Pacemaker channel dysfunction in a patient with sinus node disease

Eric Schulze-Bahr,1,2 Axel Neu,3 Patrick Friederich,4 U. Benjamin Kaupp,5 Günter Breithardt,1,2 Olaf Pongs,3 and Dirk Isbrandt3

1Genetics of Arrhythmias, Molecular Cardiology Section, Institute for Arteriosclerosis Research, University of Münster, Münster, Germany; 2Department of Cardiology and Angiology, Hospital of the University of Münster, Münster, Germany; 3Institute for Neural Signal Transduction, Center for Neurobiology Hamburg (ZMNH), University of Hamburg, Hamburg, Germany; 4Department of Anesthesiology, University Hospital Hamburg, Hamburg, Germany; 5Institute for Biological Information Processing, Research Center Jülich, Jülich, Germany

The cardiac pacemaker current If is a major determinant of diastolic depolarization in sinus nodal cells and has a key role in heartbeat generation. Therefore, we hypothesized that some forms of “idiopathic” sinus node dysfunction (SND) are related to inherited dysfunctions of cardiac pacemaker ion channels. In a candidate gene approach, a heterozygous 1-bp deletion (1631delC) in exon 5 of the human HCN4 gene was detected in a patient with idiopathic SND. The mutant HCN4 protein (HCN4-573X) had a truncated C-terminus and lacked the cyclic nucleotide–binding domain. COS-7 cells transiently transfected with HCN4-573X cDNA indicated normal intracellular trafficking and membrane integration of HCN4-573X subunits. Patch-clamp experiments showed that HCN4-573X channels mediated If-like currents that were insensitive to increased cellular cAMP levels. Coexpression experiments showed a dominant-negative effect of HCN4-573X subunits on wild-type subunits. These data indicate that the cardiac If channels are functionally expressed but with altered biophysical properties. Taken together, the clinical, genetic, and in vitro data provide a likely explanation for the patient’s sinus bradycardia and the chronotropic incompetence.

Address correspondence to: Eric Schulze-Bahr, AG Genetics of Arrhythmias, Molekular-Kardiologie, Institut für Arteriosklerosforschung an der Westfälischen Wilhelms-Universität Münster, Domagkstrasse 3, D-48149 Münster, Germany. Phone: 49-251-83-52982; Fax: 49-251-83-52980; E-mail: heart@uni-muenster.de.

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: sinus node dysfunction (SND); sinoatrial node (SAN); cyclic nucleotide–binding domain (cNBD); hyperpolarization-activated cyclic nucleotide-gated channels (HCN); beats per minute (bpm); dual-chamber (DDD); electrocardiography (ECG); CMV promoter (PCMV); human elongation factor 1α promoter (PEF-1α); enhanced green fluorescent protein (EGFP); internal ribosomal entry site (IRES); midpoint potential (V1/2).
heart rate. Apparently, cAMP sensitivity of pacemaker channels represents a key element in the response of heart rate to sympathetically stimulation (13, 14).

Hyperpolarization-activated cyclic nucleotide-gated (HCN) subunits are the molecular components of \( I_f \) channels. Two of the four known genes encoding HCN channel subunits (15–19), HCN2 and HCN4, are predominantly expressed in the heart (20). Their in vitro properties closely resemble those of native \( I_f \) channels (15–18, 21–23). Therefore, HCN genes have been suggested as candidates underlying disorders of heartbeat generation (13, 17).

In a previous study, we excluded the presence of HCN2 mutations in a series of ten patients with SND (24). Here, we report on the investigation of the HCN4 gene in the same population with idiopathic SND. In one index patient, a heterozygous 1-bp deletion mutation was detected that gave rise to a C-terminally truncated HCN4 protein (HCN4-573X) lacking the C-terminal cNBD domain. In vitro–expressed HCN4-573X channels mediated \( I_f \)-like currents comparable to those of wild-type HCN4 but were unable to respond to increases in intracellular cAMP levels. In coexpression experiments with wild-type subunits, current properties of HCN4-573X subunits were predominant. It is likely that altered \( I_f \) channel properties in the index patient are associated with the observed clinical SND phenotype. Our investigation supports the hypothesis that idiopathic SND is in part genetically determined and may be linked to mutations in a cardiac pacemaker channel gene.

