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A Multifunctional Aromatic Residue in the External Pore Vestibule of Na^+ Channels Contributes to the Local Anesthetic Receptor

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ABSTRACT

Voltage-gated Na^+ (Na_v) channels are responsible for initiating action potentials in excitable cells and are the targets of local anesthetics (LA). The LA receptor is localized to the cytoplasmic pore mouth formed by the S6 segments from all four domains (DI–DIV) but several outer pore-lining residues have also been shown to influence LA block (albeit somewhat modestly). Many of the reported amino acid substitutions, however, also disrupt the inactivated conformations that favor LA binding, complicating the interpretation of their specific effects on drug block. In this article, we report that an externally accessible aromatic residue in the Na_v channel pore, DIV-Trp1531, when substituted with cysteine, completely abolished LA block (e.g., 300 μM mexiletine induced a use-dependent block with 65.0 $\pm$ 2.9% remaining current and -11.0 ± 0.6 mV of steady-state inactivation shift of wild-type (WT) channels versus 97.4 $\pm$ 0.7% and -2.4 ± 2.1 mV of W1531C, respectively; $p < 0.05$) without destabilizing fast inactivation (complete inactivation at 20 ms at -20 mV; $V_{1/2} = -70.0 \pm 1.6$ mV versus -48.6 ± 0.5 mV of WT). W1531C also abolished internal QX-222 block (200 μM; 98.4 $\pm$ 3.4% versus 54.0 $\pm$ 3.2% of WT) without altering drug access. It is interesting that W1531Y restored WT blocking behavior, whereas W1531A channels exhibited an intermediate phenotype. Together, our results provide novel insights into the mechanism of drug action, and the structural relationship between the LA receptor and the outer pore vestibule.

Voltage-gated Na^+ (Na_v) channels mediate the rapid transmission of depolarizing electrical impulses in excitable tissues, such as nerve, heart, and skeletal muscle, to enable coordination of processes from muscle contraction to cognition. Genetic defects in Na_v channels lead to various heritable excitability diseases such as muscle paralysis (e.g., hyperkalemic periodic paralysis, myotonia congenita), cardiac arrhythmias (e.g., LQT3 syndrome), and epilepsy, often as a result of altered channel inactivation, which subsequently causes misfiring of action potentials (Hudson et al., 1995; Keating and Sanguinetti, 1996; Hayward et al., 1997; Marban et al., 1998; Balser, 2001). It is not surprising that Na_v channels are important clinically as the primary target of numerous drugs, such as local anesthetic (LA), anticonvulsant, antiarrhythmic, and antiepileptic agents. These Na_v channel drugs exert their effects by preferentially binding to open and inactivated Na_v channels (LA block is enhanced at depolarized potentials or with repetitive pulsing or both, i.e., use dependence), thereby inhibiting Na^+ currents and ultimately reducing cellular excitability (Courtney, 1975; Hille, 1977; Hondeghem and Katzung, 1977; Bean et al., 1983). According to the modulated receptor hypothesis, state-dependent LA binding results from conformational changes of the drug receptor during gating (Hille, 1977): LA affinity is low when channels are in resting closed states but increases when they are open or inactivated (Hille, 1977; Hondeghem and Katzung, 1977). Therefore, amino acid substitutions that alter gating can indirectly modify LA block without affecting the intrinsic drug affinity. In addition, access to the binding site, which determines the rates of drug binding and unbinding from the receptor, is also state-dependent; both hydrophobic and hydrophilic pathways are available depending on the hydrophobicity of drugs (Hille, 1977; Hondeghem and

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ABBREVIATIONS: LA, local anesthetic; WT, wild-type.

Katzung, 1977; Schwartz et al., 1977; Ragsdale et al., 1994; Qu et al., 1995; Ragsdale et al., 1996). Hydrophobic LAs bind and unbind freely to Na_v channels (Hille, 1977), but permanently charged drug derivatives (e.g., QX-222 and GEA-968) can gain access to the receptor site via the hydrophilic pathway, which is available only when channels are open (Strichartz, 1973; Courtney, 1975; Hille, 1977). Amphiphilic polar LAs such as mexiletine and lidocaine can block Na_v channels via both the hydrophobic and hydrophilic pathways. Because both pathways can be modified by mutations, LA block depends on both the intrinsic binding affinity of the LA receptor as well as drug access.

Previous studies have localized the LA receptor of Na_v channels to the cytoplasmic pore mouth (Strichartz, 1973) formed by the S6 segments from all four domains (DI–DIV) (Ragsdale et al., 1994; Qu et al., 1995; Ragsdale et al., 1996; Wang et al., 2000; Yarov-Yarovoy et al., 2002; Kondratiev and Tomaselli, 2003). Single-channel recordings revealed that internally perfused QX-314, a permanently charged lidocaine congener, traverses ~70% of the transmembrane electric field from the inside to reach its binding site (Gingrich et al., 1993; Kimbrough and Gingrich, 2000), approximately equivalent to the ~21% electrical distance for external Cd^{2+} binding to cysteine-substituted P-loop residues (Backx et al., 1992; Chiamvimonvat et al., 1996a; Li et al., 2000). These observations strongly suggest that the external pore vestibule and the cytoplasmic LA receptor are in close proximity. Indeed, several P-loop residues (such as DI-Tyr401, DI-Trp402, DII-Glu755, DIII-Lys1237; rat skeletal muscle $rNa_v1.4$ numbering unless otherwise specified) have been shown to influence drug block (Kambouris et al., 1998; Sunami et al., 1997, 2000). However, the observed changes in drug block with these pore substitutions were relatively modest compared with those reported for the cytoplasmic S6 segments (Ragsdale et al., 1994). Furthermore, mutations could indirectly alter drug block by modifying gating and/or drug access (Tomaselli et al., 1995; Sunami et al., 1997, 2000; Chen et al., 2000; Lee et al., 2001). Therefore, it is often difficult to interpret the specific effect of a mutation on drug block. In this article, we report that an external aromatic pore residue, DIV-Trp1531, when substituted, uniquely abolished or attenuated LA block without destabilizing inactivation or altering drug access. The results are discussed in the context of novel insights into the mechanism of drug action and its relationship with the outer pore vestibule structure.

Materials and Methods

Site-Directed Mutagenesis and Heterologous Expression in *Xenopus laevis* oocytes. $Na_v1.4$ was subcloned into the expression vector pGW1H (British Biotechnologies, Oxford, UK) or 64T* (Krieg and Melton, 1984). Mutations were created using polymerase chain reaction with overlapping mutagenic primers, followed by full sequencing to ensure the presence and absence of desired and strayed mutations, respectively. cRNA was transcribed from SalI-linearized cDNA in 64T* using T7 RNA polymerase (Promega, Madison, WI). $Na_v1.4$ channel constructs were heterologously expressed and studied in *X. laevis* oocytes as described previously (Xue et al., 2002). In brief, stage IV–VI oocytes were surgically removed from female frogs anesthetized by immersion in 0.2% tricaine, followed by digestion with 1.5 mg/ml collagenase (type IA) in OR-2 containing 88 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6, for 30 to 40 min. Wild-type or engineered cDNA (5 ng of cDNA; nuclear injection) or cRNA (50 ng of cRNA; cytoplasmic injection) together with the rat brain β_1 subunit (α/β_1 subunit ratio was 1:7) were injected into healthy, isolated, defolliculated oocytes. Injected oocytes were stored in ND96 (frog Ringer's solution) which contained 96 mM NaCl, 2 mM KCl, 1 mM $BaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6, supplemented with 50 μg/ml gentamicin, 5 mM pyruvate, and 0.5 mM theophylline for 1 to 2 days for expression before experiments.

