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Flecainide sensitivity of a Na channel long QT mutation shows an open-channel blocking mechanism for use-dependent block

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Flecainide sensitivity of a Na channel long QT mutation shows an open-channel blocking mechanism for use-dependent block. Am J Physiol Heart Circ Physiol 291: H29–H37, 2006. First published February 24, 2006; doi:10.1152/ajpheart.01317.2005.—A long QT mutation in the cardiac sodium channel, D1790G (DG), shows enhanced flecainide use-dependent block (UDB). The relative importance of open and inactivated states of the channel in flecainide UDB has been controversial. We used a modifiable, inactivation-deficient mutant channel that contains the F1486C mutation in the IFM motif to investigate the UDB difference between the wild-type (WT-ICM) and DG (DG-ICM) channels. UDB at 5 Hz was greater in DG-ICM than WT-ICM, and IC50 values for steady-state UDB were 7.19 and 18.06 μM, respectively. When [2-(trimethyammonium) ethyl]methanethiosulfonate (MTSET) was included in the pipette and fast inactivation was disabled, IC50 was 5.04 μM for WT-ICM and 12.63 μM for DG-ICM. We measured open-channel block by flecainide directly in MTSET-treated, noninactivating ICM channels. Steady-state block was higher for DG-ICM than WT-ICM (IC50 was 2.34 μM for DG-ICM and 5.87 μM for WT-ICM), suggesting that open-channel block is an important determinant of flecainide UDB. We obtained association (k_on) and dissociation (k_off) rates for open-channel block by the Langmuir-isotherm model. They were k_off = 31.37 s⁻¹, k_on = 5.83 s⁻¹·μM⁻¹, and calculated Kd = 5.38 μM for WT-ICM (where Kd = k_off/k_on); and k_off = 24.88 s⁻¹, k_on = 9.54 s⁻¹·μM⁻¹, and calculated Kd = 2.61 μM for DG-ICM. These Kd values were similar to IC50 measured from steady-state open-channel block. Furthermore, we modeled UDB mathematically by using these kinetic rates and found that the model predicted experimental UDB accurately. The recovery from UDB had a minor contribution to UDB. Flecainide UDB is predominantly determined by an open-channel blocking mechanism, and DG-ICM channels appeared to have an altered open-channel state with higher flecainide affinity than WT-ICM.

IN THE MAJORITY OF CARDIAC TISSUE, an action potential is initiated by the opening of voltage-gated sodium channel (Na+,1.5, the predominant isoform in heart). The inward sodium current (ISNa) inactivates within milliseconds, but inward Ca2+ current sustains the plateau phase. Subsequently, outward K+ current repolarizes the membrane for the next action potential. Prototypic Na+,1.5 mutations that cause long QT syndrome (LQT3) disrupt channel inactivation and produce noninactivating, sustained inward Na+ current (late ISNa), prolonging the action potential plateau and QT intervals, and subsequently predisposes for early after depolarization (EAD) and torsades des points.

Flecainide is a class I antiarrhythmic drug that blocks Na+ channels. Its activity against late ISNa, which is observed in the majority of familial LQT3 mutations, has raised a potential therapeutic strategy for LQT3 syndrome and rekindled interest in the mechanism of flecainide block of voltage-gated Na+ channels. Flecainide blocks the sodium channel on depolarization and channel opening (14). While open-channel access to the binding site is required for channel block, determining the exact channel state to which the drug interacts during use-dependent block (UDB) has been controversial (for examples of the open-state block, see Refs. 3, 19, 20; for examples of the inactivated-state block, see Refs. 13, 14, 31).

The first LQT3 mutation identified was ΔKPQ within the intracellular D3–D4 linker (32), which is critical for fast inactivation of the channel and contains the IFM motif (11485/F1486/M1487 in Na+,1.5) (34). Several recently identified LQT3 mutations that cause late ISNa (E1789K, Y1795C, 1795insD), however, are located in the intracellular COOH-terminal segment of the channel (reviewed in Ref. 8). This region of the channel has been suggested to play a role in channel inactivation in brain and cardiac channels (16, 17). Whether late ISNa is present in a recently identified D1790G (DG) LQT3 mutation (5) from the COOH-terminal region of the channel has been somewhat controversial. An et al. (2) and Wehrens et al. (33) initially reported lack of late ISNa in DG channels. However, Baroudi and Chahine (4) observed late ISNa in DG channels. Subsequently, Tateyama et al. (29) showed that late ISNa is enhanced by protein kinase A (PKA)-dependent phosphorylation or inhibition of phosphatases and is likely responsible for QT prolongation in ECG. Flecainide, but not lidocaine, when administered intravenously, corrected QT prolongation in patients with the DG mutation (6). The exact mechanism by which flecainide corrected QT prolongation in ECG probably involves, at least in part, blockade of the phosphorylation-dependent late ISNa and the increased open-channel affinity in DG channels.

