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Article

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A common *SCN5A* polymorphism modulates the biophysical effects of an *SCN5A* mutation

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Our understanding of the genetic basis of disease has expanded with the identification of rare DNA sequence variations ("mutations") that evoke inherited syndromes such as cystic fibrosis, congenital epilepsy, and cardiac arrhythmias. Common sequence variants ("polymorphisms") have also been implicated as risk factors in multiple diseases. Mutations in *SCN5A*, the cardiac Na^+ channel gene, that cause a reduction in Na^+ current may evoke severe, life-threatening disturbances in cardiac rhythm (i.e., Brugada syndrome), isolated cardiac conduction disease, or combinations of these disorders. Conduction disease is manifest clinically as heart rate slowing (bradycardia), syncope, or "lightheadedness". Recent electrophysiologic studies reveal that mutations in particular families exhibiting cardiac conduction disease cause marked effects on several competing voltage-dependent gating processes, but nonetheless cause a mild "net" reduction in Na^+ current. Here we show that a common *SCN5A* polymorphism (H558R) in the Na^+ channel I-II interdomain cytoplasmic linker, present in 20% of the population, can mitigate the in vitro effects of a nearby mutation (T512I) on Na^+ channel function. The mutation and the polymorphism were both found in the same allele of a child with isolated conduction disease, suggesting a direct functional association between a polymorphism and a mutation in the same gene.

Introduction

Inherited mutations in the family of genes encoding voltage-gated Na^+ channel pore-forming subunits (*SCNXA*) underlie a wide spectrum of neurologic, musculoskeletal, and cardiovascular disorders. Mutations in *SCN5A,* the cardiac Na^+ channel gene, generally evoke disturbances in cardiac rhythm (1–3); however, it is increasingly clear that a great many single amino acid substitutions within the *SCN5A* coding region can evoke a broad spectrum of cardiac rhythm behavior. While patients with *SCN5A* mutations linked to either long QT syndrome or Brugada syndrome experience sudden, life-threatening arrhythmias, patients with isolated conduction disease exhibit heart rate slowing (bradycardia) that manifests clinically as syncope, or perhaps only as lightheadedness.

Functional biophysical studies of cardiac Na^+ channels with Brugada syndrome mutations invariably reveal defects in either gating function or surface membrane expression that lead to a marked reduction in Na^+ current (4–6). This causes a disproportionate hastening of repolarization in the epicardial cell layer, leading to a transmural electrical gradient that provokes reentrant arrhythmias (7, 8). More recent studies of Na^+ channels have identified particular mutations linked to isolated conduction disease; these studies have revealed defects that also lead to a reduction of Na^+ current, and in some cases the ion channel functional defect is mild (3, 9). Whereas even a single Na^+ channel mutation may cause multiple changes in gating function, each of which could individually drastically increase or decrease the Na^+ current, computational models of cardiac excitability equipped to consider the ensemble of these mutational effects may predict only a mild net decrease in Na^+ current (3).

Mutations associated with a reduction of Na^+ current may evoke Brugada syndrome, conduction disease, or both. While the severity of the Na^+ channel functional defect may underlie the ECG phenotype in some cases (3), other unrecognized factors (i.e., humoral regulation, auxiliary subunits, transcriptional regulation) are certain to play a role. A recent study identified a new Brugada syndrome locus (10) distinct from *SCN5A* and associated with progressive conduction disease, suggesting that other genes could also play a role in the manifestation of the disease phenotype.

