Channel Openings Are Necessary but not Sufficient for Use-dependent Block of Cardiac Na⁺ Channels by Flecainide: Evidence from the Analysis of Disease-linked Mutations

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ABSTRACT Na⁺ channel blockers such as flecainide have found renewed usefulness in the diagnosis and treatment of two clinical syndromes arising from inherited mutations in SCN5A, the gene encoding the α subunit of the cardiac voltage-gated Na⁺ channel. The Brugada syndrome (BrS) and the LQT-3 variant of the Long QT syndrome are caused by disease-linked SCN5A mutations that act to change functional and pharmacological properties of the channel. Here we have explored a set of SCN5A mutations linked both to BrS and LQT-3 to determine what disease-modified channel properties underlie distinct responses to the Na⁺ channel blocker flecainide. We focused on flecainide block that develops with repetitive channel activity, so-called use-dependent block (UDB). Our results indicate that mutation-induced changes in the voltage-dependence of channel availability (inactivation) may act as determinants of flecainide block. The data further indicate that UDB by flecainide requires channel opening, but is not likely due to open channel block. Rather, flecainide appears to interact with inactivation states that follow depolarization-induced channel opening, and mutation-induced changes in channel inactivation will alter flecainide block independent of the disease to which the mutation is linked. Analysis of flecainide block of mutant channels linked to these rare disorders has provided novel insight into the molecular determinants of drug action.

INTRODUCTION

Local anesthetic molecules such as lidocaine and flecainide block Na⁺ channels and have been used therapeutically to manage cardiac arrhythmias (Rosen et al., 1975; Rosen and Wit, 1983; Wit and Rosen, 1983). Despite the prospective therapeutic value of the inherent voltage- and use-dependent properties of channel block by these drugs in the treatment of tachyarrhythmias, their potential has been overshadowed by toxic side effects (Rosen and Wit, 1987; Weissnburger et al., 1991). Recently however, Na⁺ channel blockers have again proven useful for diagnosis and treatment of Brugada and LQT-3 syndromes, two inherited diseases linked to mutations in SCN5A, the gene that encodes the α subunit of the cardiac voltage-gated Na⁺ channel (Brugada et al., 1999). Na⁺ channel blockade by the flecainide is of particular interest as it had been shown to reduce QT prolongation in carriers of some LQT-3 mutations (Brugada et al., 1999), and (Benhorin et al., 2000; Windle et al., 2001) and to evoke ST segment elevation, a hallmark of the Brugada syndrome (BrS), in patients with a predisposition to the disease (Brugada et al., 2000). Thus, in the case of LQT-3, flecainide has potential therapeutic application, whereas for BrS it has proven useful as a diagnostic tool. However, in some cases, flecainide has been reported to provoke BrS symptoms (ST segment elevation) in patients carrying LQT-3 mutations (Priori et al., 2000). Furthermore, flecainide preferentially blocks some LQT-3 or BrS-linked mutant Na⁺ channels (Abriel et al., 2000; Grant et al., 2000; Nagatomo et al., 2000; Viswanathan et al., 2001). Investigation of the drug interaction with these and other LQT-3- and BrS-linked mutations may indicate the usefulness of flecainide in the detection and management of these disorders and in determining whether or not it is reasonable to use this drug to identify potential disease-specific mutations. Moreover, exploration of the relationship between altered channel structure and drug efficacy can provide new insight into molecular determinants of flecainide block of the human cardiac sodium channel.

Local anesthetic compounds, such as lidocaine and flecainide, block Na⁺ channels in a use- and voltage-dependent manner. These actions have been widely interpreted within the framework of the modulated receptor hypothesis that ion channel states can alter drug affinity and that charged and neutral drugs interact with a common receptor but gain access to it via distinct hydrophobic and hydrophilic pathways (Hille, 1977a; Hondeghem and Katzung, 1977). Use-dependent block by flecainide, but not lidocaine, depends critically on channel openings (Ragsdale et al., 1994; Qu et al., 1994; Ragsdale et al., 1995; Qu et al., 1996; Ragsdale et al., 1997; Qu et al., 1998; Ragsdale et al., 1999). Under these conditions, drug affinity changes from a lower (in the open state; UDB, use-dependent block; WT, wild-type) to a higher (in the closed state; CDB, channel-dependent block) level as the channel closes.