**Methods**

**Clinical records**

A 66-year-old woman was admitted to a community hospital with a fractured nasal bone after a severe syncope. On admission, the patient had marked sinus bradycardia (41 beats per minute [bpm]) (Figure 1a) and intermittent episodes of atrial fibrillation (Figure 1b). The plasma electrolytes were normal and the patient was not taking any medication. The patient’s physician reported that bradyarrhythmia and intermittent atrial fibrillation had been known and tolerated for over 20 years. The results of a complete invasive cardiological examination (right and left ventriculography, coronary angiography, and programmed ventricular stimulation) were normal. In the absence of detectable heart disease, the diagnosis of idiopathic SND was made and a dual-chamber (DDD) pacemaker was implanted. During routine pacemaker follow-up, the patient’s physical performance improved markedly, and there was no recurrence of syncope. An evaluation of the patient’s P-waves (as obtained from the P-wave histogram during pacemaker interrogation) showed that the majority (>98%) of the atrial rates were below 60 bpm within the first 68 days after pacemaker implantation (Figure 1c). Episodes of fast atrial rates (i.e., atrial tachyarrhythmias), either isolated or together with a slow ventricular rate response (suggesting the presence of high-grade atrioventricular conduction block), were not observed during pacemaker interrogation. During exercise electrocardiography (ECG), the patient had sinus rhythm, and the maximal heart rate was 101 bpm at a maximal work load of 150 W. This was markedly below the predicted maximal heart rate when adapted for age and gender (150–184 bpm) (Figure 1d) (25).

The family history of the female index patient was unremarkable; three of her four children (between 32 and 39 years of age) that were available for clinical and genetic investigations had normal 12-lead ECGs with sinus rhythms (65, 75, and 65 bpm, respectively) and exercise ECGs with regular chronotropic responses.

In addition to this family, nine other unrelated index families with idiopathic SND were available for genetic investigations; three had familial sinus bradycardia (without atrial fibrillation), two had “lone atrial fibrillation,” three had “sporadic” SND, and one previously reported large family had autosomal dominant sinoatrial disease (24).

**Genomic structure, chromosomal localization, and mutation detection of the HCN4 gene**

A BLAST search of the National Center for Biotechnology Information GenBank high-throughput genomic sequences database using the HCN4 cDNA (GenBank accession number AJ238850) as a probe identified a
sequence harboring HCN4 gene exons 1–8 (Figure 2; accession number AC009660). The Genebridge 4 whole-genome radiation hybrid panel (Invitrogen, Karlsruhe, Germany) was screened by PCR using HCN4-specific primers. PCR reactions were performed with 25 ng of DNA for all 93 hybrid clones and with human and hamster genomic DNA controls. Two independent PCR reactions amplifying genomic fragments of exon 1/intron 1 or exon 8, respectively, were used to analyze the chromosomal localization of the HCN4 gene. The primers were derived from genomic sequences of the HCN4 gene identified as described above. Conditions were as follows: for PCR A (primer AAAGCCGTGGAGCGCGAACAGGAG [sense] and primer CCCAGCGCAAGGCAGGAAAGTTA [antisense]), 5 minutes at 95°C (1 cycle) and 30 seconds at 95°C, 30 seconds at 65°C, and 45 seconds at 72°C (30 cycles) in buffer containing 5% DMSO and Taq polymerase (Invitrogen); and for PCR B (primer TGCCACCCCTCTGTGTTTG [sense] and primer CCTCCCTCCCCTCTCTTCTA [antisense]), 5 minutes at 95°C (1 cycle) and 30 seconds at 95°C, 30 seconds at 62°C, and 30 seconds at 72°C (30 cycles) in buffer containing 5% DMSO and Taq polymerase (Invitrogen). PCR products were separated by agarose gel electrophoresis. PCR results were submitted to the Radiation Hybrid Mapping Server at the Whitehead Institute (http://www-genome.wi.mit.edu/cgi-bin/cgi-bin/rhmapper). A cutoff of 15 was chosen for the LOD score.

