Functional Heteromerization of HCN1 and HCN2 Pacemaker Channels*

Received for publication, October 18, 2000, and in revised form, December 20, 2000
Published, JBC Papers in Press, December 27, 2000, DOI 10.1074/jbc.C000738200

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An important step toward understanding the molecular basis of the functional diversity of pacemaker currents in spontaneously active cells has been the identification of a gene family encoding hyperpolarization-activated cyclic nucleotide-sensitive cation nonselective (HCN) channels. Three of the four gene products that have been expressed so far give rise to pacemaker channels with distinct activation kinetics and are differentially distributed among the brain, with considerable overlap between some isoforms. This raises the possibility that HCN channels may coassemble to form heteromeric channels in some areas, similar to other K⁺ channels. In this study, we have provided evidence for functional heteromerization of HCN1 and HCN2 channels using a concatenated cDNA construct encoding two connected subunits. We have observed that heteromeric channels activate several-fold faster than HCN2 and only a little slower than HCN1. Furthermore, the voltage dependence of activation is more similar to HCN2, whereas the cAMP sensitivity is intermediate between HCN1 and HCN2. This phenotype shows marked similarity to the current arising from coexpressed HCN1 and HCN2 subunits in oocytes and the native pacemaker current in CA1 pyramidal neurons. We suggest that heteromerization may increase the functional diversity beyond the levels expected from the number of HCN channel genes and their differential distribution.

The newly cloned hyperpolarization-activated cyclic nucleotide-sensitive cation nonselective (HCN) ion channels give rise to currents that are almost indistinguishable or very similar to native pacemaker currents termed Iₚ in heart (1) and Iₘ in brain tissue (2). To date, three of the four HCN genes have been functionally expressed, giving rise to pacemaker currents with distinct gating properties (for review see Refs. 3–5). HCN1 channels display fast gating properties and are almost unaffected by cAMP (6), similar to Iₚ recorded from some hippocampal pyramidal cells. HCN2 channels activate slowly and are strongly modulated by cAMP (7, 8), similar to native pacemaker currents in some cardiac cells. HCN4 channels activate very slowly and also respond strongly to cAMP (9, 10), similar to Iₘ in thalamic relay neurons.

Expression patterns have confirmed that HCN1 is expressed restrictively in neurons of the neocortex, hippocampus, cerebellar cortex, and brainstem nuclei (6, 11, 12). In contrast, HCN2 is widely expressed throughout the brain with prominent labeling of thalamic and brainstem nuclei as well as in heart tissue (6, 7). Finally, HCN4 is predominantly expressed in thalamus, heart, and testis (9). This distinct distribution pattern of HCN isoforms across the brain has been suggested to attribute to functional heterogeneity of native neuronal pacemaker currents (12). Nevertheless, considerable overlap was observed between HCN1 and HCN2 using in situ hybridization, namely in hippocampal pyramidal cells and some brainstem nuclei (12). Moreover, the coexistence of HCN1 and HCN2 mRNA has been demonstrated at the single-cell level for hippocampal CA1 neurons (13), raising the possibility that heteromeric channel complexes could form in some areas.

The functional heteromerization of K⁺ channels with a tetrameric subunit stoichiometry has previously been demonstrated for voltage-gated K⁺ channels (14–19) as well as inwardly rectifying K⁺ channels (20, 21) but is currently unknown for HCN channels. HCN channel subunits have an overall structure similar to voltage-gated K⁺ channel subunits, including 6 transmembrane domains (S1–S6), a selectivity filter containing the GYG motif, and a putative voltage sensor (S4) containing a positively charged residue at every third position. Based on these structural similarities it has been suggested that HCN channels will most likely adopt the general tetrameric subunit architecture of both 6- and 2-transmembrane segment K⁺ channels (4). To investigate the possibility that functional heteromeric HCN channels may form by coassembly of different HCN isoforms, we expressed a concatenated cDNA construct encoding a covalently linked HCN1 and HCN2 subunit into Xenopus oocytes. Currents were recorded using the 2-microelectrode voltage clamp technique and were compared with the biophysical properties of channels formed upon coexpression of HCN1 and HCN2 subunits and channels formed upon expression of HCN1 and HCN2 alone. The cAMP sensitivity of the homomeric and heteromeric HCN channels was assessed by coexpression of a G-protein-coupled receptor.