Moreover, the interaction of a drug with a channel is dependent on the affinity of the drug for channel states in the presence or absence of the drug. For example, the affinity of local anesthetics for closing of the channel is increased by the drug, whereas the reverse is true for opening. Thus, drug modulation of channel gating is an important aspect of drug action, and the relationship between drug affinity and channel state has been of interest in understanding drug action.
1995), suggesting that flecainide preferentially blocks channels in the open state. Use-dependent block (UDB) by lidocaine has been more closely related to the inactivated state of the channel (Bean et al., 1983). The precise mechanism underlying this difference is not clear; however, it is very likely related to the influence of drug charge on access to receptor site(s) on the channel (Strichartz, 1973; Hille, 1977a; Chernoff and Strichartz, 1990). Lidocaine has a pKa of 7.6–8.0 and thus may be up to 50% neutral at physiologic pH, and flecainide has a pKa of ~9.3 leaving <1% neutral at pH 7.4 (Hille, 1977b; Strichartz et al., 1990). Thus, the drug charge might be an important determinant of its state specificity of Na+ channel blockade as well as its distinct kinetic properties (Strichartz, 1973; Hille, 1977a; Chernoff and Strichartz, 1990).

The results of our work indicate that flecainide block that develops with repetitive channel activity, so-called UDB indeed requires channel opening. However, the efficacy of drug action is critically related to inactivation that occurs subsequent to channel opening (Fig. 1). Several recent studies have suggested that intermediate inactivation state conformations can be arrived at by closed state inactivation pathways or via channel opening–induced inactivation (Kambouris et al., 2000; Ong et al., 2000; Wang et al., 2000). Our data support a role of inactivation in flecainide UDB, but suggest that channel opening is required so that flecainide may gain access to the site of action. These sites are altered in their voltage dependence compared with wild-type (WT) in the steady-state that is reflected in the channel availability curve, an indicator of inactivation from closed states. Our results predict, and we confirm, that mutations that alter the voltage-dependence of channel inactivation will alter flecainide UDB and that the efficacy of flecainide in treating inherited arrhythmias due to SCN5A mutations will depend critically on the effects of the mutations on the voltage-dependence of channel inactivation. Further, our data clearly show that these mutation-induced changes of gating determine drug action independent of the disease (BrS or LQT-3) to which the mutation is linked and thus indicate that flecainide block cannot be used to discriminate between BrS and LQT-3 mutant channels.

MATERIALS AND METHODS

Mutagenesis and Expression of Recombinant Na+ Channels

Mutations of SCN5A were engineered into WT cDNA cloned in pcDNA3.1 (Invitrogen) by overlap extension using mutation-specific primers and Quick Change site-directed mutagenesis kit (Stratagene) as described previously (Abriel et al., 2001). The presence of the mutation was confirmed by sequence analysis. WT and mutant Na+ channels were expressed in HEK293 cells as previously described (Abriel et al., 2001). Transient transfections were performed with equal amounts of Na+ channel α subunit and with hβ1, subcloned individually into the pcDNA3.1 (Invitrogen) vector (total cDNA 2.5 μg) using a previously described lipo-fection procedure (Abriel et al., 2001). Expression of channels was studied using patch clamp procedures 48 h after transfection.

Electrophysiology

Membrane currents were measured using whole cell patch-clamp procedures with Axopatch 200B amplifiers (Axon Instruments, Inc.). Recordings were made at room temperature (22°C). The internal pipette solution contained (in mmol/L): aspartic acid 50, CsCl 60, Na2-ATP 5, EGTA 11, HEPES 10, CaCl2 1, and MgCl2 1, with pH 7.4 adjusted with CsOH. Activation curves were determined by normalization of peak current vs. voltage to driving force determined as the difference between test voltage and measured channel reversal potential in low external Na+. UDB was induced by imposing conditioning trains of 100–300 pulses (−10 mV, 25 ms) from a −100 mV holding potential (HP) (unless otherwise specified) at frequencies between 1 and 10 Hz. This was sufficiently long to induce steady-state UDB for each construct. UDB was measured as the ratio of peak current at −10 mV after and before application of a conditioning train and is reported as the percentage block of peak current.