For mutation screening, intronic primers were developed (Table 1) that encompassed the complete coding sequence, the splice sites, and the adjacent lariat sites. Because of the large size or a high CG content, exons 1 and 8 were subdivided into 2 and 5 amplicons, respectively. Genomic DNA was extracted from peripheral lymphocytes of the patients, family members, and control individuals using standard techniques (26). A total of 181 unrelated and healthy individuals (26) served as controls for verification of sequence variations from the published HCN4 cDNA sequence. Sequencing of PCR products and separation of sequencing products were performed using the BigDye RR Terminator AmpliTaq-Kit version 3.0 and the ABI3700 system (Perkin-Elmer Applied-Biosystems, Weiterstadt, Germany).

**Table 1**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>GCGGCGCGGCTGCTCGGCC</td>
<td>CGCTCGGGCTGCGGCCAG</td>
</tr>
<tr>
<td>1-2</td>
<td>CGGCAGCATGACGGAGCAGACCTGCT</td>
<td>GAAATCTAAGGCGGCAGGAGGC</td>
</tr>
<tr>
<td>2</td>
<td>TTCTCTCTCTGCAGGACTGATGG</td>
<td>GGAGAAGACCTAGATTGATTTGG</td>
</tr>
<tr>
<td>3</td>
<td>GAGCGGAGCGGAGAGACCAGCAGG</td>
<td>GAGGAGGATGGGGACGAGGAGG</td>
</tr>
<tr>
<td>4</td>
<td>ACCGGGCTCTGGGCTAGTG</td>
<td>GGGGATGTCGAGCAGGAGGG</td>
</tr>
<tr>
<td>5</td>
<td>CTGCTCTGTCCTGGCTAGTG</td>
<td>CCTTCCCTCCCTCTCTCTCTCT</td>
</tr>
<tr>
<td>6</td>
<td>CTGCCTCCTATTGCTAGTG</td>
<td>CTGCCTCCTATTGCTAGTG</td>
</tr>
<tr>
<td>7</td>
<td>GGCTGGGCTAGTG</td>
<td>GGGGATGTCGAGCAGGAGGG</td>
</tr>
<tr>
<td>8-1</td>
<td>TCCGGGCTCTGGGCTAGTG</td>
<td>GGGGATGTCGAGCAGGAGGG</td>
</tr>
<tr>
<td>8-2</td>
<td>ACTTCTCTCTATGATGCTACAG</td>
<td>TGGCAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>8-3</td>
<td>TCCGCAGCATCAGAATGCTGCTAG</td>
<td>TGCCAAGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>8-4</td>
<td>CCAGGAGGGCTGCTAGTGCTG</td>
<td>GGGGATGTCGAGCAGGAGGG</td>
</tr>
<tr>
<td>8-5</td>
<td>GGGGCTCTGGGCTAGTG</td>
<td>GGGGATGTCGAGCAGGAGGG</td>
</tr>
</tbody>
</table>

**Figure 2**

Mutation detection in the HCN4 gene. (a) The human HCN4 gene consists of eight exons. The minimal exon size was 141 bp (exon 5), and the maximal size was 1,465 bp (exon 8); the largest intron was intron 1 (~24 kb) and the smallest intron 5 (102 bp). The functional domains of the wild-type HCN4 channel are delineated below. P, pore region; 1–6, transmembrane domains. (b) Electropherogram after direct sequencing of an index patient with SND. A heterozygous 1-bp deletion (1631delC) in HCN4 resulted in a superimposing sequence pattern consisting of the wild-type and mutant exon 5 sequence. (c) The heterozygous deletion mutation induces an EcoI restriction site in exon 5; after restriction enzyme analysis, the uncut wild-type fragment (380 bp) and two EcoI fragments (200 and 180 bp, cut mutant fragment) were found in the index patient. Her three healthy children had the wild-type configuration. The left lane shows the size standard in base pairs (pUC19 DNA/MspI). (d) Schematic topology of HCN4 channels with six transmembrane segments (S1–S6) and intracellular N- and C-termini. Because of the reading frame shift in the nucleotide sequence, a resulting premature stop codon deleted the C-terminally located cNBD in HCN4-573X that is replaced by 29 novel amino acids (thick gray line). The relative sizes of the transmembrane segments and N- or C-termini are not drawn to scale.
Electropherograms were compared with the HCN4 wild-type sequence (GenBank accession number AJ238850) using the SeqMan program 5.0 (DNASTAR Inc., Madison, Wisconsin, USA).

