Hysteresis in human HCN4 channels: A crucial feature potentially affecting sinoatrial node pacemaking

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Abstract: The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels modulate and regulate cardiac rhythm and rate. It has been suggested that, unlike the HCN1 and HCN2 channels, the slower HCN4 channel may not exhibit voltage-dependent hysteresis. We studied the electrophysiological properties of human HCN4 (hHCN4) channels and its modulation by cAMP to determine whether hHCN4 exhibits hysteresis, by using single-cell patch-clamp in HEK293 cells stably transfected with hHCN4. Quantitative real-time RT-PCR was also used to determine levels of expression of HCNs in human cardiac tissue. Voltage-clamp analysis revealed that hHCN4 current (Ih) activation shifted in the depolarizing direction with more hyperpolarized holding potentials. Triangular ramp and action potential clamp protocols also revealed hHCN4 hysteresis. cAMP enhanced Ih and shifted activation in the depolarizing direction, thus modifying the intrinsic hHCN4 hysteresis behavior. RT-PCR analysis of human sinoatrial node (SAN) tissue showed that HCN4 accounts for 75% of the HCNs in human SAN while HCN1 (21%), HCN2 (3%), and HCN3 (0.7%) constitute the remainder. Our data suggest that HCN4 is the predominant HCN subtype in the human SAN and that Ih exhibits voltage-dependent hysteresis behavior that can be modified by cAMP. Therefore, hHCN4 hysteresis potentially plays a crucial role in human SAN pacemaking activity.

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hHCN4 的 $I_h$ 存在电压依赖性的滞后现象，且受 cAMP 调制。由此推断，hHCN4 通道的滞后现象可能在窦房结起搏活动中起到了关键作用。

关键词：HCN4 通道；滞后；窦房结；cAMP

Sinoatrial node (SAN) cells generate spontaneous action potentials (APs) that spread throughout the heart to provide a coordinated, rhythmic contraction of the heart. One mechanism of SAN pacemaking is an inward, non-selective, hyperpolarization-activated cation current (referred to as $I_f$ in cardiac cells) through hyperpolarization activated cyclic nucleotide-gated (HCN) channels[1, 2]. Four molecular subtypes (HCN1-4) of HCN channels have been identified in mammals[3, 4]. HCN1, HCN2, and HCN4 are the main subtypes in the heart. HCN4 is thought to be expressed in the SAN region of numerous mammalian species[5-8], including humans[9, 10]. Overexpression of HCN channels in neonatal ventricular myocytes not only shortened cycle length but also reduced beat-to-beat variability[11]. Inactivation of the HCN4 gene and $I_f$ inhibitors have shown that HCN4 mediates sympathetic stimulation of SAN pacemaker activity[7]. Mutations in HCN4 are associated with bradycardia in patients[10] and data from a transgenic HCN4-knockout mouse support the importance of HCN4 in cardiac automaticity[13]. $I_f$ appears to minimize cycle length variability and contribute to a more stable SAN rhythm.

When the current ($I$) – voltage ($V$) curve for the activation process of an ionic current differs from that for deactivation, the difference is referred to as a “hysteresis” in the voltage-dependence of the ion channel[14-16]. HCN channels exhibit the property of hysteresis in their voltage dependence[15, 16]. hHCN4 current ($I_h$) depends on the recent history of changes in voltage. HCN channels retain some memory of voltage changes in the recent past and this has been quantitatively described using the “mode-switching” model of Mannikko et al.[15]. Ionic currents with hysteresis are studied using particular voltage-clamp protocols, altering the holding potential ($V_h$) before a test pulse allows the investigation of the effect of the $V_h$ on activation and altering the speed of voltage ramps or AP clamp pulses allow the study of recent voltage changes on activation and deactivation in a more physiologically realistic setting.

Two recent studies[15, 16] have shown voltage-hysteresis in mouse HCN1 and HCN2 and in sea urchin spHCN. It has been suggested[16] that, unlike HCN1 and HCN2, the slower HCN4 may not possess hysteresis; however this conclusion was based on short-duration pulses for rabbit HCN4 (rbHCN4). Since HCN4 has the slowest kinetics of all the HCNs, voltage-clamp protocols with short test pulses or fast ramps may be too short to activate the channel. Pulses with durations on the order of the activation kinetics of HCN4 channels are needed for sufficient activation to reveal hysteresis. Similarly, only those ramp protocols that are slow enough to activate the channels will reveal any hysteresis. Extremely long pulse durations can, on the other hand, result in channels “forgetting” the recent history of voltage changes and therefore, diminish hysteresis.

