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# Electrostatic Contributions of Aromatic Residues in the Local Anesthetic Receptor of Voltage-Gated Sodium Channels

Christopher A. Ahern, Amy L. Eastwood, Dennis A. Dougherty, Richard Horn

**Abstract**—Antiarrhythmics, anticonvulsants, and local anesthetics target voltage-gated sodium channels, decreasing excitability of nerve and muscle cells. Channel inhibition by members of this family of cationic, hydrophobic drugs relies on the presence of highly conserved aromatic residues in the pore-lining S6 segment of the fourth homologous domain of the channel. We tested whether channel inhibition was facilitated by an electrostatic attraction between lidocaine and  $\pi$  electrons of the aromatic rings of these residues, namely a cation- $\pi$  interaction. To this end, we used the in vivo nonsense suppression method to incorporate a series of unnatural phenylalanine derivatives designed to systematically reduce the negative electrostatic potential on the face of the aromatic ring. In contrast to standard point mutations at the same sites, these subtly altered amino acids preserve the wild-type voltage dependence of channel activation and inactivation. Although these phenylalanine derivatives have no effect on low-affinity tonic inhibition by lidocaine or its permanently charged derivative QX-314 at any of the substituted sites, high-affinity use-dependent inhibition displays substantial cation- $\pi$  energetics for 1 residue only: Phe1579 in rNa<sub>v</sub>1.4. Replacement of the aromatic ring of Phe1579 by cyclohexane, for example, strongly reduces use-dependent inhibition and speeds recovery of lidocaine-engaged channels. Channel block by the neutral local anesthetic benzocaine is unaffected by the distribution of  $\pi$  electrons at Phe1579, indicating that our aromatic manipulations expose electrostatic contributions to channel inhibition. These results fine tune our understanding of local anesthetic inhibition of voltage-gated sodium channels and will help the design of safer and more salutary therapeutic agents. (*Circ Res.* 2008;102:86-94.)

**Key Words:** voltage-gated sodium channels ■ unnatural amino acids ■ local anesthetics ■ cardiac arrhythmias

Voltage-gated sodium channels underlie the upstroke and propagation of the action potential in excitable cells of nerve and muscle, making them ideal targets in pharmacological interventions for cardiac arrhythmias, epilepsy, and pain. Antiarrhythmics, anticonvulsants, and local anesthetics comprise a family of sodium channel inhibitors that share chemical and structural similarity. All sodium channel isoforms, including the cardiac channel Na<sub>v</sub>1.5, are inhibited by these compounds,<sup>1-3</sup> with differences in sensitivity largely attributed to divergent biophysical aspects of gating kinetics.<sup>3</sup> At physiological pH values, these inhibitors are typically lipid-soluble cations that are highly efficacious in the treatment of hyperexcitability disorders because of the preference of the inhibitors for inactivated channels, a conformational state that is prevalent during high-frequency firing of action potentials. This state-dependent inhibition is rationalized by models that propose that inactivation exposes a high-affinity binding site.

A general deficiency of this class of compounds is their lack of specificity. For example, the antiepileptic drug car-

bamazepine may adversely effect cardiac rhythms<sup>4,5</sup> but has also been used for pain management.<sup>6</sup> Multiple sites of action, whether expressly intended or not, are common to many of these inhibitors and arise from the highly conserved nature of both the compounds and their binding site within the channel where the 3 aromatic residues of relevance for the present study are 100% conserved across eukaryotic voltage-gated sodium channel isoforms.

An abundance of experimental data implicates pore-lining residues of the S6 segment of domain 4 (D4) of the sodium channel  $\alpha$  subunit in the binding of local anesthetics. Alanine-scanning mutagenesis has exposed the crucial role of 2 D4/S6 aromatics, Phe1579 and Tyr1586 (rNa<sub>v</sub>1.4 numbering), in the block by the anticonvulsant etidocaine and the quaternary amine QX-314.<sup>1</sup> Further study has revealed a near universal importance of these 2 aromatic residues in the inhibition of sodium channels by the class 1A and 1B antiarrhythmic drugs and anticonvulsants.<sup>7</sup>

These 2 residues are ideally placed to interact with drugs that inhibit channel function. Sodium channel pore domain

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models based on the crystal structures of potassium channels predict that both residues face the inner vestibule of the permeation pathway, where they could interact readily with cationic blockers and are in close proximity to the selectivity filter where they might influence sodium permeation.<sup>8,9</sup> The mechanistic details of local anesthetic action, although poorly resolved, may involve electrostatic repulsion of sodium ions at the selectivity filter,<sup>10</sup> pore occlusion,<sup>11</sup> stabilization of nonconducting states of the channel, or a combination of all 3 mechanisms, in addition to other possibilities such as gating charge immobilization.<sup>12</sup> The additional relevance of these aromatic residues to channel function is readily seen in experiments in which outright side chain replacement, for example, with either alanine or cysteine, not only disrupts inhibition by local anesthetics but also perturbs the biophysics of channel gating,<sup>1,13–15</sup> possibly obscuring the interpretation of their involvement in channel inhibition.

How do neutral aromatic residues contribute to channel inhibition by organic cations? One proposed but untested possibility is that local anesthetics have an electrostatic attraction to the negative electrostatic potential on the face of pore-lining aromatic side chains,<sup>2</sup> a so-called cation- $\pi$  mechanism. This hypothesis can be tested directly using the *in vivo* nonsense suppression method, which allows for the site-directed incorporation of subtly altered variants of the aromatic amino acid phenylalanine.<sup>16</sup> If a cation- $\pi$  mechanism contributes to the attraction between a cationic blocker and the aromatic side chain, reduction of  $\pi$  electron density on the face of the aromatic, for example, by fluorinating the aromatic ring, will reduce the binding affinity.<sup>17</sup> A benefit of this approach is that fluorination does not substantially alter the polarizability, size, shape, or hydrophobicity of the side chain.<sup>17,18</sup> Therefore, this benign manipulation of side chain structure obviates the inconvenient functional consequences of standard mutagenesis. This strategy has been applied successfully in sodium channels to test for cation- $\pi$  interactions between an extracellular aromatic residue and either tetrodotoxin<sup>19</sup> or calcium ions.<sup>20</sup>

