cAMP Sensitivity of HCN Pacemaker Channels Determines Basal Heart Rate But Is Not Critical for Autonomic Rate Control

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Background—HCN channels activate the pacemaker current If, which is thought to contribute significantly to generation and regulation of heart rhythm. HCN4 represents the dominant isotype in the sinoatrial node and binding of cAMP was suggested to be necessary for autonomic heart rate regulation.

Methods and Results—In a candidate gene approach, a heterozygous insertion of 13 nucleotides in exon 6 of the HCN4 gene leading to a truncated cyclic nucleotide-binding domain was identified in a 45-year-old woman with sinus bradycardia. Biophysical properties determined by whole-cell patch-clamp recording of HEK293 cells demonstrated that mutant subunits (HCN4-695X) were insensitive to cAMP. Heteromeric channels composed of wild-type and mutant subunits failed to respond to cAMP-like homomeric mutant channels, indicating a dominant-negative suppression of cAMP-induced channel activation by mutant subunits. Pedigree analysis identified 7 additional living carriers showing similar clinical phenotypes, that is, sinus node dysfunction with mean resting heart rate of 45.9 ± 4.6 bpm (n=8) compared with 66.5 ± 9.1 bpm of unaffected relatives (n=6; P<0.01). Clinical evaluation revealed no ischemic or structural heart disease in any family member. Importantly, mutant carriers exhibited normal heart rate variance and full ability to accelerate heart rate under physical activity or pharmacological stimulation. Moreover, mutant carriers displayed distinctive sinus arrhythmias and premature beats linked to adrenergic stress.

Conclusions—In humans, cAMP responsiveness of If determines basal heart rate but is not critical for maximum heart rate, heart rate variability, or chronotropic competence. Furthermore, cAMP-activated If may stabilize heart rhythm during chronotropic response. 

Key Words: sinoatrial node • pacemaker • heart rate • ion channels • electrophysiology • HCN channels

The sick sinus syndrome accounts for about half of all cases necessitating pacemaker implantation. Primary sinus node dysfunction importantly contributes to the syndrome and has been related to inherited forms. According to current knowledge, If is considered the most important link to automaticity by determining the slope of diastolic depolarization of sinoatrial nodal cells and is assumed to be the key factor in generation and modulation of heart rhythm. The mammalian genome encodes 4 HCN channels (HCN1-4) that activate If in response to hyperpolarization. Comparative analysis of HCN transcription revealed remarkable differences in their distribution, believed to underlie significant aspects in generation of If current. HCN4 is the dominant HCN isotype in the human adult sinoatrial node (SAN), and HCN4 mutations are associated with inherited sinus node bradycardia. HCN channels are directly regulated by cAMP, which binds to the cyclic nucleotide-binding domain (CNBD) and elicits a positive shift in the voltage dependence of activation, with HCN4 channels showing the highest cAMP sensitivity. Recently, a patient was reported expressing a truncated HCN4 protein insensitive to changes of intracellular cAMP. Clinical features included marked sinus bradycardia and chronotropic incompetence and strongly encouraged the idea that cAMP-mediated modulation of If is the dominant mechanism for heart rate regulation. This view, however, was challenged by Cre-mediated ablation of HCN4 gene activity in adult mouse hearts and by generation of mice expressing HCN4 channels insensitive to cAMP. Adult mice lacking most of If activity showed no bradycardia but developed recurrent sinus pauses. Of note, in both mouse...
models,\textsuperscript{15,16} acceleration of heart rate appeared normal during sympathetic stimulation. Thus, the precise contribution of HCN4 to sinus node function still is unresolved, and in particular the role of HCN4 in human autonomic heart rate response has yet to be established.\textsuperscript{17,18}

In the present study, we examined 14 members of a German family with 8 members carrying a novel HCN4 mutation (HCN4-695X) associated with sinus bradycardia. An insertion of 13 nucleotides in exon 6 of the HCN4 gene generates a frame shift leading to a truncated CNBD. Bio-physical properties of mutant channels expressed in HEK293 cells revealed a complete loss of If modulation by cAMP. Heteromeric channels composed of wild-type and mutant subunits failed to respond to cAMP-like homomeric mutant channels, indicating a dominant-negative suppression of cAMP responsiveness of the If current by mutant HCN4-695X subunits. Patients expressing mutant subunits insensitive to cAMP showed sinus bradycardia and exercise-induced arrhythmias but displayed regular chronotropic competence under both physical activity and pharmacological stimulation.

