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Irreversible Block of Human Heart (hH1) Sodium Channels by the Plant Alkaloid Lappaconitine

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ABSTRACT

The roots from *Aconitum* sp. plants have long been used in Chinese herbal medicine for treating pain and various heart conditions. The principal component of *Aconitum* remedies is usually aconitine, a site 2 neurotoxin that may induce severe neurological symptoms and cardiovascular collapse. Some *Aconitum* species also contain lappaconitine, the structure of which is remarkably similar to that of aconitine. In contrast to aconitine, a sodium channel agonist, lappaconitine reportedly blocks voltage-gated sodium channels in heart tissue. The results in the present study demonstrate that lappaconitine blocks cloned human heart (hH1) sodium channels under whole-cell, voltage-clamp conditions. Lappaconitine binding has several characteristics in common with the binding of site 2 neurotoxins, such as aconitine and batrachotoxin. For example, lappaconitine binds almost exclusively to open channels, but has little affect on resting or inactivated channels. Moreover, lappaconitine binding is inhibited by bupivacaine, a tertiary amine local anesthetic. Whereas site 2 neurotoxins often irreversibly modify channel kinetics, lappaconitine irreversibly blocks the channels. Finally, channels containing lysine substitutions within the local anesthetic receptor region at residues F1760 or N1765 are resistant to block by bupivacaine or lappaconitine. Given that site 2 neurotoxins and local anesthetics have nonidentical but overlapping binding regions, these data suggest that lappaconitine irreversibly blocks hH1 channels by binding to the site 2 receptor.

Voltage-gated sodium channels generate the action potential in nerve and muscle tissue. Recent advances in molecular techniques, including site-directed mutagenesis and the formation of channel chimeras, have provided important clues into the mechanism of channel kinetics and pharmacology [see Fozzard and Hanck (1996) and Marbán et al. (1998) for review). Before the advent of molecular techniques, naturally occurring neurotoxins [see Strichartz et al. (1987) and Catterall et al. (1992) for review] were essential tools for studying sodium channel pharmacology and function because they modify channel behavior. At least six receptor sites on sodium channels have been identified by toxin binding studies. Site 1 toxins, such as tetrodotoxin and saxitoxin, block sodium channels by binding to a receptor in the pore region near the extracellular face of the channel (Tomaselli et al., 1995), whereas toxins that bind to sites 2 through 6 modify channel activation and/or open-state inactivation. Of the toxins that modify channel kinetics, those that bind to sites 2 and 3 have been studied most extensively. Site 2 toxins, such as batrachotoxin (Bartels-Bernal et al., 1977), are alkaloids that bind to open channels. These toxins gain access to the receptor from the intracellular side of the channel or possibly through the lipid phase of the channel. Site 3 toxins are peptides from scorpion (Rogers et al., 1996) and sea anemone (Benzinger et al., 1998) venoms that bind to a receptor on the extracellular face of the channel.

Neurotoxins that interact with site 2 on the sodium channel are an interesting set of alkaloids because they can be found in both animal and plant species. Batrachotoxin, from the skin of South American *Phyllobates* sp. frogs, veratridine (lily), grayanotoxin (rhododendron), and aconitine (*Aconitum* sp.) markedly affect sodium channel behavior by shifting the voltage dependence of activation to more hyperpolarized potentials, by removing or reducing channel inactivation from the open state, and by altering the ionic selectivity of the channel (Strichartz et al., 1987; Catterall et al., 1992). Channel modification by site 2 toxins is generally considered to be irreversible (Hille, 1992) although the effects of veratridine modification (reviewed by Ulbricht, 1998) can be partially removed.

Although the principal ingredient in *Aconitum* toxin is aconitine, some species also contain the structurally related lappaconitine (Fig. 1). Both toxins have a skeleton consisting of six-, seven-, and five-membered rings. Lappaconitine has a benzoyl linkage on the six-membered ring at C18, whereas aconitine has a benzoyl linkage on the five-membered ring at C14 (Hardick et al., 1996). Despite the structural similarity to aconitine, lappaconitine seems to be a sodium channel

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ABBREVIATIONS: HEK, human embryonic kidney.

blocker rather than an agonist. For example, lappaconitine inhibits the population spike in the rat hippocampal slice preparation (Ameri et al., 1996a; Ameri and Simmet, 1999) and displays a use- and frequency-dependent block of the population spike that is reversible upon washing (Ameri et al., 1996b). Furthermore, lappaconitine raises the stimulation threshold of electrically stimulated guinea pig atria (Heubach and Schule, 1998) and induces use-dependent arrhythmia in guinea pig heart, suggesting that lappaconitine has local anesthetic-like properties (Gutser et al., 1998; Heubach and Schule, 1998).

The present study focused on lappaconitine block of the whole-cell current of transiently expressed human heart sodium channels (hH1; Gellens et al., 1992). Several pieces of evidence indicated that lappaconitine binds primarily to open channels. For example, lappaconitine blocked the channels during repetitive stimulation but had little effect on either resting or inactivated channels. Use-dependent block by lappaconitine did not reach steady state even after several hundred pulses, and the rate and extent of use-dependent block did not depend on the stimulation rate (0.2 to 2 Hz). Unlike other naturally occurring sodium channel blockers, block by lappaconitine was irreversible, suggesting that lappaconitine does not bind to an extracellular receptor. Several studies have indicated that local anesthetics and site 2 neurotoxins have overlapping but nonidentical binding domains (Linford et al., 1998; Wang and Wang, 1999). To determine whether the lappaconitine binding site overlaps with the local anesthetic receptor, channels containing lysine substitutions within the local anesthetic receptor (F1760K, N1765K) were assayed for their sensitivity to bupivacaine and lappaconitine. Both mutants were highly resistant to block by either bupivacaine or lappaconitine, suggesting that lappaconitine irreversibly blocks hH1 channels by binding to the site 2 receptor.

Materials and Methods

Site-Directed Mutagenesis and Transient Transfection of HEK293t Cells. Site-directed mutagenesis was used to create lysine point mutations of the hH1-pcDNA I/amp vector (Chahine et al., 1996) at residues hH1-F1760 and hH1-N1765 as described previously for rat skeletal muscle (μ1) sodium channels (Wright et al., 1998). Human embryonic kidney (HEK) 293t cells were transfected with hH1 or mutant channel plasmid (2–5 μg) and reporter plasmid CD8-pih3m (1 μg) by the calcium phosphate precipitation method (Cannon and Strittmatter, 1993) as described previously (Wright et al., 1997, 1999). The transfected cells were replated onto 35-mm culture dishes and used for experiments up to 3 days. Transfection-positive cells, as identified by CD8 Dynabeads (Dynal, Inc., Lake Success, NY), were selected for whole-cell patch recording.

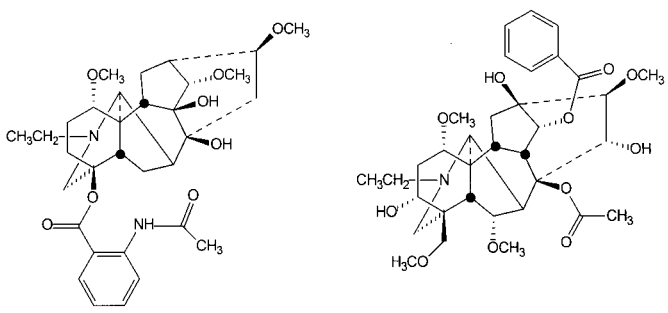

Fig. 1. The structures of *Aconitum* alkaloids lappaconitine (left) and aconitine (right).

Solutions and Chemicals. 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). For most experiments in which a reversed sodium gradient was used, the pipette solution contained 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES. For experiments in which a more conventional sodium gradient was used, the pipette solution contained 10 mM NaCl, 120 mM CsF, 10 mM EGTA, and 10 mM HEPES (titrated with cesium hydroxide to pH 7.2). Lappaconitine hydrobromide was purchased from CalBiochem (San Diego, CA), and racemic bupivacaine was purchased from Sigma Chemical Co. (St. Louis, MO). Lappaconitine was dissolved in 50% ethanol and was stored in 10 mM aliquots at −80°C; bupivacaine was dissolved in deionized water and was stored in 100 mM aliquots at −20°C. The stock solutions were diluted in extracellular saline at the appropriate concentration and were applied to the cell surface from a series of small-bore glass capillary tubes.

