Functional Analysis of Sequence Variation

The common African American polymorphism SCN5A-S1103Y interacts with mutation SCN5A-R680H to increase late Na current

Jiading Cheng,1 David J. Tester,2 Bi-Hua Tan,1 Carmen R. Valdivia,1 Stacie Kroboth,1 Bin Ye,1 Craig T. January,1 Michael J. Ackerman,2 and Jonathan C. Makielski1

1Division of Cardiovascular Medicine, Department of Medicine, University of Wisconsin, Madison, Wisconsin; and 2Departments of Medicine, Pediatrics, and Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota

Submitted 27 September 2010; accepted in final form 1 March 2011

Mutations in the SCN5A-encoded α-subunit of the voltage-dependent sodium channel Nav1.5 can alter properties of the cardiac sodium current (INa) to cause inherited arrhythmia syndromes such as long-QT syndrome (LQTS) type 3 (LQT3) and Brugada syndrome (BrS) type 1 (BrS1). S1103Y, one of the eight common (allelic frequency >0.5%) SCN5A polymorphisms with an allelic frequency of ~13% in African Americans (1), has been associated with a small risk of acquired arrhythmia, particularly in the setting of medications and hypokalemia (18), and with familial syncope, ventricular fi- late rhythm, particularly in the setting of medications and hypokalemia (18), and with familial syncope, ventricular fibrillation (5 and sudden unexplained cardiac death (SUD) (5) and sudden infant death syndrome (SIDS) (16, 21) identified S1103Y as a risk factor for increased sudden death. Electrophysiological cellular studies indicated that Y1103-containing channels had small shifts in kinetics under baseline conditions (19), but when they were exposed to cellular acidosis late INa was increased markedly (16).

Message RNA for two splice variants of SCN5A, one lacking a glutamine at position 1077 (Q1077del) and one containing the glutamine at position 1077 (Q1077), exist in the human heart at a ratio of ~2:1 (Q1077del:Q1077) (12), and the function of INa for both polymorphisms (19) and channelopath mutations (20) may depend on which splice variant background the mutation is studied. We encountered and genotyped a 23-year-old African American male SUD victim and identified two SCN5A single amino acid substitutions: the common S1103Y polymorphism and a rare missense mutation, R680H, which was reported previously in a deceased Norwegian infant. Like S1103Y, R680H had a latent dysfunctional biophysical phenotype that depended on acidosis to produce late INa (22). Hypothesizing a possible synergistic interaction, we investigated the interactions of both R680H and S1103Y in both the Q1077del and Q1077 SCN5A backgrounds as well as the pH dependence of late INa. We found that the “double hit” in combination with the environmental factor of acidosis caused a more severe pathological biophysical phenotype of late INa that critically depended on the sodium channel transcript’s status at residue 1077.

Materials and Methods

This study was approved by Mayo Clinic’s Institutional Review Board as a consented study for postmortem genetic testing.

Mutational Analysis. Genomic DNA was extracted from frozen myocardial necropsy tissue with the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA). Comprehensive open reading frame/splice site mutational analysis of the three most common susceptibility genes for LQTS and the most common susceptibility gene for BrS (KCNQ1, KCNH2, and SCN5A) was performed with polymerase chain reaction (PCR), denaturing high-performance liquid chromatography (DHPLC), and direct DNA sequencing as previously described (2). Primer sequences, PCR conditions, and DHPLC conditions are available on request.

Plasmid constructions. R680H and S1103Y were created separately and also together in the two SCN5A splice variant backgrounds designated Q1077del (GenBank access no. YA148488) and Q1077 (GenBank accession no. AC1377587) with a site-directed mutagen-
sis kit (Stratagene, La Jolla, CA). In all, eight SCN5A constructs were made and tested. The pcDNA3 plasmid vector (Invitrogen, Carlsbad, CA) was used as previously reported (12, 19). All clones were sequenced to confirm the presence of the introduced mutations and the absence of Taq polymerase-induced substitutions that may occur during PCR.

Mammalian cell transfection. The wild-type (WT) and mutant channels in these two alternatively spliced transcripts of SCN5A were transiently transfected into HEK293 cells with FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN) according to manufacturer’s instructions.

