A common human SCN5A polymorphism modifies expression of an arrhythmia causing mutation

BIN YE,1 CARMEN R. VALDIVIA,1 MICHAEL J. ACKERMAN,2 AND JONATHAN C. MAKIELSKI1
1Department of Medicine and Physiology, University of Wisconsin, Madison, Wisconsin 53792; and 2Departments of Internal Medicine, Pediatrics, and Molecular Pharmacology, Mayo Clinic, Rochester, Minnesota 55905

Ye, Bin, Carmen R. Valdivia, Michael J. Ackerman, and Jonathan C. Makielski. A common human SCN5A polymorphism modifies expression of an arrhythmia causing mutation. Physiol Genomics 12: 187–193, 2003. First published November 26, 2002; 10.1152/physiolgenomics.00117.2002.—SCN5A encodes the α-subunit of the ion channel that carries Na current in human heart. From a human cardiac cDNA library we recloned SCN5A. The new clone hH1b differed from existing clones hH1 in four and from hH1a in three positions. The common polymorphism H558R was uniquely present in hH1b. Voltage clamp study showed minor but potentially important kinetic differences between hH1b and the other clones. More dramatically, when the LQT3 mutation M1766L was introduced into the different clones, Na current was markedly reduced in the hH1 and hH1a backgrounds, whereas in hH1b the Na current was not reduced. Immunochemistry experiments showed a trafficking defect for M1766L Na channels in hH1 and hH1a but not in hH1b. The double-mutation M1766L/H558R in the hH1a background restored normal trafficking and current including persistent late current, suggesting the disease phenotype was the result of a “double hit” that included the common polymorphism, H558R. These results show that the choice of background clone must be carefully considered in mutagenesis studies. This also represents an example of intragenic complementation, the first for such a large protein.

EXPERIMENTAL PROCEDURES

RT-PCR cloning of hH1b. 5′ GATGAGAAGATGGCACAATCTCC 3′ and 5′ GTGCTGTGATCCCCGGGCTTCC 3′ primers were used to clone ~3 kb of 5′ Na,A,1.5 gene, and 5′ CACCCCCGGGATCCAGACG 3′ and 5′ TTGAGTTGCTGCTGCGGACG 3′ were used to clone ~3 kb of the 3′. Human cardiac mRNA (Clontech, Palo Alto, CA), RETRscript (Ambion, Austin, TX), and pfu DNA polymerase were used to perform RT-PCR. A reverse transcription protocol reaction was performed according to the manufacturer’s recommended procedure. PCR thermocycling involved one cycle of denaturation at 94°C for 1 min, then 35 cycles of amplification at 94°C for 1 min, 50°C for 1 min, and 72°C for 8 min, at end of one cycle of extension at 72°C for 20 min. The PCR products were cloned into pCR-BluntII-TOPO vector (In Vitrogen, Carlsbad, CA). The PCR products were sequenced with thermostable polymersases and fluorescently labeled dideoxyn terminators. The sequencing reaction samples were analyzed with automated DNA sequencers (ABI models 377XL and 377-96) at the Biotech Center of the University of Wisconsin. The sequence has been submitted to GenBank...
mammn equation using Clampfit 8 (Axon Instrument) and recovery and decay data to a two-exponential equation. The equations used are the same as Nagatomo et al. (13). Goodness of fit was determined both visually and by a sum of squares errors. One-way ANOVA was performed to determine statistical significance among three or more groups of mean data. Statistical significance was determined by P < 0.05.