Whole-Cell Voltage Clamp and Data Analysis. Whole-cell voltage clamp (Hamill et al., 1981) of transfected HEK cells was used to study macroscopic hH1 sodium currents at room temperature (21 ± 2°C). Electrode resistances ranged from 0.5 to 1.0 MΩ. Command voltages were programmed by pCLAMP 7.0 software (Axon Instruments, Burlingame, CA) and were delivered by a Warner PC501A voltage clamp (Warner Instrument Corporation, Hamden, CT). Data were sampled at 50 kHz and filtered at 5 kHz. The holding potential for all experiments was −140 mV. A previous study examined the time-dependent negative shift in the steady state inactivation curve of heterologously expressed voltage-gated sodium channels (Wang et al., 1996). As described in previous papers (Wright et al., 1997, 1999), after establishment of whole-cell voltage clamp the cells were dialyzed for 25 to 30 min before acquiring data. According to estimates by Wang et al. (1996), the steady-state inactivation curve would have shifted by about 5 to 7 mV during the time course (20–30 min) of an experiment. Most of the capacitative current was cancelled by the voltage clamp circuitry, and the remaining capacitative artifact and the leakage current were subtracted by the P/−4 method. Leakage and capacitance current subtraction (P/−4) was not employed in studies of 2-Hz use-dependent block. Voltage errors of ≤5 mV during test pulses to +30 mV were considered acceptable for the drug study (Bean, 1992). 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. Unless noted otherwise, data are presented as mean ± S.E.M.

Results

Lappaconitine Irreversibly Blocks hH1 Channels. Application of 10 to 100 μM lappaconitine produced an initial reduction in the whole-cell current (Fig. 2A, wash on), but the percentage of available channels slowly decreased during the 5-min external perfusion and never reached steady state. This phenomenon was particularly noticeable at the 60 μM (Fig. 2A, ◇) and 100 μM (Fig. 2A, △) concentrations. To determine whether the whole-cell current could be further reduced, the cells were given a 2 Hz train of 100 pulses (5 ms duration) to +30 mV. The pulse protocol reduced the percentage of available current and the reduction was concentration dependent. After 2 Hz stimulation, the first test pulse was delivered in the presence of the drug (at 7 min in Fig. 2A) and during the next 5 min, subsequent pulses were delivered as the drug was washed off by control external solution. At each

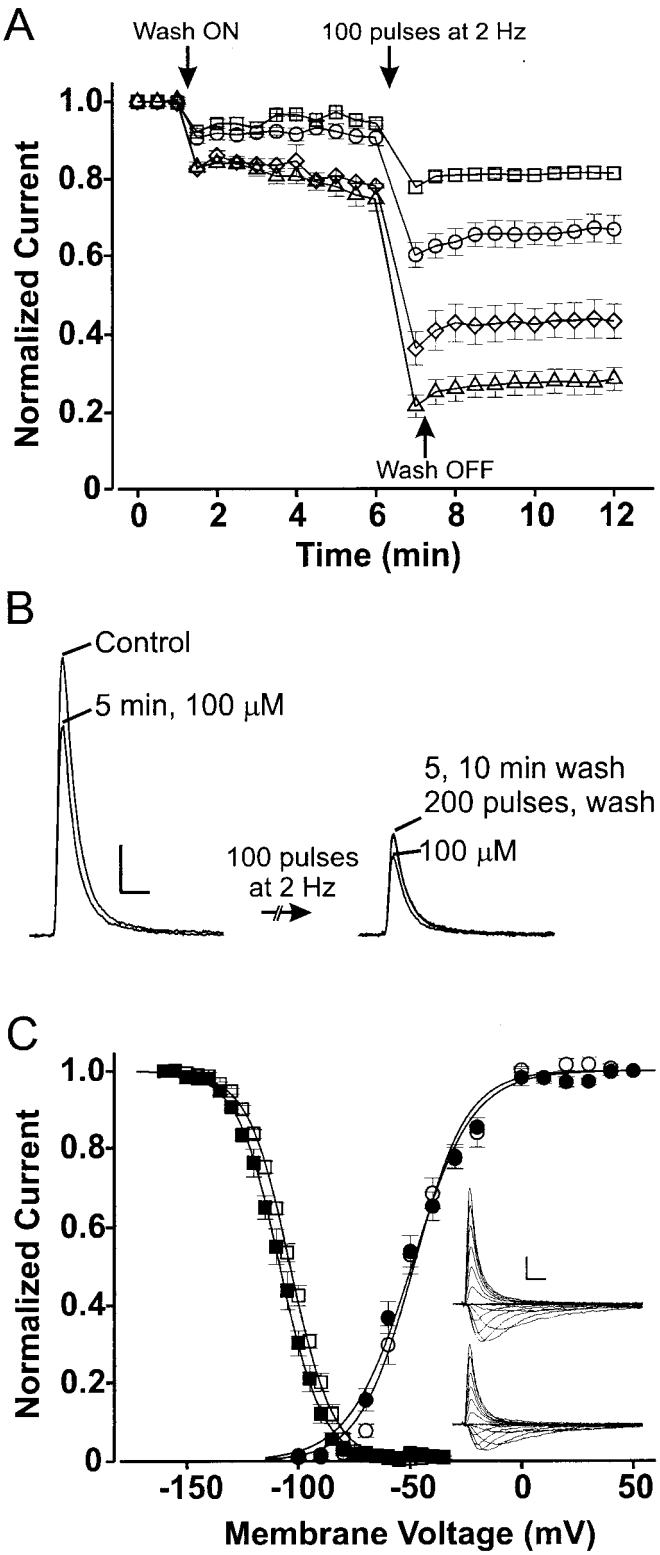

Fig. 2. Irreversible block of whole-cell hH1 currents by lappaconitine. A, lappaconitine was applied to the bath (arrow, wash on) at 10 μM (□, n = 5), 30 μM (○, n = 5), 60 μM (◇, n = 6), or 100 μM (△, n = 9), and the available current was measured during test pulses to +30 mV. Block of the hH1 current increased slowly during the initial 5-min perfusion but was markedly enhanced by delivery of a 2 Hz train of 100 pulses to +30 mV (arrow, 100 pulses at 2 Hz). The first pulse after the 2-Hz train was delivered while the cell was perfused with lappaconitine. The cells were then washed by control external saline for 5 min (*arrow*, wash off) but the blocked current did not recover. B, the traces on the left are whole-cell hH1 currents recorded in control external saline and after 5 min of exposure to 100 μM lappaconitine. The cell was then given a 2-Hz train of 100 pulses to +30 mV (broken arrow). The traces on the right show the first pulse recorded after the 2 Hz train (100 μM) and three superimposed traces recorded in control saline. The traces in control saline were recorded after 5 and 10 min of washing in control saline and after delivery of 200 additional pulses to +30 mV in control saline. Scale, 1 nA, 1 ms. C, normalized membrane conductance (circles) and normalized steady-state availability (squares) were measured before (○,□) and after (●,■) use-dependent block by 30 μM lappaconitine. Membrane conductance (g_m) was measured during 10-ms voltage steps to the voltages indicated on the abscissa; g_m was determined from the equation $g_m = I_{Na}/(E_m - E_{Na})$, and the plot was fitted with an empirical Boltzmann function. The midpoint voltage ($V_{0.5}$) and slope (k) of the Boltzmann function used to fit the data were −48.3 ± 2.4 mV and 11.8 ± 0.6 mV, respectively, for the control data (n = 4, ○). After 2 Hz stimulation in 30 μM lappaconitine (n = 4, ●) these values were −50.5 ± 2.4 mV and 13.3 ± 0.4 mV, respectively. The differences between the $V_{0.5}$ and k values of the function before and after lappaconitine block were not significant ($p > 0.05$). The currents in the inset were recorded before (top traces) and after (bottom traces) 2-Hz use-dependent block by 30 μM lappaconitine. Scale, 1 nA, 1 ms. To determine the steady-state availability of the channels, the cells were given 100 ms conditioning pulses to the voltage indicated on the abscissa and current availability was measured during a test pulse to +30 mV. The $V_{0.5}$ and k values of the Boltzmann function used to fit the data were −103.6 ± 0.8 mV and 9.6 ± 0.6 mV, respectively, for the control data (n = 5, □). After 2 Hz stimulation in 30 μM lappaconitine (n = 5, ■) these values were −108.3 ± 1.6 mV and 9.4 ± 0.3 mV, respectively. Although the change in the midpoint voltage of inactivation was significant (p = 0.03), the negative shift in channel availability after use-dependent block by lappaconitine was most probably caused by the time-dependent shift in steady-state inactivation (Wang et al., 1996).

lappaconitine concentration, the 5-min wash recovered only 5 to 7% of the blocked channels.