In this study, we used progressively fluorinated phenylalanine derivatives and the unnatural amino acid cyclohexylalanine (Cha), in which the aromatic ring is replaced by nonaromatic cyclohexane. Synthetic residues were substituted into 3 positions of the D4/S6 segment of the rNa<sub>v</sub>1.4 voltage-gated sodium channel, and in each case, channels showed robust expression with normal voltage dependence and kinetics of both activation and fast inactivation. Only 1 site, Phe1579, exhibited a strong cation- $\pi$  interaction with lidocaine. Furthermore, we found that channel inhibition by the neutral local anesthetic benzocaine was unaffected by manipulations that affected the electrostatic potential on the aromatic face of Phe1579. These experiments establish a substantial electrostatic component in the binding energy between cationic local anesthetics and a single pore-lining aromatic. This careful dissection of the energetic factors underlying the binding of these inhibitory compounds to sodium channels holds promise for the synthesis of more specific therapeutic agents for treatment of disorders of hyperexcitability.

## Materials and Methods

### Molecular Biology and Unnatural Amino Acids

The *in vivo* nonsense suppression methodology was performed as described previously.<sup>19,21,22</sup> In brief, the corresponding DNA sequences for Tyr1574, Phe1579, or Tyr1586 of the rNa<sub>v</sub>1.4 cDNA were mutated into a TAG nonsense (stop) codon by conventional mutagenesis (Stratagene), and complementary mRNA was transcribed from this cDNA (mMessage mMachine Ultra, Ambion). Unnatural amino acids were protected with nitroveratryloxycarbonyl, activated as the cyanomethyl ester, coupled to the dinucleotide dCA, and then ligated to a modified transfer (t)RNA from *Tetrahymena thermophila*. This tRNA contains the complementary anticodon to the UAG stop site within the rNa<sub>v</sub>1.4 TAG construct cRNA. As a control for spurious expression of sodium channels containing the TAG sequence, the dinucleotide dCA lacking an appended amino acid was also ligated to the tRNA. Deprotection of the aminoacylated tRNA (tRNA-aa) was performed by UV irradiation immediately before injection into stage V to VI *Xenopus* oocytes. Typically, 20 ng of tRNA-aa, 25 ng of rNa<sub>v</sub>1.4-TAG cRNA, and 2 ng of sodium channel  $\beta_1$  subunit cRNA<sup>23</sup> were coinjected in a 50-nL volume into each oocyte.

The present study used phenylalanine derivatives with 1, 2, or 3 appended fluorines, termed 3-F-Phe (single fluorination at the 3 position), 3,5-F<sub>2</sub>-Phe (fluorination at the 3 and 5 positions), and 3,4,5-F<sub>3</sub>-Phe (fluorination at the 3, 4, and 5 positions).

### Electrophysiology

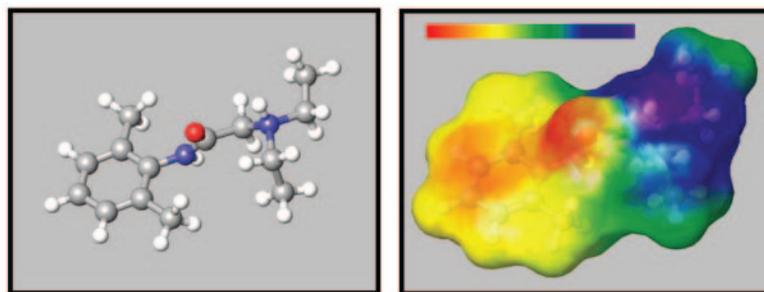
Whole oocyte sodium current was recorded with the 2-microelectrode voltage clamp technique using an OC-725C voltage clamp (Warner Instruments, Hamden, Conn) in a standard Ringers solution (in mmol/L): 116 NaCl, 2 KCl, 1 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 5 HEPES, pH 7.5. Recording electrodes were filled with a 3 mol/L KCl solution and had a resistance  $\leq$  1 M $\Omega$ . Solutions were changed with an ALA VM8 gravity-flow delivery system (ALA Scientific). A 200-mM lidocaine stock in ethanol was stored at  $-20^\circ\text{C}$ , and working dilutions were made each day from this stock. Summarized data are presented as means  $\pm$  SEM.

## Results

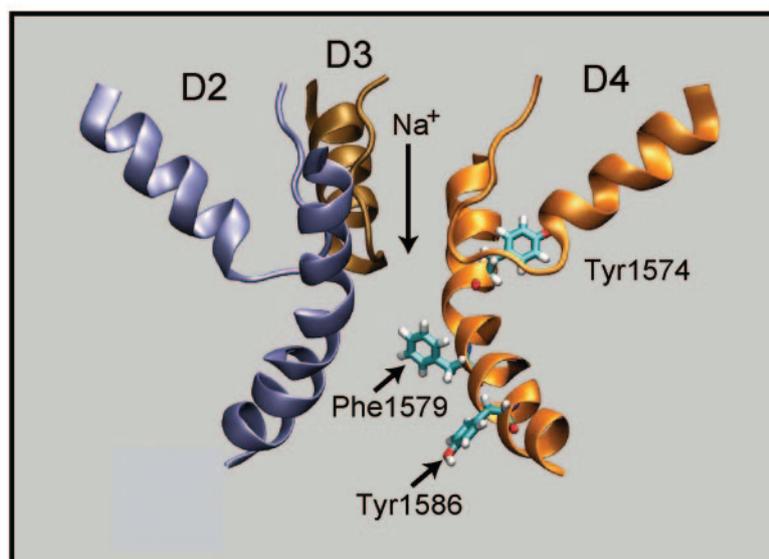
Although a cation- $\pi$  interaction has been proposed to occur between local anesthetics and D4/S6 aromatics,<sup>2</sup> this hypothesis remains entirely unsubstantiated, and we therefore tested this possibility directly. Lidocaine, like most local anesthetics, can exist in 2 forms, neutral or cationic. At physiological pH values, roughly three-quarters of lidocaine molecules carry a +1 charge, and the protonated form is a stronger pore blocker.<sup>24–26</sup> Figure 1A shows the protonated form of lidocaine and a color-coded map of the electrostatic potential on the surface of the molecule. The positive charge has an asymmetric distribution focused most strongly (blue) near the protonated nitrogen, positioned to the right in each illustration of Figure 1A.

