Human SCN5A gene mutations alter cardiac sodium channel kinetics and are associated with the Brugada syndrome

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Abstract

Background: Primary dysrhythmias other than those associated with the long QT syndrome, are increasingly recognized. One of these are represented by patients with a history of resuscitation from cardiac arrest but without any structural heart disease. These patients exhibit a distinct electrocardiographic (ECG) pattern consisting of a persistent ST-segment elevation in the right precordial leads often but not always accompanied by a right bundle branch block (Brugada syndrome). This syndrome is associated with a high mortality rate and has been shown to display familial occurrence.

Methods and results: Pharmacological sodium channel blockade elicits or worsens the electrocardiographic features associated with this syndrome. Hence, a candidate gene approach directed towards SCN5A, the gene encoding the α-subunit of the cardiac sodium channel, was followed in six affected individuals. In two patients missense mutations were identified in the coding region of the gene: R1512W in the DIII–DIV cytoplasmic linker and A1924T in the C-terminal cytoplasmic domain. In two other patients mutations were detected near intron/exon junctions. To assess the functional consequences of the R1512W and A1924T mutations, wild-type and mutant sodium channel proteins were expressed in Xenopus oocytes. Both missense mutations affected channel function, most notably a 4–5 mV negative voltage shift of the steady-state activation and inactivation curves in R1512W and a 9 mV negative voltage shift of the steady-state activation curve in A1924T, measured at 22°C. Recovery from inactivation was slightly prolonged for R1512W channels. The time dependent kinetics of activation and inactivation at −20 mV were not significantly affected by either mutation. Conclusions: Two SCN5A mutations associated with the Brugada syndrome, significantly affect cardiac sodium channel characteristics. The alterations seem to be associated with an increase in inward sodium current during the action potential upstroke. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Ventricular arrhythmias; Sudden death; Ion channels; Genetic code

1. Introduction

Primary dysrhythmias in disease entities other than the long QT syndrome (LQTS), the paradigm for ‘primary’ familial cardiac arrhythmias [1,2], although still very
uncommon, are increasingly diagnosed. Patients with a history of aborted sudden death and a distinct electrocardiographic (ECG) pattern, consisting of persistent ST-segment elevation in the right precordial leads in combination with a right bundle branch block (RBBB), in the absence of any structural heart disease, may represent another cardiac ion channel disorder [3]. The syndrome may display a familial occurrence [3], seems to be associated with a high mortality rate [4], and is increasingly recognized [4–7]. Very recently, mutations in the $\text{SCN}5\text{A}$ gene which encodes the $\alpha$-subunit of the fast cardiac sodium channel, were shown to co-segregate with electrocardiographically affected individuals within small families in which a high incidence of sudden cardiac death was present [8].

Since pharmacological sodium channel blockade elicits or worsens the electrocardiographic features associated with this syndrome [6,9,10], a candidate gene approach directed towards $\text{SCN}5\text{A}$, was followed in six individuals with the typical electrocardiographic features outlined above. In two patients, intronic mutations which could affect splicing were identified. In two other patients a missense mutation was identified in the coding region of the gene. One was a substitution of arginine 1512 by tryptophan (R1512W) in the highly conserved DIII–DIV cytoplasmic linker, a domain in which mutations associated with LQTS have been identified [11]. The other was a substitution of alanine 1924 by threonine (A1924T) in the C-terminal cytoplasmic domain, in which very recently two mutations have been reported that are involved in the LQT-3 syndrome [12,13]. Both missense mutations affected channel function. In particular, they caused a negative voltage shift of the steady-state activation curve when expressed in Xenopus oocytes.

2. Methods

2.1. Phenotypic analysis

Informed consent for genetic studies was obtained from all individuals in accordance with local institutional ethical committee guidelines. Brugada syndrome was diagnosed when the typical electrocardiographic features — at least 1 mm ST-segment elevation in leads $V_{1}–V_{2}$ or $V_{1}–V_{3}$, were present in the absence of structural heart disease [3]. An apparent right ventricular conduction delay in the ECG was not considered essential for the syndrome.