We investigated the voltage-dependence of activation and deactivation of human HCN4 channels to determine whether HCN4 exhibits hysteresis.

Adrenergic stimulation increases intracellular cAMP which binds to HCNs[17] to accelerate HCN activation and increase current amplitude, resulting in an acceleration of heart rate. We assessed the effects of cAMP on hHCN4 hysteresis. Furthermore, we quantified HCN mRNAs in human SAN and right atrial tissue. Our data suggest that HCN4 is the predominant HCN subtype in the human SAN and that $I_h$ exhibits voltage-dependent hysteresis behavior that can be modified by cAMP.

1 MATERIALS AND METHODS

1.1 Establishing the stable cell line

Human embryonic kidney (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were propagated in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated at 50%-60% confluence and this has been quantitatively described using the “mode-switching” model of Mannikko et al.[15]. Ionic currents with hysteresis are studied using particular voltage-clamp protocols, altering the holding potential ($V_h$) before a test pulse allows the investigation of the effect of the $V_h$ on activation and altering the speed of voltage ramps or AP clamp pulses allow the study of recent voltage changes on activation and deactivation in a more physiologically realistic setting.

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1.2 Electrophysiology

Cells were placed in a chamber mounted on the stage of a fluorescence-equipped microscope (Nikon, Japan). The chamber was continuously superfused (~0.5 mL/min) with
the Tyrode solution (in mmol/L): NaCl 140; KCl 5.4; CaCl₂ 1.8; MgCl₂ 1; D-glucose 5; Hapes 10 (pH adjusted to 7.4 with NaOH). Cells with GFP fluorescence were detected visually and selected for recording using the whole-cell patch-clamp configuration[19]. Glass electrodes of ~3 MΩ resistance were connected via an Ag-AgCl wire to an Axopatch 200A amplifier and a DigiData-1322 acquisition system. Once the cell membrane was ruptured to form the whole-cell configuration, cell membrane capacitance was measured in each patched cell with the pCLAMP program (Axon Instruments, Foster City, CA, USA) running on a personal computer. Whole-cell membrane capacitance and series resistance were electrically compensated (~90%) to reduce artifactual distortion. A 2 kHz corner frequency low-pass Bessel filter was used and the A/D sampling rate was 5 kHz.

\[ I_h \] was elicited using 500 or 5 000 ms test pulses from -140 mV to 0 mV in 10 mV increments every 30 s. Hysteresis was examined with various \( V_{th} \) from -90 to +30 mV[16,20]. To prevent the influence of endogenous HEK293 ionic currents[18] on HCN4 hysteresis, the modified bath solution also contained 1 mmol/L NiCl₂ and 0.2 mmol/L CdCl₂ to block Ca²⁺ currents and Ca²⁺-activated currents, 2 mmol/L BaCl₂ to block inward rectifier K⁺ currents, and 1 mmol/L 4-aminopyridine to block transient outward K⁺ currents. The recording pipette solution contained (in mmol/L): K-glutamate 130; KCl 15; K₂ATP 5; MgCl₂ 1; EGTA 5; CaCl₂ 1; Hapes 10 (pH adjusted to 7.2 with KOH). All experiments were conducted at a room temperature (~22 °C).

1.3 Data analysis

\[ I_{tail}/I_{max} \] of \( I_h \) was used to obtain current-voltage activation curves[16,17,21,22]. Normalized activation data points were fitted using a least-squares method (Origin 7.5, MicrocalTM, Northampton, MA, USA) to a Boltzmann equation to determine the midpoint of voltage for activation, \( V_{1/2} \). The effective equation for the Boltzmann was: \[ (I_{max} - I_{min})/ [1+exp((V-V_{1/2}))/k] \]. \( I_h \) from AP pulses and ramp voltage protocols was corrected for currents due to cell capacitance (\( C_m \)) by performing a linear subtraction of estimated capacitative currents (\( C_m (dV/dt) \)) and analyzed by plotting the instantaneous membrane current \( I_h \) versus the corresponding membrane voltage value. Such a plot yields an \( I-V \) curve with segments for the activating and deactivating ramps; if these two segments do not overlap, they will form an ‘I-V loop’. The difference between the activation and deactivation segments, or the ‘depth’ of the \( I-V \) loops indicates the presence of hysteresis[15,16]. All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Student’s \( t \)-test or one way analysis of variance (ANOVA) with the Scheffe’s method was applied for statistical analysis of the data. Differences were considered significant for \( P \) values less than 0.05.