Most contemporary studies of ion channels have sought to characterize the functional effects of isolated mutations linked to rare diseases. At the same time,
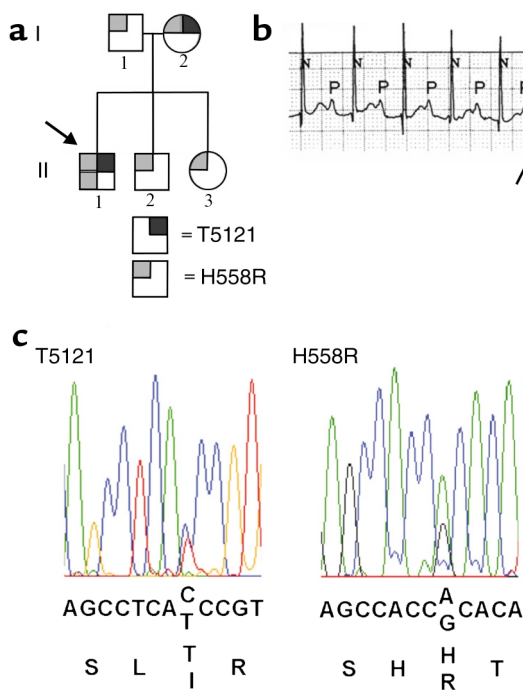
Figure 1

Genotype and ECG phenotype. (**a**) Pedigree shows affected individuals. Gray boxes represent the polymorphism, H558R, while black boxes represent the T512I mutation. While the father (I-1) was heterozygous for H558R, the mother (I-2) was heterozygous for H558R and T512I. The proband (arrow, II-1) was homozygous for H558R and heterozygous for T512I while his siblings (II-2 and II-3) were heterozygous for H558R. (**b**) ECG of proband indicating a second-degree conduction block with normal QT and QRS durations. (**c**) Sequence analysis of SCN5A reveals a change of threonine to isoleucine at position 512 and a change of histidine to arginine at position 558.

single-nucleotide polymorphisms, DNA sequence variations that are common in the population, have been implicated in phenotypic variability in physiology, pharmacology, and pathophysiology (11–14). Recently, a polymorphism in *SCN5A* (Y1102) was identified in individuals of African descent and implicated in an elevated risk for arrhythmia (15). Surprisingly, studies have not identified polymorphic alleles that modulate disease phenotypes by influencing the effects of inherited mutations within the same gene. Here we show that a common *SCN5A* allele (H558R), present in one of five people (16), mitigates the gating defect caused by a nearby mutation (T512I) in the cardiac Na+ channel DI-DII linker. The mutation and the polymorphism were both found in the same allele of a child with conduction disease, and are the first to suggest a direct functional interaction between a common polymorphism and a rare mutation within the same gene.

Method

Mutagenesis. Site-directed mutagenesis (T512I) was performed on SCN5A cDNA cloned in pSP64T as previously described (17, 18). 1795*ins*D mutant and H558R were prepared using the QuikChange Mutagenesis Kit

from Stratagene (La Jolla, California, USA), using hH1/pCGI-WT as template. H558R/T512I and H558R/1795*ins*D double mutants were made by the same mutagenesis using H558R-hH1/pCGI and 1795*ins*D/pCGI as template, respectively, for bicistronic expression of the channel protein and GFP reporter in tsa-201 or HEK 293 cells. Cells were cotransfected with an equimolar ratio of Na+ channel human β1 subunit (provided by Al George, Department of Medicine, Vanderbilt University School of Medicine).

Electrophysiology. All whole-cell Na+ currents were recorded at 21°C to limit the voltage-clamp error during activation and inactivation gating at higher temperatures. The pipette solution contained 10 mM NaF, 110 mM CsF, 20 mM CsCl, 10 mM EGTA, and 10 mM HEPES (pH adjusted to 7.35 with CsOH); the bath solution contained 145 mM NaCl, 4 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM HEPES (pH 7.35). Currents were sampled at 20 kHz using a Digidata 1200 analog to digital board (Axon Instruments Inc., Foster City, California, USA) and low-pass filtered at 2 kHz. The data were acquired using pClamp 8.0.1 (Axon Instruments Inc.) and analyzed using Clampfit (Axon Instruments Inc.). Voltage-clamp protocols are provided as insets within each figure. The results are expressed as mean ± SEM, and statistical comparisons were made by one-way ANOVA using Origin software (Microcal Software Inc., Northampton, Massachusetts, USA), with $P < 0.05$ indicating significance. Multiexponential functions were fitted to the data using the nonlinear least-squares method with Origin software.

