A Novel LQT-3 Mutation Disrupts an Inactivation Gate Complex with Distinct Rate-Dependent Phenotypic Consequences

ABSTRACT

Inherited mutations of SCN5A, the gene that encodes Na\(^+\) channel in the heart, cause congenital Long QT Syndrome variant 3 (LQT-3) by perturbation of channel inactivation. LQT-3 mutations induce small, but aberrant, inward current that prolongs the ventricular action potential and subjects mutation carriers to arrhythmia risk dictated in part by the biophysical consequences of the mutations. Most previously investigated LQT-3 mutations are associated with increased arrhythmia risk during rest or sleep. Here we report a novel LQT-3 mutation discovered in a pediatric proband diagnosed with LQTS but who experienced cardiac events during periods of mild exercise as well as rest. The mutation, which changes a single amino acid (S1904L) in the Na\(^+\) channel carboxy terminal domain, disrupts the channel inactivation gate complex and promotes late Na\(^+\) channel currents, not by promoting a bursting mode of gating, but by increasing the propensity of the channel to reopen during prolonged depolarization. Incorporating a modified version of the Markov model of the Na\(^+\) channel into a mathematical model of the human ventricular action potential predicts that the biophysical consequences of the S1904L mutation result in action potential prolongation that is seen for all heart rates but, in contrast to other previously investigated LQT-3 mutant channels, is most pronounced at fast rates resulting in a drastic reduction in the cells ability to adapt APD to heart rate.

INTRODUCTION

Congenital Long QT syndrome variant 3 (LQT-3) is a consequence of inherited mutations in SCN5A, the gene encoding the alpha subunit of the principal human heart Na\(^+\) channel and is characterized by a disruption in channel inactivation and subsequent increase in inward current during the critical plateau phase of the cardiac ventricular action potential.\(^1\)-\(^4\) The first LQT-3 mutation reported, a deletion of three amino acids (KPQ) within the inactivation gate,\(^5\) was found to promote a mode of gating in which a small percentage of channels flickered between open and closed states.\(^6\)^\(^7\) This channel phenotype, referred to as a bursting mode of gating, was shown to be consistent with the clinical phenotype, increased APD and arrhythmia risk during rest or bradycardia\(^8\) in animal\(^9\) and computational\(^10\) models, providing what has become the prototypical framework for understanding the mechanistic basis and arrhythmia susceptibility risk factors for LQT-3. Despite the fact that multiple types of mutation-altered Na\(^+\) channel activity have subsequently been shown to be able to delay cardiac repolarization and hence underlie the LQT-3 disease phenotype,\(^11\)-\(^13\) most LQT-3 mutations that have been reported and studied to date remain associated with increased arrhythmia risk at slow heart rates.

Here we report a novel SCN5A LQT-3 mutation, S1904L, which occurs within the region of the Na\(^+\)1,5 carboxy terminal (CT) domain previously found to be critical in the coordination of an inactivation gate complex consisting of the CT and inactivation gate (DIII-DIV linker).\(^14\)^\(^\text{5-16}\) Cardiac events experienced by the patient carrying this mutation were not limited to periods of rest or low sympathetic activity, but instead, also occurred during mild exercise. We find that the S1904L mutation disrupts the inactivation gate complex and causes distinct biophysical consequences, not dominated by channel bursting, but instead, by altered inactivation kinetics that are predicted to cause a novel disease phenotype wherein arrhythmogenesis is not predicted to be exacerbated by bradycardia. Our results reinforce the importance of the inactivation gate complex in coordinating physiologically correct inactivation and indicate that caution must be used...
in extrapolating risk factors from disease genotype without a more complete understanding of the physiological consequences of specific gene mutations.

**MATERIAL AND METHODS**

**Genetic screening.** The proband was bidirectionally sequenced for all the coding exons of KCNE1, KCNE2, KCNH2, KCNQ1, and SCN5A as detailed in the supplementary material.