1.4 Quantitative real-time RT-PCR analysis of HCN mRNAs in human SAN and right atrium (RA)

Intact human SAN/RA samples were obtained from Dr. Peter Molenaar (University of Queensland, Australia). Samples were obtained from healthy hearts (\( n=4 \)) not used for transplantation. This work was approved by the Queensland Government and the University of Manchester ethical committee. This investigation conforms to the principles outlined in the Declaration of Helsinki.

SAN and RA samples were micro-dissected from 30 μm frozen tissue sections and total RNA isolated using the RNeasy Micro kit (Qiagen, Valencia, CA, USA). RNA concentrations were measured using a Nanodrop spectrophotometer (Labtech International LTD, East Sussex, UK). 100 ng of total RNA from the four SAN and four RA samples was reverse transcribed with Superscript III reverse transcriptase (Invitrogen) in a 20 μL reaction using random hexamer priming. Aliquots of the resulting cDNA were diluted 10-fold in water for direct use in PCR.

The relative abundance of mRNA for the four HCN subtypes (HCN1-4) was determined with quantitative PCR (qPCR) using an ABI 7900ht instrument with Power SyBr green and QuantiTect primer assays (Qiagen) for HCN1 (QT00048020), HCN2 (QT00020902) and HCN3 (QT00039935). For hHCN4 (GenBank Accession, NM_005477), the following primers were used: forward, 5’-AGCGCAAGGGCACCCTGAAC-3’; and reverse, 5’-TGATGGTTGAGGAGGATGAA-3’. All runs were 40 cycles in duration. For all identified transcripts within each sample, at least three separate measurements were made with 1 μL aliquots of each cDNA sample. Average threshold cycle (Ct) values were used in the E⁻ΔΔE (E = efficiency of the PCR reaction) calculations. The 28S ribosomal RNA reference gene was used to normalize the data.

2 RESULTS

2.1 Shifts in hHCN4 activation with various \( V_{th} \)

We used voltage-step protocols with two durations and various \( V_{th} \) (see Fig. 1A, B) to assess the factors that affect hHCN4 activation. \( I_h \) amplitude increased with more negative test potentials. Although the \( V_{1/2} \) of activation obtained from short (500 ms) pulses do not represent the midpoint of activation in a Boltzmann fit, we use the term...
here with the understanding that it is relevant for understanding non-equilibrium behavior as in Azene et al. [16].

The “$V_{1/2}$” for short pulses was shifted from a value of (-112±2.3) mV for $V_h$ of 30 mV to (-100±10.1) mV for $V_h$ of -50 mV (Fig. 1C and Table 1, n=6, P<0.05). A more significant $V_h$-dependent shift in $V_{1/2}$ was observed with the longer (5 000 ms) test pulses (Fig. 1D, E and Table 1). Boltzmann fits of voltage dependent activation were better with the long pulses as activation approached saturating conditions and yielded “true” $V_{1/2}$ values in the biophysical sense. $V_{1/2}$, in the case of the long pulses, was shifted in the depolarizing direction, from (-94 ±2.6) mV to (-75±4.8) mV when $V_h$ was changed from 30 mV to -50 mV (n=6, P<0.05). Comparing the $V_{1/2}$ values for $V_h$ of 0 mV and -50 mV, the net change was significantly greater (Fig. 1E) for 5 000 ms duration pulses ($\Delta V_{1/2}$=25.3 mV) than for 500 ms pulses ($\Delta V_{1/2}$=11.8 mV). $I_h$ activation curves obtained using either test pulse durations were not significantly shifted for $V_h$ in the range of +30 mV to -40 mV (Fig. 1C, D, Table 1, $P>0.05$). Activation curves for both pulse durations were significantly shifted in the depolarizing direction for $V_h$ values of -70 mV and -90 mV. A significant shift in $V_{1/2}$ with a change in the $V_h$ was interpreted as an indication of the extent of hysteresis[16].