Additional washing and delivery of repetitive pulses in control external solution failed to recover additional amounts of the blocked current. In Fig. 2B, the traces on the left show the reduction of the current after a 5-min perfusion of 100 μM lappaconitine, and the traces on the right show the remaining current after 2 Hz stimulation. The smaller trace on the right was the first pulse recorded in 100 μM lappaconitine after the 2 Hz stimulation, and the three larger traces (superimposed) were recorded in control external solution. Two of the superimposed traces were recorded after washing the cell with control external solution for 5 and 10 min. The third trace was recorded after delivering 200 additional pulses to +30 mV in control solution. In three other cells, delivery of 100 additional pulses to −20 mV, which elicited a small inward current, did not increase the available fraction of channels (not shown). These data indicated that once bound to the channel, lappaconitine unbinds very slowly if at all.

Site 2 neurotoxins commonly induce a hyperpolarizing shift in channel activation. Figure 2C shows that lappaconitine had little affect on the kinetics of available channels. The conductance-voltage relationship and steady-state availability were determined in control external solution and after delivery of 100 pulses to +30 mV in 30 μM lappaconitine. To determine the conductance-voltage relationship, the cells were depolarized to the voltage indicated on the abscissa from a holding potential of −140 mV. Fig. 2C, inset, shows the currents from a representative cell before (Fig. 2C, inset, top) and after (Fig. 2C, inset, bottom) use-dependent block by 30 μM lappaconitine. In contrast to aconitine, which markedly slows the inactivation rate of the current, lappaconitine had no obvious effect on the time-constant of current decay during channel inactivation. The steady-state availability of the current (Fig. 2C, squares) also was little affected by

lappaconitine. To determine channel availability, 100-ms conditioning pulses to the voltage indicated on the abscissa were delivered to the cells and a subsequent test pulse to +30 mV was used to measure the available current. Although the midpoint voltage of steady-state availability was 4 mV more negative after lappaconitine block, the hyperpolarization was most likely caused by the time-dependent shift in steady-state inactivation that is characteristic of these channels (Wang et al., 1996). Thus, in contrast to the actions of other site 2 neurotoxins, lappaconitine blocks hH1 sodium channels and does not alter channel kinetics.

Use-Dependent Block by Lappaconitine is Dose-Dependent but Not Frequency Dependent. To examine the kinetics of use-dependent block by lappaconitine, the relative amplitude of the current during 2-Hz stimulation was plotted versus the pulse number (Fig. 3A). The extent of block by lappaconitine was dose-dependent, and the time course of the reduction in available current developed very slowly, particularly at the lower concentrations. The block did not reach steady state even at 100 μM, and delivery of an additional 200 pulses completely blocked the current ($n = 3$; data not shown).

Interestingly, the stimulation rate did not affect the extent of use-dependent block. Figure 3B shows currents recorded at 25 pulse intervals during 2-Hz (Fig. 3B, left) and 0.2 Hz (Fig. 3B, right) stimulation. The development and extent of use-dependent block by 100 μM lappaconitine was very similar despite the 10-fold difference in stimulation rate. At the 100th pulse, the fraction of available channels was 34 ± 4% ($n = 4$) at 2 Hz and was 26 ± 4% ($n = 4$, $p > 0.05$) at 0.2 Hz. Note that the cells were exposed to lappaconitine for more than 8 min at the 0.2 Hz stimulation rate compared with only 50 s at 2 Hz, suggesting that the drug has limited access to the receptor when the channel is in the closed resting state.

The traces in Fig. 3C compare the 2 Hz use-dependent block of hH1 channels by 30 μM bupivacaine (Fig. 3C, left) to the block by 30 μM lappaconitine (Fig. 3C, right). Bupivacaine block increased rapidly and reached steady state by the 20th pulse of the 60 pulse protocol ($n = 3$). For tertiary amine local anesthetics, the steady-state phase of use-dependent block represents an equilibrium between drug binding at each pulse and unbinding of the drug during the interval between the pulses (Chernoff and Strichartz, 1989). In contrast, use-dependent block of the current by 30 μM lappaconitine did not reach steady-state even after 500 pulses to +30 mV. In fact, the amount of available current was reduced after each set of 100 pulses by increments of about 28% ($n = 3$). Apparently, lappaconitine irreversibly blocks a small percentage of open channels during each pulse, thereby eliminating them from the population of available channels at subsequent pulses. Thus, the extent of block at a given lappaconitine concentration depended on the number of times the channels opened.

Lappaconitine Binding to Inactivated Channels Is Minimal. The fact that use-dependent block by lappaconitine did not depend on the stimulation rate suggested initially that channel opening and perhaps channel activation were important for drug binding and block. However, these data did not distinguish whether or not lappaconitine binding to inactivated channels during 5 ms pulses to +30 mV contributed to channel blockade. To determine whether or not inactivated channels became blocked by lappaconitine, the cells were given three 10-s pulses to −70 mV. The conditioning pulses to −70 mV, which inactivated almost all of the channels (see Fig. 2C), were separated by 30 s at the

