

A gain-of-function mutation in the cardiac pacemaker HCN4 channel increasing cAMP sensitivity is associated with familial Inappropriate Sinus Tachycardia

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Aims

Inappropriate Sinus Tachycardia (IST), a syndrome characterized by abnormally fast sinus rates and multisystem symptoms, is still poorly understood. Because of the relevance of HCN4 channels to pacemaker activity, we used a candidate-gene approach and screened IST patients for the presence of disease-causing HCN4 mutations.

Methods and results

Forty-eight IST patients, four of whom of known familial history, were enrolled in the study. We initially identified in one of the patients with familial history the R524Q mutation in HCN4. Investigation extended to the family members showed that the mutation co-segregated with IST-related symptoms. The R524Q mutation is located in the C-linker, a region known to couple cAMP binding to channel activation. The functional relevance of the mutation was investigated in heterologous expression systems by patch-clamp experiments. We found that mutant HCN4 channels were more sensitive to cAMP than wild-type channels, in agreement with increased sensitivity to basal and stimulated adrenergic input and with a faster than normal pacemaker rate. The properties of variant channels indicate therefore that R524Q is a gain-of-function mutation. Increased channel contribution to activity was confirmed by evidence that when spontaneously beating rat newborn myocytes were transfected with R524Q mutant HCN4 channels, they exhibited a faster rate than when transfected with wild-type HCN4 channels.

Conclusion

This is the first report of a gain-of-function HCN4 mutation associated with IST through increased sensitivity to cAMP-dependent activation.

Keywords

HCN4 pacemaker channel • Funny current • Channelopathies • Sinus node dysfunction • Inappropriate Sinus Tachycardia

Translational perspective

Inappropriate Sinus Tachycardia (IST) is a type of sinus node dysfunction that can be inherited and has therefore a genetic background. Based on the established role of pacemaker HCN4 channels in controlling heart rhythm, we explored the possibility that mutations of HCN4 are associated with IST. Our study shows for the first time that an inheritable form of IST, identified in an Italian family, is linked to a novel gain-of-function HCN4 mutation. Our finding provides a molecular basis to explain why one of the mechanisms thought to lead to IST involves excessive sensitivity to sympathetic stimuli.

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Introduction

Inappropriate Sinus Tachycardia (IST) is associated with fast resting sinus rates and exaggerated heart rate (HR) responses that are unrelated to physiological need. Clinical symptoms are variable and range from symptomatic palpitations and activity intolerance to multisystem complaints including recurrent syncope, lightheadedness, orthostatic intolerance and others.^{1–4} Its reported frequency in the middle-aged population is 1.16%.¹ Although its aetiology is still largely unknown, several mechanisms including excessive sympathetic or reduced parasympathetic drive, excessive intrinsic HR, dysfunctional neurohormonal modulation, ectopic activity of the SAN, and β -adrenergic-receptor autoantibodies are suggested substrates for IST.^{2–5} Channelopathies have also been suggested as potential underlying mechanisms.⁶

Hyperpolarization-activated Cyclic Nucleotide-gated (HCN4) proteins, the constitutive elements of the pacemaker ‘funny’ (f)-channels, provide a large fraction of the inward current underlying the diastolic depolarization, and have a major role in cardiac rhythm generation and autonomic modulation of HR.^{7–9} The basic biophysical properties and the physiological role of pacemaker channels have long been investigated, but there is now growing interest for clinically relevant applications of the concept of f channel-based pacemaking.^{10,11} One important clinical consequence of this concept is the finding that dysfunctional HCN4 mutations are associated with familial types of arrhythmias.^{12–15}

Several HCN4 mutations have been proposed to be arrhythmogenic. Functional studies show that all mutations investigated so far are loss-of-function, and in agreement with the established role of the channels in pacemaking, most of these mutations are indeed associated with bradycardia, although more complex arrhythmias such as AF, tachy-brady syndrome, atrioventricular (AV) block, as well as structural diseases such as noncompaction cardiomyopathy and prolapse of the mitral valve, have also been reported.^{12–14} Despite intense investigation, no gain-of-function mutations, which might be expected to cause cardiac acceleration, have been described so far. We have screened patients with tachyarrhythmias in the attempt to search for causative HCN4 mutations. Here we report the case of a gain-of-function mutation in the *hHcn4* gene found in four adults and one child of an Italian family which co-segregates with symptoms of sinus tachyarrhythmia.

Functional investigation shows that R524Q mutant channels have a higher than normal cAMP sensitivity and carry a larger than normal current during the diastolic depolarization, providing the first evidence for a gain-of-function pacemaker channel mutation associated with tachyarrhythmias.

Methods

Here we outline the major study protocols adopted. An extended methods section is available in Supplementary material online.

Including criteria

Entry criteria were as described previously² and included: symptomatic mean resting HR \geq 95 bpm during the daytime hours of 24-h Holter monitoring and/or a rapid stable symptomatic increase in resting HR $>$ 25 bpm when moving from a supine to a standing position or in response to biological stress.

Genomic DNA analysis and mutagenesis

Written informed consent was obtained from each subject prior to carrying out genetic analysis. The coding sequence of the hHCN4 gene was amplified by PCR starting from genomic DNA extracted from whole blood or saliva (Puragene Blood Kit, Qiagen). The primers were designed to amplify DNA fragments of 149 to 395 bp (Supplementary material online, Table S1) in order to screen all the coding portion of the *hHcn4* gene. The PCR reaction mixture included 100 ng of genomic DNA, 1 μ M primers and the FastStart Taq DNA Polymerase (Roche Diagnostics). The PCR cycling reaction consisted of initial denaturation for 5 min at 95°C, and 30 cycles with denaturation of 30 s at 95°C, annealing for 30 s at different temperature and extension for 30 s at 72°C.

The primers 5'TTCCCTCTCATCCACTGTCCC3' (F) and 5'GACC AATGTGCGGGTGCTCC3' (R) were used to amplify exon 4, where the pathological mutation 1571g \rightarrow a (R524Q) is located. Analysis of the amplicons was carried out by single-strand conformation polymorphism. The presence of mutations was confirmed by DNA sequencing (Bio-Fab Research).

