Molecular Characterization of the Hyperpolarization-activated Cation Channel in Rabbit Heart Sinoatrial Node*

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We cloned a cDNA (HAC4) that encodes the hyperpolarization-activated cation channel (I_f) by screening a rabbit sinoatrial (SA) node cDNA library using a fragment of rat brain I_f cDNA. HAC4 is composed of 1150 amino acid residues, and its cytoplasmic N- and C-terminal regions are longer than those of HAC1–3. The transmembrane region of HAC4 was most homologous to partially cloned mouse I_f BCNG-3 (96%), whereas the C-terminal region of HAC4 showed low homology to all HAC family members so far cloned. Northern blotting revealed that HAC4 mRNA was the most highly expressed in the SA node among the rabbit cardiac tissues examined. The electrophysiological properties of HAC4 were examined using the whole cell patch-clamp technique. In COS-7 cells transfected with HAC4 cDNA, hyperpolarizing voltage steps activated slowly developing inward currents. The half-maximal activation was obtained at \(-87.2 \pm 2.8\) mV under control conditions and at \(-64.4 \pm 2.6\) mV in the presence of intracellular 0.3 mM cAMP. The reversal potential was \(-34.2 \pm 0.8\) mV in 140 mM Na\(^+\), and 5 mM K\(^+\), versus 10 mM Na\(^+\), and 145 mM K\(^+\). These results indicate that HAC4 forms I_f in rabbit heart SA node.

The hyperpolarization-activated cation channel (I_f)\(^1\) was first described in rabbit heart sinoatrial (SA) node (1, 2). The I_f current was characterized by activation by hyperpolarizing voltage steps; mixed permeability for Na\(^+\) and K\(^+\); inhibition by extracellular Cs\(^+\), not by Ba\(^{2+}\); and a positive shift in the voltage-dependent activation curve by intracellular cyclic nucleotide (1–6).

Based on the above physiological properties, the functional roles of I_f in the SA node have been discussed in many publications. I_f is one of the inward currents that generate pacemaker depolarization (7–9). The pacemaker cells of the SA node are coupled to surrounding atrial myocytes through gap junctions. Since atrial myocytes have more negative resting membrane potentials, they hyperpolarize pacemaker cells electrotonically (10). Pacemaker cell I_f is activated under this condition; therefore, the inward I_f current is likely to limit the level of hyperpolarization of pacemaker cells (1).

Recently, three full-length (mouse BCNG-1, -2, and -4; corresponding to HAC2, -1, and -5, respectively) and one partial (mouse BCNG-3) mammalian cDNA clones encoding I_f were isolated from a mouse brain cDNA library (11–14), and one cDNA clone was isolated from sea urchin testis (SPIH) (15). However, despite the physiological significance of I_f in the SA node, its molecular characteristics still remain unclear. Therefore, we have cloned a rabbit heart SA node cDNA library and isolated a cDNA (HAC4). HAC4 is composed of 1150 amino acid residues and most likely encodes I_f. In this study, we demonstrate the amino acid sequence of HAC4, the distribution of HAC4 mRNA in cardiac tissue, and the electrophysiological properties of HAC4 heterologously expressed in COS-7 cells.