**RESULTS**

*Amino acid differences in the three complete hNa1.5 clones.* We generated a third complete hNa1.5 clone (hH1b) from human heart mRNA using RT-PCR. The hH1b clone was sequenced, and the two existing hNa1.5 clones (hH1 and hH1a) were resequenced completely. The previously reported sequences for hH1 and hH1a differ by nine amino acids with hH1 also containing one additional amino acid (glutamine at residue 1077, Q1077). Upon resequencing hH1, we found that seven amino acids in hH1 differed from the published sequence for hH1 (V120I, A180G, R552G, H987Q, W1085G, R1087E, and Q1088A, where the second letter represents the resequenced amino acid). On the other hand, resequencing hH1a confirmed the originally published sequence. Each of the seven amino acid changes in the resequenced hH1 agreed with the hH1a sequence, reducing the difference between these clones to just three amino acids (T559 vs. A559, Q1027 vs. R1027, and Q1077 vs. Q1077del, between hH1 and hH1a, respectively, see Table 1). These residues are confined to the DI-II and DII-III cytoplasmic linkers (Fig. 1). Both hH1 and hH1a contain a histidine residue due at amino acid 558 (H558), a site hosting the common polymorphism (H558R) (11, 17).

The new clone, hH1b, reported here differed from hH1 by four amino acids, R558 vs. H558, I618 vs. L618, R1027 vs. Q1027, and Q1077del vs. Q1077 (hH1b vs. hH1, respectively), in the DI-II and DII-III linker, and from hH1a by three amino acids, R558 vs. H558, T559 vs. A559, and I618 vs. L618 (hH1b vs. hH1a, respectively), confined to the DI-II linker (Table 1). Results from Blast searching of the human genome sequence at Celera database showed only two differences between hH1b and Celera (R558 vs. H558 and I618 vs. L618, Table 1) confined to the DI-II linker (Fig. 1). Four additional synonymous or “silent” base differences between

<table>
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<th>Table 1. Deduced amino acid sequence comparisons for SCN5A clones</th>
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<tbody>
<tr>
<td><strong>Amino Acid No.</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>558</td>
</tr>
<tr>
<td>559</td>
</tr>
<tr>
<td>618</td>
</tr>
<tr>
<td>1027</td>
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<td>1077</td>
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Numbering follows the original numbering of Gellens et al. (8) for hH1. The sequence designated “Celera” is from Celera human genome database using BLAST search. For Table 1, data from the resequenced hH1 were used.
between hH1 and hH1b were noted (for hH1 vs. hH1b, respectively, TGT vs. TGC for amino acid C280, ACA vs. ACC at amino acid T670, CTA vs. CTC at amino acid L789, and GAC vs. GAT at amino acid D1819).

Kinetic studies of three hNa\(_{1.5}\) clones. The \(I_{\text{Na}}\) kinetics for hH1 (8) and hH1a (10) from separate studies in the literature showed only minor differences that could be attributed to different expression systems and study techniques including solutions, temperature, and protocols. Subtle differences in kinetics such as decay rates, inactivation midpoints, and late and protocols. Subtle differences in kinetics such as decay rates, inactivation midpoints, and late and protocols. Subtle differences in kinetics such as decay rates, inactivation midpoints, and late and protocols. Subtle differences in kinetics such as decay rates, inactivation midpoints, and late and protocols. Subtle differences in kinetics such as decay rates, inactivation midpoints, and late

Summary data (Fig. 4) show that for wild type, hH1b and hH1a clones generally express more \(I_{\text{Na}}\) than the hH1 clone. Comparing the sequence variations among the three clones (Table 1), we hypothesized that the polymorphism H558R might underlie the restoration of expression in channels containing the M1766L mutation. Again using site-directed mutagenesis, we engineered the double-mutation M1766L/H558R in the hH1a clone. Now, rather than the 97% reduction in current expression observed previously for M1766L-hH1a (containing H558), the M1766L/H558R-hH1a clone (now containing R558) manifested a fully restored \(I_{\text{Na}}\) density (Figs. 3 and 4). These experiments show that the residue at position 558 in interaction with M1766L is the residue responsible for affecting expression of \(I_{\text{Na}}\).

