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Short sequence-paper

The human gene coding for HCN2, a pacemaker channel of the heart[1]

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Abstract

Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, underlying 'pacemaker' currents (I_f/I_h), are involved in pacemaker activity of cardiac sinoatrial node myocytes and central neurons. Several cDNAs deriving from four different genes were recently identified which code for channels characterized by six transmembrane domains and a cyclic nucleotide binding domain. We report here the identification of the human *HCN2* gene and show that its functional expression in a human kidney cell line generates a current with properties similar to the native pacemaker f-channel of the heart. The *hHCN2* gene maps to the telomeric region of chromosome 19, band p13.3. This is the first identification of a genetic locus coding for an HCN channel.

Keywords: Cardiac pacemaker channel; Chromosome 19; Hyperpolarization-activated channel

Cardiac pacemaker activity originates in specialized myocytes located in restricted areas of the heart which are characterized by spontaneous firing of action potentials independent of synaptic input. The 'pacemaker' I_f current is a mixed Na^+/K^+ current activated upon membrane hyperpolarization beyond the resting value, which has been extensively characterized in cardiac preparations (for review see [1]). I_f plays a key role in the generation of diastolic depolarization, a phase of the action potential responsible for repetitive activity in sino-atrial (pacemaker) myocytes, and is controlled by the second messenger cAMP. This latter mechanism underlies cardiac rhythm modulation by sympathetic and parasympathetic neurotransmitters: stimulation of β-adrenergic receptors raises intracellular cAMP levels and increases I_f by a positive shift of the current activation curve [2], thus accelerating diastolic depolarization and cardiac rate, whereas stimulation of muscarinic receptors slows rate by the opposite action [3]. The ability of cAMP to modulate the activation of the pacemaker current is mediated by its direct binding to the channel in sino-atrial node cells [4].

I_f-like currents (termed I_h) are widely expressed in other excitable cells including several types of neurons. The properties of I_h currents are well suited to control neuronal excitability and the flow of information through synaptic contacts [5].

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[1] Sequence data reported in this paper have been deposited in GenBank database under accession no. AF065164.