Family members were not screened because it was considered ethically unjustified to identify individuals with a potentially malignant genotype without the possibility to offer them appropriate treatment. In our country ICD resource limitations are such that a prophylactic implantation is currently not accepted. Hence, eventually identified asymptomatic gene carriers cannot be offered appropriate treatment.

2.2. SSCP analysis and direct sequencing of PCR products

Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. All 28 exons of $\text{SCN}5\text{A}$ were amplified by PCR using intronic primers designed by Wang et al. [14], and analyzed by single stranded conformation polymorphism (SSCP) analysis on 12.5% non-denaturing polyacrylamide gels (Pharmacia Biotech) run at 5°C and 15°C as described in GENEGEL Excel protocols (Pharmacia Biotech). PCR products presenting an aberrant conformer were re-amplified from genomic DNA, purified by micro-spin chromatography (Pharmacia Biotech) and sequenced in both directions by the fluorescent dideoxy chain termination method on an ABI 377 (Applied Biosystems) DNA sequencer.

2.3. Functional expression

Mutant sodium channel cDNAs were prepared by mutagenesis on the double-stranded pSP64T-hH1(sp) plasmid [15,16], a kind gift from A.L. George Jr., (Vanderbilt University, Nashville, TN, USA) using the QuikChange™ (Stratagene) site-directed mutagenesis kit and the following oligonucleotides: 5′GGAGCCCCATCCCCATGGCCCCC-TGAACAG3′ (sense) and 5′CTTGTTCCAGGGCCAT-GGATGCCTTC3′ (antisense) for the R1512W mutant, and, 5′CGCTTTGAAGCATACCTCCTTCCTTCC3′ (sense) and 5′GGAGAGGAGAGGATGC-TTCGAAGAGG3′ (antisense) for the A1924T mutant. Mutant clones were identified by restriction endonuclease analyses (Nco I site created by R1512W; Sph I site abolished by A1924T). A Kpn I-Bst EII fragment (nt 4378–4777) and a Kpn I-Sfi I fragment (nt 4378–6215), in the case of the R1512W and A1924T mutant constructs, respectively, was subcloned into the wild-type pSP64T-hH1(sp) plasmid, and mutant inserts and ligation regions were analyzed fully by nucleotide sequence analysis on an ABI 377 DNA sequencer to ensure that the clones selected were free of polymerase errors. Wild-type and mutant constructs were linearized with Xba I and cRNAs were synthesized using the mMessage mMachine kit (Ambion).

2.4. Electrophysiology

Stage V–VI Xenopus oocytes were isolated and injected with 16–22 ng cRNA according to standard methods [15]. The oocytes were kept in a modified Barth’s solution [17] containing in mM: NaCl 88, NaHCO$_3$ 2.4, KCl 1.0, CaCl$_2$ 0.7, MgSO$_4$ 0.8 and HEPES–NaOH 15, pH 7.6. The same solution was used in the electrophysiological experiments. Voltage clamp experiments were performed 2–3 days after injection, using a Geneclamp 500 two-electrode voltage clamp amplifier (Axon Instruments). Steady-state activation and inactivation parameters were determined using protocols similar to those published previously [11].
Fig. 1. Representative recordings (eight leads) of the ECG of patient 1, several weeks after a successful resuscitation. The right panels show the effect of oral flecainide treatment (idd 100 mg). Note the increase in ST-segment elevation in particularly lead V1. Calibration is standard (lower right corner).
Briefly, the voltage dependence of the relative Na-conductance activation was measured by applying series of voltage clamp steps to different membrane potentials ($V_{\text{test}}$) between $-120$ and $+40$ mV, starting from a holding potential ($V_{\text{H}}$) of $-100$ mV (Fig. 3A). Next, peak $I_{\text{Na}}$ at each $V_{\text{test}}$ was divided by the driving force for Na ions ($V_{\text{test}} - E_{\text{Na}}$; $E_{\text{Na}}$ = reversal potential of sodium current) and normalized to the maximum Na-conductance. Steady-state inactivation relationships were obtained by measuring the peak $I_{\text{Na}}$ during a 50 ms test step to $-30$ mV which followed a 1 second prepulse to membrane potentials between $-120$ and $-20$ mV (Fig. 3B). The peak currents measured during the test potentials were normalized to the maximum current measured following the first prepulse to $-120$ mV.

