Preferred Block of Late Sodium Current in the LQT3 ΔKPQ Mutant by the Class Ic Antiarrhythmic Flecainide

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ABSTRACT

Flecainide block of Na⁺ current (iNa) was investigated in wild-type (WT) or the long QT syndrome 3 (LQT3) sodium channel α subunit mutation with three amino acids deleted (ΔKPQ) stably transfected into human embryonic kidney 293 cells using whole-cell, patch-clamp recordings. Flecainide (1–300 mM) caused tonic and use-dependent block (UBD) of iNa in a concentration-dependent manner. Compared with WT, ΔKPQ iNa was more sensitive to flecainide, and flecainide preferentially inhibited late iNa (mean current between 20 and 23.5 ms after depolarization) compared with peak iNa. The IC50 value of peak iNa for WT was 29 ± 4 μM (n = 20) and for ΔKPQ was 11 ± 1 μM (n = 26). For ΔKPQ, UDB of late iNa was greater than for peak iNa. Recovery from block was slower for ΔKPQ than for WT. We conclude that ΔKPQ interacts differently with flecainide than with WT, leading to increased block and slowed recovery, especially for late iNa. These data provide insights into mechanisms for flecainide block and provide a rationale at the cellular and molecular level that open channel block may be a useful pharmacological property for treatment of LQT3.

Materials and Methods

Clones and Construction of ΔKPQ Mutation. The human heart Na⁺ channel clone we used (hH1a) was kindly provided by Dr. H. Hartmann (Baylor College of Medicine, Houston, TX). The nucleotide sequence of the gene-specific therapy for LQT3 (i.e., the use of drugs that target the Na⁺ channel, more specifically, late iNa) is a logical approach. Most antiarrhythmic drugs block the Na⁺ channel in a use-dependent manner by preferential binding to either the inactivated state or the open state as described in the modulated receptor model (Hille, 1977; Hondeghem and Katzung, 1977). Inactivated state blockers of the Class Ib antiarrhythmic grouping (e.g., mexiletine) inhibit late iNa at the cellular level (Wang et al., 1997), shorten action potential duration in a cellular model of LQT3 (Priori et al., 1996; Shimizu and Antzelevitch, 1997), and shorten the QT interval in LQT3 patients (Schwartz et al., 1995). We hypothesized that an open channel blocker would also be effective (perhaps more so) because of the prolonged dwell time of LQT3 channels in the open state. To test this hypothesis, we studied the Class Ic antiarrhythmic drug flecainide, a predominant open state blocker, comparing tonic block and use-dependent block (UBD) by flecainide for peak and late iNa for the wild-type (WT) human cardiac Na⁺ channel and the ΔKPQ mutant. Our findings may provide a molecular mechanism for the recently observed correction of the QTc interval in the electrocardiograms of ΔKPQ LQT3 patients by flecainide (Windle et al., 1999).

ABBREVIATIONS: LQT, long QT syndrome; ΔKPQ, LQT3 sodium channel α subunit mutation with three amino acids deleted; iNa, Na⁺ current; UDB, use-dependent block; WT, wild-type; MEM, minimal essential medium.
otide and amino acid numbering follow Hartmann et al. (1994). The ΔKPQ mutation was kindly provided by Drs. John W. Kyle and Gayle S. Tonkovitch (University of Chicago, Chicago, IL). It was made by polymerase chain reaction techniques as described previously (Nagamoto et al., 1998). The entire polymerase chain reaction-generated region was completely sequenced to confirm the deletion and ensure that no unwanted changes were made in the channel.

**Cell Preparation and Transfection.** Cells from transformed human embryonic kidney cell line 293 were used. Approximately 5 × 10^6 cells were seeded on a 60-mm diameter plate (Falcon 3001) with 3 ml of culture medium 1 day before the transfection. Culture medium was MEM complete medium containing: minimum essential medium (Eagle’s salts and L-glutamine), 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids solution, 1 mM MEM pyruvate solution, 10,000 U of penicillin and 10,000 μg of streptomycin. Transfection was carried out using a cationic liposome method. Details have been described previously (Nagamoto et al., 1998).

