A Brugada syndrome mutation (p.S216L) and its modulation by p.H558R polymorphism: standard and dynamic characterization

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Aims
The Na+ channel mutation (p.S216L), previously associated with type 3 long-QT syndrome (LQT3) phenotype, and a common polymorphism (p.H558R) were detected in a patient with an intermittent Brugada syndrome (BS) ECG pattern. The study was aimed to assess the p.S216L electrical phenotype, its modulation by p.H558R, and to identify abnormalities compatible with a mixed BS-LQT3 phenotype.

Methods and results
The mutation was expressed alone (S216L channels), or in combination with the polymorphism (S216L–H558R channels), in a mammalian cell line (TSA201). Functional analysis included standard voltage clamp and dynamic clamp with endo- and epicardial action potential waveforms. Expression of S216L channels was associated with a 60% reduction in maximum Na+ current (INa) density, attributable to protein misfolding (rescued by mexiletine pre-treatment) and moderate slowing of inactivation. INa density partially recovered in S216L–H558R channels, but INa inactivation and its recovery were further delayed. The persistent component of INa (INaL) was unchanged. Under dynamic clamp conditions, INa decreased in S216L channels and displayed a ‘resurgent’ component during late repolarization. In S216L–H558R channels, INa density partially recovered and did not display a resurgent component. INa changes during dynamic clamp were interpreted by numerical modelling.

Conclusion
The BS pattern of p.S216L might result from a decrease in INa density, which masked gating abnormalities that might otherwise result in a LQT phenotype. The p.H558R polymorphism decreased p.S216L expressivity, partly by lessening p.S216L effects and partly through the induction of further gating abnormalities suitable to blunt p.S216L effects during repolarization.

Keywords
Brugada syndrome • SCN5A • p.S216L mutation • Dynamic voltage clamp • Modelling

1. Introduction
The Brugada syndrome (BS) is considered an inherited autosomal dominant cardiac channelopathy, firstly described in 1992 and characterized by an incomplete penetrance.1 BS is characterized by a consistent ECG pattern with ST-segment elevation in the right precordial leads (V1–V3) and right bundle branch block.2 In 18–30% of BS patients, the clinical phenotype is associated with mutations in the SCN5A gene, encoding the α subunit of the voltage-dependent cardiac Na+ channel protein (Na1.5).3 Also, mutations to many other genes have been related to BS.4–9 In terms of arrhythmogenic mechanisms, BS was initially interpreted as a repolarization disorder;10 later on, mechanisms based on an impulse propagation...
disorder were also proposed. Moreover, changes in the myocardial structure, which may provide a pathological substrate to functional abnormalities, are present in many BS patients. Such a multiplicity of interpretations suggests that BS may be a complex disease whose pathogenesis is still incompletely understood.

The p.S216L mutation is located in domain I of the Na,1.5 protein on the extracellular loop connecting the S3–S4 transmembrane spans. This mutation, detected post mortem in a case of sudden infant death syndrome and expressed in TSA201 cells, was found to enhance the persistent component of \( I_{\text{Na}} \) (\( I_{\text{NaL}} \)). Accordingly, p.S216L was tentatively identified as a mutation prolonging repolarization by gain of function of Na\(^+\) channels and hypothesized to cause a type 3 long-QT syndrome (LQT3) phenotype.

The SCN5A polymorphism p.H558R has been found to segregate differentially in patient subgroups, in relation to the phenotypic expressivity of several coexisting arrhythmogenic mutations, including those with a BS phenotype. Accordingly, this polymorphism is suspected to modulate the expressivity of arrhythmogenic genotypes.

The present work reports of a BS case carrying the p.S216L mutation and the p.H558R polymorphism within the same allele. Mutant Na\(^+\) channel function, and its modulation by the p.H558R polymorphism, were evaluated by expression in TSA201 cells in the attempt to reconcile its BS phenotype with the LQT3 one previously reported for the same mutant. In keeping with the view that standard voltage-clamp protocols (V-clamp) may overlook the functional consequences of subtle gating abnormalities, the analysis also included dynamic clamp experiments and their simulation by a numerical model of human action potential. In the following presentation, constructs containing the p.S216L mutation alone, or in combination with the p.H558R polymorphism, are referred to as ‘S216L channels’ and ‘S216L–H558R channels’, respectively.

