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Prenylamine Block of Nav1.5 Channel is Mediated via a Receptor Distinct from That of Local Anesthetics

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ABSTRACT

We have shown previously that prenylamine, a calcium channel blocker, has potent local anesthetic activity in vivo and in vitro. We now characterize the tonic and use-dependent block of prenylamine on wild-type human cardiac voltage-gated sodium channels (hNav1.5) transiently expressed in human embryonic kidney 293t cells under whole-cell voltage-clamp condition. We also determine whether prenylamine and local anesthetics interact with a common binding site on the Nav1.5 channel by analyzing prenylamine block on mutant hNav1.5 channels that have substitution mutations in amino acids at the putative local anesthetic binding sites. Prenylamine exhibits tonic block at both hyperpolarizing and depolarizing potentials on hNav1.5 channels with 50% inhibitory concentrations of 9.67 ± 0.25 μM and 0.72 ± 0.02 μM, respectively. Substitutions of the amino acids at the putative local anesthetic binding site (i.e., F1760, N1765, Y1767, and N406) with lysine had much lesser effects on prenylamine block of the mutant hNav1.5 channels compared with local anesthetic block. The affinity of prenylamine was reduced at most by 5.8-fold, whereas that of bupivacaine, a known local anesthetic, was reduced by as much as 68-fold compared with wild-type by the mutations at the local anesthetic receptor site. Furthermore, equilibrium results between prenylamine-bupivacaine mixtures suggest two independent receptors. Thus, the data demonstrate that prenylamine has both tonic and use-dependent block of hNav1.5 channels similar to that of local anesthetics, but the location of the prenylamine binding site on hNav1.5 differs from that of the local anesthetic binding site.

Local anesthetics (LAs) confer their activity by blocking voltage-gated sodium channels (NaChs), which are membrane proteins involved in the generation of action potentials in excitable membranes (Catterall, 2000). LAs are known to block nerve, skeletal, muscle, and cardiac muscle NaChs. By blocking NaChs, LAs inhibit the propagation of action potentials in excitable tissues. The potency of LAs as NaCh blockers is governed by the channel state; open and inactivated (depolarized) states are favored over resting (hyperpolarized) states. The changes between low- and high-affinity channels can be explained by voltage-dependent conformational changes of the LA binding site on the NaCh (modulated receptor hypothesis) (Hille, 1977; Hondeghem and Katzung, 1977).

Mammalian NaChs consist of a large pore-forming α-subunit (230–270 kDa) and one or two smaller β-subunits (37–39 kDa), but the α-subunit alone can form functional channels when transiently expressed in human embryonic kidney cells. The α-subunit consists of four homologous domains (D1-D4), each of which has six transmembrane segments (S1–S6). It is believed currently that the NaCh is structurally organized as a pseudotetramer with the S6 segments lining the inner surface of the pore. The LA domain-interface binding site has been mapped in these S6 segments (Ragsdale et al., 1994; Wang et al., 2000). Parts of the LA receptor site on the NaCh have been delineated in the D4–S6 region as positions F1764 and Y1771 of rat brain IIA NaChs (Ragsdale et al., 1994), which correspond to positions F1760 and Y1767 in the human heart NaChs, respectively (Nau et al., 2000). These aromatic amino acids were proposed to interact with the positively charged and aromatic moieties of tertiary amine LAs (Ragsdale et al., 1994). Furthermore, positions N1769 (D4–S6) and N434 (D1–S6) of rat brain IIA NaChs, corresponding to N1765 and N406 of the hNav1.5 channel, respectively, also were shown to influence binding of the LA (Ragsdale et al., 1994; Nau et al., 1999). A recent reports from this laboratory suggests that positions S1276 and L1280 of the rat muscle NaCh D3–S6 (corresponding to positions S1458 and L1462 of hNav1.5) also may participate in LA binding (Wang et al., 2000). Others have shown that position I1469 of the rat brain type IIA NaCh D3–S6 (corresponding to position I1466 of hNav1.5) may also be involved in LA affinity to the NaCh (Yarov-Yarovoy et al., 2001). Thus,

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ABBREVIATIONS: LA, local anesthetic; NaCh, voltage-gated sodium channel; hNav1.5, human cardiac voltage-gated sodium channel; HEK, human embryonic kidney; E_{pp}, prepulse potential.

the receptor for LAs has been mapped within the S6 segments of domains D1, D3, and D4 of the NaCh.

Prenylamine, a coronary vasodilator, is a calcium channel blocker previously used for the treatment of angina pectoris (McMahon et al., 1982; Milei et al., 1982). In addition to its calcium channel blocking properties, we have shown by patch-clamp studies and by neurobehavioral examination of the sciatic nerve block in rats in a comparison of prenylamine with the known LA bupivacaine that prenylamine strongly blocks native sodium channels (NaChs) on neuronal GH_3 cells in a state-dependent manner and exhibits potent LA properties in vivo, respectively (Mujtaba et al., 2001). Although prenylamine has local anesthetic properties and inhibits neuronal voltage-gated NaChs, the affinity of prenylamine for hNav1.5 channels has thus far not been evaluated, and the location of prenylamine binding site on the NaCh has not been delineated. Here we characterize the prenylamine block in the hNav1.5 channel and determine whether this block is mediated via a receptor site on the NaCh similar to that of the LA.

Materials and Methods

Prenylamine and Bupivacaine. Prenylamine was purchased from Sigma Chemical Co. (St. Louis, MO). Bupivacaine was a gift from Astra USA, Inc. (Westborough, MA). For the electrophysiological experiments, prenylamine and bupivacaine were dissolved in dimethyl sulfoxide at 100 mM and diluted shortly before the experiments.