EXPERIMENTAL PROCEDURES

cDNA Constructs and in Vitro Transcription—The entire coding sequence for the mouse HCN1 and HCN2 channels (originally termed mBCNG-1 and mBCNG-2, respectively) were subcloned into the vector pSD64TF and pGEMHE, respectively. Plasmids were first linearized either with BamHI (for HCN1) or with SpeI (for HCN2). Next, the cRNAs were synthesized from the linearized plasmids using the large scale SP6 (for HCN1) or T7 (for HCN2) Message Machine transcription kit (Ambion). A concatenated construct encoding a covalently linked HCN1 and HCN2 subunit was engineered by digesting HCN1 with PstI (blunt) and SpeI. HCN2 was digested with SmaI (blunt) and SpeI and ligated into the corresponding sites of the previous construct. The ligation product, HCN1-HCN2, was linearized with XhoI for size verification on an agarose gel and in vitro transcription using the large scale SP6 Message Machine transcription kit. The encoded dimer lacks the last 45 residues of the HCN1 C terminus and contains a 9-amino acid linker (GDPNSPFLA) that connects HCN1-Gln⁷⁵⁶stop to HCN2-M1 (see Fig. 1A).
The human μ-opioid receptor (MOR) cDNA was subcloned into our custom high expression vector, pGEMHE (17) and linearized for transcription as previously described (22). The rat GαoQ227L cDNA clone (23) was subcloned in pcDNA1.1 and linearized with XhoI for in vitro transcription. The mouse 5-HT4 R cDNA clone (24) in its original vector (pBRK5) was first subcloned into the XhoI and HindIII sites of pSGEM, a modified version of pGEMHE (25). For in vitro transcription, 5-HT4 R/pSGEM, was linearized with NheI. The capped cRNAs were synthesized from the linearized plasmids using the large-scale T7 mMessage mMachine transcription kit.

Expression and Electrophysiological Recordings—The isolation of Xenopus laevis oocytes was as previously described (17). Oocytes were injected with 10 ng/50 nl HCN1, HCN2, or HCN1:HCN2 cRNA. For determination of cAMP sensitivity, oocytes were coinjected with 0.05 ng of MOR; 0.05 ng of GαoQ227L or 0.01 ng of 5-HT4 R; and 10 ng of HCN1, HCN2, or HCN1:HCN2 (final injection volume, 50 nl). Injected oocytes were maintained in ND-96 solution (KCl 2 mm, NaCl 96 mm, MgCl2 1 mm, CaCl2 1.8 mm, HEPES 5 mM, pH 7.5) supplemented with 50 μg/ml gentamicin sulfate. Whole-cell currents from oocytes were recorded as previously described (22). Experiments were carried out using a high potassium (HK) external solution (KCl 96 mM, NaCl 2 mM, MgCl2 1 mm, CaCl2 1.8 mm, HEPES 5 mM, pH 7.5). Reversal potentials were determined in both HK and low potassium (ND-96) external solution (NaCl 96 mM, KCl 2 mm, MgCl2 1 mm, CaCl2 1.8 mm, HEPES 5 mM, pH 7.5). Cesium sensitivity of the currents was assessed by application of a hyperpolarizing pulse to −100 mV, long enough to achieve a fully activated state (4 s for HCN1 channels, 15 s for HCN2 channels, and 4 s for tandem HCN1:HCN2 channels).

Determination of cAMP Sensitivity—In this study, we coexpressed either μ-opioid or 5-HT4 R receptors with HCN channels. Both receptors are representative members of the G-protein-coupled receptor superfamilly that display an overlapping distribution pattern with HCN1 and HCN2 isoforms in the brain (24, 26) and activate adenylyl cyclase (27, 28) and require coexpression of HCN2 subunits or by coinjection of HCN1 and HCN2 cRNA (4-s test potentials range from −40 to −110 mV in 10 mV steps; holding potential and tail potential were −40 mV). Scale bars indicate 1 μA and 1 s in all panels. C, current-voltage relationships constructed from experiments in A for homomeric HCN1 (A), homomeric HCN2 (B) and tandem HCN1:HCN2 channels (C, D). Determination of the steady-state midpoint potential of activation (V1/2) by application of hyperpolarizing test pulses with increasing duration. The same symbols were used as in C. Dashed line indicates the average between triangles and squares. Unfilled triangles are data from Ref. 12.

RESULTS AND DISCUSSION

In this study, we investigated the possible heteromerization of HCN channels by expressing a concatenated cDNA construct encoding a covalently linked HCN1 and HCN2 subunit (Fig. 1A) into Xenopus oocytes. Both HCN1 and HCN2 are members of the HCN gene family (3, 5, 6), encoding pacemaker channels with easily distinguishable kinetic properties. For comparison, all records shown in Fig. 1B were evoked using the same voltage step protocol. Homomeric HCN1 channels activate relatively fast, whereas homomeric HCN2 channels activate more slowly (Fig. 1B, Table I). Expression of cRNA encoding the dimeric construct gives rise to currents that activate somewhat slower than HCN1, but still several-fold faster than HCN2 (Fig. 1B, Table I). This finding provides important evidence that the concatenated construct encodes heteromorphic HCN1:HCN2 channels, because the unexpectedly fast kinetics cannot be accounted for by the mathematical average expected from homomeric HCN1 and HCN2 channels. Furthermore, the heteromorphic phenotype shows marked resemblance to currents that were evoked from oocytes coinjected with equal amounts of HCN1 and HCN2 cRNA (Fig. 1B). This result suggests that HCN1 and HCN2 subunits could spontaneously coassemble to form a population of channels with a subunit arrangement similar to the heteromorphic channels formed by HCN1:HCN2 dimers.