2. Methods

2.1 Clinical and genetic characterization

The proband and its relatives underwent routine cardiological examination, including standard 12-lead ECG, 24 h Holter monitoring, and echocardiogram.

Written informed consent for genetic analysis approved by our Institutional Health Department was signed from all patients. According to national guidelines, approval from the local Ethics Committee is not necessary for diagnostic testing. The investigation conformed with the principles outlined in the Declaration of Helsinki. Genomic DNA was extracted from peripheral blood and SCN5A coding sequence was analysed by DHPLC and direct sequencing as detailed in Supplementary Methods.

2.2 Site-directed mutagenesis and expression system

SCN5A constructs were made as detailed in Supplementary Methods. The mutation S216L (c.647C>T) was introduced alone and in combination with the p.H558R polymorphism (c.1673A>G), to obtain S216L and S216L–H558R constructs, respectively. All constructs were sequenced to verify the presence of the mutations and rule out spurious substitutions.

TSA201 cells were transfected with Lipofectamine 2000 (Invitrogen). The following constructs were co-transfected in equal amounts (0.5 \( \mu \)g/mL): (i) a bicistronic plasmid encoding for green fluorescent protein and

Figure 1 ECG phenotype and genotype. (A) Family pedigree and ECG tracings of the proband (arrow) and of his mother. (B) Electropherograms of SCN5A gene sequence; left, arrows indicate the c.647C>T substitution leading to the p.S216L mutation; right, arrows indicate the c.1673A>G substitution leading to the p.H558R polymorphism.
the human β1 subunit (pCGI-IRES-hβ1, kindly provided by J.R. Balser, Vanderbilt University, Nashville, TN, USA); (ii) wild-type (WT), or S216L or S216L–H558R plasmids. In order to increase INa density, in dynamic clamp experiments the amount of transfected plasmid was doubled (1 µg/mL).

2.3 Functional characterization

Whole-cell patch-clamp experiments were carried out 48 h after transfection on green fluorescent cells exhibiting peak INa larger than 1.5 nA, to minimize potential contamination by endogenous currents.23 Pipette and bath solutions are specified in Supplementary Methods. Temperature was 26°C in standard voltage-clamp experiments and 37°C in dynamic clamp experiments. Cell membrane capacitance and series resistance were compensated by 85–95%, the estimated voltage error was <3 mV in all cases. INa was identified, by digital subtraction, as the current sensitive to tetrodotoxin (TTX, 30 µmol/L). In dynamic clamp experiments, membrane potential was driven at a steady cycle length of 1 s by human endocardial or epicardial action-potential waveforms (EndoAP and EpiAP, respectively), generated by a recent numerical model of human cardiac action potential.25 INa was identified as the TTX-sensitive current. Comparison of INa during EndoAP and EpiAP waveforms within the same cell was not possible because of inadequate reproducibility of the TTX-sensitive current after repeated exposures. For normalization purposes, in each cell, dynamic clamp was preceded by evaluation of INa density (INaL) by standard voltage protocols (V-clamp).

2.4 Data analysis

V-clamp recordings were analysed as usual (see Supplementary Methods); INaL was measured as the TTX-sensitive current between 190 and 200 ms after pulse onset. In dynamic clamp experiments, TTX-sensitive current was expressed in terms of absolute density (INaL) and after normalization to INaL (INaL/INaLmax). Normalization aimed to evaluate the functional impact of changes in gating kinetics after removing the effect of changes in absolute current density. INaL was measured during four action potential (AP) phases: the AP upstroke (INaL(P0)), the AP notch (INaL(P1)), phase 2 (between AP dome and APD50, INaL(P2)), phase 3 (between APD50 and APD90, INaL(P3)) (see Figures 4 and 5). Whereas INaL(P0) was measured as its peak value, in the other phases INaL was quantified by its mean value; in addition, time to peak (TTP) was measured for INaL(P0).

2.5 Trafficking/folding analysis

Membrane expression of channel proteins was investigated by immunohistochemistry (see Supplementary Methods). To functionally assess whether the changes in INaL were due to protein trafficking/folding abnormalities, immediately after transfection cells were incubated with mexiletine (400 µmol/L), a Na+ channel blocker previously shown to rescue the phenotype of trafficking-deficient Na+ channels.26 Mexiletine was washed out 30 min before recordings.

