptor is through channel state transition as implied by the modulated receptor hypothesis (Hille, 1977). The most important parameter to gauge this modulation is the steady state inactivation curve (h_∞) as outlined in Hille's hypothesis. The voltage at -110 mV to -60 mV determines the ratio of the inactivated state to the resting state and clearly correlates with the apparent LA affinity (Bean *et al.*, 1983). Bennett *et al.* (1995) found that lidocaine block is substantially reduced in mutants with hampered fast inactivation. Their results indicate the importance of the inactivated state block by lidocaine. Recently, two saturable binding affinities for LA drugs were reported, one corresponding to the resting state at voltages ≤ -140 mV and one corresponding to the inactivated state at voltages ≥ -70 mV (Wright *et al.*, 1997).

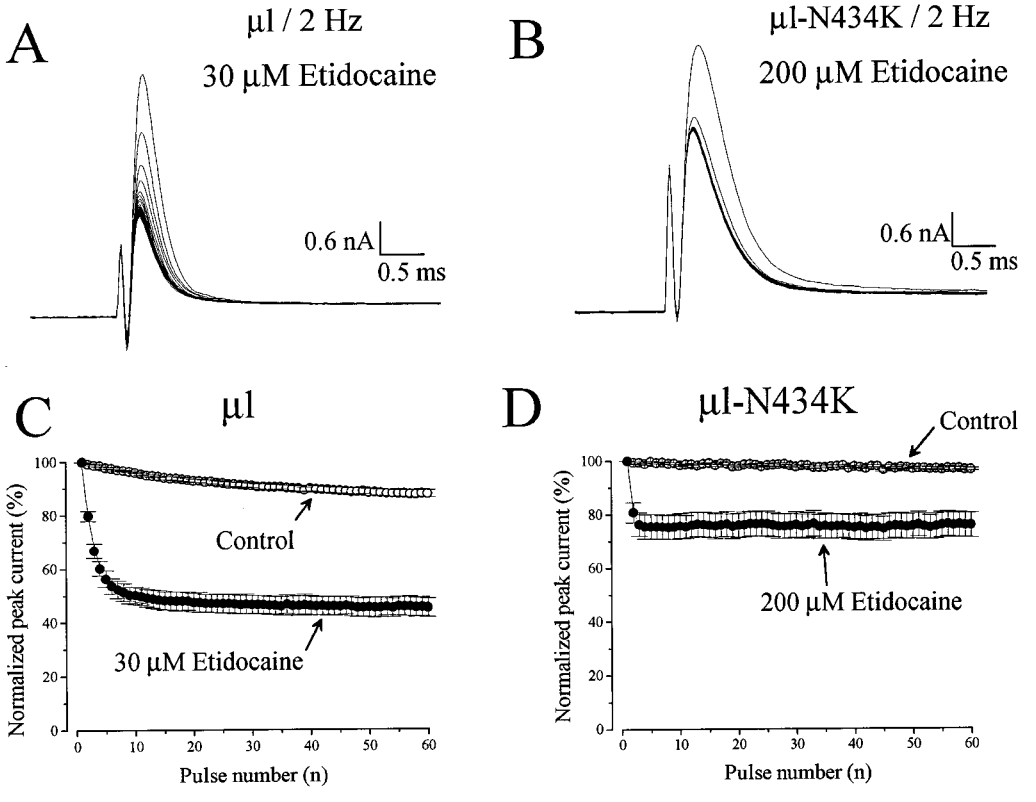

Fig. 7. Use-dependent block of wild-type and μl-N434K Na^+ channels by etidocaine. Cells were subjected to a set of 60 repetitive test pulses of +30 mV for 24 msec at 2 Hz before and after application of etidocaine. A, A typical example of wild-type Na^+ current traces in the presence of 30 μM etidocaine. B, Current traces were recorded in the presence of 200 μM etidocaine in μl-N434K Na^+ channels. This concentration, which produced about 50% tonic block of the control peak current, was chosen for comparison. The peak current amplitudes were measured, normalized with respect to the first pulse of each family, and plotted against pulse number (C). The curve was fitted by a single exponential function with a time constant of 2.36-pulse. D, The time course of the use-dependent inhibition of μl-N434K channels by 200 μM etidocaine is shown. The time constant for μl-N434K is 0.64-pulse. Little use-dependent block was found in wild-type channels or in μl-N434K mutants without drug. Holding potential was set at -100mV.