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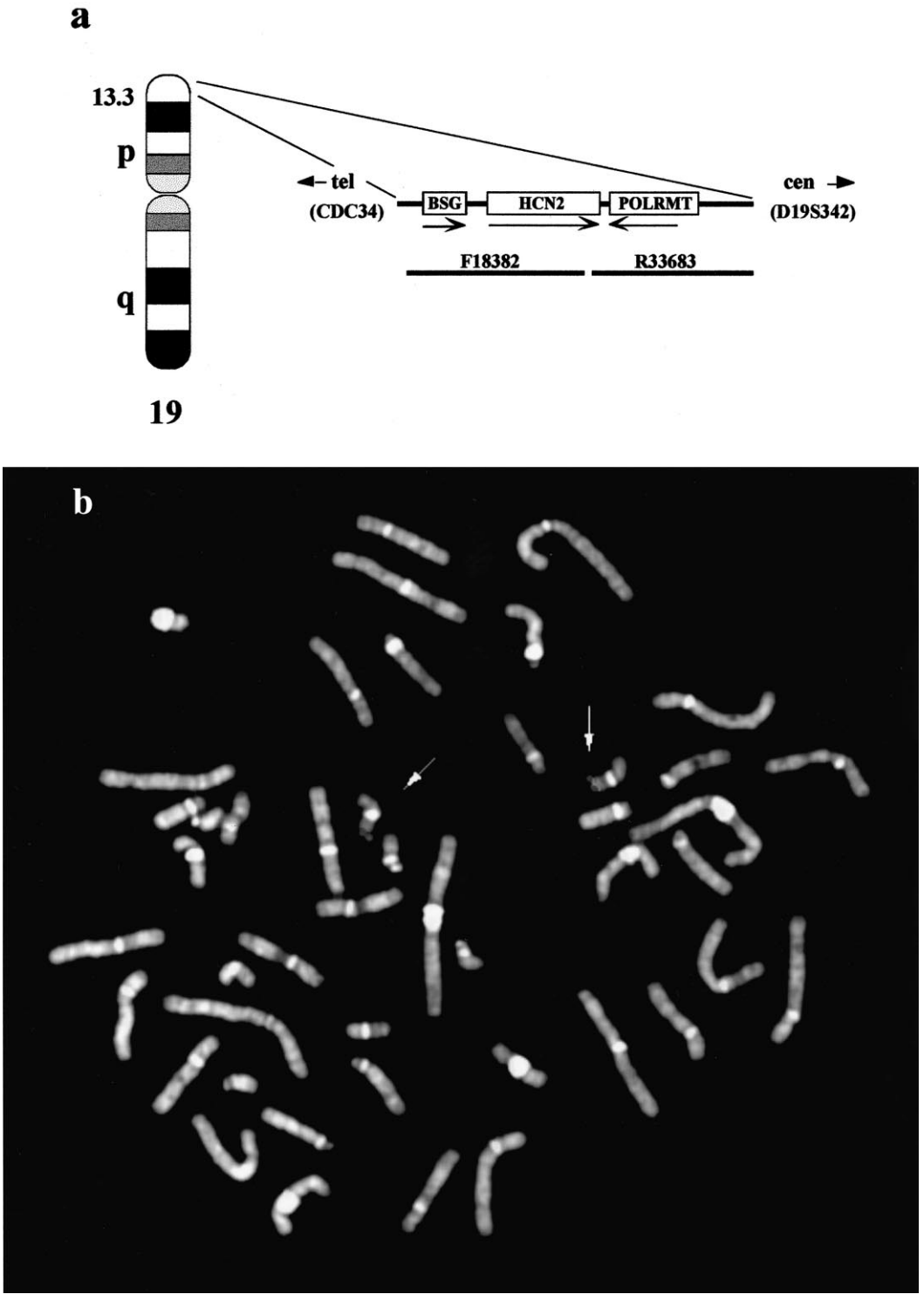

Recently, several cDNAs coding for these channels in mammals were independently cloned by different groups, and variously called BCNG1-4 [6], HAC1-3 [7] and IH1 (accession number AF065164). A cDNA for a similar channel in sea urchin sperm was named SPIH [8]. We are adopting here the HCN nomenclature proposed recently [9]. HCN proteins define a new subfamily among the cyclic nucleotide-gated

Fig. 1. The human *HCN2* gene maps to 19p13.3. (a) *HCN2* gene organization. The *HCN2* gene and the flanking markers *BSG* and *POLRMT* are sketched to scale in the upper part of the figure; an arrow below each marker indicates the direction of transcription. The orientation of the genes with respect to telomere and centromere, as well as outside markers, is indicated. Cosmids F18382 and R33683 covering this region are drawn as bars; they were both used as probes for FISH experiments. (b) FISH mapping of *HCN2* to 19p13.3. The hybridization signals are indicated by arrows. Chromosomes were stained with DAPI for identification. FISH experiments were conducted on normal human metaphase chromosomes using biotin-labeled cosmids as probes; signal was detected with Cy3-conjugated avidin (BDS). Digital images were obtained using a Zeiss Axioplan epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments, NJ). Fluorescence emitted by Cy3 and DAPI was detected using specific filter set combinations (Chroma Technology, VT) and was recorded separately as grayscale images. Pseudocoloring and merging of images were performed using Adobe Photoshop software.
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channels, which are characterized by six transmembrane domains (S1–S6) and a cAMP binding domain. The S4 domain is the voltage-sensing segment and the loop between S5 and S6 (the pore) regulates cation permeability [10].

