The Murine HCN3 Gene Encodes a Hyperpolarization-activated Cation Channel with Slow Kinetics and Unique Response to Cyclic Nucleotides*

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Hyperpolarization-activated cation channels of the HCN gene family are crucial for the regulation of cell excitability. Importantly, these channels play a pivotal role in the control of cardiac and neuronal pacemaker activity. Dysfunction of HCN channels has been associated with human diseases, including cardiac arrhythmia, epilepsy, and neuropathic pain. The properties of three HCN channel isoforms (HCN1, HCN2, and HCN4) have been extensively investigated. By contrast, due to the lack of an efficient heterologous expression system, the functional characteristics of HCN3 were by and large unknown so far. Here, we have used lentiviral gene transfer to overexpress HCN3 in HEK293T cells. HCN3 currents revealed slow activation and deactivation kinetics and were effectively blocked by extracellular Cs⁺ and the bradycardic agent ivabradine. Cyclic AMP and cGMP had no significant impact on activation kinetics but induced a 5-mV shift of the half-maximal activation voltage (V₀.5) to more hyperpolarized potentials. A negative shift of V₀.5 induced by cyclic nucleotides is an unprecedented feature within the HCN channel family. The expression of HCN3 in mouse brain was examined by Western blot analysis using a specific antibody. High levels of protein were detected in olfactory bulb and hypothalamus. In contrast, only very low expression was found in cortex. Using reverse transcriptase PCR hybrids were also detected in heart ventricle. In conclusion, the distinct expression pattern in conjunction with the unusual biophysical properties implies that HCN3 may play an unique role in the body.

The hyperpolarization-activated cation current, termed Iₜ or Iₜ, is widely expressed in heart cells and neurons. The current is best known for its prime role in the generation of rhythmic activity in cardiac and neuronal pacemaker cells (1, 2). Iₜ is also present in several types of non-pacing neurons where it contributes to various physiological functions, including the setting of the resting membrane potential, synaptic transmission, and dendritic integration. Recent evidence suggests that Iₜ is involved in diseases making the channel a promising target for drug therapy. For example, the dysfunction of cardiac Iₜ was identified in patients suffering from sick sinus brady-

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cardia (3, 4). Furthermore, it was proposed that overexpression of Iₜ in heart ventricle is associated with cardiac hypertrophy (5). Ivabradine (S-16257-2), a blocker of Iₜ, is currently considered as a novel drug in the therapy of tachycardic arrhythmia and angina pectoris (6). Iₜ is also likely to participate in neurological diseases. In particular, there is accumulating evidence that the current is involved in epileptogenesis (7, 8). Moreover, overexpression of Iₜ was observed in rat models of peripheral nerve injury suggesting a potential role of this current in driving neuropathic pain (9).

Iₜ is encoded by a family of four hyperpolarization-activated cyclic nucleotide-gated (HCN1–4) channels (1, 10). HCN channels are members of the 6-transmembrane superfamily of cation channels (11). A structural hallmark of all HCN channels is a cyclic nucleotide-binding domain in the C terminus that confers sensitivity to cAMP (12, 13). So far, the functional properties of three members of the HCN channel family (HCN1, HCN2, and HCN4) have been extensively studied using heterologous expression. Moreover, the specific physiological relevance of these channels has been defined using gene-targeting approaches in mice (8, 14, 15). By contrast, there is only sparse information on the properties of the HCN3 channel (9, 16). In situ hybridization and immunocytochemistry indicated that this channel is expressed at low levels in rat and mouse brains (17–19). The biophysical and pharmacological properties of the channel are by and large unknown.

In the present study we set out to address this important issue. We investigated the expression level of murine HCN3 in mouse brain regions using Western blot analysis. Moreover, we achieved robust expression of the channel in HEK293T cells using lentiviral expression vectors. We show that HCN3 reveals some properties shared by other HCN channel types but is unique among these channels by being rather inhibited than activated by cyclic nucleotides.