Protocols for measuring the time course of recovery from, and isochronal availability after, flecainide block were as follows. Flecainide block of channels was induced by application of a conditioning train (see above) such that >90% of channel activity was blocked. Then, for isochronal availability, a 500-ms pulse was applied to a conditioning voltage followed by measurement of current in response to a pulse to −10 mV. After a 10-s pulse-free interval at the holding potential, the trial was repeated, channel block was induced by the conditioning train, and a 500-ms conditioning pulse was imposed to a different voltage. In the case of the time course of recovery from drug block, a similar voltage trial was followed, but in this case, after application of the conditioning

![Figure 1](image-url)  
**Figure 1.** Modulated receptor scheme for preferential drug binding to inactivated or open channels. R is the rested, O the open, and I the inactivated states of drug-free Na+ channels; RD, OD, and ID are representative states for drug-associated Na+ channels.
train to block channels, current was measured in response to a −10-mV test pulse applied after a variable pulse-free interval at the holding potential. As above, voltage trials were separated by 10-s pulse-free intervals at the holding potential, and each trial consisted of application of a pulse train to induce channel block.

Pclamp 8.0 (Axon Instruments, Inc.), Excel (Microsoft), and Origin (Microcal Software) were used for data acquisition and analysis. Data are presented as mean values ± SEM. *P < 0.05 was considered statistically significant (using Student’s t test).

RESULTS

LQT-3 and BrS Mutations Alter Sensitivity to UDB by Flecainide

We have demonstrated previously that LQT-3 D1790G mutant channels are more sensitive than WT channels to UDB by flecainide (Abriel et al., 2000). To determine whether this altered sensitivity was due to mutation-induced changes in COOH-terminal structure or channel gating, we compared flecainide-induced UDB of three previously described COOH-terminal disease-linked mutant channels (D1790G, Y1795C, and Y1795H) with distinct biophysical phenotypes (An et al., 1998; Rivolta et al., 2001). Fig. 2 A illustrates this measurement for each construct and shows the superimposition of current (at −10 mV) before and after (arrows) a 1-Hz conditioning voltage pulse train in the presence of flecainide (10 μM). At this frequency, as is evident by the arrows in the figure, the inherited COOH-terminal mutations confer differing sensitivities to UDB by flecainide. This point is substantiated over a range of conditioning pulse frequencies (Fig. 2 B), at

![Figure 2](https://example.com/figure2.png)

**Figure 2.** UDB of WT and mutant Na⁺ channels by flecainide. Currents were evoked by imposing conditioning trains of 100–300 pulses (−10 mV, 25 ms) from a holding potential of −100 mV in the presence of flecainide (1 μM to 1 mM) and over a frequency range of 1–10 Hz. Pulses were applied until steady-state UDB (defined as the percent decrease in current after the pulse train relative to current before the pulse train) was achieved. (A) Examples of current traces at recorded at 1 Hz for wild-type (WT), D1790G (DG), Y1795C (YC), and Y1795H (YH) channels before and after steady-state UDB (arrows). Currents are superimposed records of first and last (100th) pulse of a conditioning train. (B) Percent UDB plotted vs. conditioning pulse frequency (1–10 Hz) in the presence of 10 μM flecainide. (C) UDB for each construct at a fixed pulse frequency (5 Hz) but over a range of flecainide concentrations (1 μM to 1 mM). The smooth curves are the best fits of the Hill equation 1/[1 + [(drug)/EC₅₀]ⁿ] to the data. The estimated EC₅₀ and n values obtained from the fits are 11.2 ± 1.4 μM, −1.27 ± 0.13 (WT); 2.8 ± 0.4 μM, −1.29 ± 0.11 (DG); 10.8 ± 1.4 μM, −1.14 ± 0.07 (YC), and 2.9 ± 0.3 μM, −1.17 ± 0.09 (YH). *P < 0.01 compared with WT, n = 3–5 cells per measurement.
fixed flecainide concentration (10 μM), and at a fixed pulse frequency (5 Hz), and over a broad flecainide concentration range (Fig. 2 C). At a fixed flecainide concentration (10 μM), ~30% of peak current was blocked in a use-dependent manner for WT channels at a conditioning pulse frequency of 1 Hz, and the block almost doubled when the pulse frequency was increased from 1 to 10 Hz (Fig. 2 B). Y1795H channels, like D1790G channels, exhibited greater UDB than WT channels. In fact, there is no significant difference in the UDB of Y1795H or D1790G channels over the frequency or concentration range studied, whereas, at the same time and same range of experimental conditions, frequency-dependent block of both D1790G and Y1795H channels is significantly greater than WT channels. In contrast, there is no significant difference between UDB for Y1795C and WT channels over the same range of experimental conditions. As indicated in Fig. 2 C, the estimated EC50 for UDB of D1790G and Y1790H channels is approximately threefold less than block of either WT or Y1975C channels. Importantly, these data indicate that UDB of peak current by flecainide cannot be used to identify LQT-3 vs. BrS mutations, as the Y1795H mutation (Rivolta et al., 2001) is linked to BrS and the D1790G mutation is linked to LQT-3 (Benhorin et al., 1998). What properties of the mutant channels cause these differences in sensitivity to flecainide UDB?