**Methods**

**Patients and Clinical Investigations**

Our study is based on a 4-generation family of German origin comprising 16 family members, of which 14 have been examined (Figure 1A through 1C). Evaluation included clinical examination, 12-lead ECG, echocardiography, 24-hour Holter recording (13 patients) and treadmill-test (13 patients). Holter recordings were analyzed by computer system (H-Scribe 4.0, Mortara) and confirmed by electrophysiological validation. Heart rate variability was measured (10 patients) by Kleiger standard deviation\textsuperscript{19} of the N-N intervals (normal $>/=100$ ms, low $>/=50$ ms). For Poincaré plots, N-N intervals obtained during 24-hour Holter recording were used. Current N-N interval (x-axis) was plotted against the subsequent N-N +1 interval (y-axis).\textsuperscript{20} Separate plots for daytime (14-hour recording) and nighttime (10-hour recording) were illustrated. Basal heart rates were evaluated by 12-lead ECG with patients at rest 10 minutes before and during measurement. In this way, shortcomings of average heart rates (Holter recording) caused by differences in physical exertion among individuals could be prevented. Four patients were further examined by dobutamine stress-MRI (increasing doses of dobutamine 10/20/30/40 $\mu$g/kg body weight every 3 minutes plus 0.25 mg atropine at the conclusion of the dobutamine challenge).

All patients gave written informed consent for clinical and genetic investigations according to the research protocol, which had been approved by the local ethics committee. The investigation conforms with the principles outlined in the Declaration of Helsinki.

**Mutation Analysis**

HCN4 exons were amplified from genomic DNA according to Schulze-Bahr et al.\textsuperscript{9} The PCR mix contained 25 ng template DNA, 25 pM of each primer, 200 $\mu$mol/L 4-dNTP, 0.7 U of HotStarTaq
Cloning and Mutagenesis of HCN4
Human HCN4 cDNA was amplified from human left ventricular mRNA (Bioca) and cloned into cytomegalovirus promoter directed expression vectors. The mutation was introduced by site-directed mutagenesis (QuickChange II Site-Directed Mutagenesis Kit, Stratagene), and wild-type and mutant sequences were verified using an ABI BioSystems 377 Prism Automated DNA Sequencer (Applied Biosystems).

Cell Culture
Human embryonic kidney (HEK293) cells were cultured in DMEM with 2 mmol/L glutamine, 10% FCS, 100 U/L penicillin-G sodium, and 100 μg/mL streptomycin sulfate in 5% CO2 at 37°C. HEK293 cells grown on glass cover slips (CS) of 12-mm diameter (Assistent Glaswarentafabrik, Hecht KG) were transfected with 0.6 μg plasmid DNA/CS. In coexpression experiments, equal amounts (0.3 μg) of wild-type and mutant plasmid DNA were transfected. Successfully transfected cells were visualized either by coexpressed DsrRed protein or CD8 antigen (0.1 μg DNA/CS) identified by anti-CD8 antibody coated Dynabeads (Invitrogen).

Electrophysiological Recordings
Membrane currents were recorded 1 to 2 days after transfection under voltage-clamp conditions using conventional whole-cell patch-clamp techniques at room temperature (21°C to 23°C) with an Axopatch 200B amplifier (Axon Instruments). Signals were analog-filtered with a low-pass Bessel filter (1-kHz corner frequency). Series resistance was compensated by 70% to 80%. Data were digitized (CED1401 micro MKII, CED), analyzed, and stored on a PC using Signal3 software (CED). Voltage commands were applied through the CED board as described below. Pipettes were pulled from borosilicate glass (inner diameter, 0.86 mm; outer diameter, 1.5 mm) with a Flaming/Brown Puller P-97 (Sutter Instruments). The patch pipettes contained (in mmol/L): KCl 130, NaCl 10, MgCl2 0.5, EGTA 1, HEPES 5, MgATP 2, NaGTP 0.1, and Phosphocreatine 5, pH 7.4 (KOH). The bath solution contained (in mmol/L): KCl 30, NaCl 110, CaCl2 1.8, MgCl2 0.5, and HEPES 5; pH 7.4 (NaOH). Intracellular and extracellular solution osmolarity was adjusted with glucose to 290 and 300 mOsmol/L, respectively.

To evaluate functional properties of HCN4 channels, different voltage protocols were used. For activation characteristics, voltage steps ranging from −50 to −140 mV (holding potential: −40 mV; increment: 10 mV; interval: 30 seconds) were applied. To prevent influence of strong negative potentials on membrane stability and recording conditions, the duration of negative voltage steps was reduced with increasing hyperpolarization from 24 seconds (−50 mV) to 3 seconds (−140 mV). Each voltage step was followed by a final command step to −140 mV, which established an identical driving force for each sweep. The fast current component was used as a direct measure of the steady-state conductance at the preceding command potential. Deactivation properties were assessed using a double pulse protocol. From a holding potential of −40 mV, 2 successive pulses to −120 mV were applied, separated by increasing time intervals in the range of 1 to 7 seconds (increment, 1 second). The reversal potential (Erev) was determined by activating hyperpolarizations (−120 mV; 3 seconds), subsequently followed by depolarizing voltage steps ranging from −120 to +20 mV (increment, 10 mV). Initial current amplitudes after the second command voltage pulse were plotted against the corresponding voltage. Erev was estimated by approximating the intersection with the voltage axis with a third-order polynomial function.

