Cardiac-Specific External Paths for Lidocaine, Defined by Isoform-Specific Residues, Accelerate Recovery From Use-Dependent Block

Peter J. Lee, Akihiko Sunami, Harry A. Fozzard

Abstract—Local anesthetic antiarrhythmic drugs block voltage-gated Na\(^+\) channels from the cytoplasmic side. In addition, cardiac Na\(^+\) channels can be also blocked by the membrane-impermeant local anesthetic QX via external paths not present in skeletal muscle or brain channels. Introduction of cardiac isoform-specific residues into wild-type skeletal muscle or brain channels creates access paths for external QX block. These paths should affect the characteristics of use-dependent block by influencing drug on- and off-rates. We investigated the effects of these external paths on drug kinetics of lidocaine, a lipophilic drug of clinical relevance, by studying use-dependent block using a two-electrode voltage clamp in *Xenopus* oocytes. Recovery from use-dependent block was slowed when cardiac isoform-specific residues important for external QX access were mutated to skeletal muscle or brain isoform-specific residues. As the fraction of charged lidocaine was decreased by raising external pH, differences in recovery kinetics diminished, indicating that these mutations mostly influenced block by charged lidocaine molecules. Data were fit into a model in which bound drug distributes into charged and neutral forms based on its pK\(_a\) and external pH with separate dissociation paths and recovery-time constants. These isoform-specific mutations altered the recovery-time constants for the charged molecules with smaller effects on those for the neutral molecules. We conclude that the external egress paths created by isoform-specific residues influence the drug kinetics of lidocaine, and these residues define cardiac-specific external paths for local anesthetic drugs. (*Circ Res.* 2001;89:1014-1021.)

Key Words: voltage-gated Na\(^+\) channel  •  lidocaine  •  electrophysiology  •  antiarrhythmic drugs

Class I local anesthetic (LA) antiarrhythmic drugs (reviewed in Butterworth and Strichartz\(^1\)) block Na\(^+\) current by binding to voltage-gated Na\(^+\) channels. LA drugs in clinical use are tertiary amines and exhibit both tonic and use-dependent block (UDB) of Na\(^+\) current. UDB by LA drugs is the hallmark of their antiarrhythmic activity; it enables these drugs to be more effective when the frequency of action potentials is high such as in ventricular tachycardia. During UDB, blocked channels accumulate because of incomplete recovery of drug-bound channels during diastole. Slowed recovery of drug-bound channels can be explained by a combination of the modulated-receptor hypothesis\(^2,3\) and the guarded-receptor model.\(^4,5\) The modulated-receptor hypothesis proposes that higher affinity for LA drugs during activated and inactivated gating states slows unbinding of drug and, consequently, recovery of the drug-bound channels. The guarded-receptor model emphasizes that a state-dependent availability of the drug access path to and from the binding site influences apparent binding and unbinding kinetics.

LA drugs block Na\(^+\) channels by binding to a site within the pore below the selectivity filter and above the activation gate at the inner pore mouth.\(^6,7\) Specific S6 residues from domains 1, 3, and 4 (D4S6) have been identified as parts of the binding site.\(^8–11\) Hille\(^2\) has proposed two paths to and from the binding site, a fast hydrophobic path for neutral drug and a slower hydrophilic path for charged drug. One hydrophilic path common to all channel isoforms is the inner pore, guarded by the activation gate. Lidocaine is a tertiary amine with a pK\(_a\) near the physiological level of pH, and it exists in both cationic and neutral forms, allowing it to use both pathways. Onset and recovery-rate constants for UDB by lidocaine increase as the external pH is raised, but internal pH has little effect.\(^12,13\) Thus, external pH can influence kinetics of UDB for tertiary amine drugs by influencing the fraction ionized and the relative use of fast hydrophobic and slow hydrophilic paths.