2.6 Numerical modelling

Numerical simulations were performed by a recently published model of human ventricular action potential,25 based on Hodgkin–Huxley (HH) formalism. The original model was used to generate EndoAP and EpiAP waveforms to be used in dynamic clamp experiments. Three sets of INa model parameters were generated by fitting all the gating properties of WT, S216L, and S216L–H558R constructs, detected by V-clamp analysis (see Supplementary Methods). INaL was then modelled during dynamic clamp with EndoAP and EpiAP waveforms. A detailed description of the model is given in Supplementary Methods.

3. Results

3.1 Case report: clinical and genetic profile

A Caucasian 10-year-old male (proband) was referred to the arrhythmology unit for palpitations (at rest, more frequent after meals); his clinical history did not include syncope or other symptoms suggesting haemodynamically significant arrhythmias. ECG at admission showed sinus rhythm at 75 bpm, PR 160 ms, QRS 93 ms, right bundle branch block with ST segment elevation of 2 mm in lead V1 and 4.5 mm in lead V2 with negative T-waves in leads V1 and V2, and QTc 460 ms. The proband’s ECG abnormalities are compatible with...
the type 1 BS pattern (Figure 1A). During hospitalization, the BS pattern proved to be intermittent, the ECG periodically reversing to normality without identifiable reasons. Echocardiography showed the absence of structural cardiac disease. At the time of programmed electrical stimulation, the patient was displaying type 1 BS pattern. Ventricular fibrillation could not be induced with up to three extrastimuli delivered to the right ventricular apex and outflow tract. The proband's mother (age 37 years) was healthy and had an entirely normal ECG (sinus rate 62 bpm, PR 138 ms, QRS 93 ms, QTc 430 ms, Figure 1A); she underwent a provocative test with flecainide, which was negative. The proband's father (age 39 years) was also healthy and had a normal ECG. Family history was negative for events that could be related to arrhythmias.

Analysis of the coding region of the proband's SCN5A gene, comprehensive of intron–exon boundaries, identified a heterozygous missense mutation in exon 6. This consisted of a c.647C>T nucleotide variation (Figure 1B, left), leading to the replacement of serine (S) to leucine (L) at protein residue 216 (S216L). Sequence analysis also revealed the presence of the common polymorphism H558R in SCN5A exon 12 (Figure 1B, right), involving a substitution of histidine (H) with arginine (R) at position 558 in the intracellular loop connecting domains I and II of the channel protein. The reported frequency of this variant in the general population is between 19 and 24%. Genetic screening of the family revealed that the proband's mother was a carrier of both the p.S216L mutation and the p.H558R polymorphism. Because the mutation and the polymorphism were absent in the proband's father, they were both inherited from mother's chromosome. Since recombination is a very rare event after fertilization, expression of mutation and polymorphism on the same allele is more likely to represent the proband's condition and was reproduced in heterologous expression studies.

### 3.2 Functional characterization

To evaluate the electrophysiological consequences of the p.S216L mutation alone, and associated with the p.H558R polymorphism, three TSa201 cell groups were transfected with WT, S216L, and S216L–H558R constructs, respectively. In the following description, the functional phenotype of mutant channels is expressed relative to that of WT ones.

### 3.3 Standard V-clamp analysis

The first set of experiments was devoted to traditional voltage-clamp analysis, whose results are summarized in Table 1. Peak current/voltage (I/V) relationships of S216L channels showed that $I_{Na}$...
Figure 3 Standard V-clamp analysis—$I_{\text{Na}}$ gating. (A) Steady-state activation and fast inactivation curves. (B) steady-state slow-inactivation curves (solid lines are Boltzmann fit of data points). (C) Voltage-dependency of ‘fast’ inactivation time constants. Lower and upper curve groups represent fast and slow components, respectively. (D) Time course of recovery from inactivation. Voltage protocols are shown in the inset of each panel. Statistical analysis is reported in Table 1. *$P < 0.05$ vs. WT.