We investigated defective trafficking as a possible mechanism for the failure of M1766L to express well in the hH1a background by using confocal microscopy and by imaging the Na channel labeled with the FLAG epitope (Fig. 5). The regular bright-field images on the left in Fig. 5 show the location of the nucleus and the cell periphery, and the fluorescent images on the right show the immunolocalization of the Na channel protein. The nontransfected cell showed minimal fluorescence, whereas transfections with wild-type hH1a and hH1b showed localization at the periphery of the cell (Fig. 5, B and C). The M1766L mutation in the hH1a background showed a perinuclear localization pattern with no peripheral expression, consistent with trapping in the endoplasmic reticulum (3). When M1766L was expressed in the hH1b clone, the normal peripheral fluorescent pattern was restored (Fig. 5E). When M1766L was expressed in the modified hH1a clone now containing R558 as in hH1b rather than H558, normal trafficking to the cell periphery was also observed (Fig. 5F). The results shown in Fig. 5 were typical for 3–5 pictures taken of nonoverlapping cells in 3 independent expression experiments. These data are consistent with the functional data (Fig. 3) and suggest a
trapping defect as the mechanism for the decreased current expression. M1766L represents the third documented trafficking defect for SCN5A, with the others being R1232W (3) and R1432G (4), and it is the first trafficking defective mutation for SCN5A that has been shown to be rescued by a drug.

**DISCUSSION**

We report a third complete sequence of the hNav1.5 α-subunit designated hH1b. Amino acid sequences for the three clones hH1, hH1a, and hH1b, together with the sequence from the Celera database, differ in up to five amino acid positions (Table 1). A difference found only in the hH1b clone, R558 rather than H558, is also a previously reported amino acid polymorphism (11) with /H1101120–30% of white humans heterozygous for H558R in a population study (17). A separate analysis of healthy subjects indicates that /H1101130–40% of individuals are heterozygous for H558R with no significant difference in the allelic frequency between whites and blacks (M. J. Ackerman, unpublished data). It should be noted that the term mutation refers to any heritable or spontaneous germ line (sporadic) change in DNA sequence. The term common polymorphism refers to sequence variations occurring among populations of individuals of the same or different ethnicities with a certain allelic frequency (usually >1%) that may underlie differences in health. Clinicians and physiologists also use the term polymorphism to denote a mutation occurring with some frequency in the population that does not cause overt disease or protein dysfunction (11, 15), which is also called a nonpathogenic mutation (15).

Another difference found only in hH1a, A559 rather than T559, is not a known polymorphism; that is, no population studies have been done. A third difference found only in hH1b, I618 rather than L618, is also not a known polymorphism, but the conservative change of a leucine to an isoleucine is a known high-frequency spontaneous change. The fourth and fifth differences (Q1027 and Q1077) are both found only in hH1 and are not known to be polymorphisms. The latter, the insertion of Q at 1077 in hH1, occurs at an intron-exon boundary and could represent alternative splicing. Aside from the known polymorphism at position 558, the Celera sequence agrees with the “majority” and differs from hH1 at two positions (1027 and 1077), from hH1a at one position (559), and from hH1b at one position (619). These differences may be cloning errors, or they may be actual polymorphisms. Further population studies are

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**Fig. 2.** Whole cell Na current (I_{Na}) for hH1, hH1a, and hH1b. A: representative traces were recorded with test potentials of 24-ms duration from ~120 to +60 mV from a holding potential of ~120 mV. B: current-voltage relationship (left), “steady-state” inactivation relationship (middle), and recovery from inactivation relationship (right) for I_{Na}. Diagrams depicting the protocols are inset into each plot. In every case the test step (the second depolarization for middle and right) was 24 ms in duration. The conditioning (first) step duration for the recovery protocol was 1 s. The y-axis in each case is peak I_{Na} normalized to the maximal peak I_{Na} obtained in the protocol. Solid symbols represent the mean data for between 6 and 10 experiments (see Table 2 for exact n numbers) with hH1 (square), hH1a (circle), and hH1b (triangle), and the bars represent SD.
Table 2. Kinetic parameters for three SCN5A clones