Results

Clinical characteristics and genetic findings. We studied a family who came to medical attention when the proband, a 2-year-old at the time of initial examination (for pedigree see Figure 1a, II-1), was evaluated for an irregular heartbeat. A second-degree atrioventricular (AV) block was detected and treated with a pacemaker. The ECG trace (Figure 1b) shows a background heart rate of approximately 130 beats per minute (normal for a 2-year-old). The QRS duration and QT interval are normal. On conducted beats, the PR interval is relatively stable and prolonged to 200–240 ms (normal for age 2 years is less than 150 ms), a pattern suggestive of type 2 conduction system (Purkinje) block. In addition, the ECG shown (arrow) indicates an intermittent blocked beat, with shortening of the PR interval on the beat immediately following block, a feature suggestive of type 1 (AV) block. The parents and siblings have a normal ECG (normal QRS, PR, and QT intervals), and there was no evidence of maternal antibody–associated heart block (19).

SCN5A was evaluated as a candidate gene, and mutations were sought using direct, bidirectional sequencing of the coding region. Sequence analysis revealed a change of cytosine to thymine (C1535T), resulting in an amino acid change from threonine to isoleucine (T512I) at codon 512, and a change of adenine to gua-
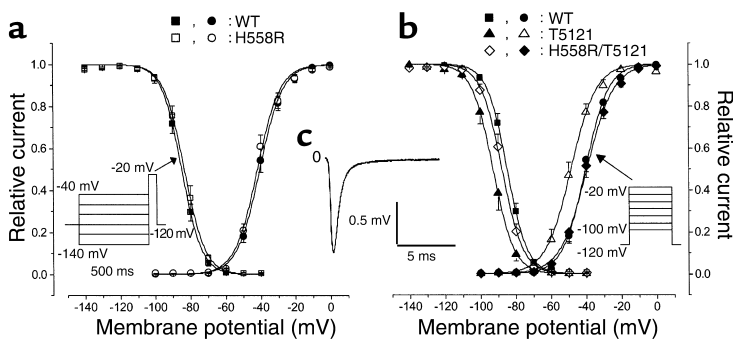
Figure 2

Steady-state gating parameters. (**a**) Voltage dependence of activation and inactivation of wild type and H558R obtained using the protocols shown in the inset and fitted to a Boltzmann function. (**b**) Activation and inactivation parameters of wild type, T512I, and H558R/T512I fitted to a Boltzmann function. Note the hyperpolarizing shifts in activation and inactivation curves as a result of the mutation as well as their restoration by H558R. (**c**) Wild-type and T512I I_{Na} transients obtained during depolarization to –20 mV from a holding potential of –120 mV are normalized to illustrate similarity in fast inactivation.

nine (A1673G), resulting in an amino acid change from histidine to arginine (H558R) at codon 558 (Figure 1c). The nucleotide change C1535T creates a unique BstF5I restriction site, while the nucleotide change A1673G creates an AciI restriction site, allowing independent confirmation of both sequence changes (T512I and H558R). The father was heterozygous for H558R, while the mother was heterozygous for H558R and T512I with both sequence changes on the same allele. The proband (Figure 1a, II-1) was homozygous for H558R and heterozygous for T512I. His siblings (Figure 1a, II-2, II-3) were heterozygous for H558R and did not carry T512I. To verify that the polymorphism and mutation were on the same allele, exon 12 (from the proband) was PCR amplified and subcloned using the TA cloning method (Invitrogen Corp., San Diego, California, USA). The four individual clones were then evaluated by restriction digest with BstF5I and AciI; two clones contained both T512I and H558R (inherited from the mother) and two contained H558R (inherited from the father).

