An LQTS6 MiRP1 Mutation Suppresses Pacemaker Current and is Associated with Sinus Bradycardia

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An LQTS6 M54T Mutation and Sinus Node Dysfunction. Background: Sinus node (SN) dysfunction is observed in some long-QT syndrome (LQTS) patients, but has not been studied as a function of LQTS genotype. LQTS6 involves mutations in the hERG β-subunit MiRP1, which also interacts with hyperpolarization-activated, cyclic nucleotide gated (HCN) channels—the molecular correlate of SN pacemaker current (I\(_f\)). An LQTS registry search identified a 55-year male with M54T MiRP1 mutation, history of sinus bradycardia (39–56 bpm), and prolonged QTc.

Objective: We tested if LQTS6 incorporates sinus bradycardia due to abnormal I\(_f\).

Methods: We transiently co-transfected neonatal rat ventricular myocytes (to study currents in a myocyte background) with human HCN4 (hHCN4, primary SN isoform) or human HCN2 (hHCN2) and one of the following: empty vector, wild-type hMiRP1 (WT), M54T hMiRP1 (M54T). Current amplitude, voltage dependence, and kinetics were measured by whole cell patch clamp.

Results: M54T co-expression decreased HCN4 current density by 80% compared to hHCN4 alone or with WT, and also slowed HCN4 activation at physiologically relevant voltages. Neither WT nor M54T altered HCN4 voltage dependence. A computer simulation predicts that these changes in HCN4 current would decrease rate and be additive with published effects of M54T mutation on hERG kinetics on rate.

Conclusions: We conclude that M54T LQTS6 mutation can cause sinus bradycardia through effects on both hERG and HCN currents. Patients with other LQTS6 mutations should be examined for SN dysfunction, and the effect on HCN current determined. (J Cardiovasc Electrophysiol, Vol. pp. 1-7)

HCN4, HCN2, KCNE2, long-QT syndrome 6, M54T mutation, MiRP1, pacemaker current, sinus node dysfunction, sinus bradycardia

Introduction

The long-QT syndrome (LQTS) predisposes to cardiac arrhythmias and is characterized by prolongation of the QT interval on ECG. One of the leading causes of sudden death in the young, LQTS is caused by mutations in genes encoding ion channels controlling ventricular repolarization, or mutations in proteins that affect the function of these channels. Patients with LQTS may present with syncope, seizures, or sudden death.

Some studies suggest that average resting heart rate is lower in LQTS patients, especially children compared with normal control subjects.1–3 In the study by Schwartz et al. in 1985 proposing the first set of diagnostic criteria for LQTS, low heart rate in children is one of the minor criteria.4 In the 1993 update to this study, a resting heart rate below the second percentile still remains a diagnostic criteria.5 In addition to being a strong clue to diagnosis in the pediatric age group, sinus bradycardia is a risk factor for cardiac events in family members of LQTS patients.6

Molecular genetic studies have identified up to 12 genetic loci associated with LQTS.7 Mutations in potassium channel subunits KVLQT1 (LQTS1), hERG (LQTS2), KCNE1 (LQTS5), and KCNE2 (LQTS6) diminish the potassium current and cause delayed repolarization of the myocardium,6–10 prolonging the QT interval. Of these, the KCNE2 gene encodes a single transmembrane domain protein, MinK related Peptide 1 (MiRP1), which assembles with pore-forming α subunits to establish the attributes of characteristic channels in vivo. MiRP1 functions as a β subunit of hERG (human ether-a-go-go channel) potassium channel,11 and together these proteins form the functional channel that generates I\(_{Kr}\), rapid delayed rectifier potassium current. The QT prolongation in LQTS6 is ascribed to the effect of MiRP1 mutations on I\(_{Kr}\).

MiRP1 combines with and alters the function of not only hERG,11 but also other K channels (Kv4.2,12 KvLQT1,13,14 Kv1.515) as well as the HCN (hyperpolarization activated cyclic nucleotide gated channel) family of pacemaker channels.16,17 MiRP1 has been shown to modulate HCN
channel expression and gating properties in myocytes and heterologous expression systems. However, these effects vary with different isoforms and species. High levels of both MiRP1 and HCN subunits (predominantly HCN4, with HCN1 or HCN2 also present, depending on the species) are expressed in the cardiac SN, where pacemaker current contributes to impulse initiation. Mutations in the HCN gene are associated with familial sinus bradycardia. The question of whether human MiRP1 mutations would differentially affect HCN current compared to WT hMiRP1 protein in a manner that could contribute to sinus bradycardia has not been addressed.