For functional studies, both in HEK293 cells and neonatal rat myocyte cultures we used hHCN4 cDNA cloned into the pcDNA 1.1 vector. The mutation of interest was incorporated into the hHCN4 cDNA sequence by means of a commercial kit (QuikChange® Site-Directed Mutagenesis, Agilent Technologies). The primers used were: 5'CTGG ACTCCTCCCAGCGCCAGTACCAG3' (F) and 5'CTGGTACTGG CGCTGGGAGGAGTCCAG3' (R).

Statistical analysis

Activation and dose–response curves were compared using the Extra sum of squares *F* test followed by one-way ANOVA and post hoc tests. Group comparisons were made with unpaired Student's *t*-test or one-way ANOVA followed by post hoc tests. *P*-values of 0.05 or less were considered to indicate statistical significance. Statistical analysis were carried out with OriginPro 9.1, OriginLab Corporation, USA and GraphPad Prism 5, GraphPad Software, USA. Unless otherwise indicated data are expressed as mean \pm SEM.

Results

R524Q mutation in a family with Inappropriate Sinus Tachycardia

Forty-eight patients affected by IST (four of whom with familial history of IST) were tested for the presence of mutations in the coding region of the cardiac pacemaker channel gene *Hcn4*. The screening led to the identification in an adult female (proband) of the mutation 1571g \rightarrow a, which generates the missense aminoacidic substitution R524Q in exon 4 of one allele (Supplementary material online, Figure S1). Genetic analysis was then extended to eight other members of the family and the mutation was identified in three more adults and in one child (Figure 1A).

The mutation 1571g \rightarrow a (R524Q) was not identified in a group of 200 healthy subjects and we therefore excluded the possibility of a DNA polymorphism. One case is reported for a single allele over 1092 individuals from 14 populations in the 1000 Genomes Project.¹⁶ Analysis with the SIFT algorithm¹⁷ identified this mutation as deleterious, with a normalized probability to be tolerated of 0.0002.

Arginine 524 is conserved among all human HCN isoforms and throughout the animal kingdom from insects to man, but not in the echinoderm *Strongylocentrotus purpuratus* and the cnidarian