Electrophysiological measurements. Macroscopic voltage-gated \( I_{\text{Na}} \) was measured 24 h after transfection with the standard whole cell patch-clamp method at 21–23°C in HEK293 cells. The extracellular (bath) solution contained the following (in mM): 140 NaCl, 4 KCl, 1.8 CaCl2, 0.75 MgCl2, and 5 HEPES and was adjusted to pH 7.4 with NaOH. The intracellular (pipette) solution contained the following (in mM): 120 CsF, 20 CsCl2, 2 EGTA, 5 NaCl, and 5 HEPES and was adjusted to pH 7.4 or 6.7 with CsOH. Microelectrodes were manufactured from borosilicate glass with a puller (P-87, Sutter Instrument, Novato, CA) and were heat polished with a microforge (MF-83, Narishige, Tokyo, Japan). The resistances of microelectrodes ranged from 1.0 to 2.0 M\( \Omega \). Voltage clamp data were generated with pClampex 10.2 and analyzed with Clampfit 10.2 (Molecular Devices, Sunnyvale, CA). Membrane current data were digitalized at 100 kHz, low-pass filtered at 5 kHz, and then normalized to membrane capacitance. Standard voltage clamp protocols are presented with the data, and data were measured and analyzed as described previously (7, 12, 19) and with additional details provided in Figs. 2–4.

Statistical analysis. All data points are reported as means and SE. Determinations of statistical significance were performed with a Student’s \( t \)-test for comparisons of two means or with analysis of variance (ANOVA) for comparisons of multiple groups. Statistical significance was determined by a value of \( P < 0.05 \).

RESULTS

Case report of sudden cardiac death. A 23-year-old African American man collapsed while playing football. He was transported immediately to a hospital, where he was pronounced dead. Postmortem examination demonstrated no obvious pathological changes to explain the SUD of this apparently healthy

Fig. 1. Postmortem genetic testing identifies SCN5A mutations in sudden unexplained death (SUD). DNA sequence chromatograms superimposed on locations in the amino acid channel topology are shown. The cis- or trans-status could not be determined in the victim’s heart because the quality of the RNA isolated from the submitted autopsy sample was not adequate for long-range PCR.

Fig. 2. Representative whole cell current traces of wild-type (WT) and variant sodium channels. Currents were recorded at membrane potentials between -120 and +60 mV in 10-mV increments from a holding potential of -140 mV as depicted in the protocol inset. A: representative whole cell current traces under baseline pH. B: representative whole cell current traces under internal acidosis condition (pH 6.7).
Iated late CR680H/S1103Y showed significant 2.1-, 3.4-, and 3.6-fold in-cant difference from WT, but R680H, R680H/H11001† The late (Fig. 2 could be obtained from family members. conserved across species. Regrettably, no other information tions coexpressed together (R680H/S1103Y). However, the cis- or trans-status could not be determined in the victim’s heart because the quality of the RNA isolated from the submitted autopsy sample was not adequate for long-range PCR; therefore we tested all combinations. R680H was absent in >1,300 reference alleles and demonstrated various levels of conservation across species. Regrettably, no other information could be obtained from family members.

Electrophysiology. We studied WT, the single mutations R680H and S1103Y expressed separately, the two single mutations coexpressed together (R680H + S1103Y) but on separate plasmid, and the double mutation R680H/S1103Y in the same plasmid, using the Q1077del cDNA that reflects the most abundant alternatively spliced SCN5A transcript (~65%) in human hearts (12). Representative families of current traces at normal pH (Fig. 2A) and with acidosis (Fig. 2B) showed no obvious differences, and summary data for peak I\textsubscript{Na} density (Tables 1 and 2) showed no significant differences. Representative traces on an expanded amplitude scale to depict late I\textsubscript{Na} (Fig. 3, A and B) showed an obvious increase in late I\textsubscript{Na} under acidic pH (Fig. 3B) compared with normal pH (Fig. 3A) as summarized in Fig. 3C. At baseline pH the late I\textsubscript{Na} for S1103Y by itself showed no significant difference from WT, but R680H, R680H+S1103Y, and R680H/S1103Y showed significant 2.1-, 3.4-, and 3.6-fold increases, respectively (Fig. 3C; Table 1). The degree of accentuated late I\textsubscript{Na} for the combined mutants is comparable to that observed in patients established to have LQT3-associated muta-
tions in SCN5A (3). Moreover, late I\textsubscript{Na} for both R680H+S1103Y and R680H/S1103Y was significantly greater than that of R680H alone (P < 0.05). The double variants, whether on the same allele or on separate alleles that were coexpressed, had an interactive effect to produce increased late I\textsubscript{Na}. Lowering the intracellular pH to 6.7 significantly increased late I\textsubscript{Na} compared with baseline for each group tested except for WT (Fig. 3C; Tables 1 and 2), but the effect was especially marked for both R680H+S1103Y and R680H/S1103Y, where the already increased (relative to WT) late I\textsubscript{Na} was doubled.