Quaternary amine QX analogues of LA drugs are membrane-impermeant and are inactive from the outside of neuronal and skeletal muscle isoforms of Na\(^+\) channels.\(^2,7,14\) In contrast, external QX is effective in blocking cardiac Na\(^+\) channels,\(^15\) and isoform-specific residues determine this external access path. Qu et al.\(^16\) identified an isoform-specific
residue in the upper part of D4S6 (Thr in heart, Val in brain, and Cys in \( \mu \lambda \)) that is a determinant for external QX block, and we identified an isoform-specific residue within the P-loop of domain 1 (Cys in heart, Phe in brain, and Tyr in \( \mu \lambda \)).\textsuperscript{14} It is important to determine if this external access path also influences the action of lipophilic LA antiarrhythmic drugs in clinical use. In addition, better definition of the factors controlling LA kinetics may assist both in understanding the action of currently used drugs and in development of better drugs.

In our study, we characterize the properties of these paths defined by the P-loop (C373) and D4S6 (T1752) isoform-specific residues in human heart channel, hH1a, by studying recovery from UDB by lidocaine. We show that these isoform-specific residues influence the lidocaine recovery kinetics and define cardiac-specific external paths for lidocaine. The data are fit into a mathematical model\textsuperscript{17} to separate the recovery-time constants for charged and neutral lidocaine molecules. From this result, we propose a model that suggests the impact of the external path on the drug kinetics.

### Materials and Methods

#### Clones, Molecular Biology, and Expression of Na\(^+\) Channels in Xenopus Oocytes

The hH1a \( \alpha \)-subunit (a gift from Dr. J.A. Hartmann, University of Maryland, Baltimore, Md) is one of three clones of the normal human heart channel and differs in its amino acid numbering by one from the hH1 clone.\textsuperscript{19} The mutants h-C373Y, h-T1752V, and h-(C373Y/T1752V) were prepared by oligonucleotide-directed mutagenesis with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). The \( \mu \)-subunit (provided by Dr. J.R. Moorman, University of Virginia, Charlottesville, Va) and the P-loop mutant \( \mu \)-Y401C were prepared as described previously.\textsuperscript{14,15} Stage V and stage VI Xenopus oocytes were isolated and injected with \( \approx 50 \) to 100 nanograms of cRNA, in vitro-synthesized by a T7 or SP6 transcription system (Ambion), using hH1a or rat skeletal muscle \( \mu \)-subunit clones. An equimolar cRNA of the human \( \beta \) subunit was included in all injections. The oocytes were incubated at 16°C for 24 to 72 hours before the experiments.

#### Electrophysiology and Data Analyses

Experiments used two-electrode whole-cell voltage-clamp of Xenopus oocytes using a Dagan CA-1 system (Dagan Corporation) with pCLAMP6 or pCLAMP8 software (Axon Instruments, Inc) at 50 to 60 kHz and filtered at 2 kHz. The experimental chamber of \( \approx 200 \) \( \mu \)L was filled with a buffer containing (in mmol/L) NaCl 90, CaCl\(_2\) 1, MgCl\(_2\), 1, KCl 2.5, and Hepes (OR2) 5 at 23°C to 25°C with a flow rate of 0.6 mL/min during all experiments. Buffer pH was adjusted with NaOH. Lidocaine solution was made in water at 100 mmol/L stock solutions in small aliquots and stored at \(-20\)°C until dilution into OR2 at the time of experiments. The electrodes were filled with 3 mol/L KCl with resistances between 0.3 and 0.8 MΩ. Leak currents were not subtracted, but oocytes displaying leak currents >0.05 of the peak current estimated from current-voltage relationship experiments were not used. The capacitance transients were adjusted with series resistance compensation. Data from the current-voltage relationship (−90 to +50 mV) were normalized and fit to the Boltzmann equation,

\[
I(t) = G_{\text{max}}(t - V_{\text{rev}})/(1 + e^{(V_{\text{1/2}} - t)/s})
\]

where current I is a function of t, to obtain the following: \( G_{\text{max}} \), maximum conductance; \( V_{\text{rev}} \), reversal potential; \( V_{\text{1/2}} \), half-maximal voltage; and s, slope factor. Likewise, steady-state availability (−130 to −30 mV) experiments were fit to the Boltzmann equation to obtain \( G_{\text{max}} \), \( V_{\text{1/2}} \) and s. The test pulses were to −20 mV for 10 ms.