**Electrophysiological Recordings.** I Na was recorded using the whole-cell patch clamp method. The bath solution contained 140 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 0.75 mM MgCl₂, and 5 mM HEPES, pH 7.4, set with NaOH. The pipette solution contained 120 mM CsF, 10 mM CsCl, 5 mM EGTA, and 5 mM HEPES, pH 7.4, set with CsOH. Electrodes were made from borosilicate glass with a puller (P-87; Sutter Instrument, Novato, CA) and heat-polished with a microforge (MF-83; Narishige, Tokyo, Japan). They had a final resistance of less than 1.2 MΩ in the whole-cell recording when filled with the pipette solution. Cells for study were placed in a Plexiglas chamber with continuously flowing bath solution mounted on an inverted microscope (Nikon, Tokyo, Japan) in a Faraday cage. Membrane currents were recorded with an Axopatch 200 amplifier (Axon Instruments Inc., Burlingame, CA) and data were acquired using pClamp v6.03. Data were digitized at 100 kHz and low pass filtered at 10 kHz.

Adequate voltage control for whole-cell currents was achieved using low resistance pipettes, highly conductive solutions, and series resistance compensation. To further minimize possible errors arising from loss of voltage control, we selected clonal lines expressing lower I Na density. The small size and low capacitance (<10 pF) of these cells allowed for very rapid charging of the membrane capacitance. We required the presence of two indices of voltage control: a graded slope of the activation curve (Boltzmann slope factor > 5.0) and scaling of currents of different amplitudes to the same test potential as in steady-state inactivation and recovery protocols. These indirect measures have been validated in other preparations (Makielski et al., 1987). With the whole-cell, ruptured patch technique, intrinsic kinetic changes with time have been reported (Makielski et al., 1987; Wang et al., 1996a). A shift in steady-state inactivation of <0.5 mV/min was observed in our preparation. To minimize the effects of this shift, flecainide was applied rapidly and block was measured within 5 to 7 min except for recovery protocols.

**Data Analysis.** Leak subtraction of peak and late currents was calculated by extrapolating holding currents at subthreshold potentials (≤100 mV) to the potential of interest (Makielski et al., 1987). Data were fit to model equations using nonlinear regression (pClamp v6.03 or SigmaPlot 3.0). Parameter estimates are reported ± S.E. of the parameter estimate; statistical differences in parameters were determined by a t statistic. Goodness of fit was judged both visually and by the sum of squared errors. The number of exponential components that best fit the data were determined by an F-ratio test (P < .05) to account for the increased number of free parameters. Experiments at different concentrations of flecainide were done in separate cells at the same time after patch rupture. Summary data are expressed as means ± S.E. n represents the number of cells studied. In figures, the symbols represent mean data and the error bars are shown only when they exceed the size of the symbol.

**Results**

**Tonic Block for Peak and Late INa is Greater for ΔKPQ.** Tonic block is defined as the decrease in INa in the presence of drug and in the absence of previous depolarizations or with a frequency of depolarizations sufficiently slow that no UDB developed. INa in response to a 24-ms depolarization to −20 mV from a holding potential of −150 mV was measured in control solutions and 5 to 7 min after exposure to various concentrations of flecainide (see example records in Fig. 1, A and B). Tonic block of peak INa was assessed by dividing the peak INa for the first depolarization in flecainide by the control peak INa. The concentration dependence of block (Fig. 1, C and D) was then fit with a single site-binding equation. The half concentration (IC50) value for block of peak INa was 127 ± 5.6 μM for WT (n = 20) and 80 ± 9.0 μM for ΔKPQ (n = 31), a significant difference (P < .001). To assess the tonic block of late current, the average value of (INa) between 20 ms and 23.5 ms after the depolarization was measured in control and flecainide (see Fig. 1, A and B, for examples) and analyzed in the same way as for peak INa (Fig. 1, C and D). The IC50 value for block of late INa was 44 ± 1.6 μM for WT (n = 20) and 19 ± 1.6 μM for ΔKPQ (n = 31), a significant difference (P < .001).