MATERIALS AND METHODS

Lentiviral HCN2 and HCN3 Expression Vectors—The mouse HCN3 (mHCN3) coding sequence was excised as a 2.7-kb HindIII-SpeI fragment from the plasmid mHCN3/pcDNA3 (16). The mHCN3 was cloned via XbaI and SpeI sites into pBluescript II KS plasmid containing the IRES-EGFP coding sequence (Clontech). Lentiviral plasmid LV-HCN3 was prepared by replacing the FPF coding sequence with the XbaI-Sall HCN3-IRESEGFP fragment. A schematic representation of the HCN3 lentiviral ex...
pression vector is given in Fig. 2. The lentiviral HCN2 expression vector LV-HCN2 was constructed accordingly by cloning the coding region of mHCN2 (20) into the LV vector. Recombinant lentivirus as well as lentiviral particles were prepared as described previously (21).

Cell Culture—HEK293T cells (DSMZ, Braunschweig, Germany) infected with lentiviruses as previously described (21) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C with 10% CO₂ in T25, gelatin-fibronectin-coated flask (Sigma). For electrophysiological experiments, glass coverslips were coated in 24-well dishes with poly-1-lysine (Sigma), and 30,000 cells/well were seeded.

RT-PCR—Total RNA was isolated using TRIzol (Invitrogen) and subsequently treated with DNase I (Roche Applied Science). First strand cDNA was synthesized from 5 μg of RNA with the Superscript II H-Kit (Invitrogen) using oligo(dT) primers. HCN3 was amplified from 0.5 μl of cDNA using following primers and conditions: 5’-GTCGCCCGGGGCTGGAT-3’ (forward), 5’-CCCTCCACTGTTGTATGTC-3’ (reverse); 40 cycles at 60 °C. Amplicons were separated on 5% polyacrylamide gels, stained with ethidium bromide, and visualized on a Gel Doc 2000 system (Bio-Rad). The primer pairs were intron-spanning to avoid amplification of genomic DNA.

Generation of Anti-HCN3 Antibody and Western Blot—Polyclonal rabbit antibodies against the C-terminal (amino acids 552–779) region of murine HCN3 was generated by immunization (Grazmach Laboratories; Schwabhausen, Germany) with a His-Tag fusion protein expressed and purified using the QiaExpress-Kit (Qiagen, Germany) and affinity-purified using the Amino-Link-Kit (Pierce).

To determine the specificity of the anti-HCN3 antibody, membrane proteins were isolated from HEK293 cells transfected with mHCN1, mHCN2, or mHCN4 as described previously (22) and subjected to Western blot analysis with anti-HCN3. HEK293T cells transduced with LV-HCN3 were analyzed using the same protocol.

Tissue from various mouse brain regions was dissected and snap frozen in liquid nitrogen. Samples were homogenized on dry ice using a mortar and pestle, boiled in lysis buffer (2% SDS, 50 mM Tris) for 10 min, and centrifuged (15 min at 16,000 g) to remove cell debris. Proteinase K (Roche) was used to lyse the remaining tissue and to degrade RNA. Proteins were separated by a 10% SDS-PAGE gel and electrotransferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in TBS (10 mM Tris, pH 7.4, 150 mM NaCl) and incubated with primary antibodies. The blots were then washed and visualized using a SuperSignal West Pico kit (Pierce). Proteins were analyzed by densitometric scanning using a computer program (ImageJ).