Figure 4 Dynamic clamp analysis—absolute $I_{\text{Na}}$ density. (A) Upstroke phase ($P_0$): AP waveforms and representative $I_{\text{Na}}$ recordings during the AP upstroke; time to peak $I_{\text{Na}}$ (TTP) for the three constructs is compared in the histogram after pooling of EndoAP and EpiAP data. *$P < 0.05$ vs. WT. (B) Notch phase ($P_1$): comparison of EndoAP and EpiAP waveforms (left) and $I_{\text{Na}}$,$P_1$ recorded under each waveform from the three channel constructs (right). *$P < 0.05$ vs. EpiAP. (C and D) Repolarization ($P_1$–$P_3$): averaged $I_{\text{Na}}$ recordings during EpiAP and EndoAP; $I_{\text{Na}}$ recordings are aligned with the respective AP waveforms; dashed lines denote the repolarization phases ($P_1$–$P_3$). Confidence intervals of average $I_{\text{Na}}$ recordings are not shown for clarity; statistical comparison of $I_{\text{Na}}$ during the various AP phases is reported in Table 2. In all panels, $I_{\text{Na}}$ is expressed as current density (pA/pF).
density was decreased at all membrane potentials to approximately 40% of the WT value. The reduction in INa density was partially reversed (to approximately 60% of WT) in S216L–H558R channels (Figure 2A and B). Density of the persistent component (INaL) also differed between the three channel types; however, differences mirrored those in total INa; therefore, the INaL/INa ratio was roughly similar for the three constructs. If anything, the INaL/INa ratio was slightly decreased in S216L channels, a change which did not achieve significance, but was predicted by model simulations (Figure 2C). Therefore, the increase in INaL density previously described was absent in both S216L and S216L–H558R channels.

The voltage-dependency of steady-state activation was normal in S216L channels, but slightly shallower in S216L–H558R channels (larger slope factor); no significant changes were observed in mid-activation potential (V1/2) (Figure 3A and Table 1).

The voltage-dependency of steady-state inactivation was tested with conditioning pulses of short (100 ms) and long (60 s) duration, to analyse the contribution of inactivation and ‘slow inactivation’ gating, respectively. Both inactivation components were similar among the three channel constructs (Figure 3A and B). The overlap between activation and availability curves, suitable to support INa ‘window’ current, was unchanged (Figure 3A). Inactivation was also analysed in terms of time course, which was best fit by a double exponential. Its faster component was slightly slower in S216L channels and only at very negative potentials. On the other hand, both inactivation components were significantly delayed in S216L–H558R channels over a wider range of potentials (negative to 0 mV) (Figure 3C). The same pattern was observed for the faster component of recovery from inactivation, which was slowest in S216L–H558R channels (Figure 3D).

3.4 Dynamic clamp analysis

This set of experiments aimed to evaluate the impact of the mutation on INa expressed during actual endocardial and epicardial action potentials. In this setting, recordings could be performed at physiological temperature, an important factor in evaluating the effect of changes in gating kinetics.

The upstroke phase (INaP0) was identical between EpiAP and EndoAP waveforms; accordingly, data from the two waveform types were pooled (Figure 4A and B). Peak INa elicited by the AP upstroke (non-instantaneous depolarization, 37 °C) was considerably smaller than that measured at the same potential during step depolarization at room temperature (e.g. ~35 pA/pF vs. ~147 pA/pF for WT at 30 mV). Furthermore, in spite of the marked Imax reduction resulting from the mutation, absolute peak INa density during the upstroke was not significantly different between the three channel constructs (Table 2). TTP was larger in S216L–H558R and S216L channels than in WT ones (Table 2); these differences were in the same rank as those in inactivation time constants (S216L–H558R ≃ S216L ≈ WT). Altogether, these findings suggest that, during the non-instantaneous phase 0 depolarization, kinetic features of S216L and S216L–H558R channels may compensate for the differences in Imax. This view is supported by the results of modelling (see below and Supplementary material online, Figure S3).

Phase 1 repolarization and the subsequent notch (P1) differed between EpiAP and EndoAP (Figure 4B) and INa was smaller in...
Table 2 Dynamic clamp analysis