Electrophysiology and Experimental Protocols. All electrical recordings were performed at room temperature. The bath solution was ND96. Stock solutions of 100 mM mexiletine, lidocaine, QX-222, and QX-314 were made in deionized H_2O. Drug stock was added to the bath solution at the concentrations indicated. A 5-min interval was allowed for equilibration whenever the drug concentration was changed. Intracellular application of QX-222 or QX-314 to oocytes was performed by injecting 100 nl of a 2 mM drug solution 10 min before experiments. Assuming an oocyte volume of 1 μl (Ragsdale et al., 1994; Qu et al., 1995), the final QX concentration applied intracellularly was ~200 μM. Data were collected using custom software. Capacity transients were removed using the p/4 leak subtraction protocol.

Use-dependent block was induced by applying a continuous train of 20 ms step depolarizations to −10 mV from rest at a stimulation frequency of 1, 5, and 10 Hz in the presence of mexiletine or lidocaine. Test potentials for W1531C and W1531A channels were −20 mV because the reversal potentials of these channels were close to 0 mV (Tsushima et al., 1997a). Holding potential was −100 mV. Steady-state, use-dependent block was assessed as the fraction of block of the 15th pulse compared with the first pulse (i.e., $1 - I_{Pulse\ 15}/I_{Pulse\ 1}$, where $I_{Pulse\ 1}$ and $I_{Pulse\ 15}$ represent the peak currents measured during the 1st and 15th pulses, respectively).

Steady-state activation curves were constructed from current-voltage relationships recorded from a holding potential of −120 mV using the equation $m_\infty = g/g_{max}$, where g was obtained from the current-voltage relationship by scaling the peak current (I) by the net driving force using the equation $g = I/(V_t - E_{rev})$. V_t is the test potential and E_{rev} is the reversal potential. Current-voltage data were fit to the equation $I = m_\infty \times g_{max} \times (V - E_{rev})$. Steady-state activation and inactivation curves were obtained by fitting the corresponding data with the Boltzmann function m_∞ or $h_\infty = 1/\{1 + \exp[(V_t - V_{1/2})/k]\}$ where V_t is the test potential, $V_{1/2}$ is the half-point of the relationship, and k ($= RT/zF$, where R is the gas constant, T is the absolute temperature, z is the charge number of the electrode reaction, and F is the Faraday constant) is the slope factor. Recovery from inactivation of $Na_v1.4$ channel constructs was examined using a standard two-pulse protocol (500-ms depolarizing pulse to −20 mV followed by repolarization to −100 mV for a varying period before a second depolarizing "test" pulse to −20 mV). A 500-ms pulse was chosen to ensure drug equilibrium without significantly inducing intermediate inactivation (Balser et al., 1996a; Featherstone et al., 1996). The holding potential was −100 mV. Recovery data were fit with a biexponential function.

Tonic block was determined by measuring Na_v currents in response to 50 ms depolarization from a holding potential of −120 mV at 0.25 Hz stimulation in the presence and absence of drugs, and the data were fit with an isothermal binding equation with the Hill coefficient equals 1. All data are presented as mean ± S.E.M. Statistical significance was determined using the student-t test with $p < 0.05$ representing significance.

Results

Screening of Cysteine Pore Mutants for Lidocaine Sensitivity. DI-Trp402, DII-Trp756, DIII-Trp1239 and DIV-Trp1531, which are at homologous positions in the four domains, form a tryptophan ring in the extracellular pore vestibule of $Na_v1.4$ channels (Fig. 1). Although W402C has been

previously demonstrated to attenuate lidocaine block, presumably by accelerating exit from the intermediate inactivated state that has high affinity for lidocaine (Kambouris et al., 1998), the role of other pore-lining tryptophans (Pérez-García et al., 1996; Tsushima et al., 1997b) has not been tested. Given the potential of the tryptophan ring to interact with the aromatic moiety of LA via π-electrons (as a result of their proximity to the cytoplasmic drug receptor), we first screened for changes in lidocaine block of W756C, W1239C, and W1531C channels to test whether they participate in LA block. Indeed, significant reductions in use dependence were observed with W402C [percentage of current remaining at 15th pulse (i.e., $I_{Pulse\ 15}/I_{Pulse\ 1}$ = 65 ± 1.8% versus 49 ± 2.5% of WT with 300 μM lidocaine at 10 Hz)] and W1239C (65 ± 1.9%) channels; W1531C was unique in that the substitution completely abolished use-dependent lidocaine block (95.5 ± 0.4%). Given the dramatic change in drug block observed with W1531C channels and the role of DIV in formation of the LA receptor (Ragsdale et al., 1994), we therefore focused on investigating the role of this DIV pore tryptophan in drug binding.

Use-Dependent Block of W1531C by Mexiletine. As a first step to explore in detail the effect of W1531C on LA block, we next examined the effects of stimulation frequency and drug concentration on use-dependent block of $Na_v1.4$ channels by mexiletine, a clinically used oral lidocaine analog, with local anesthetic and antiarrhythmic actions similar to lidocaine (Xu et al., 1992). Figure 2 shows the effect of 100 and 300 μM mexiletine on Na^+ currents through WT and W1531C channels during a train of depolarizing pulses. Under our control drug-free conditions, no significant use-dependent reduction was observed in either WT or W1531C channels ($p > 0.05$). Mexiletine (100 μM) did not induce noticeable use-dependent block of WT at 1 Hz ($p > 0.05$) but did cause progressive reduction of Na_v currents after depolarizations subsequent to pulse 1 at the higher stimulation frequencies of 5 and 10 Hz. Application of 300 μM mexiletine further increased use-dependent block of WT $Na_v1.4$ channels at 1, 5, and 10 Hz. In contrast to WT, the substitution W1531C significantly attenuated mexiletine-induced use-dependence at all the concentrations (including 500 μM and 1 mM at 10 Hz) and frequencies tested ($p > 0.05$). Prolonging the depolarizing test pulse from 20 to 100 ms (to increase the population of inactivated channels) enhanced steady-state use-dependent block (assessed as $I_{Pulse\ 15,\ 300\ \mu M\ Mex}/I_{Pulse\ 1,\ 300\ \mu M\ Mex} - I_{Pulse\ 15,\ drug\text{-}free}/I_{Pulse\ 1,\ drug\text{-}free}$) of WT $Na_v1.4$ channels by 300 μM mexiletine at 5 Hz to 32.3 ± 4.9% (n = 4) but caused only a modest 13.3 ± 1.1% (n = 4) block for W1531C channels under the same experimental conditions (Fig. 2B, center).

Effects of Mexiletine on Steady-State Fast-Inactivation of $Na_v1.4$ Channels. Several mechanisms (alterations of drug affinity, drug access, gating, or a combination) may contribute to changes in LA block. Although the inactivation time course (τ_h = 2.5 ± 0.1 ms at −20 mV, n = 12) of W1531C $Na_v1.4$ channels, an index of their rate of entry into fast-inactivation was notably slowed compared with that of WT (τ_h = 1.3 ± 0.1 ms at the same voltage; n = 10), the DIV pore construct completely inactivated within 20 ms indicating no disruption of inactivation (compare Figure 2A). Indeed, Fig. 3 shows that the mid-point ($V_{1/2}$) of steady-state inactivation for W1531C channels (−70.0 ± 1.6 mV, n = 4) was negatively shifted compared with that of WT channels (−48.6 ± 0.5 mV, n = 5; $p < 0.05$) suggesting that fast-inactivated W1531C