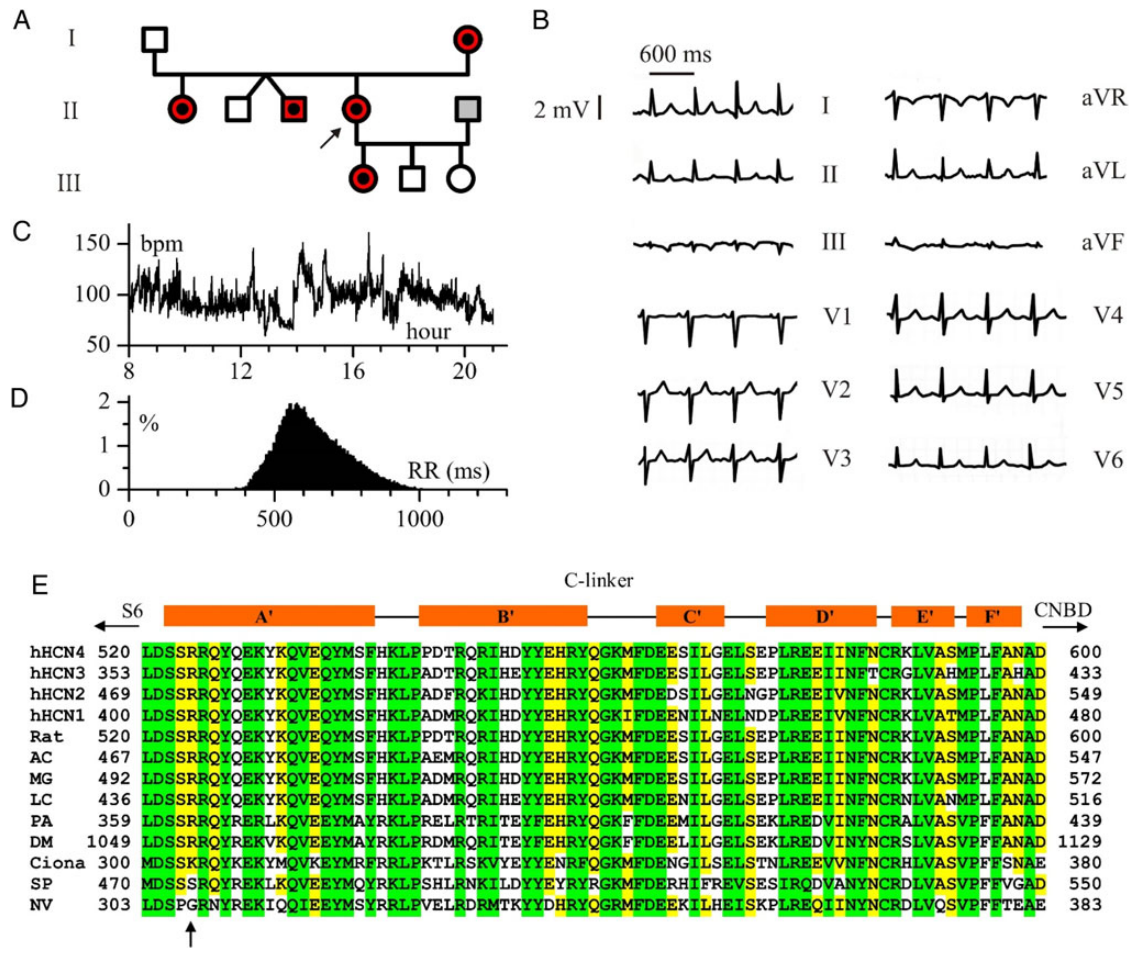


Figure 1 HCN4 mutation associated with Inappropriate Sinus Tachycardia. (A) Pedigree of a three-generation family with Inappropriate Sinus Tachycardia (the arrow indicates the proband). Individuals with Inappropriate Sinus Tachycardia symptoms and/or unjustified sinus tachycardia are labelled by red symbols; dots indicate members of the family carrying the heterozygous mutation R524Q, empty symbols are wild-type, grey symbol indicates a genetically unrelated individual. The twins II-2 and II-3 are heterozygous. (B) Twelve-lead ECG recorded at baseline in the proband; the heart rate is 103 bpm and the P wave is indicative of sinus node origin. (C) Plot of daytime heart rate (from 8 am to 9 pm) recorded in the proband during Holter monitoring; the mean rate was 98.5 ± 14.2 bpm (mean \pm SD). (D) Histogram of daytime R–R intervals recorded during Holter monitoring. (E) Sequence alignment of the C-linker region of human HCN isoforms and of corresponding regions of homologous channel proteins from different species. Red bars above the sequences indicate α -helices. Significance of symbols is as follows: Rat, *Rattus norvegicus*; AC, *Anolis carolinensis*; MG, *Meleagris gallopavo*; LC, *Latimeria chalumnae*; PA, *Panulirus argus*; DM, *Drosophila melanogaster*; Ciona, *Ciona intestinalis*; SP, *Strongylocentrotus purpuratus*; NV, *Nematostella vectensis*. Arginine 524 of hHCN4 (arrow) is in the region most proximal to the S6 domain.

Nematostella vectensis, while a synonymous mutation (to lysine) is found in the urochordate *Ciona intestinalis* (Figure 1E).

The clinical profile of the proband (II-4 in Figure 1A) was characterized by prolonged periods of symptomatic sinus tachycardia (Figure 1B), frequent palpitations at rest and/or during effort and anxiety, orthostatic intolerance with syncope (Supplementary material online, Figure S2), reproducible dyspnoea on effort limiting or causing early termination of any physical activity, and light-headedness. At electrocardiogram (ECG) Holter-monitoring, daytime HR (8 am to 9 pm) had a mean \pm SD value of 98.5 ± 14.2 bpm (Figure 1C and D). The patient was diagnosed as IST after excluding other mechanisms potentially causing compensatory tachycardia such as: structural heart disease, neuroendocrine disorder, postural hypotension, fever, anaemia, pregnancy, and medications.

Among the other family members also carrying the mutation, all adults (I-2, II-1, II-3, Figure 1A) were affected by palpitations at baseline and anxiety, while the young girl (III-1) was asymptomatic. In individuals II-1, II-3, II-4, and III-1, episodes of symptomatic or asymptomatic unjustified sinus rhythm acceleration and deceleration were repeatedly recorded. In individual I-2 (proband's mother) multiple ECG recordings carried out in a 5-year period confirmed the presence of sinus tachycardia with resting HRs in the range of 100 bpm (103.3 bpm in the ECG of Figure 2A; see Supplementary material online, Table S2). The proband's sister (II-1) also presented with orthostatic intolerance and light-headedness; Holter monitoring revealed multiple phases of sudden unexplained sinus tachycardia unrelated to physical activity during both day and night (Figure 2B).

