

BRIEF REPORT

Familial Sinus Bradycardia Associated with a Mutation in the Cardiac Pacemaker Channel

Raffaella Milanesi, Ph.D., Mirko Baruscotti, Ph.D.,
Tomaso Gneccchi-Ruscone, M.D., and Dario DiFrancesco, Ph.D.

SUMMARY

We found that sinus bradycardia in members of a large family was associated with a mutation in the gene coding for the pacemaker HCN4 ion channel. Pacemaker channels of the sinoatrial node generate spontaneous activity and mediate cyclic AMP (cAMP)–dependent autonomic modulation of the heart rate. The mutation associated with bradycardia is located near the cAMP-binding site; functional analysis found that mutant channels respond normally to cAMP but are activated at more negative voltages than are wild-type channels. These changes, which mimic those of mild vagal stimulation, slow the heart rate by decreasing the inward diastolic current. Thus, diminished function of pacemaker channels is linked to familial bradycardia.

BRADYCARDIA IS CONVENTIONALLY DEFINED AS A HEART RATE LOWER than 60 beats per minute. Asymptomatic sinus bradycardia is usually harmless and is often a sign of good physical conditioning. On the other hand, symptomatic sinus bradycardia, such as that associated with the sick-sinus syndrome, can be a life-threatening condition and deserves prompt medical attention. The fact that sinus bradycardia can be inherited¹ indicates that it can have a genetic basis.

The pacemaker (“funny”) current (I_f) of sinoatrial-node myocytes determines the slope of the diastolic depolarization of pacemaker cells and thus has a key role in the generation and autonomic regulation of sinus rhythm and rate.² Because of their specific involvement in pacemaking, f-channels are the target for the pharmacologic control of heart rate. Indeed, substances acting by specific f-channel blockade slow the heart rate without side effects, such as the negative inotropic effects typical of beta-blockers or calcium antagonists.³ This property is particularly useful in the treatment of coronary heart disease.⁴

The f-channels are encoded by the hyperpolarization-activated, cyclic nucleotide-gated (HCN) channel gene family.^{5,6} Of the four known HCN subunits (1 through 4), HCN4 is the most highly expressed in the mammalian sinoatrial node.⁷ We therefore screened a panel of persons with asymptomatic or symptomatic bradycardia (including sick-sinus syndrome and atrioventricular block) for mutations in the *hHCN4* gene. The *hHCN4* gene is located on chromosome 15 (15q24–25)⁸; the genes coding for other channels contributing to the electrical activity of sinoatrial-node cells, including the L- and T-type calcium channels and delayed-rectifying potassium channels, are all located on different chromosomes.⁹

We report here on a family with a hereditary form of asymptomatic bradycardia associated with a mutation in the pacemaker-channel α -subunit HCN4. Functional

From the Department of Biomolecular Sciences and Biotechnology, Laboratory of Molecular Physiology and Neurobiology, University of Milan, Milan (R.M., M.B., D.D.); and the Department of Cardiology, Merate Hospital, Merate, Italy (T.G.-R.). Address reprint requests to Dr. DiFrancesco at the Department of Biomolecular Sciences and Biotechnology, Laboratory of Molecular Physiology and Neurobiology, University of Milan, Via Celoria 26, 20133 Milan, Italy, or at dario.difrancesco@unimi.it.

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analysis revealed that the mutant channels are activated at voltages more negative than those at which wild-type channels are activated; as a result, they supply less current during diastolic depolarization, which in turn results in slowing of the heart rate. The mutation mimics the effect of moderate vagal stimulation.

METHODS

Our research protocol was reviewed and approved by the institutional review board for the Department of Biomolecular Sciences and Biotechnology, University of Milan.