### Results

#### Gating Kinetics

Although effects of the access path on UDB may be made by direct comparison between the cardiac skeletal muscle and brain channels, these isoforms have different gating properties that also influence LA block.\textsuperscript{22,23} Consequently, we compared lidocaine-induced UDB in the background of the cardiac isoform between the wild-type that has known QX access and mutants containing a single residue substitution in the D4 P-loop and D4S6 that should reduce access. In addition, we studied the reverse mutations in the skeletal muscle isoform for comparison with prior QX studies. To accommodate the difference in steady-state availability curves, we set the holding potentials of −120 mV for hH1a clones and −100 mV for \( \mu \)-1 clones.

Recently, several reports suggested a close relationship between intermediate inactivation (\( I_M \)) and lidocaine block of the Na\(^+\) channel.\textsuperscript{20,21} Recovery-time constants for \( I_M \) did not significantly differ among our wild-type and mutant cardiac channels (Table 1). Furthermore, the recovery-time constants for \( I_M \) did not shorten when pH was raised (57.5±11.6, 58.2±4.8, 68.8±8.1, and 71.4±10.8 ms for h-WT, h-C373Y, h-T1752V, and h-(C373Y/T1752V), respectively; n=5 to 6), unlike those for recovery from lidocaine block. Therefore, the differences in recovery from lidocaine UDB described next likely did not result from differences in \( I_M \) among the clones.

#### P-Loop Isoform-Specific Mutation Attenuates Lidocaine Recovery From UDB

The isoform-specific residue Cys373 in the outer vestibule of hH1a, which permits external QX access,\textsuperscript{14} is thought to be in the hydrophilic path for ion permeation because it interacts well with guanidinium toxins and with external Cd\(^{2+}\).\textsuperscript{24,25} If the QX path defined by C373 in the heart channel provides an egress path for lidocaine not present in \( \mu \)-1, mutation to the \( \mu \)-1-specific residue h-C373Y would be expected to slow the recovery from UDB compared with wild-type hH1a. Lidocaine has a pK\(_a\) of 7.86 in bulk solution (see next section) and exists in both charged and neutral forms in physiological pH. If this path is hydrophilic, the difference in kinetics will be more pronounced at lower pH where the charged form of lidocaine predominates and will become less significant as pH is raised.
Recovery from UDB by lidocaine can be fit by two exponential terms,

\[
c(t) = a_1 e^{-(t/\tau_1)} + a_2 e^{-(t/\tau_2)} + b_n
\]

where \(c(t)\) is current after \(t\) milliseconds of recovery after the train of pulses to induce UDB; \(\tau_1\) and \(\tau_2\) are time constants for fast and slow components, respectively; \(b_n\) is the current at \(t = \infty\) (equals the tonic-block level); and \(a_1\) and \(a_2\) are amplitudes for fast and slow components, respectively. The fast time constant relates to recovery from inactivation by drug-free channels, and the slower time constant is thought to reflect unbinding of the drug.\(^{26}\) The faster time constants ranged from 2 to 3 ms without consistent patterns and were not altered significantly by lidocaine (not shown). The slower time constants reflecting the drug unblock are listed in Table 2. As predicted, the recovery from UDB was slowed by the mutation h-C373Y (Table 2 and Figure 1A). At higher pH, the recovery-time constants for both wild-type (h-WT) and h-C373Y decreased and the differences disappeared, suggesting that the effect of C373Y was mostly on the charged drug. The reverse mutation in \(\mu_1, \mu_1-Y401C,\) accelerated the recovery of UDB. Therefore, the external QX path defined by the isoform-specific P-loop residue significantly influenced recovery kinetics for lidocaine. Furthermore, the effect was mostly on charged lidocaine, suggesting that this external path is hydrophilic.