Statistics and Data Analysis
All results are depicted as arithmetic mean ± SEM; a 2-tailed unpaired Student t test followed by a Welch correction to account for unequal variances was used for statistical analysis. Multiple comparisons were performed using 1-way ANOVA; differences were judged significant at a P < 0.05 significance level. Limitations include small sample size and data from a single family. All current traces were leak-subtracted off-line. Data were tested for normality (Kolmogorov-Smirnov test). Time constants were obtained by fitting a single exponential function to individual current traces that reached steady state. Voltage dependence of activation was calculated using a simplified Goldman-Hodgkin-Katz current equation.

Results

Genetic Screening
In the course of our mutation screening program, 416 patients with cardiac arrhythmias were screened for arrhythmia-related gene mutations by analyzing exon sequences of HCN4, KCNQ1, KCNH2, SCN5A, ANK2, KCNE1, and KCNE2 genes by SSCP gel and subsequent DNA sequence analysis. Among others, the novel HCN4-695X mutation was identified (Figure 2). Of note, no HCN4 mutation was detected among 526 healthy individuals.
Presentation of Patients Carrying the HCN4-695X Mutation

The index patient (III-6, 45 years, Figure 1A) was admitted to our clinic because of a marked sinus bradycardia and recurrent ventricular premature beats (VPB) noticed during routine medical examination. Apart from sporadic symptoms of slight dizziness and episodes of palpitations, she reported no discomfort. The resting ECG showed a sinus bradycardia (41 bpm; Figure 3A); QTc intervals were within normal range (Table 1). Holter recording revealed sinus rhythm without pauses >2 seconds and heart rates ranging from 34 bpm at night to 147 bpm during exercise. Numerous VPB and episodes of distinctive sinus arrhythmia were apparent and could be related to the onset of physical activity. Regular left ventricular function (ejection fraction 61%) was confirmed by echocardiography and MRI. Treadmill test up to 200 W showed a regular chronotropic competence. The patient exhibited numerous VPB and ventricular bigeminy throughout heart rate acceleration, followed by stabilization of heart rhythm at maximum rate levels. During dobutamine-stress MRI, normal heart rate response was documented. Contractility increased globally without signs of an ischemic heart disease.

Pedigree analysis (Figure 1A) revealed 7 additional family members carrying the HCN4-695X mutation (Figure 1B). All were aware of remarkably slow heart rates since childhood. Patient III.2 displayed multiple atrial premature beats (APB) and episodes of severe sinus arrhythmia during exercise (Figure 3B and 3C) and under dobutamine stress. Notably, increasing heart rate several times fell off from 80 to 100 bpm to 40 to 60 bpm before rising again, causing nausea and chest discomfort. Syncopy suggestive of rhythmogenic events was absent from all mutant carriers except patient III.4, who had a history of recurrent and severe events with consecutive forehead injury. Because of documented episodes of sinus bradycardia up to 27 bpm, pacemaker implantation was recommended, but the patient refused. Clinical circumstances of syncope provided additional hints toward seizures (ie, prodromi, enuresis, stiffness followed by clonic jerking) and neurological examination confirmed generalized grand mal epilepsy. Treatment with antiepileptic drugs valproat and levetiracetam suppressed further events demonstrating neu-
found to be associated with a variety of clinical phenotypes, including sinus bradycardia, arrhythmias, and cardiac dysfunction.

**Main Clinical Findings**

Common to all mutant carriers is a marked sinus bradycardia. This was observed in 5 of 7 mutant carriers and linked to regular exercise (Figure 3A and 3B). An increased susceptibility to premature beats (APB) and episodes of distinctive sinus arrhythmia were apparent in 4 of 7 mutant carriers, and linked to the onset of adrenergic stress (Figure 3C through 3E). An increased susceptibility to premature beats (APB, VPB) and episodes of distinctive sinus arrhythmia were observed in patients with sinus bradycardia. Clinical evaluation did not show ischemic or structural heart disease in any family member. The rather mild clinical symptoms including sporadic dizziness and palpitations but lacking cardiac syncpe or presyncope allowed for management without pacemaker implantation (for clinical data, see Table 1).

**Clinical Classification of Family Members**

Family members were classified as clinically affected when resting heart rates were <60 bpm and minimum heart rates were <40 bpm. Patients III.2 and II.3 did not fully meet the criteria as minimum heart rates exceeded 40 bpm, but genetic testing identified them as mutant carriers. Hence, they were classified as phenotypically nondistinctive (Table 1).