**UDB by Flecainide Requires Channel Openings**

Mutations could alter UDB during a pulse train either by altering the amount of drug that binds to and dissociates from open or resulting inactivated channels during the depolarizing pulses or by altering the rate at which the drug dissociates between the pulses as the channels return to the resting state. We reported previously that the D1790G mutation slows the recovery from flecainide block at negative voltages (Abriel et al., 2000). Here, we next sought to determine the contribution of channel openings to flecainide UDB, and applied the voltage dependence of channel activation (determined as in MATERIALS AND METHODS) is shown for the four constructs investigated. n = 3–4 cells per measurement.

![Figure 3. Influence of inherited mutations on the voltage dependence of flecainide UDB.](image-url)
protocol illustrated in Fig. 3 for channels encoded by each construct. Currents were measured before (control) and after (test) applying conditioning pulses (100 pulses, 25 Hz) of varying amplitude in the presence of 10 μM flecainide, a protocol similar to that used previously by Ragsdale et al. (1996). A 1-s delay was imposed between the conditioning train and test pulse to allow drug-free channels to recover from inactivation that developed during the train. Normalized block was determined as the fraction of test pulse current (normalized to control current) reduced by the conditioning train for each construct. Normalized block was plotted vs. conditioning pulse amplitude in Fig. 3. Drug-free currents (open symbols) are not affected by the protocol, indicating full recovery from inactivation during the pulse free interval at −100 mV. In contrast, the conditioning train induces block for each construct and the threshold for block is near −40 mV (arrow in figure). Fig. 3 B shows activation curves for WT and the three COOH-terminal mutant channels to confirm that the threshold for channel openings (arrow in Fig. 3 B) is virtually indistinguishable from the threshold for UDB. Importantly, there is no UDB if the conditioning voltages vary from −80 mV to −50 mV, despite the fact that for each of the constructs tested closed-state inactivation channel availability changes from 100% to 0% over this
voltage range. Thus, channel openings are necessary for flecainide UDB of WT as well as D1790G, Y1795H, and Y1795C channels. Therefore, use-dependent flecainide block does not appear to develop from the closed state, excluding the possibility that increased closed-state inactivation underlies this process (Kambouris et al., 2000; Viswanathan et al., 2001).

Fig. 2 illustrates the effect of COOH-terminal mutations on UDB, whereas Fig. 3 demonstrates the necessity of channel opening for the development of this block. To rule out the possibility that flecainide UDB is due to block of open channels, we tested the effect of channel mean open time (MOT) on drug affinity. If flecainide blocks open channels, then it follows that
mutation-induced changes in MOTs would be expected to parallel mutation-induced changes in UDB (Anno and Hondeghem, 1990). Channel mean open times are significantly shorter for D1790G and Y1795H, and significantly longer for Y1795C channels compared with WT channels (Fig. 4, A and B). If flecainide were to block open channels, we would expect that D1790G and Y1795H would exhibit less UDB than Y1795C channels. However, we find the opposite: Y1795C are less sensitive to flecainide UDB than either Y1795H or D1790G channels. Together, these results strongly suggest that flecainide requires channels to open first in order to bind, but is not dependent on the open state to promote block. This possibility is further supported by the data presented in Fig. 4 C, which compares the time course of the onset of block for WT and Y1795C channels. Here the development of block is clearly faster for Y1795C channels, but the steady-state block is the same as WT. This effect might be expected if entry through open channels is a prerequisite for block, but that affinity of block is not determined by the open state of the channel. Our data raise another interesting possibility that the resulting inactivated state may be playing an important role in flecainide block.