Electrophysiology—Currents were measured 2–3 days after infection with recombinant lentivirus using whole-cell patch clamp technique. Patches were equilibrated for at least 2 min before experiments to minimize rundown of current. The standard extracellular solution was composed of 110 mM NaCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.4, adjusted with NaOH. Whole-cell currents were recorded with a recentrified, ivabradine (S 16257-2) and Cs⁺ blocking solution containing 110 mM NaCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 110 mM CsCl, 5 mM HEPES, pH 7.4, adjusted with KOH. For determining the cAMP and cGMP sensitivity of HCN channels intracellular solution was supplemented with 0.5 mM cAMP or cGMP. The extracellular solutions were exchanged by a local solution exchanger. The different solutions reached the cell membrane within less than 100 ms. All recordings were obtained at room temperature. Data were acquired at 10 kHz using an Axopatch 200B amplifier and pClamp 8 (Axon Instruments). Voltage clamp data were stored on the computer hard disk and analyzed off-line by using Clampfit 8 (Axon Instruments) and Origin (Origin Lab Corporation). For determination of the voltage of half-maximal activation (V₅₀) currents were elicited by hyperpolarizing the membrane from 3 s to voltages ranging from −140 to −20 mV (in 10-mV increments) from a holding potential of −40 mV followed by a 500 ms step to −140 mV. Amplitude of tail currents, determined immediately after the disappearance of the capacitive transient, were normalized to the maximal current (Iₘₐₓ) and plotted as a function of the preceding membrane potential. The data points were fitted with the Boltzmann function, \( I - I_{\text{min}} \times \exp [(V - V_{\text{m}}) / k]) \), where \( I_{\text{min}} \) is an offset caused by a non-zero holding current, \( V_{\text{m}} \) is the test potential, \( V_{\text{m}} \) is the voltage of half-maximal activation, and \( k \) is the slope factor. Time constants of channel activation (\( \tau_{\text{on}} \)) of HCN2 and HCN3 channels were determined by monoeponential function fitting the current evoked during hyperpolarizing voltage pulses to appropriate voltage.
HCN3 Encodes a Hyperpolarization-activated Current with Slow Kinetics—EGFP-positive cells were used for electrophysiological analysis. HCN3 currents were compared in side-by-side experiments with HCN2 overexpressed in HEK293T cells using lentiviral vectors. The current density obtained for HCN3 was approximately five times smaller than the current density in HCN2-expressing cells (27 ± 5 pA/pF, n = 29 for HCN3 versus 139 ± 26 pA/pF, n = 26 for HCN2). Fig. 3, A and B show representative whole-cell current traces elicited by a family of hyperpolarizing steps from a holding potential of −40 mV. Both currents are composed of a fast component I_{act} and a slowly activating sigmoidal component. The relative amplitude of the I_{act} was consistently larger in HCN3 (13 ± 2% of total current, n = 19) than in HCN2 (5.0 ± 0.7%, n = 20). HCN3 activated and deactivated with significantly slower kinetics than HCN2. The activation time constants at a fully activating membrane potential (−140 mV) were τ_{act} = 470 ± 30 ms (n = 13) for HCN3 and τ_{act} = 330 ± 50 ms (n = 9) for HCN2. By contrast, HCN2 and HCN3 did not differ from each other, with respect to their voltage of half-maximal activation (V_{0.5} = −95 mV, Fig. 3D). In addition, the reversal potential obtained from the I-V curve of the fully activated channel was not different between the two channels (−27 mV, data not shown) indicating that both channels share the same ion selectivity.

HCN3 currents revealed the typical pharmacological profile of native and heterologously expressed H_{i,n} channels. The channel was readily blocked by 2 mM extracellular Cs⁺ (Fig. 4A). Likewise, the bradycardic drug ivabradine (6) almost completely inhibited the fully activated current at a concentration of 30 μM. This concentration was used previously to block specifically H_{i,n} in sino-atrial node cells (24) (Fig. 4B).

Cyclic Nucleotides Shift the Activation Curve of HCN3 to More Negative Voltages—We next went on to test the effect of cAMP on lentivirally expressed HCN channels. Fig. 5, A and B show representative normalized current traces of fully activated HCN2 and HCN3 channels (at −140 mV) obtained either with 2 mM extracellular Cs⁺. B, blocking effect of 30 μM ivabradine on the HCN3 current at −140 mV (n = 5 and 6, respectively).