The International Long-QT syndrome Registry, with its expanding number of genotyped families, has provided an opportunity to study the clinical aspects and explore the genotype–phenotype relationships in LQTS. A search of the Long-QT syndrome Registry identified a 55-year-old patient with the M54T MiRP1 mutation (first reported by Abbott et al.) with sinus bradycardia. This patient’s previously healthy daughter died suddenly at the age of 13. No abnormalities were found at autopsy, but a subsequent blood test confirmed the M54T mutation. Following this result, the index patient tested positive for the M54T mutation. A 12-lead ECG from this patient at age 51 years shows heart rate of 39 bpm (Fig. 1) with a corrected QT interval of 476 milliseconds. This patient had telemetry strips from age 7 years that showed underlying sinus bradycardia with heart rates between 31 and 56 bpm.

We thus asked if this M54T MiRP1 mutation associated with LQTS6 affects HCN function differently than the wild-type hMiRP1 (WT). This difference if present could possibly explain SN dysfunction in this subgroup of LQTS patients. If there is no difference it could mean that any observed SN dysfunction in LQTS6 patients arises from effects on SN targets other than HCN. Previous studies have used different species isoforms to study effects of MiRP1 on HCN; e.g., Qu et al. tested the effects of rat MiRP1 on mouse HCN2. Abbott et al. have shown that when different species of MiRP1 interact with hERG, the functional outcome is different. Therefore, in this paper we study the effects of human WT MiRP1 and its M54T mutant on human HCN isoforms.

Methods

Molecular Cloning and Mutagenesis

Mammalian expression vectors

The complete human HCN2 and human HCN4 sequences were packaged in pDC515 and pDC516 (Microbix Biosystems, Inc., Mississauga, Ontario, Canada), respectively, to create the pDC515hHCN2 and pDC516hHCN4 vectors driven by CMV promoter. For hMiRP1, the pCMVNeo vector (a gift from Dr. Geoffrey Abbott, University of California, Irvine) was used as a carrier. To identify transfected cells in all groups, the gene encoding the green fluorescent protein (GFP) was co-transfected using the pmaxGFP vector (Amxa Nucleofector kit, Lonza Group Ltd., Basel, Switzerland).

The M54T mutant was created using Quickchange Site-directed mutagenesis kit (Stratagene). In the M54T mutant, threonine replaces methionine in the transmembranous region. The primers (Genelink) designed for mutagenesis are shown in Table 1. Successful mutagenesis and the mutant DNA sequence were verified by sequencing at the Columbia University DNA sequencing facility.

Preparation of Genomic DNA and Sequencing of HCN4

Informed consent was obtained from the subject. The protocol for performing skin biopsies on patients and families with inherited heart disease has been reviewed and approved by Columbia University Institutional Review Board (IRB-AAAD5685). A 3 mm punch biopsy was obtained from the subject and dermal fibroblasts were derived in tissue culture. Genomic DNA was derived from cultured fibroblasts using QiAmp DNA Mini Kit (Qiagen Inc, Valencia, CA, USA). HCN4 coding sequences were amplified by PCR using FastStart Taq polymerase (Roche). Primers selected are listed in Table 1. Successful mutagenesis and the mutant DNA sequence were verified by sequencing at the Columbia University DNA sequencing facility.

Cell Culture and Transfection

Cultures of newborn rat ventricular myocytes (NRVM) were prepared as previously described. Cultures were quickly removed and ventricles were dissociated by a standard trypsinization procedure. Myocytes were harvested and plated in protamine-sulfate coated dishes. On the day of the experiment, the cell monolayer was resuspended by brief

<table>
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<th>Primers</th>
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<td></td>
<td>Reverse</td>
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exposure to 0.1% trypsin and the cells then re-plated onto fibronectin-coated coverslips.

Transfection was performed on day 0 by electroporation using the Rat Cardiomyocyte Neo Nucleofector® Kit (Lonza Group Ltd.). The NRVM (2 × 10⁶ cells) were transfected with a total of 3.5 μg of DNA as follows: either pDC516hHCN4 (1.5 μg) or pDC515hHCN2 (1.5 μg) with one of the following: (1) pCINeoWThMiRP1 (1.5 μg), (2) pCINeoM54ThMiRP1 (1.5 μg), (3) pDC516 (“Empty”; 1.5 μg empty plasmid) to maintain equal DNA load during electroporation. To identify the transfected cells for electrophysiology, all the cell groups were co-transfected with GFP (green fluorescent protein; 0.5 μg).