Site-Directed Mutagenesis. Human heart cDNA was obtained from Dr. Roland Kallen (University of Pennsylvania, Philadelphia, PA). Mutagenesis of the hNav1.5 clone was performed with the Transformer Site-Directed Mutagenesis Kit (BD Clontech, Inc., Palo Alto, CA). Two primers (a mutagenesis primer and a restriction primer) were synthesized and used to generate the desired mutants. The restriction primer had a sequence of 5′-GGAATTCTGCAGAATTCCATCACACTGG-3′, in which the restriction site *Eco*RV in the polylinker region has been changed to *Sac*I. In vitro synthesis was performed for 4 h, with one addition of dNTPs and T4-DNA polymerase during the reaction. The potential mutants were identified as *Eco*RV-resistant plasmids and confirmed by DNA sequencing using appropriate primers near the mutated region. Mutant NaChs that had point mutation at sites S1458K and L1462K did not express enough current (<500 pA) for further experimentation.

Transient Transfection. The culture of HEK293t cells and their transient transfection were performed as described previously (Cannon and Strittmatter, 1993). Cells were first grown to 50% confluence in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin and streptomycin solution (Sigma, St. Louis, MO), 3 mM taurine, and 25 mM HEPES (Invitrogen). Transfection of these cells with hNav1.5 (10 μg of wild-type and 5 μg of mutants) and reporter plasmid CD8-pih3m (1 μg) was accomplished by a calcium phosphate precipitation method in a Ti25 flask. Cells were replated 15 h after transfection, maintained at 37°C in a 5% CO_2 incubator, and used for experiments after 1 to 4 days. Transfection-positive cells were identified by immunobeads (CD-8 Dynabeads, Dynal, Lake Success, NY).

Electrophysiology and Data Acquisition. The whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used to record macroscopic Na^+ currents in cells coated with CD8 immunobeads at room temperatures ranging from 21 to 23°C. Pipette electrodes were fabricated with a tip resistance ranging from 0.8 to 1.2 MΩ. Command voltages were controlled by pCLAMP8 software (Axon Instruments, Inc. Union City, CA) and delivered by a List-EPC7 patch-clamp amplifier (List Electronics, Darmstadt/Eberstadt, Germany). Pipette electrodes were filled with an internal solution containing 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES titrated with CsOH to pH 7.2. The external solution consisted of 85 mM choline chloride, 65 mM NaCl, 2 mM $CaCl_2$, and 10 mM HEPES titrated with tetramethylammonium hydroxide to pH 7.4. We have previously used similar solutions and protocols to measure outward Na^+ current and to minimize the effects of series resistance artifact (Wright et al., 1999; Nau et al., 2000; Gerner et al., 2001; Wang et al., 2001). After the establishment of whole-cell configuration, cells were dialyzed for 20 to 30 min to equilibrate with the pipette solution before data were acquired. All cells requiring drug perfusion were perfused with drug until a steady-state block was reached. Time-dependent shifts in the midpoint voltage of sodium channel availability during experiments (30–60 min after membrane rupture) would have been approximately 5 to 7 mV (Wang et al., 1996). Data were filtered at 5 kHz, sampled at 50 kHz, collected, and stored with pCLAMP software (Axon Instruments). Voltage error was generally <3 mV at +30 mV after compensation for series resistance. Leak and capacitance currents were subtracted by P/−4 protocol, which was not applied in the use-dependent block of Na^+ currents. Cells producing current greater than 10 nA were excluded from the study. Results of analyses from the experiments are presented as mean ± S.E. Curve fitting was performed by Origin (OriginLab Corp., Northampton, MA). An unpaired Student's t test and a one-way analysis of variance were used to evaluate the significance of changes produced by the drugs on the tonic and the use-dependent block. A probability value (p) of <0.05 was considered statistically significant.

Results

Voltage-Dependent Block of Human Heart NaChs by Prenylamine. The voltage-dependent affinity of prenylamine for hNav1.5 channels was assessed by delivering conditioning pulses of 10 s ranging from −180 mV to −50 mV and measuring the Na^+ current remaining at +30 mV test pulse (Fig. 1A, inset). Conditioning pulses of 10 s were used to allow steady-state binding of drug, and a 100-ms interval separated each conditioning and test pulse to allow drug-free channels to recover from fast inactivation. At hyperpolarized prepulse voltages <−150 mV, 10 μM prenylamine produced approximately 41% tonic block of peak Na^+ current. Furthermore, at prepulse voltages >−140 mV, 10 μM prenylamine produced a strong block of peak Na^+ current, which reached a steady-state level of approximately 99% block between prepulse voltages of −100 mV and −50 mV (Fig. 1B). Thus, prenylamine binding with the hNav1.5 is voltage-dependent, with low-affinity binding at more hyperpolarized prepulse voltages and high-affinity binding at more depolarized prepulse voltages.