Generation of wild-type and mutant HCN4 expression constructs

Immunocytochemistry. Full-length wild-type HCN4 cDNA was cloned into the vector pcDNA1Amp (Invitrogen), and the identified mutation was introduced by site-directed mutagenesis. Subsequently, N-terminal epitope sequences (wild-type, HA epitope; mutant, myc epitope) were added to the cDNAs. The pcDNA1Amp-HCN4 vector (1 µg) was used for transient expression of either tagged wild-type or mutant channels or cotransfection of both constructs (Figure 3) in COS-7 cells (American Type Culture Collection, Rockville, Maryland, USA).

Electrophysiological experiments. For transient expression and electrophysiological characterization of either wild-type or mutant channels in COS-7 cells (Figures 4 and 5), full-length wild-type or mutant HCN4 cDNA was cloned into the first polylinker of the expression vector pBud-CE4.1 (Invitrogen). Expression was therefore driven by the CMV promoter (PCMV). In order to enable selection of transfected cells, a cDNA encoding EGFP (enhanced green fluorescent protein; Clontech, Palo Alto, California, USA) was inserted into the second polylinker of pBud-CE4.1 that already contained untagged full-length (wild-type or mutant) HCN4 cDNA in the first polylinker. For coexpression experiments (Figure 6), another expression plasmid (pCo-HCN4) was constructed that contained the mutant HCN4-573X cDNA in the first polylinker of pBud-CE4.1 (under control of PCMV) and the wild-type HCN4 cDNA in the second polylinker (under control of human elongation factor 1α promoter (PEF-1α); Figure 6a). In addition, an internal ribosomal entry site (IRES) EGFP element was cloned 3′ to wild-type HCN4 to allow identification of transfected cells. Hence, EGFP expression was coupled to the expression of wild-type HCN4, since both open reading frames were located on one transcript.

RT-PCR. The relative amounts of wild-type and mutant HCN4 transcripts in coexpression experiments (Figure 6b) were determined as follows: total RNA from 10⁶ cells transiently transfected with either pCo-HCN4, 1540

The Journal of Clinical Investigation | May 2003 | Volume 111 | Number 10

Table 2
Exon/intron boundaries of the HCN4 gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>cDNA nucleotides</th>
<th>Amino acid</th>
<th>Splice donor site</th>
<th>Splice acceptor site</th>
<th>Intron size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1–785</td>
<td>1–262</td>
<td>...cccccgcccaTGGGACAAAC...</td>
<td>...GTGACCTCACAGgagggcc...</td>
<td>23.7 kb</td>
</tr>
<tr>
<td>2</td>
<td>786–1209</td>
<td>263–403</td>
<td>...cctccgga-ATTTTTACTGG...</td>
<td>...GTGGGAAGAGgtagggct...</td>
<td>11.5 kb</td>
</tr>
<tr>
<td>3</td>
<td>1210–1371</td>
<td>404–457</td>
<td>...ctctggcagATCTTCCAACA...</td>
<td>...CCAGAAAGgtgatagc...</td>
<td>2.3 kb</td>
</tr>
<tr>
<td>4</td>
<td>1372–1590</td>
<td>458–530</td>
<td>...ctctccgaaAAACACTCTCT...</td>
<td>...CCAGGAGAAGgtgatagc...</td>
<td>4.3 kb</td>
</tr>
<tr>
<td>5</td>
<td>1591–1732</td>
<td>531–579</td>
<td>...ccggccccTACAAGGAGG...</td>
<td>...CCCTGGGGAGGgtgagggc...</td>
<td>102 bp</td>
</tr>
<tr>
<td>6</td>
<td>1733–1978</td>
<td>580–659</td>
<td>...tgccctggcAGAGATCATCA...</td>
<td>...TACCTTGGAGgtgagggc...</td>
<td>701 bp</td>
</tr>
<tr>
<td>7</td>
<td>1979–2143</td>
<td>660–714</td>
<td>...gcctccccAGATCTGCTCT...</td>
<td>...GACCAGGAGGgtgatagc...</td>
<td>139 bp</td>
</tr>
<tr>
<td>8</td>
<td>2144–3609</td>
<td>715–1203</td>
<td>...gctccccgGCAAGAAGAAG...</td>
<td>...CAATCTATAGgtgatagc...</td>
<td>3′-UTR</td>
</tr>
</tbody>
</table>

The gene contains eight exons; the cDNA is composed of 3,609 nucleotides resulting in 1,203 amino acids. The intronic splice sites adhere to the “AG-GT” rule; these nucleotides are underlined. Coding nucleotides are marked in capital letters. UTR, untranslated region.