Flecainide showed increased UDB of DG channels without change in tonic block (1). What is the underlying mechanism for the increased UDB in DG channels in the experimental setting? Could this difference be used to clarify the mechanism...
of flecainide UDB of the channel? In our study, we determined the
factors that influenced flecainide UDB in WT and DG
d channels empirically. We used wild-type (WT) and DG
channels with and without fast inactivation to assess the contribu-
tion of open and inactivated channels toward UDB by using the
F1486C mutation within the IFM motif (above). We then
determined the relative importance of those factors in flecain-
ide UDB by mathematical modeling. We found that the open-
channel affinity for flecainide was increased in DG channels,
and using mostly the open-channel blocking parameters, we
could mathematically model UDB of WT and DG channels
accurately, indicating that the increased open-channel affinity
was mainly responsible for the increased UDB in DG channels.
This suggests that flecainide UDB involved an open-channel
blocking mechanism of the sodium channel.

MATERIALS AND METHODS

Channel constructs. The hH1α-α-subunit was a gift from Dr. H. A.
Hartmann (11). Compared with the hH1 clone (9) used by Abriel et al.
(1), hH1α is different from hH1 at four amino acid positions, including
the deletion of Q1077. These differences do not result in significant
differences in channel properties (15) (our data compared with pub-
dished data, not shown). For historical reasons, we used the amino acid
numbering of hH1 in our proposal. For example, D1790 of hH1
occurs at the 1789th position in hH1α, but we referred the DG
mutation as D1790G. Human β1-cDNA was cloned and sequenced
to confirm the amino acid sequence. In all experiments,
β1 was coex-
pressed with the α-subunit. Typically, to express both in transfection,
two separate plasmids containing the α- and β-subunits are mixed and
cotransfected. However, assuring adequate expression of the β1-
subunit for each α-subunit being expressed is difficult because there
is no independent method of quantifying its expression. Thus we con-
structed an expression vector, which utilizes the internal ribosome
eragy sequence (IRES) to produce bicistronic messages to ensure a 1:1
(α:β1) mRNA ratio. Briefly, the vector contains a CMV promoter
that drives a bicistronic mRNA containing the β1-cDNA in the 5’-side
of the IRES and the α-cDNA in the 3’-side of IRES. The vector also
has a separate GFP expression cassette. In transient transfection, cells
that take up the plasmid can be identified by fluorescence within 18 h.
Cells with green fluorescence were patched between 18 and 48 h. Almost
all cells (>95%) patched contained inward sodium current
between 500 pA and 5.0 nA (an improvement from transfection with a
mixture of multiple plasmids). The presence of sodium current indicates
production of intact bicistronic mRNAs because a truncated
version lacks the poly(A) tail and is held in the nucleus. We believe
that this is an improved approach for coexpressing a consistent
amount of β1. The DG mutation was introduced into the WT channel
in pRC/CMV (Invitrogen, Carlsbad, CA) and subcloned into the IRES
vector above. The Fl486C (IFM→ICM) mutation was introduced
into the WT channels by oligonucleotide-directed mutagenesis with the
Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA) and
subcloned into the IRES constructs containing the WT and DG
channels.

Channel expression. HEK-293 cells were maintained in DMEM
supplemented with 10% fetal bovine serum in humidified CO2
incubators at 37°C. For transfection experiments, the Superfect Transfe-
recording chamber containing
City, CA). HEK-293 cells were treated with trypsin and placed in a
recording chamber containing ~0.1 ml of appropriate external buffer.
All recordings were carried out at room temperature (22–25°C). Tip
resistance of electrodes was between 0.6 and 1.2 MΩ. The standard
bath solution contained (in mM) 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2,
10 HEPES, pH 7.4 with NaOH. Pipettes contained (in mM) 35 NaCl,
105 CsF, 10 EGTA, 10 HEPES, 1 MgCl2, pH 7.3 with CsOH. This
yielded a reversal potential (Vrev) of +35 mV at room temperature.
Leak current and series resistance corrections were done electroni-
ally. The quality of voltage-clamp was judged by examination of
data, including the Vrev from current-voltage relationship experiments.
Cells expressing more than 5 nA of current at room temperature were
not used in order to minimize voltage-clamp errors.

Before all experiments, the holding potential was −140 mV.
Unless specified, UDB experiments involved repetitive 10-ms pulses to
−10 mV from −140 mV at 5 Hz. A UDB protocol in drug-free
conditions preceded the drug block experiments and showed <5%
current loss in all instances. In all experiments, the whole cell
configuration was held at −140 mV for at least 8 min, and when
flecainide was applied, cells were held at −140 mV and superfused
for at least 2 min for complete dialysis. For each cell, only one dose
of flecainide was used to avoid accumulation of drug effects. When-
ever possible, the flecainide was washed out, and current levels were
checked. The majority of experiments involved long periods of
voltage clamp because of slow flecainide recovery, and washing out
the flecainide was frequently not possible. In one of four experiment sets
(consisting of drug-free channel gating and UDB protocols followed by
flecainide block and recovery protocols), washout data were
available. In all cases, the full recovery of current levels was observed.