A previous report[16] showed that mouse HCN1 channels do not exhibit hysteresis with long test pulses (3 000 ms). This is most likely due to these faster channels having a shorter “memory”. Compared with other HCN subtypes, HCN4 channels are activated at a much slower time scale and require a longer time to reach an equilibrium level[16, 21]. To examine the dependence of hHCN4 hysteresis with longer test pulses, we used test pulse durations of up to 13 s

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Fig. 1. HCN4 activation with different holding potentials ($V_h$). A, B: Superimposed traces of hHCN4 currents ($I_h$) were evoked using different voltage pulses with $V_h$ of 0, -40, or -70 mV. Note the two different pulse durations: 500 ms (A) and 5000 ms (B) and corresponding time scales in the abscissas (X-axes). C, D: Activation curves plotted using normalized tail currents versus the test pulse voltages. Currents were activated by 500 ms (C) or 5 000 ms (D) test pulses from -140 mV to 0 mV (see protocols in A and B) for $V_h$ from 30 mV to -90 mV (see symbol legends). Tail currents were measured at the dotted lines shown in the protocols in panel A and B and normalized to the corresponding maximal tail currents of -140 mV. E: $V_{1/2}$ of HCN4 activation plotted versus $V_h$ for 500 ms (○) and 5 000 ms (●) test pulses.
Figure 2. Activation of hHCN4 channels with longer test pulses. A: Voltage clamp waveforms: 13 s test pulses from -140 mV to 0 mV in 10 mV increments every 30 s with a holding potential of 30 mV. B: Superimposed current traces of \( I_h \) evoked using the waveforms shown in panel A. C: The magnified current trace from the square area in panel B shows that the activation reaches a steady-state or equilibrium level >10 s after hyperpolarization from 30 mV to -140 mV. D: Activation of HCN4 channels with the various holding potentials obtained from the tail currents plotted versus the corresponding test voltages. Tails currents were measured at the dashed line in panel A and normalized to the corresponding maximal tail currents of -140 mV.

Values shown are mean ± SEM. Numbers in the parentheses are the numbers of cells in each group. Test pulses of two durations (500 ms and 5 000 ms) were used (see protocols in Fig. 1). Tail currents were normalized and plotted against test voltages (see Fig. 1). Statistical significance was determined using ANOVA analysis. *\( P<0.05; ** P<0.001 \) compared to corresponding values at the voltages ≥ -50 mV for 5 000 ms test pulses. Note: the \( V_{1/2} \) values of the activation of HCN4 channels for the pulse duration 500 ms with the holding potentials at -70 mV and -90 mV are not applicable, because more than 50% channels were not deactivated even at the most positive voltage (0 mV) test pulse used in our protocol (see Fig. 1). The \( V_{1/2} \) values for 13 000 ms test pulses (see Fig. 2) were obtained from that representative myocyte (#, holding potential at -60 mV).
the faster HCN1, HCN2, and spHCN channels\cite{23} and suggests that hHCN4 channels share this property with the faster channels, albeit at a slower time scale.

2.2 Hysteresis of hHCN4 during AP pacing

As dynamic AP clamp can be a powerful tool used in studying gene-based biopacemaker\cite{24} and as the cardiomyocyte membrane potential changes constantly during a cardiac cycle, we used AP waveform voltage-clamp protocols (Fig. 3B) to assess the role of hHCN4 hysteresis during a cardiac cycle. Pacing rates between 6 and 60 beats/min (bpm) were used. At 6 bpm, $I_h$ increased steadily during phase 4 membrane depolarization (Fig. 3A, upper panel), most likely due to the slow kinetics of hHCN4. In contrast, at 30 or 60 bpm, $I_h$ shows deactivation during phase 4 membrane depolarization (Fig. 3A, middle and low panels). AP-induced $I_h$ was plotted against the corresponding voltage values to detect differences during the activation and deactivation segments of cardiac pacemaker cell APs. Figure 3C shows $I-V$ loops from -30 mV to -90 mV of $I_h$ evoked by AP clamps. The current activated by AP clamp was confirmed as $I_h$ which was blocked by Cs (Fig. 3D). Hysteresis is more obvious in the 10-s cardiac cycle, consistent with the idea that, up to a point, increased activation of HCNs reveals hysteresis.