EXPERIMENTAL PROCEDURES

Molecular Biology—The cloning procedure was performed as described previously (16). Briefly, cDNA templated by mRNA isolated from rabbit heart was used as a DNA template for polymerase chain reaction amplification. The 5’ (P1) and 3’ (P2) sequences were derived from mouse HAC2 (13) and are as follows (N represents A/G/C/T): P1, 5’-ATG(C/T)TNTG(T/C)AT(T/C/A)GGNTA(T/C)GG-3’; and P2, 5’-AT/A/G/TAMCNGG(T/C)TG(A/G)AA-3’. Polymerase chain reaction amplification was performed according to the following schedule: five cycles at 94 °C for 1 min, 46 °C for 1 min, and 72 °C for 2 min, followed by 26 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The polymerase chain reaction products were electrophoresed on a 1% agarose gel, excised, and purified with QIAEX II (QIAGEN Inc.) for subsequent subcloning and sequence determination. Through this procedure, we identified a clone for a rat homologue of mouse HAC2, pR1. A cDNA library was prepared from rabbit heart SA node regions. Using pR1 as a probe, 5 × 10^6 phase clones of the cDNA library were hybridized for the isolation of a new clone (washed with 2 × SSC at 55 °C). Five positive clones were isolated and sequenced. All of them were identical. A representative clone, pHI1, was subjected to sequence determination. Both strands of the cDNA sequence were determined by the chain termination method (BigDye Terminator Cycle Sequencing, Applied Biosystems, Inc.). The clone contains 3396 base pairs of cDNA that comprises a large open reading frame. However, the cDNA does not possess a termination codon in the C-terminal region. Therefore, we performed 3’-rapid amplification of cDNA ends (RACE) polymerase chain reaction amplification, CLONTECH) and obtained the C-terminal region of the clone by using probeprinting Taq polymerase (LA Taq, TaKaRa). We added the C-terminal region amplified by the probeprinting Taq polymerase to pH1I by using a SacI restriction site. Northern blots, prepared with 2 μg of poly(A)^+ mRNA isolated from the indicated rabbit tissues (atrium does not contain the SA node region), were probed in hybridization solution (Life Technologies, Inc.) and 50% formamide at 42 °C with a radiolabeled DNA fragment derived from the coding region of pH1I (corresponding to amino acids 707–1116), washed with 0.1% SSC and 0.1% SDS at 65 °C, and exposed to x-ray film at −80 °C with an intensifying screen for 84 h.

Functional Expression and Electrophysiological Measurements—HAC4 cDNA and green fluorescent protein S65A cDNA (a gift from Dr. K. Moriyoshi) were subcloned into a DNA template for vectors (Promega), and the mixture of vectors were transfected into COS-7 cells using LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s instructions. The vector amounts were 1.6 μg/35-mm dish for HAC4 and 0.4 μg/35-mm dish for green fluorescent protein. COS-7 cells (Riken) were cultured on coverslips in Dulbecco’s modified essential medium...
supplemented with 10% fetal calf serum (Life Technologies, Inc.) and antibiotics.

36–48 h after transfection, a coverslip was transferred to the recording chamber on an inverted microscope (TMD300, Nikon), and patch-clamp experiments were carried out in green fluorescent protein-positive cells using Axopatch 200B amplifier (Axon Instruments, Inc.). The data were directly recorded on the hard disk of an IBM-PC compatible computer through an AD converter (Digipack 1200, Axon Instruments, Inc.) and were analyzed using commercially available software (pClamp6 and Clampex7, Axon Instruments, Inc.). The data points represent the means ± S.E. The statistical difference was evaluated using Student's unpaired t test.

The composition of the bathing solution was 140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES. The pH was adjusted to 7.4 with NaOH. Appropriate amounts of KCl, CsCl, and BaCl₂ were added in some experiments. The pipette solution contained 130 mM KCl, 5 mM HEPES, 5 mM EGTA, 5 mM MgATP, and 5 mM disodium creatine phosphate. The pH was adjusted to 7.4 with KOH. The final K⁺ concentration was ~145 mM. In some experiments, 0.3 mM cAMP was added to the pipette solution, and the pH was readjusted to 7.4 with KOH. All patch-clamp experiments were carried out at 35 °C by perfusing the bathing solution through a water jacket.

**RESULTS**

cDNA Cloning of HAC4—The transmembrane region of rat HAC2 was amplified by the polymerase chain reaction method with a rat brain cDNA mixture and was used to screen a rabbit SA node cDNA library. The most 5’ methionine codon in the positively hybridizing clone (pIH1) initiated an open reading frame that did not contain a termination codon in the 3’-terminal region. 3’-Rapid amplification of cDNA ends was performed to obtain the 3’-terminal region, and pIH1 lacked 103 base pairs of the 3’-coding region. The 3’-terminal region was amplified by proofreading T7 polymerase and attached to pIH1. The sequence (HAC4) (Fig. 1) predicts a protein of 1150 amino acid residues with six transmembrane domains, a pore region, and a cyclic nucleotide-binding domain. HAC4 shows 85–90 and 80–96% identities to HAC1–3 in the transmembrane region and the cyclic nucleotide-binding domain, respectively. HAC4 is most related to mouse BCNG-3. Although full-length mouse BCNG-3 cDNA has not been cloned, the partial sequence of mouse BCNG-3 was 96% homologous to the transmembrane region of HAC4. The predicted N- and C-terminal regions of HAC4 are notably longer than those of the rest of the HAC family. The last five amino acid residues (corresponding to positions 1146–1150) at the C terminus were conserved in all HAC clones. The amino acid sequence corresponding to positions 1045–1055 was also conserved in all HAC clones. The rest