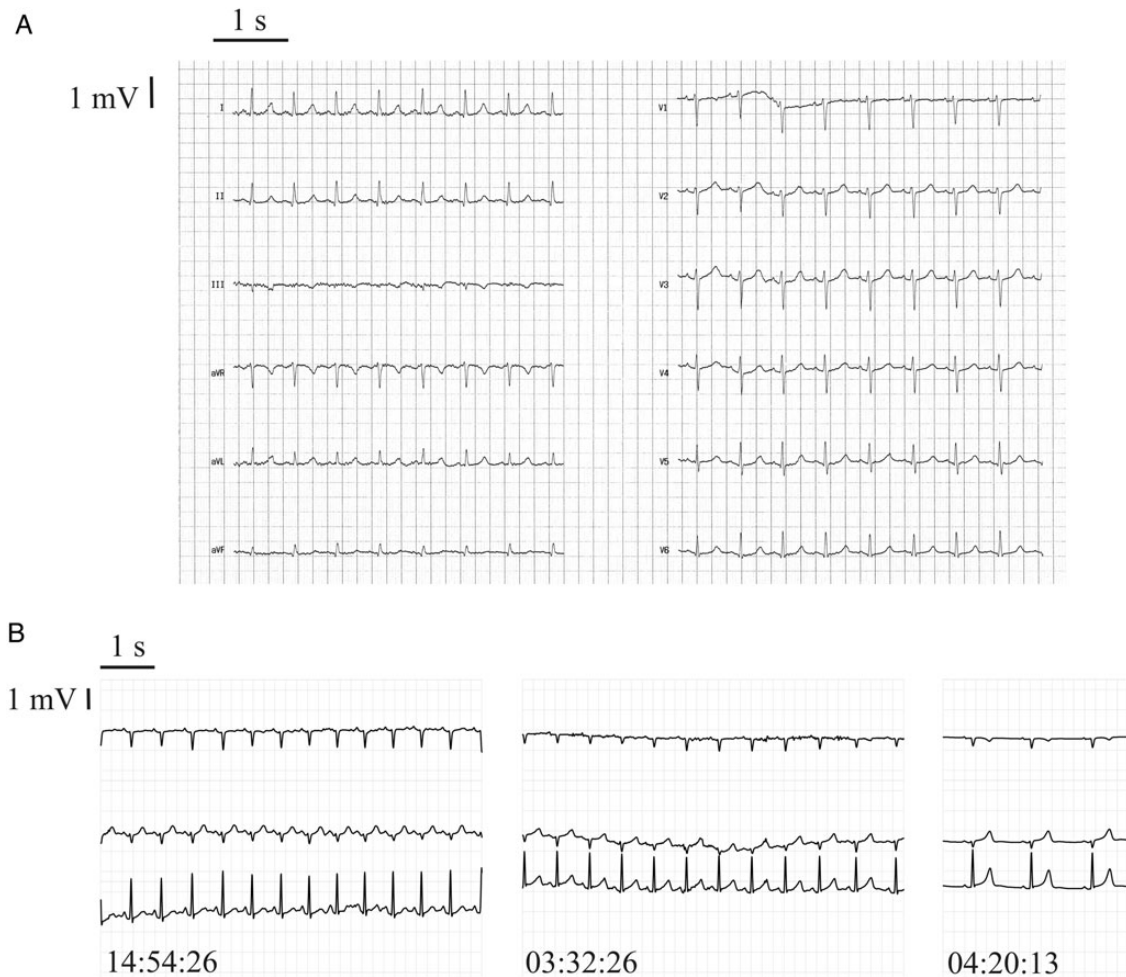


Figure 2 Episodes of unjustified sinus tachycardia recorded at rest from the proband's mother (I-2) and sister (II-1). (A) Twelve-lead electrocardiogram recordings at rest from the proband's mother; the mean heart rate was 103.3 bpm. (B) Stretches of Holter recording showing the occurrence in the proband's sister (II-1) of episodes of sinus tachycardia at rest during daytime (left, 111.5 bpm) and night (middle, 94.3 bpm). P wave morphology was the same as that recorded during normal sinus rhythm (right, 56.6 bpm).

Individual II-3 is an adult male with a history of recurrent syncope associated with binodal dysfunction characterized by sinus bradycardia and phases of first and second degree LW AV block associated with enhanced vagal tone (Figure 3A and C and Supplementary material online, Table S2). Despite the prevalent bradycardia, this individual presented several unexpected bursts of increasing HR at rest. Phases of sinus tachycardia typically manifested with 'warm-up' characteristics and were abruptly interrupted by phases of bradycardia (Figure 3B and C), likely induced by a robust vagal discharge.¹⁸ Although these events were particularly frequent at nighttime (Figure 3C), they were also observed during the day (Figure 3B) where they were associated with transient symptomatic sinus tachycardia.

Individual III-1 (proband's daughter) is a healthy young girl who did not report specific cardiac symptoms. Nonetheless, Holter monitoring revealed phases of anomalous tachycardia (Supplementary material online, Figure S3) unrelated to any physical activity or emotional stress.

No signs of IST or other cardiac arrhythmic abnormalities were found in the adult (I-1, II-2) or young (III-2, III-3) family members carrying the HCN4 wild-type genetic profile.

A summary of clinical profiles of all members of the family is given in Supplementary material online, Table S2.

Functional analysis by heterologous expression in HEK293 cells

The R524 residues of human HCN4 channels are located in the initial part of the C-linker (Figures 1E and 4), a region connecting the S6 transmembrane domain to the cyclic nucleotide binding domain (CNBD) which functionally couples the binding of the second messenger cAMP to channel activation; in the tetrameric channel assembly they form a ring of positively charged amino acids which surrounds the internal mouth of the channel (Figure 4B). As shown in Figures 1 and 4B, the residue R524 is located in the first portion of the A' α -helix of the C-linker, in close proximity to the S6 domain.

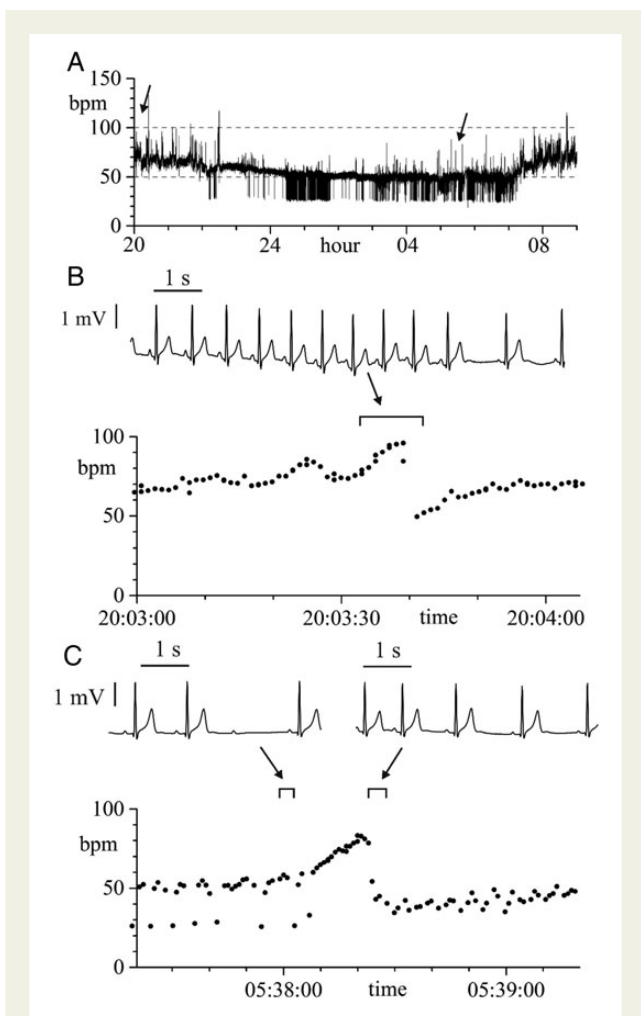


Figure 3 ECG-Holter recordings from the proband's brother (II-3). (A) Plot of heart rate (from 8 pm to 9 am) during Holter monitoring. Sinus rhythm was prevalently bradycardic with several episodes of 2:1 AV block particularly during sleep. Arrows indicate correspondence with enlargements in (B) and (C). (B) Time course of heart rate (bottom) and corresponding electrocardiogram recording (top) associated with symptomatic palpitation and anxiety at rest during the day, as reported by the patient. (C) Time course of heart rate (bottom) and corresponding electrocardiogram recordings (top) showing an episode of asymptomatic sinus tachycardia recorded at night during sleeping (top, right). Sinus node tachycardia occurs on a baseline condition of sinus bradycardia during which several episodes of I and II degree AV block were present (top, left). Atrioventricular blocks are associated with enhanced vagal tone, as shown by the increase in PP interval duration in correspondence with the AV block. PP intervals prior to, during, and after block were (ms): 1060, 1040, 1040, 1160, 1140 (block), 1128, and 1012. P wave morphology was unchanged during the whole recording. 'Warm-up' events characterized by inappropriate sinus tachycardia followed by sudden rate slowing are apparent in both panels. See Supplementary material online, Table S2 for further clinical information.