Concentration-inhibition experiments were performed next to assess more fully the potencies of prenylamine in blocking hyperpolarized and depolarized hNav1.5 channels. Based on the results from Fig. 1, prepulse voltages of −180 mV and −70 mV were used to measure the dose response of prenylamine on hyperpolarized and depolarized channels, respectively. The 50% inhibitory concentration (IC_{50}) value for hyperpolarized and depolarized channels was 9.67 ± 0.25 μM, and 0.72 ± 0.02 μM, respectively. Thus, prenylamine was approximately 12 times more potent in hNav1.5 channels at depolarizing potentials than at hyperpolarizing potentials (Fig. 2). The Hill coefficients calculated for the hyperpolarized and depolarized states were 2.1 ± 0.1 and 2.0 ± 0.1, respectively. A Hill coefficient of approximately 2.0 implies that two prenylamine molecules must bind before the

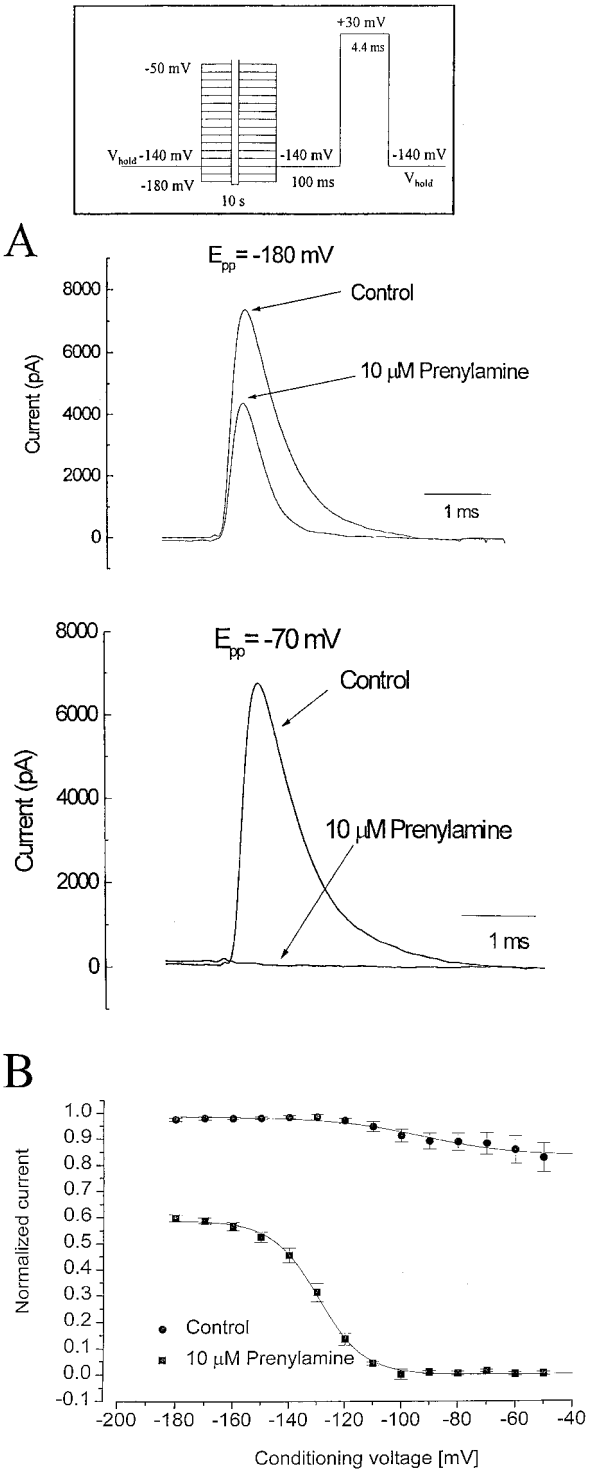

Fig. 1. Voltage-dependent block of hNav1.5 channels by prenylamine. Conditioning prepulses (10 s each) ranging in amplitude from −180 mV to −50 mV were applied. After a 100-ms interval at −140 mV, Na^+ currents were evoked by the delivery of the test pulse to +30 mV. Currents were normalized to the control currents obtained with a prepulse to −180 mV. A, representative current tracings for 10 μM prenylamine are shown for the hyperpolarized state (conditioning E_{pp} = −180) and for the depolarized state (E_{pp} = −70 mV). B, normalized Na^+ current in the absence (control) or presence of 10 μM prenylamine was plotted against conditioning prepulse potential. Data were fitted well with a Boltzmann function ($1/[1 + \exp((V_{0.5}-V)/K_E)]$). The average $V_{0.5}$ value (50% availabilities) and K_E (a slope factor) values for the fitted Boltzmann functions were −92.1 ± 5.6 mV and 15.7 ± 4.8 mV, respectively, for control and −129.5 ± 0.4 mV and 8.1 ± 0.4 mV, respectively, for prenylamine.

NaCh is blocked and therefore suggests that there might be at least two binding sites for at least two prenylamine molecules. Alternatively, a high Hill coefficient may result from the possibility that there is a nonlinear relation between the aqueous concentration of prenylamine and the effective concentration near the binding site if the effective prenylamine concentration increases more than the aqueous prenylamine concentration in a nonlinear manner (Meeder and Ulbricht, 1987). Therefore, a high Hill number may not truly reflect the number of binding sites. The results here indicate that prenylamine has a low affinity at hyperpolarizing potentials and a high affinity at depolarizing potentials for hNav1.5 channels with possibly two binding sites.

Use-Dependent Block of NaChs by Prenylamine. In addition to a tonic block exhibited by prenylamine when the cell is stimulated infrequently, prenylamine also exhibits a use-dependent block when the cell is stimulated frequently. When hNav1.5-transfected HEK cells were depolarized to +30 mV for 24 ms repetitively at a frequency of 5 Hz, a strong use-dependent block (57%) occurred in the presence of 3 μM prenylamine compared with the block in control cells (Fig. 3). The time course of this use-dependent block was fitted by a single exponential function with a rate constant of 0.39 per pulse. Thus, prenylamine, as shown previously for native NaCh on neuronal cells (Mujtaba et al., 2001), also exhibited use-dependent block of hNav1.5 channels.