By contrast, perfusion with 0.5 mM cAMP did not significantly alter the kinetics of the HCN3 current (τ_{act} = 470 ± 30 ms (n = 13); τ_{act} = 510 ± 30 ms (n = 11)). Cyclic AMP shifts the voltage dependence of all HCN channels characterized so far to more depolarized potentials. Indeed, the voltage curve of lentivirally expressed HCN2 was
about +10 mV more positive in the presence than in the absence of cAMP (V_{0.5} = −98 ± 1 mV versus −85 ± 1 mV (n = 13 and 9, respectively); Fig. 5F). Interestingly, the effect of cAMP on the HCN3 activation curves (Fig. 5D) was profoundly different from that on HCN2 (Fig. 5C). cAMP reduced the steepness of the I-V curve (k (−cAMP) = 9.6 ± 0.6 mV (n = 18); k (+cAMP) = 11.8 ± 0.7 mV (n = 15)). As a consequence, the half-maximal activation potential was slightly but significantly shifted to more negative values (V_{0.5} = −95 ± 1 mV versus −100 ± 2 mV (n = 18 and 15, respectively); p < 0.05; Fig. 5F).

Cyclic GMP modulated HCN3 currents in a similar manner as cAMP did. Intracellular application of 0.5 mM cGMP did not significantly alter the kinetics of the fully activated HCN3 current (τ_{act} (−cGMP) = 470 ± 30 ms (n = 15); τ_{act} (+cGMP) = 520 ± 50 ms (n = 17) at −140 mV; Fig. 6A). At less hyperpolarizing conditions cGMP tended to slow down HCN3 current kinetics (Fig. 6B). However, this effect was not statistically significant (p > 0.05). In the presence of cGMP the same behavior was observed (data not shown). Like cAMP, cGMP shifted the voltage dependence of HCN3 activation slightly to more negative values (V_{0.5} = −95 ± 1 mV versus −101 ± 1 mV (n = 18 and 17 in the absence and presence of cGMP, respectively; Fig. 6C). This effect was statistically significant (p < 0.05) and resulted from the change in the steepness of the I-V curve (k (−cGMP) = 9.6 ± 0.6 mV (n = 18); k (+cGMP) = 11.3 ± 0.6 mV (n = 17)).

Thus, with respect to V_{0.5} values, cyclic nucleotides regulated HCN2 and HCN3 in opposite direction. Because the voltage dependence of HCN2 activation determined in our study was entirely consistent with previous data on that channel the unique behavior of HCN3 was genuine and not an artifact resulting from the lentiviral expression system used.

**DISCUSSION**

Our knowledge of the HCN channel family has increased dramatically since the first cloning of the channels 7 years ago (20, 26, 27). Despite the principal progress made, one member of the family, HCN3, was enigmatic up to now. In this study, we set out to determine for the first time the basic characteristics of this channel.

Using lentiviral gene transfer we achieved robust expression of the channel in HEK293T cells. Although we consistently found i_{h} in cells that were successfully infected (as indicated by green fluorescence of coexpressed EGFP), current densities of HCN3-expressing cells were about five times smaller than those seen with HCN2. By contrast, protein levels of HCN3 were not significantly different from those of HCN2 (data not shown). Thus, the smaller amplitude of HCN3 currents probably reflects an intrinsic property of the HCN3 channel molecule (e.g., lower open probability or smaller single-channel conductance) or alternatively, may point to the absence of channel components (e.g., auxiliary subunits) that are missing in our expression system and are necessary for normal channel activation. Finally, the possibility that the discrepancy observed is because of lower cell surface expression of HCN3 must be also considered.

HCN3 currents activated significantly slower than HCN2 currents expressed under the same conditions. The HCN3 activation constant was well within the range of that observed for HCN4 (25). It should be noted that the τ_{act} determined for HCN2 in the present study (330 ms at −140 mV) was also somewhat larger than previously found (20) suggesting that the expression system may exert some minor influence on the channel kinetics.