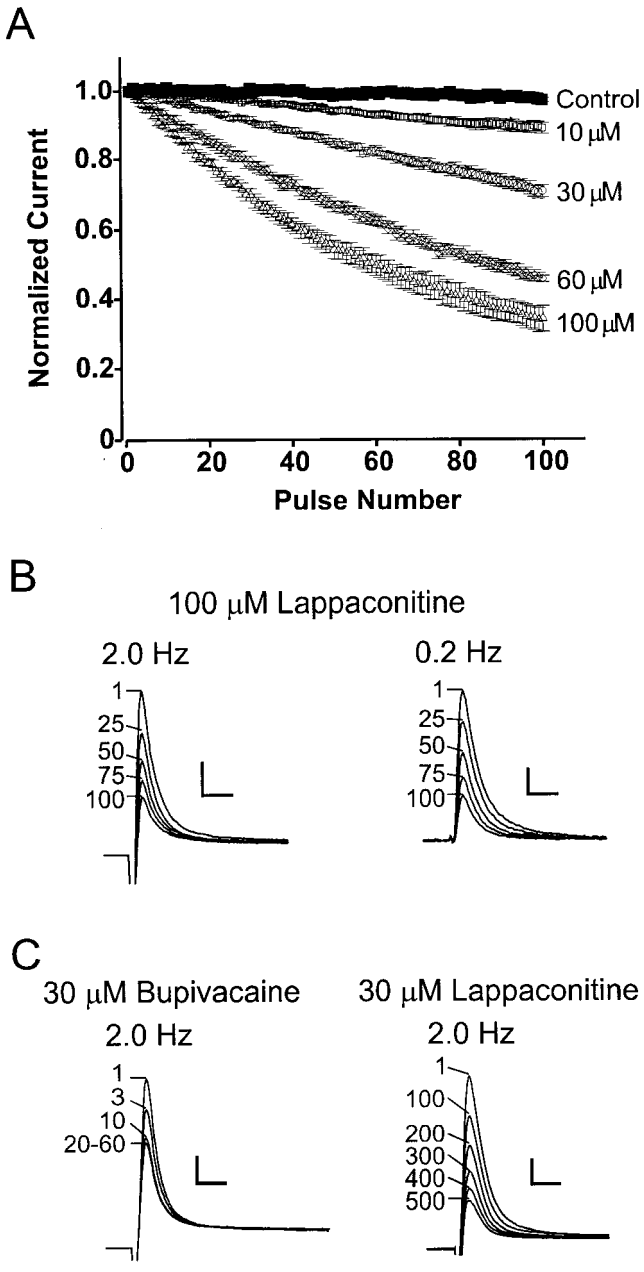

Fig. 3. Use-dependent block of hH1 current by lappaconitine. A, use-dependent block was monitored during a 2-Hz train of 100 pulses to +30 mV. In control saline, the percentage of available channels did not change during the 100-pulse train. At the 100th pulse of the 2-Hz stimulation, the current amplitudes in 10 μM, 30 μM, 60 μM, and 100 μM lappaconitine were 89 ± 2%, ($n = 5$), 71 ± 2% ($n = 5$), 46 ± 1% ($n = 5$), and 34 ± 4% ($n = 4$), respectively, of the first pulse. B, block of hH1 current at +30 mV by 100 μM lappaconitine during 2-Hz (left) and 0.2-Hz (right) stimulation. For clarity, only the first pulse and every 25th pulse of the 100-pulse protocol are shown. Block of the current was very similar despite the large difference in stimulation frequency. C, use-dependent block by 30 μM bupivacaine (left) and 30 μM lappaconitine (right). For bupivacaine block, the 1st, 3rd, and every 10th pulse of the 60-pulse protocol are shown; for lappaconitine block, the 1st and every 100th pulse of the 500-pulse protocol are shown. Note that use-dependent block by bupivacaine reached steady state by the 20th pulse, whereas block by lappaconitine did not reach steady state even after 500 pulses. Scale in B and C, 1 nA, 1 ms.

holding potential to allow the channels to recover from small amounts of slow inactivation. Figure 4A shows that 100 μM lappaconitine blocked hH1 channels to a much lesser degree during 10-s conditioning pulses to −70 mV (○) than during 2 Hz stimulation to +30 mV (△). The available current after washout of the drug was about the same as the available current before delivery of the inactivating pulses to −70 mV. Furthermore, the conditioning pulses to −70 mV would almost certainly have caused a large fraction of the channels to enter preactivated states, yet lappaconitine had little effect on the available current. These data suggested that lappaconitine is essentially incapable of binding to inactivated channels and like aconitine and other site 2 neurotoxins, the drug gains access to the receptor primarily when the channel is open.

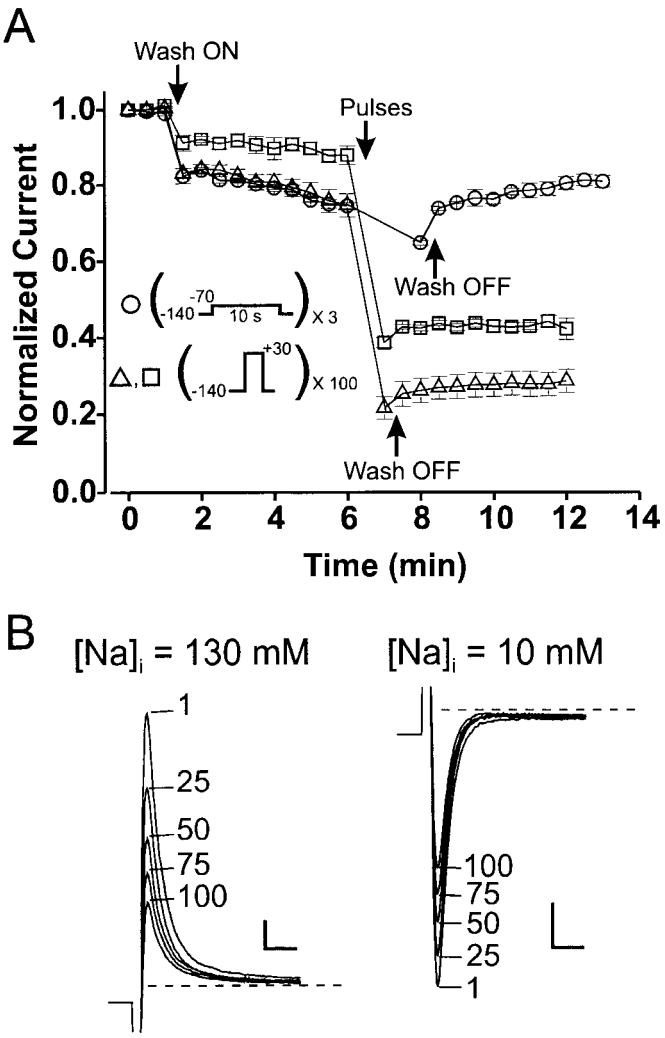

Fig. 4. Insensitivity of inactivated channels to lappaconitine and the effect of the sodium gradient on the irreversible block. A, the same general procedures described in Fig. 2A were followed to examine block of inactivated channels by 100 μM lappaconitine. The inset in the figure shows the pulse protocol delivered after the 5-min perfusion of drug. Block of inactivated channels (○) was measured by delivering three 10-s conditioning pulses to −70 mV and then measuring the available current during a test pulse to +30 mV. The conditioning pulses were separated by 30 s to allow the channels to recover from small amounts of slow inactivation. The amount of available current was then monitored during test pulses to +30 mV in control saline. Note that 100 μM lappaconitine blocked a much larger percentage of channels during a 100-pulse train to +30 mV (△, same data as Fig. 2A) than during 10-s depolarizations to −70 mV. Furthermore, 100 μM lappaconitine blocked a smaller percentage of channels when the driving force on sodium was inward at +30 mV (□, 65 mM $[Na]_o$/10 mM $[Na]_i$) compared with when the driving force on sodium was outward at +30 mV (△, 65 mM $[Na]_o$/130 mM $[Na]_i$). Nevertheless, the extent of block under either condition was irreversible. B, comparison of use-dependent block when the driving force on sodium was outward (left) or inward (right) at +30 mV. During a 2-Hz train of 100 pulses, 100 μM lappaconitine blocked about 75% of the current when the driving force was outward compared with about 45% when the driving force was inward. The current amplitudes were measured from the steady-state current levels (dashed lines) following open channel inactivation. Scale, 500 pA, 1 ms.

To determine whether the reversed sodium gradient (65 mM $[Na]_o$/130 mM $[Na]_i$) contributed to the irreversible block, use-dependent block of the channels was examined under conditions in which the driving force on sodium was inward at +30 mV. The squares in Fig. 4A represent the averaged data from three experiments in which 100 μM lappaconitine block was measured using a 65 mM $[Na]_o$/10 mM $[Na]_i$ gradient. As in the experiments with the reversed sodium gradient, channel availability was measured during test pulses to +30 mV. Under these conditions block of the resting channels during the initial 5-min perfusion of lappaconitine did not steadily increase, as was consistently observed in the reversed sodium gradient, and the fraction of available channels at the conclusion of the 5 min perfusion was greater at 88 ± 3%. In addition, a larger fraction of channels was available after 2 Hz stimulation to +30 mV and subsequent 5-min wash in control external solution (42 ± 3%, n = 3) than were available after use-dependent block in the reversed sodium gradient (28 ± 3%, n = 9). Furthermore, an inward driving force on sodium at +30 mV reduced the extent of use-dependent block by lappaconitine (Fig. 4B). Note that although the extent of use-dependent block was reduced in the 65 mM $[Na]_o$/10 mM $[Na]_i$ gradient, blocked channels did not recover after several minutes of washing in control saline, indicating that the reversed sodium gradient was not responsible for the irreversible block. As demonstrated for quaternary derivatives of lidocaine (Cahalan and Almers, 1979), these data showed that external sodium ions influence lappaconitine binding.