Fast inactivation was disabled by including 1 mM [2-(trimethyl-
ammonium)ethyl]methanethiosulfonate bromide (MTSET) (Anatrace,
Maumee, OH) in the pipette. MTSET was prepared just before the
experiment. We selected cells that showed <15% of current decay at
the end of a 100-ms pulse at −10 mV for analyses. Details of other
experiments are described in RESULTS and in corresponding figure
legends.

Data analysis. Data are reported as means value ± SE. Data were
analyzed with Clampfit (Axon Instruments, Union City, CA) and
Origin 7 (Originlab, Northampton, MA) software. Two sets of data
were compared by using paired or unpaired Student’s t-test, and P <
0.05 was used to define statistical significance.

RESULTS

Rationale for design of channel constructs. First, we con-
confirmed the findings by Abriel et al. (1) using hH1α. At 2 Hz,
steady-state UDB level (ssUDB) and τUDB (UDB onset time
constant in pulse numbers) were 0.301 ± 0.041 and 8.1 ± 1.1
for WT and 0.515 ± 0.076 and 31.9 ± 5.8 for DG channels
with 10 µM flecainide (P < 0.05 for both ssUDB and τUDB,
n = 5). With 2 µM flecainide, they were 0.084 ± 0.018 and
22.7 ± 3.3 for WT and 0.220 ± 0.048 and 53.4 ± 5.9 for DG
channels (P < 0.05 for both parameters, n = 4 and 6 respec-
tively). Thus, under our experimental conditions, which used
hH1α and −140 mV as a holding potential, DG channels
showed increased UDB compared with WT. One facet of
flecainide UDB that was not described in previously published
studies was UDB onset kinetics (1, 13, 14). UDB developed
more slowly in DG channels (longer τonset) although ssUDB
was higher, in both 2 µM and 10 µM flecainide. The onset
kinetics of UDB is determined by multiple factors, including
drug affinities to various channel states and drug access/egress
paths. To evaluate these possibilities better, we inserted the
Fl486C mutation within the IFM motif of the D3–D4 linker
[the fast-inactivation particle (34)] into the WT and DG
channels (WT-ICM and DG-ICM). With the Fl486C mutation, the
flecainide UDB difference between WT-ICM and DG-ICM
channels persisted (Fig. 1, A, C, and D and time constants for
DG-ICM channels were more susceptible to UDB by flecainide than WT-ICM channels. Recent data suggest that flecainide reaches the binding site through the cytoplasmic pore after crossing the membrane when superfused (13). By including MTSET in the pipette, we removed fast inactivation, continuously exposing the open state of the channels during the UDB pulses (7, 12, 23, 30). This approach allowed for the direct comparison of channel block by flecainide with or without fast inactivation (with or without MTSET) in a common channel background. Previous studies have shown that internal MTSET by itself does not affect $I_{Na}$ (24, 35).

Flecainide UDB of ICM channels with fast inactivation intact. As with WT and DG channels, DG-ICM was more sensitive to flecainide UDB (higher ssUDB at 5 Hz) than WT-ICM although the UDB developed more slowly (Fig. 1, A, C, and D, and time constants for UDB in Table 1). Therefore, WT-ICM and DG-ICM channels were qualitatively similar to WT and DG channels with regard to flecainide UDB.

Increased UDB in DG-ICM (and DG) channels can result from higher flecainide affinity to the closed, open, or inactivated states. A closed state with a higher affinity is unlikely because the first-pulse block (resting block) was unchanged in DG-ICM (Table 1). To minimize the contribution from the inactivated state, we measured UDB of the two channels (WT-ICM and DG-ICM) with 0.6-ms pulses, during which development of an inactivated state (both fast or slow inactivated state) is unlikely, and found that DG-ICM channels still showed higher ssUDB ($0.24 \pm 0.04$ at 5 $\mu$M flecainide, $P < 0.05, n = 4$) than WT-ICM ($0.12 \pm 0.04, n = 3$). The result indicated that the main difference in flecainide UDB between WT-ICM and DG-ICM channels was likely to be related to the open-channel state. Without the inactivated state, DG-ICM channels were still blocked more than WT-ICM channels.

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**Fig. 1.** Use-dependent block (UDB) ± [2-(trimethylammonium) ethyl]methanethiosulfonate bromide (MTSET) of WT-ICM and DG-ICM channels. A: UDB for wild-type (WT)-ICM and D1790G LQT3 mutation (DG)-ICM without MTSET was induced by applying a train of 100 depolarizing pulses (−10 mV/10 ms) at a frequency of 5 Hz. The currents were measured after application of flecainide (2, 5, 20, 55 $\mu$M). B: UDB for WT-ICM and DG-ICM with MTSET was induced by applying a train of 100 depolarizing pulses (−10 mV/10 ms) at a frequency of 5 Hz. The currents were measured after application of flecainide (2, 5, 20, 55 $\mu$M). C: top: UDB without MTSET (flecainide, 20 $\mu$M). First-pulse block ($P_0$, no drug; $P_1$, first pulse of UDB train) was minimal. $P_{ss}$, a pulse of the steady-state UDB (ssUDB). C, bottom: when MTSET was added, current decay was abolished, but the difference in UDB persisted. Peak current (*) levels are used for UDB (20 $\mu$M). D: $IC_{50}$ for ssUDB obtained from the dose-response curves. $IC_{50}$ for flecainide without MTSET is 18.06 $\mu$M (WT-ICM) and 7.19 $\mu$M (DG-ICM); $IC_{50}$ for flecainide with MTSET is 12.63 $\mu$M (WT-ICM) and 5.04 $\mu$M (DG-ICM). ICM refers to modifiable, inactivation-deficient mutant channel that contains the F1486C mutation in the IFM motif.
Table 1. Parameters for first-pulse block and UDB