## Table 1. Clinical Data of Family Members

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age, y</th>
<th>ECG at Rest</th>
<th>Holter Recording</th>
<th>Max. HR</th>
<th>Structural Heart Disease</th>
<th>Other Diagnoses</th>
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<td></td>
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<td>QTC, ms</td>
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<td>Dob Stress</td>
<td>Arrhythmia</td>
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<td>III.1</td>
<td>F</td>
<td>33</td>
<td>51 102 424</td>
<td>42 145 58</td>
<td>157 n/a</td>
<td>n/a</td>
<td>SA, APB</td>
</tr>
<tr>
<td>III.2</td>
<td>F</td>
<td>29</td>
<td>51 85 390</td>
<td>35 145 59 260</td>
<td>172 138</td>
<td>n/a</td>
<td>SA, APB</td>
</tr>
<tr>
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<td>F</td>
<td>21</td>
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<td>n/a</td>
<td>n/a</td>
<td>VPB</td>
</tr>
<tr>
<td>II.2</td>
<td>M</td>
<td>66</td>
<td>38 102 423</td>
<td>35 112 60 n/a</td>
<td>142 n/a</td>
<td>n/a</td>
<td>VPB</td>
</tr>
<tr>
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<td>27 106 47 n/a</td>
<td>166 n/a</td>
<td>n/a</td>
<td>VPB</td>
</tr>
<tr>
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<td>56 149 86 102</td>
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<td>n/a</td>
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<tr>
<td>IV.1</td>
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<td>46 168 77 191</td>
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<tr>
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<td>53 148 76 146</td>
<td>157 n/a</td>
<td>n/a</td>
<td>VPB</td>
</tr>
<tr>
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<td>170 n/a</td>
<td>n/a</td>
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<td>42 181 62 233</td>
<td>179 n/a</td>
<td>n/a</td>
<td>VPB</td>
</tr>
</tbody>
</table>

HR indicates heart rate; Min, minimal; Max, maximal; Avg, average; HRV, heart rate variability; kSD, Kleiger standard deviation; Dob, dobutamine; n/a, not applicable; and bigeminy, ventricular bigeminy.

Values are measured in bpm if not labeled differently.

The clinical symptoms included sporadic dizziness and palpitations, but lacking cardiac syncpe or presyncope allowed for management without pacemaker implantation. For clinical data, see Table 1.

**Clinical Classification of Family Members**

Family members were classified as clinically affected when resting heart rates were <60 bpm and minimum heart rates were <40 bpm. Patients III.2 and II.3 did not fully meet the criteria as minimum heart rates exceeded 40 bpm, but genetic testing identified them as mutant carriers. Hence, they were classified as phenotypically nondistinctive (Table 1). Patient II.3 was aware of a marked bradycardia since childhood and because of Hashimoto thyroiditis recently was treated with levothyroxin, leading to a suppressed thyroid stimulating hormone level (0.15 mU/L) that could explain the relatively high minimum heart rate (44 bpm) compared with other mutant carriers. Moreover, 3 noncarriers (II.4, III.8, and IV.2) displayed slow heart rates (Table 1) linked to regular exercise. Patient II.4 intensely participates in endurance sports, his son (III.8) is a regular runner, and his grandson (IV.2) competitively plays in a youth soccer team. However, all 3 did not meet the criteria to be classified as clinically affected.
Functional Characterization of Homomeric HCN4 and HCN4-695X Channels