**Use-Dependent Block of Peak INa Is Greater for ΔKPQ.** Use-dependent block of peak INa was produced by imposing a pulse train of 200 depolarizations of 24-ms duration from −150 mV to −20 mV at 5 Hz. Under control conditions, application of this pulse train produced negligible (<5%) change in peak INa (data not shown). Figure 2A shows an example of UDB by 100 μM flecainide for WT and ΔKPQ. Figure 2B shows summary data for the relative amplitude of peak INa (compared with peak INa in the first pulse) in various concentrations of flecainide plotted versus the pulse number in the train. UDB of peak INa was deeper but developed more slowly for ΔKPQ than for WT. The time course of UDB was evaluated by fitting the peak currents to a one- or two-component exponential decay as shown in Fig. 2B with summary data in Table 1.

The steady-state UDB was evaluated as the decrease in peak INa for the 200th depolarizing pulse compared with the first depolarization pulse. Dose-response curves for the UDB of peak INa (Fig. 3) were fit to a single site-binding equation and yielded an IC50 value of 29 ± 4.4 μM for WT (n = 20) and 11 ± 1.0 μM for ΔKPQ (n = 26), again showing that ΔKPQ is significantly (P < .001) more sensitive to UDB by flecainide than WT. The steady-state UDB of the late currents were also analyzed. For WT UDB, the late currents were too small to be measured reliably (Fig. 3); for ΔKPQ, they could only be measured for the lower doses (n, Δ, n = 18). The results show that the late current amplitude was preferentially blocked. Note that UDB for the late current does not follow a single-site-binding curve, nor, on closer inspection, does UDB for peak current. The amplitude of UDB has a complex dependence on uptake and recovery from block during repetitive depolarization and repolarization (e.g., Starmer, 1987). Thus the fits to the peak current should be regarded as descriptive and not mechanistic.

**Recovery from Use-Dependent Block of Peak INa Is Slower for ΔKPQ.** We measured recovery rates after UDB induced by a train of 100 24-ms depolarizing steps to −20 mV at 25 Hz in 10 μM flecainide. The pulse train was followed by a variable recovery interval (Δt) and a test depolarization (Fig. 4, inset). Summary results for normalized peak INa in
response to the test depolarization are plotted versus a logarithmic scale of the recovery interval (Fig. 4). For control conditions, two components of recovery are apparent, with >95% of the recovery occurring within 10 ms for both WT and ΔKPQ. This is consistent with rapid recovery from inactivation of unblocked channels (An et al., 1996; Wang et al., 1996c). The small amplitude of the slow recovery component in control (<5%) indicates that the relatively short (24 ms) conditioning depolarizations are not sufficiently long to induce slow recovery (Shander et al., 1995). In 10 μM flecainide, three components of recovery are apparent (Fig. 4) for both WT and ΔKPQ. The fastest component (<10 ms) is consistent with recovery of unblocked channels from inactivation, an intermediate component (10 ms–1000 ms) is consistent with a rapid recovery from flecainide block, and the slowest component (>1000 ms) is consistent with a slow recovery from flecainide block. For recovery intervals <10 ms, recovery was faster for ΔKPQ compared with WT consistent with recovery from inactivation of unblocked channels. For recovery intervals >10 ms, the recovery rate from flecainide block was decreased for ΔKPQ compared with WT, especially for the first component of recovery. Results from recovery in the presence of 3 μM flecainide were similar to those of 10 μM (data not shown). This slower recovery for ΔKPQ is consistent with the deeper UDB found for the mutant (Fig. 2) and it is also consistent with the slower development of block. Quasi-equilibrium for the amplitude of UDB is reached when the number of channels blocked during a pulse is equal to the number of channels being unblocked during the recovery interval. Slower rates of interaction during either interval will slow the process of achieving this quasi-equilibrium (Starmer, 1987).

**Use-Dependent Block of Late I_{Na} Is Greater for ΔKPQ.** Fig. 5 shows the time course of UDB of peak and late (averaged between 20 ms and 23.5 ms) I_{Na} for 3 mM flecainide. At this concentration, little tonic block was exhibited and it is near the levels achieved in patients treated for arrhythmia. For ΔKPQ, the late component of I_{Na} was more sensitive to UDB by flecainide than was peak I_{Na} as also shown in Fig. 3.