We investigated the kinetic properties of channels by patch-clamp analysis of HEK293 cells transfected with wild-type (wt) and mutated *hHCN4* cDNA. Both channel types were expressed

with similar efficiency; mean current densities measured at -145 mV were: -50.7 ± 11.5 ($n = 16$), -56.5 ± 11.7 ($n = 14$), and -48.2 ± 6.9 pA/pF ($n = 21$) for wt, heteromeric wt-R524Q and homomeric mutant R524Q channels, respectively (not significantly different, $P > 0.05$).

However, the mutation increased availability of the pacemaker current by shifting to more depolarized voltages the activation curve of whole-cell currents by 4.2 mV in heteromeric wt-R524Q channels and 7.6 mV in homomeric mutant R524Q channels (Figure 5A, top).

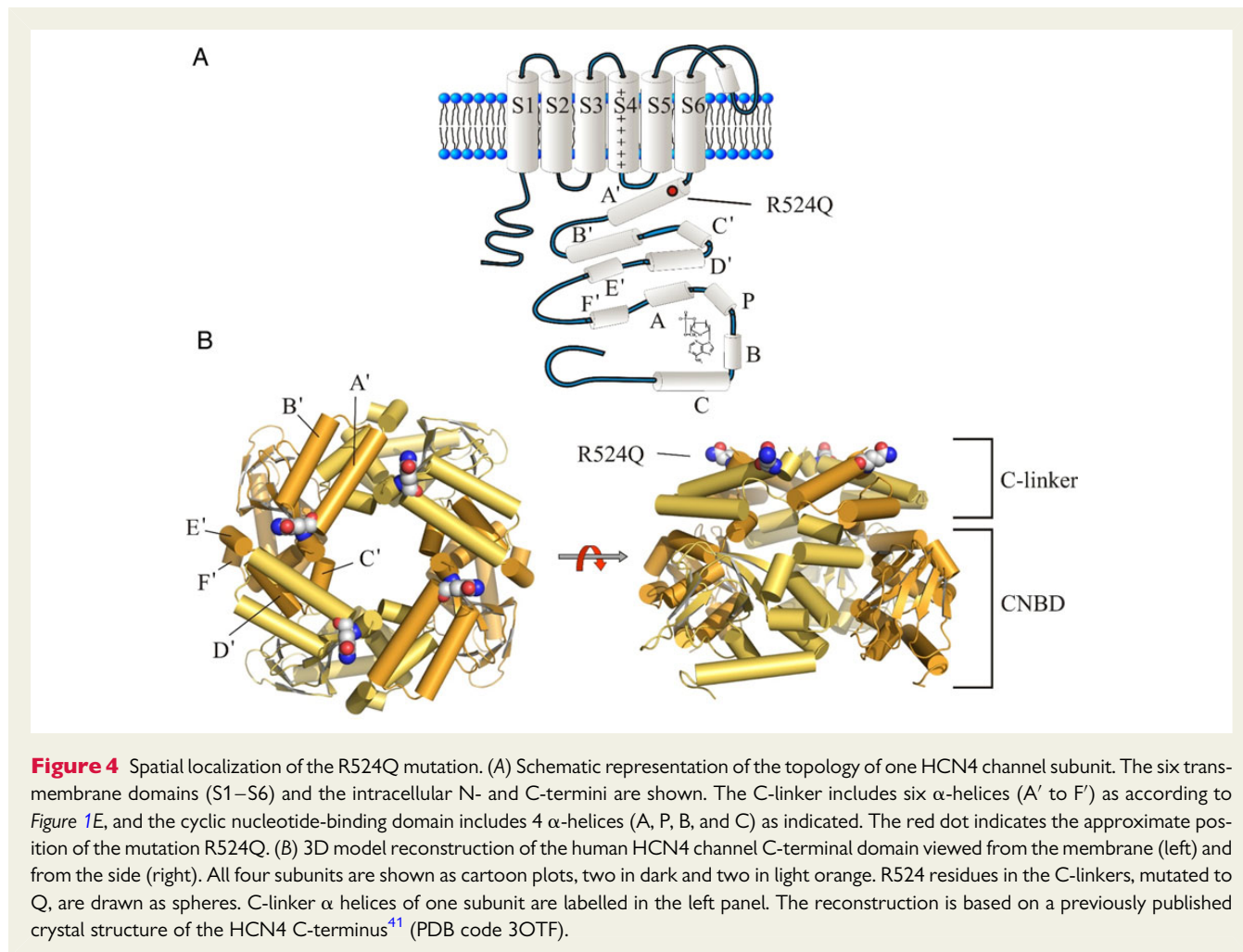
We reasoned that differences in the half-activation ($V_{1/2}$) values of activation curves could reflect intrinsic new properties of mutant channels or differences in the sensitivity to basal cytoplasmic cAMP. To verify this, we exposed cells to a saturating cAMP concentration (10 μ M) in the whole-cell pipette solution. Under these conditions, all activation curves overlapped and their $V_{1/2}$ values did not differ significantly (Figure 5A, bottom); thus, while the cAMP-induced shift of $V_{1/2}$ in wt channels (13.0 mV) had the expected size,^{19,20} it was significantly reduced in wt-R524Q (8.3 mV) and R524Q channels (4.6 mV) ($P < 0.05$; Figure 5B).