**FIG. 1.** Amino acid sequence of HAC4 and comparison with other HAC channels. HAC1–3 were cloned from mouse, whereas HAC4 was from rabbit. Alignments were generated by eye; dashes represent gaps introduced to optimize the alignment. The six predicted transmembrane domains (S1–S6), the pore region (P), and the cyclic nucleotide-binding domain (CNBD) are underlined. Amino acid residues that are conserved between HAC4 and the rest of the HAC family are boxed. The asterisks indicate stop codons. Amino acid numbers for the full-length coding sequences are given on the right.

**FIG. 2.** Northern blot analyses of HAC4 mRNA distribution. Poly(A)⁺ mRNA (2 μg), isolated from the indicated tissue sources, was loaded in each lane. Sizes (kilobases) are indicated to the left. HAC4 mRNA (top arrow) was mainly expressed in the SA node, but not clearly in the atrium, ventricle, forebrain, or cerebellum. Glyceraldehyde-3-phosphate dehydrogenase (bottom arrow) was used as a control.
of the C-terminal regions were remarkably diverse.

Expression Patterns of HAC4 mRNA—To examine the expression of HAC4 mRNA, we performed Northern blotting (Fig. 2). Since the C-terminal regions are diverse among HAC family members, we synthesized a radiolabeled DNA fragment derived from the coding region corresponding to amino acids 707–1116. It is clear from Fig. 2 that HAC4 was the most highly expressed in the SA node among the cardiac tissues examined. Unlike HAC1–3, HAC4 was not significantly expressed in brain. The size of the mRNA for HAC4 was estimated to be 7.1 kilobases, although presumably a partially processed transcript was detected.

Voltage-dependent Gating of HAC4—Fig. 3A shows representative current traces of HAC4 expressed in COS-7 cells. When a voltage step was more negative than −60 mV, slowly activating inward currents were activated. Such a current was not observed when COS-7 cells were transfected with an empty vector (data not shown). The current activation appeared to be the sum of two exponential time courses. For example, time constants of current activation were 384 ± 71 and 2275 ± 319 ms at −110 mV (n = 6). These values were similar to those reported for the I_f current of rabbit SA node cells (17). Fig. 3B shows the current-voltage (I-V) relationship. The closed circles indicate the amplitude of the initial current measured at the beginning of hyperpolarizing pulses. The open circles are the steady-state I-V relationship measured at the end of pulses. It is clear from Fig. 3B that the threshold of current activation was between −60 and −70 mV.

The voltage dependence of current activation was further analyzed by measurements of outward tail currents (Fig. 3C). Under control conditions, hyperpolarizing voltage steps to −40 and −50 mV did not elicit the outward tail current. When the voltage step was −60 mV, a miniature tail current was recorded. The amplitude of the tail current increased as the conditioning voltage became more negative and saturated at −140 mV. It is well known that intracellular cAMP produces a positive shift in the voltage dependence of the activation of the I_f and HAC1 currents in a dose-dependent manner. The maximal effect was obtained at −0.1 mM cAMP (4, 13). In accordance with these reports, an obvious tail current was elicited by hyperpolarization to −50 mV in the presence of intracellular 0.3 mM cAMP (Fig. 3C, right panel). In Fig. 3 (C and D), the outward tail current (I_{tail}) measured at 0 mV was normalized by the maximal value (I_{max}) in the presence (▼) and absence (▲) of 0.3 mM cAMP. In each experiment, we fitted the Boltzmann function to the data points: I_{tail}/I_{max} = 1/(1 + exp (V_m − V_h)/S), where V_m is the test potential, V_h is the membrane potential for half-maximal activation, and S is the slope factor. Under control conditions, V_h = −87.4 ± 2.8 mV, and S was 9.6 ± 0.8 mV (n = 10). In the presence of 0.3 mM cAMP, the value of V_h shifted toward the positive direction (−64.4 ± 2.6 mV (n = 7); p < 0.01), whereas S did not change significantly (7.9 ± 0.4 mV (n = 7); p > 0.05). The shift in V_h was 23 mV, which was clearly larger than those in other mammalian clones such as mouse BCNG-1 (corresponding to HAC2; 2 mM cAMP) (12) or HAC1 (12 mM cAMP) (13).