**TABLE 1. Clones and Inactivation**

<table>
<thead>
<tr>
<th></th>
<th>h-WT</th>
<th>h-C373Y</th>
<th>h-T1752V</th>
<th>h-(C373Y/T1752V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I_0)</td>
<td>0.539±0.023/2.57±0.11</td>
<td>0.493±0.056/2.51±0.18</td>
<td>0.516±0.066/2.44±0.27</td>
<td>0.489±0.038/4.76±0.96</td>
</tr>
<tr>
<td>(a_I/\tau_1)</td>
<td>0.176±0.027/66.7±3.7</td>
<td>0.131±0.016/56.2±9.5</td>
<td>0.132±0.019/63.6±9.6</td>
<td>0.229±0.040/68.4±7.4</td>
</tr>
<tr>
<td>(a_I/\tau_2)</td>
<td>0.308±0.026/522±98</td>
<td>0.377±0.051/403±11</td>
<td>0.352±0.058/357±50</td>
<td>0.283±0.008/626±57</td>
</tr>
</tbody>
</table>

SSI

- \(V_{1/2}\) slope (lidocaine): -72.2±0.6/4.5±0.2
- \(V_{1/2}\) slope (lisocaine: -89.6±1.5/5.0±0.2

In upper half are time constants and amplitudes for recovery from fast, intermediate (I M) (see text), and slow inactivation. Normalized currents are fitted to an equation, \(I(t) = a_1[1 - \exp(-t/\tau_1)] + a_2[1 - \exp(-t/\tau_2)] + a_3[1 - \exp(-t/\tau_3)]\), where \(a_1, a_2,\) and \(a_3\) are amplitudes and \(\tau_1, \tau_2,\) and \(\tau_3\) are time constants of recovery. Time constants of recovery are expressed in milliseconds.

**TABLE 2. Time Constants for Recovery From UDB**

<table>
<thead>
<tr>
<th>pH</th>
<th>h-WT</th>
<th>h-C373Y</th>
<th>h-T1752V</th>
<th>h-(C373Y/T1752V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.20</td>
<td>207±7</td>
<td>265±8 (+28.0%)</td>
<td>244±10 (+17.9%)</td>
<td>304±13 (+46.9%)</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.001)</td>
<td>(&lt;0.05)</td>
<td>(&lt;0.05)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>7.60</td>
<td>138±6</td>
<td>162±8 (+17.4%)</td>
<td>163±8 (+18.1%)</td>
<td>182±8 (+31.9%)</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.05)</td>
<td>(&lt;0.05)</td>
<td>(&lt;0.05)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>8.00</td>
<td>99±6</td>
<td>115±6 (+16.2%)</td>
<td>113±3 (+14.1%)</td>
<td>114±7 (+15.2%)</td>
</tr>
<tr>
<td></td>
<td>(NS)</td>
<td>(NS)</td>
<td>(NS)</td>
<td>(NS)</td>
</tr>
<tr>
<td>8.30</td>
<td>87±3</td>
<td>89±2 (+2.3%)</td>
<td>92±2 (+5.7%)</td>
<td>98±3 (+12.6%)</td>
</tr>
<tr>
<td></td>
<td>(NS)</td>
<td>(NS)</td>
<td>(NS)</td>
<td>(&lt;0.05)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Time constants of recovery are expressed in milliseconds. Percent changes in comparison to the respective wild-type channels are noted in the parentheses next to the time constants. The \(P\) values for comparison to the wild-type channels are in parentheses below each time constant. h-WT, n=6; h-C373Y, n=6 to 7; h-T1752V, n=8 to 8; h-(C373Y/T1752V), n=6; \(\mu_1\)-WT, n=5 to 7; and \(\mu_1\)-Y401C, n=5 to 6.