Bupivacaine Inhibits Lappaconitine Block. Previous studies have demonstrated that local anesthetics allosterically inhibit batrachotoxin binding to voltage-gated sodium channels (Postma and Catterall, 1984; Wang and Wang, 1999); that is, channels blocked by local anesthetic cannot open and become modified by batrachotoxin. As shown in Figs. 2–4, lappaconitine binds weakly to resting or inactivated channels, and the kinetics of use-dependent block are very slow. In contrast, the onset of use-dependent block by bupivacaine is relatively rapid because local anesthetics have a high affinity for open channels as well as inactivated channels (Hille, 1992). To examine whether use-dependent block by bupivacaine could reduce lappaconitine binding, the two drugs were applied together before delivery of the 2-Hz pulse protocol. In these experiments, the bupivacaine concentration was varied and the lappaconitine concentration was always 100 μM. The traces in Fig. 5A show the irreversible block produced by application of 100 μM lappaconitine alone (Fig 5A, top), and the reduction in irreversible block when the channels were exposed to both 100 μM lappaconitine and 8 μM bupivacaine (Fig 5A, middle) or to 100 μM lappaconitine and 300 μM bupivacaine (Fig 5A, bottom). Delivery of 100 pulses at 2 Hz again reduced the current, but a much larger fraction of channels recovered at the higher bupivacaine con-

centration (Fig. 5A, right traces). The plot in Fig. 5B shows the time course of these experiments for bupivacaine concentrations of 1, 8, 60, and 300 μM. These data clearly demonstrated that block of the channels by bupivacaine inhibited lappaconitine binding as measured by the reduction in irreversible block by lappaconitine.

Fig. 6 shows the dose dependence of the interaction between bupivacaine and lappaconitine. Each data set was best fitted by the Hill equation and had a Hill coefficient of about 1, indicating that one drug molecule binds to one channel. The IC_{50} values of resting block (at −140 mV) by bupivacaine alone (Fig. 6, ■) and resting block by bupivacaine plus 100 μM lappaconitine (Fig. 6, ♦) were similar at 65 and 73 μM, respectively. To determine the extent of use-dependent block by bupivacaine alone, the cells were given a 2 Hz train of 100 pulses (5 ms in duration). The available current during the steady-state phase of 2 Hz block by bupivacaine alone (Fig. 6, ●) was normalized to the current amplitude measured in control external solution. The IC_{50} value for bupivacaine during this summed block of resting channels and channels blocked during the 2 Hz train was 9 μM. As shown in Fig. 5B, the fraction of channels recovered after the use-dependent protocol increased with increasing bupivacaine concentration. For each bupivacaine concentration, the fraction of available channels at the conclusion of the 5-min wash in control external solution was renormalized according to the fraction of available channels after use-dependent block by 100 μM lappaconitine alone. These data were plotted as the fraction of recovered channels in Fig. 6 (right ordinate, △). The IC_{50} value for the fraction of recovered channels after use-dependent block by bupivacaine and lappaconitine was

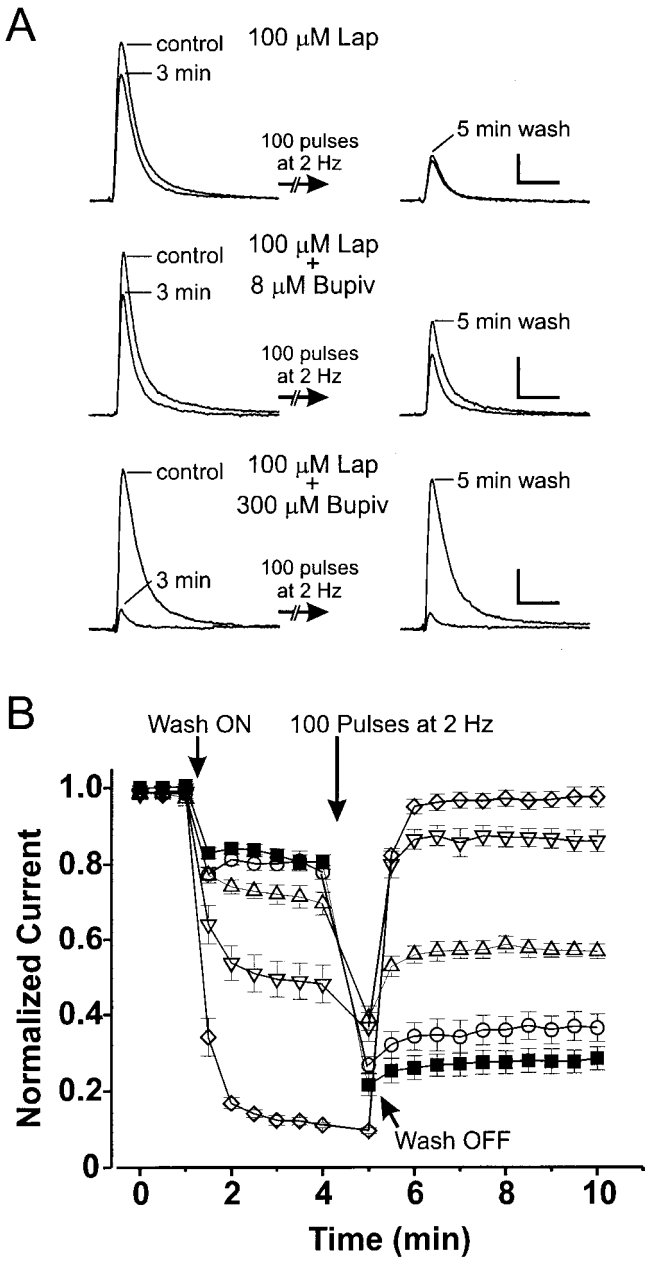

Fig. 5. Bupivacaine inhibits the irreversible block by lappaconitine. A, the traces on the left of each panel show the tonic block after 3-min perfusion of 100 μM lappaconitine alone (top), 100 μM lappaconitine and 8 μM bupivacaine (middle), and 100 μM lappaconitine and 300 μM bupivacaine (bottom). At the conclusion of the 3-min perfusion, the cells were given a 2-Hz train of 100 pulses. The traces on the right of each panel show the first pulse recorded immediately after the 2 Hz train (unlabeled) and after a 5-min wash in control saline. Increases in the concentration of bupivacaine increased the fraction of channels that were protected from irreversible block by lappaconitine. Scale in all panels, 1 nA, 2 ms. B, average data for experiments like those shown in A. In addition to perfusion of 100 μM lappaconitine alone (■), bupivacaine (open symbols) was applied (along with 100 μM lappaconitine) at four concentrations [1 μM, ○ (n = 4); 8 μM, △ (n = 4); 60 μM, ▽ (n = 5); 300 μM, ◇ (n = 5)]. Increases in the concentration of bupivacaine reduced the fraction of channels that were irreversibly blocked by 100 μM lappaconitine during 2 Hz stimulation.