Figure 1B shows a side view of a portion of the putative local anesthetic binding site in a model of the voltage-gated skeletal muscle sodium channel rNa<sub>v</sub>1.4 adapted from Lipkind and Fozzard.<sup>8</sup> The pore helices and extracellular pore loops of domains D2 to D4 and the opposing transmembrane S6 segments of domains D2 (silver) and D4 (orange) are shown in an open-channel conformation. Note the proximity of the residues Phe1579 and Tyr1586 to each other and to the selectivity filter above them. Figure 1B is intended to represent a side view of the aqueous vestibule in which lidocaine binds and to illustrate that lidocaine is large enough ( $\approx 11 \times 6 \text{ \AA}$ ) to interact simultaneously with Phe1579 and Tyr1586

A



B



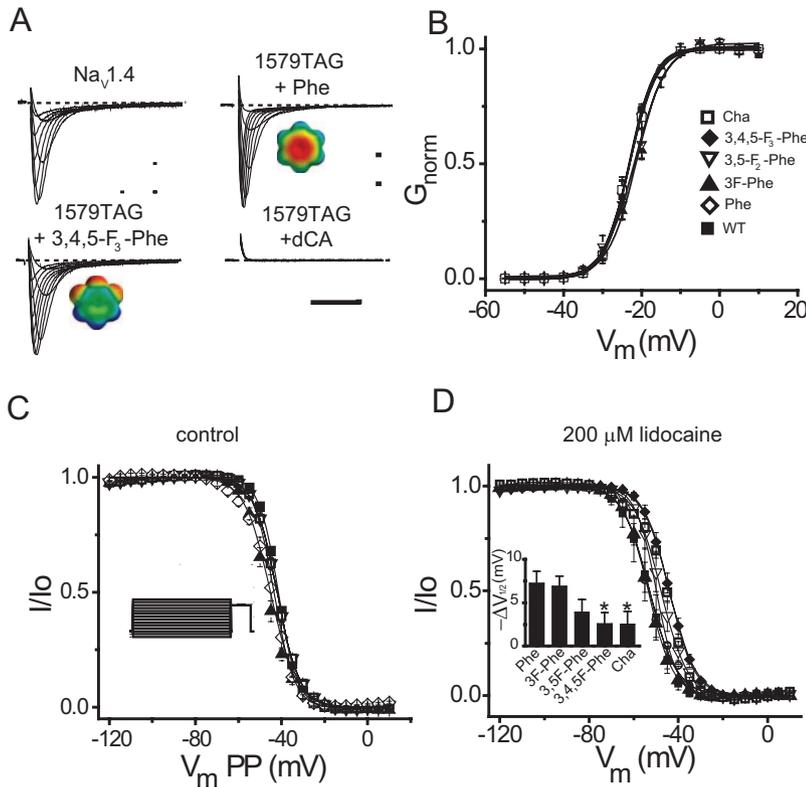
**Figure 1.** Lidocaine and the sodium channel pore. A, Structure and the electrostatic surface of protonated lidocaine. Left, The structure of lidocaine optimized at the HF/6-31++G\*\* level, with carbon, hydrogen, nitrogen, and oxygen shown as gray, white, blue, and red, respectively. Right, Results of an ab initio determination of the electrostatic surface of protonated lidocaine in water. The red-to-blue spectrum shown on the scale bar corresponds to a range of 0 to +150 kcal/mol. B, Side view of a putative local anesthetic receptor in a voltage-gated sodium channel. The image is adapted from the sodium channel pore model of Lipkind and Fozzard,<sup>8</sup> as described in the text.

( $\approx 10$  Å apart) in this structural model. By contrast, Tyr1574 is on the “backside” of the S6 segment facing away from the permeation pathway.

Although standard point mutants of Phe1579 typically alter the biophysical properties of the channel,<sup>1,13,15</sup> the unnatural mutants we generated by *in vivo* nonsense suppression resulted in channels that were indistinguishable from wild-type rNa<sub>v</sub>1.4 with respect to kinetics of activation and inactivation. Figure 2A shows families of normalized sodium currents from oocytes expressing wild-type rNa<sub>v</sub>1.4 or channels with the indicated amino acid at position 1579. Inward currents shown in Figure 2A were elicited in increments of 5-mV depolarizations from  $-30$  mV to  $+10$  mV from a holding potential of  $-100$  mV. Figure 2A, lower right, shows that oocytes coinjected with 1579TAG cRNA and a tRNA lacking an appended amino acid did not generate measurable sodium currents, ruling out counterfeit channel expression attributable to enzymatic manipulation of the coinjected tRNA within the oocyte. Insets in Figure 2A show the consequences of trifluorination on the electrostatic potential of benzene, where red and blue represent negative and positive, respectively, and green is neutral. Peak conductance–voltage relationships for activation are shown in Figure 2B for wild-type rNa<sub>v</sub>1.4 or channels with the indicated

amino acids at the 1579 position. There was no change in either voltage dependence or slope of activation for any of the mutants (Table I in the online data supplement at <http://circres.ahajournals.org>). Moreover, replacement of Phe1579 with either serially fluorinated derivatives of phenylalanine or nonaromatic Cha resulted in insignificant effects on steady-state inactivation (Figure 2C). We also incorporated the same series of side chains at Tyr1586, a site roughly 2 turns downstream along the S6  $\alpha$  helix. Like the 1579 site, the incorporation of unnatural amino acids was well tolerated, producing channels with healthy expression and normal gating (supplemental Table I).