These data suggest an increased cAMP sensitivity of mutant channels, the shifts observed in whole-cell conditions in Figure 5A reflecting higher response to basal cytoplasmic cAMP of mutant vs. wt channels. To confirm this hypothesis, we investigated the action of cAMP in inside-out macro patches. As shown in Figure 5C, mean activation curves of wt, wt-R524Q, and R524Q channels measured with a voltage ramp protocol in a cAMP-free intracellular solution overlapped, indicating that the mutation did not modify the intrinsic voltage dependence of channels in the absence of cAMP. A cAMP concentration of 0.1 μ M, on the other hand, was more effective on mutant than on wt channels (Figure 5D, top). Measuring for a fuller assessment of the cAMP dependence the dose-response relationships of the $V_{1/2}$ shift against cAMP concentration for the three channel types (Figure 5D, bottom) showed that indeed mutant channels have a higher cAMP sensitivity.

We additionally verified that ivabradine 3 μ M blocks wt and R524Q channels in a similar way (wt: $68.0 \pm 4.1\%$, $n = 6$; R524Q: 64.0 ± 8.1 , $n = 6$; $P > 0.05$).

R524Q mutant proteins increase automaticity in neonatal cardiac myocytes

The data in Figure 5 show that R524Q channels respond more strongly to cAMP and provide a basis to predict an abnormally fast, β -adrenergic sensitive cardiac rhythm of patients carrying the mutation. For a direct evaluation of the effects of the mutation on beating rate, we transfected wt and/or mutant channels into rat neonatal cardiac myocytes²¹ (Figure 6). Cardiomyocytes were co-electroporated with wt and/or mutant channel cDNA and with GFPmax-containing plasmids to allow for the identification of green cells. HCN4 channel expression was equally robust in all groups and mean current densities measured at -125 mV values were not significantly different: -39.4 ± 8.2 ($n = 6$), -34.6 ± 6.6 ($n = 6$), and -37.6 ± 6.1 ($n = 7$) pA/pF for wt, wt-R524Q, and R524Q channels, respectively ($P > 0.05$); for comparison, cells expressing the GFPmax reporter only had a current density of



-3.6 ± 0.8 pA/pF ($n = 9$; *t*-test with Welch correction, $P < 0.05$ vs. control). Transfected cells acquired a constant, regular rate as previously shown.²¹

We compared the activity of cells expressing wt and mutant channels, and found that the latter beat faster (Figure 6B and D). Relative to wt (65.6 ± 3.7 bpm, $n = 9$), the mean spontaneous rate was 34.7% (88.4 ± 9.0 bpm, $n = 9$) and 72.2% faster (113.0 ± 5.8 bpm, $n = 10$) in cells expressing wt-R524Q and R524Q channels, respectively ($P < 0.05$; Figure 6D). Video detection measurements of rate contraction were also performed, and mean rates of 65.2 ± 5.4 bpm ($n = 10$) and 96.6 ± 9.3 bpm ($n = 10$, 48.2% acceleration) were measured from wt and R524Q-channel expressing cells, respectively (Supplementary material online, Video S1).

A faster spontaneous rate is expected in the presence of a larger pacemaker current. We thus checked if in cardiomyocytes, as in HEK293 cells, the current availability was increased by the mutation and indeed found that the fractional current activation increased in mutant channels in the mid-activation range (see -75 mV records in Figure 6A) and that mean activation curves were shifted to more positive voltages by 3.9 and 7.3 mV in wt-R524Q and R524Q channels, respectively (Figure 6C).

Discussion

We report here the first evidence for a heterozygous gain-of-function mutation in the pacemaker HCN4 channel associated with symptoms of IST.

All arrhythmogenic mutations in HCN4 reported so far are loss-of-function and, accordingly, are mostly associated with bradycardia.^{12–14}

In this work, all family members carrying the mutation R524Q displayed unjustified phases of symptomatic or asymptomatic tachycardia, both at rest and during sleep, as assessed by objective evaluations (ECG and Holter monitoring) and by subjectively reported symptomatology. Different clinical profiles likely reflect the presence of incomplete penetrance and variable expressivity as it occurs in almost all channelopathies.^{22–24} Phenotypical heterogeneity could derive, for example, from individual variability in the autonomic control of HR. A similar mechanism, proposed to explain differential arrhythmic risk in LQT1 patients, has been recently identified, for example, in KCNQ1 A341V mutation carriers.²³ Similarly, studies carried out by Berge *et al.*²⁵ and by Priori *et al.*²⁶ estimated penetrance values of 41 and 16%, respectively, in LQTS-associated and in BrS-associated genes.