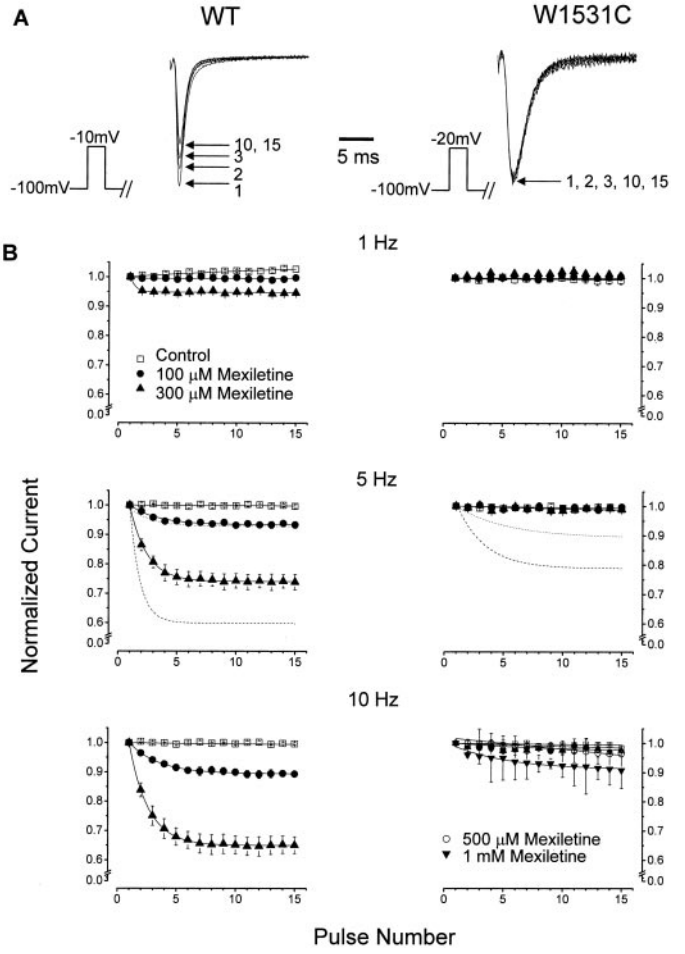

Fig. 2. Use-dependent block by mexiletine of WT $Na_v1.4$ and W1531C channels. A, representative current traces normalized to the 1st pulse such that peaks at the baseline are equal. The numbers 1, 2, 3, 10, and 15 indicate the 1st, 2nd, 3rd, 10th, and 15th pulses, respectively, of the 10-Hz train in the presence of 300 μM mexiletine. B, time course of development of use-dependent block of WT $Na_v1.4$ and W1531C by 100 and 300 μM mexiletine at stimulation frequencies of 1, 5, and 10 Hz. Peak Na^+ currents were normalized to that measured during the first pulse and plotted against the pulse number. Use-dependent block was induced by depolarizing cells to −10 or −20 mV for 20 ms from rest at a holding potential of −100 mV at different frequencies (see *Materials and Methods*). Dotted and dashed lines indicate use dependence recorded with 100-ms pulses in the absence and presence of 300 μM mexiletine at 5 Hz, respectively. Data shown represent mean ± S.E. of three to five individual cells.

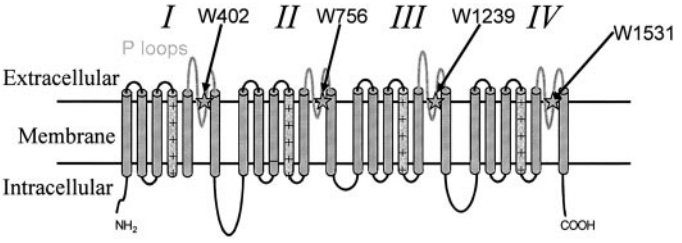

Fig. 1. Putative membrane topology of Na^+ channels with four homologous domains (*I–IV*), each containing six transmembrane segments (S1–6). The pore loops are located between S5 and S6. The tryptophan residue (Trp1531) examined in this study is located in the ascending limb of the pore loop of domain IV.

channels were stabilized rather than destabilized by the substitution. Furthermore, steady-state activation was not altered by the substitution ($V_{1/2}$: -28.8 ± 0.7 mV versus -33.7 ± 2.6 mV of WT; $p > 0.05$). The steady-state gating parameters are summarized in Table 1.

To explore the mechanisms by which W1531C affects LA block, we studied the changes in drug binding energetics by measuring shifts in $V_{1/2}$ of inactivation or channel availability by mexiletine (Fig. 3, Table 1). For WT channels, mexiletine significantly shifted $V_{1/2}$ in the hyperpolarizing direction as anticipated for preferential drug binding to the inactivated state with the addition of LA (Courtney, 1975; Hille, 1978) ($\Delta V_{1/2}$ -4.0 ± 0.4 mV, $n = 3$ and -11.0 ± 0.6 mV, $n = 3$ for 100 and 300 μM, respectively; $p < 0.05$). In contrast, mexiletine did not produce appreciable shift in $V_{1/2}$ of W1531C channels ($\Delta V_{1/2}$ -2.1 ± 0.9 mV, $n = 3$ and -2.4 ± 2.1 mV, $n = 3$ for 100 and 300 μM, respectively; $p > 0.05$) suggesting disruption of drug binding to inactivated channels.

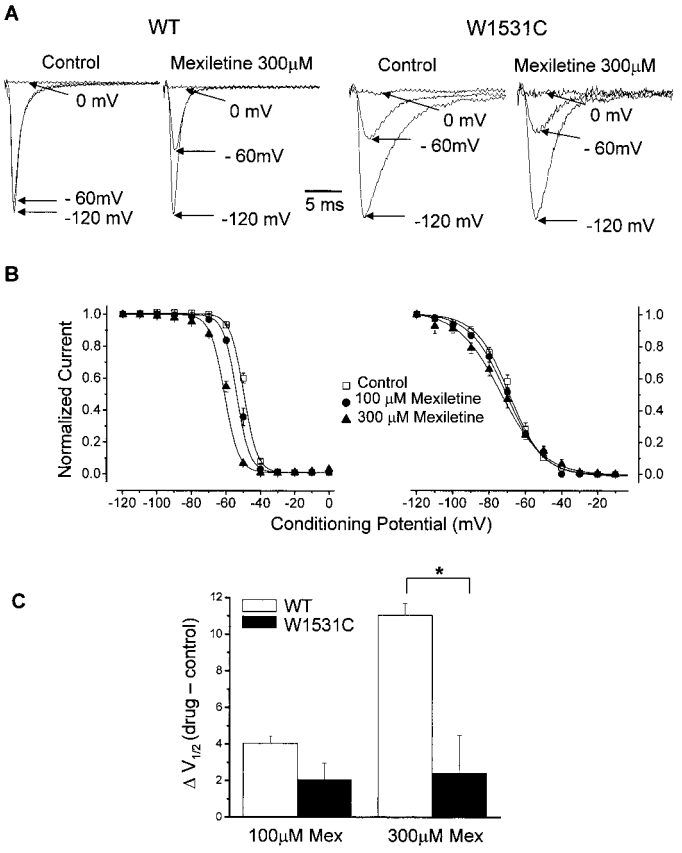

Fig. 3. Mexiletine-dependent shifts in the voltage dependence of channel availability for WT $Na_v1.4$ and W1531C channels. A, representative whole-cell currents recorded at -20 mV after the channels were prepulsed to various voltages for 500 ms (see *Materials and Methods*) in the presence of 300 μM mexiletine. The prepulse potentials -120, -60, and 0 mV are indicated. Channel availability was significantly reduced in WT $Na_v1.4$ channels after the application of 300 μM mexiletine, whereas W1531C channels showed no appreciable change in channel availability in response to mexiletine. B, channel availability curves of WT $Na_v1.4$ and W1531C in the absence and presence of 100 and 300 μM mexiletine. Data points were fitted with the modified Boltzmann function (see *Materials and Methods*). C, bar graph showing the shifts in half-maximal inactivation ($\Delta V_{1/2}$) by 100 and 300 μM mexiletine for WT and W1531C channels. $\Delta V_{1/2}$ was determined from $V_{1/2,\ \text{mexiletine}} - V_{1/2,\ \text{control}}$ using $V_{1/2}$ values obtained from fits of the individual data as described in B. Each data point represents the mean ± S.E.M. of three to five experiments. *, statistical significance ($p < 0.05$) compared with WT $Na_v1.4$ channels.