**Evidence for Interactions with Inactivated Channels**

The COOH-terminal tail of the Na$^+$ channel α subunit plays a critical role in control of inactivation (Mantegazza et al., 2001; Cormier et al., 2002). Point mutations in the COOH terminus can shift the voltage-dependence and alter kinetics of inactivation (An et al., 1998; Benhorin et al., 1998; Bezzina et al., 1999; Wei et al., 1999; Akai et al., 2000; Veldkamp et al., 2000; Rivolta et al., 2001). If UDB by flecainide is associated with preferential interaction with the inactivated state, a shift in voltage-dependent availability by flecainide would be expected (Bean et al., 1983; Snyders and Hondeghem, 1987). We therefore first determined the influence of flecainide on WT Na$^+$ channel availability in the absence and presence of conditioning pulses (Fig. 5 A). Because the kinetics of drug unblock are so slow (see below), we did not measure steady-state availability in the presence of drug. Instead, we measured channel availability using prepulses of fixed intervals (500 ms, inset Fig. 5). Because channel openings are required for flecainide UDB, the availability curve of drug-associated channels was measured after conditioning trains (protocol shown as inset Fig. 5). Both curves were fitted with Boltzmann equations to determine the influence of flecainide on the voltage for which half of the channels are available to conduct ($V_{1/2}$) and the slope factor for the curve ($V_k$). Under drug-free conditions, the conditioning train has only a small effect on WT channel availability due to channel inactivation induced by the conditioning pulses (Fig. 5 A, ○, drug-free no train; □, drug-free plus conditioning train). However, in the presence of flecainide (10 μM), channel availability is markedly altered after (■), but not in the absence of conditioning pulses (●). After conditioning pulses, $V_{1/2}$ is shifted in the presence of flecainide in the hyperpolarizing direction (−87.4 ± 3.0 mV [flecainide] vs. −73.3 ± 3.0 mV [control], n = 5, P < 0.01) and $V_k$ is reduced (10.1 ± 2.0 mV [control] vs. 7.3 ± 1.0 mV [flecainide], P < 0.01). Normalization of the availability curves in the presence of flecainide (dotted curves, Fig. 5 A) clearly shows the effect of the drug on the voltage-dependence of channel availability, similar to the effects of both flecainide and quinidine on native Na$^+$ channels in guinea pig myocytes (Anno and Hondeghem, 1990; Snyders and Hondeghem, 1990). Together, these results are consistent with preferential flecainide block of inactivated channels, but only after channels are opened during the conditioning train.

**Altered Channel Availability Alters Flecainide Block**

These results suggest that mutations that affect the voltage-dependence of channel availability may influence flecainide block through interactions with inactivated channels. We took advantage of the effects of the D1790G, Y1795H, and Y1795C mutations on the voltage-dependence of channel availability (An et al., 1998; Rivolta et al., 2001) to test this prediction. Fig. 5, B–D, summarizes these experiments and shows strong correlation between mutation-induced changes in drug-free availability and the voltage-dependence of channel availability in the presence of drug. In the case of the Y1795C mutation, which has little effect on the voltage-dependence of inactivation compared with WT channels (compare open triangles, Fig. 5 C, with open circles, Fig. 5 A), flecainide-altered availability is virtually identical to flecainide-altered availability of WT channels. However, in the case of both D1790G and Y1795H channels, which shift inactivation of drug-free channels in the hyperpolarizing direction (compare open circles, Fig. 5 B, and triangles, Fig. 5 D, with open circles, Fig. 5 A), the channel availability curve is markedly shifted to more negative voltages after conditioning pulses in the presence of drug. Furthermore, after normalization (dotted curves, Fig. 5, B and D) and estimation of Boltzmann parameters for availability in the presence of flecainide, it is clear that channel availability in the presence of flecainide is approximately the same for both mutations.

The flecainide-altered availability curves presented in Fig. 5 reflect mutation-induced differences in the number of channels that recover from drug-block for fixed time periods (500 ms) at different conditioning voltages. Hence, these curves may be considered isochronal (500 ms) for recovery from block and inactivation rather than true steady-state availability curves in
the presence of drug, and the parameters describing the curves (see Table I) only estimate channel availability in the presence of drug.

To estimate more accurately times needed for steady-state recovery from drug block, we next measured the time course of recovery from UDB by flecainide for WT and the three mutant channel constructs. In these experiments, we applied a conditioning train of pulses for a fixed duration and frequency to induce flecainide block of channels. As illustrated in Fig. 6, in the absence of drug two components of recovery were apparent, with >75% of the recovery occurring within 100 ms for both WT and mutant channels. This is consistent with rapid recovery from inactivation of unblocked channels. In the presence of flecainide, the recovery of functional channels is very different: D1790G (DG) and Y1795H (YH) channels recover very slowly. Even at 10 s at the holding potential (−100 mV) under pulse-free conditions, only a small fraction of the flecainide-blocked current recovers. The slower recovery kinetics for drug-bound DG and YH channels are consistent with enhanced UDB found for these mutants.