<table>
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<th>Parameter</th>
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<th>hh1a</th>
<th>hh1b</th>
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<tr>
<td>Activation V1/2</td>
<td>-44±5</td>
<td>-40±7</td>
<td>-43±6</td>
</tr>
<tr>
<td>Slope factor</td>
<td>5±1</td>
<td>5.5±0.6</td>
<td>5.5±0.7</td>
</tr>
<tr>
<td>Inactivation V1/2</td>
<td>-95±4.8</td>
<td>-86±5.8</td>
<td>-90±8.8</td>
</tr>
<tr>
<td>Recovery τn [ms]</td>
<td>8±5</td>
<td>5±3</td>
<td>5±2</td>
</tr>
<tr>
<td>Recovery A [s]</td>
<td>0.31±0.1</td>
<td>0.25±0.1</td>
<td>0.24±0.1</td>
</tr>
<tr>
<td>Decay τ [ms]</td>
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<td>1.5±0.5</td>
<td>1.3±0.5</td>
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<tr>
<td>Decay A [s]</td>
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<td>6.0±3</td>
<td>4.0±1</td>
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<tr>
<td>Decay n</td>
<td>0.71±0.1</td>
<td>0.65±0.1</td>
<td>0.56±0.2</td>
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The fitted kinetic parameters from n experiments were averaged and are reported as means ± SD. Three SCN5A clones, hh1, hh1a, and hh1b, show minor differences in activation, inactivation and recovery from inactivation kinetics. These parameters were obtained from fitting the individual experiments as in Fig. 2 to the appropriate model equations (13). For the Boltzmann fits (activation and inactivation) the parameters are V1/2 midpoint and slope factor. For the double exponential fits (recovery and current decay) the parameters are: τn, the fast time constant; τs, the slow time constant; A, the fractional amplitude of slow component; and Af, the fractional amplitude of fast component. All parameters were analyzed by one-way ANOVA across the three clones. *Statistically significant values.

needed to make this determination and to more definitively determine the “correct” sequence for Na<sub>1.5</sub>. We have shown that the three clones have very similar but not identical kinetic function (Table 2 and Fig. 2). The midpoint of inactivation was significantly more negative for hh1 (−95 mV) than it was for hh1a (−86 mV), with hh1b being in the middle (−90 mV) and not significantly different from either one within the limitations of this study. This difference has possible significance, because with the lack of difference in activation midpoint, the more positive the inactivation relationship, the more “window” current. Window current has been implicated in the control of repolarization for SCN5A mutants (1). Relatively subtle differences in the early rate of decay of INa current may also play a role in determining repolarization by effects on other currents and the early plateau time course (6, 16). Here caution is urged in interpretation of the voltage clamp data, as decay rates are exquisitely sensitive to voltage control problems. In our analysis, however, decay experiments were selected to have low and similar current densities with activation slopes >5.5. We conclude that the kinetic profiles are very similar but that subtle differences in the wild-type clones may be important for predisposition to arrhythmia. The differences in these channels, should they prove to be polymorphisms in the population, may be candidates for predisposition to acquired repolarization abnormalities.

The most dramatic difference between the three clones, however, is the rescue of the expression defect for M1766L when expressed in the hh1b background (Fig. 3) compared with hh1 and hh1a. The known polymorphism H558R and the variation L618I are the only differences between hh1 or hh1a compared with hh1b (Table 1). The double mutation experiments provide strong evidence that amino acid at position 558 was responsible for the profound differences in trafficking and INa expression level. The term “intragenic complementation” refers to the case where a mutation at one locus in a gene interacts with a mutation at another locus to restore function of the gene product. The phenomenon has been widely reported in nature, but previous examples in humans have involved small multimeric proteins where separate alleles restored enzyme function (18). The restoration of protein function by H558R does, however, fit the original definition of intragenic complementation (7) and represents a unique example. How could amino acids located over 1,200 residues away from each other interact to restore function? Amino acids 558 and 1766 are 1,200 residues away from each other interact to restore function of the gene product. The phenomenon has been widely reported in nature, but previous examples in humans have involved small multimeric proteins where separate alleles restored enzyme function (18). The restoration of protein function by H558R does, however, fit the original definition of intragenic complementation (7) and represents a unique example. How could amino acids located over 1,200 residues away from each other interact to restore function?