**Molecular biology.** Gene constructs used for production of C-terminus protein fragments for biochemical studies were made with pET-SUMO vector (Invitrogen) containing gene fragment 1773-1937 of Na\textsubscript{v}1.5 for the wild type (ΔCT\textsubscript{WT}) backbone and the same residues but incorporating the S1904L mutation (ΔCT\textsubscript{MT}). A truncated C-terminal construct (1786-1863 of Na\textsubscript{v}1.5 WT; ΔCT\textsubscript{trunc}) was cloned into pGEX4T-3 vector.\textsuperscript{17} PCR amplified Na\textsubscript{v}1.5 DIII-DIV linker gene fragment (1471–1523) was cloned into pET28a(+) vector. Expression and purification of proteins. All constructs were expressed in *E. coli* BL-21DE3 cells (Stratagene). SUMO-fusion gene constructs were cotransformed with calmodulin (CaM). Tag- and CaM-free proteins (ΔCT\textsubscript{WT} and ΔCT\textsubscript{MT}) fractions were checked on SDS-PAGE followed by Coomassie blue staining. Purification procedures, including CaM separation, are detailed in supplementary material.

**Far Western blotting.** Interactions between DIII-DIV linker and purified ΔCT\textsubscript{WT}ΔCT\textsubscript{MT} and ΔCT\textsubscript{trunc} protein (15 μg each) were detected using a modified Far Western blot technique are detailed in supplementary material.

**Isothermal titration calorimetry.** Isothermal titration calorimetry (ITC) was performed using a MicroCal Omega-ITC titration microcalorimeter at 25°C. Purified wild type or mutant CT at a concentration of 60 μM was placed in the 1.4-ml calorimeter cell and His-tagged Na\textsubscript{v}1.5 DIII-DIV linker peptide (836 μM) was added sequentially in 10 μl aliquots (for a total of 25 injections) at 3-min intervals. Analysis was carried out (described previously in ref. 18).

**Electrophysiology.** Wild-type and mutant human sodium channel α subunits were coexpressed with human β1 subunits in HEK 293 cells using Lipofectamine (Invitrogen), and currents were measured with whole cell patch clamp procedures (previously described in ref. 19). Solutions and basic stimulation protocols are detailed in the supplementary materials. Data are represented as mean ± S.E.M. Statistical significance was determined using an unpaired Student’s t test; p < 0.05 was considered statistically significant.

For single channel experiments, pipettes were coated with Sylgard (Dow Chemical, Midland, MI) to decrease noise and capacitance of the glass. Electrode resistance was typically 5–9 MΩ when filled with internal solution. Solutions and pulse protocols are detailed in supplemental material. Idealization of single channel currents and the measurement of open time were carried out with the program SKM (QUB suite, Univ. Buffalo).\textsuperscript{20,21}

**Computational methods.** Sodium channel kinetics were modeled in the context of a Markov model based on the previous work of Clancy et al.,\textsuperscript{22} as described in detail in supplementary methods. Both macroscopic currents and single channel events are simulated with this approach. For single channel simulations, dwell time (T) for any state is as follows:

\[
T = -\ln r / \sum_{\alpha} \alpha
\]

(Eqn. 1)

where r is a random number [0,1] and \(\alpha_k\) are the transitions out of the current state j into all neighboring states (k). After time T, the channel enters a neighboring state, again governed by a random number, where the probability for entering every given state j is given by:

\[
\alpha_j / \sum_{\alpha} \alpha_j
\]

(Eqn. 2)

The random number generator is seeded to the internal clock to insure each run is unique. The Markov model is then inserted into a human ventricular action potential model\textsuperscript{23} and paced with twice threshold stimuli at a variety of pacing intervals to examine the cellular consequence of the mutation.

**RESULTS**

**Patient history.** The proband was a 12-year-old male who was healthy until the age of six when he had an episode of chest pain while walking to school. His ECG demonstrated a QTc of 464–469 ms and he had a history of palpitations, at rest and associated with activity such as playing basketball (Fig. 1A). He has never had any episodes of syncope, pre-syncope, cardiac arrest, or torsades de pointes. His family history is significant for a maternal half sister with a history of syncope as a child and a mother who had symptoms of palpitations and shortness of breath while taking indapamide, a medication associated with prolongation of the QT interval\textsuperscript{24} (Fig. 1B). The family is from Barbados. Sequence analysis for KCNQ1, HERG, SCN5A, KCNE1, and KCNE2 identified a c.5711 c→T S1904L mutation in SCN5A and no other gene variants. Screening of other family members, identified his symptomatic mother and maternal half sister as carriers of the S1904L mutation (Fig. 1B). Subsequent screening of 270 asymptomatic Caribbean subjects including 80 subjects from Barbados revealed no carriers of the S1904L mutation.