TABLE 1
Steady-state activation (m^∞) and inactivation (h^∞) and the effects of mexiletine on the recovery from inactivation of WT $Na_v1.4$ and W1531C channels
Recovery data were fit with a biexponential function (see *Materials and Methods*). Data represent the mean ± S.E.M. of three to five oocytes.

Channel	$V_{1/2}^{m^\infty}$	$V_{1/2}^{h^\infty}$	Control			100 μM Mexiletine			300 μM Mexiletine		
			τ_{fast}	τ_{slow}	A_{slow}	τ_{fast}	τ_{slow}	A_{slow}	τ_{fast}	τ_{slow}	A_{slow}
$Na_v1.4$	−33.7 ± 2.6	−48.6 ± 0.5	1.1 ± 0.0	43.1 ± 4.7	5.6 ± 0.7	2.0 ± 0.3*	447.6 ± 72.0*	35.2 ± 6.0*	4.2 ± 0.2*	724.0 ± 51.0*	78.0 ± 5.4*
W1531C	−28.8 ± 0.7	−70.0 ± 1.6†	2.3 ± 0.6	105.2 ± 38.3†	31.2 ± 8.6†	5.7 ± 1.6	151.5 ± 56.8†	30.3 ± 5.1†	12.7 ± 0.3*†	156.1 ± 24.8†	40.0 ± 8.5†

* Statistical significance ($p < 0.05$) with respect to control values of the corresponding channels.
† Statistical significance ($p < 0.05$) from WT values.

Effects of Mexiletine on Recovery from Fast-Inactivation of WT and W1531C channels. Next, we examined the effects of Trp1531 on the kinetics of LA block by examining recovery from inactivation in the presence and absence of mexiletine. Figure 4A shows that WT $Na_v1.4$ channels recovered from inactivation with two time constants under control conditions. The fast component ($\tau_{fast,\ con}$) accounted for the majority (A_{fast}>95%) of the total recovery; the comparatively minor slow component ($\tau_{slow,\ con}$) probably reflects channels recovering from the intermediate slow-inactivated states in response to 500-ms depolarizations (Featherstone et al., 1996). In the presence of 100 or 300 μM mexiletine, the proportion of channels recovering quickly from fast-inactivation (i.e., A_{fast}) was substantially reduced. However, $\tau_{fast,\ mex}$ was very similar in magnitude to $\tau_{fast,\ con}$, consistent with the idea that this component represents the fast recovery of drug-free channels; in contrast, $\tau_{slow,\ mex}$ reflects the recovery rate of mexiletine-bound channels (Bean et al., 1983). Therefore, $A_{fast,\ mex}$ and $A_{slow,\ mex}$ can provide a measure of the proportion of drug-free and -bound channels, respectively. Figure 4B shows the effect of mexiletine on the recovery from inactivation for W1531C channels. Under control conditions, like WT, the recovery of W1531C channels from inactivation displayed two distinct phases, except that the proportion of W1531C channels recovering slowly made up a larger component of the total recovery (A_{slow} = 31.2 ± 8.6%; $p < 0.05$). In accordance with disrupted drug binding, application of 100 and 300 μM mexiletine altered neither the time constants nor their relative amplitudes ($p > 0.05$). All fitted parameters for recovery from inaction of WT and W1531C channels with and without mexiletine are summarized in Table 1.

W1531C Abolished Mexiletine Binding to Intermediate Slow-Inactivated Channels. Because DI-W402C attenuates lidocaine block, presumably by accelerating exit from the intermediate inactivated state, which has high LA affinity (Kambouris et al., 1998), we tested whether the same mechanism also applies to W1531C channels by examining the effects of mexiletine and the substitution W1531C on entry into (intermediate) slow-inactivation of $Na_v1.4$ channels (Fig. 5). The protocol employed is given in the inset. To examine the time-dependent development of intermediate slow-inactivation, $Na_v1.4$ channels were prepulsed from a holding potential of −120 to −20 mV for a variable period of time from 2 ms to 10 s to induce inactivation. This depolarizing prepulse (P1) was followed by a short, 20-ms hyperpolarization to −120 mV to allow recovery from fast- but not slow-inactivation. A test depolarizing pulse (P2) was then applied to assess any accumulation of intermediate slow-inactivation as current reduction relative to that recorded during the prepulse. Figure 5A shows that 300 μM mexiletine substantially accelerated the entry of WT channels into the intermediate slow-inactivated state(s), consistent with the notion that LAs are allosteric effectors of inactivation (Balser et al., 1996b). It is interesting that entry of W1531C channels into intermediate slow-inactivation was enhanced (Fig. 5B) despite their rate of entry into the fast-inactivated state(s) was slowed by the substitution (compare Figure 2A). Unlike WT channels, however, mexiletine failed to facilitate the entry of W1531C channels into intermediate slow-inactivation. Taken together, these results suggest that mexiletine also did not bind intermediate slow-inactivated W1531C channels. Therefore, although Trp402 and Trp1531 are at the "equivalent" positions in the DI and DIV P-loops, respectively, they exert functionally distinctive effects on LA block.

Trp1531 Substitutions Altered Neither External Nor Cytoplasmic LA Access. To investigate whether drug access to the LA receptor was modified by the substitution W1531C, we employed the permanently and positively charged membrane-impermeant quaternary lidocaine derivative QX-222. QX-222 binds to the LA receptor only via the hydrophilic pathway, making it a useful probe for assessing access to the drug binding site (Frazier et al., 1970; Strichartz, 1973; Alpert et al., 1989). In addition, we also constructed W1531A and W1531Y channels to probe the role of the chemical structure of the side chain at position 1531 in LA block of $Na_v1.4$ channels. In the absence of QX-222, neither WT, W1531C, W1531A, nor W1531Y channels exhibited use-dependence when a train of 100-ms depolarizing pulses was applied at 1 Hz ($p > 0.05$; data not shown). Figure 6, A and B, shows that application of ~200 μM intracellular QX-222 induced significant use-dependent reduction of WT Na_v current (46.0 ± 3.2%, n = 4 at the 60th pulse). In contrast, no detectable block was observed when the same concentration of QX-222 was internally applied to W1531C channels ($p > 0.05$). It is interesting that, similar to WT, W1531Y channels were potently blocked by internal QX-222. W1531A channels had an intermediate phenotype. Therefore, use-dependent QX-222 block follows the sequence WT~W1531Y ≫ W1531A > W1531C suggesting that both

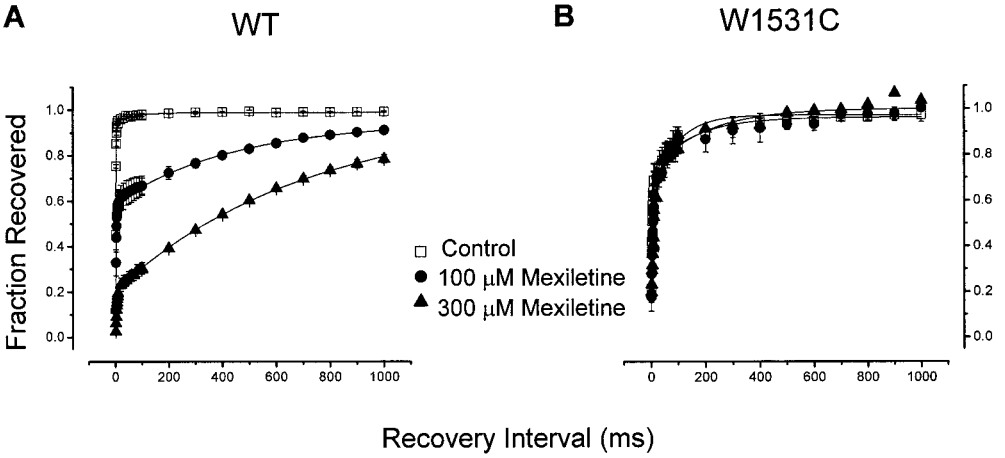

Fig. 4. Effects of mexiletine on the recovery from inactivation of WT $Na_v1.4$ and W1531C channels. Recovery from inactivation in the absence and presence of 100 and 300 μM mexiletine. A typical two-pulse protocol was used and the data points were fit with a bi-exponential function (see *Materials and Methods*). Recovery of WT channels was slowed by mexiletine application and displayed two distinct time constants (see text). Mexiletine did not significantly affect recovery of W1531C channels. Data shown represent mean ± S.E. of three to five individual cells.

aromaticity and hydrophobicity of the native residue at position 1531 are important for drug block.