A phenomenological hallmark of local anesthetic action on sodium channels is a hyperpolarizing shift of the steady-state inactivation relationship.<sup>24,27</sup> To test a possible role for cation- $\pi$  energetics at Phe1579, we incubated oocytes expressing the indicated channel type for 5 minutes in 200  $\mu$ mol/L lidocaine at  $-100$  mV and then measured steady-state inactivation (Figure 2D). Consistent with previous studies, incubation with lidocaine caused an approximate  $-7$ -mV shift for Phe1579 channels,<sup>27,28</sup> but this effect was serially diminished with each added fluorine to a minimum of  $-2.5$  mV for either 3,4,5-F<sub>3</sub>-Phe1579 or Cha1579 (inset). These results suggest that the reduction of negative electro-



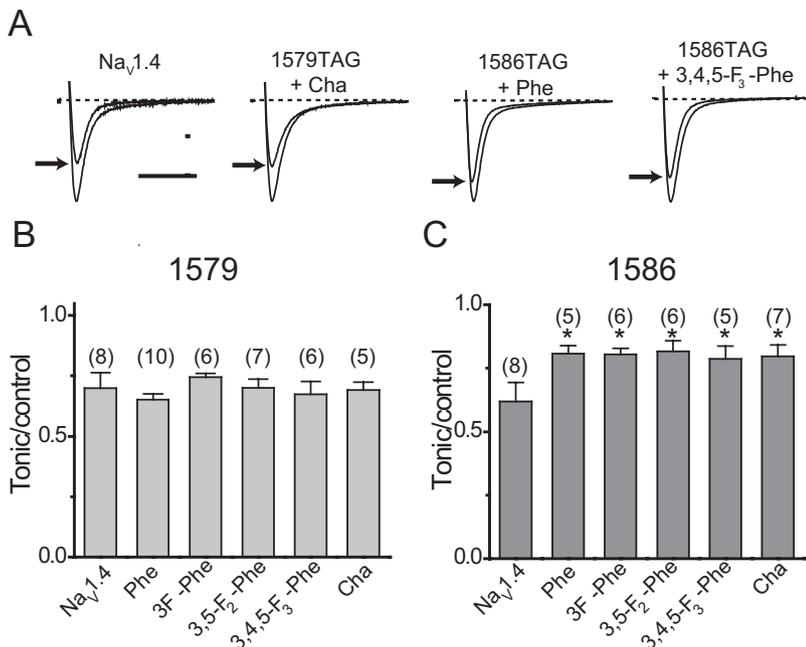
**Figure 2.** Unperturbed gating properties of rNa<sub>v</sub>1.4 channels containing unnatural amino acids at 1579. A, Families of sodium currents for the indicated channel types elicited by 5-mV steps from -30 to +25 mV from a holding potential of -100 mV. Scale bar=5 ms. Insets show the electrostatic potentials of benzene and trifluorobenzene, with red as negative (-20 kcal/mol) and blue as positive (+20 kcal/mol). No currents were seen when cRNA from Phe1579TAG was coinjected with a tRNA lacking an appended amino acid. B, Peak conductance-voltage relationships for indicated side chains at the 1579 site. C, Steady-state inactivation curves generated from the inset protocol. Holding potential, -100 mV; 500-ms prepulse; 2-ms reset at -100 mV; -10 mV test potential. D, Same protocol as C, after a 5-minute incubation with 200 μmol/L lidocaine. Inset summarizes the shift in steady-state voltage dependence caused by lidocaine for the indicated channel types. \**P*<0.05.

static potential on the aromatic face of the residue at position 1579 weakens the binding of lidocaine to inactivated channels.

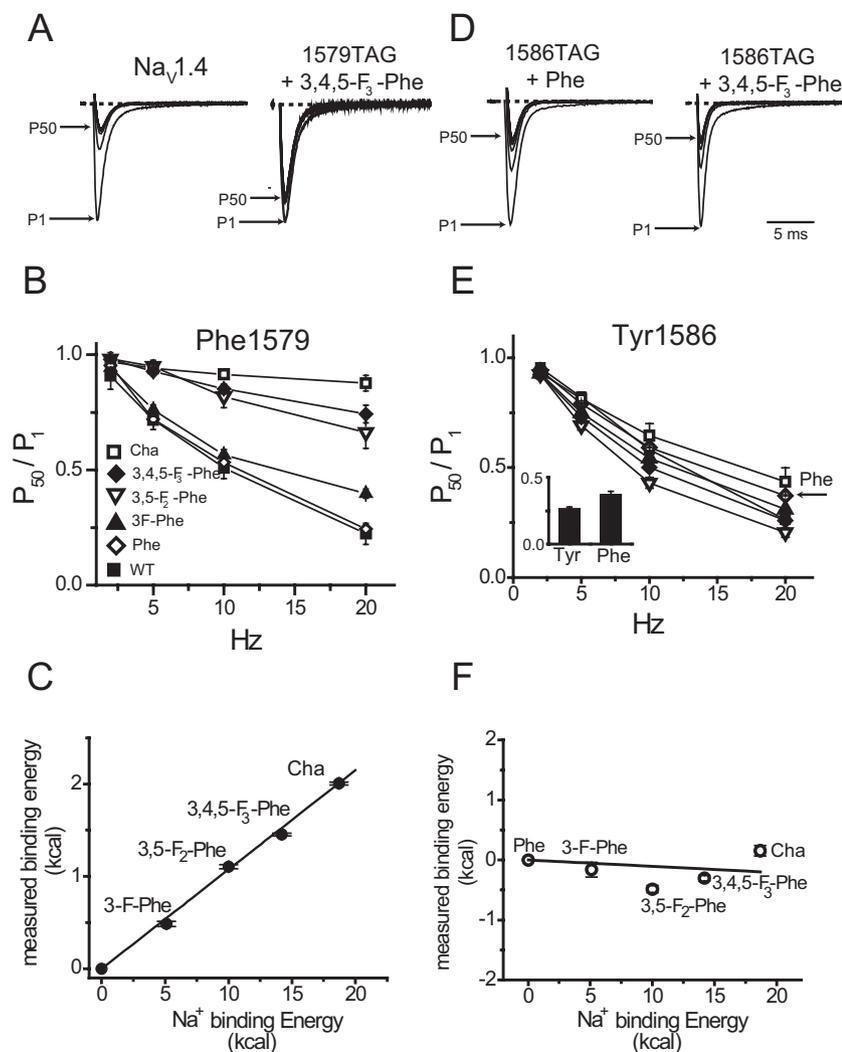
**Tonic Inhibition**

Local anesthetics can interact with sodium channels under either stimulated or resting conditions, the latter with lower affinity. Systematic replacement of the equivalents of rNa<sub>v</sub>1.4 Phe1579 and Tyr1586 in neuronal isoforms with nonaromatic

residues reduces resting affinity for the local anesthetics etidocaine<sup>1</sup> and tetracaine.<sup>2</sup> We therefore investigated the possibility of a contribution of cation- $\pi$  energetics to this tonic inhibition, which we define operationally as the fractional reduction of peak current in response to a low rate of depolarization (15-ms pulses to -20 mV at 0.05 Hz). Figure 3A shows representative sodium currents before and after 5-minute continuous perfusion of 200 μmol/L lidocaine. Wild-type rNa<sub>v</sub>1.4 channels and those containing Cha1579