Development and Recovery of Depolarized hNav1.5 Channels from Block by Prenylamine. Because the affinities of prenylamine and LAs are higher for the depolarized than the hyperpolarized state of the NaCh, the time course of development and recovery from the depolarized state was assessed. The development of drug block was determined by applying conditioning prepulses to −70 mV of variable duration (0–20 s) followed by a 100-ms interval at the holding potential of −140 mV before the test pulse to +30 mV to allow full recovery of fast inactivation. Block by 3 μM prenylamine developed with a time constant of 0.32 ± 0.02 s

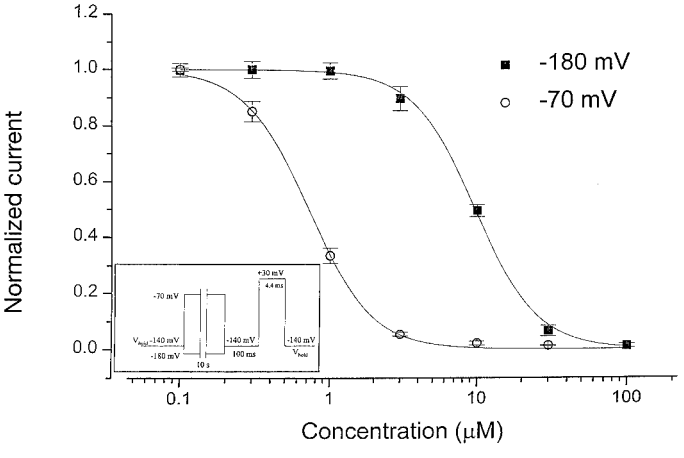

Fig. 2. Dose-response curves for hyperpolarized and depolarized sodium channels. The pulse protocol is shown in the inset. The hyperpolarized state affinity for prenylamine on NaChs was measured with a prepulse of −180 mV for 10 s, and the depolarized state affinity was measured with a prepulse of −60 mV for 10 s. The peak amplitudes of Na^+ currents, evoked by a test pulse to +30 mV for 4.4 s, were measured at various drug concentrations, normalized with respect to the peak amplitude in control, and plotted against the drug concentration. Data are reported as the mean ± S.E. (n = 6 for all groups). Solid lines represent fits to the data with the Hill equation.

(Fig. 4). The recovery from block was determined by applying a test pulse to +30 mV from a holding potential of −140 mV at various times after a 10-s conditioning prepulses to −70 mV. As determined from Fig. 5, currents in the absence and presence of drug recovered with fast and slow time constants. Control currents showed fast and slow time constants of 7.5 ± 0.5 ms and 0.8 ± 0.6 s, respectively. In the presence of 3 μM prenylamine, hNav1.5 channels recovered with fast and slow time constants of 5.9 ± 8.1 ms and 2.28 ± 0.07 s, respectively. The fractional amplitude of the slow phase of recovery for prenylamine was 98% because of the slow dissociation of prenylamine from channels blocked during the conditioning prepulse. In comparison, it has been reported previously that for bupivacaine in the presence of 10 μM (*R*)(+)-bupivacaine, a large portion (77%) of the current recovered with a slow time constant of 2.1 s and a small portion (23%) recovered with a fast time constant of 7.3 ms (Nau et al., 2000).

Block of Mutant NaChs by Prenylamine. To determine whether prenylamine and LAs share a common receptor site on the NaCh, we used site-directed mutagenesis to generate mutant hNav1.5 channels, which have been previously shown to reduce affinity for LAs, especially in the depolarized state, and compared the prenylamine voltage-dependent block of these mutants with that of wild-type. Individual residues constituting the LA binding site were substituted with lysine in homologous segments in D1–S6, D3–S6, and D4–S6 of the hNav1.5 channels. Previous studies have shown that positions F1760, N1765, Y1767, I1466, and N406 are involved in LA binding (see Introduction). The activation and inactivation kinetics of mutants F1760K, N1765K, Y1767K, and N406K have previously been reported (Nau et al., 2000). Control currents were normalized to the current obtained with a prepulse to −180 mV. Currents obtained in the presence of drug were normalized to the current obtained in control with the corresponding prepulse potential (E_{pp}). As reported previously for the common LA, bupivacaine (Nau et al., 2000) substitution at position F1760 with lysine (F1760K) reduced the voltage-dependent block of bupivacaine, and no two distinguishable binding affinities were detected, unlike the wild-type hNav1.5 channels (Fig. 6), where block reached a plateau at prepulse potentials ≤ −150 mV and ≥ −100 mV. Thus, high-affinity binding of bupivacaine to the depolarized channels was virtually eliminated. The estimated IC_{50} values of bupivacaine block of F1760K mutant showed a 4.5-fold reduction in affinity for hNav1.5 channels at hyperpolarizing potentials and a 68.5-fold reduction in affinity at depolarizing potentials (Table 1). On the contrary, prenylamine voltage-dependent block had distinguishable binding affinities for all the mutant NaChs similar to the wild-type (Fig. 7, A–F). At a concentration of 10 μM, prenylamine block of wild-type NaChs reached a plateau at hyperpolarizing E_{pp} of ≤ −150 mV and depolarizing E_{pp} ≥ −100 mV. In mutation F1760K, block of hyperpolarized and depolarized channels reached a plateau at E_{pp} ≤ −140 mV and ≥ −80 mV, respectively. The estimated IC_{50} values show a 1.7-fold reduction in affinity at hyperpolarized potentials and a 4.0-fold reduction in affinity at depolarized potentials compared with that of the wild type (Table 1). Similarly, for