SEQUENCE ANALYSIS AND MUTAGENESIS

All subjects gave written informed consent before undergoing genetic analysis. Screening for mutations was performed on genomic DNA samples extracted from whole blood, saliva, or both (Puragene, Gentra Systems). The primers were designed to amplify DNA fragments of 200 to 350 bp in order to screen all of the coding portion of *hHCN4*. The polymerase-chain-reaction (PCR) products were analyzed by single-strand conformation polymorphism (SSCP) analysis. We used the primers 5'ATGCCTCATCCTGAGTCCTG3' (F) and 5'CTCACCAATGCGGTCCAG3' (R) to amplify exon 7 by Pfu polymerase (Stratagene). Mutagenesis was identified by DNA sequencing (MWG Biotech). A control group of 373 healthy subjects was used to exclude DNA polymorphisms. The *hHCN4* complementary DNA was inserted in the eukaryotic expression vector pcDNA 1.1 (Clontech Laboratories), and the point mutation (2016 C→A) was generated by a PCR overlap method. The oligonucleotide used to incorporate the mutation into *hHCN4* was 5'ACAGCCAGAGTGAGGGCC3'.

ELECTROPHYSIOLOGICAL METHODS

Wild-type and mutant human (*h*) *HCN4* channel complementary DNA was transfected for transient functional expression into HEK293 cells with a plasmid containing green fluorescent protein, as described previously.¹⁰ From one to five days after transfection, the cells were dispersed by trypsin and plated on 35-mm plastic petri dishes. A dish was placed under the stage of an inverted microscope, and GFP-expressing cells were selected for patch-clamp analysis at room temperature (25° to 26°C). The cells were initially superfused with Tyrode's solution containing 140 mM sodium chlo-

ride, 5.4 mM potassium chloride, 1.8 mM calcium chloride, 5.5 mM D-glucose, and 5 mM HEPES-sodium hydroxide buffer (pH 7.4). The pipettes used in the whole-cell patch-clamp experiment contained 10 mM sodium chloride, 130 mM potassium chloride, 1 mM egtazic acid (EGTA), 0.5 mM magnesium chloride, 2 mM ATP (sodium salt), 0.1 mM guanosine triphosphate (sodium salt), 5 mM phosphocreatine, and 5 mM HEPES-potassium hydroxide buffer (pH 7.2). The control extracellular solution in whole-cell experiments contained 110 mM sodium chloride, 30 mM potassium chloride, 1.8 mM calcium chloride, 0.5 mM magnesium chloride, and 5 mM HEPES-sodium hydroxide buffer (pH 7.4); 1 mM barium chloride, 2 mM manganese chloride, 100 μ M nickel chloride, and 20 μ M nifedipine were added to improve dissection of the pacemaker current. The pipettes used in inside-out macropatch¹¹ experiments contained 70 mM sodium chloride, 70 mM potassium chloride, 1.8 mM calcium chloride, 1 mM magnesium chloride, 1 mM barium chloride, 2 mM manganese chloride, and 5 mM HEPES-sodium hydroxide buffer (pH 7.4); a solution containing 130 mM potassium aspartate, 10 mM sodium chloride, 2 mM calcium chloride, 5 mM EGTA-potassium hydroxide, and 10 mM HEPES-potassium hydroxide buffer (pH 7.2; pCa [the negative log of the concentration of Ca²⁺ ions] 7) perfused the intracellular sides of the patches.

For the investigation of mutant channels, HEK293 cultures were split into two halves, one to express control channels and one to express mutant channels, and the two halves were treated by identical procedures; experiments comparing the properties of mutant and control channels were always performed on cells matched according to the day of culture.

The activation curves for *hHCN4* currents recorded under whole-cell conditions were obtained by standard activation and deactivation protocols and analyzed by the Boltzmann equation, $y = 1 / \{1 + \exp[(V - V_{1/2}) / s]\}$, where y is the fractional activation, V is the voltage in millivolts, $V_{1/2}$ is the half-activation voltage in millivolts, and s is the inverse slope factor in millivolts. Mean activation curves were obtained by fitting individual curves from each cell to the Boltzmann equation and averaging half-activation voltages and inverse slope factors. The activation curves in inside-out macropatches, the time constants of current activation and deactivation, and the shifts induced

by cAMP were calculated as previously reported.¹² The dose–response curves of cAMP-induced shifts in inside-out macropatches were analyzed by the Hill equation, as follows: $S \div S_{\max} = 1 \div [1 + (k_{1/2} \div \text{cAMP concentration})^h]$, where S is the shift, $k_{1/2}$ is the half-maximal concentration, and h is the Hill factor. Each patch was exposed to cAMP only once. The holding potential was -35 mV in all experiments.

