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Supplementary material

Digenic Heterozigosity in *SCN5A* and *CACNA1C* Explains the Variable Expressivity of the Long QT Phenotype in a Spanish Family

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SUPPLEMENTARY MATERIAL

METHODS

Genetic Analysis

Complete clinical evaluation, including 12-lead electrocardiogram (ECG), of the family members was performed at our hospital. The study was approved by the local ethics committee and conforms to the principles outlined in the Declaration of Helsinki. Each participant gave written informed consent.

Genetic analysis was performed by using procedures previously described.[1-3] In the deoxyribonucleic acid (DNA) samples, a library of 9 genes (*KCNQ1, KCNH2, SNC5A, KCNJ2, KCNJ8, CACNA1C, AKAP9, KCNE1,* and *KCNE2*) involved in long QT syndrome (LQTS) was sequenced using an Illumina 1500 Hiseq next-generation sequencing platform. The sequence of all coding exons and intronic flanking regions was obtained. Targeted enrichment was performed using Agilent SureSelect. Design of the capture baits was performed using Agilent eArray. Bioinformatic analysis was performed by a pipeline designed and validated in-house. Fragment coverage was analyzed. Variant pathogenicity was graded according to its presence in a previously associated or candidate gene, the *in silico* predicted impact on the protein using widely used software (Polyphen, SIFT, MutationTaster), the degree of conservation of the affected residue measured by multiple ortholog alignment using Alamut software (version 2.4.5; Interactive Biosoftware, Rouen, France) and its presence in public databases of the general population such as dbSNP, Exome Aggregation

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Consortium (ExAC),[4] or the NHLBI GO Exome Sequencing Project database. Potentially pathogenic variants were confirmed using Sanger sequencing.

Mutagenesis and Cell Transfection

The p.S1961N substitution in Cav1.2 (NP_000710.5) was introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene, United States) and confirmed by direct DNA sequencing.[1-3,5,6] Chinese ovary hamster cells were transiently transfected with the cDNA encoding native (WT) or mutated Cav1.2 channels and the ancillary subunits (α2δ and β) (1:1.7:4 ratio), together with the cDNA encoding the CD8 antigen (0.5 µg) by using FUGENE XtremeGene (Roche Diagnostics, Switzerland).[5] In another group of experiments, cells were cotransfected (0.5:0.5 ratio) with the cDNA encoding WT and p.S1961N Cav1.2 channels (together with the genes encoding the α2δ and β subunits) to simulate the heterozygous condition of the patients. After 48 hours, cells were incubated with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450; Dynal, Norway). Most of the cells that were beaded also had channel expression. The cells were removed from the dish with a cell scraper and the cell suspension was stored at room temperature (21ºC - 23ºC) and used within 12 hours for electrophysiological experiments.[1-3,5,6]

Patch-clamping

Currents were recorded at room temperature using the whole-cell patch-clamp configuration following previously described methods.[1-3,5-7] Recording pipettes were pulled from 1.0 mm o.d. borosilicate capillary tubes (GD1, Narishige Co, Ltd, Japan) using a programmable patch micropipette puller (Model P-2000 Brown-Flaming, Sutter Instruments Co, United States) and were heat-polished with a microforge (Model MF-830, Narishige). Micropipette resistance was kept below 3.5 MΩ when filled with the internal solution and immersed in the external solution. In all experiments, series resistance was compensated manually by using the series resistance compensation unit of the Axopatch-200B amplifier, and usually ≥ 80% compensation was achieved. Uncompensated access

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resistance and cell capacitance of Chinese ovary hamster cells were 1.8 ± 0.4 MΩ and 16.2 ± 1.2 pF (n = 24), respectively. Therefore, considering the peak L-type Ca current (I_{CaL}) amplitude generated by WT Cav1.2 channels (-521 ± 60 pA, n = 15), no significant voltage errors (< 5 mV) due to series resistance were expected with the micropipettes used. Currents were filtered at half the sampling frequency and stored on the hard disk of a computer for subsequent analysis by using pCLAMP software (Molecular Devices, United States).

To record the I_{CaL}, cells were perfused with an external solution containing (mM): N-metil-D-glucamine 130, HEPES 10, KCl 5, $MgCl_2$ 1, $CaCl_2$ 15 (pH 7.35 with HCl). In some experiments, Ba was used as charge carrier to record Ba currents (I_{Ba}) and, therefore, an equimolar substitution between $BaCl_2$ and $CaCl_2$ was made. The internal solution contained (mM): $CsCl_2$ 120, $MgCl_2$ 2, ATP-Mg 2, HEPES 10, $CaCl_2$ 5, EGTA 10 (pH 7.25 with CsOH).