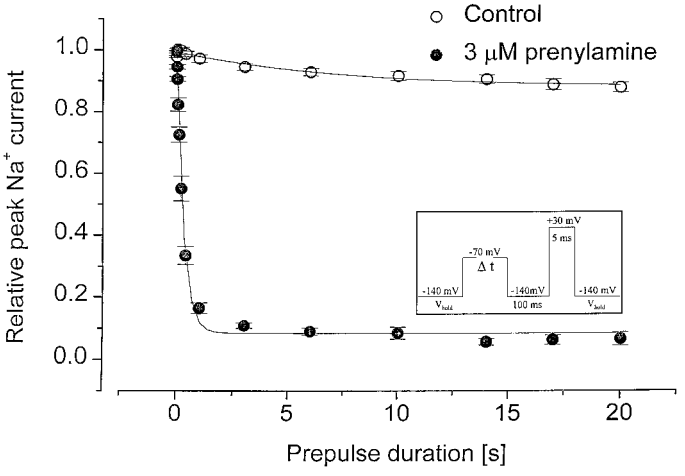

Fig. 4. Development of 3 μM prenylamine block of depolarized hNav1.5 channels. For the development of block, the prepulse duration at −70 mV was varied, and the peak current at the test pulse was measured, normalized to the initial peak amplitude (t = 0), and then plotted against the prepulse duration. The data were fitted by a single-exponential function.

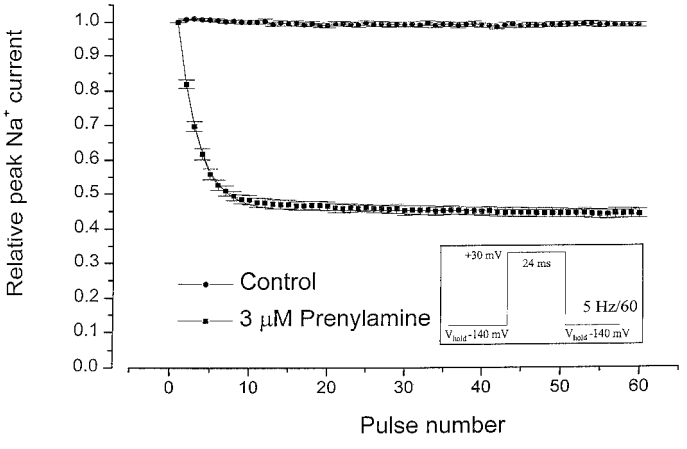

Fig. 3. Use-dependent block of hNav1.5 channels by prenylamine. From the holding potential of −140 mV, a test pulse of +30 mV was evoked for 24 ms. This cycle was obtained for a total of 60 pulses at a frequency of 5 Hz. The peak amplitude of each data set was normalized with respect to the peak amplitude of the first pulse of the set and plotted against pulse number. Lines drawn through the data points are the best fit of single-exponential function, with a time constant of 2.56 ± 0.06 pulse for prenylamine.

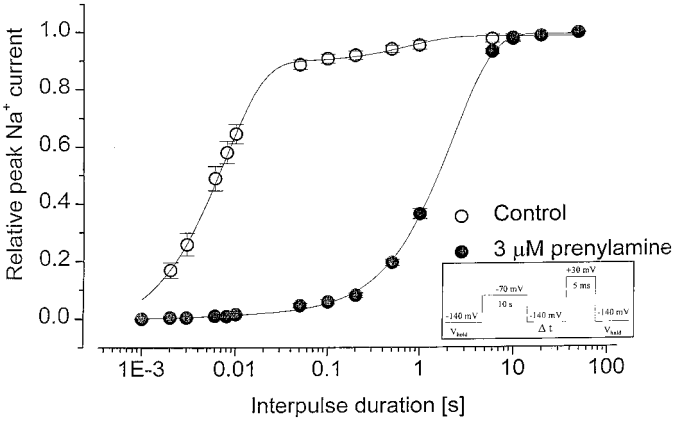

Fig. 5. Recovery from 3 μM prenylamine block of depolarized hNav1.5 channels. For recovery from block, the interpulse duration at −140 mV was varied and the peak current at the test pulse was measured, normalized with respect to the peak amplitude without the prepulses, and plotted against the interpulse duration. The data were fitted by a double-exponential function.

mutations N1765K, Y1767K, N406K, and I1466K, which are amino acid positions that have been shown to participate in LA binding, there is clear evidence of a voltage-dependent block by prenylamine. The estimated IC_{50} values for these mutants are shown in Table 1. Also, there are shifts of approximately 20 and 40 mV in the curves for F1760K and N1765K, respectively, toward the depolarized direction for prenylamine voltage-dependent block that are perhaps caused by the intrinsic inactivation kinetics of the particular mutant (Nau et al., 2000). Furthermore, other mutations, such as N927K and L931K, which have been shown to modulate batrachotoxin affinities (Wang et al., 2001), and mutation L409K, which is near the LA receptor site, showed no major reduction in binding affinities (Table 1). Most of the mutants showed a reduced affinity toward prenylamine but at a level far less than that toward bupivacaine or other LAs. Thus, prenylamine blocks hNav1.5 mutants that have reduced affinities for LAs in a voltage-dependent manner similar to the block of wild-type NaChs, which suggests that the prenylamine receptor site differs from that of the known LAs.