Electrophysiology

The whole-cell HCN current from myocytes was recorded at 35°C. Extracellular solution contained (mM): NaCl, 140; NaOH, 2.3; MgCl₂, 1; KCl, 10; CaCl₂, 1; HEPES, 5; glucose, 10; MnCl₂ (2 mM), and BaCl₂ (4 mM) were included to eliminate Iₖ and Iₖ₁, respectively; pH 7.4. The pipette solution included (mM): aspartic acid, 130; KOH, 146; NaCl, 1; CaCl₂, 2; EGTA-KOH, 5; Mg-ATP, 2; HEPES-KOH, 10; pH 7.2. The pipette resistance was typically 3–5 MΩ. An Axopatch-200B amplifier and pClamp9.2 software (Molecular Devices) were used for data acquisition and analysis.

The normalized plot of tail current versus test voltages was fit to a Boltzmann function, and the midpoint, and slope factor were determined from the fitting. Time constants of activation were obtained by fitting the early time course of current traces with a mono-exponential function; the initial delay and any slow activation phase were not fit. Deactivation time constants were obtained by a single exponential fit of the time course of the current trace at each test voltage after activation by a prepulse to –125 mV. For both activation and deactivation, the duration of the trace being fit was at least 3 times the measured time constant to ensure accuracy. Data analysis was done using Origin software version 7. Pooled data are expressed as mean ± SEM; n denotes number of cells. Significant statistical significance was determined by the t-test or ANOVA, as appropriate; P < 0.05 was considered significant.

HCN4 current was defined as the time-dependent component taken at the end of 9-second hyperpolarizing test voltage steps from –35 to –115 mV with increments of 10 mV from the holding potential of –35 mV. The hyperpolarizing step was followed by a 9-second step to –110 mV to record the tail current and then a 0.5-second pulse to –5 mV to ensure full deactivation. Current-voltage relationships were obtained by measuring the current amplitude after application of 9-second hyperpolarizing test pulses and normalization to the fully activated current at –110 mV. The HCN2 protocol varied from the above in that the hyperpolarizing step duration was 6 seconds and the tail current was measured at –100 mV where current was fully activated, reflecting the faster activation kinetics of this isoform.

Computer Simulation

We employed the sinoatrial node model of Kurata et al. The magnitude, midpoint, and slope of the pacemaker current activation curve were modified to match the experimentally observed effect of the M54T mutation on HCN4. We also modified Iₖ to reflect the published report of the effect of M54T on this current. The Kurata model represents significant improvements over earlier models as it provides well-integrated explanations of the electrophysiological behavior of primary pacemaker cells in the rabbit SN and incorporates Iₖ not included in previous models and expression of kinetics of Iₖ, in addition to other currents.

Results

Effect of M54T MiRP1 on Biophysical Properties of HCN4 Currents Expressed in NRVM

Since we had previously shown that HCN channel biophysics varies with cellular context, we studied biophysical properties of HCN4 and HCN2 current in NRVM to provide a cardiac background. HCN2 or HCN4 was

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M54T decreases HCN4 amplitude

Representative current recordings in NRVM for +WT and +M54T group are shown in Figure 2A and B. Tail currents were measured at −110 mV where the current was fully activated and to avoid contaminating currents at less negative voltages. Co-expression of M54T (15 ± 1.4 pA/pF) decreases HCN4 tail current density by 80% compared with HCN4 alone (81 ± 13 pA/pF) as well as with +WT (85 ± 7 pA/pF) as shown in Figure 2C. The magnitudes of the time dependent currents for M54T are significantly different from the HCN4 and +WT groups (n = 6–10) by ANOVA, P < 0.001. WT co-expression did not have a significant effect on the HCN4 current amplitude.

M54T slows activation kinetics of HCN4 current

Figure 2D plots average time constants of activation as a function of voltage. It is evident that the M54T mutant, when co-expressed with HCN4, slows the kinetics in comparison with HCN4 alone or +WT. These differences are statistically significant for M54T at −65 and −75 mV (by pairwise multiple comparison, Bonferroni t-test, P < 0.05; n = 6–10), voltages relevant to the membrane potential in sinus node (SN). Tau at −65 mV was 2246 ± 142 milliseconds for M54T compared with 1443 ± 271 milliseconds for HCN4 or 1167 ± 135 for +WT; n = 6–10, P < 0.01. We did not find any statistically significant difference between kinetics of the HCN4 group and +WT group. We separately studied the effect of the M54T mutation on HCN4 deactivation kinetics and found no significant difference between the 3 groups (Fig. 2E). Tau at −65 mV was 2660 ± 204 milliseconds for M54T compared with 2506 ± 202 milliseconds for HCN4 or 2721 ± 288 for +WT; n = 8–10, P > 0.05. Further, the M54T mutant did not affect voltage dependence of HCN4 current. The midpoints of activation of the 3 groups: HCN4 alone, +WT, +M54T, were −69 ± 4, −71 ± 3, −68 ± 3 mV (P > 0.05 by ANOVA, n = 6–10). There also was no statistically significant difference between the groups in the slope factors of the fractional activation curves (data not shown).