2.3 Relationship between hHCN4 hysteresis and voltage-clamp speed

We tested hHCN4 voltage hysteresis further using ramp voltage-clamp protocols, ramping from -40 mV to -140 mV to activate $I_h$ and back to -40 mV to deactivate it (Fig. 4B). Figure 4A shows the different amplitudes of $I_h$ elic-

![Fig. 3. Hysteresis in the voltage-dependence of hHCN4 currents in response to a train of action potentials. A: Raw current traces (colored) were elicited by action potential clamp waveforms (black, also see panel B) of various cycles: 6 beats per minute (bpm) (10 s duration, red brown), 30 bpm (2 s, red), and 60 bpm (1 s, blue). C: Time data from panel A replotted as current-voltage ($I-V$) loops. The upper part (arrow going down) of each loop corresponds to the repolarization phase of the action potential or the activation of hHCN4 and the lower part of the loop (arrow going up) corresponds to the phase 4 depolarization. Note the hysteresis with 10 s cardiac cycle. The arrows indicate the direction of activation and deactivation. D: Cesium (Cs) block of hHCN4 currents. hHCN4 currents elicited by 5 s test pulses from 0 to -130 mV every 30 s are shown in the absence (Control and Washout) and presence of 5 mmol/L Cs.](image-url)
Yong-Fu Xiao et al: Hysteresis in hHCN4 Channel

The fastest ramp (400 mV/s) protocol elicited small currents (Fig. 4A) while the slowest (10.5 mV/s) evoked the largest $I_h$. Figure 4C and D show $I-V$ loops for hHCN4 activation and deactivation. The slow deactivation process resulted in residual currents at the end of the pulse and the $I-V$ loop was “open” when the voltage was ramped back to -40 mV. The increased hysteresis in response to slow ramps can be attributed to stronger activation and also to the fact that hHCN4 deactivation is even slower than activation. $I-V$ loops narrowed with increasing ramp speeds to the point of almost complete overlap at the fastest (400 mV/s) ramps (Fig. 4C, D). Such an overlap suggests insufficient activation of hHCN4 channels during the fast ramp rather than a lack of hysteresis. These results also explain the inability to detect hysteresis behavior in HCN4 channels in previous studies using short activation pulse durations.

2.4 Voltage-dependent deactivation of hHCN4 channels

In order to examine the contribution of deactivation to hHCN4 hysteresis, the $V_H$ was set at -70 mV, at which point approximately 50% of hHCN4 channels are activated (Table 1), followed by voltage steps to various depolarized potentials to deactivate channels. A final pulse to -140 mV was used to assess the extent of deactivation (insets in Fig. 5A, B). Figure 5A and B show that deactivation depended strongly on depolarizing potentials and pulse durations. Figure 5C shows representative current traces elicited by the shortest (a, 50 ms) and longest (b, 2 000 ms) deactivation pulses (inset, Fig. 5C). Data points were...
fitted with the single exponential equation yielding time constants (τ) of 362 and 739 ms for depolarizing voltages of 30 mV and 0 mV, respectively (Fig. 5D). Human HCN4 deactivation was much slower with a depolarizing voltage of -30 mV, and even slower and incomplete at -50 mV (Fig. 5D). These results suggest that hHCN4 deactivation kinetics is strongly dependent on the depolarizing pulse voltage. This observation is important in understanding the mechanisms behind hysteresis in hHCN4 channels.

2.5 Effects of cAMP on hHCN4 hysteresis

We tested the ability of cAMP to modulate hHCN4 channel hysteresis using extracellular application of the membrane-permeable cAMP analog, 8-Br-cAMP. 8-Br-cAMP (200 μmol/L) enhanced the maximal amplitude of Ih by 39.4% ± 13.4% (n=11, P<0.01) elicited by 5 000 ms voltage-step pulses from -40 mV to -130 mV. Figure 6A shows that 200 μmol/L 8-Br-cAMP increased Ih evoked by a voltage ramp protocol. The activation of the I-V loop of Ih was shifted in the depolarizing direction in the presence of cAMP.