MEASUREMENTS OF HEART RATE

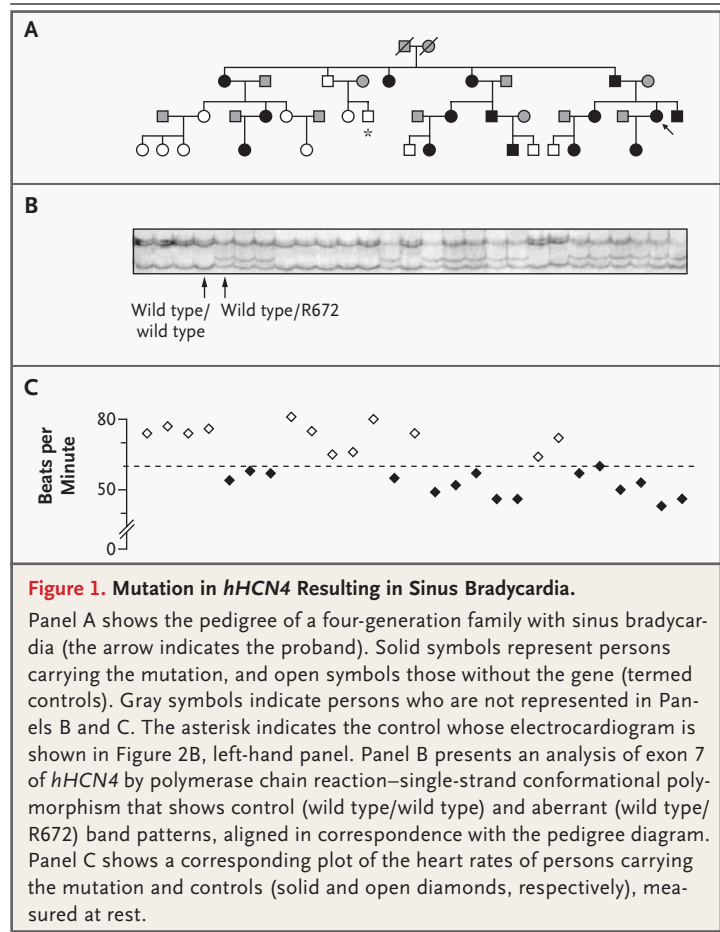
Heart-rate measurements were performed during the daytime (between 10 a.m. and 6 p.m.) with the patient at rest. The rates for children 10 years of age or younger were normalized to adult rates by linear scaling according to published data (Table A1.46 in Macfarlane and Lawrie¹³). The mean adult rates used for normalization were calculated by averaging the rates for the age groups 18 through 29 years, 30 through 39 years, 40 through 49 years, and 50 years or more from Table A1.1 in Macfarlane and Lawrie.¹³ These rates (\pm SE) were 71.0 ± 0.7 beats per minute for men and 76.7 ± 0.2 beats per minute for women.

STATISTICAL ANALYSIS

Data were compared by the independent Student's t -test or two-way analysis of variance, and P values of 0.05 or less were considered to indicate statistical significance. Genetic-linkage analysis and lod-score calculations were performed with the MLINK program of the LINKAGE software package.¹⁴