The protocol to obtain the I_{CaL} current-voltage (I-V) relationships consisted of 500 ms pulses in 10 mV increments from -80 mV to potentials between -50 and +70 mV.

The charge crossing the membrane estimated from the integral of the current traces was also measured and was normalized to the cell capacitance. Activation curves were constructed plotting the normalized conductance (G) as a function of the membrane potential. The conductance was estimated by the equation (#1):

$G = I/(V_m - E_{rev})$

where G is the conductance at the test potential V_m, I represents the current amplitude at V_m, and E_{rev} is the reversal potential. To determine the E_{rev}, the current density-voltage relationships were fitted to a function of the form (#2):

$I = (V_m - E_{rev}) * G_{max} * (1+\exp[V_m - V_h]/k)^{-1}$

where I is the current amplitude elicited at the test potential V_m and G_{max} is the maximum conductance. To construct inactivation/availability curves a protocol consisting of 500 ms pulses in 10 mV increments from -70 mV to potentials between -90 and +50 mV, followed by a test pulse at +20 mV was applied. Current amplitude obtained with the test pulse was normalized to the largest

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current and was plotted as a function of the voltage command of the conditioning pulse. Fitting a Boltzmann function to the activation/conductance-voltage and inactivation curves yielded the midpoint (V_h) and the slope (k) of the curves. Inactivation time constants (τ) were obtained by fitting a monoexponential (for I_{Ba}) or biexponential (for I_{CaL}) functions to the decay of current traces recorded at +20 mV. On each experiment, current amplitude was normalized to membrane capacitance to obtain current density.

Mathematical Modelling of Ventricular Action Potential

To simulate the shapes of ventricular action potential, we employed the O'Hara-Rudy model of a human ventricular action potential (AP) previously validated and used for similar purposes.[8,9] Simulated APs were implemented with MATLAB R2016a (Mathworks Inc, Natick, MA, United States) using the ode15s integration algorithm. In each simulation the model was paced to steady state (1000 beats) and under these conditions, it was stable and reproduced the results obtained by O'Hara et al. in the original description of the model.[8] The model was run at different driving frequencies (0.1, 1, 2, and 3 Hz) under baseline conditions (WT) or by incorporating the specific changes in I_{NaL} and I_{CaL} produced by p.R1644H Nav1.5 and p.S1961N Cav1.2 mutations, respectively, alone or when both modifications were incorporated simultaneously in the model. The APD at 20% (APD_{20}), 50% (APD_{50}) and 90% (APD_{90}) of repolarization were measured on each group.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Paired or unpaired *t* test or 1-way ANOVA followed by the Newman-Keuls test were used to assess statistical significance where appropriate. To take into account repeated sample assessments, data were analyzed with multilevel mixed-effects models. A value of $P < .05$ was considered statistically significant.

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Table of the supplementary material

Summary of all Nonsynonymous Exonic Variants Identified in the Patient

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Gene	Genotype	Ancestral allele	Variant	dbSNP_ID	MAF	Aminoacid substitution	Transcript	Provean prediction	SIFT prediction
AKAP9	Heterozygous	G	T	rs6964587	0.3829	M463I	NM_005751.4	Neutral	Tolerated
AKAP9	Heterozygous	A	AAAC	rs10644111	0.3989	Insertion of Q at residue 1335	NM_005751.4	Neutral	Unknown
AKAP9	Heterozygous	A	G	rs6960867	0.3593	N2792S	NM_005751.4	Neutral	Tolerated
AKAP9	Homozygous	C	T	rs1063242	0.9960	P2979S	NM_005751.4	Neutral	Tolerated
CACNA1C	Heterozygous	G	A			S1961N	NM_000719.6	Unknown	Unknown
KCNE1	Heterozygous	T	C	rs1805127	0.6477	S38G	NM_000219.3	Neutral	Tolerated
KCNH2	Heterozygous	T	G	rs1805123	0.1871	K897T	NM_000238.3	Neutral	Tolerated
SCN5A	Heterozygous	C	T	rs28937316		R1644H	NM_198056.2	**Deleterious**	**Damaging**

Only nonsynonymous variants with a quality index (as provided by SAMtools) > 50 were included.

MAF, minor allele frequency as provided in ExAC Browser Beta.[4]