<table>
<thead>
<tr>
<th>[Flecainide]</th>
<th>1st-pulse block</th>
<th>ssUDB</th>
<th>τ_{on}</th>
<th>1st-pulse block</th>
<th>ssUDB</th>
<th>τ_{on}</th>
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<tbody>
<tr>
<td></td>
<td>WT-ICM</td>
<td></td>
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<td>DG-ICM</td>
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<tr>
<td>2 μM</td>
<td>0.074±0.026</td>
<td>0.177±0.039</td>
<td>9.3±1.0</td>
<td>0.046±0.018</td>
<td>0.206±0.002</td>
<td>3.06±0.40†</td>
</tr>
<tr>
<td>5 μM</td>
<td>0.118±0.027</td>
<td>0.325±0.025</td>
<td>6.26±0.62</td>
<td>0.036±0.007</td>
<td>0.357±0.022</td>
<td>1.53±0.13†</td>
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<td>20 μM</td>
<td>0.156±0.035</td>
<td>0.490±0.019</td>
<td>4.08±0.42</td>
<td>0.172±0.032</td>
<td>0.580±0.010</td>
<td>0.60±0.07†</td>
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<td>55 μM</td>
<td>0.198±0.034</td>
<td>0.691±0.017</td>
<td>1.93±0.17</td>
<td>0.189±0.025</td>
<td>0.730±0.021</td>
<td>0.41±0.01†</td>
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<tr>
<td></td>
<td>DG-ICM</td>
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<tr>
<td>2 μM</td>
<td>0.069±0.022</td>
<td>0.302±0.030*</td>
<td>13.2±1.6*</td>
<td>0.076±0.024</td>
<td>0.314±0.022*</td>
<td>2.65±0.32†</td>
</tr>
<tr>
<td>5 μM</td>
<td>0.080±0.024</td>
<td>0.486±0.044*</td>
<td>9.69±1.53*</td>
<td>0.089±0.021</td>
<td>0.556±0.027*</td>
<td>1.59±0.21†</td>
</tr>
<tr>
<td>20 μM</td>
<td>0.108±0.023</td>
<td>0.616±0.015*</td>
<td>5.44±0.29*</td>
<td>0.179±0.042</td>
<td>0.696±0.021†</td>
<td>0.57±0.04†</td>
</tr>
<tr>
<td>55 μM</td>
<td>0.160±0.035</td>
<td>0.758±0.022*</td>
<td>3.56±0.35*</td>
<td>0.200±0.033</td>
<td>0.812±0.018*</td>
<td>0.39±0.07†</td>
</tr>
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</table>

Values are means ± SE. First-pulse block and use-dependent block (UDB). WT-ICM is wild-type ICM; DG-ICM has D1790G long QT syndrome (LQT3) mutation. First-pulse block was relatively small and generally increased with drug concentration ([flecainide]); (n=3–5 for all except DG-ICM without [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (–MTSET) at 20 μM (n=6) and 55 μM (n=7). In UDB experiments (10–ms pulses from −140 mV to −10 mV at 5 Hz), peak-current levels were normalized to the first pulse of the train. UDB onset time constants (τ_{on}) were estimated by a one-exponential equation, \( I(t) = Ae^{-kt} + C \), where \( I \) denotes relative current in a function of time; \( n \) is the pulse number, \( k \) is the time constant of UDB onset in number of pulses, and \( C \) is the steady-state UDB level (ssUDB). UDB developed more slowly in DG-ICM channels when fast inactivation was intact. With (+ MTSET), there was no difference in τ between WT-ICM and DG-ICM. However, in both cases, ssUDB values were higher in DG-ICM. For both channels, all drug concentrations sped up UDB (shorter \( \tau_{on} \) when MTSET was applied (P < 0.05). The higher the drug concentration, the faster the UDB (P < 0.05 for each concentration) (n = 4–6). *P < 0.05 for comparison between WT-ICM and DG-ICM (+MTSET or −MTSET). †P < 0.05 for comparison between −MTSET and +MTSET (for either WT-ICM or DG-ICM).