RESULTS

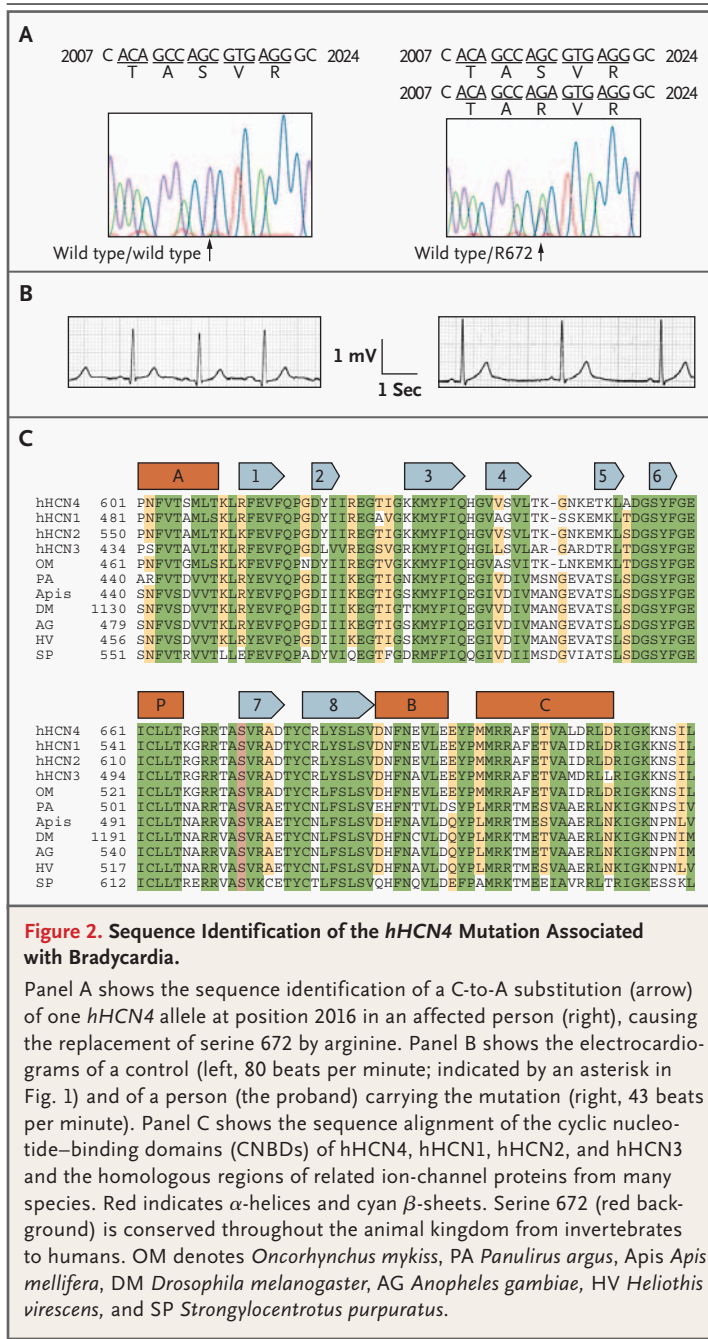
We screened 52 persons with bradycardia for mutations anywhere in the coding region of the pacemaker-channel gene *hHCN4*. This procedure led us initially to identify a missense mutation in exon 7, S672R, in one member of an Italian family (the proband) with asymptomatic sinus bradycardia (heart rate, 43 beats per minute). We then collected and examined DNA from a total of 27 members of the same family. PCR–SSCP analysis showed that the S672R mutation cosegregates with the bradycardic phenotype, indicating an autosomal dominant pattern (Fig. 1). The heart rate varied from 43 to 60 beats per minute in persons with the mutated gene (mean value for 15 persons, 52.2 ± 1.4 beats per minute) and from 64 to 81 beats per minute in those with the wild-type gene



(mean value for 12 persons, 73.2 ± 1.6 beats per minute; $P < 0.001$ by Student's t -test) (Fig. 1 and 2B). The presence of the S672R mutation was confirmed by electropherograms (Fig. 2A). The same mutation was absent in 746 control chromosomes from unrelated persons of Italian origin.

To quantify the cosegregation of bradycardia with the *hHCN4* gene, we calculated the two-point lod score for the family shown in Figure 1. Since no estimates of the frequency of the gene for sinus bradycardia exist, for the purposes of this study we assumed a frequency of 0.005 for this gene and a penetrance of 0.9. The maximum lod score was 5.47 (at $\theta = 0$, the range of θ is 0 to 0.5, step 0.01), indicating tight linkage. Changing the penetrance over the range from 0.7 to 1.0 generated maximum lod scores (at $\theta = 0$) in the range of 5.07 to 5.66.