We identified the human *HCN2* gene and deduced the coding sequence of the protein. Database search using information on the mouse brain-specific HCN1/BCNG1 protein revealed several human ESTs, including one related but not identical to the 3′ region of mouse HCN1/BCNG1 cDNA (GenBank accession no. N72770). We designed oligos and obtained a 548 nt fragment, related to HCN2, amplified from human cerebellum polyA$^+$ RNA by RT–PCR (using oligos FOR 5′-TTCCACATGACCTATGACC-3′ and REV 5′-CGGCAGTTGAAGTTGAC-3′ on oligo dT− retrotranscribed RNA). A new database search using this sequence as a query revealed the existence of a novel human genomic sequence, deriving from a sequencing project at LLNL (USA). Two contiguous but non-overlapping cosmids F18382 (AC005559, AC005577) and R33683 (AC004449), partly sequenced, were obtained from LLNL and their sequence was analysed with the GRAIL2 program for exon/intron predictions. We found that the two cosmids included the 5′ and 3′ portions of the human *HCN2* gene, respectively, and that the gap between them was covered by the *HCN2* fragment previously retrieved by reverse transcription–polymerase chain reaction (RT–PCR) from human cerebellum. Based on the above information we deduced the coding sequence of the human HCN2 protein (GenBank accession no. AF065164).

The human *HCN2* gene that we characterized is the first mammalian gene identified which codes for an HCN channel, since only cDNAs have been described so far. The gene spans at least 26 kb. The two cosmids F18382 and R33683 contain seven protein-coding exons. The genomic region between the cosmids (which we could not amplify by PCR) contains one or more exons, whose protein-coding sequence is completely derived from the cDNA. An additional putative noncoding exon is predicted by the GRAIL2 program at the 5′ end of the gene.

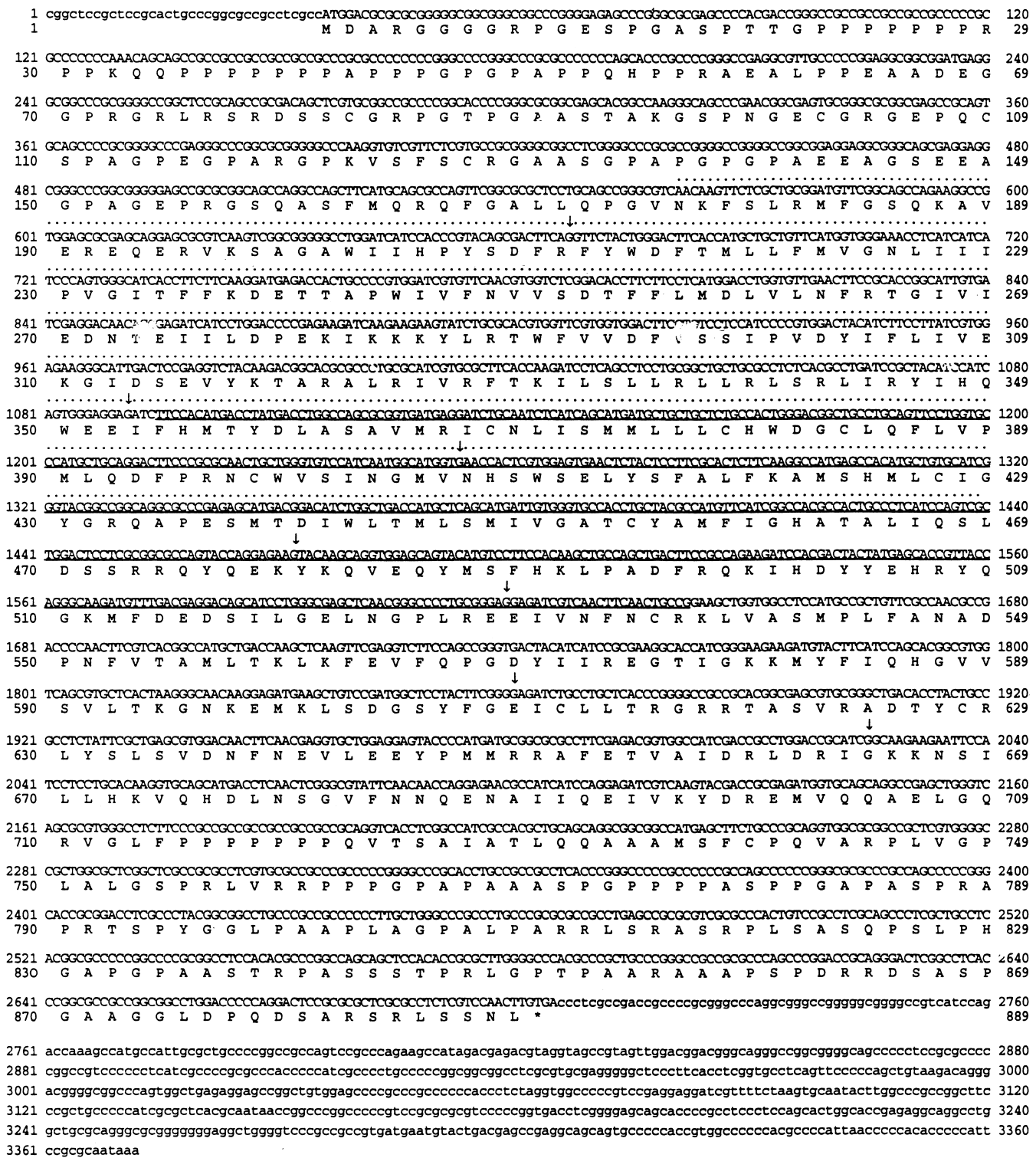
Sequence analysis of the two cosmids reveals that *HCN2* is flanked by *BSG* (basigin precursor; 7 kb upstream of *HCN2*) and *POLRMT* (DNA-directed RNA polymerase mitochondrial precursor; 1.5 kb downstream of *HCN2*) (Fig. 1a