Activation and inactivation curves were fitted with a Boltzmann equation to establish the potential for half-maximal activation or inactivation ($V_{1/2}$) and slope factors ($k$) for these parameters. The experiments were performed at $10^\circ\text{C}$ and at room temperature ($22^\circ\text{C}$) to ensure reliable voltage clamping of these fast currents.

Recovery from inactivation was measured at $22^\circ\text{C}$ using a two pulse protocol in which a conditioning pulse to $-30$ mV, allowing inactivation, was followed by a test pulse (also to $-30$ mV) after a variable recovery interval ranging between 3 and 83 ms. The fraction of channels that had recovered following the various time intervals, was calculated by dividing the peak Na currents evoked by the test pulse by the peak current measured during the conditioning pulse. Holding potential between conditioning and test pulses was $-100$ mV. Averaged data were fitted to a single exponential to obtain the time constant of recovery from inactivation.

3. Results

Structural heart disease was excluded by normal echocardiograms in six out of six patients (6/6), coronary angiograms (performed in 5/6), left and right ventriculograms (5/6) and stress tests (6/6). Electrolytes were normal in all patients. The typical electrocardiographic...
features, viz. at least 1 mm ST-segment elevation in leads V1–V2 or V1–V3, were present in all six patients. Right ventricular conduction delay was only present in patients 4 and 6. Patients 1, 3–6 were successfully resuscitated, patient 2 was without complaints. Fig. 1 shows eight selected leads of an ECG taken from patient 1. The typical ST-elevation pattern in V1–V2 was enhanced by sodium channel blockade in this patient (fecainide, Fig. 1, right panel), as well as in patients 3 and 5 (not shown) who also received these drugs during the hospitalization phase.

SSCP analysis of the entire coding region of SCN5A in six patients identified four different aberrant conformers in patients 1–4. None of these conformers was present in 100 alleles from unrelated control individuals. DNA sequence analysis in patient 1 revealed heterozygosity for a C->T transition in the first nucleotide of codon 1512, resulting in an Arg->Trp substitution (R1512W; Fig. 2, top panel) within the highly conserved DIII–DIV cytoplasmic linker (Fig. 2, lower panel). DNA sequence analysis in patient 2 revealed heterozygosity for a G>A substitution in the first nucleotide of codon 1924, resulting in the substitution of Ala by Thr (A1924T; Fig. 2 middle panel) within the cytoplasmic C-terminal domain of the protein (Fig. 2, lower panel). Patient 3 was heterozygous for a C>T substitution at position −24 within intervening sequence 2 (IVS2 −24). This substitution (G[C->T]CTAAT) occurred within a site that bears a strong resemblance to the lariat branch site consensus sequence (YNYYRAY) implicated in splicing, which is typically located 20–50 bp upstream of the 3’ splice site. Finally, patient 4 was heterozygous for a C>T substitution at IVS24 +28.

To determine the functional consequences of the R1512W and A1924T mutations, wild-type and mutant sodium channel proteins were expressed in Xenopus oocytes by injecting the corresponding cRNAs. All three cRNAs resulted in expression of functional channels, since in each case typical rapidly activating and inactivating Na-currents were recorded under voltage clamp conditions (Fig. 3). Maximum current amplitudes ranged between 2 and 12 μA. To assess the electrophysiological mechanisms in this syndrome, we compared the voltage dependence of the activation and inactivation kinetics of currents carried by wild-type (INa-WT) and mutant sodium channels (INa-R1512W or INa-A1924T).

At 10°C the V1/2 of INa-R1512W activation and inactivation curves each were shifted about 2.5 mV to more negative voltages as compared to INa-WT. For INa-A1924T these shifts were larger: −7.5 and −4.4 mV, respectively (Fig. 4A and Table 1). The slope factors for the voltage dependence of inactivation for wild-type and mutant channels were similar.