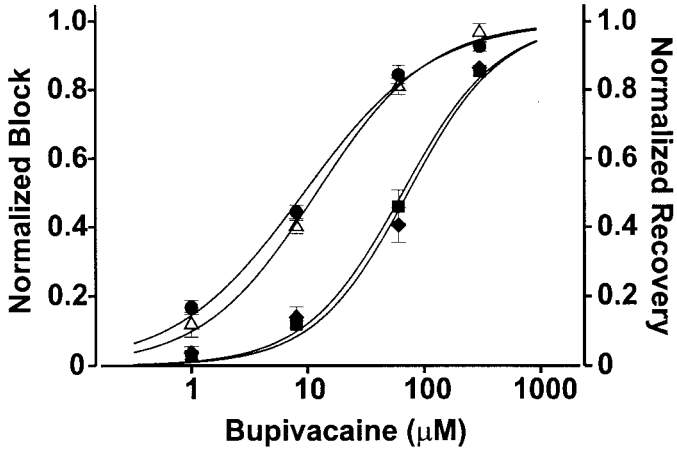

Fig. 6. Bupivacaine dose-response curves. The solid symbols on the plot correspond to the normalized block of the current (left ordinate) at each bupivacaine concentration. Resting block (−140 mV) by bupivacaine (■) and by bupivacaine plus 100 μM lappaconitine (♦) had similar IC_{50} values of 65 ± 6 μM (Hill coefficient, h = 1.1 ± 0.1) and 73 ± 14 μM (h = 1.1 ± 0.2), respectively. The plot also contains the dose-response data for steady-state use-dependent block by bupivacaine alone (●). The available current during the steady-state phase of 2-Hz use-dependent block was normalized to the available current in control saline. The IC_{50} value for this sum of resting and phasic block under these conditions was 9.3 ± 1.2 μM (h = 0.8 ± 0.1). The triangles represent the fraction of channels recovered (right ordinate) at each concentration of bupivacaine following use-dependent block (in the presence of 100 μM lappaconitine). The data at each concentration of bupivacaine are the data points shown at the conclusion of the 5-min wash in Fig. 5B (i.e., 10-min time point). The normalized fraction of available current at each bupivacaine concentration was renormalized according to the fraction of channels available after block by 100 μM lappaconitine alone and subsequent 5 min wash (Fig. 5B, ■, 10-min time point). The IC_{50} value for the fractional recovery of channels was 11.9 ± 0.9 μM (h = 0.9 ± 0.1), and was thus similar to the IC_{50} value for the summed block (resting plus use-dependent block) by bupivacaine alone. For all curves, the errors for the IC_{50} values and Hill coefficients are the errors of the best fit to the data.

12 μM, very similar to the IC_{50} value of the summed block by bupivacaine alone. As one would expect, the accumulation of bupivacaine block (resting block and use-dependent block) at each concentration reduced the fraction of available channels to which lappaconitine could bind. Although tonic block of the channels by bupivacaine undoubtedly contributed to the fraction of recovered channels, the more rapid binding of bupivacaine during the use-dependent protocol made the largest contribution to the increased recovery from block.

Lysine Point Mutations in the Local Anesthetic Receptor Region Render hH1 Channels Resistant to Lappaconitine. To establish the proximity of the binding sites for lappaconitine and bupivacaine, I examined bupivacaine and lappaconitine block using channels containing point mutations within the local anesthetic receptor region. As demonstrated for the μ1 isoform (Wright et al., 1998), hH1 channels containing lysine substitutions at F1760 (μ1-F1579K) or N1765 (μ1-N1584K) expressed well in HEK293t cells (Fig. 7A). Figure 7B shows the normalized conductance-voltage relationships, and Fig. 7C shows the normalized steady-state availability relationships of wild-type hH1 channels and the two lysine mutants. As with the comparable mutations in μ1 channels (Wright et al., 1998), the midpoint voltages of the conductance-voltage and steady-state availability relationships for the mutant channels were significantly different ($p < 0.05$) from those of the wild-type channels. Nevertheless, the channels otherwise appeared to open and inactivate normally. In contrast to the comparable lysine mutation in the μ1 isoform (μ1-N1584K), hH1-N1765K channels did not exhibit an unusually large maintained current at +30 mV.

The sensitivity of F1760K and N1765K channels to racemic bupivacaine was first examined to determine whether the lysine mutations conferred resistance to local anesthetic block. Bupivacaine (30 μM) blocked approximately 45% of hH1 channels during the steady-state phase of 2-Hz use-dependent block (Fig. 8A). In contrast, both F1760K and N1765K channels were extremely resistant to use-dependent block. The lysine point mutations also reduced steady-state bupivacaine block of resting channels and inactivated channels (Fig. 8B). To determine resting channel block, a 10-s conditioning pulse to −160 mV was followed by 100 ms at the holding potential and the available current was measured during a subsequent test pulse to +30 mV. As shown in the traces in Fig. 8B, left, resting F1760K and N1765K channels were about half as sensitive to 30 μM bupivacaine compared with hH1 channels. A similar pulse protocol was used to measure inactivated channel block except that the 10-s conditioning pulse was to −70 mV. The conditioning pulse to −70 allowed bupivacaine binding to inactivated channels to reach steady-state, and the 100-ms interval at the holding potential permitted inactivated but drug-free channels to recover from fast inactivation (Wright et al., 1997). Compared with hH1 channels, inactivated F1760K and N1765K channels were > 20-fold less sensitive to 30 μM bupivacaine (right column in Fig. 8B). These data were consistent with the >20-fold reduction in cocaine block of inactivated μ1-F1579K and N1584K channels (Wright et al., 1998).

In addition to reducing local anesthetic block, lysine substitutions at homologous residues in the μ1 isoform eliminate channel modification by batrachotoxin (Wang and Wang, 1999). To confirm that the lysine substitutions also conferred resistance to lappaconitine block, the channels were exposed

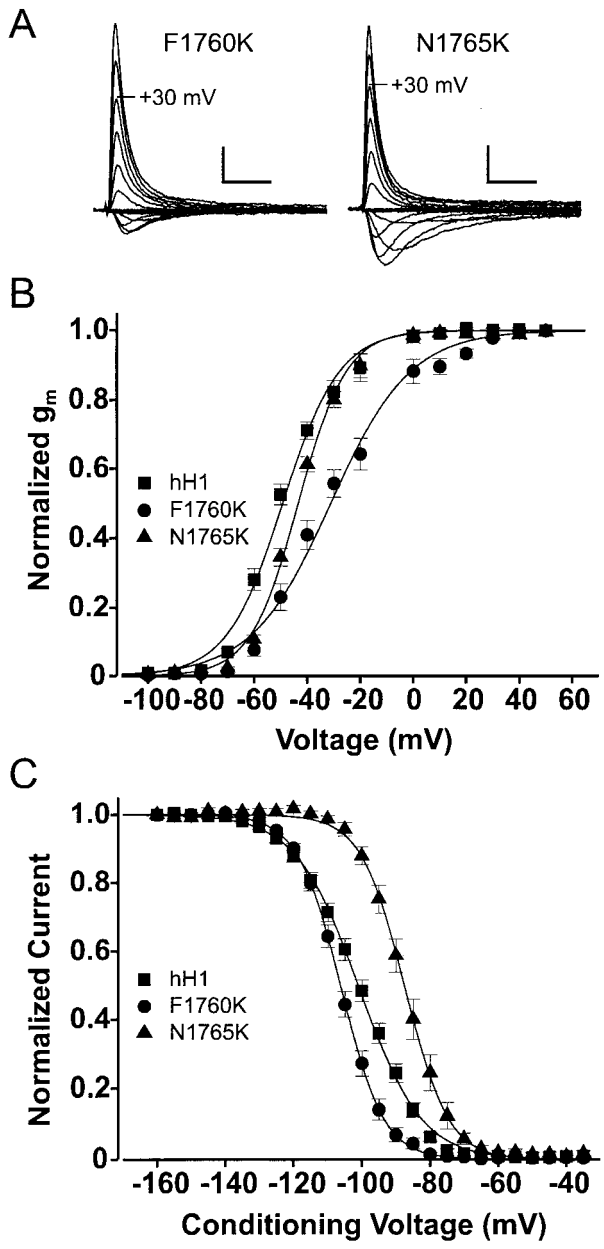

Fig. 7. Normalized conductance-voltage and steady-state inactivation relationships of hH1 channels containing lysine substitutions. A, channels containing lysine substitutions within the local anesthetic receptor at residues F1760 or N1765 express well in HEK cells. The currents were recorded during 10-ms voltage commands ranging in amplitude from −100 mV to +50 mV. Scale, 1 nA, 2 ms. B, normalized membrane conductance plotted versus the amplitude of the 10-ms voltage step. As described in the Fig. 2 legend, the data were fitted with a standard Boltzmann function to obtain the midpoint voltage ($V_{0.5}$) and slope (k) of the data. For hH1 ($n = 7$), the mean $V_{0.5}$ and k values were −49.0 ± 1.4 mV and 10.6 ± 0.8 mV, respectively. For F1760K ($n = 7$), the mean $V_{0.5}$ and k values were −31.2 ± 2.6 mV* and 14.4 ± 0.5 mV*, respectively. For N1765K ($n = 8$), these values were −43.2 ± 0.9 mV* and 9.1 ± 0.8 mV. * indicates $p < 0.05$ compared with hH1. C, normalized steady-state availability function (h_∞) for the channels. Cells were held at −140 mV and were given 100-ms conditioning pulses ranging in amplitude from −160 mV to −35 mV followed by a test pulse to +30 mV. The mean $V_{0.5}$ values (50% availability) and k values for the fitted Boltzmann functions were: −101.2 ± 1.2 mV and 8.9 ± 0.4 mV, respectively for hH1 channels ($n = 10$). The $V_{0.5}$ and k values for F1760K channels ($n = 9$) were −106.2 ± 1.0 mV and 6.1 ± 0.3 mV, respectively, and for N1765K channels ($n = 6$) these values were −87.5 ± 1.5 mV and 6.1 ± 0.2 mV, respectively. The mean $V_{0.5}$ and k values of both mutants were all significantly different from the mean values for hH1 ($p < 0.05$).