Hyperpolarization-activated currents of HCN4 mutant and wild-type-channels were analyzed in transiently transfected HEK 293 cells (Figure 5A).23,26 The voltage for half maximal activation ($V_h$) of mutant HCN4 695X channels ($-80.1 \pm 2.7$ mV; $n=14$) appeared more positive than $V_h$ of wild-type HCN4 channels ($-87.5 \pm 3.3$ mV; $n=15$), although the difference did not reach statistical significance ($P=0.09$), whereas the slopes of the activation curves ($V_c$) apparently were different (see Table 2; Figure 5B,C). In the presence of intracellular cAMP (10 μmol/L), $V_h$ of HCN4 channels was shifted to more positive potentials by $\approx 15$ mV ($-73.2 \pm 1.8$ mV; $n=16$; $P<0.01$; Figure 5B). Mutant channels, however, lacked a positive shift in the activation curve ($V_h = -83.1 \pm 2.4$ mV; $n=15$), indicating cAMP insensitivity (Figure 5C). The time constant of activation ($\tau$) was highly voltage-dependent and similar in wild-type and mutant channels (Figure 5D and 5E). Control recordings performed from a population of cells with a cAMP-free pipette and then repeated for another population of cells using a cAMP-containing pipette revealed that 10 μmol/L cAMP to the pipette solution substantially accelerated activation of HCN4 channels (from $\tau_{-120}$ mV=1.66±0.10 seconds; $n=13$ to $\tau_{-120}$ mV cAMP=0.88±0.07 seconds; $n=12$; $P<0.01$; Figure 5D) but failed to accelerate mutant channels (from $\tau_{-120}$ mV=1.19±0.16 seconds; $n=14$ to $\tau_{-120}$ mV cAMP=1.37±0.10 seconds; $n=15$; $P=0.35$; Figure 5E). Thus, because of their cAMP insensitivity, mutant channels activate slower and at more negative potentials in the presence of cAMP than HCN4 channels. In the absence of cAMP, however, mutant channels activate at more positive potentials than wild-type channels and activate faster at $-120$ mV (Table 2). Channel deactivation properties were derived from a 2-pulse protocol. The initial instantaneous current after the second activation step ($I_{INS}$) reflects incomplete deactivation that vanished with prolonged interpulse periods (Figure 6A).23 The time-dependent decrease of this current ($\Delta I_{INS}$, normalized to $I_{NS}$) showed no significant difference between HCN4 and HCN4-695X channels (Table 2; Figure 6B,C). In the presence of cAMP, however, $\Delta I_{INS}$ of HCN4 channels increased from 47±7% ($n=10$) to 65±4% ($n=11$; $P=0.04$) at 1-second interpulse intervals, and similar differences appeared at other interpulse intervals (Figure 6B). HCN4-695X channels, in contrast, showed no cAMP-mediated effect on deactivation (Table 2 and Figure 6C). Thus, in the presence of cAMP, wild-type HCN4 channels stay longer in the open state than mutant channels. Therefore, it is conceivable that more HCN4 channels are activated during the plateau phase of action potentials, leading to a faster repolarization and an increased heart rate. Mutant channels, however, lacked this modulation. Despite these kinetic alterations, the reversal potential of mutant and wild-type channels were equivalent (Table 2 and Figure 6D and 6E).

Heteromeric Channels Composed of HCN4 and HCN4-695X Subunits

To mimic the heterozygous cellular phenotype of patients carrying the HCN4 mutation, HEK293 cells were cotransfected using identical amounts of plasmid DNA encoding HCN4 and HCN4-695X subunits. Under basal conditions, cells expressing heteromeric channels showed a half-maximal potential of activation ($V_h$) of $-89.6 \pm 3.3$ mV ($n=12$), similar to the value determined for HCN4 channels ($-87.5 \pm 3.3$ mV; $n=15$; Figure 7) but significantly more negative than homomeric mutant channels ($-80.1 \pm 2.7$ mV; $n=14$; $P=0.01$). Voltage sensitivity (ie, $V_C=-12.2 \pm 1.3$ mV) and time constant of activation (1.47±0.13 seconds) in heteromeric channels were intermediate between wild-type and homomeric mutant channels with nonsignificant differences (see Table 2 and Figure 7).
Peak currents were similar in amplitudes, ranging from \(-1000\) to \(-5000\) pA. Thus, heteromeric channels showed normal functional behavior under basal conditions. However, in the presence of cAMP they lacked a shift of activation voltage (basal: \(-89.6\pm3.3\) mV; \(n=12\), with cAMP: \(-85.7\pm3.7\) mV; \(n=14\); \(P=0.42\)) and displayed unchanged slope of activation curves (Table 2 and Figure 7), indicating a dominant-negative suppression of cAMP-induced HCN4 channel activation by mutant subunits.

### Table 2. Electrophysiological Properties of HCN4 Wild-Type and Mutant Channels Expressed Either in Homomeric or Heteromeric Conformation

<table>
<thead>
<tr>
<th></th>
<th>cAMP-Free</th>
<th></th>
<th>cAMP-Modulated</th>
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<tbody>
<tr>
<td>(V_h, \text{mV})</td>
<td>(V_c, \text{mV})</td>
<td>(\tau_{1/2 \text{m}}), s</td>
<td>(E_{\text{rev}}, \text{mV})</td>
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<tr>
<td>HCN4</td>
<td>(-87.5\pm3.3)</td>
<td>(-14.0\pm1.3)</td>
<td>(1.66\pm0.10)</td>
</tr>
<tr>
<td>n=15</td>
<td>n=15</td>
<td>n=13</td>
<td>n=10</td>
</tr>
<tr>
<td>HCN4-695X</td>
<td>(-80.1\pm2.7)</td>
<td>(-10.5\pm0.9)</td>
<td>(1.19\pm0.16)</td>
</tr>
<tr>
<td>n=14</td>
<td>n=14</td>
<td>n=14</td>
<td>n=6</td>
</tr>
<tr>
<td>*wt</td>
<td>*wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCN4/HCN4-695X</td>
<td>(-89.6\pm3.3)</td>
<td>(-12.2\pm1.3)</td>
<td>(1.47\pm0.13)</td>
</tr>
<tr>
<td>*695X</td>
<td></td>
<td></td>
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</table>

Half-maximum of activation (\(V_h\)) and slope factor (\(V_c\)), as derived from the Boltzmann function, time constant of activation at \(-120\) mV (\(\tau_{1/2 \text{m}}\)), normalized instantaneous current (\(\Delta_{\text{ins}}\)), reversal potential (\(E_{\text{rev}}\)), significantly different from wild-type (*wt), significantly different from HCN4-695X (*695X).