The HCN channels are six transmembrane-domain channels that are dually activated by voltage hyperpolarization and cAMP,^{2,5} and the



native cardiac pacemaker f-channels are composed mainly of hHCN4 subunits.⁷ The cAMP activates the channels by direct binding¹¹ and partial removal of an intrinsic inhibitory mechanism,^{15,16} resulting in a depolarizing shift of the open probability curve¹²; this shift underlies the modulation of the heart rate by autonomic neurotransmitters.¹⁷⁻¹⁹

Since the S672R mutation is located within

the cyclic nucleotide-binding domain (CNBD) of hHCN4 (Fig. 2C), it might affect cAMP binding and thus interfere with the autonomic modulation of heart rate. The fact that S672 is highly conserved throughout the animal kingdom (Fig. 2C) supports the view that it has an essential functional role.

To evaluate the functional effect of the S672R mutation, we transfected wild-type and mutated hHCN4 complementary DNA into HEK293 cells (Fig. 3A). The mutant S672R channels were expressed in HEK293 cells as efficiently as were wild-type channels. However, the S672R mutant channels were activated at more negative voltages than were wild-type channels. The half-activation voltages, as calculated from the Boltzmann equation, were -76.1 ± 1.3 mV for the wild-type channels and -84.5 ± 1.5 mV for the mutant channels ($P=0.002$ by Student's *t*-test), with a negative shift of 8.4 mV; inverse slope factors did not change significantly (10.2 ± 0.8 and 11.2 ± 0.8 mV for wild-type and mutant channels, respectively; $P=0.41$) (Fig. 3A, left-hand panel). Moreover, the mutant channels were deactivated faster than were the wild-type channels, as shown by plotting mean activation and deactivation time-constant curves for wild-type and mutant channels (Fig. 3A, right-hand panel). The deactivation time constants were significantly faster in mutant than in wild-type channels, according to two-way analysis of variance ($P<0.001$). These properties of the mutant channels are compatible with a reduced contribution of the pacemaker current to diastolic depolarization.

A negative shift of the activation range could be due to a reduced ability of the basal cAMP concentration in HEK293 cells to activate S672R channels; this possibility is suggested by the localization of the mutation within the CNBD (Fig. 2C), close to residues (such as R669) affecting cAMP binding.²⁰ However, cAMP-induced shifts of the current activation curve measured in inside-out patches were similar for wild-type and mutant channels (Fig. 3B, left-hand panel); fitting with the Hill equation (lines) yielded maximal shifts of 9.79 and 9.67 mV, half-maximal concentrations of 1.53 and 1.66 μ M, and Hill slopes of 0.787 and 0.982 for wild-type and mutant channels, respectively.

Since the S672R mutation does not interfere with cAMP-induced channel activation, the negative shift of the activation curve in Figure 3A prob-