Wildtype

M1766L

M1766L/R558

Fig. 3. Current magnitude for wild-type hh1, hh1a, and hh1b clones, for the arrhythmia mutation M1766L expressed in each clone, and for the double mutation. Examples of INa traces (protocol inset) for each construct show that M1766L expressed very poorly in hh1 and hh1a but expressed normally in hh1b. Expression levels for the double-mutant M1766L/H558R in hh1a, however, were normal.
lyase, mutations at different loci on separate monomers interact to affect the thermodynamic stability of the protein (18). Further study is required to elucidate the precise structural determinants as well as the mechanism of this restoration in hNav1.5.

We have shown that the “background” in which SCN5A mutations are expressed may impact profoundly the results of studies obtained in heterologous expression systems. The polymorphism also probably played a role in the pathogenesis of the arrhythmia syndrome in the patient presenting with M1766L (14). We had previously shown that the patient had a prolonged QT interval, and that the “mexiletine-rescued” M1766L-hH1a showed a typical late I Na (14), but we were puzzled why the patient would manifest QT prolongation in the absence of mexiletine. In light of our observation that hH1b rescued M1766L expression, we reexamined the patient’s genomic DNA and found that in addition to the pathogenic mutation, M1766L, the patient was indeed heterozygous for the H558R polymorphism. Because the patient manifested QT prolongation, it might be assumed that one allele contained both R558 and L1766, resulting in a rescued Na current with increased late current. The functional evidence provided herein suggests a “double hit” requiring both mutations on the same allele. Unfortunately, no postmortem tissue permitting the isolation of SCN5A mRNA transcript from the decedent was available to confirm this speculation, only blood in EDTA preservative. Because of the large number of intervening base pairs we could not determine from the available genomic DNA whether or not they were on the same allele. Also, linkage analysis was not possible because the M1766L mutation was a spontaneous germ line mutation.

If the M1766L mutation were to reside on the more common H558-containing allele, then this would be predicted to cause loss of function and Brugada syndrome or conduction system disease. This could be one mechanism for the observation that some mutations can present with different clinical phenotypes in different persons (5). A “double hit” involving mutations R1232W and T1620M was previously implicated to cause a trafficking defect for the Brugada syndrome (3).

Caution must always be used in extrapolating basic studies to the clinical setting. The question of whether the expression levels and the kinetic behavior of different wild-type and mutated hNav1.5 channels in these experimental expression systems faithfully recapitulate their function in intact heart remains a common

**Fig. 4.** Summary data for current density for experiments as depicted in Fig. 3. I Na values were normalized to cell capacitance, and mean values are shown as a column with SD bar, with the number of experiments indicated above the bar. To assure voltage control, cells with total currents >2 nA were excluded. Approximately 25% of wild-type currents were >2 nA and were excluded; no M1766L currents were excluded. The effect of this exclusion would tend to underestimate the I Na suppression by M1766L; the actual effect is larger.

**Fig. 5.** Images of fluorescent-labeled HEK293 cells that were nontransfected (A), transfected with wild-type channels (B and C), or transfected with mutant channels (D–F). For each experiment a bright-field image (left) and a fluorescent image (right) are shown. A: the nontransfected HEK293 cell with a setting of maximum laser power and a gain of 1900 shows only minor background florescence. B: HEK293 cell transfected with wild-type hH1a showed peripheral localization. C: HEK293 cell transfected with wild-type hH1b also showed peripheral localization. D: HEK293 cell transfected with M1766L in the hH1a clone showed peripheral localization within the cell near the nucleus. E: HEK293 cell transfected with M1766L in the hH1b clone showed peripheral localization as in the wild type. F: HEK293 cell transfected with the double-mutation M1766L/H558R-hH1a showed expression at the cell periphery like wild type.
limitation. Nevertheless, our findings provide a caveat for those using heterologous expression systems that the choice of background clone is important. Also our findings suggest that the particular channel background sequence in patients may be important in defining functional properties of putative arrhythmia-causing mutations in SCN5A. The search for “modifier genes” must include the disease-causing gene itself.

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