**Figure 3.** Electrostatic contributions are not involved in tonic inhibition for Phe1579 and Tyr1586. A, Normalized control and tonically inhibited currents (indicated by arrow) elicited by test pulses to -10 mV; holding potential, -100 mV. Scale bar=5 ms. Tonic inhibition reached equilibrium after 5 minutes of continuous perfusion of 200 μmol/L lidocaine, as measured by 15-ms pulses every 20 seconds. B, Electrostatic potential of Phe1579 does not contribute to lidocaine binding. The number of cells are in parentheses. C, Mutation of Tyr1586 to phenylalanine relieves tonic block, but any additional manipulation of  $\pi$  electrons has no consequence. \**P*<0.05 vs wild-type rNa<sub>v</sub>1.4.



**Figure 4.** Use-dependent inhibition relies on a cation- $\pi$  interaction between lidocaine and Phe1579, not Tyr1586. A, Normalized representative current traces in the presence of 200  $\mu\text{mol/L}$  lidocaine from repeated depolarizations to  $-10$  mV from  $-100$  mV for  $r\text{Na}_v1.4$  or channels with 3,4,5-F<sub>3</sub>-Phe at the 1579 site. The first 10 and the 50th trace of 50 total pulses are indicated. B, Frequency profile for use-dependent inhibition for Cha and fluorinated phenylalanine derivatives. C, Linear relationship between experimental data and theoretical predictions for a cation- $\pi$  interaction. Dissociation constants estimated at 20 Hz were used to calculate the energetic effect of manipulating aromatic electrostatic potential at Phe1579. Binding energy is defined as  $0.58\ln(K_{i,\text{mut}}/K_{i,\text{wt}})$  kcal/mol, where  $K_{i,\text{mut}}/K_{i,\text{wt}}$  is the fractional increase in the inhibitory constant caused by an unnatural mutant compared with benzene/phenylalanine. Theoretical data on the abscissa are based on the calculated interaction between a single sodium ion and the indicated benzene derivative.<sup>22</sup> D, Absence of a cation- $\pi$  contribution at Tyr1586 to lidocaine inhibition. The normalized representative traces are as in Figure 4A. Complete loss of the negative electrostatic potential of the aromatic ring has little effect on use-dependent inhibition. E, Frequency profile of use-dependent inhibition for the 1586 position, as in B. Arrow represents Y1586F. Inset shows the relief of inhibition for the Y1586F mutation at 20 Hz. Neither serial fluorination nor replacement by Cha further disrupts lidocaine block. F, Energy plot as in C details the complete lack of a cation- $\pi$  phenotype at 1586.

showed indistinguishable tonic inhibition. The results for all the side chains introduced at the 1579 position are shown in Figure 3B and demonstrate that a cation- $\pi$  interaction with this residue is not a factor in the tonic inhibition by lidocaine.

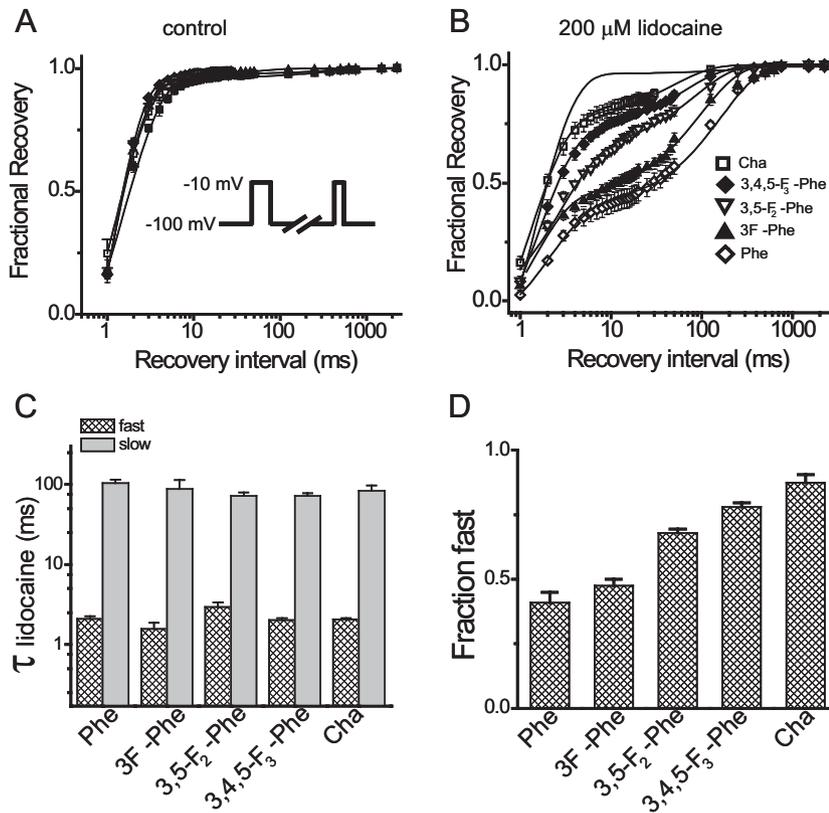
We also investigated the role of Tyr1586 in tonic inhibition by lidocaine. Consistent with a previous study,<sup>2</sup> removal of the hydroxyl group by mutation to phenylalanine alleviated the tonic inhibition from  $30 \pm 6\%$  to  $19 \pm 3\%$ . Figure 3C shows that further manipulation of this side chain to withdraw  $\pi$  electrons had no additional effect on the tonic inhibition. These data show that although the hydroxyl group of Tyr1586 plays a role in inhibition, lidocaine does not interact directly with its aromatic face under resting conditions.