Flecainide UDB of ICM channels with fast inactivation disabled. To clarify further which channel state was responsible, we measured flecainide UDB after disabling fast inactivation by including MTSET (1 mM) in the pipette. Removal of fast inactivation resulted in a small increase in ssUDB. There was a trend toward higher ssUDB with MTSET, but P > 0.05 (except for DG-ICM at 20 μM, P = 0.05) (Fig. 1, B–D, and Table 1). After fast inactivation was disabled, flecainide UDB achieved steady state quickly. DG-ICM channels were still blocked more than WT-ICM channels even in the absence of fast inactivation. Therefore, the fast-inactivated state is not blocked more than WT-ICM channels even in the absence of fast inactivation. The open-channel affinity for flecainide was increased in DG-ICM channels. The increased affinity was due to a combination of both increased on rate (\( k_{on} \)) and decreased off rate (\( k_{off} \)), estimated in the Langmuir-isotherm model (Fig. 2D). The dissociation constants \( K_d \) for WT-ICM and DG-ICM calculated from \( k_{off}k_{on} \) were in good agreement with measured IC50 values from the dose-response curve (Fig. 2C). These \( k_{off} \) and \( k_{on} \) values are also in reasonable agreement with results by others using the IFM/QQQ mutation, considering differences in experimental designs [Ref. 10, HEK-293 system, but no β1 and at −20 mV (vs. our −10 mV); Ref. 20, Xenopus oocyte system and no β1].

UDB recovery. Recovery from UDB is an important determinant of ssUDB and τ_{on}. Abriel et al. (1) showed that UDB recovery was slowed in DG channels. Recovery was not complete after 10 s for either WT or DG channels at −100 mV, and the recovery time constants were not estimated (Fig. 4 in Ref. 1). We measured recovery from flecainide UDB after a 100-pulse train (5 Hz) (Fig. 3A). The first pulse in the recovery protocol occurs 50 ms after the UDB train, allowing the channels to recover from fast inactivation. In both WT-ICM and DG-ICM channels, near-complete recovery from UDB was achieved at −140 mV, and accurate estimation of the recovery time constants was possible. Two-exponential fitting was necessary for accurate modeling of the UDB recovery, which was not dependent on the drug concentration (not shown) and was slowed in DG-ICM channels, mainly due to prolongation of the longer time constants (A2, ~70–80%, Fig. 3A and Table 3). The faster components (τ ~ 1 s) were not significantly different between WT-ICM and DG-ICM. Inter-
Fig. 2. Open-channel block after one 100-ms pulse. A: WT-ICM + MTSET. B: DG-ICM + MTSET. A (WT-ICM) and B (DG-ICM) are examples of open-channel block in the presence of 20 μM flecainide. Time-dependent block is evident, and the time course of current decay was fitted with a one-exponential decay function, $Ae^{-t/	au_{	ext{open}}}$ + C, where $A$, $t$, and $C$ are amplitude, duration of open channel exposure to flecainide, and steady-state open channel block, respectively. ($\tau_{\text{open}}$, Table 2). Steady-state block is $b(a + b)$. First-pulse block, $(d - c)/d$, is relatively small (Table 1). C: dose-response curve with IC$_{50}$ values for steady-state open-channel block. D: effective blocking rate ($1/\tau_{\text{open}}$) plotted vs. flecainide concentration. DG-ICM$\text{MTSET}$ and WT-ICM$\text{MTSET}$ refer to DG-ICM with MTSET and WT-ICM with MTSET, respectively. Blocking rate ($1/\tau_{\text{open}}$) increased linearly with flecainide concentration where the slope and $y$-intercept represent the association ($k_{\text{on}}$) and dissociation ($k_{\text{off}}$) rate constants, respectively. $1/\tau_{\text{open}} = k_{\text{on}} \times D + k_{\text{off}}$. For WT-ICM, $k_{\text{off}} = 31.37$ s$^{-1}$ and $k_{\text{on}} = 5.83$ s$^{-1}$·μM$^{-1}$. For DG-ICM, $k_{\text{off}} = 24.88$ s$^{-1}$ and $k_{\text{on}} = 9.54$ s$^{-1}$·μM$^{-1}$. With fast inactivation disabled, the flecainide affinity for open channels was higher for DG-ICM than for WT-ICM. Calculated $k_{\text{d}}$ ($k_{\text{off}}/k_{\text{on}}$) was 5.38 μM for WT-ICM and 2.61 μM for DG-ICM, in good agreement with measured IC$_{50}$ values in C.

Table 2. Parameters for open-channel block

<table>
<thead>
<tr>
<th>Flecainide</th>
<th>WT-ICM + MTSET</th>
<th>DG-ICM + MTSET</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ssOCB $\tau_{\text{open}}$ ms</td>
<td>ssOCB $\tau_{\text{open}}$ ms</td>
</tr>
<tr>
<td>2 μM</td>
<td>0.329±0.012</td>
<td>0.484±0.017*</td>
</tr>
<tr>
<td>5 μM</td>
<td>0.441±0.035</td>
<td>1.47±1.8</td>
</tr>
<tr>
<td>20 μM</td>
<td>0.734±0.005</td>
<td>6.84±0.12</td>
</tr>
<tr>
<td>55 μM</td>
<td>0.842±0.009</td>
<td>2.84±0.09</td>
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</table>

Values are means ± SE. Steady-state fractional open-channel block (ssOCB) and time course of open-channel block ($\tau_{\text{open}}$) are shown (e.g., Fig. 2). See Fig. 2 legend and text for details. *P < 0.05; n = 4–5.

attribute the difference in UDB between WT-ICM and DG-ICM channels solely to UDB recovery when the interpulse recovery periods were 190 ms (5 Hz with 10-ms pulses).