Ion Pore Properties of HAC4—The I_f current of the SA node and other HAC clones is inhibited by extracellular Cs^+ and divalent cations (3, 7, 12, 13, 15, 17, 18). We examined the effects of Cs^+ and Ba^{2+} on HAC4 currents as shown in Fig. 4A. The magnitude of the block was estimated by measuring the amplitude of time-dependent currents in the presence and absence of blockers. At −90 mV, 3 mM Cs^+ partially blocked the HAC4 current. The magnitude of the block was 70.8 ± 5.4%, which was smaller than reported for mouse BCNG-1 and HAC1. The outward tail current was also suppressed by extra-

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**Fig. 3. Voltage-dependent gating of HAC4.** A, a representative current recording of the HAC4 current. The pulse protocol is shown in the top. The dotted line indicates zero current level. The initial current and the steady-state current were measured at the times indicated (● and ○), respectively. B, I-V relationships measured at ● and ○. The data points were averaged from 6 to 10 cells. C, expanded traces of the outward tail current. Two sets of current traces were recorded in different cells. The voltage of conditioning pulse is indicated at the corresponding tail current. The amplitude of the tail current (I_{tail}) was the difference between the peak current measured at the time indicated by the marks and the steady-state current at the end of the 0-mV voltage step. D, voltage-dependent activation curves measured in the presence (▼; n = 7) and absence (▲; n = 10) of 0.3 mM cAMP. Abscissa, voltage of conditioning hyperpolarization; ordinate, I_{tail} normalized by the maximal value in each experiment (I_{max}). V_h and S (see “Results”) were measured in each experiment, and the fitted lines were drawn using their average values. V_h = −87.4 ± 2.8 mV (control) and −64.4 ± 2.6 mV (0.3 mM cAMP; p < 0.01); S was 9.6 ± 0.8 mV (control) and 7.9 ± 0.4 mV (0.3 mM cAMP; p > 0.05). In all experiments, the extracellular potassium concentration was 5 mM.
cellular Cs\(^+\). 3 mM Ba\(^{2+}\) blocked only 9.6 ± 2.0\% of the HAC4 current.

It is well known that both Na\(^+\) and K\(^+\) permeate \(I_f\) in native pacemaker cells (3, 5, 6). In Fig. 4B, we examined the ion selectivity of HAC4 by measuring the reversal potential, which was determined by intersections between the \(I-V\) curve of initial tail currents and the \(I-V\) curve of outward currents overlap each other. C, replacement of extracellular Na\(^+\) by Li\(^+\). In 140 mM Na\(^+\) and 140 mM Li\(^+\) solutions, the K\(^+\) concentration was 5 mM. Three sets of current traces were recorded in the same cell. It should be noted that the outward tail currents in 140 mM Li\(^+\) solution are clearly larger than those in 140 mM Na\(^+\) solution. The pulse protocol was same as described for B. The dotted lines indicate the zero current level.