We analyzed the kinetic parameters of activation (Fig. 4, A and C) and inactivation (Fig. 4, B and D) at baseline pH (Fig. 4, A and B) and pH 6.7 (Fig. 4, C and D) for all variants and compared these data with WT. Parameter values for the fits of activation and inactivation fits at baseline pH (Table 1; Fig. 4, A and B) showed a 6-mV negative shift in activation midpoint for S1103Y, but otherwise no significant differences in activation, inactivation, and recovery from inactivation parameters were noted. At pH 6.7, compared with WT, none of the variants showed a significant difference in activation (Fig. 4C; Table 2) while S1103Y, R680H+S1103Y, and R680H/S1103Y caused a statistically significant depolarizing shift (4–6 mV) in channel inactivation (Fig. 4D; Table 2). Fast inactivation properties of all variants were analyzed by two-exponential fits of the decay phase of macroscopic sodium current measured at various test potentials. Only R680H/S1103Y had significantly larger fast time constant (τ\textsubscript{f}) values across a wide range of test potentials than WT at pH 6.7 (data not shown). For recovery from inactivation, S1103Y, R680H+S1103Y, and R680H/S1103Y exhibited faster recovery from inactivation and had significantly smaller time constant (τ\textsubscript{i} and slow time constant (τ\textsubscript{s}) values compared with WT channels under low-pH conditions (Table 3).

Table 2. Biophysical properties of WT or variant sodium channels in Q1077del background in HEK293 cells under internal acidosis condition (pH 6.7)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Peak I\textsubscript{Na}</th>
<th>Activation</th>
<th>Inactivation</th>
<th>Late I\textsubscript{Na}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pA/pF</td>
<td>n</td>
<td>V\textsubscript{1/2}, mV</td>
<td>K</td>
</tr>
<tr>
<td>WT</td>
<td>−223 ± 34</td>
<td>18</td>
<td>−42.9 ± 1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>S1103Y</td>
<td>−215 ± 40</td>
<td>12</td>
<td>−41.1 ± 1.4</td>
<td>4.0</td>
</tr>
<tr>
<td>R680H</td>
<td>−264 ± 38</td>
<td>11</td>
<td>−41.8 ± 1.8</td>
<td>5.1</td>
</tr>
<tr>
<td>S1103Y/R680H</td>
<td>−205 ± 23</td>
<td>16</td>
<td>−44.0 ± 0.6</td>
<td>4.0</td>
</tr>
<tr>
<td>S1103Y/R680H</td>
<td>−234 ± 32</td>
<td>16</td>
<td>−41.2 ± 0.9</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Values are means ± SE for n experiments. All parameters were analyzed with 1-way ANOVA followed by a Tukey test. *P < 0.05 vs. WT; †P < 0.01 vs. WT, P < 0.05 vs. S1103Y or R680H.
Fig. 3. pH dependence of late current in WT and mutant constructs in Q1077del background. A: representative traces showing increased late sodium current ($I_{Na}$) associated with variants compared with WT under baseline pH condition. The corresponding peak $I_{Na}$ were WT = $-3.044$ pA, S1103Y = $-4.936$ pA, R680H = $-5.546$ pA, S1103Y + R680H = $-5.263$ pA, and S1103Y/R680H = $-5.209$ pA. B: representative traces showing increased late $I_{Na}$ associated with S1103Y, R680H, S1103Y + R680H, and S1103Y/R680H compared with WT under pH 6.7. The corresponding peak $I_{Na}$ were WT = $-3.809$ pA, S1103Y = $-4.170$ pA, R680H = $-4.663$ pA, S1103Y + R680H = $-4.285$ pA, and S1103Y/R680H = $-4.655$ pA. C: summary data of late $I_{Na}$ normalized to peak $I_{Na}$ after leak subtraction. Late $I_{Na}$ was measured as the mean current between 600 and 700 ms after the initiation of the depolarization from $-140$ mV to $-20$ mV for 750 ms (see protocol insets) after passive leak subtraction. Numbers of experiments are indicated above bars. Data were analyzed with 1-way ANOVA followed by a Tukey test. *$P < 0.05$ vs. WT at baseline pH; $SP < 0.05$ vs. S1103Y or R680H at baseline pH; †$P < 0.05$ vs. WT at pH 6.7; $#P < 0.05$ vs. S1103Y or R680H at pH 6.7. ‡$P < 0.05$ vs. the same variant at baseline pH (Student’s t-test).