Figure 3
Immunostaining of HCN4 and HCN4-573X in COS-7 cells. Wild-type and HCN4-573X channels were transiently expressed in COS-7 cells. For detection, wild-type HCN4 was HA-tagged and HCN4-573X was myc-tagged. Both channels showed comparable immunostains (a and b) with immunoreactivity detectable in perinuclear regions and at the plasma membrane. When coexpressed (c–f), the immunostaining patterns did not change and completely overlapped, and they were particularly concentrated in the cell membrane (f).
pcDNA1Amp-HCN4, or pcDNA1Amp-HCN4-573X was isolated (Purescript RNA isolation Kit, Biozym, Oldendorf, Germany) and reverse-transcribed (SuperScript II, Invitrogen). HCN4 cDNA fragments were amplified by PCR using 1 µl of reverse transcription reaction (forward primer, 5′-GGCATGTCCGACGTCTGGCTCAC-3′; reverse primer, 5′-TCACGAAGTTGGGGTGCACTATTGG-3′). To differentiate between wild-type and mutant PCR fragments, we digested the fragments with EciI (MBI Fermentas, St. Leon-Roth, Germany) (see Figure 2c) and separated them by agarose gel electrophoresis. A CCD camera-acquired image (Biometra, Göttingen, Germany) of the ethidium bromide–stained gel was analyzed using AIDA image analysis software (Raytest, Sprockhövel, Germany).

**Cell culture, transfection, and immunostaining**

For immunocytochemical localization, COS-7 cells were transiently transfected with wild-type and/or mutant cDNAs. COS-7 cells were seeded on coverslips in 35-mm dishes 1 day before transfection, which was performed using 3.5 µl of lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol and 1.5 µg of the respective plasmid DNA. Two days after transfection, cells were fixed on coverslips with 2% paraformaldehyde. After a blocking incubation (1 hour at 37 °C with 3% goat serum and 5% bovine serum albumin [Sigma-Aldrich, Deisenhofen, Germany] in phosphate-buffered saline), immunostaining was performed with the affinity-purified anti-myc monoclonal antibody (dilution, 1:100; Roche Diagnostics, Mannheim, Germany) and the polyclonal anti-HA tag antibody (dilution, 1:500; Babco, Richmond, California, USA). Alexa546-labeled anti-rabbit IgG or Alexa488-labeled anti-mouse IgG (dilution, 1:2000; Molecular Probes, Eugene, Oregon, USA) served as secondary immunofluorescent reagents. Confocal images were obtained in sequential scan mode using a Leica TCS NT laser scanning microscope (Leica, Bensheim, Germany).

**Electrophysiological recordings**

Transfection of COS-7 cells for electrophysiological analyses was performed as described above but without coverslips. Two days after transfection, cells were divided in order to yield a density of 10^4 cells in a 35-mm dish. These cells were analyzed the following day. Currents were recorded at room temperature (20–22 °C) in the whole-cell patch-clamp configuration using an EPC9 patch-clamp amplifier and Pulse+Pulsefit software (HEKA Elektronik, Lambrecht, Germany). The extracellular solution contained (in mM) 135 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 5 HEPES, 10 glucose, and 20 sucrose (pH 7.4 with NaOH). The intracellular solution contained (in mM) 120 K-gluconate, 20 KCl, 10 HEPES, 10 EGTA, 2 MgCl2 (pH 7.3 with KOH), and 1 mM 8-Br-cAMP where indicated. Series resistances ranged from 4–7 MΩ and were electronically compensated (60–80%). Cells were clamped at –20 mV and hyperpolarized to potentials from –40 mV to –120 mV for 5 seconds.

**Figure 4**

Functional characterization of homomeric HCN4 and HCN4-573X channels in COS-7 cells under control conditions. Representative current traces of wild-type (a) or HCN4-573X (c) channels elicited by 5-second-long hyperpolarizing voltage steps from –40 mV to –120 mV under control conditions. Relative open probability of wild-type (b) and HCN4-573X (d) channels was dependent on test-pulse duration (gray circles, 2 seconds; black circles, 5 seconds; white circles, 15 seconds). Absence of error bars indicates errors smaller than the symbol size.