In this study, we have cloned a cDNA (HAC4) and demonstrated that HAC4 encodes \(I_f\). HAC4 is composed of 1150 amino acid residues. Both the cytoplasmic N- and C-terminal regions of HAC4 are longer than those of HAC1–3. We could not exclude the possibility that the N terminus of HAC4 was not complete because we have not obtained a clone that has an in-frame stop codon in front of the assigned start codon. However, the N-terminal region contained enough length and showed weak but significant homology compared with other HAC clones. Moreover, the cDNA of HAC4 generated robust functional \(I_f\) currents when expressed in COS-7 cells. These findings might support the idea that the cDNA of HAC4 contains the entire coding sequence. The C-terminal region of HAC4 showed remarkably low homology compared with other HAC clones. The amino acid sequence of the transmembrane region of HAC4 was 89\% homologous to HAC1 (mouse BCNG-2), 86\% to HAC2 (mouse BCNG-1), 85\% to HAC3 (presumably a splicing variant of mouse BCNG-4), and 97\% to mouse BCNG-3 (14). The partial sequence of the N-terminal region (82 amino acid residues) of mouse BCNG-3 was also homologous to HAC4 (89\%). Therefore, HAC4 may be the rabbit homologue of mouse BCNG-3. It is difficult, however, to conclude this matter because only a partial sequence of mouse BCNG-3 has been determined (506 amino acids), and in particular, the amino acid sequence of the C-terminal region of mouse BCNG-3 has not been determined.

Although HAC4 and mouse BCNG-3 are highly homologous, their expression patterns seem different; in mouse, BCNG-3 is expressed in cardiac tissue, but not in the SA node. BCNG-3 is also expressed in mouse brain and skeletal muscle (12). In contrast, HAC4 mRNA is highly expressed in rabbit heart SA node. In other parts of cardiac tissues, brain, and skeletal muscle, HAC4 signals are not significant. We do not have ready explanations for this difference. In Northern blotting, we used the sequence that corresponds to amino acids 707–1116. Because the amino acid sequence of the corresponding region of mouse BCNG-3 had not been determined, we could not exclude the possibility that the C-terminal region was not homologous between HAC4 and mouse BCNG-3. Another possibility may be that the difference expression pattern is simply due to the difference of species.

In accordance with the distribution of HAC4 mRNA, the electrophysiological properties of HAC4 closely resembled those of the \(I_f\) current reported in native pacemaker cells isolated from rabbit SA node. In native pacemaker cells, particularly during \(\beta\)-adrenergic stimulation, the separation of \(I_f\) and delayed rectifier K\(^+\) (\(I_{\text{IK}}\)) currents was difficult because the activation of \(I_f\) was overlapped by the deactivation of \(I_{\text{IK}}\) (1, 19).
In this study, there was no time-dependent current in COS-7 cells other than the heterologously expressed HAC4 current, which enabled us to measure voltage-dependent gating of $I_f$ more precisely. The threshold of current activation was between $-60$ and $-70$ mV under control conditions and between $-40$ and $-50$ mV in the presence of intracellular 0.3 mM cAMP. The $V_h$ of HAC4 ($-87.4$ mV) was more positive than that of HAC1 ($-103$ mV) or mouse BCNG-1 (HAC2; $-100$ mV). In HAC4, the positive shift in $V_h$ was $23$ mV in the presence of 0.3 mM cAMP, which was clearly larger than that in HAC1 (12 mV by 0.1 mM cAMP) or mouse BCNG-1 (2 mV by 3 mM cAMP). In native pacemaker cells, the activation curve of $I_f$ was considerably different from cell to cell (3, 8). This variety may be explained in two ways. Intracellular cAMP concentration may varyably different from cell to cell (3, 8). This variety may be explained in two ways. Intracellular cAMP concentration may differ from cell to cell; alternatively, $I_f$ in the SA node may be a heteromultimer composed of different HAC subunits, and their composition may be different from cell to cell, although we do not have information on the expression level of other HAC clones in rabbit SA node. In the latter case, it appears reasonable to expect that the heteromultimer may possess a property intermediate between those of HAC4 and other HAC family members.

The functional role of $I_f$ in the SA node is to generate pacemaker depolarization (7, 8, 9) and to limit the hyperpolarization of pacemaker cells caused by electronic coupling with atrial myocytes (1, 10). The significance of $I_f$ in the generation of pacemaker potential still appears to be a matter of debate (17, 20–23). Targeting of the HAC gene should provide a clue to addressing this question. To conduct such an experiment, it is essential to determine the molecular characteristics of $I_f$ in the SA node. In this study, we have demonstrated that HAC4 forms $I_f$ in the SA node. This molecular identification of HAC4 in the SA node would facilitate the understanding of the physiological function of $I_f$.

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