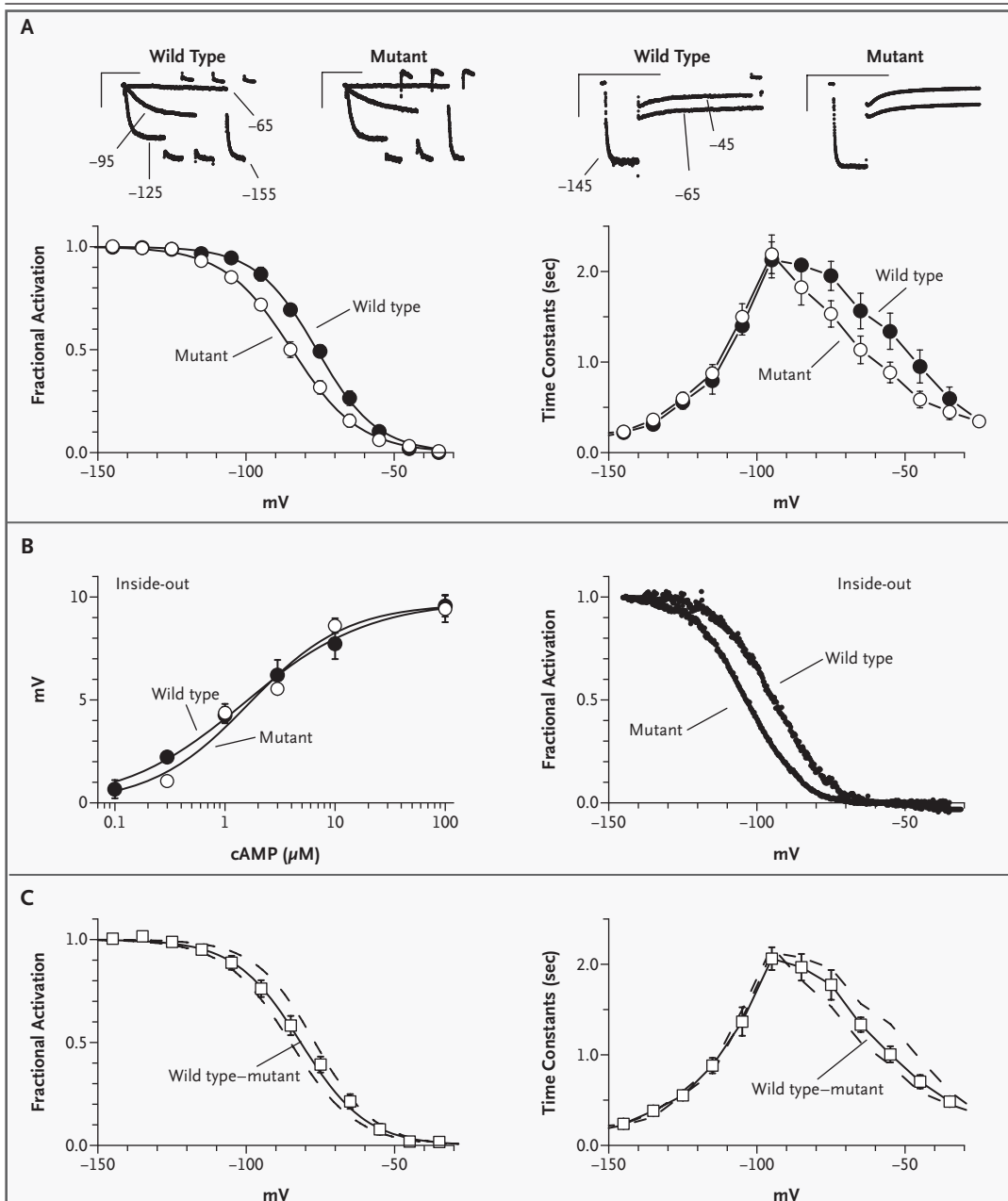


Figure 3. Kinetic Properties of Mutant hHCN4 Channels.

Panel A (left) shows the mean activation curves for wild-type channels (solid circles, six cells) and homomeric S672R mutant hHCN4 channels (open circles, eight cells) expressed in HEK293 cells. Sample traces recorded at the voltages indicated are shown at the top; the vertical scale bars represent current (50 picoamperes per picrofarad) and the horizontal scale bars, time (5 seconds). Panel A (right) shows the time-constant curves of activation (less than -90 mV) and deactivation (greater than or equal to -90 mV) obtained as averages of five curves for wild-type channels and nine curves for mutant channels. Panel B (left) shows mean cAMP-dependent shifts of the activation curve measured in inside-out macropatches expressing wild-type channels (solid circles, 27 patches) and mutant channels (open circles, 25 patches). Each data point is the average of three to nine exposures. Panel B (right) shows the mean activation curves of wild-type channels (averaged from five curves) and mutant channels (averaged from nine curves) measured in inside-out macropatches by slow voltage ramps.¹² Panel C (left) shows the mean activation curve for heteromeric wild-type-mutant channels (solid line, seven cells). Panel C (right) shows the time constants of activation and deactivation, as averaged from eight curves. The curves for wild-type and homomeric mutant channels, which are also shown in Panel A, are plotted here as broken lines. Mean \pm SE values are plotted in Panels A, B (left), and C.

ably reflects a constitutive new biophysical property of the channel caused by the mutation. This was confirmed by measuring activation curves in inside-out macropatches in the absence of cAMP: the $V_{1/2}$ of the mutant channels was -94.4 mV, 9.1 mV more negative than that of the wild-type channels (-103.5 mV), and s was 8.7 mV in both cases (Fig. 3B, right-hand panel). Thus, S672R channels are constitutively activated at voltages about 8 to 9 mV more negative than are wild-type channels.