**Figure 5**

Functional characterization of homomeric wild-type and HCN4-573X channels in COS-7 cells in the presence of 8-Br-cAMP. Shown are representative current traces of wild-type (a) or HCN4-573X (c) channels elicited by 5-second-long hyperpolarizing voltage steps from –40 mV to –120 mV in the presence of 8-Br-cAMP. Voltage dependence of wild-type (b) and HCN4-573X (d) conductances in the absence (black circles) or presence (white circles) of 1 mM 8-Br-cAMP. Absence of error bars indicates errors smaller than the symbol size.
In addition, 2-second test pulses up to –140 mV and 15-second test pulses up to –110 mV were applied. The voltage dependence of activation was analyzed by fitting the Boltzmann function \( \frac{I - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}} = \frac{1}{1 - \exp\left\{\frac{V - V_{1/2}}{\text{slope factor}}\right\}} \) to tail currents at 20 mV or 30 mV. Time constants of activation were determined by fitting single exponentials to current traces of 15-second duration. All data are given as means ± SEM.

Results

Genomic structure and chromosomal localization of the \( \text{HCN4} \) gene

The genomic structure of the \( \text{HCN4} \) gene was defined on the basis of several genomic contigs identified by a database search of the NCBI GenBank htgs database. Eight exons, comparable to the structure of the \( \text{HCN2} \) gene (17), were identified (Table 2 and Figure 2a). In agreement with the chromosomal in situ hybridization (18), the \( \text{HCN4} \) gene was localized to chromosome 15q23–q24.1 near the microsatellite D15S215 (data not shown).

Mutation detection in the \( \text{HCN4} \) gene

We sequenced the \( \text{HCN4} \) genes of 10 unrelated index patients. For one index patient, we detected a heterozygous single base-pair deletion (1631delC) in exon 5 of the \( \text{HCN4} \) gene (Figure 2b). The deletion gave rise to a new \( \text{EciI} \) restriction site that was also used for mutation detection (Figure 2c). Restriction digests and sequencing of the DNA from the three available healthy children of this index patient did not show the mutation. Screening of genomic DNA from a sample of the general population (362 chromosomes) failed to detect the mutation, making the presence of a polymorphism unlikely.

The 1631delC mutation in exon 5 caused a reading frame shift that gives rise to a shortened open reading frame (P544fsX30) and thereby eliminated the \( \text{HCN4} \) cNBD. The mutant protein is predicted to be only 572 amino acids long (HCN4-573X) and ends with an \( \text{HCN4} \)-unrelated sequence of 29 amino acids (residues 544–572) (Figure 2d).

Immunostaining and intracellular trafficking of wild-type and mutant \( \text{HCN4} \) subunits

To investigate the subcellular localization and intracellular trafficking of \( \text{HCN4-573X} \), COS-7 cells were transiently transfected with tagged \( \text{HCN4} \) constructs. Subsequent confocal microscopy showed similar immunostaining patterns of comparable intensities with antibodies directed against tagged \( \text{HCN4} \) or \( \text{HCN4-573X} \) proteins. Prominent immunoreactivity was detected in perinuclear regions and at the plasma membrane.
membrane of COS-7 cells (Figure 3, a and b). The HCN4 and HCN4-573X immunostaining patterns completely overlapped when wild-type and mutant HCN4 subunits were coexpressed (Figure 3, c to e). The observed overlapping immunoreactivity in the cell membranes (Figure 3f) suggested that the subunits were colocalized. Thus, the immunohistochemical results indicated that intracellular trafficking and processing of HCN4 and HCN4-573X proteins were similar.