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>S216L</th>
<th>S216L–H558R</th>
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<tr>
<td><strong>EpiAP + EndoAP</strong></td>
<td></td>
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</tr>
<tr>
<td>$P_0$</td>
<td>$I_Na$ (pA/pF)</td>
<td>$-35.3 \pm 5.4$</td>
<td>$-26.9 \pm 3.9$</td>
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<tr>
<td>$I_Na/I_{max}$ (%)</td>
<td>$5.2 \pm 0.8$</td>
<td>$9.3 \pm 1.3^*$</td>
<td>$7.2 \pm 0.9$</td>
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<tr>
<td>TTP (ms)</td>
<td>$1.08 \pm 0.01$</td>
<td>$1.13 \pm 0.01^*$</td>
<td>$1.15 \pm 0.01^*$</td>
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<tr>
<td><strong>EpiAP</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$P_1$</td>
<td>$I_Na$ (pA/pF)</td>
<td>$-0.37 \pm 0.9^*$</td>
<td>$-0.48 \pm 0.9^*$</td>
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<tr>
<td>$I_Na/I_{max}$ (%)</td>
<td>$0.13 \pm 0.04$</td>
<td>$0.11 \pm 0.03$</td>
<td>$0.15 \pm 0.02$</td>
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<td>$P_2$</td>
<td>$-0.11 \pm 0.04^*$</td>
<td>$-0.20 \pm 0.03^*$</td>
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<tr>
<td>$I_Na/I_{max}$ (%)</td>
<td>$0.07 \pm 0.02$</td>
<td>$0.05 \pm 0.02$</td>
<td>$0.06 \pm 0.01$</td>
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<tr>
<td>$P_3$</td>
<td>$-0.31 \pm 0.07$</td>
<td>$-0.07 \pm 0.02^*$</td>
<td>$-0.07 \pm 0.02^*$</td>
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<tr>
<td>$I_Na/I_{max}$ (%)</td>
<td>$0.04 \pm 0.02$</td>
<td>$0.15 \pm 0.06$</td>
<td>$0.02 \pm 0.004^*$</td>
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<tr>
<td><strong>EndoAP</strong></td>
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<tr>
<td>$P_1$</td>
<td>$-0.32 \pm 0.09^*$</td>
<td>$-0.48 \pm 0.1$</td>
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<tr>
<td>$I_Na/I_{max}$ (%)</td>
<td>$0.07 \pm 0.01$</td>
<td>$0.08 \pm 0.01$</td>
<td>$0.18 \pm 0.07$</td>
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<tr>
<td>$P_2$</td>
<td>$-0.11 \pm 0.03$</td>
<td>$-0.27 \pm 0.06^*$</td>
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<tr>
<td>$I_Na/I_{max}$ (%)</td>
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<td>$0.03 \pm 0.007$</td>
<td>$0.1 \pm 0.03$</td>
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<tr>
<td>$P_3$</td>
<td>$-0.19 \pm 0.07$</td>
<td>$-0.12 \pm 0.06$</td>
<td></td>
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<tr>
<td>$I_Na/I_{max}$ (%)</td>
<td>$0.002 \pm 0.01$</td>
<td>$0.08 \pm 0.03^*$</td>
<td>$0.03 \pm 0.01$</td>
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$^a$ P = 0.05 vs. EpiAP.
$^b$ P < 0.05 vs. WT.
$^c$ P < 0.05 vs. S216L.

EndoAP for WT channels. For S216L and S216L–H558R channels, the current during EpiAP was reduced to a greater extent, thus blunting the difference between the two AP waveform types (Figure 4B).

During the AP plateau (P2) $I_Na$ density was minimal in S216L channels and, particularly in EndoAP, partially recovered in S216L–H558R channels (Figure S1). The same parameter sets were used in modelling $I_Na$ during dynamic clamp, with the only exception of the P3 ‘resurgent’ component during phase 3 ($I_Na/\gamma_3$, Figure S2). Because of its high variability, $I_Na/\gamma_3$ enhancement achieved statistical significance in all comparisons only when results from EndoAP and EpiAP were pooled (Figure S5), but a similar trend was observed in the two waveform types (Figure S5 and Table 2). The resurgent component and its variability were largely suppressed in S216L–H558R channels (Figure S5 and D and Table 2).

3.5 Numerical modelling

The purpose of modelling was to test whether the mutation-induced changes in $I_Na$ profile, observed in dynamic clamp experiments, could be explained by the observed gating abnormalities within the HH formalism (which assumes independence of the channel gating particles). A single set of model parameters was adequate to reproduce all the gating properties, detected by V-clamp experiments, within each of the channel constructs (Supplementary material online, Figure S1). The same parameter sets were used in modelling $I_Na$ during dynamic clamp. The latter focused on the consequences of channel kinetic properties; therefore, the results were analysed in terms of normalized current ($I_Na/I_{max}$).