**Flecainide Blocks the Open State of hH1.** To confirm the affinity of flecainide for open state channels, we compared the amount of UDB for peak I_{Na} with various pulse durations in 100 μM flecainide. If flecainide has important additional affinity for the inactivated state, then prolonging the depolarization would be expected to increase the level of block. Pulse trains of depolarizations to −20 mV with various pulse durations (1, 2, 5, 10, 20, 100, 200 ms) were applied from a holding potential of −150 mV and the recovery interval was kept constant at 200 ms. The fractional UDB in both WT and ΔKPQ saturated within the first 10 ms of the depolarization and less than 5% of block occurred after further pulse prolongation (Fig. 6). Although data such as these have...
traditionally been interpreted as indicating open state block (Anno and Hondeghem, 1990; Nitta et al., 1992), an alternative explanation that cannot be excluded includes preferential binding to another transiently available state, such as a preopen state.

**Discussion**

This study is the first description of the effects of a Vaughan-Williams Class Ic antiarrhythmic drug, the predominantly open channel blocker flecainide, on \( I_{Na} \) through human cardiac \( Na^+ \) channels and on \( I_{Na} \) through the \( \Delta KPQ \) mutant of the LQT3 syndrome. Consistent with previous studies of flecainide block in nonhuman \( Na^+ \) channels (Anno and Hondeghem, 1990), our results show that flecainide blocked \( I_{Na} \) in both a tonic and use-dependent manner and that recovery from block was slow and had multiple components. For the first time, we have characterized block of the late component of \( I_{Na} \), the current flowing after peak \( I_{Na} \) that

![Fig. 2](image-url). Time course of use-dependent block of peak \( I_{Na} \) in response to pulse trains for WT and \( \Delta KPQ \) hH1a channels. Pulse trains consisted of depolarizations of 24-ms duration from \(-150\) mV to \(-20\) mV applied at 5 Hz. A, superimposed current recordings in response to a pulse train in the presence of flecainide 100 mM. \( I_{Na} \) from the 1st, 2nd, 5th, 10th, 20th, 50th, 100th, and 200th pulse in the train are shown. B, time course of UDB for WT and \( \Delta KPQ \) channels. Drug concentrations of 1 (•), 3 (○), 10 (▲), 30 (□), 100 (■), and 300 μM (□) flecainide. Relative amplitude of \( I_{Na} \) for each pulse number was calculated as the peak \( I_{Na} \) for the pulse number divided by the peak \( I_{Na} \) for the first pulse in that train. Symbols represent the mean of from three to five measurements; S.E. is shown as a bar. Solid lines represent fitting with a one or two exponential function with the parameters of the fit shown in Table 1.

**TABLE 1**

Parameters for Fitting UDB for 5-Hz Pulse Trains by Flecainide

<table>
<thead>
<tr>
<th>Concentration μM</th>
<th>n</th>
<th>( \tau_f ) (pulses⁻¹)</th>
<th>( \tau_s ) (pulses⁻¹)</th>
<th>( A_f \times 10^{-2} )</th>
<th>( A_s \times 10^{-2} ) base (× 10⁻²)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>3</td>
<td>4</td>
<td>54.5 ± 4.6</td>
<td>87.4 ± 1.9</td>
<td>54.7 ± 7.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4</td>
<td>16.6 ± 6.8 (n = 4)</td>
<td>87.7 ± 20 (n = 3)</td>
<td>82.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4</td>
<td>8.5 ± 0.9</td>
<td>54.7 ± 7.7</td>
<td>60.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4</td>
<td>4.1 ± 0.4</td>
<td>69.0 ± 24</td>
<td>42.0 ± 3.6</td>
</tr>
<tr>
<td>ΔKPQ</td>
<td>300</td>
<td>4</td>
<td>2.0 ± 0.11</td>
<td>49.5 ± 12</td>
<td>26.8 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>121 ± 21</td>
<td>82.5 ± 3.5</td>
<td>54.7 ± 7.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>88.3 ± 14</td>
<td>74.8 ± 0.9*</td>
<td>74.8 ± 0.9*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>66.7 ± 4.7*</td>
<td>47.8 ± 4.0*</td>
<td>47.8 ± 4.0*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5</td>
<td>49.9 ± 1.2*</td>
<td>28.9 ± 1.4*</td>
<td>28.9 ± 1.4*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
<td>10.6 ± 0.5*</td>
<td>14.9 ± 0.8*</td>
<td>14.9 ± 0.8*</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>4</td>
<td>4.8 ± 0.4*</td>
<td>5.6 ± 0.6*</td>
<td>5.6 ± 0.6*</td>
</tr>
</tbody>
</table>