T512I and H558R: competing effects on voltage-dependent gating. We expressed *SCN5A* wild-type, T512I, and H558R channels in tsa-201 cells to allow whole-cell voltage-clamp measurements. Figure 2c shows a typical Na⁺ current (I_{Na}) recorded during a step depolarization to –20 mV from a holding potential of –120 mV. We measured peak I_{Na} at varying activating voltages (protocol inset, Figure 2b) and after conditioning steps to a range of inactivating voltages (protocol inset, Figure 2a). The voltage dependence of activation (circles) and inactivation (squares) for wild-type (filled symbols) and H558R (open symbols) channels were plotted (Figure 2a), and voltage-dependent parameters were derived by fitting a Boltzmann function to the data (Table 1). Over a broad range of membrane potentials, activation and inactivation of wild-type and H558R channels were similar. In contrast, the voltage-dependence of activation (open triangles in Figure 2b) and inactivation (filled triangles in Figure 2b) of T512I was shifted negatively by 8–9 mV (Table 1). Since the proband was heterozygous for T512I and homozygous for H558R, we also investigated the gating of the double

mutant H558R/T512I. Figure 2b shows activation (filled diamond) and inactivation (open diamond) of the H558R/T512I construct. Surprisingly, H558R eliminated the negative shift in activation and inactivation.

Enhanced inactivation and conduction disease. Given that the polymorphism entirely corrected the T512I-induced effects on the voltage dependence of gating, we considered whether residual kinetic changes in H558R/T512I could explain the slow conduction phenotype observed. Upon depolarization, Na⁺ channels rapidly open and "fast inactivate" within a few milliseconds, resulting in a transient inward current. To determine whether the rate of fast inactivation gating was modified by the mutation, we compared the decay of wild-type and T512I I_{Na} upon step depolarization to test potentials ranging from –40 mV to 0 mV. Figure 2c shows representative I_{Na} recordings from wild-type and T512I channels during a step depolarization from a holding potential of –120 mV to –20 mV. At all voltages tested, the rate of decay was similar.

Recent studies have demonstrated a pathophysiologic role for slower kinetic components of inactivation that develop throughout the time course of the cardiac action potential and delay recovery from inactivation between heartbeats (5, 6, 20). In Brugada syndrome, enhanced "slow" inactivation significantly delays the recovery of Na⁺ channels between stimuli, causing a cumulative loss of function, particularly at rapid heart rates due to the shortened diastolic interval (5). We examined slow inactivation of T512I mutants using a dual-pulse protocol to mimic consecutive stimuli

Table 1

Voltage dependence of activation and inactivation

		Inactivation		Activation	
		$V_{1/2}$	k	$V_{1/2}$	k
Wild type	(n = 11)	–85.0 ± 1.2	5.2 ± 0.2	–40.7 ± 1.4	5.9 ± 0.4
H558R	(n = 10)	–83.2 ± 1.4	5.0 ± 0.3	–42.1 ± 1.3	5.7 ± 0.5
T512I	(n = 7)	–92.5 ± 1.7ᴬ	4.8 ± 0.3	–49.4 ± 1.8ᴬ	6.6 ± 0.3
H558R/T512I	(n = 10)	–87.2 ± 1.3ᴮ	5.2 ± 0.1	–40.0 ± 1.8ᶜ	6.8 ± 0.3

Data points shown in Figure 2 were fit using a Boltzmann function of the form y = [1 + exp{$(V – V_{1/2})/k$}]⁻¹, where $V_{1/2}$ is the half maximal voltage of activation and k is the slope factor. Activation and inactivation of wild type and H558R were similar, while T512I shifted these parameters negatively by 8–9 mV. [A]P < 0.01 compared with wild type. H558R eliminated the negative shift in activation and inactivation caused by T512I. [B]P < 0.05 compared with T512I. [C]P < 0.01 compared with T512I.
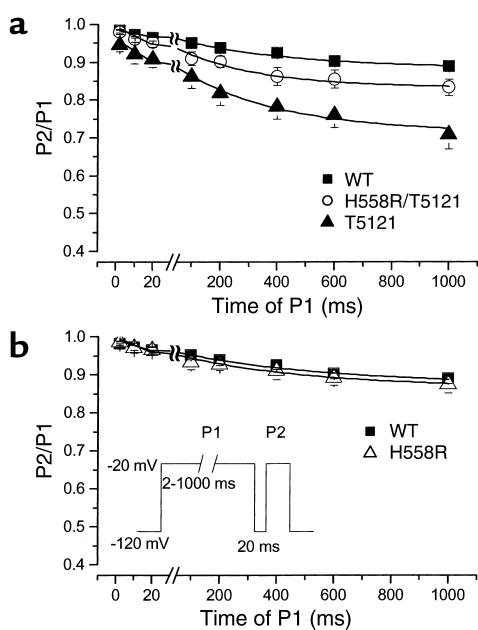
Figure 3
Development of slow inactivation. (**a**) Slow inactivation was evaluated using the two-pulse protocol shown in the inset in **b**. Plot shows the ratio of P2/P1 for wild-type, T512I, and H558R/T512I as a function of duration of P1 pulse. Data points were fitted using a two-exponential function. While T512I dramatically enhanced slow inactivation, H558R attenuated slow inactivation caused by T512I alone. (**b**) Slow inactivation of wild type and H558R. Inset shows the protocol used for evaluation. Slow inactivation of H558R was not different from wild type.