Although the results presented to date are entirely consistent with a disruption or destabilization of the drug-channel complex, it is still possible that the observed reduction in drug block arise from alterations in drug access to the LA receptor site (e.g., creation of an external drug access pathway for bound drug to escape) (Ragsdale et al., 1994; Qu et al., 1995). To differentiate between these possibilities, we performed the following experiments. Consistent with previous studies, extracellular application of 500 μM QX-222 to WT $Na_v1.4$ channels resulted in no significant Na^+ current blockade 5 min after drug application ($I_{QX}/I_{Control}$ = 100.0 ± 3.6%, n = 4, Fig. 6C), despite potent block by internal QX-222. Likewise, 500 μM external QX-222 also did not significantly block W1531C, W1531A, and W1531Y channels, which were insensitive, intermediate, and highly sensitive to internal QX-222, respectively, over the same period of time (Fig.

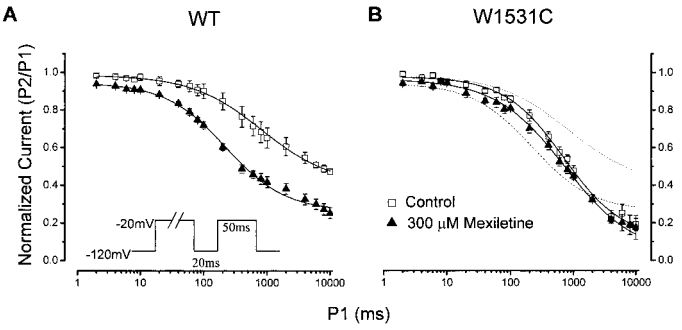

Fig. 5. Time-dependent development of slow inactivation for WT $Na_v1.4$ and W1531C channels. Inset shows the electrophysiological protocol. Normalized current is plotted as a function of the prepulse duration (see text for details). A, for WT $Na_v1.4$ channels, 300 μM mexiletine accelerated their entry into the intermediate slow-inactivated state(s). B, in contrast, mexiletine failed to facilitate the entry of W1531C channels into intermediate slow-inactivation.

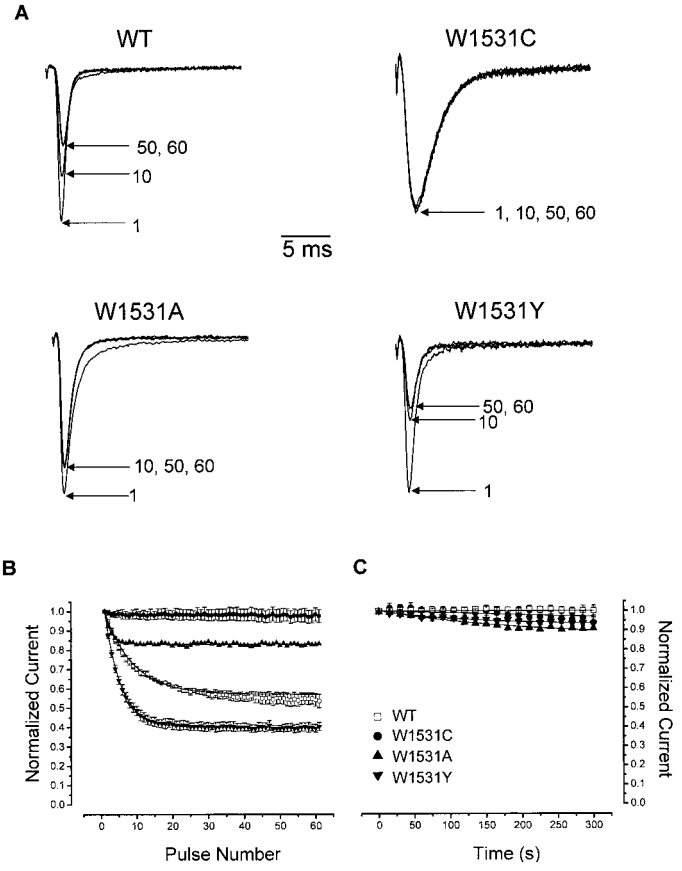

Fig. 6. Effects of substitutions at residue Trp1531 on block by intracellular and extracellular QX-222. A, representative current traces normalized to the 1st pulse such that peaks at the baseline are equal. The numbers 1, 10, 50, and 60 indicate the 1st, 10th, 50th, and 60th pulses, respectively, when a train of 100-ms depolarizing pulses was applied at 1 Hz in the presence of intracellular 200 μM QX-222. B, use-dependent block of WT $Na_v1.4$ and Trp1531 mutant channels by intracellular QX-222. Steady-state block of W1531C and W1531A channels by internal QX-222 was abolished and attenuated, respectively compared with WT. C, normalized Na^+ current plotted against exposure time. Exposure of WT, W1531C, W1531A, and W1531Y channels to 500 μM external QX-222 by superfusion to oocytes expressing the corresponding channels resulted in no detectable reduction in current in all channels.

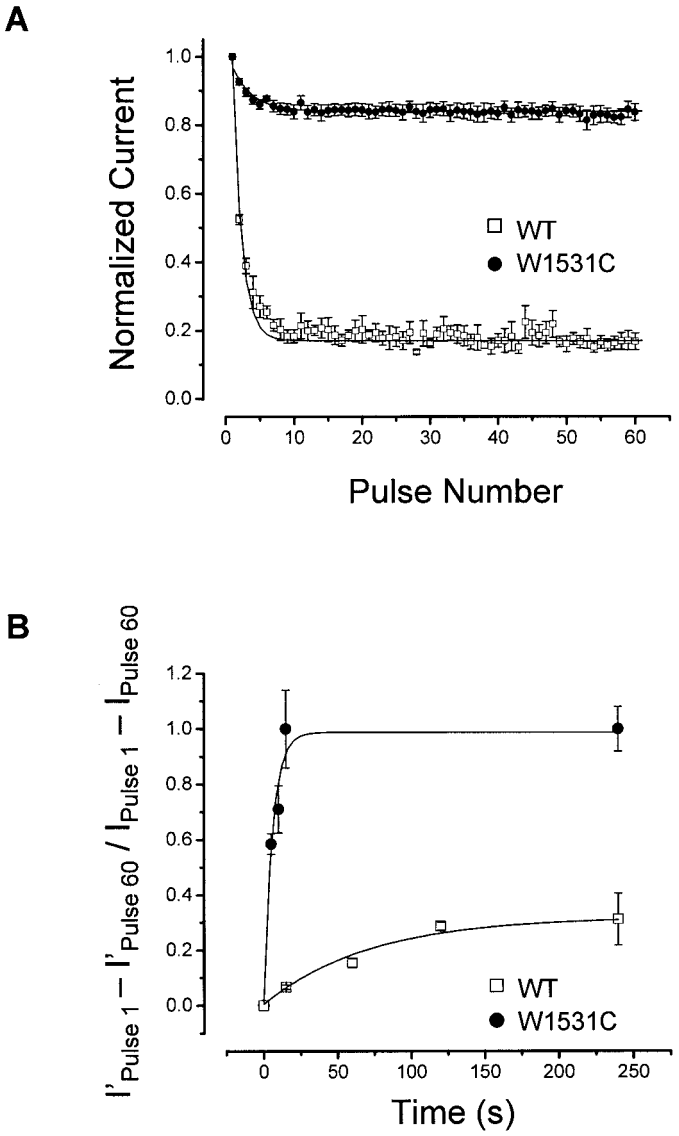

Fig. 7. A, use-dependent block of WT $Na_v1.4$ and W1531C channels by internally applied QX-314. Use-dependent block of W1531C by internal QX-314 was greatly attenuated compared with that of WT. B, the time course of recovery from block was obtained by plotting the ratio of differences between the 1st and 60th pulses before and after recovery (i.e., $(I'_{Pulse1} - I'_{Pulse\ 60})/(I_{Pulse\ 1} - I_{Pulse\ 60})$ where I − represents the current elicited after a particular interval of hyperpolarization) against the duration of hyperpolarization at −140 mV.