**D4S6 Isoform-Specific Residue Attenuates Recovery From UDB**

Qu et al\(^{16}\) showed that when an isoform-specific residue in D4S6 was switched from valine in brain to threonine in heart channel, recovery from QX block slowed. The impact of “closing” this D4S6 path by T1752V mutation in the heart channel, judged by the attenuation of recovery kinetics, was somewhat less than that of the P-loop path and it diminished at pH 8.30. The S6 mutant showed a relatively constant degree of attenuation between pH 7.20 and pH 8.00, suggesting that the S6 mutation may have affected a path used by both charged and neutral forms of lidocaine (Table 2). We propose two possible models for our findings. First, there is one external path, which is influenced more significantly by the P-loop residue than by the S6 residue. Alternatively, the P-loop and S6 residues define two independent paths. The latter fits with our recent observation that cysteine-modifying MTSEA treatment of \(\mu_1\)-Y401C decreased external QX block, but MTSEA treatment of \(\mu_1\)-(Y401C/C1572T) did not effectively lessen external QX block additionally (A.S. and H.A.F., unpublished data, 2000). These two observations suggest that C1572T mutation in \(\mu_1\), analogous to T1752 in hH1a, opened up a nonpore QX path, which did not overlap with that of the Y401C mutation. Taken together, this raises the interesting possibility that the D4S6 path defined by
A. h-WT vs. h-C373Y

<table>
<thead>
<tr>
<th>pH</th>
<th>h-WT</th>
<th>h-C373Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.20</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>7.60</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>8.30</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

B. μ1-WT vs. μ1-Y401C

<table>
<thead>
<tr>
<th>pH</th>
<th>μ1-WT</th>
<th>μ1-Y401C</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.20</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>7.60</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>8.30</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Figure 1. Recovery from UDB. A, Mutation in the P-loop residue slows recovery from UDB in the heart channel. Difference in the recovery diminishes at higher pH. Current levels were normalized to \( b \), obtained by two-exponential fitting described in the text. For clarity, only the time points from 2 ms to 5 seconds are shown. Smaller graphs show magnified portions of the graph between 2 and 200 ms, where the differences are most apparent. Data points are plotted as mean ± SEM. See Table 2 for numbers of experiments. B, Converse mutation in μ1 accelerates recovery from UDB, which also diminishes as pH is raised.

T1752, like that defined by C1572 in μ1, may represent a separate nonpore "protein path" that influences unblock of both charged and neutral forms of lidocaine. However, there are no direct data in support of two separate external paths for lidocaine at the present time.

In Qu et al., substitution of D4S6 threonine residue for valine in the rat heart clone attenuated external QX block, but not to the level of the brain channel, likely reflecting the contribution of the P-loop path in the external QX block. "Shutting down" both P-loop and S6 external QX paths with a double mutant, h-(C373Y/T1752V), resulted in a more significant effect on the recovery kinetics (Table 2) that disappeared as the external pH was raised. Interestingly, the effects of the P-loop and D4S6 paths seemed to be additive. Thus, there seems to be little overlap in the impact on drug egress between the P-loop and S6 residues, perhaps suggesting two independent paths.

Tonic and UDB

Tonic block levels were similar for h-WT and h-C373Y and for μ1-WT and μ1-Y401C at a given pH (Table 3 and Figure 2). UDB at 20 Hz (10-ms pulses) resulted in further decrease of current with the steady-state block reaching near 90%. Each clone showed enhanced tonic block and less UDB at pH 8.30 compared with pH 7.20, resulting in a similar level of final steady-state block (tonic plus UDB) (Figure 2). The h-T1752V and h-(C373Y/T1752V) also showed similar degree of tonic and UDB (Table 3). \( V_{1/2} \) values of steady-state inactivation with and without lidocaine were not significantly altered in these mutants compared with h-WT (Table 1). The
results suggest that the mutations did not cause significant alterations in intrinsic affinities for the drug. We modeled the onset kinetics of UDB by fitting the normalized data with a single exponential term,

\[ b(n) = b_\text{n\_tonic} + (1 - b_\text{n\_tonic}) e^{-\frac{n}{\tau}} \]

where \( b(n) \) indicates block at the \( n \)th pulse; \( b_\text{n\_tonic} \), the steady-state level of remaining fraction of current after UDB (reflecting UDB only); and \( \tau \), the first-order time constant of decay. In Table 3, time constants and amount of UDB, \((1 - b_\text{n\_tonic})\) in Equation 3, are listed. First-order onset time constants of UDB were increased in h-C373Y, h-T1752Y, and h-(C373Y/T1752V) compared with h-WT. The change in h-C373Y (versus h-WT) was not statistically significant, perhaps because of the difficulty of measuring these short time constants with a pulse train of 20 Hz, or 50 ms per episode. As in recovery, onset kinetics of UDB also accelerated as the pH was raised, and the effects of the mutations disappeared at pH 8.30.