To determine whether or not both WT and mutant channels eventually recover fully from drug block, even in the absence of channel openings, we further measured the recovery from UDB for WT and DG
channels over extremely long pulse-free intervals at 
\(-120\) mV. These data, summarized in Fig. 7, indicate 
that after extremely long periods (100 s) at this voltage, 
even DG channels recover from UDB.

We then fit the data summarized in Fig. 6 with func-
tions containing two exponential components under 
two different conditions. First, we allowed the fitting 
routine (MATERIALS AND METHODS) to vary not only the 
time constants and relative amplitudes of the two expo-
nential components, but also the estimated steady-state 
fraction of current that recovers. Because the recovery 
process is so slow (Fig. 7) over the limited time course 
of the experiments in Fig. 6, this method underesti-
mates the total fraction of recovered current and hence 
also underestimates any slow time constant of recovery 
that may contribute to this process. In a second ap-
proach, we took the data of Fig. 7 and assumed that, 
given enough time, all channels would eventually re-
cover so the steady-state fraction of recovered current 
was set to 1 and fixed in the fitting routine. In a second 
approach, we took the data of Fig. 7 and assumed that, 
given enough time, all channels would eventually re-
cover so the steady-state fraction of recovered current 
was set to 1 and fixed in the fitting routine. This pro-
ceedure results in estimates of slow time constants that are 
much longer than those obtained with the former 
methodology, but based on the data of Fig. 7, are likely 
to be a more accurate estimation. The data summa-
rized in Table I provide a range of time constants for 
recovery from block which shows a clear mutation-
induced slowing of the recovery from drug block that is 
most pronounced for YH and DG channels. Again, this 
very slow recovery precludes quantitative measurement 
of the voltage-dependence of steady-state inactivation 
in our cells (Fig. 5), but it does allow us to analyze the 
recovery data in two ways (see Table I). In the first row, 
corresponding to flecainide block, are functions fit to 
the actual data in Fig. 7 (where we allow the fitting rou-
tine to extrapolate the fraction of steady-state recov-
ered current. We have also included (next row) the fits 
to data assuming complete recovery from block (equi-
librium value = 1), which we believe reflects true 
steady-state conditions. In either case, the results show 
clear mutation-induced slowing of recovery that paral-
lels the mutation-induced shifts of drug-free availability 
(Table II).

These data are consistent with the modulated recep-
tor framework in which drug-bound channels transition 
from a high affinity–inactivated state to a lower af-
finity drug-bound and rested state from which drug dis-
associate from the channel (Hille, 1977a; Bean et al., 1983).

Our results thus implicate a crucial role of the inactivated state of the Na\(^+\) channel in the development of flecainide UDB. To test if this is indeed the case, we extended the analysis of flecainide UDB to a series of Na\(^+\) channel mutations that alter the voltage-dependence of channel availability and that have been described in more detail in other studies. As predicted, we found significantly greater UDB by flecainide for mutations that cause negative shifts in the voltage-dependence of channel availability (inactivation) and less UDB for mutations shift availability in the positive direction. Furthermore, mutations that cause negative shifts in channel availability (DG, YH) also markedly slow the recovery from drug block of these channels. These data, summarized in Tables I and II, support the hypothesis that the development of UDB by flecainide is due to preferential block of inactivated channels.

### Table I

<table>
<thead>
<tr>
<th>Mutations</th>
<th>A1 (fraction recovering at (\tau_1))</th>
<th>(\tau_1) (ms)</th>
<th>A2 (fraction recovering at (\tau_2))</th>
<th>(\tau_2) (ms)</th>
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<td>DG control</td>
<td>1</td>
<td>0.45</td>
<td>148</td>
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<tr>
<td>WT flecainide</td>
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<td>0.31</td>
<td>103</td>
<td>0.17</td>
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### Table II