6C), suggesting that these substitutions did not create a low energy pathway between the LA receptor and the extracellular solution.

Unlike W1531A channels, the complete absence of external and internal QX-222 block of W1531C channels did not enable us to draw definitive conclusions about possible alterations of drug access (or the lack thereof). Therefore, we employed the more potent derivative QX-314. As anticipated, ~500 μM intracellular QX-314 blocked 83.1 ± 2.5% (n = 6) of WT currents but induced only a modest yet significant 16.2 ± 2.5% (n = 6) use-dependent reduction of peak I_{Na} at steady-state ($I_{Pulse\ 60}$) in W1531C channels (Fig. 7A). To assess the recovery of WT and W1531C channels from internal QX-314 block, a variable interval of hyperpolarization to −140 mV was given to open the intracellular gate for the trapped drug molecule to unbind (Strichartz, 1973; Yeh and Tanguy, 1985; Starmer et al., 1986). Consistent with previous reports, recovery from internal QX-314 block of WT $Na_v1.4$ channels was extremely slow (Strichartz, 1973; Yeh and Tanguy, 1985; Sunami et al., 1997) with 31.2 ± 9.4% (n = 3) of the blocked current recovered over a 240-s hyperpolarization period (Fig. 7B). In contrast, 58.4 ± 3.8% (n = 3) of W1531C recovered within 5 s. Complete recovery was achieved within 15 s with a recovery time constant of 5.9 ± 1.1 s (versus 81.0 ± 25.5 s for WT). It is noteworthy that the same concentration of QX-314, when applied externally, blocked neither WT nor W1531C channels (data not shown), as was the case for QX-222.

To determine whether drug access to the LA receptor from the cytoplasmic side was altered in W1531C channels, we also examined the ability of QX-222 to bind to inactivated channels (Fig. 8). The protocol used is shown in the inset. $Na_v1.4$ channels were prepulsed from a holding potential of −120 to −55 mV for a variable period of time from 1 ms to 10 s to induce inactivation without channel opening (Lawrence et al., 1991). At −55 mV, more than 80% of the channels had entered the fast-inactivated state at steady state (compare Figure 3B). This prepulse was followed by a hyperpolarizing pulse of 300 ms at −120 mV to allow recovery from fast-inactivation. The small reduction of current after depolarizing prepulses longer than 100 ms under control QX-222-free conditions probably reflects accumulation of intermediate inactivation (Bennett et al., 1995; Featherstone et al., 1996). After collecting control data, the same oocytes were then injected with 100 nl of 2 mM QX-222 for 10 min (final concentration, ~ 200 μM), and the protocol was repeated with a repetition interval of 25 s (to ensure complete recovery from use-dependent block). If internal QX-222 has access to the LA receptor when channels were inactivated, accumulated block would be expected. However, the onset of current reduction after QX-222 injection was not different from the control. Identical results were also obtained with Qx-314 (data not shown). Similar studies in WT $Na_v1.4$ channels were not possible because of the extremely slow recovery of this channel from internal QX-222 or QX-314 block (compare Figure 7B) (Strichartz, 1973; Yeh and Tanguy, 1985; Sunami et al., 1997).

Tonic Block Was Enhanced by W1531C. Our results support the hypothesis that the LA receptor of W1531C channels is inaccessible to intracellular QX-222 when channels are inactivated or when QX-222 was applied externally. Although hydrophobic and amphiphilic LAs preferentially bind to inactivated Na_v channels, they also bind to the resting state, thereby producing tonic block (Hille, 1977; Hondeghem and Katzung, 1977). Assessment of tonic block was accomplished by comparing the peak Na^+ current amplitude (recorded after step depolarizations from a holding potential of −120 mV at a low stimulation rate of 0.25 Hz) before and after application of various concentrations of mexiletine. Figure 9A shows the effects of 1 mM mexiletine on whole-cell currents of $Na_v1.4$ and W1531C channels. The fraction of current remaining after mexiletine application was plotted against drug concentration to obtain the dose-response curves of WT and W1531C channels. Interestingly, W1531C

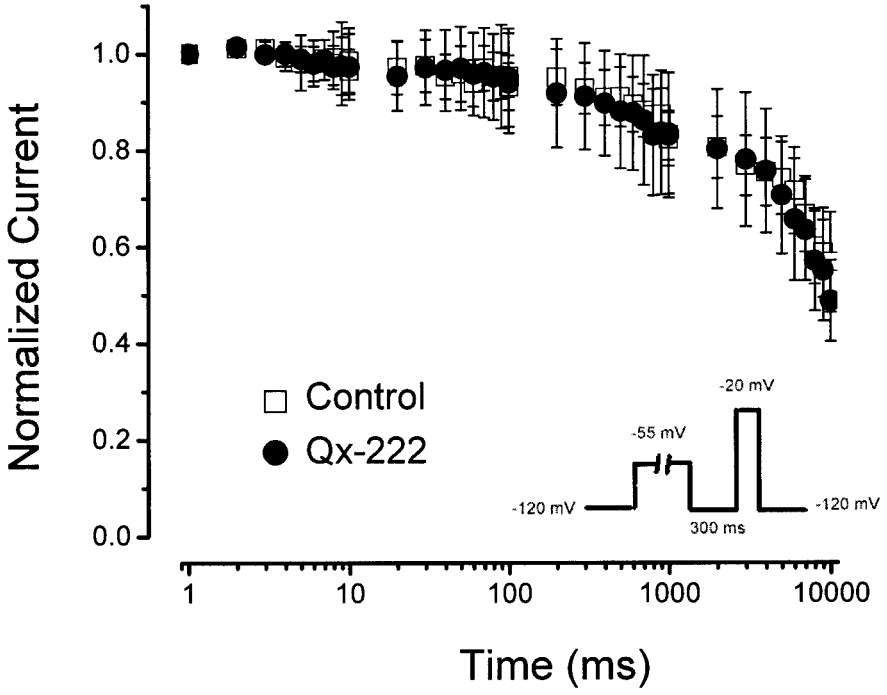

Fig. 8. QX-222 does not bind to the inactivated states of W1531C channels. Inset shows the electrophysiological protocol used in these experiments. Channels were prepulsed to −55 mV for a variable period of time from −120 mV to induce inactivation without opening. A recovery interval of 300 ms was given to allow recovery from fast-inactivation. Under control conditions (□); the reduction of current after 100 ms was caused by accumulation of slow inactivation. Oocytes were then injected with 100 nl of 2 mM QX-222 as described previously (see *Materials and Methods*). The onset of current reduction with intracellular QX-222 (●) was not different from control, suggesting that QX-222 did not bind to the inactivated states of these channels.

channels were more sensitive to tonic block by mexiletine than WT. Assuming a 1:1 stoichiometry (Bean et al., 1983; Nuss et al., 1995; Balser et al., 1996b), the half-blocking concentrations (IC_{50}) were 2974.3 ± 1.3 and 1286.0 ± 1.4 μM for WT and W1531C channels, respectively (Fig. 9B).