### Modeling of the Drug Egress Paths

Extracellular pH influences ionization of bound lidocaine molecules into charged and neutral forms based on the p\( K_a \).

\[ \text{charged } \Rightarrow \text{neutral} \]

In a simplified model of drug and channel kinetics (Figure 3A), recovery (or unbinding of drug) of the individual drug-bound channel can be described in a first-order exponential decay function with a rate constant of \( 1/\tau \). The apparent rate constant for the scheme in Figure 3A is as follows:

\[ k_{\text{apparent}} = 1/\tau_{\text{apparent}} = \left( \text{fraction of BL}^+ / \text{charged} \right) + \left( \text{fraction of BL}^0 / \tau_{\text{neutral}} \right) + \left( \text{fraction of BL}^- / \tau_{\text{dissociation}} \right) \]

After rearranging, it becomes:

\[ \tau_{\text{apparent}} = \left( 1 + R \right) / \left( R / \tau_{\text{neutral}} + 1 / \tau_{\text{dissociation}} \right) \]

where \( R \) denotes the fraction of BL\(^0\)/BL\(^+\) and equals, by Henderson-Hasselbalch relationship,

### Table 3. Tonic and UDB

<table>
<thead>
<tr>
<th>pH</th>
<th>h-WT</th>
<th>h-C373Y</th>
<th>h-T1752V</th>
<th>h-(C/Y+T/V)</th>
<th>( \mu_1 )-WT</th>
<th>( \mu_1 )-Y401C</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.20</td>
<td>0.364±0.018</td>
<td>0.376±0.012</td>
<td>0.357±0.026</td>
<td>0.403±0.019</td>
<td>0.452±0.010</td>
<td>0.463±0.025</td>
</tr>
<tr>
<td>8.30</td>
<td>0.536±0.014</td>
<td>0.553±0.017</td>
<td>0.550±0.014</td>
<td>0.547±0.006</td>
<td>0.542±0.019</td>
<td>0.593±0.027</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Tonic block is described as the fraction of current blocked by lidocaine. \( \tau \) values, expressed in milliseconds, denote first-order time constants of UDB onset as noted in the text. \((1 - b_\text{n\_tonic})\) values denote fraction of current blocked by the train of UDB after tonic block. Thus, total fraction of current blocked after the tonic and UDB can be calculated by \( (\text{tonic block}(1 - b_\text{n\_tonic})) n = 6 \) to \( 8 \) for all data points.

*\( P < 0.05 \) compared with the wild-type channel.
pKa values of lidocaine in a bulk solution depend on the pH points (pH 6.9 and 7.4), despite different experimental conditions and methods; \( \tau_{\text{charged}} \) and \( \tau_{\text{neutral}} \) were 476 and 43 ms, respectively. Interestingly, a similar treatment for h-C373Y yielded \( \tau_{\text{charged}} \) and \( \tau_{\text{neutral}} \) of 704 and 68.6 ms, respectively (Figure 3B and Table 4). We conclude, in a more quantitative manner than comparing time constants, that the isoform-specific mutation C373Y altered the egress path for charged lidocaine, blocked channel with neutral lidocaine, charged lidocaine, neutral lidocaine, and unblocked/recovered channel, respectively. PKa* denotes the apparent pKₐ of the bound lidocaine at the binding site. \( k_{\text{charged}} \) and \( k_{\text{neutral}} \) represent first-order rate constants for charged and neutral lidocaine, respectively. B, Fitting of the recovery-time constants listed in Table 2 with the mathematical model discussed in the text, where charged and neutral lidocaine distribute according to pKₐ of bound lidocaine (in this case, 7.86) and recover/unblock with separate time constants and paths. Closed circles represent data for h-WT and open circles for h-C373Y. All data are shown as mean±SEM. The result of fitting is also shown within the graph. See also Table 4 and the text. C, Fitting the data for converse mutation in \( \mu \)T shows that \( \tau_{\text{charged}} \) has significantly decreased with a relatively small change in \( \tau_{\text{neutral}} \).