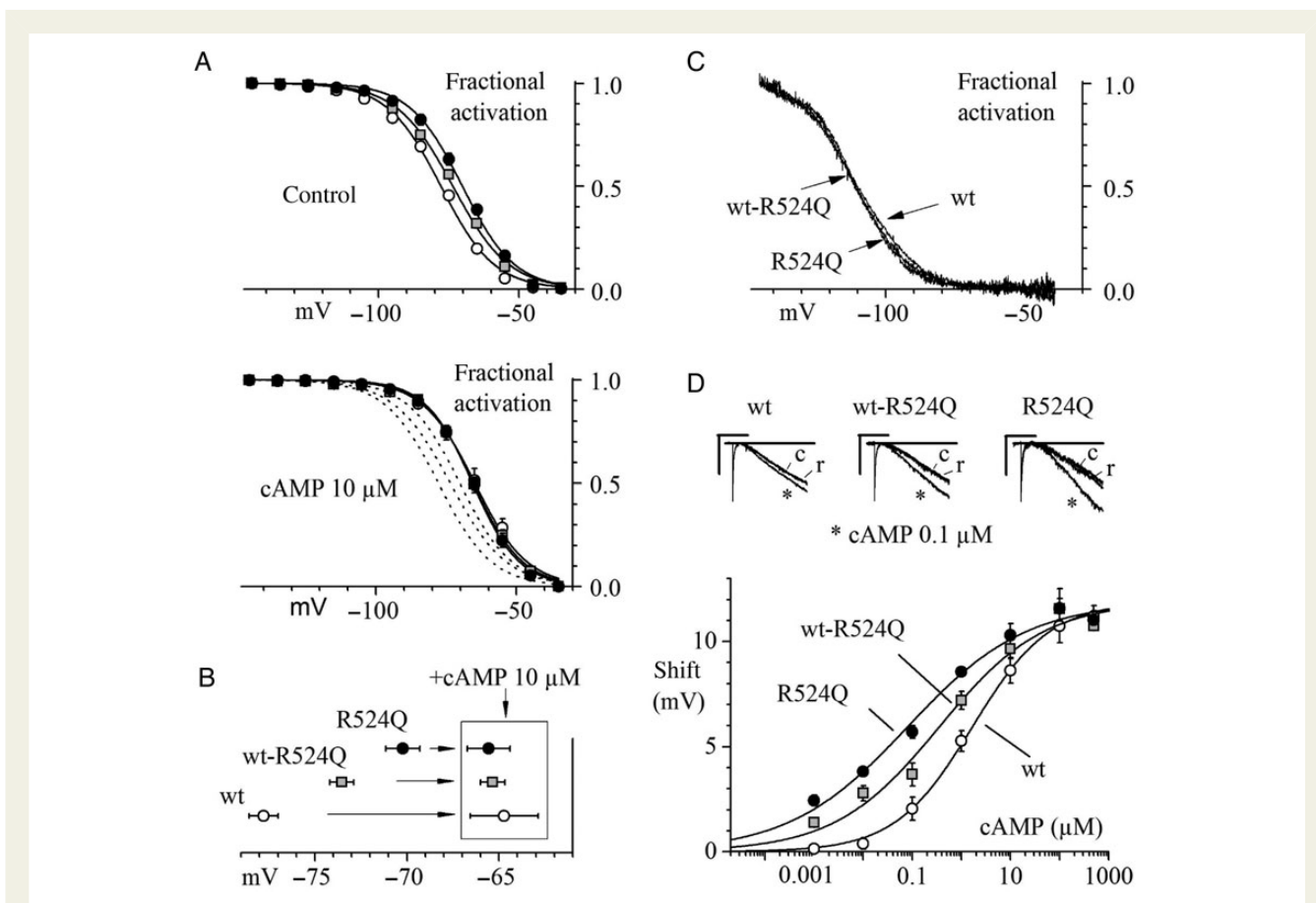


Figure 5 Wild-type and mutated channel expression in HEK293 cells. (A) Mean activation curves of HCN4 current measured in HEK293 cells in control (upper) and in the presence of 10 μM cAMP in the whole-cell pipette (lower). Datapoints are means from $n = 6-10$ cells. The values of $V_{1/2}$ from Boltzmann fitting (full lines) are: -77.8 , -73.6 , and -70.2 mV (top) and -64.8 , -65.3 , and -65.6 mV (bottom) for wild-type (open circles), heteromeric wt-R524Q mutant (grey squares) and homomeric R524Q mutant channels (filled circles), respectively. All curves were significantly different in control, but not in the presence of cAMP. Curves in the upper panel are replotted as broken lines in the lower panel. (B) Mean $V_{1/2}$ calculated from Boltzmann fitting of single-cell activation curves ($n = 6-10$ cells). Values in the presence of cAMP are boxed. (C) Mean activation curves of wt, wt-R524Q, and R524Q channels averaged from 9, 7, and 9 curves, respectively, as measured in inside-out macropatches by slow voltage ramps⁴² in the absence of cAMP. (D) Dose-response relationships of cAMP dependence of activation curve shift measured in inside-out macropatches expressing wt, wt-R524Q, and R524Q channels. Each datapoint is the average of 3-10 exposures. Parameters of Hill fitting of these curves are: $K_d = 1.67$, 0.35 , and 0.080 μM and $h = 0.55$, 0.41 , and 0.36 for wt, wt-R524Q, and R524Q channels, respectively. Maximal shift was fixed to $S_{\text{max}} = 11.9$ mV for all curves. Top panels: sample inside-out traces in control (c), during perfusion with cAMP (*) and upon return (r). Horizontal bar: 1 s; vertical bar: 20 pA.

Inappropriate Sinus Tachycardia has recently received much medical attention since several laboratories have reported that patients resistant to treatment with conventional rate-decreasing medications can be successfully treated with ivabradine, a specific inhibitor of the If current.^{2,27-30} The evidence that the If current is an effective pharmacological target in IST treatment highlights on the one hand its role as a major mechanism for HR control, and supports on the other its potential involvement in IST aetiology. Residue R524 is located in the first α -helix (A') of the C-linker, a stretch of 81 residues connecting the cytoplasmic end of the S6 segment to the CNBD. Previous crystallographic and functional studies of mHCN2 channels have shown that C-linkers contribute essentially to the structural re-arrangements involved in the cAMP-induced modulation of the channel.³¹⁻³³

We found that substitution of the positively charged arginine with the polar, uncharged glutamine does not modify the intrinsic voltage dependence of channel activation (Figure 5C), but does increase its sensitivity to cAMP (Figure 5A-D). From the dose-response curves in Figure 5D the half-maximal cAMP concentration was decreased ~ 4.6 -fold (from 1.67 to 0.35 μM) in heterozygous wt-R524Q, and >20 -fold (from 1.67 to 0.080 μM) in homozygous R524Q mutant channels.