The sequence around the initiation codon ATG is in good accordance with the Kozak consensus (A/GCCATGG) ([11], Fig. 1). A canonical polyadenylation signal, AATAAA, included at the 3′ end of the sequence shown in Fig. 1a, was found in some human ESTs (data not shown).

In order to map the newly characterized *HCN2* gene, fluorescence in situ hybridization (FISH) experiments were conducted on normal human metaphase chromosomes using biotin-labeled cosmids F18382 and R33683 as probes. Both hybridization signals were found to be located on the telomeric region of chromosome 19, band p13.3 (Fig. 1b). These results agree with the mapping done by LLNL.

The human *HCN2* produces a 3.4 kb transcript which codes for a 889-amino-acid protein. The encoded protein is a channel with six transmembrane domains and a cyclic nucleotide binding domain (Fig. 2a). A BLASTX search using the complete cDNA revealed that it is clearly homologous to mouse HCN2 (90% identity at protein level) and

a



b

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hHCN2  MDARGGGGRPGESPGASPTTGPPPPPPPRPPKQQPPPPPPPAPPPGPGPAPPQHPPRAEALPPEAADEGGPRGRLRSRDSSCGRPGTPGA   90
mHCN2  ----------D---TT-AP--------PA-P-PQ---    ----N-TTPSH           --S---P---A--C----A-     ---   72
mHCN1                                                                    M---GKPNSASNSRDD-NSVFPS    23
mHCN3                                                                                   MEEEAR     6

hHCN2  ASTAKGSPNGECGRGEPQCSPAGPEGPARGPKVSFSCRGAASGPAPGPGPAEEAGSEEAGPAGEPRG  SQASFMQRQFGALLQPGVNKF 178
mHCN2  -  ---GA----------   ---------------------S    A----------------  -----L--------------- 151
mHCN1              KAPATG-VAADKRLGT-P GGGAA-KEH-NSVCFKVDGGG-E-P--SFEDAE-PRR-YG------TSM-------- 98
mHCN3  PAAGAGEAATPAR    ETP-AAP AQA-          ----G                    VPE  -APEPKR--L-T----T----  59