The S1904L mutation disrupts an inactivation complex. The location of the S1904L mutation was of interest to us because it falls within a structured region of the Na\textsubscript{v}1.5 CT domain (Fig. 2A)\textsuperscript{14,16} that has been shown to interact with the inactivation gate (DIII-DIV linker) and contribute to minimization of late Na\textsupscript{+} channel currents.\textsuperscript{15} We thus asked whether the S1904L mutation (1) affects interactions between the Na\textsubscript{v}1.5 CT and DIII-DIV linker (2) alters inactivation. We used two biochemical techniques to address question (1). Here, to minimize possible interaction perturbations caused by the large molecular tag GST (26 kDa) attached to a peptide of the DIII-DIV linker (MW 6kDa), previously used to detect interactions,\textsuperscript{15,25} we used tag-free purified CT domain proteins and purified DIII-DIV linker peptide (1471-1523 of Na\textsubscript{v}1.5) with a much smaller penta-His (<1 kDa) tag. The purified C-termini proteins consisted of residues 1773–1937 for the wild type (ΔCT\textsubscript{WT}) backbone and the same residues but incorporating the S1904L mutation for the mutant protein(ΔCT\textsubscript{MT}). A further truncated C-terminal construct (1786–1863 of Na\textsubscript{v}1.5) (ΔCT\textsubscript{trunc}) was also purified and used as a control because this protein contains only the first four predicted helices shown previously not to interact with the DIII-DIV linker.\textsuperscript{15}
Far Western blotting (FWB), a modified blot overlay technique, confirmed a direct interaction between the DIII-DIV linker and ΔCTWT protein that is significantly reduced by the S1904L mutation (Fig. 2B). ITC, which measures the heat created upon complex formation when proteins interact, revealed a 1:1 binding stoichiometry and a micromolar dissociation constant (Kd = 5 ± 2.5 μM) for the DIII-DIV linker peptide and ΔCTWT protein (Fig. 2C). Substitution of the S1904L mutant CT resulted in such low affinity binding that, under similar conditions, we were not able to resolve a binding affinity indicating dissociation constant far less than that for the ΔCTWT protein. A prohibitively large amount of DIII-DIV linker would have been required to generate a comparable binding isotherm for the S1904L mutant. Interestingly, in both of the experimental approaches described above, both ΔCTWT and ΔCTMT purified proteins were CaM-free providing evidence that CaM is not an obligatory component for the direct interaction between the DIII-DIV linker and the CT domain.

The S1904L mutation slows the onset of inactivation. We next studied the effects of the S1904L mutation on Na+ channel gating in HEK 293 cells expressing both the beta subunit hβ1 and either WT or S1904L NaV1.5. The mutation at S1904L did not affect the expression of the channel in the membrane (data not shown). Figure 3A compares families of averaged whole cell current traces for both WT (left panel) and S1904L (right panel) channels. The current traces suggest that the S1904L mutation slows the time course of the onset of inactivation, an effect confirmed in Figure 3D, which plots the half-time of decay of peak current vs. test voltage from -20 mV to +20 mV. Activation curves (Fig. 3B), generated from individual data traces show that the mutation does not alter the voltage dependence of channel activation (WT V1/2 = -24.9 ± 0.72 mV, k = 6.88 ± 0.57 mV).
Mutation-induced channel phenotype is predicted to be an increase in late reopenings but not bursting. Using a modified version of the Markov model scheme, the S1904L mutant channel activity can be estimated by simply reducing the transition rate $\alpha_5$ by roughly 30% and transition rate $\alpha_4$ by a factor of 4. The model reproduces the characteristics of the macroscopic recordings; namely, the slight slowing of time to half inactivation and slowly decaying late Na$^+$ current (B and C). Interestingly, for simulations of single channel activity, the parameters used to fit the macroscopic data also predicts a mutation-induced increase in late re-openings (0.012 for WT and 0.0592 for S1904L) as seen in the bar graph in (D) and the example computed traces from (E and F).