The third region to modulate LA affinity is through BTX binding. BTX-modified Na^+ channels display a greatly reduced binding affinity toward lidocaine and QX-314 (Moczydlowski *et al.*, 1986; Zamponi and French, 1993). It is not clear whether this effect is caused by the removal of the inactivated state, changes in the h_∞ curve, shifts in activation, or other gating changes by BTX binding. Our results show clearly that the two BTX-resistant mutants, μ1-N434K and μ1-N437K, have a reduced binding affinity for etidocaine in both resting and inactivated Na^+ channels. In addition, BTX-resistant channels are functional, and the μ1-N434K mutant exhibits little change in gating kinetics. Hence, this reduction in LA binding is not a consequence of direct gating changes. We hypothesize that the reduction in LA binding is caused by changes in the microenvironment (such as charge-charge repulsion or conformational changes) of the LA receptor induced by the mutation at the BTX binding site. These changes in microenvironment need not cause apparent gating changes. This finding is significant because it also explains why BTX-producing *P. terribilis* frogs survive in the wild without apparent gating changes in their BTX-resistant Na^+ channels (Daly *et al.*, 1980). Whether Na^+ channels in *P. terribilis* frogs show a reduced LA affinity remains to be examined.

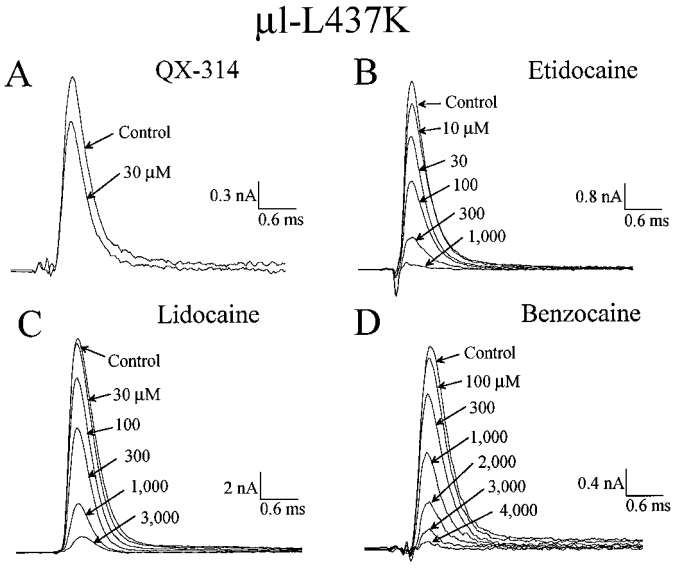

Fig. 8. Superimposed μl-L437K Na^+ current traces before and after treatment with QX-314 (A), etidocaine (B), lidocaine (C), and benzocaine (D). Experiments were performed as described in Fig. 2. Except for QX-314, which was perfused internally, all drugs were applied externally.

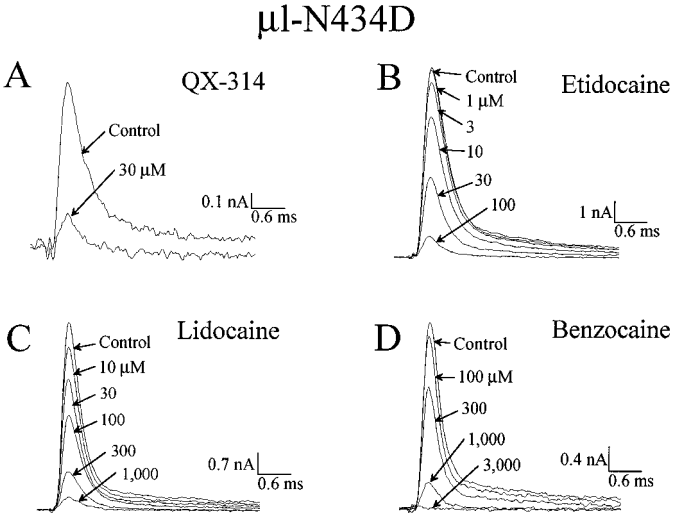

Fig. 9. Superimposed μl-N434D current traces before and after treatment with QX-314 (A), etidocaine (B), lidocaine (C), and benzocaine (D). Experiments were performed as described in Fig. 2. Except for QX-314, all drugs were applied externally.

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