<table>
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<th>Mutations</th>
<th>(\Delta V_{1/2}) for inactivation (mV)</th>
<th>(\Delta V_{1/2}) for activation (mV)</th>
<th>UDB percentage at 5 Hz</th>
<th>Linked disease</th>
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<td>WT</td>
<td>5.04 ± 0.68 (n = 5)</td>
<td>0.9 ± 0.3 (n = 5)</td>
<td>73.5 ± 3.04(^a) (n = 4)</td>
<td>BeS (Rivolta et al., 2001)</td>
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<td>Y1795H</td>
<td>-10.50 ± 0.88 (n = 5)</td>
<td>0.9 ± 0.3 (n = 5)</td>
<td>73.5 ± 3.04(^a) (n = 4)</td>
<td>BeS (Rivolta et al., 2001)</td>
</tr>
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<td>Y1795G</td>
<td>-10.21 ± 0.37(^a) (n = 6)</td>
<td>1.4 ± 0.5 (n = 6)</td>
<td>79.56 ± 2.90(^a) (n = 5)</td>
<td>LQT-3 (Benhorin et al., 1998)</td>
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<td>I885stop</td>
<td>-11.0 ± 0.6 (n = 9)</td>
<td>0.9 ± 0.4 (n = 12)</td>
<td>80.0 ± 2.0(^a) (n = 3)</td>
<td>BrS (Rivolta et al., 2001)</td>
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<td>Y1795E</td>
<td>-10.40 ± 0.9(^a) (n = 6)</td>
<td>1.7 ± 1.4 (n = 6)</td>
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<td>-10.56 ± 1.3(^a) (n = 6)</td>
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<td>Y1795C</td>
<td>-2.30 ± 0.4 (n = 8)</td>
<td>-1.0 ± 0.4 (n = 8)</td>
<td>49.33 ± 0.16 (n = 4)</td>
<td>LQT-3 (Abriel et al., 2001)</td>
</tr>
<tr>
<td>E1295K</td>
<td>5.2 ± 0.2(^a) (n = 7)</td>
<td>3.4 ± 0.3(^a) (n = 7)</td>
<td>37.0 ± 4.97(^b) (n = 4)</td>
<td>LQT-3 (Abriel et al., 2001)</td>
</tr>
</tbody>
</table>

Data are mean ± SEM.

\(^a\)P < 0.01.

\(^b\)P < 0.05.

### Discussion

**Molecular Determinants of Flecainide Block**

Blockade of voltage-dependent Na\(^+\) channels has long been recognized as a therapeutic approach to the management of many cardiac arrhythmias, but with considerable risk of toxic side effects (Rosen et al., 1975). The discovery that mutant forms of Na\(^+\) channels linked to inherited human cardiac arrhythmias might make distinct targets for Na\(^+\) channel blocking drugs (An et al., 1996; Wang et al., 1997; Dumaine and Kirsch, 1998; Kambouris et al., 2000; Nagatomo et al., 2000; Viswanathan et al., 2001) has stimulated reinvestigation of the molecular determinants of Na\(^+\) channel blockade in the heart. In this study, we have used several disease-linked Na\(^+\) channel mutations that alter key biophysical properties and drug sensitivity of encoded channels in order to elucidate the mechanism by which mutation-induced changes in channel properties underlie altered apparent affinity of drug block. We fo-
cused on the Na\(^+\) channel blocker flecainide, which is distinguished in its UDB activity, because it has been shown to be very effective in correcting QT prolongation in carriers of some LQT-3 mutations (Benhorin et al., 2000; Windle et al., 2001), and is also used clinically to detect candidates for Na\(^+\) channel mutations underlying BrS (Brugada et al., 2000). From our experimental data, we conclude that flecainide UDB occurs as a consequence of stabilization of the drug-bound inactivated state of the channel after access to a drug binding site that is permitted only after channels open, and that disease-linked mutations that alter the voltage-dependence of inactivation alter flecainide UDB.

Voltage-dependent block of Na\(^+\) channels by local anesthetics and related drugs has been well described within the framework of the modulated receptor hypothesis which proposes that allosteric changes in a drug receptor occur when changes in voltage induce changes in channel conformation (states) (Hille, 1977a; Hondeghem and Katzung, 1977). High-affinity binding of the local anesthetics lidocaine and mexiletine to the inactivated state of the Na\(^+\) channel has been shown to require two critical amino acid residues, Phe-1764 and Tyr-1771, located in the IVS6 transmembrane segment in the brain IIA and cardiac Na\(^+\) channels (Ragsdale et al., 1994, 1996; Li et al., 1999; Weiser et al., 1999). Evidence for binding to the inactivated state was provided in part by the voltage-dependence of frequency-dependent block. The voltage-threshold for lidocaine binding was found to coincide with the voltage dependence of channel transitions from closed (rested) to inactivated states. In contrast, the threshold for flecainide UDB was much closer to the voltage that promotes channel activation (25 mV more positive than lidocaine), corresponding to transitions from closed (rested) to open states (Ragsdale et al., 1996). Thus, these data suggested that, while lidocaine preferentially interacted with inactivated channels, flecainide was more likely to bind with high affinity to open channels, consistent with earlier investigations in native cells (Anno and Hondeghem, 1990).