At 22°C (Fig. 4B and Table 1), the negative shifts of the V1/2 of activation at 22°C were more pronounced than at 10°C: −5.1 mV for INa-R1512W and −9.0 mV for INa-A1924T. The V1/2 of inactivation at 22°C for INa-R1512W was shifted by −3.8 mV, but for INa-A1924T it was identical to that of INa-WT. In addition, the slope of the inactivation curves was increased in particular. The negative shifts in V1/2 for activation of the mutant sodium channels found at 22°C, suggest an enhancement of excitability of cardiomyocytes expressing these channel mutants.

At 10°C the mutations did not noticeably alter time
Fig. 3. Examples of Na-currents measured at 10°C after injection of wild-type or mutant SCN5A cRNA in Xenopus oocytes. (A) Left panel: selected recordings of $I_{\text{Na,wt}}$ currents elicited by the voltage clamp protocol used to determine the voltage dependence of activation. Holding membrane potential ($V_h$) was $-100$ mV, with increasingly depolarizing test-pulses ($V_{\text{test}}$) more $I_{\text{Na,wt}}$ was activated. Maximum peak inward current of $-12$ µA was measured at $V_{\text{test}}$ between $-30$ and $-20$ mV. Right panel: peak $I_{\text{Na,wt}}/V_{\text{test}}$ relationship from which the voltage dependence of activation was calculated (see Methods). Reversal potential for the sodium current ($E_{\text{Na}}$) was estimated from its $x$-axis intercept as indicated. (B) Left panel: selected recordings of $I_{\text{Na,A1924T}}$ currents elicited by the voltage clamp protocol used to determine the voltage dependence of inactivation. Only the last 3 ms of the inactivating prepulse ($V_{\text{prepulse}}$) are shown. Peak $I_{\text{Na-A1924T}}$ measured during the $-30$ mV test-potential was maximal ($-6.8$ µA) when $V_{\text{prepulse}}$ was more negative than $-120$ mV. With increasingly depolarizing $V_{\text{prepulse}}$, $I_{\text{Na-A1924T}}$ decreased to zero beyond prepulses more positive than $-65$ mV. Right panel: relationship between $V_{\text{prepulse}}$ and $I_{\text{Na-A1924T}}$ derived from all data points from the experiment shown in the previous panel.
Fig. 4. Comparison of the voltage dependence of activation and inactivation of wild-type (WT) and mutant (R1512W and A1924T) cardiac sodium channels. Data were fitted with a Boltzmann equation to estimate the potential for half-maximal activation or inactivation (V½) and slope factor (k). Scale bars represent S.E., accuracy of fits exceed 99%. (A) Voltage dependence of wild-type and mutant cardiac sodium channels measured at 10°C. Inactivation of R1512W and A1924T channels was shifted -2.4 and -4.4 mV, respectively, compared to WT channels. Activation of R1512W and A1924T channels was shifted -2.7 and -7.5 mV, respectively. (B) Voltage dependence of wild-type and mutant cardiac sodium channels measured at 22°C. Inactivation of R1512W channels was shifted -3.8 mV compared to WT channels. The voltage dependence of inactivation of A1924T channels was virtually similar to that of WT channels. Activation of R1512W and A1924T channels was shifted -5.1 and -9.0 mV, respectively.

dependent channel kinetics during a voltage step from -100 mV to -20 mV, as is shown in Fig. 5A. At this temperature, the gating behavior was well described by the classical Hodgkin and Huxley relationship \( m^3 h \). The time constants for the activation-parameter \( m \) and inactivation-parameter \( h \) were \( \sim 1.3 \) ms and 13 ms, respectively. At 22°C activation was already so fast that reliable fitting with \( m^3 h \) was not possible. Inactivation was best fitted by a double exponential, indicating the presence of a fast and a slow component. As shown in Fig. 5B, only R1512W channels were marginally different from wild-type channels and showed a slight increase in the \( \tau_{fast} \) value of inactivation.