Effect of WT and M54T MiRP1 on Biophysical Properties of HCN2 Currents Expressed in NRVM

M54T did not alter HCN2 amplitude

To determine if the effects of M54T were specific to the HCN4 isoform, we repeated these studies with hHCN2.
In contrast to HCN4, when measuring HCN2 tail currents (at –100 mV), the M54T mutant (67 ± 5 pA/pF) had no significant effect on HCN2 amplitude in comparison with HCN2 (78 ± 7 pA/pF) or +WT (70 ± 2 pA/pF), P > 0.05 by ANOVA, n = 6–10 (Fig. 3A).

**M54T mutant slows activation kinetics of HCN2 current**

Normalized current traces of sample cells of the HCN2 and +M54T group at –75 mV are presented in Figure 3B showing slower activation kinetics with the +M54T group. In Figure 3C, the HCN2 alone and +WT curves did not differ significantly; however, there was a statistically significant difference between the M54T curve in comparison with the former 2 groups (P < 0.05 by ANOVA, n = 6–10). For M54T, tau at –65 mV was 1190 ± 126 milliseconds compared with 705 ± 46 milliseconds for HCN2 or 725 ± 29 for +WT; n = 6–10, P < 0.01. Thus, similar to what was observed for HCN4, the activation kinetics for HCN2 were slower when co-expressed with M54T mutant in comparison with HCN2 or +WT.

In addition to amplitude and activation kinetics, the other biophysical property we studied was voltage dependence. The mean V1/2 for the HCN2 was –64 ± 3 mV versus +WT (–66 ± 3 mV) or +M54T (–66 ± 2 mV); no statistical difference was found between the 3 groups, n = 6–10. The slope factor of the fractional activation curves did not differ significantly between the groups (data not shown).

**Computer Simulation of Effect of M54T Mutation on SN Automaticity**

To investigate the possible impact of the change of amplitude and kinetics of HCN by the M54T mutant on spontaneous rate, we employed the Kurata computer model of SN. This model is based on the rabbit SN, the tissue with the most complete data set since the rabbit SN is the prototypical experimental model for the study of SN channel function. As such, our goal was not to quantitatively replicate what occurs in a human subject, but rather to determine if our experimentally measured changes in pacemaker current could qualitatively slow rate. Simulated action potentials are shown in Figure 4. M54T mutation decreased the HCN4 amplitude by 80% and slowed activation kinetics. Using these HCN experimental data, the cycle length (CL) with M54T mutation was longer at 331 milliseconds compared to control whose CL was 307 milliseconds. The M54T mutant effect on Ikr increased the CL to 324 milliseconds. This is secondary to M54T slowing the deactivation kinetics of Ikr when co-expressed with hERG in comparison with WTMiRP1 co-expressed with hERG.²⁶ Combining both the effects of Ii and Ikr, the CL changed to 358 milliseconds reflecting a greater slowing of rate than the individual effects of Ii or Ikr.

**HCN4 Sequence Analysis from Subject Patient**

The heterologous expression studies demonstrate that the M54T mutation of MiRP1 can result in altered HCN4 current, and the computer simulation suggests that when this is combined with the known effect of this mutation on hERG the result is a measurable slowing of rate. However, it is possible that there are other genetic factors in our subject that contribute to the marked bradycardia. The most obvious candidate would be a mutation in HCN4, since such mutations have been linked to familial sinus bradycardia.²² We therefore sequenced the HCN4 gene in our subject. We identified 3 single-nucleotide polymorphisms (SNPs; Table 3), one of which leads to a missense (G36E) and 2 to synonymous mutations (L520L and P1200P), and all previously reported (http://www.ncbi.nlm.nih.gov/projects/SNP). Of the 3 SNPs found, only 107G→A, generating the missense mutation G36E, has a minor allele frequency just below 0.05, the value assumed to separate rare variants from common polymorphisms.³¹ To our knowledge, no pathological effect of any of these SNPs is reported in the literature.