HCN4 activation curves from tail current analysis show depolarizing shifts in the presence of 200 μmol/L 8-Br-cAMP (Fig. 6B). V1/2 shifts were significant (Fig. 6C) for 5 000 ms test pulses with V_H of either 0 mV (P<0.001, n=5) or -40 mV (P<0.05, n=9). V1/2 changes did not reach statistical significance for 500-ms pulses (Fig. 6C), as it was impossible to determine V1/2 values reliably when cAMP caused large shifts in activation curves (control, n=4; cAMP, n=2; control, n=7; cAMP, n=4; for V_H=0 mV and V_H=-40 mV, respectively). Nevertheless it can be observed that cAMP has a consistent modulatory effect on hHCN4 hysteresis.

Fig. 5. Voltage-dependent deactivation of hHCN4. Membrane potential was held at -70 mV to partially activate hHCN4 channels. Depolarizing voltages (A, 30 mV; B, -50 mV) with 50 ms pulse-duration increments were applied to deactivate hHCN4 channels and a final hyperpolarizing pulse to -140 mV to reactivate Ih. A and B: Superimposed hHCN4 current traces elicited by two test protocols (insets). C: Current traces elicited by 50 ms and 2 000 ms depolarizing pulses to 30 mV to deactivate hHCN4 channels. HCN4 currents were measured at the time-points indicated by the arrows marked a and b. The values of the initial currents were plotted against the durations of the depolarizing pulses (D) for various depolarizing voltages of 30, 0, -30 and -50 mV. Data points generated by 30 and 0 mV test pulses were fit well by a single exponential function term, but not those generated by -30 or -50 mV pulses.
Fig. 6. Effects of cAMP on hHCN4 activation and deactivation. A: I-V loops show an increase in $I_h$ amplitude and earlier activation with 200 μmol/L 8-Br-cAMP (red trace). The ramp protocol was composed of a 10 s hyperpolarization from the holding potential of 0 mV to -140 mV followed by a 10 s depolarization from -140 mV back to 0 mV (see inset). I-V loops of control are in black and cAMP treatment in red. cAMP modulation of hHCN4 hysteresis was also observed with step test pulses (B, C).

B: Activation curves were plotted with the normalized tail currents on the ordinates versus the test pulse voltages on the abscissas for the test pulse durations of 5 000 ms. The cell membrane potential was held at 0 mV. C: Changes in $V_{1/2}$ shifts in the absence and presence of cAMP at the holding potentials of 0 mV and -40 mV with 500 ms (open bar) and 5 000 ms (filled bar) test pulses. Data shown are mean±SEM of the delta changes before and after cAMP. *P<0.05; **P<0.01; ***P<0.001; comparison between with and without cAMP.

2.6 Quantitative real time RT-PCR analysis of HCNs in human SAN and atrial tissues

Previous studies have used RNase protection assays [9] and in situ hybridization [6] to study levels of expression of HCNs in rabbit, rat and mouse SAN. A recent report used RT-PCR analysis to indicate that, in qualitative terms, hHCN4 was expressed at higher levels in human SAN. In order to assess the role of hHCN4 channels in human SAN more quantitatively, we analyzed mRNA samples for the presence of the four HCN subtypes in intact human SAN and RA tissues using quantitative real time RT-PCR. Figure 7 shows that all four HCN subtypes were found in human SAN and RA tissue. The averaged mRNA levels of HCN1 ($P<0.05$) and HCN4 ($P<0.01$) were significantly greater in the SAN than in RA and hHCN4 was the most abundant isoform in the SAN. In contrast, the mRNA level of HCN2 was significantly higher in the RA than in SAN ($P<0.01$). HCN3 levels in both tissues were very low, both, in absolute value and in relative terms, less than 1% of the total HCN expression. On average, HCN4 accounted for 75% of the total HCN content in sinoatrial node (SAN) samples and 19% in right atrial (RA) samples. Data shown are mean±SEM ($n=4$). Significant differences between RA and SAN are shown. *P<0.05; **P<0.01.
of the total HCN expression in human SAN and 19% of
that in the RA. In SAN samples, HCN1, HCN2, and HCN3
accounted for 21%, 3%, and 0.7% of the total HCN mRNA.
While levels of expression of HCN2 were low in SAN
samples, it accounted for the highest proportion (48%),
almost half, of HCNs in the RA samples with HCN1 (32%),
HCN3 (0.9%), and HCN4 (19%) constituting the
remainder. These results clearly demonstrate that HCN4 is
the predominant subtype in the human SAN.