### Use Dependence

Use-dependent inhibition of sodium channels is manifest as progressively reduced current with increased stimulation frequency. Although experiments using site-directed mutagenesis show that use-dependent block by local anesthetics involves side chain contributions from residues in each of the four sodium channel domains,<sup>1,29-31</sup> the presence of an aromatic phenylalanine, tyrosine, or tryptophan at the position aligned with 1579 is required for use-dependent inhibi-

tion,<sup>2</sup> suggesting that a cation- $\pi$  interaction may play a substantial role at the binding site. Figure 4A shows representative sodium currents in response to 20-Hz depolarizations to  $-10$  mV from a holding potential of  $-100$  mV in the presence of 200  $\mu\text{mol/L}$  lidocaine. In each example, the first and 50th pulses are labeled. Note that the first pulse represents a channel inhibited in its resting state. In the absence of lidocaine, sodium currents are stable in response to 20-Hz stimulation with no run-down in amplitude (data not shown). Quite dramatically, trifluorination of Phe1579 nearly abolishes the use-dependent inhibition by lidocaine (Figure 4A and 4B). Similar results were obtained at a 10-fold lower lidocaine concentration, at which tonic inhibition was negligible (supplemental Figure I), eliminating a substantial role of rapid open-channel block.

Evidence of a cation- $\pi$  interaction between Phe1579 and lidocaine in the inactivated state is presented in Figure 4B, in which monotonic relief of inhibition can be seen for each added fluorine, with Cha substitution producing almost complete relief. This effect is most apparent at high frequencies, where inactivated states would be expected to dominate. Although use-dependent inhibition by lidocaine is an obvious phenotype, it is an inherently complicated process, and as



**Figure 5.** Recovery from lidocaine inhibition affected by a cation- $\pi$  interaction with Phe1579. A, Recovery from inactivation in absence of lidocaine has an indistinguishable double-exponential time course among phenylalanine derivatives. B through D, Fluorination or Cha substitution increases the fractional amplitude of the fast component of recovery in 200  $\mu$ mol/L lidocaine without affecting the recovery time constants.

such, the progressive current reduction seen in Figure 4A and 4B is likely a combination of interaction of lidocaine with both inactivated and open states. Although acknowledging this caveat, we calculated an operationally defined inhibitory constant  $K_i$  from the fraction of current remaining at the completion of 50 pulses delivered at 20 Hz in the presence of 200  $\mu$ mol/L lidocaine. This apparent inhibitory constant was used to calculate the loss in binding energy, resulting from the serial fluorination of phenylalanine or outright replacement by Cha. The result of this analysis is shown in Figure 4C. The abscissa is derived from ab initio estimates of the energetic effect of fluorination on the cation- $\pi$  interaction between benzene and a Na<sup>+</sup> ion.<sup>17</sup> As predicted for a cation- $\pi$  interaction, a stepwise reduction in electrostatic potential on the face of the aromatic results in a monotonic reduction in lidocaine inhibition.<sup>32</sup> Given the ambiguities presented by both the charge asymmetry of lidocaine and the interpretations of use dependence protocols, the linear relationship between our experimental results and the theoretical predictions for a canonical cation- $\pi$  system is striking, confirming such an interaction between Phe1579 and lidocaine.

The results are remarkably different when the same series of phenylalanine derivatives are substituted into the nearby aromatic Tyr1586 (Figure 4D). The complete data set in Figure 4E lacks both the trend and the magnitude of effect that was observed at Phe1579. The inset of Figure 4E shows the disruptive consequence of exchanging of phenylalanine for tyrosine, an effect greater than any further manipulation of the phenylalanine side chain. Figure 4E and 4F shows that, contrary to the situation for Phe1579 only  $\approx$ 11 Å away, perturbation of  $\pi$  electrons at Tyr1586 has little consequence

on use-dependent lidocaine inhibition, ruling out a cation- $\pi$  interaction at this site.

We then tested whether lidocaine might interact with a third D4/S6 aromatic residue, Tyr1574 (Figure 1B), positioned roughly 10 Å upstream from Phe1579 but predicted to lie on the opposite face of the S6 helix. Alanine substitution at this site results in a modest loss of both resting and use-dependent inhibition by etidocaine in Na<sub>v</sub>1.2,<sup>1</sup> suggesting that it may play a subtle role in stabilizing the inhibitor in its binding pocket. We incorporated a single unnatural amino acid at this site, 3,4,5-F<sub>3</sub>-Phe, because trifluorination effectively ablates the negative electrostatic potential of the face of an aromatic ring.<sup>20,32</sup> As for the other 2 sites we examined, unnatural amino acid incorporation was well tolerated and produced channels with robust expression and voltage-dependent gating similar to that of wild-type rNa<sub>v</sub>1.4 channels (data not shown). Trifluorination at this site had no effect on tonic ( $36 \pm 6\%$  versus  $39 \pm 8\%$ ) or use-dependent inhibition ( $84 \pm 4\%$  versus  $74 \pm 3\%$  at 20-Hz stimulation;  $P=0.08$ ) for rNa<sub>v</sub>1.4 and 3,4,5-F<sub>3</sub>-Phe 1574 channels, respectively. Therefore, only 1 of the 3 D4/S6 aromatic residues, Phe1579, shows a robust cation- $\pi$  interaction with lidocaine.

### Recovery

Another experimental manifestation of lidocaine action is the emergence of a slow component in the recovery from fast inactivation.<sup>28</sup> In fact, use-dependent inhibition is largely a consequence of slowed recovery from inactivation. In the absence of lidocaine, all Phe1579 mutants recover rapidly from a 15-ms pulse to -10 mV (Figure 5A), but clear differences arise in the presence of 200  $\mu$ mol/L lidocaine

(Figure 5B). For each variant of Phe1579, the recovery time course can be fit by a double-exponential relaxation. A plausible explanation for this biophysical behavior is that the fast and slow components represent unblocked and lidocaine-engaged channels, respectively. Figure 5C and 5D show that whereas the fast and slow time constants from the biexponential fits are the same across all channel types, the fractional weight of the fast, unblocked component is serially increased with fluorination and replacement by Cha. Therefore, fluorination appears to reduce the fraction of channels that trap lidocaine when they inactivate, whereas the recovery rate of the drug-modified channels is insensitive to the unnatural mutations. These results, like those describing use-dependent inhibition, demonstrate a cation- $\pi$  interaction between lidocaine and Phe1579. By contrast, neither fluorination of Phe1586 nor replacement with Cha had a measurable effect on the recovery time course (data not shown), consistent with the use-dependent inhibition experiments summarized in Figure 4F.