Unlike UDB recovery, recovery from inactivation was faster in DG-ICM channels (Fig. 3B). A two-exponential fit of the recovery showed significant differences in the time constants between WT-ICM and DG-ICM channels (Fig. 3 legend). Therefore, the slowed flecainide-UDB recovery in DG-ICM channels does not appear to be due to slowed recovery from inactivation. Recovery from UDB (100 10-ms pulses to −10 mV at 5 Hz) without flecainide was fast and complete for both WT-ICM and DG-ICM with time constants 1–2 ms ($P$ = not significant; not shown).

Mathematical modeling of UDB. How much does open-channel block, relative to UDB recovery, contribute to flecainide UDB? To estimate the importance of open-channel block in UDB, we used mathematical modeling to simulate flecainide UDB of WT-ICM and DG-ICM clones. Because we found that the first-pulse block is relatively small (Table 1), and Liu et al.
Fig. 3. Recovery from UDB (A) and recovery from inactivation (B). Recovery kinetics were fitted to a 2-exponential equation, \( R(t) = 1 - A_1e^{-\lambda_1t} - A_2e^{-\lambda_2t} - C \), where \( R(t) \) is the fractional current at time \( t \), and \( A_1 \) and \( A_2 \) are amplitudes of exponentials, \( \lambda_1 \) and \( \lambda_2 \) are the recovery time constants, and \( C \) is the fraction of channels that are not fully recovered. A: recovery from UDB was measured by a test-pulse (1-ms pulse duration/−10 mV) after a train of 100 UDB pulses at 5 Hz after varying lengths of time. (see Table 3 for fitting parameters). B: recovery from inactivation was measured by a pulse to −10 mV after a 1-s inactivating pulse to −10 mV and variable length of recovery period at −140 mV. The amplitudes and time constants (\( A_1, \tau_1, A_2, \tau_2 \)) for inactivation recovery were: for WT-ICM, 0.325, 2.01 ms, 0.675, 89.77 ms; for DG-ICM, 0.439, 1.13 ms, 0.561, 69.27 ms.

(14) concluded that channel opening was necessary for flecainide block, we considered the receptor to be guarded at a hyperpolarized membrane potential (−140 mV). We used the equations described by Starmer and Grant (25) and Starmer et al. (26) under the guarded-receptor model (Eqs. 1, 2, and 5 in Fig. 4C) and the equation that describes the bimolecular-binding model (Eq. 3 in Fig. 4C). The recurrence equation can be solved to give the fraction of blocked channels associated with the \( n \)th stimulus (Eq. 1 in Fig. 4C). The uptake rate \( \lambda \) is a linear combination of the two reciprocal time constants (Eq. 2 in Fig. 4C). When recovery potentials are sufficiently negative and drug block is relatively small, the steady-state blockade is simplified and can be described by Eq. 5 in Fig. 4C. When MTSET is used, the open-channel state is continuously exposed during each pulse. The equations used by Starmer and Grant (25) are not necessarily unique to the guarded-receptor model and can be used to model any UDB when several key features of flecainide UDB validate the assumptions required for Eq. 4 in Fig. 4C.

First, we note that the uptake rate \( \lambda \) (Eq. 2 in Fig. 4C) is influenced by both \( t_d/\tau_4 \) and \( t_e/\tau_3 \), although \( t_d/\tau_4 \) is significantly greater than \( t_e/\tau_3 \). This is consistent with the slow UDB recovery (Table 3, with time constants in s) observed in our study and with the conclusion by Ramos and O’Leary (20) that flecainide is trapped by the activation gate. As shown in Fig. 4D, the UDB-onset kinetics (\( t_{UDB} \)) and ssUDB can be accurately predicted (compare the modeled parameters to those in Table 1). More interesting is whether the model can predict UDB when fast inactivation is intact where access to and egress from the receptor site is not continuous. We examined this by assuming that flecainide is a “pure” open-channel blocking drug. We used the same \( t_d \) as above (\( k_{on} \) and \( k_{off} \) from the open-channel block; Fig. 4C, Eq. 2) and adjusted the \( t_d \) (duration of open-channel exposure to flecainide in each pulse, “effective exposure time”) by calculating the area under the current tracing. As shown in Fig. 1, DG-ICM channels show faster current decay than WT-ICM channels. A similar observation was also true for WT and DG channels (not shown). Therefore, the effective exposure time is shorter for DG-ICM channels than that for WT-ICM when fast inactivation is intact (without MTSET, 1.93 ± 0.33 ms for WT-ICM and 1.23 ± 0.10 ms for DG-ICM, \( P < 0.05 \), \( n = 10 \); with MTSET, 8.25 ± 0.25 ms for WT-ICM and 7.77 ± 0.35 ms for DG-ICM, \( P \) = not significant, \( n = 13 \)). In this manner, the model estimated