hHCN2  SLRMFGSQKAVEREQERVKSAGAWIIHPYSDFRFYWDFTMLLFMVGNLIIIPVGITFFKDETTAPWIVFNVVSDTFFLMDLVLNFRTGIV 268
mHCN2  ---------------------------------------------------------------------------------------- 241
mHCN1  ------------K------T--F--------------LI--IM-----V---------TEQ--T-------A---V--L--IM-----T- 188
mHCN3  ---V---H----I----------------------LI--LL-----IVL--------E-NSP-------L------L--V-------- 149
                                  S1                                S2
hHCN2  IEDNTEIILDPEKIKKKYLRTWFVVDFVSSIPVDYIFLIV  EKGIDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHM 356
mHCN2  ---------------------------------------  ---------------------------------------------- 329
mHCN1  N--SS------KV--MN--KS------I------------  ---M------------------------------------------ 276
mHCN3  V-EGA--L-A-RA-RTR------L--LI----------V-EL-PRL-A--------------------------------------- 239
                        S3                                S4
hHCN2  TYDLASAVMRICNLISMMLLLCHWDGCLQFLVPMLQDFPRNCWVSINGMVNHSWSELYSFALFKAMSHMLCIGYGRQAPESMTDIWLTML 446
mHCN2  ----------------------------------SD---------------------------------------------------- 419
mHCN1  --------V--F---G-----------------L-----PD----L-E---D--GKQ--Y----------------A---V--S-L-I--- 366
mHCN3  --------V--F---G----------------------SD----M-R---H--GRQ--H----------------Q---VG-P-V------ 329
                  S5                                Pore
hHCN2  SMIVGATCYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYMSFHKLPADFRQKIHDYYEHRYQGKMFDEDSILGELNGPLREEIVNFNCR 536
mHCN2  ---------------------------------------------------------------------------------------- 509
mHCN1  ------------V-------------------------------M---------------I---EN--S--ND-------------- 456
mHCN3  ----------------------------------------T--R--E-------------E------SE------I--T-- 419
           S6
hHCN2  KLVASMPLFANADPNFVTAMLTKLKFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGNKEMKLSDGSYFGEICLLTRGRRTASVRADT 626
mHCN2  ---------------------------------------------------------------------------------------- 599
mHCN1  ----T-----------------S--R--------------AV-----------AG-I--SS-----T------------K------- 546
mHCN3  G---H-----H---S----V----R--------LVV---SV-R--------LL---AR-ARDTR-T------------------------ 509
                                                                  CNBD
hHCN2  YCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDRIGKKNSILLHKVQHDLNSGVFNNQENAIIQEIVKYDREMVQQAE LG         708
mHCN2  ----------------------------------------------------S--------------------------  --         681
mHCN1  ---------------------------------------------Q-F-K---T--------E-LKQ---H------AIPPINYPQMTAL 636
mHCN3  ----------H--A----F-----------M---R---------QRK RSEPSP-   SSGGVME-HL-QH--D-ARGVRG-A         588

hHCN2  QRVGLFPPPPPPPQVTSAIATLQQAAAMSFCPQVARPLVGPLALG  SP     RLVRRPPPGPA    PAAASP  GPPPPAS        778
mHCN2  ------ ------------------V-------------------  --     -----A----L    -P----  - --A--        750
mHCN1  NCTSSTTT-TSRMRTQ-PPVYTATSLSH-NLHSPSPSTQT-QPSAIL--CSYTTAVCSP-IQS-LATRTFHY---TASQLSLMQQPQQQL 726
mHCN3  PGT-ARLSGK-VLWEPLVH-P--A--VT-NVAIALTHQR---P-SPD--ATLLA-SA--SAGS--SPLV-VR-G-LLARG-W--       672