H558R/T512I significantly hastened recovery from inactivation compared with T512I alone ($P < 0.05$, Figure 4a and Table 3). These results also point to a modulatory role of the H558R polymorphism on the slow inactivation gating characteristics of the mutation.

To investigate whether H558R modulation of slow inactivation is localized to companion mutations in the DI-DII linker, we engineered the C-terminal mutation 1795*ins*D and H558R in the same cDNA and expressed them in HEK 293 cells. The 1795*ins*D mutation enhances slow inactivation and elicits Brugada syndrome, as reported previously (5) (Figure 4b). H558R had no effect on the kinetic features of 1795*ins*D (Figure 4b), suggesting that the modulatory role of H558R cannot be generalized to remote channel loci and may be specific to nearby mutations within the DI-DII linker.

Discussion

Mutations of the human cardiac Na$^+$ channel gene (*SCN5A*) underlie multiple cardiac diseases, including long QT syndrome and Brugada syndrome. While most of the mutations that cause long QT syndrome result in a gain of Na$^+$ channel function, mutations that cause Brugada syndrome invariably result in a loss of Na$^+$ channel function. Recent studies have identified yet another class of mutations in the Na$^+$ channel that cause isolated conduction disease. Tan et al. (3) provided the first evidence that a mutation in *SCN5A* causes competing shifts in activation and inactivation gating, where the net effect is a small reduction in Na$^+$ current — sufficient to cause conduction slowing, but not Brugada syndrome. While this suggests that manifestation of a particular rhythm phenotype (Brugada syndrome or conduction

(inset, Figure 3b). As the duration of the first depolarization pulse (P1) was increased, the extent of slow inactivation increased, as indicated by the fractional reduction in peak I_{Na} during the P2 pulse relative to that recorded in the P1 pulse. A 20-ms repolarization to –120 mV was interposed between P1 and P2 to allow recovery from fast, but not slow, inactivation. Figure 3a shows that with increasing P1 duration, the current elicited by the P2 pulse decreased due to slow inactivation. For the wild type, the extent of slow inactivation was similar to previously published results (21). In contrast, T512I markedly increased the development of slow inactivation (Figure 3a), whereas H558R did not change the extent to which slow inactivation developed (Figure 3b). Given that H558R appeared to moderate the effect of T512I on the voltage dependence of inactivation gating (Figure 2), we hypothesized that H558R may also limit the extent to which slow inactivation develops with T512I. To test this, we assessed the development of slow inactivation of H558R/T512I (Figure 3a). We found that H558R/T512I significantly enhanced slow inactivation relative to the wild type but to a lesser extent than T512I alone (see Table 2), lending support to our hypothesis.