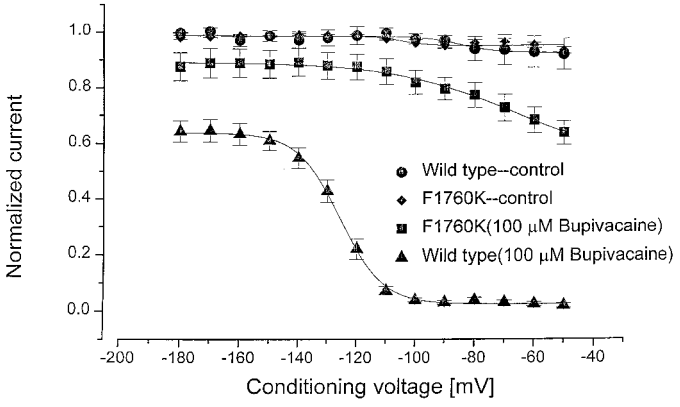

Fig. 6. Voltage-dependent block of hNav1.5 wild-type and mutant channels by bupivacaine. Similar pulse protocol was used as in Fig. 1. Normalized Na^+ currents of mutant and wild-type hNav1.5 channels in the absence (control) or presence of 100 μM bupivacaine were plotted against condition prepulse potentials. Control currents were normalized to the current obtained with a prepulse to −180 mV. Currents obtained in the presence of bupivacaine were normalized to the current obtained in control with the corresponding prepulse potential. Data were fitted well with a Boltzmann function $(1/[1 + \exp((V_{0.5}-V)/K_E)])$ where $V_{0.5}$ is the 50% availability value and K_E is the a slope factor value.

Block of hNav1.5 Channels by Prenylamine-Bupivacaine Mixture. To confirm our mutagenesis data, we performed binding experiments using a mixture of prenylamine and bupivacaine. Assuming that separate receptors are present for prenylamine and bupivacaine and that a channel is blocked if either of the prenylamine or bupivacaine receptors are occupied, for a two-site model, we can calculate the fraction of blocked channels using the equation $Y = Y_{bup} + Y_{pre} - Y_{bup}Y_{pre}$, where Y is the fraction of blocked channels, Y_{bup} and Y_{pre} are the fractions of prenylamine and bupivacaine receptors, respectively, that are occupied at equilibrium in a prenylamine-bupivacaine mixture, and $Y_{pre}Y_{bup}$ is the probability that a channel is doubly blocked (Wagner and Ulbricht, 1976). As shown in Fig. 8A, the experimental values for drug combination exceed those calculated for additive effect, differences that are more noticeable at the hyperpolarizing potentials for a drug concentration of 10 μM prenylamine and 100 μM bupivacaine. Also, at the hyperpolarizing potential of −180 mV, the wash-in prenylamine (10 μM) block of the NaChs that were pretreated with bupivacaine (100 μM was directly proportional to prenylamine wash-in block without bupivacaine pretreatment (Fig. 8B), suggesting that bupivacaine already bound to its receptor site does not alter the wash-in rate of prenylamine. Block by prenylamine and bupivacaine was reversible individually and in combination (data not shown). Furthermore, if we assume prenylamine and bupivacaine bind to the same receptor in a one-to-one fashion, the opposite scenario can be tested, as would be expected in the case of competition. The fraction of blocked channels (Y) in the bupivacaine-prenylamine mixture, in the case of a similar receptor, can be calculated using the equation $Y = (C_{pre} + C_{bup})/(C_{pre} + C_{bup} + 1)$, where $C_{pre} = Y_{pre}/(1 - Y_{pre})$ and $C_{bup} = Y_{bup}/(1 - Y_{bup})$ (Wagner and Ulbricht, 1975, 1976). As shown in Fig. 8, the calculated values of block for competition is less than the experimental values and the values calculated for independent sites at each prepulse potential, more noticeable at the hyperpolarizing prepulse potentials. Thus, the experimental block of Na^+ current of prenylamine-bupivacaine mixture exceeds that calculated from the blocking effect of the individual drugs under the assumption of two independent sites.

TABLE 1

Estimated IC_{50} values for block of hNav1.5 wild-type and mutant NaChs by prenylamine and bupivacaine

The IC_{50} values for prenylamine block of wild-type channels were derived from concentration-inhibition experiments at both the hyperpolarized state (−180 mV) and depolarized state (−70 mV). The IC_{50} values for prenylamine block of mutant channels and bupivacaine block of channels were estimated by the formula $Y = Y_{max} / [1 + (X / IC_{50})^{n_H}]$, where Y is the current remaining, Y_{max} is the maximum current without drug, X is the drug concentration (3 μM to 100 μM), and n_H is the Hill coefficient.

	Hyperpolarized State IC_{50}	Ratio Mutant/Wild-type	Depolarized State IC_{50}	Ratio Mutant/Wild-type
	μM		μM	
Bupivacaine				
Wild-type	177.8		2.6	
F1760K	809.1	4.5	179.0	68.5
Prenylamine				
Wild-type	9.7		0.7	
F1760K	16.5	1.7	2.9	4.0
N1765K	16.4	1.7	1.6	2.2
Y1767K	14.2	1.5	1.5	2.1
I1466K	13.6	1.4	0.6	0.8
N406K	14.6	1.5	4.2	5.8
L409K	16.9	1.7	4.2	5.8
N927K	8.9	0.9	0.9	1.3
L931K	13.6	1.4	2.3	3.2

Discussion

The data presented in this report show that prenylamine is a potent hNav1.5 channel blocker under voltage-clamp conditions and suggest that the binding site of prenylamine differs from that of the LAs.