hHCN2               PPGAPASPRAPRTSPYGGLPAAPL  AG   PALPARRLSRASRPLSASQPSLPHG  APGPAASTRPASSSTPR   846
mHCN2               --A--S---------- -V-GS-ATRV-   -------------------------VP--S----A---------   821
mHCN1  PQSQVQQTQTQT(Q)37   -QT-GS-T       -KNEVHKSTQ--HNTN-TKEV-----------EV   STLI- --HPTVGESLA 834
mHCN3               TSRL--P-AR TLHASLSRTGRSQVSLLGPP-GGG----GPRG-----------QRATGD-   -P-RKG-       739

hHCN2                           LGPTPAARAAAPSPDRRDSASPGAAGGLD       PQDSARSRLSSNL 889
mHCN2                           ---A-T--T----------------S---       -L----------- 863
mHCN1  SIPQPVAAVHSTGLQAGSRSTVPQRVT-FRQMSSG-IP-NRGVPPAPP-P--VQRESPSVLNTDP-AEKP-FA--- 910
mHCN3                          GSERLPPSGLL-KPPGTVQPPRSSVPEPVTPRGPQISANM          779
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Fig. 2. The human HCN2 transcript and protein. (a) Sequence of the human HCN2 transcript (GenBank accession number AF065164). The ORF is uppercase, the 5′ and 3′ UTRs lowercase. The amino acid sequence is shown underneath the nucleotide sequence. The stop codon is marked by an asterisk. The arrows indicate the exon/intron junctions where known. The part of the sequence which is underlined corresponds to a RT–PCR product obtained from human cerebellum as follows: polyA$^+$ mRNA (Fast Track, Invitrogen) was retrotranscribed (First Strand c-DNA Synthesis kit, Boehringer) with oligo dT (5′-CAATCTGAAGTGGGAGCGGCCGCTTTTTTTTTTTTTTTTTT-3′) and amplified by PCR with oligos FOR (5′-aggtaccTTCCACATGACCTATGACC-3′, coding strand) and REV (5′-actcgagCGGCAGTTGAAGTTGAC-3′, noncoding strand). The part of the sequence marked by upper dots was derived from a commercially available cDNA (human brain cerebellum Marathon-ready cDNA Clontech) by PCR with oligos REV2 (5′-CCAGCTTCCGGCAGTTGAAGTTGA-3′, noncoding strand) and nested REV3 (5′-CGTTGAGCTCGCCCAGGATGCTGTC-3′, noncoding strand). The rest of the sequence was obtained by analysis of sequences from cosmids F18382 and R33683 and removal of putative introns. Additional sequence information from the LLNL Web Site was used to close the gap between the two cosmid sequences. (b) Multiple alignment of human HCN2 and mouse HCN1, 2, 3 proteins [7]. Residues identical to hHCN2 are indicated by a dash. A space indicates a gap. A stretch of 37 consecutive glutamines in mHAC2 is indicated as $(Q)_{37}$. The functional domains of the proteins are underlined (S1–S6: transmembrane domains, Pore and CNBD: cyclic nucleotide binding domain).