The mutation R524Q involves the change of electrical charge distribution in the C-linker. Although our study did not address the structural basis of the effects of the R524Q mutation, it is worth noting that several previously reported arrhythmogenic missense, loss-of-function hHCN4 mutations of C-terminus residues (such as K530N, D553N, S672R, and P883R) involve a change of electric

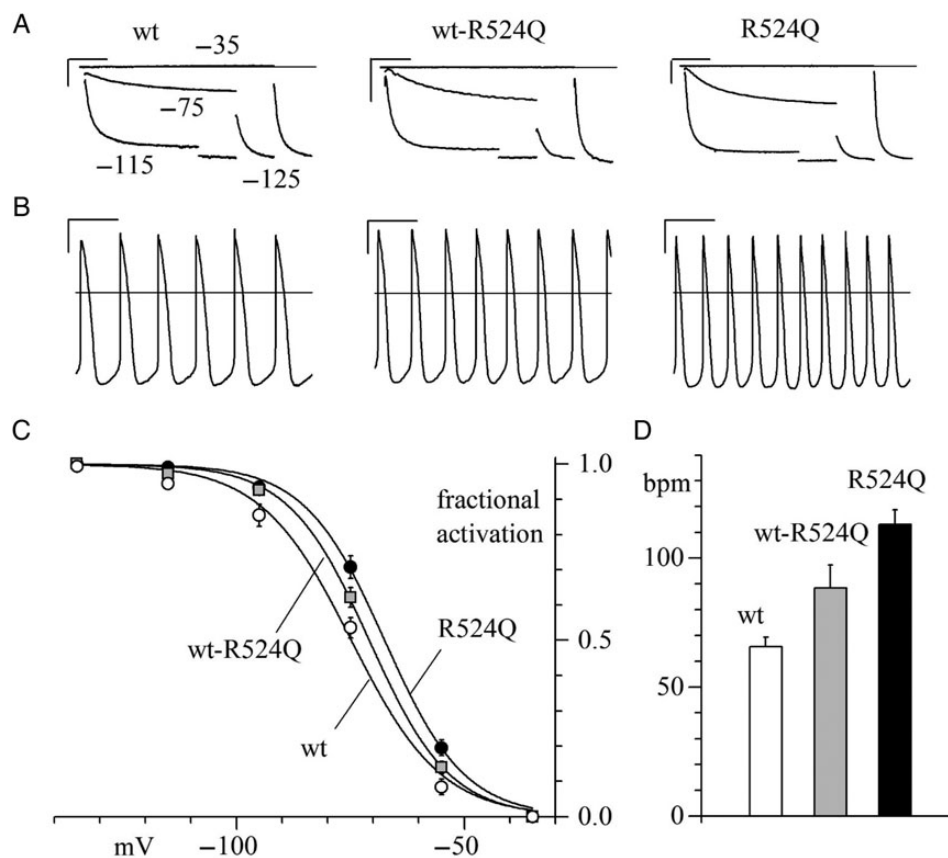


Figure 6 Wild-type and mutated channel expression in rat neonatal myocytes. (A) Sample current and (B) free-running voltage traces recorded in cells expressing wt (left), wt-R524Q (middle), and R524Q channels (right). Currents in (A) were measured during two-step protocols to the voltages indicated. In (A) and (B): horizontal bar: 2 and 1 s; vertical bar: 500 pA and 20 mV, respectively. (C) Mean activation curves (each point is the average of data from $n = 4-10$ cells). The values of $V_{1/2}$ from Boltzmann fitting were: -74.8 , -70.9 , and -67.5 mV for wt, wt-R524Q, and R524Q curves, respectively. (D) Mean spontaneous rates were 65.6 ± 3.7 ($n = 9$), 88.4 ± 9.0 ($n = 9$), and 113.0 ± 5.8 bpm ($n = 10$) in cells expressing wt, wt-R524Q, and R524Q channels, respectively. All values are significantly different from each other ($P < 0.05$).

charge,³⁴ and that several findings indicate that channel gating is affected by the electric charge distribution in the C-terminus.^{32,35-37}

Also interestingly, cAMP is known to have an exaggerated activating action on the HCN-homologous channel of the sea urchin (SPIH), where the residue homologous to arginine 524 is a serine (SP in Figure 1E).³⁵

In contrast with our data, the K530N mutation previously reported by Duhme *et al.*³⁸ is to be classified as loss-of-function since it is associated with a strong negative shift of the activation voltage dependence of the pacemaker current,^{13,14} which represents the rational mechanistic link to the manifestation of Brady-arrhythmias reported in their work. Interestingly, the authors report also a larger than normal response to cAMP of the heteromeric wt + mutant channel (though not of the homomeric mutant channel); this lends further support to the view that the C-linker region comprising R524 and K530 residues is involved in mediating the cAMP-induced modulation of the channel.

Whichever the mechanism involved, our data show that the R524Q mutation causes a higher cAMP sensitivity, resulting in a rightward shift of the activation curve (Figures 5 and 6), a property

that mimics the effect of β -adrenergic stimulation.³⁹ Accordingly, newborn myocytes pace faster when transfected with mutant rather than wt channels (Figure 6).

Exaggerated β -receptor stimulation operated by autoantibodies and the associated cAMP elevation are known causes of IST.^{5,40} Our data therefore provide an explanation for the link between β -receptor stimulation by autoantibodies and IST.

Because of the properties of R524Q channels, our data support the view that individuals carrying this mutation are in a permanent state of higher than normal channel activation and/or associated rhythm acceleration. Our results thus provide a basis to explain the faster intrinsic HR and the hypersensitivity to sympathetic stimulation typical of IST.^{2,4} They also explain why ivabradine, a known 'pacemaker' channel blocker used in the therapy for angina and heart failure, is today considered as an emerging new therapy for IST.^{2,4} R524Q is the first example of a gain-of-function mutation in hHCN4 associated with IST, and may represent a special case of a more general mechanism of HCN4-linked cardiac tachyarrhythmias. Our results introduce the concept that IST can have a genetic basis and raises the possibility that inheritable IST traits are less infrequent than previously believed.

Authors' contributions

M.B., A.B., and D.D.F.: performed statistical analysis; M.B., A.B., and D.D.F.: handled funding and supervision; M.B., A.B., R.M., M.P., A.B., T.G.-R., E.B., and L.V.-S.: acquired the data; M.B., A.B., R.C., and D.D.F.: conceived and designed the research; M.B., A.B., E.B., R.C., and D.D.F.: drafted the manuscript; M.B., A.B., A.B., T.G.-R., E.B., L.V.-S., R.C., and D.D.F.: made critical revision of the manuscript for key intellectual content.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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