### Figure 5. Expression of HCN4 wild-type or HCN4-695X mutant subunits in HEK293 cells. A, Pulse protocol with representative whole-cell current traces on top. B, Voltage-dependent activation curves (B and C) and the potential of half-maximal activation (\(V_h\); marked by arrowheads). In the presence of cAMP (open symbols), the activation curve of wild-type is shifted significantly by \(\sim15\) mV toward more positive potentials. C, No cAMP modulation was observed in the mutant (arrowheads indicate the corresponding \(V_h\) of wild-type-channels). D, In the presence of cAMP, the time constants of activation were significantly smaller in wild-type channels than in mutant channels (E). Asterisks mark significant differences.

### Discussion

In mammals, cardiac pacemaker activity, controlling spontaneous excitation, originates in the SAN and is mediated by expression of the hyperpolarization-activated and nucleotide-gated ion channel HCN4.6–8 Consistent with this, human HCN4 mutations are associated with inherited sinus node bradycardia.9–12 A study of a single patient expressing HCN4 mutant subunits insensitive to cAMP binding suggested that \(I_f\) is crucial for chronotropic heart rate response.9 However, a
more sophisticated model for spontaneous excitation and heart rate modulation comprising interaction of transporters and intracellular calcium release\textsuperscript{18,27} raised fundamental concerns regarding the implication of $I_f$ in the generation, maintenance, and regulation of heart rhythm in vivo.\textsuperscript{17} In the present study, we present a novel, cAMP-insensitive HCN4-695X mutation associated with a new clinical phenotype and address the physiological role of $I_f$ current.

**Suggested Mechanisms Causing Sinus Bradycardia in HCN4-695X Mutant Carriers**

Biophysical properties of homomeric HCN4-695X channels revealed insensitivity to cAMP. This deficit also was observed in the heteromeric conformation (Figure 7) that most suitably reflects the heterozygous situation of our patients, indicating a dominant-negative suppression of the channel’s cAMP responsiveness by the mutant subunit. In the absence of cAMP, however, heteromeric channels showed regular activation curves similar to wild-type (Figure 7)—mirroring nonphysiological conditions. For example, DiFrancesco and Mangoni\textsuperscript{28} reported basal cytoplasmic cAMP levels of $0.2 \mu$mol/L in unstimulated SAN cells leading to a positive shift of 7 to 8 mV in the $I_f$ activation curve, whereas saturating cAMP level (100 $\mu$mol/L) induced a total shift of 14.6 mV. Similarly, our experiments showed that virtually saturating levels of 10 $\mu$mol/L cytoplasmic cAMP\textsuperscript{28} caused a total shift of 14.3 mV for wild-type HCN4 channel. These observations suggest that in vivo, mimicked by heteromeric channel expression in our experiments (Figure 7), the negative shifted $I_f$ activation curve in the resting state cannot be rescued by stimulation of basal cAMP,\textsuperscript{28} which functionally reduces $I_f$ contribution to depolarization resulting in a decreased basal heart rate. In addition, deactivation of HCN4 channels is less efficient in the presence of cAMP, indicating that an extended open state may be advantageous for faster repolarization and an increased heart rate. Thus, accelerated deactivation of mutant HCN4-695X channels, caused by

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**Figure 6.** Deactivation properties and I/V relation of homomeric HCN4 wild-type and mutant channels. A, Voltage protocol and representative current response used to investigate deactivation properties. The evoked instantaneous current (at the beginning of the second pulse) reflects a portion of channels not deactivated during the given time interval. B, In wild-type channels, $I_{INS}$ increased in the presence of cAMP (open squares) compared with basal condition (filled squares), which was significant ($P=0.04$) at 1-second interval. C, Mutant channels lack cAMP modulation. D, Voltage protocol and representative current response used to determine the IV relation (E). The intersections of these IV relations with the voltage axis are proportional to the reversal potentials, which were not different between both channel types (F).