Current-voltage relationships were constructed from the records in Fig. 1B and are compared in Fig. 1C. It should be noted that the 4-s test pulses applied to record the traces shown in Fig. 1B do not allow HCN2 channels to obtain a steady-state level of activation because of their relatively slow time course of activation (12). To make a valid comparison of the voltage dependence of activation between homomorphic and heteromorphic HCN1 and HCN2 channels, we calculated half-maximal values of activation (V1/2) from experiments with voltage steps of increasing duration (Fig. 1D). These results show that the use of relatively short hyperpolarizing pulses shifts the estimates of
the $V_{1/2}$-values to more negative potentials. Comparison of calculated $V_{1/2}$-values shows that heteromeric HCN1:HCN2 channels activate at membrane potentials intermediate to homomeric HCN1 and HCN2 channels for pulses $\leq 5$ s. However, heteromeric HCN1:HCN2 channels activate at membrane potentials more negative than the average of steady-state $V_{1/2}$-values for homomeric HCN1 and HCN2 channels (average indicated with a dashed line), for pulses long enough to achieve steady-state levels of activation ($> 10$ s). This result would suggest that a voltage-dependent transition in the opening of the heteromeric HCN1:HCN2 channel complex is limited by the isoform that activates at the most negative membrane voltages, as in the case of HCN2.

As could be observed from the current traces in Fig. 1B, heteromeric HCN1:HCN2 channels activate with a faster time course than the average of homomeric HCN1 and HCN2 channels predicts. For clarification, we have superimposed traces from homomeric and heteromeric channels recorded during a hyperpolarizing step to $-90$ mV (Fig. 2A). The current trace recorded from oocytes coexpressing HCN1 and HCN2 channels perfectly matches with the trace obtained from the tandem HCN1:HCN2 channels, suggesting that channels are formed with a subunit stoichiometry similar to the defined subunit arrangement of the tandem-linked channels. Next, we quantified the kinetics of activation by fitting current traces during hyperpolarizing voltage steps (as shown in Fig. 1B) with two exponential functions (12). The calculated fast and slow time constants of activation were plotted as a function of the applied test potential and are shown in Fig. 2, B and C, respectively. Values calculated for the traces in Fig. 2A were $\tau_{fast} = 214 \pm 20$ ms ($n = 6$) and $\tau_{slow} = 909 \pm 54$ ms ($n = 6$) for tandem HCN1:HCN2 channels and $\tau_{fast} = 168 \pm 8$ ms ($n = 6$) and $\tau_{slow} = 944 \pm 49$ ms ($n = 6$) for coexpressed HCN1 and HCN2 channels. Homomeric HCN1 and HCN2 channels as well as heteromeric HCN1:HCN2 channels have slow time constants of activation that are 5- to 10-fold higher than fast time constants. Compared with the respective time constants for homomeric HCN1 channels, homomeric HCN2 channels have fast and slow time constants that are $\approx 10$-fold higher. These results are in accordance with recently reported data on pacemaker channels in the mouse CNS (12). Heteromeric HCN1:HCN2 channels have the time constants of activation that are significantly faster than the average of homomeric HCN1 and HCN2 channels predicts (indicated with a dashed line). Heteromeric HCN1:HCN2 channels activate similarly to homomeric HCN1 channels, with fast and slow time constants of activation that are only about 3-fold higher. This result suggests that HCN1 subunits dominate the activation rate of the heteromeric channel complex.