3 DISCUSSION

Mannikko et al. proposed a quantitative model for the
mechanism underlying hysteresis: HCN channels exist in
two modes with different voltage dependencies. In this
“mode-shift” model, the voltage-dependence of HCN chan-
nels depends on prior activity, and this “memory” effect is
observed as hysteresis. Azene et al. showed that non-
equilibrium activation of the mouse HCN1 (mHCN1) chan-
nel reveals hysteresis properties. We found that hyster-
esis in the voltage-dependence of hHCN4 channels is marked-
dly dependent on $V_{\text{h}}$ (Fig. 1 and Table 1) and durations,
including depolarizing ramp speeds (Fig. 4). As would be
expected in a process with a finite-duration “memory” of
the recent past, prolonged duration (13 s) voltage-step pulses resulted in a greatly reduced degree of hysteresis
(Fig. 2) in hHCN4 channels. Human HCN4 activation is
slow and saturating currents for a test potential of -140
mV requires at least 10 s under our recording condition at
a room temperature (~22 °C). However, hHCN4 activa-
tion to reach a steady-state level can be much shorter at
the body temperature (37 °C). We conclude that near-equ-
librium activation of hHCN4 channels due to prolonged
pulse durations reduces hysteresis. This result is consist-
tent with the previous finding that mHCN1 channels ex-
hibit no hysteresis when activation reaches an equilibrium
level. At the other extreme, hysteresis was not observed for
100-ms duration voltage-step pulses nor for 400 mV/s
ramps (Fig. 4) due to insufficient activation.

Although Azene et al. proposed that the slow gating
and pronounced delay in rbHCN4 activation attenuated the
extent of its hysteresis, a more recent study indicates that
HCN4 may undergo mode shifts similar to those that give
rise to hysteresis in the spHCN channel. Our data clearly
indicate that hHCN4 does exhibit hysteresis. One explana-
tion for this apparent disagreement is the difference in spe-
cies in the two studies, i.e. rabbit versus human. Another is
the difference in voltage-clamp analysis. Azene et al. used
a train of 500 ms-duration APs to assess rbHCN4 hyster-
esis (Fig. 4 in reference). Short-duration APs that result
in insufficient channel activation will not reveal the latent
hysteresis phenomenon in HCN4. To avoid this situation,
we varied the durations of voltage pulse steps, ramp clamps
and AP waveforms. Our results showing a lack of hysteresis
with short duration waveforms is consistent across the
protocols in our experiments as well as previous results.
Our results showing hysteresis with longer-duration wave-
forms is consistent across the various protocols and with
hysteresis being caused by a “memory” effect. Finally, we
(Fig. 1, 2) used tail currents to analyze hHCN4 hysteresis
as Elinder et al. have shown that in channels like HCN2
and HCN4, with relatively slow activation and fast mode-
shift transitions, mode shift effects are not readily observ-
able except in the tail current kinetics.

We found that cAMP increased $I_h$ and shifted the activa-
tion segment of hHCN4 $I-V$ loops in the depolarizing di-
rection (Fig. 6). Our results are consistent with previous
findings on cAMP-induced shifts in HCN voltage-depend-
dence. Our data suggest, further, that cAMP can modify
the extent of hysteresis in hHCN4 channels.

The deactivation time constant ($\tau$) of hHCN4 channels
doubled when the depolarized potential was changed from
30 mV ($\tau = 362$ ms) to 0 mV ($\tau = 739$ ms, Fig. 5). hHCN4
deactivation was even slower for depolarizing steps to -30
mV or to -50 mV, suggesting that part of hHCN4 hyster-
esis may be attributed to incomplete deactivation. For
example, at -50 mV, only 8% of $I_h$ deactivated after 2 s
(Fig. 5B, D). Longer durations and more depolarizing volt-
ages are required to deactivate HCN channels for more
negative holding potentials. Therefore, hHCN4 hysteresis
at $V_{\text{h}}$ of -70 or -90 mV with 5 000 ms test pulses in Fig. 1
most likely results from incomplete channel deactivation,
as deactivation is considerably slower at these potentials.
The slow deactivation also explains the depth of $I-V$ loops
seen with AP clamp (Fig. 3) and ramp protocols (Fig. 4).