Finally, in order to produce channels heteromeric for wild-type and mutant α -subunits, such as those occurring in heterozygotes, we cotransfected identical amounts of wild-type and S672R complementary DNA into HEK293 cells and measured the resulting currents (Fig. 3C). The properties of heteromeric wild-type-mutant channels were intermediate between those of wild-type and homomeric S672R channels. The mean activation curve had a $V_{1/2}$ of -81.0 ± 1.6 mV, with a shift of -4.9 mV relative to the control ($P=0.042$ by Student's t test); s was 11.2 ± 0.7 mV ($P=0.38$) (Fig. 3C, left-hand panel); the deactivation time constants were significantly different from those of wild-type channels according to two-way analysis of variance ($P=0.008$) (Fig. 3C, right-hand panel).

Shifting of the I_f activation curve underlies physiologic frequency changes in pacemaker cells.² Moderate vagal activity slows the heart rate by a muscarinic-induced negative shift of the I_f activation curve,¹⁸ a mechanism contributing to the basal slow rate associated with vagal tone¹⁹; this effect is accompanied by a marked acceleration of channel deactivation¹⁸ and is fully explained by a reduction of intracellular cAMP.¹¹ The effects of the heterozygous S672R mutation on hHCN4 channels (a shift of -4.9 mV in the activation curve and acceleration of deactivation) are therefore analogous to those of a low dose (10 to 30 nM) of acetylcholine.¹⁹ This led us to speculate that the bradycardic rate slowing in persons heterozygous for the S672R mutation might be similar to the slowing induced in freely beating pacemaker myocytes by 10 to 30 nM acetylcholine. Published data indicate that the slowing in pacemaker rate caused by acetylcholine at these concentrations varies between 15 and 41 percent.¹⁹ Indeed, in the family investigated, the bradycardic mutant rates were on average 29 percent slower than nonbradycardic rates (Fig. 1), a result compatible with the above concept.

DISCUSSION

We report that familial sinus bradycardia is associated with a mutation in the HCN4 pacemaker-channel α -subunit. HCN4 is the major HCN isoform contributing to native f -channels in the sinoatrial node, the natural cardiac pacemaker region.⁷ Defective HCN4 channels have been found to be associated with disorders of cardiac rhythm, but previous reports either were based on a single patient²¹ or described a complex array of rhythm disturbances without clarifying which specific effect was mechanistically related to the mutation.²²

The f -channels control pacemaker activity by generating the diastolic depolarization phase of the action potential, and they mediate the chronotropic action of autonomic neurotransmitters.² They are activated by cAMP¹¹; by increasing cAMP levels, β -adrenergic stimulation increases diastolic I_f and consequently steepens diastolic depolarization, thus causing an acceleration of the heart rate¹⁷; the opposite mechanism operates when muscarinic stimulation by acetylcholine decreases cAMP levels and I_f and slows the heart rate.^{18,19}

The hHCN4 mutation associated with bradycardia, S672R, is located in the CNBD (Fig. 2C), a region composed of several residues that affect cAMP binding.²³ We found, however, that the S672R mutation does not affect cAMP-induced channel activation, but it does modify channel kinetics by shifting the current activation range to hyperpolarized voltages and slowing current deactivation; these changes mimic those caused by a low concentration (10 to 30 nM) of acetylcholine¹⁹ and lead to a reduced flow of inward current during diastolic depolarization and hence to slowing of the heart rate.

Finally, it is known that several mutations may alter the kinetics and cAMP dependence of HCN channels^{24,25}; furthermore, HCN kinetics are modified by β -subunits.⁶ Thus, the mutation reported here may represent a specific case of a broader mechanism for sinus bradycardia based on constitutive inhibition of pacemaker channels.

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