Local anesthetic properties of prenylamine have been shown previously in vivo (Mujtaba et al., 2001). Like LAs, in vitro prenylamine elicits both tonic and use-dependent block activities both in neuronal cells that contain native NaChs (Mujtaba et al., 2001) and in hNav1.5 channels transfected into HEK cells. Block of the hNav1.5 channels was voltage-dependent, with a 12-fold difference in affinity between the hyperpolarized state (IC_{50}, 9.7 μM) and depolarized state (IC_{50}, 0.72 μM). The NaCh displayed a nonunity stoichiometry in prenylamine binding with a Hill coefficient $\approx$ 2, meaning that two prenylamine molecules are required to block one NaCh. We have shown previously that prenylamine has a Hill coefficient >1 in the inactivated state when tested on native sodium channels that are constitutively expressed on rat neuronal GH_3 cells (Mujtaba et al., 2001). This suggests that at least two binding sites for prenylamine are present on hNav1.5 channels. Both prenylamine sites are probably different from the LA site, because the bupivacaine-prenylamine mixture equilibrium experiments showed no competition between the two drugs (Fig. 8). Because some of the mutant NaChs show a small reduction in affinity to prenylamine compared with the wild-type NaCh (Table 1), however, certain regions of the prenylamine receptor site may overlap that of the LA receptor site. Alternatively, a higher Hill coefficient may be attributable to the difference of aqueous concentrations and the effective concentrations near the binding site of the prenylamine receptor (Meeder and

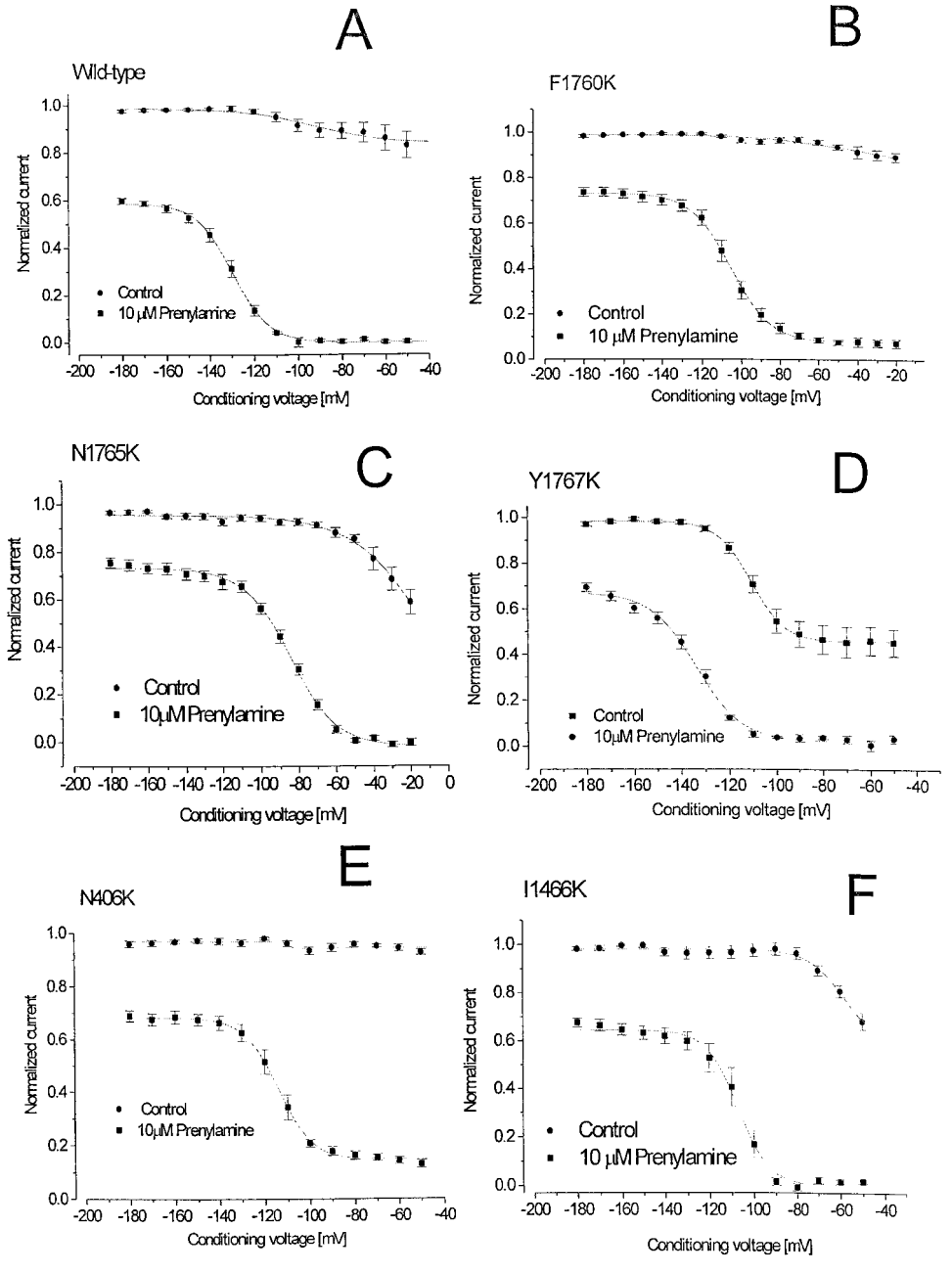

Fig. 7. Voltage-dependent block of hNav1.5 wild type and mutant channels by prenylamine, using a pulse protocol similar to that used in Fig. 1. Normalized Na^+ currents of wild-type and various mutant hNav1.5 channels in the absence (control) or presence of 10 μM prenylamine were plotted against condition prepulse potentials. Control currents were normalized to the current obtained with a prepulse to −180 mV. Currents obtained in the presence of prenylamine were normalized to the current obtained in control with the corresponding prepulse potential. Data were fitted well with a Boltzmann function as described in the legend for Fig. 6.

Ulbricht, 1987). Furthermore, it has previously been noted that Hill coefficients >1 are difficult to interpret and may not reflect the true number of binding sites (Weiss, 1997). However, from the results of these experiments, we can state that prenylamine blocks wild-type hNav1.5 channels and, like LAs, displays voltage-dependent binding affinity.