The most surprising feature of HCN3 was its unique re-
sponse to cyclic nucleotides. In contrast to all other HCN channel types described so far, the activity of HCN3 was not enhanced by these second messengers. Rather, cAMP and cGMP changed the slope of the voltage dependence of activation, inducing a slight but significant shift (−5 mV) of the activation curve to more negative voltages. The effect of cyclic nucleotides was apparent at membrane voltages more negative to −90 mV. Hence, cAMP and cGMP inhibited rather than activated the HCN3 current. According to a cyclic allosteric model (28) the free energy available from ligand binding should be coupled to enhance gating, which in the case of HCN channels, should result in the shift of I-V curve to more positive values and faster kinetics (29). Such a phenomenon was indeed observed for HCN2 and HCN4 (30). However, in the case of HCN3, despite the requirement of rather strong hyperpolarization and slow kinetics of gating, the binding of cyclic nucleotides resulted in the reverse effect, shifting the I-V curve to more negative values. This apparent discrepancy could be reconciled in terms of the recent ligand-gating model (12, 31, 32). According to this model, the binding of cAMP controls the transition in the oligomerization state of cyclic nucleotide-binding domain between the 4-fold symmetric tetrameric gating ring (enhancing the gating) and 2-fold symmetric dimer of dimers (suppressing gating). This transition should be facilitated through the interactions in the C-linker, connecting the cyclic nucleotide-binding domain with the gate. Interestingly, it has been shown recently that a tripeptide mutation in the C-linker of HCN2 reversed the polarity of ligand gating (33). The mutation converted cAMP from an agonist that facilitated channel opening into an inverse agonist that inhibited channel opening. Therefore, it is plausible that the observed inhibitory effect of ligand binding on gating of HCN3 could result from the formation of the 4-fold symmetric tetrameric gating ring in the absence of cAMP because of the amino acid composition of its C-linker or its cyclic nucleotide-binding domain. Similarly to the situation observed in the mutated HCN2 channel (33), ligand binding would facilitate the transition into 2-fold symmetric dimer of dimers, suppressing gating.

Using a polyclonal antibody raised against the C terminus of the channel we demonstrated expression of the HCN3 protein in restricted areas of the brain. The protein was not detected in heart ventricle although the HCN3 mRNA was readily identified in this tissue. This finding suggests that HCN3 is expressed only at low levels in heart. Like other HCN channels, native HCN3 is N-linked glycosylated (22, 34). In HCN2, the glycosylation site has been recently determined (22). The identified residue (Asn-380) is highly conserved in the HCN channel family and, hence, may also confer glycosylation of HCN3.

The highest expression levels of HCN3 were found in the olfactory bulb and hypothalamus, intermediate levels were found in the amygdala and hippocampus, and low levels were found in the retina and cortex. This expression pattern is in line with a recent report describing the localization of HCN channels in the same regions of the rat brain (19). The expression of HCN3 in hypothalamus is of particular pharmacological interest because neurons of this brain region are involved in the regulation of important physiological functions, including the tone of the autonomous nervous system, circadian rhythm, and hormone secretion (35). To our knowledge, native Iₜ currents displaying the specific properties of HCN3 (slow kinetics and inhibitory rather than stimulatory effect of cyclic nucleotides) have not yet been identified in vitro. Several reasons could account for this discrepancy. For example, it seems possible that HCN3 heteromerizes with other HCN isoforms (22, 36, 37) or interacts with additional auxiliary subunits (1, 38) that could modulate current properties. Alternatively, the overlapping expression of HCN channel isoforms in the brain (19) opens the possibility of co-localization of different homomeric HCN channels within the same cell. The macroscopic current from such cells would be composed of contributions from different homomeric HCN channels. Genetic deletion of HCN3 in mice will be required to define the exact physiological relevance of HCN3 in distinct brain regions.

Finally, we demonstrate that HCN3 is inhibited by ivabradine, a member of a novel class of Iₜ blockers that is considered for clinical use as bradycardic agent. Like the structurally related cilobradine (39), ivabradine is quite unselective among different homomeric HCN channels because it also blocks HCN1, HCN2, and HCN4.2 It will be desirable to develop isoform specific HCN channel blockers. The lentiviral HCN3 expression system described in this study provides an important tool to achieve this goal.

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