<table>
<thead>
<tr>
<th>Parameters for recovery from UDB</th>
<th>( A_1 )</th>
<th>( \tau_1, \text{s} )</th>
<th>( A_2 )</th>
<th>( \tau_2, \text{s} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ICM −MTSET</td>
<td>0.3097±0.028</td>
<td>1.09±0.22</td>
<td>0.6903±0.028</td>
<td>62.6±3.45</td>
</tr>
<tr>
<td>DG-ICM</td>
<td>0.1818±0.017*</td>
<td>1.40±0.13</td>
<td>0.8182±0.017*</td>
<td>99.5±4.6*</td>
</tr>
<tr>
<td>+MTSET</td>
<td>0.291±0.035</td>
<td>0.98±0.12</td>
<td>0.709±0.035</td>
<td>67.3±4.5</td>
</tr>
<tr>
<td>WT-ICM</td>
<td>0.169±0.025*</td>
<td>1.61±0.39*</td>
<td>0.831±0.025*</td>
<td>107.4±4.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Recovery from UDB was measured by test pulses at varying times from 5 ms up to 600 s after the completion of a UDB protocol. In all experiments shown, current recovery during the protocol was >99%. A 2-exponential equation, \( R(t) = 1 - A_1e^{-\lambda_1t} - A_2e^{-\lambda_2t} - C \) was used to derive the time constants and amplitudes, where \( I(t) \) is the fractional current of time \( t \), and \( A_1 \) and \( A_2 \) are amplitudes of exponentials, \( \lambda_1 \) and \( \lambda_2 \) are the recovery time constants, and \( C \) is the fraction of channels that are not fully recovered. Recovery was not dependent on drug concentration, and the amplitudes were normalized. Time constants in s (not ms) and relative amplitudes are listed. Recovery was not changed when fast inactivation was disabled (+MTSET), but the difference between WT-ICM and DG-ICM persisted. *\( P < 0.05 \) for WT-ICM vs. DG-ICM; \( n = 7–8 \).

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The primary purpose of our study was to clarify controversies in the basic mechanism of flecainide UDB by examining the drug-channel interaction of a mutant channel that shows increased flecainide UDB, i.e., we reasoned that whatever difference existed in drug-channel interaction between the WT and DG channels would be important in the flecainide UDB. In our study, we used ICM channels (WT-ICM and DG-ICM), with which we can selectively disable the fast inactivation and compare UDB in a common channel background. WT-ICM and DG-ICM channels were qualitatively similar to WT and DG channels with regard to flecainide UDB. We found that the open-state affinity of DG channels for flecainide was higher than that of WT and that this increased affinity primarily underlies the difference in UDB. This suggests an open-channel mechanism for flecainide UDB.

Recent studies also demonstrated data in favor of an open-channel mechanism for flecainide block. Observation of time-dependent open-channel block supports direct interaction between the open-state and flecainide (Refs. 10 and 20 and our current study). In the recent two studies (10, 20), the “inactivation-deficient” (IFM→QQQ) channel was used to measure the open-channel block. However, the IFM→QQQ channels may not be directly comparable to wild-type channels, and the contribution of fast inactivation toward flecainide UDB and slow recovery from the drug block cannot be directly compared. In the absence of MTSET, WT-ICM and DG-ICM channels exhibited essentially intact fast inactivation and current-decay properties similar to those of WT and DG channels.

In the study by Viswanathan et al. (31), evidence for an “inactivated-state” blocking mechanism has involved correlation to enhanced “closed-state” and intermediate inactivation. Direct interaction between flecainide and these inactivated states were not demonstrated. In addition, the mutant channels showing the enhanced closed-state inactivation also had sig-
significant late \(I_{Na}\), which could have been blocked by flecainide. Liu et al. (14), using several Na\(^+\) channel disease mutations with distinct flecainide sensitivities, concluded that channel opening is necessary but flecainide binds tightly to “an inactivated state.” They estimated mean open times (0.50 ± 0.02 ms for WT and 0.42 ± 0.03 ms for DG). However, they did not consider the possibility of a change in flecainide affinity to the open-state between WT and DG [calculated open-state \(k_{a}\) (\(k_{a}\) = \(k_{a}\))] were 5.38 \(\mu\)M for WT-ICM and 2.61 \(\mu\)M for DG-ICM], which is the main finding in our study. Slowed recovery from flecainide block in the above studies (and our current study) may also represent differential voltage- and state-dependant “trapping” of the drug (20) and not necessarily an increased affinity to an inactivated state. A neutral form of flecainide, which does not depend on the pore access path for block, has been shown to shift the steady-state availability (inactivation) curve (13). However, it is not clear whether the behavior of the neutral drug truly represents that of flecainide.

We used a holding potential of −140 mV, sufficiently hyperpolarized to deactivate sodium channels fully. Our choice of such a hyperpolarized holding potential may have lessened the contribution from an inactivated state toward flecainide UDB. At −140 mV, all channels are uniformly in the closed state; it is approximately −35 mV more negative than half-average voltage (\(V_{1/2}\)) of the steady-state availability (SSA) curves of DG channels (not shown), similar to the conditions in the study by Abriel et al. (1). UDB recovery was nearly complete (Fig. 3A) within 200 s and was accurately estimated with a two-exponential function. Even at −140 mV, the difference in UDB recovery between WT-ICM and DG-ICM channels persisted, similar to the results by Abriel et al. (1) at −100 mV.