A Bar graph representing mean ± S.E.M. ratio of late current at 200 ms to peak current for both WT and S1904L.

and S1904L $V_{1/2} = -26.2 \pm 2.2$ mV, $k = 7.12 \pm 0.46$), but has very minor effects on steady-state inactivation. We found a small (-5 mV) but significant mutation-induced shift in the $V_{1/2}$ of steady state inactivation (WT $V_{1/2} = -61.3 \pm 1.64$ mV, $k = 6.40 \pm 0.71$ and S1904L $V_{1/2} = -66.2 \pm 0.86$ mV, $k = 7.31 \pm 0.38$) (Fig. 3C), and no significant effect on the recovery from inactivation following brief (50 ms) conditioning pulses (Fig. 3E). Figure 3F compares averaged TTX-sensitive currents (normalized to peak current) at two time bases and current gains. At low gain and a narrow time window, the mutation-induced slowing of inactivation is clear (arrow, Fig. 3F). High gain and a slower time scale (inset), reveal mutation-increased late current at 200 ms ($I_{NaL}$). This mutation causes nearly a fivefold increase in $I_{NaL}$ as illustrated in Figure 3G, which compares the average of $I_{NaL}$/peak measured at 200 ms during pulses to -10 mV (WT = 0.08 ± 0.02% and S1904L = 0.38 ± 0.06%). We found, however, that $I_{NaL}$ decreased with prolonged depolarization (data not shown), suggesting that the underlying mechanism might not be a mutation-induced increase in channel bursting which would not be expected to decrease with time. We thus explored predictions of computational models of altered channel gating that are consistent with our experimental data.

Mutation-induced channel phenotype is predicted to be an increase in late reopenings but not bursting. Using a modified version of the Markov model developed by Clancy et al. S1904L kinetics were fit by changing the appropriate rate constants as illustrated in Figure 4A. To reproduce the slowing of time to half inactivation, the transition rate out of the open state into the fast-inactivated state ($\alpha_2$) was reduced by roughly 30%. Importantly, the altered slow inactivation was best fit by decreasing the rate leaving the fast inactivated state into slower, more buried inactivated states.
The S1904L mutation increases the probability of channel reopening during prolonged depolarizations. (A) Cell-attached recordings of WT and S1904L channels. Recordings were obtained from pulses to -30 mV for 100 ms at a 1 Hz frequency from a holding potential of -120 mV. Five consecutive sweeps from a representative recording are shown for each case. (B) Bar graph showing the frequency with which a sweep has a channel that reopens after the first 30 ms of the test pulse (n = 4 cells and >1200 sweeps for WT and n = 5 cells and >1300 sweeps for S1904L). (C and D) MOT histogram generated from 400 depolarizations from -120 mV to -30 mV for both WT and S1904L channels. The average of the fits of the MOT histograms for both WT and mutant are shown in the graph. (E and F) Diary illustrating the Po determined during each 100 ms depolarization for WT (left) and S1904L (right) from representative recordings with 2 channels each.

Figure 5. The S1904L mutation increases the probability of channel reopening during prolonged depolarizations. (A) Cell-attached recordings of WT and S1904L channels. Recordings were obtained from pulses to -30 mV for 100 ms at a 1 Hz frequency from a holding potential of -120 mV. Five consecutive sweeps from a representative recording are shown for each case. (B) Bar graph showing the frequency with which a sweep has a channel that reopens after the first 30 ms of the test pulse (n = 4 cells and >1200 sweeps for WT and n = 5 cells and >1300 sweeps for S1904L). (C and D) MOT histogram generated from 400 depolarizations from -120 mV to -30 mV for both WT and S1904L channels. The average of the fits of the MOT histograms for both WT and mutant are shown in the graph. (E and F) Diary illustrating the Po determined during each 100 ms depolarization for WT (left) and S1904L (right) from representative recordings with 2 channels each.