**Inherited Mutations Reveal the Role of Channel Openings in Use-dependent Block of Na\(^+\) Channels by Flecainide**

Our results indicate that the threshold for flecainide UDB closely parallels that for channel activation (Fig. 3), but additional experiments in this study are not consistent with open state block. The potency of flecainide UDB did not decrease with mutation-induced shortening of channel MOT, but instead was increased by mutations that shortened MOT. In contrast, all mutations that decreased channel availability at negative potentials (caused hyperpolarizing shifts in the voltage-dependence of channel inactivation) increased the sensitivity of the encoded channels to flecainide block. Most convincingly, the E1295K mutation, which has an opposite effect on the voltage-dependence of inactivation (Abriel et al., 2001), reduces the channel sensitivity to flecainide UDB (Table II). The simplest interpretation of these results is that flecainide interacts preferentially with inactivated channels, but that channel openings are required for drug access to its receptor site. The results shown in Fig. 4 C (WT vs. YC) support the view that channel opening is required for drug access because the onset of drug block is faster for channels with prolonged MOT (YC). A similar conclusion has recently been reached regarding mexiletine block of myotonia and periodic paralysis mutations of skeletal muscle sodium channels (Desaphy et al., 2001). An interesting observation is that the drug dissociation rate during steady-state pulse trains (Fig. 4 C) is apparently much faster than is observed in the recovery from drug block at hyperpolarized potentials (Fig. 7), because if recovery from open inactivated channels were as slow as recovery from closed inactivated channels, block during repetitive pulse trains would increase until all channels were blocked (Chernoff, 1990; Chernoff and Strichartz, 1990). This suggests flecainide cannot leave its binding site as easily from closed inactivation states as it can from open inactivation states.

**Ionized Flecainide may Limit Access to Sites**

Lidocaine has a pKa of 7.6–8.0 and thus may be up to 50% neutral at physiologic pH (Hille, 1977b; Strichartz et al., 1990). In contrast, flecainide has a pKa of ~9.3, leaving <1% neutral at pH 7.4 (Hille, 1977b; Strichartz et al., 1990). Thus, one possibility underlying differences in the voltage-dependence of flecainide- and lidocaine-induced modulation of cardiac Na\(^+\) channels is restricted access to a common site that is caused by the ionized group of flecainide. Channel opening would then be a prerequisite for flecainide, but not lidocaine, UDB. Our data, consistent with this scheme, suggest that flecainide, like lidocaine, interacts preferentially with inactivated channels and that mutations that promote inactivation (shift channel availability in the hyperpolarizing direction) enhance flecainide UDB. Interestingly, in a study of the molecular determinants of local anesthetic block of sodium channels, Ragsdale et al. (1996) reported weak functional overlap of in the effects of mutations that disrupt lidocaine and flecainide block of brain Na\(^+\) channels.

Recent studies of both flecainide and lidocaine block of cardiac sodium channels have revealed roles of an intermediate inactivated state (Chen et al., 2000; Kambouris et al., 2000; Viswanathan et al., 2001). Although similar to the state-dependent interactions we describe, there are key differences between the voltage-dependence of flecainide block we describe (block due to open state inactivation) and previously described block
that develops over voltages below the threshold for channel opening (Viswanathan et al., 2001). Differences between these mechanisms may be due to drug interactions via multiple pathways. Clearly, UDB reported in this study demands that channels first open before drug block occurs and hence cannot occur via the closed inactivated transition.

**Flecainide Sensitivity Is Mutation-, but not Disease-, specific**

The mutations used in the present study were discovered by genetic analysis of both LQT-3 and BrS patients (Benhorin et al., 1998; Abriel et al., 2001; Rivolta et al., 2001), but mutation-altered flecainide sensitivity segregates with channel but not disease phenotype. This is most apparent in comparing the sensitivities of Y1795H (BrS) and D1790G (LQT-3) channels to flecainide: both are fourfold more sensitive to flecainide than WT channels (Fig. 2), despite being linked to two different diseases. On the other hand, LQT-3 Y1795C channels respond to flecainide block with the same sensitivity as WT channels, but are fourfold less sensitive than the LQT-3 mutant D1790G channels to flecainide block. Clearly in the case of the two inherited arrhythmias, LQT-3 and BrS, it is possible for one (flecainide) drug to cross disease boundaries and have similar pharmacological profiles for mutant channels that are distinct from the profiles of WT channels. Thus, it is not surprising that in some cases drug-induced effects in patients may be similar for some BrS and/or LQT-3 mutations (Priori et al., 2000; Cerrone et al., 2001).

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