In our study, we found two significant differences in flecainide UDB between the WT and DG channels, increased open-channel affinity to flecainide and slowed recovery from UDB. The fast-inactivated state appeared to have little influence on flecainide block because the lack of it did not decrease UDB or prolong UDB recovery. Direct involvement of a slow-inactivated state seems unlikely because the time constants for the open-channel block ranged from 1 to 30 ms (Table 2), while slow-inactivated states have been associated with longer time constants when fast inactivation is disabled.

We used the guarded-receptor model (25, 26) to mathematically model the flecainide UDB so that the observed differences in open-channel affinities and UDB recovery accounts for the differing flecainide UDB in the WT-ICM and DG-ICM channels. The model predicted flecainide UDB accurately by using open-channel flecainide affinities and kinetic constants for UDB recovery during the interpulse intervals without contribution from any inactivated states. We believe that the difference in open-channel affinity for flecainide is the predominant factor in the increased UDB in DG channels because the recovery from UDB during each interpulse interval is relatively small. Consistent with this is the observation that, in physiological conditions, flecainide slows conduction but does not prolong refractoriness significantly (22). In fact, when the faster component of UDB recovery (see RESULTS and Table 3) was omitted in the mathematical modeling, \(A\) was completely dominated by the open-channel parameters (\(\tau_d\)). Prediction of \(\tau_{UDB}\) was still very accurate, and ssUDB levels were overestimated (i.e., the model predicts higher flecainide UDB than observed) only by ~10–20% in both WT-ICM and DG-ICM channels (data not shown), suggesting that the open-channel affinity for the drug predominantly underlies flecainide UDB.

Interestingly, the modeled \(\tau_{UDB}\) underestimated (that is, UDB develops faster in the model) the experimental observations. There are couple possibilities for this. In UDB experiments, on repolarization at the end of each pulse, a small fraction of the drug might egress quickly from the pore-receptor site before the activation gate closes. This would result in prolonging \(\tau_{UDB}\) (number of pulses needed to reach the steady state) in experiments. Such loss would not be reflected in \(k_{a}\) and \(k_{off}\) values obtained from the single-pulse open-channel experiments. Second, the 10-ms pulse duration in UDB may not be long enough to “induce” the channel state that binds to flecainide with a high affinity. This may be consistent with the fact that \(\tau_{open}\) (Fig. 2 and Table 2) ranges beyond 10 ms. We propose that the high-affinity site likely occurs after 0.6 ms of membrane depolarization because UDB with 0.6-ms pulses resulted in significant decrease in ssUDB, suggesting that a preopen state is not likely to be involved. The time course of block is still too fast for direct involvement of other inactivated states in flecainide binding.

We demonstrated an open-channel blocking mechanism for flecainide UDB by mathematical modeling. However, there are several limitations to our study. We conducted all of our experiments at room temperature. At more physiological temperatures, channel gating and drug-block kinetics may be different. For example, the majority of such studies are carried out at room temperature (~22°C), which is 15°C lower than the physiological body temperature. Conductance-voltage and SSA curves shift toward more depolarized potentials as temperature rises (18). We chose the holding voltage of −140 mV in part to accommodate such shifts. The hyperpolarized holding potential may have also unfairly excluded certain inactivated or closed states of the channel in our protocols. This also directly limits addressing physiological significance of our findings but made it possible to separate channel states clearly for our mathematical model.

One issue that we did not address in our study is how flecainide corrected QT prolongation in patients with DG mutations. QT prolongation by DG mutation is likely caused by late \(I_{Na}\) that was observed in DG channels in experiments by Baroudi and Chahine (4) and under phosphorylation-promoting conditions in the study by Tateyama et al. (29). In the study by Baroudi and Chahine (4), fluoride ion, which can inhibit phosphatase activities, was included in the patch pipette solutions (cytoplasmic side) and may have promoted late \(I_{Na}\). In our experiments, we also observed late \(I_{Na}\) (fluoride in the pipette solution) that was −2.4% of the peak current in DG channels, which was not present in WT channels. However, although implicitly included, the late \(I_{Na}\) contributed little toward the “effective exposure time” that was used in our mathematical modeling. The mechanism of correcting QT prolongation by flecainide likely involves both blockade of the late \(I_{Na}\) and the increased open-channel affinity in DG channels that we showed in our current work. The main focus of our study was not to elicit the mechanism of flecainide’s ability to correct QT prolongation but to investigate the fundamental drug-channel interaction between flecainide and Na\(_{1.5}\), specifically DG-ICM vs. WT-ICM.
In conclusion, we propose that the open-channel blocking mechanism is responsible for flecainide UDB and that increased open-channel affinity underlies the increased UDB in DG channels with a minor contribution from slowed recovery from UDB. We believe that our results provide definitive evidence for an open-channel blocking mechanism for flecainide UDB of Na⁺. Further investigation of identifying the structural features of DG channels responsible for the increased open-channel flecainide affinity remains to be done.

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