Functional expression of wild-type and mutant HCN4 channels

The whole-cell configuration of the patch-clamp technique was used to functionally characterize HCN4-573X channels transiently expressed in COS-7 cells. Upon hyperpolarizing steps in membrane potential (−40 mV to −120 mV for 5 seconds), HCN4-573X channels mediated, like wild-type HCN4 channels, hyperpolarization-activated currents with typical I features (17, 18) (Figure 4). Activation kinetics of HCN4 and HCN4-573X currents did not differ significantly and were reasonably well fit with a single exponential function ($\tau_{1/2}$ = 2377 ± 546 ms [n = 11] versus 2028 ± 433 ms [n = 12]) (Table 3). In agreement with a previous report (17), HCN4 channels did not reach steady state during the 5-second activation protocol (Figure 4a). However, the relative open probabilities of HCN4 and HCN4-573X channels taken from instantaneous outward tail currents were reliably described by Boltzmann functions. The midpoint potentials ($V_{1/2}$) of HCN4 and HCN4-573X channels were very similar (HCN4, −89.6 ± 1.3 mV [n = 11]; HCN4-573X, −88.7 ± 1.2 mV [n = 11]; $P = 0.61$) for the 5-second hyperpolarization protocol (Figure 4 and Table 3). Because of the slow activation kinetics of HCN channels, $V_{1/2}$ strongly depended on the test-pulse duration. Two-second test pulses yielded $V_{1/2}$ values of −107.0 ± 2.0 mV [n = 7] for HCN4 versus −110.2 ± 1.2 mV [n = 10] for HCN4-573X ($P = 0.21$). Test pulses of 15 seconds resulted in $V_{1/2}$ values of −70.2 ± 2.2 mV [n = 7] for HCN4 versus −76.0 ± 0.8 mV [n = 8] for HCN4-573X ($P = 0.043$) (Figure 4). Thus, for long activation protocols, the midpoint of activation of HCN4-573X channels was approximately 6 mV more negative than that of wild-type channels.

The deactivation kinetics of HCN4 channels displayed a sigmoidal time course. In contrast, the deactivation kinetics of HCN4-573X channels were exponential and therefore more closely resembled those of HCN1 and HCN2 than of HCN4 channels.

A hallmark of HCN channels is their regulation by cyclic nucleotides. Since the HCN4-573X channels lacked the C-terminal cNBD, we investigated the effect of increased cAMP levels on HCN4-mediated currents. We used the nonhydrolyzable cAMP analog 8-Br-cAMP to assess the sensitivity of HCN4 channels to intracellular cAMP levels and compared cAMP-treated cells with cells under control conditions.

| Table 3 |

Electrophysiological characteristics of wild-type, mutant, and coexpressed HCN4 channels

<table>
<thead>
<tr>
<th></th>
<th>HCN4</th>
<th>HCN4-573X</th>
<th>HCN4/HCN4-573X</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>(C)</td>
</tr>
<tr>
<td>$V_{1/2}$ (mV)</td>
<td>−89.6 ± 1.3</td>
<td>−88.7 ± 1.2</td>
<td>−85.0 ± 1.3</td>
</tr>
<tr>
<td>Slope factor (mV)</td>
<td>9.1 ± 0.3</td>
<td>7.2 ± 0.4</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td>$\tau_{1/2} (ms)$</td>
<td>2377 ± 546</td>
<td>2028 ± 433</td>
<td>2032 ± 266</td>
</tr>
<tr>
<td>$V_{1/2}$ plus cAMP (mV)</td>
<td>−74.2 ± 1.2</td>
<td>−85.7 ± 1.9</td>
<td>−85.5 ± 2.5</td>
</tr>
<tr>
<td>Slope factor plus cAMP (mV)</td>
<td>10.3 ± 0.2</td>
<td>7.6 ± 0.2</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>$\tau_{1/2}$ (ms) plus cAMP</td>
<td>1483 ± 216</td>
<td>2676 ± 413</td>
<td>1806 ± 172</td>
</tr>
</tbody>
</table>

Values are given as means ± SEM. $V_{1/2}$ values shown were obtained from 5-second test pulses. For statistical analysis, a heteroscedastic two-tailed Student’s t test was applied. *$P < 0.001$. **$P = 0.206$. ***$P = 0.879$. 