Fig. 4. pH effects on gating kinetics of WT and mutant constructs in Q1077del background. A: voltage dependence of activation for WT and variants under baseline pH condition. Activation was measured with the protocol shown in the inset. The curves were fit with a Boltzmann function in which $G_{Na} = [1 + \exp(V_{1/2} - V/K)^{-1},$ where $G$, $V_{1/2}$, and $K$ are conductance, midpoint, and slope factor, respectively. $G_{Na}/V_{rev} = I_{Na}/(I_{Na} - V_{rev})$ where $V_{rev}$ is the reversal potential and $V$ is the membrane potential. B: steady-state inactivation for WT and variants under baseline pH condition. Steady-state availability from inactivation was measured with the protocol shown in the inset and was determined by fitting the data to the Boltzmann function $I_{Na} = I_{Na\max} [1 + \exp((V - V_{1/2})/K)^{-1}].$ $V_{1/2}$ and $K$ are midpoint and slope factor, respectively, and $V_{rev}$ is the membrane potential. C: voltage dependence of activation for WT and variants under pH 6.7. None of the variants altered steady-state activation parameters significantly. D: steady-state inactivation for WT and variants at pH 6.7. S1103Y, S1103Y + R680H, and S1103Y/R680H caused a statistically significant depolarizing shift in inactivation by 4 – 6 mV. See Tables 1 and 2 for summary of parameter fits and $n$ values.

Fig. 3. pH dependence of late current in WT and mutant constructs in Q1077del background. Wang et al. (22) also found that, in the Q1077 background, some SCN5A mutations including R680H exhibited no significant increase of late $I_{Na}$ that had been seen in the Q1077del background. To determine whether the minor alternatively spliced transcript exerts an effect on these case variants, we also tested R680H, S1103Y, R680H/S1103Y, and R680H/S1103Y in the Q1077-containing background. Compared with SCN5A-Q1077 WT, all variants showed no significant difference in both peak and late $I_{Na}$, activation, and inactivation (Table 4).

variant Q1077 background compared with Q1077del background. Wang et al. (22) also found that, in the Q1077 background, some SCN5A mutations including R680H exhibited no significant increase of late $I_{Na}$ that had been seen in the Q1077del background. To determine whether the minor alternatively spliced transcript exerts an effect on these case variants, we also tested R680H, S1103Y, R680H/S1103Y, and R680H/S1103Y in the Q1077-containing background. Compared with SCN5A-Q1077 WT, all variants showed no significant difference in both peak and late $I_{Na}$, activation, and inactivation (Table 4).

DISCUSSION

The common polymorphism SCN5A-S1103Y is a proarrhythmic, sudden cardiac death-predisposing risk factor in African Americans. Clinical, molecular (5, 6, 16, 18, 21), and functional (16, 18) investigations indicate that S1103Y may increase risk for arrhythmia susceptibility in the presence of environmental risk factors such as acidosis, medications, hypokalemia, and structural heart disease.

In the present study, we showed that Y1103-containing channels exhibit a statistically significant negative shift in
activation and that the rare R680H mutation causes a mild increase in late $I_{\text{Na}}$ as previously reported (18, 22). The new findings in this study are that S1103Y interacts with R680H to significantly increase late $I_{\text{Na}}$, and that this marked accentuation occurs whether the two mutations reside on the same allele or on separate alleles and then coexpressed together. Loss-of-function mutations in SCN5A have been "rescued" by the interaction with another common polymorphism, H558R (17), on separate alleles and then coexpressed together. Loss-of-function mutations in SCN5A have been "rescued" by the interaction with another common polymorphism, H558R (17), function in cardiac sodium channel deserves further investigation.

This study has several limitations. Heterologous systems do not faithfully recapitulate the actual cellular phenotypes because they lack subunits and interacting proteins found in myocytes. Despite this limitation we have described current dysfunction that is plausibly pathogenic. Also, the exercise phenotype of this case is different from conventional LQT3

<table>
<thead>
<tr>
<th>Samples</th>
<th>Recovery at Baseline pH</th>
<th>Recovery at pH 6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_r$, ms</td>
<td>$\tau_s$, ms</td>
</tr>
<tr>
<td>WT</td>
<td>1.8 ± 0.3</td>
<td>45.1 ± 6.2</td>
</tr>
<tr>
<td>S1103Y</td>
<td>2.2 ± 0.3</td>
<td>45.0 ± 8.8</td>
</tr>
<tr>
<td>R680H</td>
<td>1.7 ± 0.2</td>
<td>34.2 ± 3.0</td>
</tr>
<tr>
<td>S1103Y+R680H</td>
<td>1.8 ± 0.2</td>
<td>42.1 ± 4.1</td>
</tr>
<tr>
<td>S1103Y/R680H</td>
<td>1.7 ± 0.2</td>
<td>37.7 ± 3.0</td>
</tr>
</tbody>
</table>