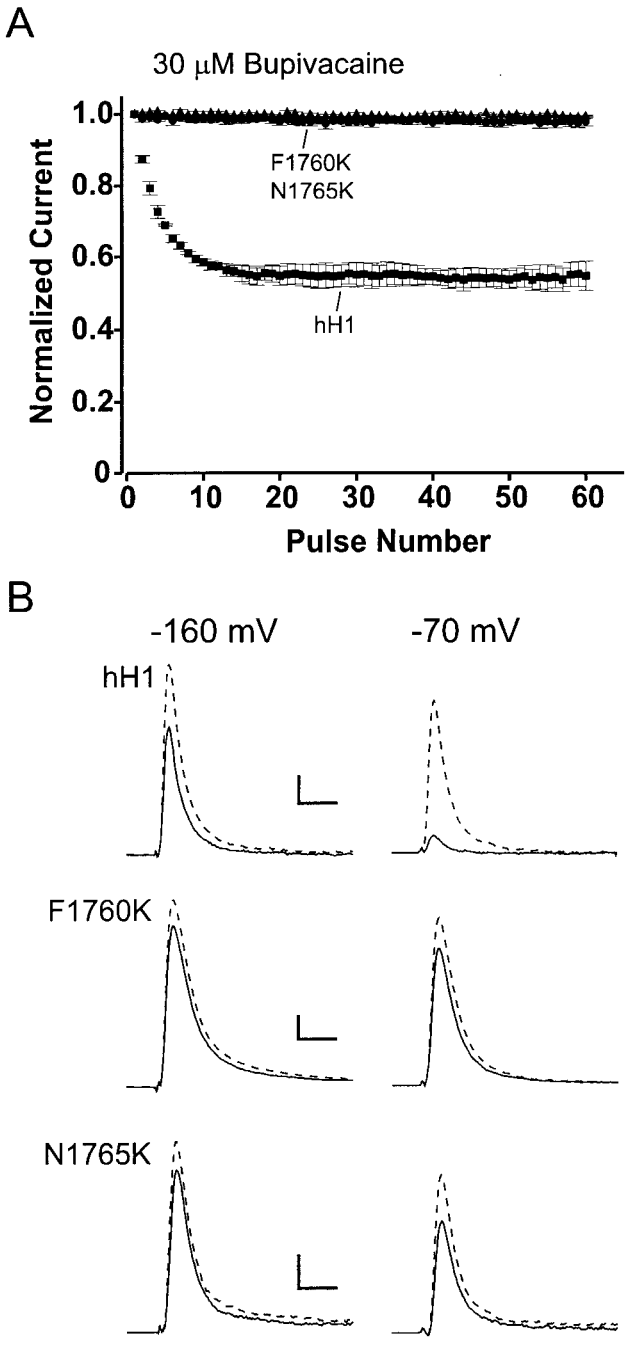

Fig. 8. Resistance of lysine mutants to block by 30 μM bupivacaine. A, the plot shows the use-dependent block of hH1 channels by 30 μM bupivacaine. The fraction of hH1 channels blocked during the steady-state phase of the 60 pulse protocol was 45%. In contrast, 30 μM bupivacaine reduced the fraction of available F1760K and N1765K channels by only 1–2%. B, steady-state block of hH1 and lysine mutants by 30 μM bupivacaine. The pulse protocol consisted of a 10-s conditioning pulse to −160 mV or to −70 mV followed by a 100 ms interval at −140 mV, and a subsequent test pulse to +30 mV. The conditioning pulse to −160 mV was used to estimate resting channel block, whereas the conditioning pulse to −70 mV was used to estimate inactivated channel block. The traces show the available current in control saline (dashed traces) and in 30 μM bupivacaine (solid traces). Bupivacaine blocked 26.6 ± 1.8% of resting hH1 channels (n = 4) and blocked 10.8 ± 3.2% and 13.9 ± 2.5% of resting F1760K (n = 3) and N1765K channels (n = 5), respectively. Bupivacaine blocked 87.5 ± 1.0% of inactivated hH1 channels (n = 4) and blocked 18.5 ± 6.3% and 24.0 ± 4.2% of inactivated F1760K (n = 3) and N1765K channels (n = 5), respectively. Compared with hH1 channels, the block of the mutant channels by 30 μM bupivacaine was significantly less (p < 0.05). Scale, 500 pA, 1 ms.

to 100 μM lappaconitine for 5 min and then given a 2-Hz train of 100 pulses to +30 mV. Figure 9 shows that both F1760K and N1765K channels were highly resistant to use-dependent block by 100 μM lappaconitine. Thus, the substitution of lysine for native residues in the local anesthetic receptor region reduced block by a local anesthetic as well as lappaconitine. The relationship between the local anesthetic receptor region and site 2 neurotoxin binding will be addressed under *Discussion*.

Discussion

Site 2 neurotoxins typically induce negative shifts in sodium channel activation and reduce or eliminate channel inactivation from the open state. This report focused on the interaction between the plant alkaloid lappaconitine and hH1 sodium channels. Despite the structural similarity to aconitine, lappaconitine irreversibly blocked the channels and did not modify channel kinetics. Lappaconitine did not interact with the site 1 receptor as do other naturally occurring channel blockers such as tetrodotoxin. The results in the study suggested that lappaconitine blocked hH1 channels by binding to the site 2 receptor. Like most other site 2 toxins, lappaconitine 1) binds only with open channels, 2) binds irreversibly, and 3) does not bind to channels containing lysine substitutions within the local anesthetic receptor.

Site 1 neurotoxins such as tetrodotoxin or saxitoxin block sodium channels by binding to a receptor on the extracellular region of the domain 1 pore loop between segments 5 and 6 (Tomaselli et al., 1995). As one would expect for toxins that bind on the extracellular surface of the channel, block by site 1 toxins is almost always reversible. In contrast, several minutes of washing in control external solution or repetitive pulses delivered in control solution failed to alleviate lappaconitine block. Thus, lappaconitine is a naturally occurring sodium channel blocker that binds to a receptor that is not on the extracellular surface of the channel.