The relative amplitude of peak \( I_{Na} \) was fit with a single-component (\( A_f \times \exp(-t/\tau_f) + \text{base} \)), or two-component (\( A_f \times \exp(-t/\tau_f) + A_s \times \exp(-t/\tau_s) + \text{base} \)) decay; \( t \), pulse number; \( \tau_f \) and \( \tau_s \), time constants of fast and slow components; \( A_f \) and \( A_s \), fractional amplitudes of fast and slow components. Single component fits are reported where the F-ratio did not support fitting with two components (see Materials and Methods), and the \( \tau \)'s for the single component fits were arbitrarily assigned to the \( \tau_f \) column. \( n \) represents the number of experiments. Data = mean ± S.E. Significant difference compared with WT at \( P < .01 \) (*) and \( P < .05 \) (†).
influences action potential duration especially for the ΔKPQ channel where this late component is increased. For ΔKPQ compared with WT, the open channel blocker flecainide 1) preferentially inhibited the late component of INa in both a tonic and use-dependent manner, 2) blocked peak and late INa, with higher affinity, and 3) caused deeper UDB consistent with a demonstrated decrease in the recovery rate for flecainide for ΔKPQ current.

The ΔKPQ Mutant Has an Intrinsically Increased Affinity for Flecainide. The modulated receptor (Hille, 1977; Hondegem and Katzung, 1977) and the guarded receptor (Starmer, 1987) models provide two frameworks for understanding antiarrhythmic drug block in terms of intrinsic association and dissociation rates for ion channels. Our results show different affinities of flecainide for WT versus ΔKPQ channels and for peak versus late INa in ΔKPQ. Do
these results represent intrinsic affinity differences for drug binding to the channel protein, or are they instead secondary to the altered kinetics of ΔKPQ? Flecainide has been considered to be an open channel blocker (Anno and Hondeghem, 1990; Nitta et al., 1992), having higher affinity for the open state than the resting or inactivated states. Our data confirm for hH1 that flecainide has preferential affinity for a state transiently available at the beginning of the depolarization, consistent with open state block (Fig. 6). We originally hypothesized that flecainide might have a greater blocking effect on ΔKPQ because of an increased dwell time in the open state, rather than an intrinsic change in drug binding affinity. The results shown in Figure 6, however, suggest that use-dependent flecainide block occurred predominantly within the first 10 ms of depolarization for both WT and ΔKPQ channels. Therefore, additional binding to a persistent open state is unlikely to be an important mechanism for the higher affinity block in ΔKPQ channels. This suggests, instead, that the ΔKPQ channel has an intrinsically higher open state affinity for flecainide than WT.

The interpretation that the ΔKPQ mutant has an intrinsically increased affinity for flecainide is supported by additional findings. UDB of peak INa by flecainide was deeper (Fig. 2) and recovery from UDB was slower (Fig. 4) for ΔKPQ. Anno et al. (Anno and Hondeghem, 1990) interpreted flecainide recovery in terms of the modulated receptor hypothesis by suggesting that unbinding occurred rapidly via the open state and more slowly via the resting and inactivated states. In the present study, the ΔKPQ channel showed a dramatically reduced first component of recovery from flecainide block (between 10 ms and 3 s) compared with WT. Following the interpretation of Anno et al. (Anno and Hondeghem, 1990), our results show that flecainide unbound more slowly from the open state for the ΔKPQ channel, supporting the hypothesis that flecainide has an intrinsically higher affinity for the open state.