### Discussion

The outside QX access paths associated with P-loop and D4S6 cardiac isoform-specific residues also influence lidocaine block, as shown here for UDB kinetics. The more obvious effect is on the recovery rate from UDB, which plays an important role in the clinical actions of the tertiary amine LA drugs. Onset kinetics were also changed, but limitations of the protocols made these changes less accurate and consistent. Limited access/egress paths would be expected to result in higher level of UDB. We did observe a trend of increased UDB in our mutants [Table 3, see (1–b)] at pH 7.20 for the heart channels], even with limitations in the protocols.

The effects of access paths on lidocaine appear to be related to the charged form of the drug, which most closely resembles the permanently charged QX analogues. This is consistent with the expectation that the P-loop mutation C373Y alters a hydrophilic path. This interpretation fits well the theoretical framework proposed by Broughton et al. The role of the hydrophilic path was clear for the P-loop-induced path, but less clear for the D4S6 path. The two paths seemed to be affected differently by pH change, and they are additive, leading us to suggest that the paths are separate, with the D4S6 path being through the protein itself. However, it remains equally possible that there is only one external access path, which is more influenced by the P-loop residue than the H9270}

### Table 4. Fitted Time Constants for Charged and Neutral Forms of Lidocaine

<table>
<thead>
<tr>
<th></th>
<th>h-WT</th>
<th>h-C373Y</th>
<th>h-T1752V</th>
<th>h-(C373Y/T1752V)</th>
<th>( \mu )-WT</th>
<th>( \mu )-Y401C</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \tau_{\text{charged}} )</td>
<td>394</td>
<td>704</td>
<td>526</td>
<td>1030</td>
<td>954</td>
<td>484</td>
</tr>
<tr>
<td>( \tau_{\text{neutral}} )</td>
<td>64.9</td>
<td>68.6</td>
<td>71.2</td>
<td>72.1</td>
<td>128</td>
<td>118</td>
</tr>
</tbody>
</table>

Recovery-time constants in milliseconds from the mathematical fitting described in the text are listed. The values reflect the fit with an assumed lidocaine pKₐ of 7.86.
Figure 4. Schematic model of lidocaine recovery paths. Diagrammatic representation for recovery paths for charged lidocaine in each channel clone is shown with the corresponding recovery-time constants (and rate constants in parentheses). Derivation of each value is described in the text in detail. Thick dashed arrows represent the sum of all the paths for neutral lidocaine. Names of clones are as described in the text, except for h-(C373Y/T1752V), which is noted as h-C/Y+T/V. Shaded oval figures with plus sign represent lidocaine molecules blocking the channel. Oval figures with Y and V represent tyrosine and valine residues, respectively, and circles with C and T represent cysteine and threonine residues, respectively.

D4S6 residue. The much faster hydrophobic path also made it more difficult to measure the outside access path(s) with alkalinization.

How important is the external path? If we assume that the mutations used in these experiments had no direct effects on the inside access path and that the C373Y and T1752V paths are additive as suggested from our results, either from sharing little overlap or being separate, we can estimate their relative effects on recovery rates. For example, in h-WT the overall recovery process through the external hydrophilic path is described as follows:

\[ \frac{1}{\tau_{\text{charged}}} = \frac{1}{\tau_{\text{P-loop}}} + \frac{1}{\tau_{\text{S6}}} + \frac{1}{\tau_{\text{cytoplasmic}}} \]

where \( \tau_{\text{charged}} \), \( \tau_{\text{P-loop}} \), \( \tau_{\text{S6}} \), and \( \tau_{\text{cytoplasmic}} \) represent rate constants for overall, P-loop, S6, and cytoplasmic paths, respectively; and \( \tau_{\text{P-loop}} \), \( \tau_{\text{S6}} \), and \( \tau_{\text{cytoplasmic}} \) represent time constants for P-loop, S6, and cytoplasmic paths, respectively. The fitted time constants are taken from Table 4 and the results are shown in Figure 4. At this time, we draw one common external path for both the P-loop and S6 residues due to lack of direct evidence for their spatial independence.