The time constant of recovery from inactivation at 22°C was 8 ms for Na–WT and A1924T channels, but somewhat prolonged (9.6 ms) for R1512W channels (Table 1).
4. Discussion

Ventricular fibrillation in the absence of structural heart disease is classified as ‘primary electrical disease’. Such a diagnosis places the arrhythmogenic substrate in the excitable and conducting properties of the heart. Indeed, genes encoding ion channels have been demonstrated to underlie various forms of the congenital long QT syndrome (for reviews see [1,2]). In their initial report on a distinct clinical and electrocardiographic entity, consisting...
Recovery from inactivation

Inactivation

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<th>Temperature (°C)</th>
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<td>V_{1/2} (mV)</td>
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<td>22</td>
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R1512W channels this would be counteracted by the negative voltage shift of the steady state activation.

To explain the ECG of the Brugada patients carrying the R1512W or the A1924T sodium channel mutations on the basis of an increase in $I_{N\alpha}$, we tentatively suggest the following mechanism. Altered $I_{N\alpha}$ will have an impact on $I_{Na}$ (and $I_{CaL}$) characteristics. A larger action potential overshoot due to increased $I_{Na}$ may result in increased $I_{CaL}$, potentially also leading to a negative shift of the epicardial phase 1 nadir. The resultant epicardial action potential shortening and related ECG changes have been discussed above. Interestingly, in the family described by Chen et al. and in the patients 1 and 2 with mutations in SCN5A exons we describe here, no RV conduction delay is observed [8], which also argues against a substantial reduction of $I_{Na}$.

Alternatively, to explain our results still assuming a reduction in $I_{Na}$, the shift of the activation curves towards the resting membrane potential at physiological temperature could be such that the fraction of open sodium channels during diastole is increased. This would cause some persistent depolarization and consequently a reduced action potential upstroke.

In patients 3 and 4, we detected intronic mutations which may possibly affect splicing. It has been shown that changes in the consensus sequence at splice sites or lariat branch-point regions cause exon skipping. Moreover, intron or exon mutations (including silent mutations) outside these regions can also affect splicing [23–26]. Whenever these mutations lead to alternative splicing, it is likely that non-functional channels will ensue. Chen et al. also described SCN5A gene mutations in small Brugada families leading to premature truncation of the sodium channel protein [8]. It may be speculated that in these particular cases heterozygosity for the mutation will lead to a reduction in expression of functional sodium channels. In these cases the Brugada phenotype would result from a reduction of $I_{Na}$.

The fact that no SCN5A mutations were detected in two patients (5 and 6) suggests genetic heterogeneity for this disorder. However, the SSCP methodology (the most widely used screening method) for mutation detection can detect between 88 and 90% of mutations [27] and therefore one cannot entirely exclude presence of SCN5A mutations within this gene in these individuals.

4.1. Limitations of the study

In this study we have not shown co-segregation of the SCN5A mutations with the typical electrocardiographical features. However, we believe that the mutations described in this study could be causally linked to the disease for two reasons. First, Chen et al. recently described mutations in the SCN5A gene that co-segregated with the clinical syndrome [8]. Second, the estimated effect of the functionally studied mutation [8], and of our mutations is a consistent increase in window current. However, the impact of these changes on action potential morphology and the resulting ECG in the patients, in which the mutant sodium channels are presumably heterozygously expressed, is uncertain. A particular complicating factor is the potential temperature dependence of Na-channel gating parameters. Expression studies at physiological temperature in mammalian cell lines and direct measurements on epicardial cells from affected patients may be the only way to provide answers to these uncertainties. We have not studied the interaction with the putative β-subunit [28] for the sodium channel encoded by SCN5A, which could be affected in the case of the A1924T mutant channel, as has been shown for the other C-terminal mutation D1790G.

In conclusion, functional studies in a heterologous expression system of Brugada syndrome related mutant sodium channels, reveal significant shifts of the activation curves in the negative direction. These shifts could result in increased sodium current during the action potential upstroke. Our results may provide the basis for new insights into the mechanisms of arrhythmogenesis in patients with idiopathic VF.

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References