Discussion

Trp1531 and LA Block. LAs are commonly used to treat a variety of diseases (including LQT3) that involve abnormal Na_v channel function. The major finding of this study was that the replacement of the aromatic residue Trp1531, located in the ascending portion of the P-loop in DIV, with cysteine or alanine abolished or markedly attenuated LA-induced use dependence, shift in steady-state fast-inactivation, and delay in the recovery rate from inactivation. Because gating interacts profoundly with LA binding, mutagenesis experiments of Na_v channels to study drug block are often difficult to interpret. For instance, gating was shown to be altered by numerous amino acid substitutions in the putative receptor site S6 and the P region, independent of their superimposed drug effects (Kondratiev and Tomaselli, 2003). In fact, LA block phenotype can be altered by mutations in channel regions that are distant from the putative receptor site (Fan et al., 1996; Li et al., 2002). Although several P-loop substitutions from other domains have also been shown to influence LA block (Sunami et al., 1997; Kambouris et al., 1998), Trp1531, however, is unique in that its substitution by cysteine completely *abolished* LA block without destabilizing inactivation and altering drug access. Indeed, the slower recovery from inactivation and the hyperpolarizing shifts of steady-state fast-inactivation of W1531C and W1531A channels suggest that these substitutions stabilize inactivation. Therefore, these changes in the gating properties of Trp1531 channels should enhance LA block, as predicted from a simple gating-drug block coupling effect [as has been described for the enhanced lidocaine block of the paramyotonia congenital mutation, R1448C, in skeletal muscle of Na_v channels (Fan et al., 1996)]. However, this was obviously not the case for Trp1531 mutations. Modification of drug access can affect use-dependent block and recovery from inactivation by changing the rates for drug binding and unbinding without shifting steady-state inactivation (Bean et al., 1983). However, QX-222 access from the extracellular or cytoplasmic face was not altered by Trp1531 substitutions. Therefore, it is unlikely that the dramatic changes in LA block observed were secondary to changes in gating and drug access.

Molecular Picture of LA Block. Our data raise the intriguing possibility that Trp1531 constitutes part of the dynamic drug receptor. This hypothesis is appealing for several reasons. First, reduced drug block could not be secondary to alterations in gating because none of the Trp1531 substitutions studied disrupts inactivation. Second, Trp1531 is an aromatic residue capable of forming π-electron interactions, which are known to be crucial for high-affinity LA block of Na channels (Zamponi and French, 1993; Ragsdale et al., 1994; Sheldon et al., 1995). Furthermore, Trp1531 is a determinant of ionic selectivity; it has long been thought that LAs bind deep in the pore from the cytoplasmic side up to the selectivity region (Hille, 2001; Sunami et al., 1997; Tsushima et al., 1997a). Although Trp1531 channels cannot discriminate among monovalent cations (Chiamvimonvat et al., 1996b; Tsushima et al., 1997a), they are impermeable to organic cations such as tetramethylammonium (R. A. Li and P. H. Backx, unpublished data) whose chemical structure mimics that of lidocaine, consistent with the finding that QX compounds do not block from the extracellular side. Finally, the external pore vestibule is adjacent to the cytoplasmic LA receptor pocket (e.g., benzocaine action remains potent even when the drug is covalently anchored to DI-Y401C) (Li et al., 1999): the electrical distance (δ) for LA block is 0.7 to 0.8 from the inside (Gingrich et al., 1993), whereas the externally accessible Trp1531 has δ ~0.25 from the outside for Cd^{2+} block (Chiamvimonvat et al., 1996a). At first glance, the external accessibility of Trp1531 does not seem to reconcile with the cytoplasmic location of the LA receptor (Ragsdale et al., 1994, 1996). Perhaps Trp1531 forms the "roof" of the cytoplasmic pore vestibule that contains the drug receptor (Fig. 10A). On the other hand, Trp1531 may translocate from the extracellular to the cytoplasmic face (or "flip" at the interface) during inactivation and/or upon drug binding (Fig. 10, B-C), which subsequently leads to intimate hydrophobic or π-electron interactions of the aromatic drug portion with Trp1531 and other residues comprising the LA receptor (such as those in DIV S6). Previously, two distinct modes of fast and discrete-slow single-channel block of skeletal muscle and cardiac Na_v channels by lidocaine and QX-314 have been observed (Gingrich et al., 1993; Zamponi and French, 1993). Based on these results, it has been suggested that low-affinity rapid QX-314 block serves as an intermediate to catalyze

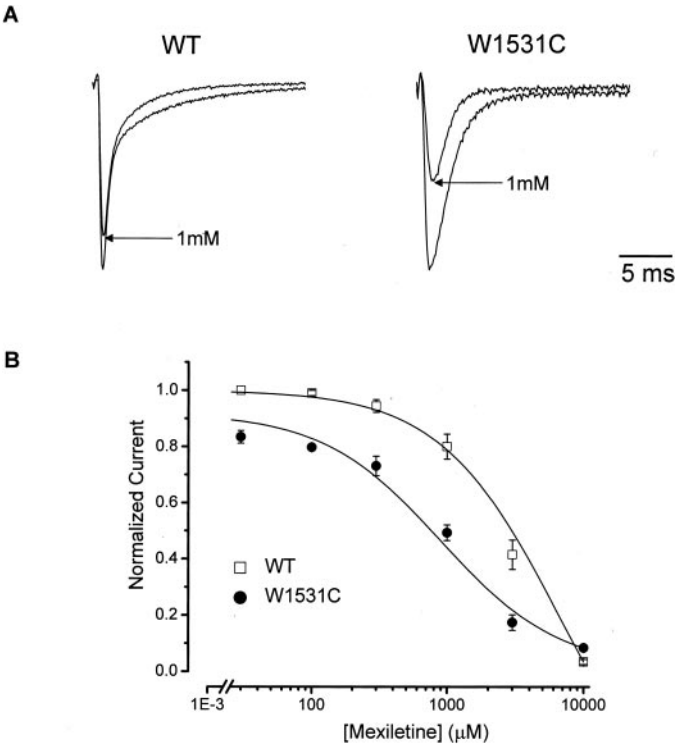

Fig. 9. Tonic block by mexiletine of WT $Na_v1.4$ and W1531C channel. A, representative Na^+ currents through WT $Na_v1.4$ and Trp1531 channels in the absence and presence of 1 mM mexiletine. The currents were elicited by depolarizing cells to −10 mV for $Na_v1.4$ and −20 mV for W1531C for 50 ms from −120 mV at 0.25 Hz. B, dose-response curves showing tonic block of WT and W1531C channels by mexiletine. That of W1531C channels is shifted leftward.

the formation of a high-affinity discrete block complex (Gingrich et al., 1993). According to our model, Trp1531 is critical for the transition from low- to high-affinity conformations, consistent with the notion that *DIV* P-loop is most flexible relative to P-loops from other domains (Tsushima et al., 1997b). Furthermore, our proposed drug binding mechanism is also in accordance with the model that LAs act as allosteric effectors of inactivation (Balser et al., 1996b). Indeed, dynamic rearrangements of the Na channel pore, similar to those reported for voltage-gated K^+ (K_v) channels, have been demonstrated to link to slow inactivation and use dependence by molecular motions of the outer charge ring (Glu403, Glu758, Asp1241, and Asp1532) (Xiong et al., 2003) as well as state-dependent accessibility of F1236C in the DIII P-loop to externally applied methanethiosulfonate ethylammonium (Ong et al., 2000). When DIV Trp1531 is substituted, high-affinity LA binding is attenuated or abolished (Fig. 10C). In fact, ionic selectivity for monovalent cations is also altered (Tsushima et al., 1997a), presumably because of disruptions of the selectivity filter caused by these mutations. However, drug binding and selectivity can be retained with the conservative substitution W1531Y.