**Figure 7.** Voltage dependence of heteromeric channels composed of HCN4 wild-type and mutant subunits after coexpression in HEK293 cells. Normalized peak current amplitudes, fitted by a Boltzmann equation, in the absence (filled triangles) or presence (open triangles) of 10 $\mu$mol/L cAMP clearly demonstrate the lack of cAMP modulation. Arrowheads indicate Boltzmann fits obtained from wild-type channels (dotted lines).
The absence of heart-rate–lowering effects of ivabradine in calcium-Clock” System

Substituted in Part by the “Voltage-/Calcium-Clock” System in particular drive the pace, current data support the idea that activated If at the healthy state provides a backup depolarizing force preventing arrhythmia during threshold conditions of heart rate acceleration. However, unlike the HCN4-D553N mutation associated with QT prolongation and polymorphic ventricular tachycardia, our mutant carriers displayed normal QTc intervals and lacked severe ventricular arrhythmias.

Adrenergic Stimulation of HCN Channels May Ensure Stable Heart Rhythm

Four, respectively, 5 of 7 mutant carriers, exhibited sinus arrhythmia and premature beats (APB, VPB), including ventricular bigeminy, observed at the initial period of heart rate acceleration (Figure 3C through 3F). A recent study reported of sinus pauses in adult transgenic mice lacking HCN4 mainly during transition from stimulated to basal cardiac states and discussed that If might function as a depolarization reserve that ensures rhythmogenic state during conditions of changing heart rate. Because of the lack of If activation by cAMP-insensitive HCN4-695X subunits, arrhythmogenic potential of our mutant carriers might be unmasked at the onset of adrenergic stimulation. Although additional cAMP-responsive contributors (“voltage-/calcium-clock” system) in particular drive the pace, current data support the idea that activated If at the healthy state provides a backup depolarizing force preventing arrhythmia during threshold conditions of heart rate acceleration. However, unlike the HCN4-D553N mutation associated with QT prolongation and polymorphic ventricular tachycardia, our mutant carriers displayed normal QTc intervals and lacked severe ventricular arrhythmias.

Neurological Disorders of HCN4-695X Mutant Carriers

Both affected males (II.2 and III.4) showed neurological disorders. Patient II.2 was diagnosed with a schizophrenic syndrome. His son (III.4) had febrile seizures during childhood and recurrent syncope caused by generalized grand mal epilepsy. Recently, an increased susceptibility to future seizures was reported in rats exhibiting slowed kinetics of the

The absence of heart-rate–lowering effects of ivabradine in HCN4-573X mutant mice suggested that loss of cAMP sensitivity completely abolished functional If activity that could not be restored by other HCN isotypes. In another study, Cre-mediated ablation of HCN4 in adult mice induced no impairment of β-adrenergic heart rate acceleration even in the presence of If blockers, demonstrating that HCN1 and HCN2 were unable to replace HCN4. Furthermore, HCN2 knockout mice showed no significant alteration of heart rate regulation, neither at rest nor under adrenergic stimulation. Similarly, HCN1 and HCN3 channels, which are resistant to cAMP, appear unable to substitute for HCN4. Thus, it seems unlikely that other HCN isoforms contribute significantly to continued heart rate modulation in HCN4-695X mutant carriers. This indicates that cardiac If current is not critical for autonomic control of heart rate in humans. In support of this possibility, other ionic currents contribute to a “voltage-clock” that closely interacts with rhythmic ryanodine receptor-mediated intracellular calcium release (“calcium-clock”). These other currents are mediated by L- and T-type Ca\(^{2+}\) channels (\(I_{\text{CaL}}, I_{\text{CaT}}\)), the delayed rectifier (\(I_{\text{K}}\)), and the Na\(^{+}/Ca^{2+}\) exchanger (\(I_{\text{NCX}}\)). Furthermore, the contribution of these currents to pacemaker function is modulated by protein kinase A or calmodulin-dependent protein kinase II (CaMK II) phosphorylation. A coordinated system of both clocks has been suggested to drive cardiac automaticity and to largely determine autonomic chronotropism. However, we do not exclude the possibility that cAMP-stimulated If contributes to autonomic heart rate control, although mutant carriers in our study suggested only a minor influence.

Loss of HCN4 Activity Might Not Be Restored by Other HCN Isoforms But Is Likely to Be Substituted in Part by the “Voltage-/Calcium-Clock” System