Mutagenesis data showed that hNav1.5 mutants with greatly reduced affinity to LAs were blocked in the presence of prenylamine (Fig. 7), especially at depolarized potentials. Mutants N1765K and F1760K showed a shift in the curve for the voltage-dependent block toward the depolarized direction, which could be caused by the intrinsic inactivation kinetics of the mutant NaCh due to the point mutation as shown previously (Nau et al., 2000). Alternatively, prenylamine may have a receptor site that overlaps with that of LAs. Most of the mutant NaChs that have low affinity for LAs showed a 1.4- to 5.8-fold decrease in affinity for prenylamine (Table 1), suggesting that certain regions of the LA site may overlap that of the prenylamine receptor site. However, the difference between bupivacaine and prenylamine block of mutant NaChs that have low affinity for LAs, especially in the depolarized state, is significant in that prenylamine blocked mutant NaChs with distinct affinity states at hyperpolarizing and depolarizing potentials, whereas bupivacaine virtually eliminated high-affinity binding to channels at depolarized potentials. There was a 68.5-fold difference in affinity at depolarized potentials between wild-type and F1760K with respect to bupivacaine block, whereas the level of difference between the wild-type and mutant channels was much less significant for prenylamine. Thus, mutation of the LAs receptor site on the hNav1.5 channels does not reduce the activity of prenylamine block, suggesting that prenylamine has an alternative site for binding on the NaCh.

Our prenylamine-bupivacaine mixture experiments confirmed the mutagenesis data in that prenylamine and bupivacaine act via separate pharmacological receptors. The calculated values for the block with the bupivacaine-prenylamine mixture, if one assumes separate and independent receptor sites for drugs, were close to those of the experimental values, most noticeably at the hyperpolarizing voltages (Fig. 8). In fact, the experimental values showed potentiation compared with the calculated values. Furthermore, the wash-in of prenylamine in the presence of bupivacaine was proportional to the wash-in of prenylamine alone; thus, bupivacaine does not alter the time course of prenylamine block, suggesting that a separate receptor site is being blocked by prenylamine. Wagner and Ulbricht previously used similar calculation and experimental methods to show that procaine and saxitoxin bind to separate sites on the NaCh (Wagner and Ulbricht, 1976). Thus, our equilibrium results are consistent with the finding that prenylamine and LAs have independent receptor sites on the NaCh.

Although we have not yet delineated the specific location of the prenylamine binding site(s) on the NaCh, we do know from the data presented here that prenylamine and LAs do not have similar receptor sites on hNav1.5 channels. This finding has both clinical and academic relevance in that regions of the NaChs other than the traditional LA receptors site can be targeted to enhance the design of LAs and to improve therapeutics for sodium channel-associated pathologies such as cardiac arrhythmias and cardiac deaths related to the LQT-3 form of the long QT syndrome.

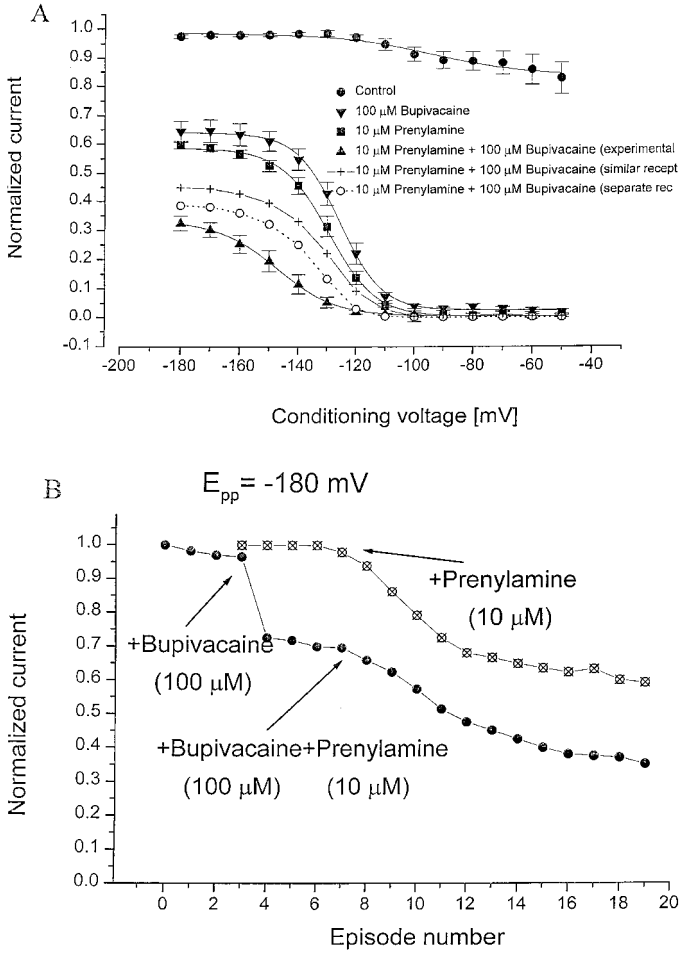

Fig. 8. A, block of hNav1.5 channels by prenylamine-bupivacaine mixture, using a pulse protocol similar to that used in Fig. 1. Normalized Na^+ currents in the absence (control) or presence of prenylamine, bupivacaine, and bupivacaine-prenylamine mixture were plotted against condition prepulse potentials. Values of block for similar and separate receptor site of bupivacaine-prenylamine mixture were calculated based on individual block of the NaCh of each drug (see *Results*), and the fraction of block remaining ($1 - Y$) was plotted against prepulse potentials. Data were fitted well with a Boltzmann function as described in the legend for Fig. 6. B, wash-in of bupivacaine, prenylamine, and bupivacaine-prenylamine mixture at the prepulse of −180 mV, using a pulse protocol similar to that used in Fig. 2. Arrow, start of wash-in of each drug or drug mixture.

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