The final fit is shown in Figure 4, panel B, where a single steady-state step depolarization from -100 mV to -10 mV is shown for the WT (black lines) and mutant (blue lines) channels. Panel C illustrates the increased contribution in late Na+ current in mutant (0.34%) versus WT (0.003%). Using the fit to macroscopic data in single channel simulations resulted in an increased probability in channel late openings as measured by event frequency in panel D (0.012 vs 0.059). Data here are shown as computed from 10,000 sweeps with voltage step from -120 mV holding potential to a -10 mV, 100 ms depolarizing pulse every 1 second. Panels E and F are example traces from simulations of single channels showing the late re-openings summarized in the bar graph.

Single channel measurements confirm model predictions. We next carried out single channel experiments to test directly the predictions of the computations and summarize them in Figure 5. Figure 5A, which shows five consecutive sweeps of representative cell attached single channel recordings from both WT (3 channels in the patch) and S1904L (5 channels in the patch) channels, reveals the mechanism of the mutation-induced late current and the slowing inactivation kinetics: S1904L channels have an increased propensity to reopen during a prolonged depolarization. To compare this effect with channel bursting, a biophysical property reported for other previously-investigated LQT-3 mutations, we monitored the frequency with which channels reopen after initial channel openings as well as the propensity of the channel to enter a bursting mode of gating (see Supplemental Methods). For the calculation of burst and reopening frequencies, 9 patches were examined for both S1904L and WT with channel number ranging from 1 channel per patch to a maximum of 7 channels per patch. The number of channels per patch was estimated by counting overlapping unitary current measured during a large number of test sweeps (400 per patch). We defined bursting as a series of openings or a prolonged opening that occur at times after 30 ms at a fixed test potential and for which the channel does not dwell in the closed state for longer than 3 ms. Similarly, we limited our analysis of late re-openings to times later than 30 ms. As summarized in Figure 5B, S1904L channels were found to have an increased frequency of late re-openings at -30 mV compared with WT channels (WT = 0.007 ± 0.001, S1904L = 0.08 ± 0.001). This phenomenon was also seen during voltage pulses to -10 mV (WT = 0.001 ± 0.002, S1904L = 0.03 ± 0.008). We also measured a non-significant increase in the probability that S1904L channels enter the bursting mode of gating (WT = 0.07±0.03% and S1904L = 0.3 ± 0.1%, p > 0.11) that is far less than other LQT-3 mutants, such as the deletion mutant ΔKPQ, that has previously been shown to enter the bursting mode in 3.5% of the sweeps recorded.

To extend the characterization of the mutant channel, open probability (Po) and mean open time (MOT) were calculated for four patches in both the WT and S1904L cardiac sodium channels. One of the WT patches contained a single channel, and the remaining seven patches each contained two channels. Open time histograms were constructed for openings at -30 mV and are illustrated in
Figure 6. Action potential prolongation by S1904L mutation. (A and B) Shown are the computed action potentials for the S1904L mutant (red traces) as compared to WT (black traces) and a classic LQT-3 mutation, the KPQ deletion mutant (ΔKPQ, blue traces). (C) In contrast to ΔKPQ cells, S1904L mutants prolonged APD more significantly at short cycle lengths rather than long suggesting a different disease phenotype.

DISCUSSION

From biochemical analysis to distinct clinical phenotype. Particularly because point mutations in NaV1.5 have been shown to underlie a variety of cardiac diseases, it is not surprising that there is phenotypic variability within the population of LQT3 patients.30 In this study, we have examined a novel mutation in the NaV1.5 CT domain associated with a clinically prolonged QT interval and palpitations that is of interest both for its effects on intramolecular interactions in the channel alpha subunit, its distinct effects on Na+ channel activity, and for its predicted arrhythmia risk profile which, in both cases, differ from previously characterized LQT-3 mutations.

The analysis of the biochemical, biophysical, and clinical consequences of the S1904L mutation in Nav1.5 has provided additional insight into the molecular mechanisms underlying human cardiac electrical activity. Our biochemical experiments in which we used simplified peptide fragments of both the NaV1.5 inactivation gate (DIII-DIV linker peptide) and CT domain constructs has confirmed the interaction of these two critical channel structures. We find an interaction characterized by a dissociation constant, comparable to that of the previously-described interaction between the inactivation gate and the inner mouth of the channel pore in brain Na+ channels,31 which is disrupted by the S1904L mutation. Our results thus confirm and expand upon earlier studies reporting inactivation gate and CT interactions,13,16,25 and report the first disease-associated mutation that disrupts the complex, confirming the physiological significance of the complex.