The detailed kinetics of recovery from inactivation were evaluated using a paired-pulse protocol wherein the hyperpolarizing interval before the second depolarization pulse (P2) was varied (Figure 4a, inset). Both wild-type and mutant data followed a biphasic time course, reflecting components of recovery attributable to fast and slow inactivation. The kinetics of H558R recovery were indistinguishable from wild type (data not shown, but see Table 3). However, recovery of T512I was significantly slower than the wild type (Figure 4a and Table 3). Once again, the double mutation

Table 2
Development of slow inactivation

		A_1	A_2	τ_{fast} (ms)	τ_{slow} (ms)
Wild type	($n = 9$)	0.03 ± 0.005	0.08 ± 0.007	13 ± 5	583 ± 61
T512I	($n = 7$)	0.07 ± 0.01	0.22 ± 0.01^A	7 ± 3	565 ± 59
H558R/T512I	($n = 6$)	0.04 ± 0.009	0.12 ± 0.01^B	10 ± 1	410 ± 83

Data points shown in Figure 3a were fit using a two-exponential function of the form $y = y_0 + A_1 e^{-t/\tau_{fast}} + A_2 e^{-t/\tau_{slow}}$, where y_0 is the offset, A_1 is the amplitude of the fast component, A_2 is the amplitude of the slow component, and τ_{fast} and τ_{slow} are the time constants of fast and slow components, respectively. The amplitude (A_2) of the slow component was significantly greater in the mutant than in the wild type ($^A P < 0.01$ compared with wild type). However, the time constant (τ_{slow}) was similar to that of the wild type. H558R attenuated slow inactivation resulting from T512I but was still greater than wild type ($^B P < 0.05$ compared with wild type).
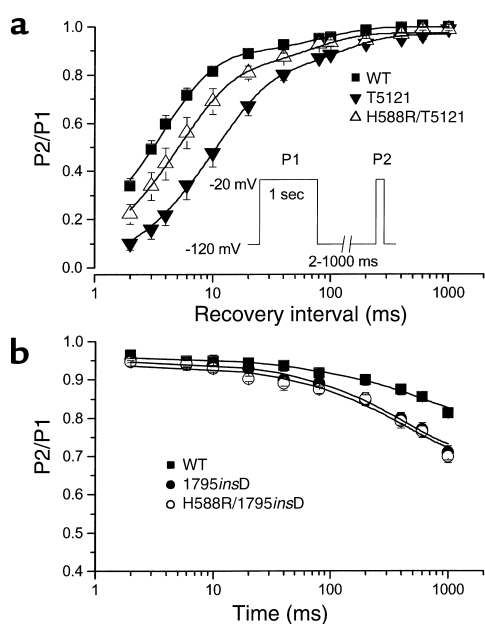
Figure 4

Recovery from inactivation. (**a**) Recovery was evaluated using the two-pulse protocol shown in the inset. Data points were fitted using a two-exponential function with the fast time constant corresponding to recovery from fast inactivation and the slow time constant corresponding to recovery from slow inactivation. Plotted are the results obtained from wild type, T512I, and H558R/T512I. (**b**) Development of slow inactivation of wild type and 1795insD using the protocol shown in Figure 3b inset. It is observed that H558R had no effect in attenuating the slow inactivation caused by 1795insD.

defect) may depend on the interplay between competing gating lesions, it is also likely that other, unrecognized factors contribute to manifestation of a particular disease phenotype. Recent studies have identified a novel gene locus for Brugada syndrome (10) on chromosome 3p22-25, distinct from the known Na⁺ channel loci and overlapping with a previously reported condition arising from locus 3p22-25 — dilated cardiomyopathy with conduction disease (22). Although the functional role of this gene locus has not been identified, the study suggests that other as yet unidentified genes could play a role in the manifestation of disorders in cardiac excitability.

We identified a novel mutation, T512I, in the Na⁺ channel gene of a 2-year-old boy diagnosed with second-degree AV conduction block. The T512I mutation, when heterologously expressed, caused hyperpolarizing shifts in activation and inactivation, and also enhanced slow inactivation. However, the common polymorphism H558R also found in this child's Na⁺ channel gene, which had no effect on wild-type Na⁺ current, attenuated the gating effects caused by mutation T512I. The polymorphism *entirely* restored the voltage-dependent activation and inactivation voltage shifts caused by the T512I mutation, but only partially restored

the kinetic features of slow inactivation. No other family member carried the loss-of-function mutation (T512I) except the mother, who also carried the corrective H558R variant on the same allele (Figure 1a).