The model failed to accurately reproduce the actual $I_Na/I_{max}$ profiles experimentally observed during dynamic clamp (Supplementary material online, Figure S2). In particular, during repolarization, modelled $I_Na$ differed between EpiAP and EndoAP only during the phase 1–2 notch ($P_1$). Nevertheless, the model was successful in reproducing the pattern of $I_Na/I_{max}$ changes observed in S216L and S216L–H558R channels during dynamic clamp, with the only exception of the $P_3$ ‘resurgent’ component (Supplementary material online, Figure S3).

3.6 Membrane channel expression and its rescue

This set of experiments tested the hypothesis that protein trafficking or folding abnormalities contributed to the changes in $I_Na$ density between the three constructs.

At immunostaining, WT channels appeared to be smoothly distributed along cell borders; perinuclear staining was absent (Figure 6A, leftmost panel). In both S216L and S216L–H558R transfected cells, the staining was more irregular and perinuclear localization prevailed (Figure 6B, second and third panels), being almost exclusive in S216L transfected cells.

The hypothesis of a trafficking defect was functionally tested by evaluating whether $I_Na$ could be rescued by incubation with the Na+ channel ligand mexiletine. Peak $I_Na$ I/V relations for the three channel constructs were compared between control and mexiletine treated cells (Figure 6B) to detect changes in maximal $I_Na$ density (mexiletine was washed-out before measurements). Incubation with
mexiletine did not change maximal $I_{\text{Na}}$ density in cells transfected with WT channels, but significantly increased it in both S216L and S216L–H558R transfected cells, thus leading to almost complete rescue of mutation effects on maximal current density.

4. Discussion

The main effects of the p.S216L mutation, as detected by the standard voltage-clamp approach, included: (i) a substantial decrease in $I_{\text{Na}}$ density and Na\textsubscript{v}1.5 membrane localization, the former being rescued by incubation with mexiletine; (ii) a moderate slowing of $I_{\text{Na}}$ inactivation at negative potentials, which did not affect the $I_{\text{Na}}$/ $I_{\text{NaP3}}$ ratio. Coexpression of the p.H558R polymorphism partially reversed the mutation’s effects on $I_{\text{Na}}$ density, but it delayed inactivation and its recovery. The $I_{\text{Na}}$ ‘window’ was unmodified by the p.S216L mutation alone and tended to be reduced by the S216L–H558R genotype.

Decreased $I_{\text{Na}}$ density might result from a change in channel gating properties, or from a decrease in the number of functional channels expressed in the membrane. It has been reported that binding of mexiletine to misfolded mutant Na\textsuperscript{+} channels may restore their function,\textsuperscript{26} probably through stabilization of the properly folded structure. The almost complete rescue of mutant $I_{\text{Na}}$ by incubation with mexiletine (Figure 6B) suggests misfolding of the mutant channel as the cause of reduced current density. The observation that $I_{\text{Na}}$ was reduced, as opposed to suppressed, suggests that the p.S216L mutation caused structural instability, leading to an equilibrium between properly folded and misfolded forms.

Immunostaining experiments showed that the WT Na\textsubscript{v}1.5 localization pattern was altered by the p.S216L mutation (Figure 6A), to indicate that misfolding also affected Na\textsubscript{v}1.5 trafficking to the membrane. Whereas the S216L–H558R genotype was associated with a milder reduction of $I_{\text{Na}}$ density (intermediate between WT and S216L channels), its localization pattern was still compatible with abnormal trafficking; however, immunostaining may be inadequate to detect quantitative changes in membrane channel expression.

Mutation-induced changes in absolute $I_{\text{Na}}$ during repolarization (dynamic clamp experiments) were dominated by the overall decrease in $I_{\text{Na}}$ density (Figure 4). The latter was partially compensated by p.H558R coexpression. Compensation of differences in absolute $I_{\text{Na}}$ density by normalization revealed further changes of functional significance in current profile (Figure 5). The relationship between gating features measured with the standard voltage-clamp approach and dynamic $I_{\text{Na}}$ profiles deserves to be analysed.

During the action potential upstroke, TTP varied in the three channel constructs according to inactivation slowing (measured by standard voltage clamp). Indeed, an enhanced lag between activation and inactivation, as that potentially resulting from inactivation slowing, is expected to prolong TTP as shown by model simulations (Supplementary material online, Figure S3). On the other hand, the model slightly underestimated overall TTP, possibly because activation rate, too fast to be measured experimentally, was arbitrarily set.