Lappaconitine has antiepileptiform activity and exhibits a use- and frequency-dependent antagonism of the population spike in hippocampal slice preparations (Ameri et al., 1996a,b; Ameri and Simmet, 1999). In addition, lappaconitine induces bradycardia in heart preparations (Gutser et al., 1998; Heubach and Schule, 1998), suggesting that the drug has class I local anesthetic properties. In retrospect, the idea that lappaconitine has local anesthetic properties seems rea-

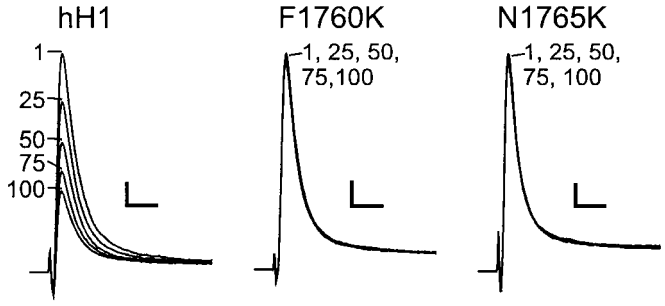

Fig. 9. Resistance of lysine mutants to block by 100 μM lappaconitine. As shown in Fig. 3B (different cell), 100 μM lappaconitine markedly reduced the available hH1 current during a 2-Hz train of 100 pulses. In contrast, the same concentration of lappaconitine had little effect on the available current of F1760K (n = 5) or N1765K (n = 3) channels. For clarity, only the 1st and every 25th pulse of the 100-pulse protocol are shown. Scale, 500 pA, 1 ms.

sonable given that local anesthetics, antiepileptiform drugs, and site 2 neurotoxins have binding domains that seem to overlap (Catterall, 1999). Several pieces of evidence in the present study indicated that block of hH1 channels by lappaconitine differed from that of tertiary amine local anesthetics and more closely resembled the block by quaternary derivatives of lidocaine, such as QX-314 and QX-222. Lappaconitine almost exclusively preferred open channels and had a low affinity for resting or inactivated channels. Consistent with an open channel block, altering the stimulation rate by an order of magnitude did not markedly affect the time course or extent of use-dependent block. Similarly, QX-314 and QX-222 bind preferentially to open channels and have little affect on closed channels. The reversibility of use-dependent block by QX compounds seems to differ depending on the preparation. QX-222 block of cardiac sodium channels in dog Purkinje cells is only partially reversible after several minutes of washing (Hanck et al., 1994). In contrast, frog (Strichartz, 1973) or squid (Yeh and Tanguy, 1985) sodium channels recover in seconds to minutes from QX-222 or QX-314 block, and the recovery from use-dependent block by QX-314 can be accelerated by small repetitive depolarizations (Strichartz, 1973). In contrast, lappaconitine block was essentially irreversible during the time course of the experiments and >100 pulses in control solution did not elicit any use-dependent unblock. These data suggest that bound channels may become locked in a conformation that ultimately prohibits reopening and drug unbinding.

Lappaconitine Binding within the Site 2 Domain. The structures of aconitine and lappaconitine are remarkably similar, which alone suggests that the toxins share a binding domain. Evidence obtained from other preparations supports the notion that the compounds share a common receptor. For example, lappaconitine competitively inhibits aconitine-induced increases in synaptosomal intracellular calcium (Gutser et al., 1998) and aconitine-induced arrhythmia in guinea pig heart (Heubach and Schule, 1998), suggesting that the toxins bind to the same receptor or at least that the binding sites overlap.

Previous studies have demonstrated that local anesthetics noncompetitively inhibit the binding of site 2 neurotoxins (Postma and Catterall, 1984; Wang and Wang, 1999). Likewise, batrachotoxin modification lowers channel affinity for lidocaine (Zamponi et al., 1993) and for (+) optical isomers of bupivacaine and cocaine (Wang and Wang, 1992). Studies that have described the allosteric binding interactions between batrachotoxin and local anesthetics benefited from the fact that site 2 toxins typically activate the channels and remove inactivation, whereas local anesthetics block the channels. In the present report, lappaconitine exhibited use-dependent block of the channels, albeit with much slower kinetics than use-dependent block by bupivacaine. As shown in Fig. 6, the inhibition of lappaconitine binding by bupivacaine was not caused exclusively by tonic bupivacaine block. Rather, the more rapid kinetics of use-dependent bupivacaine block protected blocked channels from lappaconitine access by removing them from the fraction of available channels. Thus, as reflected in Fig. 6, the dose-response data of use-dependent block by bupivacaine alone and the fraction of channels protected from irreversible lappaconitine block were similar. Although the conclusions from these experiments are simplistic, the data indicated that bupivacaine binding inhibits lappaconitine binding and further confirmed that lappaconitine binds only to open channels. Although the precise mechanism for the allosteric interactions between the binding sites for local anesthetics and site 2 toxins has not been deduced, one possible explanation for the interaction between bupivacaine and lappaconitine is that bupivacaine binding induces an allosteric change in the channel that inhibits lappaconitine binding. The inhibition of lappaconitine block by bupivacaine could be explained if, at closed channels, the anesthetic has better access to the local anesthetic receptor than lappaconitine has to the site 2 receptor. More rapid bupivacaine binding during repetitive stimulation could then induce an allosteric change in channel conformation that inhibits toxin binding. Alternatively, bupivacaine and lappaconitine may compete for binding sites that are in close proximity. The fact that the mutant channels were resistant to both bupivacaine and lappaconitine is consistent with the idea that local anesthetics and site 2 neurotoxins bind to nonidentical receptors within an overlapping region (Linford et al., 1998).

Homologous amino acid residues within D4-S6 of rat brain (RBIIA; Ragsdale et al., 1994) and rat skeletal muscle (μ1; Wright et al., 1998) sodium channels are critical determinants for local anesthetic block. In addition, alanine substitution within the local anesthetic receptor at RBIIA-F1764 inhibits batrachotoxin modification of the channels (Linford et al., 1998). Lysine substitution within the local anesthetic receptor region of μ1 channels at residues μ1-F1579 or N1584 inhibits modification of the channels by batrachotoxin (Wang and Wang, 1999), and lysine substitution at μ1-F1579 reduces channel modification by grayanotoxin (Kimura et al., 2000). Consistent with the results obtained using other channel isoforms, hH1 channels containing lysine substitution at F1760 or N1765 were resistant to block by bupivacaine or lappaconitine. With respect to batrachotoxin binding, the more critical of the two residues is probably hH1-N1765, which faces away from the pore region according to the Ragsdale et al. (1994) model of sodium channel D4-S6. Alanine substitution at hH1-N1765 or the homologous site in μ1 channels (N1584) reduces batrachotoxin modification to a much greater extent than does alanine substitution at μ1-F1579 (Wang and Wang, 1999). The importance of sodium channel S6 segments is further supported by more recent studies that implicate a neighboring residue within D4-S6 (μ1-V1583; Vedantham and Cannon, 2000), as well as residues within D3-S6 (Wang et al., 2000) as critical binding regions for batrachotoxin. In the present study, lysine substitution at hH1-F1760 may disrupt lappaconitine binding simply by introducing a positive charge within the vicinity of the lappaconitine binding site. On the other hand, the mutation at N1765 may inhibit lappaconitine block because the residue is part of the site 2 receptor.

Although the data in the present study demonstrate that lappaconitine binds to open channels, the mechanism of block is not entirely clear. Indeed, no study has determined definitively the access route for site 2 neurotoxins. Like other site 2 toxins, the structure of lappaconitine indicates that the drug is lipid soluble and can easily cross the membrane. Once inside the cell, the toxin could access the receptor using more than one pathway. For example, lappaconitine could simply enter the pore from the intracellular side while the channel is open, bind to the receptor, and block the pore. Alternatively,

the toxin could access the receptor through the lipid phase of the channel. The conformational changes associated with channel opening would then permit drug binding with the receptor, and the gating mechanism of channels bound by the toxin could become locked in a configuration that prevents subsequent opening. One other alternative is a combination of the two possibilities above. That is, the drug may enter the pore during channel opening, bind to the receptor, and lock the channel in a nonconducting state. Although I cannot rule out any of these possibilities to explain lappaconitine binding and block, an allosteric modification of the gating mechanism would be consistent with the action of other site 2 neurotoxins.

The most abundant neurotoxin in *Aconitum* plants is usually aconitine, with lappaconitine and several other toxins in the minority. The fact that the same plant may synthesize two compounds that bind to the same receptor, but have opposing and essentially irreversible effects, is difficult to reconcile. The two toxins could target specific isoforms of sodium channel; however, this possibility has not been fully explored. Although several of the side groups on the diterpene skeleton may prove to be important in toxin action, the location of the benzoyl linkage is particularly interesting and may partially explain the contrasting effects of aconitine and lappaconitine at voltage-gated sodium channels.

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