The absence of heart-rate–lowering effects of ivabradine in HCN4-573X mutant mice suggested that loss of cAMP sensitivity completely abolished functional If activity that could not be restored by other HCN isotypes. In another study, Cre-mediated ablation of HCN4 in adult mice induced no impairment of β-adrenergic heart rate acceleration even in the presence of If blockers, demonstrating that HCN1 and HCN2 were unable to replace HCN4. Furthermore, HCN2 knockout mice showed no significant alteration of heart rate regulation, neither at rest nor under adrenergic stimulation. Similarly, HCN1 and HCN3 channels, which are resistant to cAMP, appear unable to substitute for HCN4. Thus, it seems unlikely that other HCN isoforms contribute significantly to continued heart rate modulation in HCN4-695X mutant carriers. This indicates that cardiac If current is not critical for autonomic control of heart rate in humans. In support of this possibility, other ionic currents contribute to a “voltage-clock” that closely interacts with rhythmic ryanodine receptor-mediated intracellular calcium release (“calcium-clock”). These other currents are mediated by L- and T-type Ca\(^{2+}\) channels (\(I_{\text{CaL}}, I_{\text{CaT}}\)), the delayed rectifier (\(I_{\text{K}}\)), and the Na\(^{+}/Ca^{2+}\) exchanger (\(I_{\text{NCX}}\)). Furthermore, the contribution of these currents to pacemaker function is modulated by protein kinase A or calmodulin-dependent protein kinase II (CaMK II) phosphorylation. A coordinated system of both clocks has been suggested to drive cardiac automaticity and to largely determine autonomic chronotropism. However, we do not exclude the possibility that cAMP-stimulated If contributes to autonomic heart rate control, although mutant carriers in our study suggested only a minor influence.

### Table 3. Summary of Clinical Results

<table>
<thead>
<tr>
<th>Family Members</th>
<th>Carriers</th>
<th>Noncarriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mean Age, 40.1 Years)</td>
<td>35.9±5.6</td>
<td>47.2±5.9</td>
</tr>
<tr>
<td>Heart rate at rest, bpm</td>
<td>45.9±4.6</td>
<td>66.5±9.1</td>
</tr>
<tr>
<td>Average heart rate, bpm</td>
<td>56.4±4.8</td>
<td>71.6±10.3</td>
</tr>
<tr>
<td>Maximum heart rate, bpm</td>
<td>160.3±12.6</td>
<td>171.8±18.7</td>
</tr>
<tr>
<td>Heart rate variability, kSD</td>
<td>248.8±62.7</td>
<td>190.0±70.6</td>
</tr>
</tbody>
</table>

kSD indicates Kleiger standard deviation.

Averages of carriers/noncarriers are characterized as arithmetic mean±SEM. Individual data sets are displayed in Table 1.

cAMP insensitivity, favors bradycardia. In combination, both mechanisms may account for 31% reduction of basal heart rate (~21 bpm) observed in mutant carriers (Table 3). Interestingly, in the absence of cAMP, the activation curve of homomeric mutant channels was shifted in the positive direction although compared with the wild-type difference did not reach statistical significance. This shift of voltage sensitivity, however, might be explained by an intrinsic sterically inhibitors of channel activity by the vacant CNBD in wild-type channels that, because of a truncated CNBD, is abolished in homomeric mutant channels (Figure 2).
hippocampal Iₜ resulting from recurrent febrile seizures during development. Similar mechanisms may have unmasked proepileptic changes in patient III.4, possibly caused by the mutant HCN4-695X genotype. Future studies will reveal a possible impact of HCN4 mutant channels on pathogenesis of neurological disorders with special attention to sex-related effects because all female carriers of our study lacked neurological symptoms.

Conclusion

We describe a novel HCN4 mutation that abolishes the channel’s cAMP sensitivity. Mutant carriers showed marked sinus bradycardia at rest with preserved heart rate variability and chronotropic competence. Moreover, distinctive sinus arrhythmias and premature beats emerged during chronotropic response. Because of a mild clinical phenotype without rhythmogenic syncope and high-grade ventricular arrhythmia, neither pacemaker nor implantable cardioverter-defibrillator implantation was required. We provide clinical and mechanistic evidence that cAMP-mediated modulation of Iₜ determines basal heart rate but is not critical for β-adrenergic receptor–induced control of pacemaker activity. Furthermore, Iₜ might be important to prevent arrhythmogenic state during threshold conditions of adrenergic stimulation by providing backup depolarization capacity.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Pharmacological $I_f$ inhibition is clinically used to treat patients with stable angina and sinus tachycardia. However, the role of $I_f$ in sinus node function, and particularly mechanisms underlying autonomic heart rate regulation, are incompletely understood. According to the BEAUTIFUL study, blockage of $I_f$ current in patients with stable coronary artery disease and left ventricular systolic dysfunction reduced resting heart rate by 6 bpm (8%), a minor effect related to the reduction of 21 bpm (31%) observed in HCN4-695X carriers. Our results suggest that mutant carriers might tolerate sinus bradycardia owing to their preserved ability to accelerate heart rate according to physical requirements. Thus, cardiac-specific blockage of $I_f$ may allow for significant reduction of basal heart rate without adversely affecting chronotropic competence. This suggestion has important clinical implications. Improvements in drug selectivity will reduce extracardiac side effects as well as nonspecific channel inhibition and may offer a more effective blockage of $I_f$ pacemaker currents in the future. On the other hand, as shown in the present study, functional inactivation of $I_f$ might unmask arrhythmogenic potential during adrenergic stimulation. These considerations are relevant to safety concerns other than bradycardia associated with high-dose $I_f$-blockage.
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