### Role of Charge

To further test the role of electrostatics in the interaction between a local anesthetic and Phe1579, we examined 2 nontitratable, structurally similar derivatives of lidocaine, 1 neutral (benzocaine) and the other permanently charged (QX-314). Both compounds are postulated to have overlapping binding sites with other local anesthetics and, more importantly, to rely on the presence of an aromatic side chain at Phe1579.<sup>26,33</sup> Benzocaine does not cause use-dependent inhibition in sodium channels.<sup>24,33</sup> Replacement of Phe1579 by either 3,4,5-F<sub>3</sub>-Phe1579 or Cha1579, both of which effectively abolish the negative electrostatic potential on the aromatic face of phenylalanine, had no effect on tonic inhibition by 1 mmol/L benzocaine (fraction of inhibition was  $0.49 \pm 0.02$ ,  $0.46 \pm 0.04$ ,  $0.44 \pm 0.02$ , respectively, for wild-type and the 2 unnatural mutants). If the binding site of benzocaine overlaps that of lidocaine, our data suggest that the fundamental effect of these manipulations of the aromatic ring of Phe1579 involves electrostatics, leaving benzocaine inhibition unaffected.

The lack of an effect of 3,4,5-F<sub>3</sub>-Phe1579 on tonic inhibition by lidocaine (Figure 3) raises the possibility that lidocaine is deprotonated (uncharged) in its tonically inhibited state. This possibility can be tested directly with permanently charged QX-314 that causes both tonic block and use-dependent inhibition.<sup>1,27,34</sup> If the tonically blocked state involves an intimate interaction between QX-314 and Phe1579, then it should be substantially affected by trifluorination of the aromatic side chain. QX-314 was injected directly into voltage-clamped oocytes to yield an approximate cytoplasmic concentration of 0.5 mmol/L. This concentration produces dramatic resting-state and use-dependent (1 Hz) inhibition in wild-type channels, and only the use-dependent component is ameliorated in the 3,4,5-F<sub>3</sub>-Phe1579 mutant (Figure 6A), similar to our results with lidocaine. The time course of use dependence and the extent of block for the 2 oocytes shown in Figure 6A are plotted in Figure 6C. The summarized data in Figure 6E and 6F show that trifluorinating Phe1579 has a similar effect on binding energy for

lidocaine and QX-314, suggesting comparable modes of inhibition. The faster rate of use-dependent inhibition in the unnatural mutant suggests an increased dissociation rate for QX-314 in the drug-bound inactivated state. Our results further show that the tonically blocked state does not involve a close interaction between the blocker and Phe1579, and yet a substantial cation- $\pi$  interaction underlies use-dependent inhibition by QX-314.

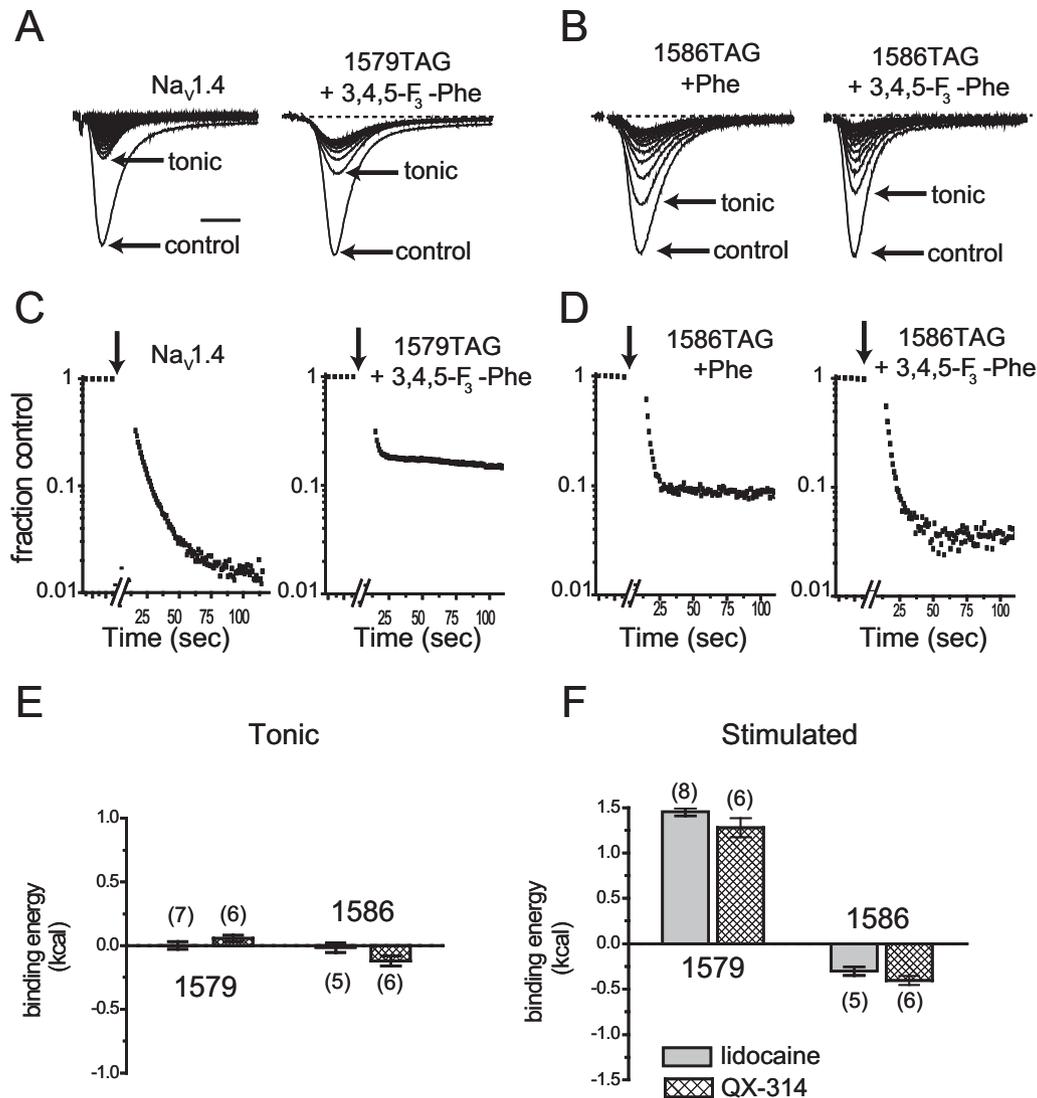
By contrast with Phe1579, trifluorination of Phe1586 causes a mild (<0.5 kcal/mol) stabilization of QX-314 block in both the tonic and stimulated states (Figure 6D through 6F). The effect on stimulated-state inhibition is energetically indistinguishable for lidocaine and QX-314 (Figure 6F).