Independently of the action potential type, $I_{\text{Na}}$ during terminal repolarization ($I_{\text{NaP3}}$) and its variability were markedly increased in S216L channels, thus generating a distinctive ‘resurgent’ component. In S216L–H558R channels, both the amplitude and the variability of $I_{\text{NaP3}}$ were reverted to control values (Figure 5C and D). If sufficiently large, resurgent $I_{\text{Na}}$ during terminal repolarization would facilitate early afterpotentials (EADs), thus supporting an arrhythmogenic mechanism more commonly associated with LQTS than with BS. A resurgent current behaviour during fast repolarization is generally explained as recovery of inactivation of a small proportion of channels and their immediate reactivation;\textsuperscript{29} therefore, its enhancement might depend by the mutation-induced gating abnormalities detected by V-clamp experiments. However, their implementation in dynamic clamp simulations of S216L channels predicted a decrease in $I_{\text{NaP3}}$ instead (Supplementary material online, Figure S3). While this discrepancy might result from the simplifications implicit in the model formalism, the observation that S216L also increased $I_{\text{NaP3}}$ variability favours the hypothesis that a random mechanism, a form of gating instability, may underlie the observed $I_{\text{NaP3}}$ enhancement in S216L channels. Such gating instability was absent in S216L–H558R channels, in which both $I_{\text{NaP3}}$ amplitude and variability were comparable to those of WT channels (Figure 5C and D). The model predicted a decrease in $I_{\text{NaP3}}$ between WT and S216L–H558R channels (Supplementary material online, Figure S3), as expected from the delay in channel recovery from inactivation (Figure 3D). The latter might contribute to blunt the resurgent component but, as it was also present in S216L channels, may not be sufficient to justify suppression of the S216L phenotype.
The main peculiarity of S216L–H558R channels was partial recovery of the reduction in absolute $I_{Na}$ density caused by the p.S216L mutation (Figure 2B). This effect was also visible under dynamic clamp conditions as restoration of $I_{NaP1}$ and $I_{NaP2}$ amplitudes (Figure 4C, Table 2) and might contribute to reduce expressivity of the mutation. The restoring effect of p.H558R coexpression was almost complete for EndoAP waveforms and only partial for EpiAP ones (Figure 4D, Table 2). The model successfully predicted the overall changes in $I_{NaP1}$ and $I_{NaP2}$ (Supplementary material online, Figure S3), but failed to distinguish between EpiAP and EndoAP waveforms. This might reflect inadequacy of the model formalism to reproduce the effect of the spike-and-dome voltage profile on $I_{Na}$ time course. However, it should be stressed that subtle differences between EpiAP and EndoAP were detected by comparison of different, and relatively small, cell groups; thus they should be interpreted with caution.

4.1 Relationship with the ECG phenotype

In its classical description, the BS ECG pattern consisted of a repolarization abnormality, with substantially preserved impulse propagation. The $I_{Na}$ abnormality found to be associated with BS, and best suited to account for this pattern, was an acceleration of fast inactivation, without major changes in peak $I_{Na}$ density. Moreover, the BS ECG pattern has also been found in association with mutations affecting mainly peak $I_{Na}$ density and interpreted by numerical simulations as a large delay in transmural propagation.

According to V-clamp analysis, the S216L mutant is characterized by a significant reduction in maximal $I_{Na}$ density ($I_{Na(max)}$) and its gating features do not predict selective $I_{Na}$ reduction during early repolarization. This phenotype would be more likely to cause BS through conduction slowing than by altering repolarization. On the other hand, dynamic clamp analysis and its numerical simulation suggest that differences in $I_{Na(max)}$ may be minimized during the AP upstroke if inactivation is simultaneously slowed; indeed, $I_{NaP0}$ difference between WT and mutant channels was small (Figure 4A) and did not achieve significance (Table 2).

This may explain the absence of overt conduction disturbance in the proband’s ECG (normal PQ and QRS intervals). $I_{Na(max)}$ reduction in S216L channels had a larger impact on $I_{Na}$ density during repolarization (P1 and P2); however, this defect was partially rescued in S216L–H558R channels by the additional gating changes introduced by coexpression of the p.H558R polymorphism (Figure 4C and D and Table 2).