**Clinical Implication**

We had identified our index patient, a 55-year-old Caucasian male with LQTS6. The patient’s daughter died of sudden death at age 13. Subsequent postmortem genetic testing revealed the M54T mutation. She did not have any ECG prior to her death. The mother’s ECG was normal and she tested negative for the mutation. Our patient who tested positive for the M54T mutation had resting bradycardia in the thirties from a young age. Holter monitoring showed an average heart rate of 43 bpm, with lowest heart rate of 30 bpm and highest rate of 125 bpm along with 17 pauses. The underlying rhythm was sinus bradycardia. Our results are consistent with the bradycardia arising, at least in part, from the effect of the M54T mutation on Ii and Ikr currents contributing to SN automaticity.

**Discussion**

Long-QT syndrome type 6 is a rare type of LQTS. The majority of the patients have LQTS mutations in the first 3 genes (KVLQ1T, hERG, and SCN5A). Nonetheless, MiRP1
protein has been found in the SN and interacts with the pacemaker channels, functioning as a β subunit. The mutations in this gene affecting the pacemaker channel function are interesting as these modulate HCN properties different than the WT. Most SN studies in LQTS patients have been done in patients with LQTS 1–3. We focused on LQTS6 mutation M54T and its effect on biophysical properties of HCN isoforms, providing an underlying mechanism for sinus bradycardia seen in our index patient. None of the previous studies have explored pacemaker current as a cause of sinus bradycardia in LQTS.

We over-expressed HCN channels in NRVM to investigate the biophysical properties of HCN currents alone, when co-expressed with WT, and in comparison with co-expression of HCN and the M54T mutant. We used this preparation to provide a myocardial context, since we previously demonstrated that heterologous HCN currents differ biophysically when expressed in myocytes versus non-myocytes. However, we did not use these automatic cells to mimic SN function, since there are clear differences in factors contributing to automaticity in NRVM and SN, such as the contribution of Na current and absence of Ca-stimulated adenyl cyclase isoforms in NRVM. These rat cells do, however, express endogenous HCN and MiRP1 that could interact with the exogenous human species we are expressing. However, we previously demonstrated that the exogenous HCN2 and MiRP1 proteins are expressed in these cells at levels far above those of endogenous proteins. Thus, the endogenous rMiRP1 cannot saturate the high levels of expressed hHCN4 in these experiments. Our results show that: (1) M54T slows activation kinetics of HCN2 and HCN4 at physiologically relevant diastolic potentials, but does not alter HCN4 deactivation kinetics, (2) M54T markedly decreases HCN4 but not HCN2 current amplitude, (3) voltage dependence of neither HCN2 nor HCN4 was altered by M54T.

These findings were used in generating a SN computer simulation. The simulation predicts that the observed changes in HCN4 current slow rate 7%, that the published change in HCN4 function and thus is a plausible explanation for the bradycardia. The Kurata computer simulation predicts that the altered current will slow SN rate, and that this effect is additive with that of MiRP1-M54T on hERG current. However, the bradycardia in the computer simulation is modest, suggesting there may be effects of MiRP1-M54T on other currents in SN and/or limitations of the existing rabbit based SN computer model. Additional studies will be required to establish a causal relation between the effects of M54T on SN currents and the marked bradycardia observed in this patient.

Bradycardia remains an important risk factor for sudden cardiac events in patients with LQTS. A recent Scientific Statement from the American Heart Association (AHA) recommends that hospital patients should receive QT interval monitoring if certain conditions are present: sinus bradycardia is one of those conditions. This study is the first to explore the cause of this risk factor in LQTS6 at a cellular level as an effect on pacemaker current. The functional effects of these mutants on pacemaker channel could translate into subtle clinical differences in SN function that may be otherwise missed in the LQTS6 group. However, we believe there are broader implications for other mutations related to LQT or other arrhythmias where the mutation lies not in a channel but in an interacting subunit, because such subunits often interact with multiple ion channels and therefore can have secondary effects. Comparable secondary effects could occur in other LQT subtypes, namely type 4 and 9 where ankyrin and caveolin 3 subunits also interact with HCN channels. The data suggest that as arrhythmia predisposition genotyping becomes more common, asymptomatic individuals with potentially pathological MiRP1 variants in such pedigrees should be aware of important associated abnormalities like sinus bradycardia. Further, there are other LQTs types that affect nonchannel proteins and which may impact the HCN or other SN currents; these also may merit exploration in terms of SN function.

References