If drug molecules bind to the inactivated state, the slowed inactivation time course of W1531C channels (compare Fig. 2A) may contribute to the lack of use-dependence in that channels will be considerably less inactivated during each depolarizing pulse of the train. Furthermore, one might also argue that nonaromatic substitutions of Trp1531 intrinsically mimic LA action (or normal drug-bound channels) by negatively shifting the steady-state fast-inactivation (see Ta-

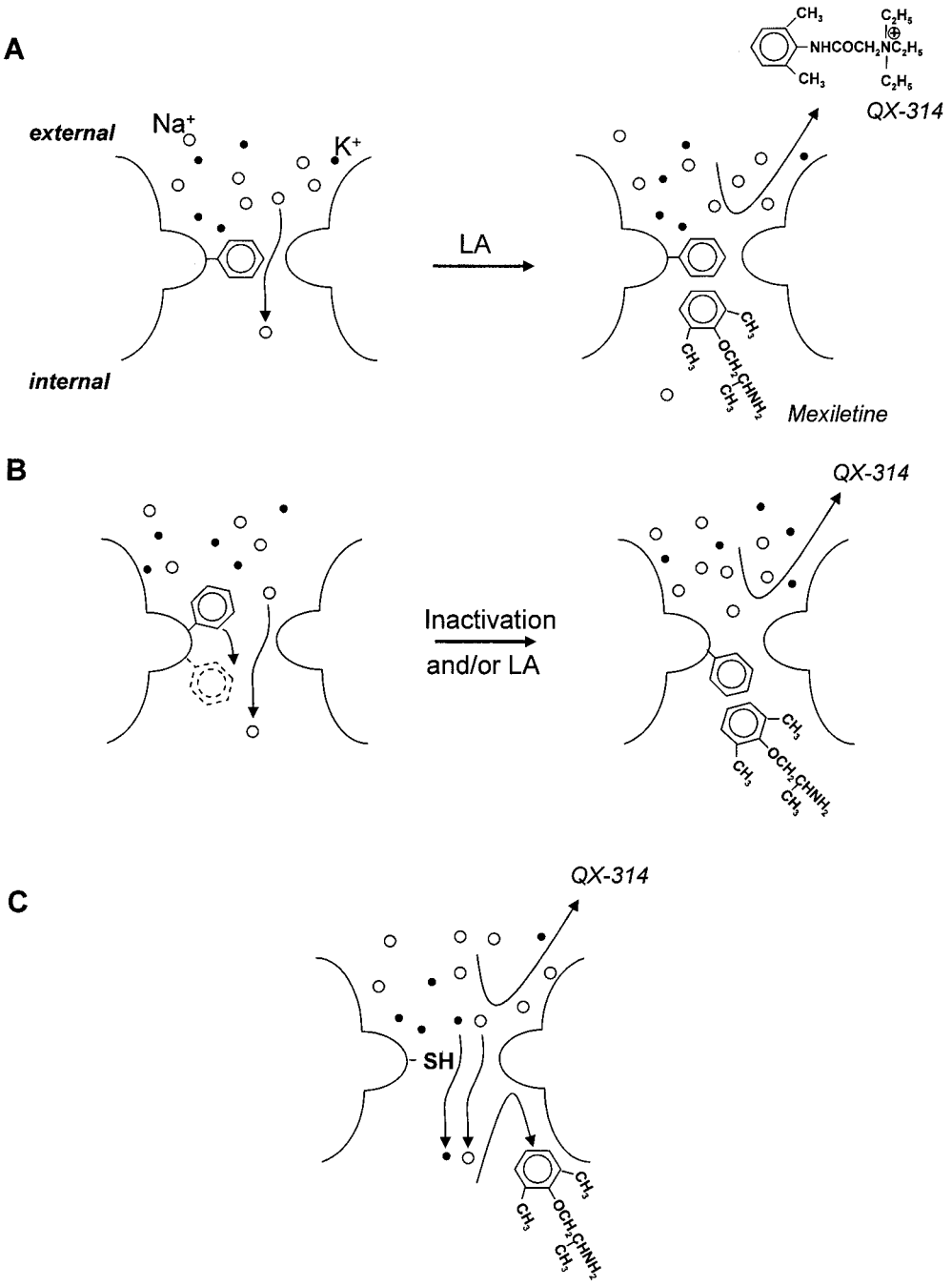

Fig. 10. Schematic diagram illustrating the interactions between mexiletine and the local anesthetic receptor in the $Na_v1.4$ channel pore. A, Trp1531 from the DIV P-loop is located at the interface between the extracellular and cytoplasmic sides by forming the "roof" of the inner pore vestibule that also contains the drug receptor. QX314 does not block from the outside. B, Trp1531 either "flips" at the interface or translocates from the extracellular to the cytoplasmic face during inactivation and/or upon drug binding. C, high-affinity LA binding is abolished when the native tryptophan is substituted by a cysteine (or an alanine). Ionic selectivity for monovalent cations is also lost (Tsushima et al., 1997a), presumably as a result of the disrupted selectivity filter.

ble 1) and decelerating the rate of recovery from inactivation, thereby saturating any possible drug effects. However, relative to WT channels, mexiletine-induced use-dependence of W1531C channels only very modestly increased even with 5-fold prolongation of depolarizing pulse (from 20 ms to 100 ms), suggesting that drug binding to fast-inactivated W1531C channels was indeed significantly attenuated. In addition, our preliminary experiments further indicate that substituting the equivalent tryptophan in the human cardiac $Na_v1.5$ channels, Trp1712, with cysteine altered neither the inactivation time course nor steady-state inactivation yet also rendered the cardiac channels insensitive to drug block (F. Tsang and R. A. Li, unpublished data). Although the differences between $Na_v1.4$-W1531C and $Na_v1.5$-W1712C channels can be attributed to the fundamental differences in gating properties between the two isoforms, our observations collectively support the notion that the changes in drug effects observed were not secondary to changes in gating. Thus, it is possible that the DIV pore tryptophan constitutes part of the drug receptor.

W1531C enhanced tonic block despite its attenuating effects on LA-induced use dependence, $\Delta V_{1/2}$, and recovery from inactivation. Similar changes have been previously observed in channels with residues replaced in DIV S6 (Ragsdale et al., 1994). Bean et al. (1983) suggest that tonic block antagonizes use-dependent block at depolarized holding potentials because channels with drug bound to the closed and/or open (i.e., rapid block upon channel opening) states are not available for further drug binding to the inactivated state, thus leading to an apparent reduced use dependence. Further experiments are needed to differentiate among the two possibilities.

Aromaticity Is Critical for Na^+ Channel Function and Pharmacology. Remarkably, DIV-Trp1531 seems to govern several major Na_v channel properties, whereas the analogous Trp402, Trp756, and Trp1239 in DI-III are not as significant. When the DIV aromatic residue is replaced with the nonaromatic amino acid cysteine or alanine, channels do not discriminate among monovalent cations (such as Na^+, K^+, NH_4^+, and Cs^+). Trp1531 is also an important determinant for μ-conotoxin (Li et al., 1997) as well as LA block. However, replacing Trp1531 with tyrosine or phenylalanine (i.e., W1531F or W1531Y) maintains WT phenotypes. These results suggest that Trp1531 plays a multifunctional role and that its aromaticity is crucial for normal channel function and pharmacology (Li et al., 1997; Tsushima et al., 1997a) and are consistent with the notion that DIV is a particularly important domain.

Conclusion

In summary, our study demonstrates that the DIV aromatic residue Trp1531 in the external pore vestibule of $Na_v1.4$ channels, which has previously been shown to play a critical role in ionic selectivity (Chiamvimonvat et al., 1996a; Tsushima et al., 1997a) and μ-conotoxin binding (Li et al., 1997), also prominently influences LA block. The observed reductions in drug sensitivity were not caused by changes in gating and drug access. We propose that Trp1531 dynamically constitutes part of the LA receptor. Together, these data provide novel structural and functional insights into the external pore of Na_v channels in relation to LA block, which may be helpful for future molecular-based drug design.

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