### Discussion

We investigated whether 3 D4/S6 aromatic residues contribute to local anesthetic block through the electrostatic attraction of their aromatic  $\pi$  electrons. In contrast with previous studies using traditional site-directed mutagenesis, incorporation of unnatural amino acids at each site resulted in channels with normal gating, substantially simplifying the interpretation of their role in pore block. A cation- $\pi$  interaction with lidocaine during use-dependent inhibition was observed for only 1 site, Phe1579, whereas ablation of the negative electrostatic potential on the face of either Tyr1574 or Tyr1586 had relatively little consequence. The roles of these tyrosine residues in block cited by previous reports may therefore be attributable to the fact that replacement with a nonaromatic amino acid is a more drastic alteration of side chain chemistry and often changes the biophysical properties of the channel.<sup>1,13–15</sup> Fluorination of phenylalanine, by contrast, has no effect on channel gating at any of the 3 D4/S6 positions. Although our results suggest that cationic blockers have an electrostatic attraction for the  $\pi$  electrons of residue 1579, it may be surprising that replacement of this aromatic residue with negatively charged glutamate or aspartate obliterates use-dependent inhibition by lidocaine.<sup>10</sup> A possible explanation for these apparently contradictory observations is that in an aqueous milieu an organic cation typically has a higher affinity for an aromatic ring than an acidic residue.<sup>35</sup> This is attributable to the substantial energetic penalty for dehydrating the acidic residue.

Our study provides several structural insights into the local anesthetic binding site in the high-affinity state created by high-frequency stimulation. The cation- $\pi$  interaction at Phe1579 suggests that the positive charge of lidocaine, concentrated in the vicinity of its titratable amine group, is situated near the aromatic face of Phe1579 in the inactivated state. By contrast, Tyr1574 and Tyr1586 have neither cation- $\pi$  interactions nor  $\pi$ - $\pi$  stacking<sup>36</sup> interactions with lidocaine, as either would be disrupted by fluorination of the aromatic ring. Furthermore, Cha lacks the quadrupole moment of an aromatic, and therefore its substitution should reduce any attraction with the aromatic moiety of lidocaine through  $\pi$ - $\pi$  stacking, ruling out this type of attraction between the blocker and the tyrosine residues.

Electrostatic manipulations of Phe1579 affect use-dependent but not tonic inhibition. This contrasts with the interaction between the cationic sodium channel blocker



**Figure 6.** Use-dependent and tonic block by QX-314. A, Control is a representative trace at  $-10$  mV (0.1 Hz), after which 25 nL of 20 mmol/L QX-314 was injected to give an estimated final concentration of 0.5 mmol/L QX-314, assuming a 1- $\mu$ L volume for the oocyte. The oocyte was held at  $-100$  mV for 5 minutes without depolarization to allow for diffusion, followed by one hundred 1-Hz, 15-ms depolarizations, the first of which represents the tonic block. The right recording is from an oocyte expressing trifluorinated Phe1579. B, Comparable experiments for residue Phe1586. C and D, Normalized peak currents from recordings in A and B. Arrow indicates time of QX-314 injection. Time break is 5 minutes. E and F, Effect of trifluorination on both lidocaine and QX-314-binding energy; the number of cells are in parentheses. Lidocaine data are from Figures 3 and 4.

tetrodotoxin and an extracellular pore aromatic, Tyr401 in rNa<sub>v</sub>1.4.<sup>19</sup> In the case of tetrodotoxin, both the tonic and use-dependent block are weakened identically with serial fluorination, implying that a similar physical interaction is present in both states. In the present study, our results suggest that tonic and use-dependent block either represent differences in the lidocaine binding site or arise from differences in the charged state of lidocaine (protonated versus deprotonated). The latter possibility is ruled out by our experiments with the permanently charged QX-314 (Figure 6). Both tonic and use-dependent block are observable with QX-314, but trifluorination of Phe1579 alleviates only use-dependent block. The composite results show not only that tonic and stimulated states represent distinct conformations but also that in the tonic state, lidocaine is not close to any of the 3 aromatic residues we examined. Although lack of a cation- $\pi$

interaction does not rule out other energetic attractions (eg, hydrophobic, charge transfer, or induced dipoles in the aromatic), an intimate contact between an aromatic side chain and a charged blocker would be expected to be affected by the dramatic manipulations of electrostatic potential we introduced (changes of  $>600$  mV within 2  $\text{\AA}$  of the aromatic ring<sup>20</sup>).

What, then, accounts for the differences between tonic and use-dependent inhibition? Because the 2 inhibited states have the same voltage dependence, equivalent to a blocker residing 70% of the way into the electric field from the cytoplasmic side,<sup>37</sup> we assume that lidocaine occupies a comparably deep site within the pore in both states. A cation- $\pi$  interaction only appears between Phe1579 and a charged blocker during repeated depolarizations, indicating that stimulation moves this side chain closer to the blocker. The electrostatic invis-

ibility of Phe1579 in the tonic state suggests that its side chain does not point into the aqueous cavity of the pore and that it is unveiled, for example, by a rotation of the D4/S6 helix during a conformational transition that accompanies inactivation.<sup>29,38–40</sup> Note that this mechanism contrasts with a previous proposal in which the pore lining remains stationary while the blocker moves.<sup>37</sup> Future experiments should resolve the gating motions that underlie the exposure of a high-affinity receptor.

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### Disclosures

None.

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