The index patient’s $V_{1/2}$ values were −70.2 ± 8.0 mV (n = 7) for HCN4 versus −76.0 ± 0.8 mV (n = 8) for HCN4-573X ($P = 0.043$) (Figure 4). Thus, long activation protocols, the midpoint of activation of HCN4-573X channels was approximately 6 mV more negative than that of wild-type channels.
We previously investigated the embryonic and adult stages (29, 30). In humans, a contribution of If channels in the pacemaking activity of the SAN has been discussed controversially (31, 32). It is likely that slow and/or fast hyperpolarization-activated pacemaker currents represent key determinants of spontaneous diastolic depolarization in the SAN. The fast component of If is most likely HCN2-dependent and the slow component HCN4-dependent (17, 18). We previously investigated the HCN2 gene as a candidate for SND. However, we did not find HCN2 gene mutations in a study population with idiopathic SND (24). The present finding of a mutation in the HCN4 gene of one index patient suggests that a genetically determined alteration of cardiac pacemaker channel activity causes SND.

The identified mutation eliminates the functionally important cNBBD in the derived HCN4 subunit sequence because of a truncation of the C-terminus. Two recent reports showed a role for the C-terminus of HCN1 and HCN2 channels in the control of voltage and cAMP-mediated channel gating (33, 34). The results are compatible with our findings in electrophysiological recordings on transfected COS-7 cells, in which C-terminally truncated HCN4 channels showed regular intracellular trafficking and mediated If-like currents but were unresponsive to increases in the intracellular cAMP concentration. Since the patient’s HCN4 mutation was found in a heterozygous state, we also coexpressed wild-type and mutant HCN4 subunits. Like homomeric HCN4-573X channels, coexpressed HCN4-573X/HCN4 channels did not respond to a rise in intracellular cAMP with a shift in their current-voltage relationship. In addition, the exponential time course of channel deactivation resembled that of HCN4-573X channels. Taken together, these data suggested a dominant-negative effect of mutant HCN4 on wild-type channel function. Despite intensive clinical examination of the index patient, there was no obvious cause for SND, nor was there any other disease-associated condition. Thus, the patient with the HCN4 gene mutation was classified as having idiopathic disease. Since the patient’s family history revealed no further information regarding SND, we were not able to follow the genetic transmission of the mutation to other family members and thus cannot provide further genetic evidence that the mutation is disease causing. The index patient’s phenotype was characterized by a remarkably low heart rate resulting from sinus bradycardia (Figure 1a), intermittent episodes of atrial fibrillation (Figure 1b), and chronotropic incompetence during exercise ECG even under maximum work load (Figure 1d). After pacemaker implantation, retrospective evaluation of the atrial rates over a period of several months was possible. In the P-wave histograms obtained after pacemaker interrogation, consistently low atrial rates were noted (Figure 1c).

Our in vitro expression data strongly support the notion that the patient suffered from SND due to the expression of mutant HCN4 channels, which are insensitive to intracellular cAMP and have altered activation and deactivation properties. Chronotropic incompetence is likely to occur when If channels cannot respond to increases in intracellular cAMP concentrations during β-adrenergic stimulation. The sinus bradycardia observed under resting conditions, on the other hand, might be explained by the altered deactivation kinetics of HCN4-573X channels; because of the extremely slow activation kinetics of HCN4 channels, cumulative current activation during repetitive heart beat cycles, as demonstrated for KCNQ1 channels (35), might occur. Faster closure (deactivation) of If channels during sinus nodal action potentials therefore possibly decreases channel availability in the subsequent cycle, thereby slowing the heart rate. Detailed modeling studies would be necessary to underscore this hypothesis. Considering the broad phenotypic spectrum of arrhythmias associated with familial SND (2, 4–7), it is likely that additional genes and molecular mechanisms for this “idiopathic” disease will be defined.
Note added in proof: Further experimental support for a role of HCN channels in cardiac pacing was recently provided by Fikret et al. (36) who showed that these channels are critical for spontaneous beating of rat neonatal cardiomyocytes.

Acknowledgments

We gratefully acknowledge the excellent technical work of Ellen Schulze-Bahr, Marielles Hesse, Kathrin Sauter, Andrea Zaisser, and Anne Schneider-Darlison. We are indebted to the patients and individuals that participated in the present study, which was in accordance with the ethical standards in the revised version of the Declaration of Helsinki and with ethical recommendations given by the local ethics committee. This work was partially supported by grants from the Innovative Medizinische Forschung of the University of Muenster (IMF; SC 2.1_99_25), from the Deutsche Forschungsgemeinschaft (PF1625/1-1), and from Fondation Leducq (Paris, France).