We start with h-(C373Y/T1752V), in which the S6 and P-loop paths are negligible. Thus, \( \tau_{\text{cytoplasmic}} = \tau_{\text{charged}} = 1030 \) ms. In h-T1752V, the P-loop path (Cys373) path is “opened,” resulting in speeding up of recovery from UDB for charged molecules. Impact of opening up the P-loop path can be estimated. \( \tau_{\text{charged}} = \frac{1}{\tau_{\text{charged}}} = \frac{1}{(1/526) + (1/1030)} = 1070 \) in a similar manner, from h-C373Y, \( \tau_{\text{P-loop}} = 2220 \). In h-WT, both of the external paths are open. Then, \( \tau_{\text{external}} = \frac{1}{(1/\tau_{\text{P-loop}}) + (1/\tau_{\text{S6}}) = (1/1030) - (1/1030) - (1/1030) = 1.56 \times 10^{-3} \) ms, or \( \tau_{\text{external}} = 0.638 \) ms.

The \( \mu l \) Na\(^+\) channel isoform has different gating kinetics, so it is difficult to compare its UDP recovery rates directly with those of the cardiac isoform. Nonetheless, the direction of change in recovery for the P-loop mutation Y401C is as expected. Given the different gating kinetics and holding potentials in our protocols, it is difficult to expect that the recovery-time constants for \( \mu l \)-WT would be the same as those for h-(C373Y/T1752V). However, we cannot rule out the possibility that the cardiac isoform has another as yet unidentified residue contributing to its outside access path(s).

Hille\(^a\) estimated the narrowest part of the Na\(^+\) channel pore to be \( =3\) Å by \( 5\) Å. It is difficult to imagine how molecules such as lidocaine or QX are able to access the binding site located below the selectivity filter through the pore from outside, as implied by previous studies\(^7,14,16\) and our present report. Moorman et al\(^29\) proposed a model in which all bound drug unblocks in a neutral form through a hydrophobic route alleviating the need for permeation through the narrow selectivity filter. In their model, deprotonation rate of the bound drug is the rate-limiting step in recovery from drug block. Our data from h-WT fit their model fairly well, but it could not accommodate the data from our P-loop, D4S6, and double mutants; the deprotonation rates no longer became rate-limiting (not shown). Indeed, Wendt et al\(^30\) by deuterium exchange experiments, reported that proton exchange was rapid and not rate-limiting. Recently, Huang et al\(^31\) observed that large hydrophobic molecules of diameter up to \( 15\) Å could permeate through the Na\(^+\) channel with certain selectivity filter mutants and suggested that hydrophobic interphases contiguous to the pore can facilitate permeation of large molecules. Perhaps the charged aliphatic portion of the LA drug uses the direct route through the pore through the selectivity filter and the rest of the molecule “slips out” of the pore through interphases between P-loops and S6 helices.\(^32\)

Our schematic model presented above, although semiquantitative, offers an opportunity to visualize the paths important in lidocaine UDB in terms of their impact on drug kinetics. We conclude that, in the heart channel, the external paths for lidocaine may play a more significant role in drug kinetics than the cytoplasmic path. Drugs using such external paths would be specifically influenced by their ionization states, ability/inability to cross membrane, and any factors affecting the external channel structures. Drugs that block the channel only in the open state might be more profoundly affected when hydrophilic pore paths are altered. Recently, Grant et
reported data suggesting that disopyramide and flecainide use different paths to block and unblock the Na\(^+\) channel during UDB. It is likely that the relative potencies of the different egress paths described in our study are drug-specific. Understanding the nature of the access/egress paths specific to an antiarrhythmic drug is likely to be crucial in understanding its characteristics of UDB of the Na\(^+\) channel.

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