While additional studies will be required to firmly establish causality between conduction block and modest changes in slow inactivation, we offer the following hypotheses for linkage between the biophysical observations and the observed phenotypes. The patient described here exhibits AV conduction slowing (prolonged PR intervals) but no intraventricular or intra-atrial conduction defect (normal P wave and QRS duration). Recent studies have associated similar Na⁺ channel slow-inactivation gating abnormalities with AV conduction defects in patients not exhibiting evidence of other conduction slowing (9). These results contrast markedly with the phenotype of another mutation (G514C) associated with slow AV conduction, as well as delayed conduction throughout the atria and ventricles, including broad P waves, PR interval prolongation, and widening of the QRS complex (3). It is possible that enhanced slow inactivation produced by H558R/T512I, which would cause Na⁺ channels to recover from inactivation more slowly during diastole than wild-type channels do (Figure 3a), provides a mechanism whereby AV conduction is slowed in preference to atrial or ventricular conduction. Mutations that preferentially delay recovery from inactivation by enhancing slow inactivation could disproportionately affect cells with longer inherent action potential duration (Purkinje cells). In contrast, as in the case of G514C, mutations targeting the channel *activation* process could affect the myocardium more uniformly, as has been observed (3). Consistent with this idea, it is noteworthy that in the present study H558R entirely eliminated the T512I effect on activation gating (Figure 2b), but only partly corrected the slow inactivation defect. Greater accumulation of Na⁺ channel slow inactivation upon successive stimuli in Purkinje cells, with their longer action potential duration and smaller consequent diastolic interval (23), could lead to greater loss of Na⁺ channel function in these cells at rapid heart rates (130 beats per minute, basal heart rate in the proband), and thereby produce isolated AV conduction delay. Moreover, a premature stimulus could

Table 3

Recovery from inactivation

		A_1	A_2	τ_{fast} (ms)	τ_{slow} (ms)
Wild type	(n = 7)	0.87 ± 0.01	0.11 ± 0.01	3.8 ± 0.3	85 ± 7
H558R	(n = 7)	0.86 ± 0.03	0.12 ± 0.03	4.1 ± 0.4	74 ± 5
T512I	(n = 6)	0.82 ± 0.03	0.16 ± 0.03	11.6 ± 1.6[A]	137 ± 14[A]
H558R/T512I	(n = 7)	0.85 ± 0.01	0.14 ± 0.01	5.5 ± 1.1[B]	83 ± 17[C]

Data points shown in Figure 4a were fit using a two-exponential function of the form $y = y_0 + A_1(1 - \exp^{-t/\tau_{fast}}) + A_2(1 - \exp^{-t/\tau_{slow}})$. While the kinetics of recovery of the wild type and H558R were similar, T512I significantly delayed recovery of both components compared with the wild type. [A]$P < 0.01$ compared with wild type. H558R significantly hastened recovery from inactivation resulting from T512I. [B]$P < 0.01$ compared with T512I. [C]$P < 0.05$ compared with T512I.

also further compromise the Purkinje diastolic interval and lead to dramatic loss of Na$^+$ current and result in all-or-none repolarization and conduction block.

In adulthood, the mother's electrophysiologic phenotype (by history), ECG, and 24-hour ambulatory monitoring was entirely normal. It is possible that the slower heart rate of the mother and consequent longer diastolic interval provides sufficient time for recovery from slow inactivation and thereby did not result in a disease phenotype. While it is unknown whether she displayed ECG abnormalities similar to the proband's during childhood, reduced penetrance of abnormalities in adults with *SCN5A* mutations has been previously described (3, 24). This may also relate to unidentified factors controlling ion channel function and expression.

There is increasing awareness of the role of common polymorphisms in altering gene function and in susceptibility to disease. Studies have linked gene polymorphisms to elevated risk for cystic fibrosis, Alzheimer disease (12, 13), and even heart disease (25, 26). In addition to their role in disease, polymorphisms are also thought to confer sensitivity to drug therapy (27), as well as proarrhythmic risk from drug therapy (15, 28). This study provides the first example in which a polymorphism in the same gene as a rare mutation alters the biophysical effect of the mutation on the channel protein.

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