Tonic block is also greater for the ΔKPQ channel. Tonic block is usually interpreted as being caused by drug binding to the resting state; our data therefore support increased affinity of flecainide for the resting state of ΔKPQ. Alternatively, rapid drug binding to a preopen state, or to the open state before peak current is reached (Starmer et al., 1991), could also account for tonic block. Thus, the demonstrated increased affinity of flecainide for preopen or open states could account for both the increased tonic and UDB for the ΔKPQ channel. Our data do not distinguish between these possibilities.

Implications for the Antiarrhythmic Drug Binding Site. These data also provide additional evidence that the III-IV linker of the Na+ channel participates as part of the binding site for antiarrhythmic drugs in addition to the previously implicated Domain IV S6 (Ragsdale et al., 1996). The binding site for flecainide is thought to be the same, or to at least overlap, the binding site for other antiarrhythmics and local anesthetic drugs such as lidocaine (Ragsdale et al., 1996). Modification of three key amino acids (IMF to QQQ) on the III-IV linker of the channel produced a channel that inactivated slowly and had a reduced affinity for lidocaine (Bennett et al., 1995). A naturally occurring mutation (T1313M) in the III-IV linker of the skeletal muscle Na+ channel also slowed inactivation and decreased lidocaine affinity and UDB (Fan et al., 1996). For T1313M, the decreased block was shown to be caused by an intrinsic change in channel affinity for the drug rather than secondary to the changes in inactivation properties, implicating the III-IV linker as a part of the binding site. Our results with flecainide show an increased intrinsic (that is, not explained by the altered channel kinetics) affinity difference for ΔKPQ over WT, supporting the view that the III-IV linker forms part of the binding site for the drug.

Clinical Implications and Limitations of the Study. Mexiletine, generally considered to be an inactivated state blocker, has attracted the most attention as a therapeutic agent for LQT3 patients (Schwartz et al., 1995; Shimizu and Antzelevitch, 1997; Wang et al., 1997). The present study provides direct evidence that supports open channel block as a pharmacological model for the treatment of LQT3 patients. Compared with antiarrhythmic drugs such as flecainide, Class Ib drugs, such as mexiletine, provide less UDB at slow heart rates because of more rapid recovery kinetics. These kinetic differences offer a theoretical advantage for flecainide as a gene-specific therapy in patients with LQT3. Flecainide has been beneficial in a skeletal muscle disease in which Na+ channel mutants have increased late I Na (Rosenfeld et al., 1997). Recently, it has been shown to correct the QTc interval in the electrocardiograms of ΔKPQ LQT3 patients (Windle et al., 1999). Caution must be used in extrapolating the IC50 values reported here to the clinical setting because of the experimental conditions required by the voltage clamp technique. These include the hyperpolarized holding potential (~150 mV) used to minimize the effects of time-dependent kinetic shifts (Makielski et al., 1987; Wang et al., 1996a) and the lower temperature (Johns et al., 1989; Makielski and Falleroni, 1991) used to facilitate voltage control. Two additional considerations may also affect the clinical application of these results. First, in the CAST study, flecainide was associated with increased mortality in patients after myocardial infarction (CAST investigators, 1989). Patients with LQT, however, generally do not have structural heart diseases and thus flecainide might be used safely. Second, flecainide is not entirely specific for I Na. At relatively high concentrations, flecainide suppressed other currents, such as ATP-sensitive potassium current (Wang et al., 1995b), rapidly activating delayed-rectifier potassium current (Follmer et al., 1992; Wang et al., 1996b), the transient outward current (Wang et al., 1995b), and L-type calcium current (Scamps et al., 1989). Nonetheless, the efficacy of flecainide to shorten the QT interval in patients (Windle et al., 1999) suggests that late I Na block is a predominant mechanism contributing to the shortening.

In conclusion, this study provides new insight into mechanisms of drug block of the human heart Na+ channel and the ΔKPQ mutant channel of LQT3. Flecainide has higher intrinsic binding affinity for the mutant channel and a preference for blocking the late current. This may account for the correction of the QTc interval in the electrocardiograms of ΔKPQ LQT3 patients (Windle et al., 1999).

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