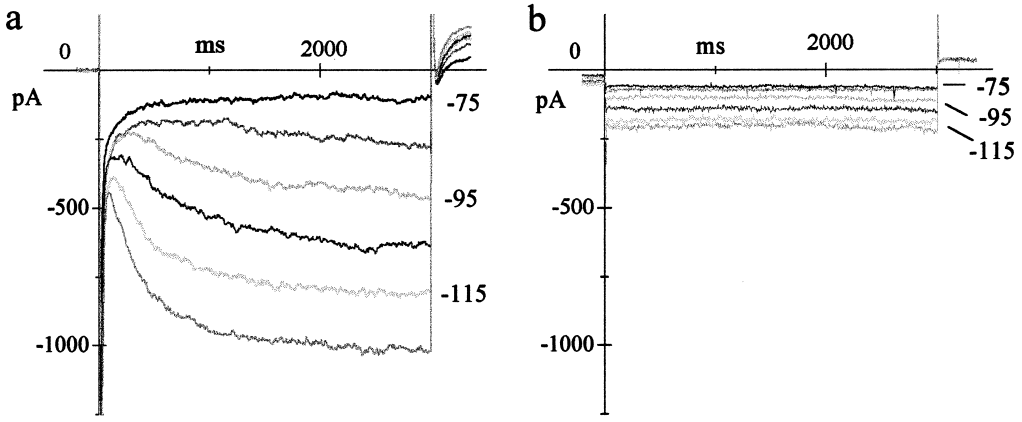

Fig. 3. Expression of *HCN2* in Phoenix cells. *hHCN2* minigene comprised the sequence between the *Kpn*I site 0.7 kb upstream of the translation initiation site and the *Pvu*II site in the 3′ UTR, cloned into the *Kpn*I site of pcDNA3.1 (Invitrogen). The construct was transiently expressed in Phoenix cells transfected using a Ca^{2+}-phosphate transfection protocol. 24–36 h after transfection, cells were dispersed by trypsinization and plated onto plastic Petri dishes at low density prior to electrophysiological measurements. Cells were perfused at room temperature by a high-K^+ Tyrode solution containing (mM): NaCl, 140; KCl, 30; $CaCl_2$, 1.8; $MgCl_2$, 1; D-glucose, 5.5; Hepes–NaOH, 5; pH 7.4, and patch-clamped in the whole-cell configuration. Patch pipettes contained (mM): NaCl, 70; KCl, 70; $CaCl_2$ 1.8; $MgCl_2$, 1; $BaCl_2$, 1; $MnCl_2$, 2; Hepes–KOH, 5; pH 7.4. (a) Traces recorded during 3-s hyperpolarizing steps from a holding potential of −35 mV to the range −75 to −125 mV, showing activation of the inward hyperpolarization-activated component. The current was sensitive to Cs^+ (5 mM CsCl, not shown). (b) Traces recorded during 3-s steps to the same voltage range (holding potential of −35 mV) from an untransfected cell.

highly similar to mouse HCN1 (68% identity at protein level) (Fig. 2b). At the DNA level, the homology between human HCN2 and mouse HCN2 (EMBL database accession no. AJ225122) extends beyond the coding sequence in the 5′ UTR.

HCN2 has been reported to be expressed in both brain and heart [6,7]. To express human *HCN2* we used a minigene which was derived partially from the genomic clones and partially from mRNAs retrotranscribed from human cerebellum, as indicated in Fig. 2a. The expression vector was pcDNA3.1(+) (Invitrogen), which carries a CMV strong promoter. This construct was co-transfected with a GFP-containing plasmid into modified HEK293 cells [12] for transient expression. We used patch clamp analysis on isolated cells to investigate the ability of expressed clones to yield an hyperpolarization-activated current. Visual inspection of transfected cells indicated a transfection efficiency of about 20%. In Fig. 3a, traces recorded from a transfected cell in the voltage range −75 to −125 mV from a holding potential of −35 mV show the presence of a hyperpolarization-activated current with properties similar to the native cardiac I_f in SA node myocytes [13]. The current was reduced by perfusion with 5 mM CsCl (not shown), in agreement with the known blocking action of this cation [14]. In Fig. 3b, records from an untransfected cell are shown for comparison. No hyperpolarization-activated current with properties similar to I_f was recorded in untransfected cells ($n = 19$).

In conclusion, we have sequenced and mapped on chromosome 19p13.3 the human *HCN2* gene whose heterologous expression produces a hyperpolarization-activated current with properties similar to the native cardiac 'pacemaker' current, I_f.

No loci responsible for cardiac dysfunctions have so far been localized to chromosome 19 band 13.3, but the identification of *HCN2* in this region will prompt reconsideration of those cardiac pathologies which do not appear to be associated to mutations in previously identified loci. Knowledge of the exon/intron structure will allow amplification of *HCN2* exons for analysis of possible harmful mutations. Also, an abnormal expression of I_f current in hypertrophic human ventricle has been reported recently [15] and awaits confirmation at the molecular level.

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