The biophysical hysteresis behavior of hHCN4 is likely
to play a role in “remembering” SAN membrane potential
changes in the recent past, allowing dynamic fine-tuning of
the pacemaking rate. Slowing down heart rate to <60
bpm (>1 s cycle length) would increase hHCN4 hysteresis
(Fig. 3) which leads to increasing heart rate (Fig. 8). Since hHCN4 activation is slow, fast heart rates (>100 bpm) will decrease $I_h$ and reduce
HCN4 hysteresis (Fig. 3 and 4). Thus hysteresis provides
a negative feedback mechanism based on recent events in
the past to stabilize heart rate (Fig. 8). Since HCN4 is the
predominant HCN subtype in human SAN (Fig. 7), its
voltage-dependent hysteresis is likely to play a critical role.

Yong-Fu Xiao et al.: Hysteresis in hHCN4 Channel

Fig. 8. Proposed model of regulation of SAN pacemaking by HCN hysteresis and cAMP modulation. Any slowing down of the heart rate (to the left of the diagram) increases hysteresis during an action potential (top part of the diagram) thus increasing residual $I_f$. The increased $I_f$ accelerates SAN pacemaking rate which, in turn, causes reduced hysteresis behavior and a concomitant reduction in residual $I_f$. These changes occur over a few SAN action potentials while cAMP modulates this hysteresis behavior on a longer time-scale of a few minutes. An increase in cAMP increases overall hysteresis, $I_f$ and, thus, pacemaking rate. The self-regulating negative feedback inherent in hHCN4 hysteresis opposes this rate increase. Similarly, a reduction in cAMP decreases $I_f$ and slows down the heart but, once again, the inherent hysteresis of hHCN4 will stabilize heart rate variability.

In heart-rate regulation. In addition, heart rate and rhythm can be modulated by numerous neuronal, humoral, or mechanical factors in a complicated way, but sympathetic stimulation increases intracellular cAMP levels, which modify HCN hysteresis and increase $I_f$, thus increasing heart rate (Fig. 8). While cAMP-mediated effects may last several minutes, intrinsic hHCN4 channel hysteresis regulates heart rate on a beat-to-beat basis. Therefore, hHCN4 hysteresis and cAMP modification of hysteresis may be working together to fine-tune SAN pacemaking rate (Fig. 8).

In the current study, hHCN4 kinetics was assessed at a room temperature and its hysteresis occurred relatively slow. However, hHCN4 hysteresis can be accelerated at the body temperature. Further, other HCN isoforms, such as HCN1 and HCN2, coexist with HCN4 and make the molecular heteromers of native $I_f$ channel complex. The stoichiometric contributions to functional HCN channels are still unknown, but HCN4 is the dominate isoform and its expression can be used as a tool to map and identify the cardiac SAN pacemaker region [26]. HCN1 and HCN4 are most likely the subunits to form hetero-HCN-channels in human SAN, because these two are the major isoforms there (Fig. 7). A recent study also showed that different composition ratio of the HCN isoforms significantly affected HCN current kinetics [27]. Importantly, hysteresis has been demonstrated for both HCN1 [36] and HCN4 [21]. Therefore, hHCN4 hysteresis potentially plays an important role in human SAN activity in vivo.

Recently, genetically engineered cardiac biopacemakers have been examined in experimental animal models [28-34], including approaches that use gene transfer of HCN1, HCN2 and HCN4 [29, 30, 33, 35, 36]. HCN hysteresis is likely to play a significant role in HCN-generated biological pacemakers. We have demonstrated in this study that HCN4 is the predominant HCN subtype in human SAN and have shown that hHCN4 channels exhibit significant voltage-dependent hysteresis which can be modulated by intracellular cAMP. Hysteresis is an essential property of hHCN4 and helps to maintain, fine-tune and regulate heart rate in a dynamic, continuous and beat-to-beat fashion. Knowledge and insight from this and future studies could be useful in optimizing HCN gene constructs for biological pacemakers.

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REFERENCES