Values are means ± SE for $n$ experiments. Time course of recovery from inactivation was analyzed by fitting data with a 2-exponential (exp) function: normalized $I_{\text{Na}}(t) = A_f[1 - \exp(-t/\tau_f)] + A_s[1 - \exp(-t/\tau_s)]$, where $i$ is time, $A_f$ and $A_s$ are fractional amplitudes of fast and slow components, respectively, and $\tau_f$ and $\tau_s$ fast and slow time constants, respectively. *P < 0.05 vs. WT (1-way ANOVA followed by a Tukey test).

Table 4. Biophysical properties of WT or variant sodium channels in Q1077 background

<table>
<thead>
<tr>
<th>Samples</th>
<th>Peak $I_{\text{Na}}$</th>
<th>Activation</th>
<th>Inactivation</th>
<th>Late $I_{\text{Na}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pA/pF</td>
<td>$n$</td>
<td>V1/2, mV</td>
<td>K</td>
</tr>
<tr>
<td>WT</td>
<td>$-267 \pm 54$</td>
<td>7</td>
<td>$-39.6 \pm 2.5$</td>
<td>4.0</td>
</tr>
<tr>
<td>S1103Y</td>
<td>$-246 \pm 56$</td>
<td>5</td>
<td>$-41.2 \pm 1.2$</td>
<td>4.0</td>
</tr>
<tr>
<td>R680H</td>
<td>$-213 \pm 43$</td>
<td>5</td>
<td>$-42.2 \pm 0.6$</td>
<td>4.0</td>
</tr>
<tr>
<td>S1103Y+R680H</td>
<td>$-228 \pm 32$</td>
<td>11</td>
<td>$-40.1 \pm 0.7$</td>
<td>4.0</td>
</tr>
<tr>
<td>S1103Y/R680H</td>
<td>$-235 \pm 69$</td>
<td>7</td>
<td>$-40.4 \pm 1.3$</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Values are means ± SE for $n$ experiments. All parameters were analyzed by 1-way ANOVA followed by a Tukey test. Compared with WT, all $P > 0.05$. 
late current-induced arrhythmia mechanisms, but we did not study the possible effect of increased sympathetic stimulation on late $I_{Na}$ in this case, because of the limitations of heterologous systems, or of rate dependence on late $I_{Na}$.

In summary, the SCN5A biological phenotype associated with this SUD case suggests that pathogenicity may involve $I_{Na}$ a “double hit” involving a rare mutation, R680H, and a common polymorphism, S1103Y, 2) in the most common alternatively-spliced SCN5A transcript, with 3) acidosis as a contributing environmental trigger. Although the voltage clamp data provide plausible mechanisms for the presumed arrhythmia in this SUD case, the association is conjectural as it was not supported by a linkage study or other corroborative evidence of causality. This report, however, does lend further support to the proarrhythmic, sudden death-predisposing potential of the S1103Y common polymorphism in African Americans and to our knowledge is the first report of an intragenic interaction for S1103Y. This may have implications for screening and prevention of arrhythmia as well as postmortem genotyping of SUD cases in African Americans. In addition, the data show that separate SCN5A α-subunits interact to affect function and suggest they may assemble together as part of the NaV1.5 macromolecular complex (9).

ACKNOWLEDGMENTS

We thank Jessie Ou, Neal Haas, and Qing Zhou for technical assistance.

GRANTS

This work was supported by the University of Wisconsin Cellular and Molecular Arrhythmia Research Program (J. C. Makielski), the Mayo Clinic Windland Smith Rice Comprehensive Sudden Cardiac Death Program (M. J. Ackerman), the established Investigator Award from the American Heart Association (to M. J. Ackerman), National Heart, Lung, and Blood Institute Grants HL-42569 (to M. J. Ackerman), HL-60723 (to C. T. January), and HL-71092 (to J. T. January) and Grant 30973367 (to J. Cheng) from the National Natural Science Foundation of China.

DISCLOSURES

M. J. Ackerman is a consultant for PGxHealth and chairs their FAMILION Medical/Scientific Advisory Board (approved by Mayo Clinic’s Medical-Industry Relations Office and Conflict of Interests Review Board). In addition, the data show that separate SCN5A α-subunits interact to affect function and suggest they may assemble together as part of the NaV1.5 macromolecular complex (9).

REFERENCES