Perhaps more importantly, however, is the physiological significance of the subtle changes in Na+ channel gating caused by this perturbation in channel structure, gating changes that were resolved at the single channel level. The single channel functional consequences of the mutation are primarily reflected in an increased probability that mutant channels will reopen in a manner that can be distinguished from channels entering a bursting mode of gating.22,32 This subtle difference in the mutation-altered gating of the channel, while difficult to distinguish in whole cell recordings, which report increased late Na+ currents in both cases, is predicted to have important distinct effects on ventricular cellular activity, effects that were revealed by computational analysis.

Computational modeling predicts a distinct cellular phenotype. Our initial modeling focused on the slowing of the onset of macroscopic current fast inactivation by the S1904L mutation (Fig. 4), but our analysis of macroscopic (whole cell) currents led us to predict changes in single channel gating, predictions that were verified by
experiment. In our analysis of macroscopic currents, we found that the S1904L-altered gating could be accounted for largely by reduction of the transition rate out of the open state into the fast-inactivated state. This accounted for early kinetic events (the first 5 ms) during the onset of inactivation. Together with this change in transition rate out of the open state, slowing the rate leaving the fast inactivated state into slower, more buried, inactivated states was needed to more completely reproduce the slowly decaying late component of Na⁺ channel activity during more prolonged depolarization. Together, these kinetic changes, in turn, predicted that S1904L channels would experience an increased probability of channel reopening after entering the fast-inactivated state, a prediction confirmed by our experimental single channel recordings.

Importantly, the model predictions and the experimental measurements described mutation-altered gating that could be distinguished from channels predominantly entering a bursting mode. This distinction, which may appear as a subtle difference in gating, turned out to have very important physiological consequences. Previously-analyzed LQT-3 mutations, such as the ΔKPQ or Y1790C mutations, which increase late currents by promoting a bursting mode of gating are associated with a disease phenotype that is exacerbated at slower heart rates. 8,10,22,33 In contrast, our modeling and single channel data predict that the S1904L mutation predict a mild disease phenotype that is most pronounced at fast rates.

A clinical phenotype with unusual risk profile consistent with extrapolation from altered single channel gating. The clinical history of the proband is remarkably consistent with the computational predictions based on single channel analysis. It had suggested that the altered channel activity might be expected to be modestly different from wild type channel activity and perhaps have a novel relationship between dysfunctional channel activity and stimulation rate. Clues came from both a modestly-prolonged QT interval (QTc of 464–469 ms) and from the fact that cardiac events that were reported were not restricted to periods of rest and/or sleep, but occurred as well during mild exercise. The proband’s family history is significant for a mother with indapamide associated palpitations and a maternal half-sister with several episodes of syncope and both of these relatives were found to be carriers of S1904L SCN5A. Our functional analysis had been motivated in large part the hypothesis that, in fact, channel activity altered by this mutation might provide new insight into a novel mechanism underlying Na⁺ channel-dependent action potential prolongation in the heart. Our interest was also stimulated by the physical location of the mutation within the putative structure of the Na⁺ 1.5 C-T domain. Our biochemical and biophysical analysis support the proposed hypothesis and provide additional evidence for the physiological importance of the interaction between the III-IV linker and C-T domain in Na⁺1.5 channels.

CONCLUSIONS

The results of this study thus reinforce the physiological importance of the molecular interactions between the Na⁺1.5 channel inactivation gate and C-T domain, an interaction that is necessary to ensure proper Na⁺ channel inactivation, particularly during prolonged depolarization. The results also demonstrate the importance of understanding functional consequences of disease-causing mutations of ion channels and the potential problems of extrapolating disease-associated arrhythmia risk and possibly pharmacological therapy based only on identification of a gene variant without more detailed understanding of gene product function. Investigations of gene mutations in LQTS have demonstrated, without question, the necessity of linking genotype to clinical phenotype in order to reasonably predict arrhythmia risk factors, 8,34 but the present study indicates the importance of both biological and in silico analysis of the physiological consequences of specific gene mutations in the overall evaluation of risk factors associated with specific channelopathies of the heart.

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