When the decrease in $I_{Na(max)}$ was compensated by normalization, S216L channels revealed the tendency to generate a significant residual component. This property suggests that, if $I_{Na}$ density were restored by recovery of channel trafficking, the p.S216L mutation might be associated to perturbations of terminal repolarization, an abnormality typical of long QT(LQT) phenotypes. Mixed BS-LQT3 phenotypes have been described for several SCN5A arrhythmic mutations. The common observation of a higher p.H558R prevalence in SCN5A mutation carriers has been variably interpreted. Whereas some authors interpreted p.H558R as a risk factor, others viewed it as a protective factor, maintained by positive selection in a high-risk population. The present findings are consistent with previous ones in which p.H558R expression was found to rescue gating and trafficking abnormalities induced by various mutations. This supports the view that p.H558R polymorphism has been evolutionarily selected for its protective effect.

The observation that a single genetic variant may compensate diverse gating abnormalities, caused by different mutations, is puzzling and the underlying mechanism is unknown. In the present case, the p.S216L mutation and p.H558R polymorphism likely affected the same allele, as in previous instances of rescue of gating abnormalities. Rescue of channel trafficking, also observed in the present study, has been previously reported to occur also when mutation and polymorphism affected different alleles. Therefore, both cis and trans modulation mechanisms may be involved in the protective effect of p.H558R.

4.3 Study limitations

Under physiological conditions, membrane current and potential mutually interact in a closed feed-back loop. Under dynamic clamp conditions, changes in current cannot feed-back to determine potential course, which is fixed (open loop). Therefore, prediction of action potential changes (i.e. the electrical phenotype) from the mutation-induced changes in $I_{Na}$ is somewhat arbitrary. Nevertheless, this technique allows to analyse the actual $I_{Na}$ behaviour during dynamic changes in membrane potential at physiological temperature.
As neither of these conditions apply to standard voltage clamp, dynamic clamp can help in disclosing features of mutant currents of potential importance in cardiac electrical activity.

Comparison of EndoAP and EpiAP waveforms within individual cell was impossible because of insufficient stability of the preparation relative to the small amplitude of \(I_{\text{Na}}\) during repolarization. Therefore, the possibility that causes other than action potential contour were involved in EpiAP vs. EndoAP differences was considered. In order to avoid systematic artefacts, EpiAP and EndoAP waveforms were applied to alternate cells within each experimental session, thus being homogeneously represented among cell batches and over time. While dynamic \(I_{\text{Na}}\) profiles are represented as averages from multiple cells (Figures 4 and 5) \(I_{\text{Na}}\) parameters were measured from individual recordings and subjected to statistical analysis (Table 2).

Caution should be used when translating data obtained from heterologous expression studies to the clinical phenotype, because many factors modulating channel function in myocytes are not necessarily present in TSA201 cells. Moreover, as genotyping was limited to ologous expression studies to the clinical phenotype, because many most likely interpretative model. The decrease in INa density masks recordings and subjected to statistical analysis (This work was supported by PRIN 2007 (to A.Z.), and grants from Funding Conflict of interest: videring pSP64T and hb/pirGFP constructs. Acknowledgements Supplementary material is available at Cardiovascular Research online. Acknowledgements We thank Pasquale Vergara for help with clinical data collection and interpretation. We are grateful to A.L. George and J.R. Balser for providing pSP64T and hb/pirGFP constructs. Conflict of interest: none declared. Funding This work was supported by PRIN 2007 (to A.Z.), and grants from Medtronic Italy (to M.F.) and the Italian Istituto Superiore di Sanità (ISS-526D/55 to C.P.).

4.4 Conclusions The association between the p.S216L mutation and the BS phenotype may be primarily accounted for by a decrease in \(I_{\text{Na}}\) density, which leads us to postulate the ‘conduction abnormality’ as being the most likely interpretative model. The decrease in \(I_{\text{Na}}\) density masks gating abnormalities, other than \(I_{\text{Na}}\) enhancement, that might otherwise result in a LQT phenotype. Coexpression of the h558R polymorphism is likely to decrease S216L expressivity: this occurs partly through reversal of S216L effects and partly because of the induction of further abnormalities suitable to blunt S216L effects during repolarization (dynamic conditions). The same gating abnormalities might differentially affect the \(I_{\text{Na}}\) profile during epicardial and endocardial action potentials.


References