Another striking feature that discriminates between homomeric HCN1 and HCN2 channels is their differential sensitivity to cAMP (6, 7). Hormones and neurotransmitters acting through the second messenger cAMP finely tune the activation of the pacemaker current by shifting the voltage dependence of activation along the voltage axis (1, 2). The underlying mechanism involves direct binding of cAMP to the channel cyclic nucleotide binding domain (29). Similar to studies using excised inside-out patches (6, 7), we found that activation of a coexpressed G-protein-coupled receptor, either the 5-HT4a or $\mu$-opioid receptor, shifts the $V_{1/2}$-value by 14.9 mV toward more positive potentials for homomeric HCN2 channels and no shift for homomeric HCN1 channels (Fig. 3A). Intriguingly, heteromeric HCN1:HCN2 channels display a cAMP sensitivity that is intermediate to homomeric HCN1 and HCN2 channels, with a 7.2 mV shift toward more positive potentials in response to activation of coexpressed G-protein-coupled receptor. Representative current traces from experiments using homomeric and heteromeric channels coexpressed with $\mu$-opioid or 5-HT4a receptors are shown in Fig. 3, B and C, respectively. Based on the structural similarities of the 6 transmembrane domains, the pore-forming region and cytoplasmic N and C termini of HCN channels with other K+ channels, it has previously been suggested that HCN channels will most likely conform to the tetrameric subunit arrangement of both the 6- and 2-transmembrane domain K+ channels (4). With respect to our results with HCN1:HCN2 tandems, this idea would suggest that functional channels are likely to be formed by coassembly of two HCN1:HCN2 tandems, adopting a HCN1/HCN2/HCN1/HCN2 or HCN1/HCN2/HCN2/HCN1 subunit stoichiometry. Our experimental results on the cAMP sensitivity of hetero-

### Table I

Comparison between HCN clones and $I_h$ in CA1 hippocampal neurons

<table>
<thead>
<tr>
<th></th>
<th>$V_{1/2}$ shift of $V_{50}$</th>
<th>$\tau_{fast}$, $\tau_{slow}$ at 120 mV</th>
<th>$V_{50}$, S (2-s pulses)</th>
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<tbody>
<tr>
<td></td>
<td>mV</td>
<td>ms</td>
<td>mV</td>
</tr>
<tr>
<td>HCN1</td>
<td>0.02 ± 1.13 $^a$</td>
<td>23.7 ± 2.9</td>
<td>-66.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.3 ± 1.4 $^b$</td>
<td>138 ± 11</td>
<td>-13.2 ± 0.06</td>
</tr>
<tr>
<td>HCN2</td>
<td>15.4 ± 1.0 $^a$</td>
<td>287.2 ± 8.9</td>
<td>-91.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>1.6 ± 1.1 $^b$</td>
<td>794 ± 98</td>
<td>-7.9 ± 0.5</td>
</tr>
<tr>
<td>HCN1 - HCN2</td>
<td>7.1 ± 1.2 $^a$</td>
<td>99.8 ± 7.7</td>
<td>-80.8 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>7.3 ± 1.3 $^b$</td>
<td>440 ± 16</td>
<td>-11.6 ± 1.1</td>
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<tr>
<td>$I_h$ (13)</td>
<td>64.4 ± 2.9</td>
<td>537 ± 47</td>
<td>-10.1 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$ Determined with a coexpressed $\mu$-opioid receptor and G$_{o,2}$Q227L.

$^b$ Determined with a coexpressed 5-HT4a receptor.

$^c$ Determined by including 1 mM 8-bromo-cAMP in the pipette solution.

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**Fig. 2. Analysis of activation kinetics for HCN channels.** A, superimposed current traces at $-90$ mV for homomeric HCN1 and HCN2 channels as well as heteromeric channels obtained by coinjection of HCN1 and HCN2 or tandem-linked HCN1:HCN2 channels. Records were normalized to the steady-state current amplitude after a 4-s pulse (30 s for HCN2). Also shown are algebraic sums of HCN1 and HCN2 currents in ratios of 1:5, 1:1, and 3:1. B and C, fast (filled symbols) and slow (unfilled symbols) time constants of activation determined for homomeric HCN1 (triangles) and HCN2 (squares) channels and heteromeric tandem HCN1:HCN2 channels (circles). Dashed line indicates the average between triangles and squares.
pressed G-protein-coupled receptor, either μ-opioid receptors (MOR) in the presence of a constitutively active Goα mutation or 5-HT4a receptors (see "Experimental Procedures"). A, receptor-evoked shifts of the \(V_{1/2}\) value for homomeric HCN1, homomeric HCN2, and tandem HCN1-HCN2 channels. B and C, representative current traces evoked before and after agonist application to activate MOR (B) or 5-HT4a, R (C). Records were evoked using a test pulse to \(-65\) mV for HCN1 (left), \(-70\) mV for HCN1-HCN2 tandem (middle), and \(-85\) mV for HCN2 channels (right). Holding potential and tail potential were \(-40\) mV.

meric HCN1-HCN2 channels support the idea of a tetrameric subunit arrangement, because the shift of the \(V_{1/2}\) value for heteromeric HCN1-HCN2 channels (7.2 mV) is exactly half of the shifts determined for homomeric HCN1 (0.2 mV) and HCN2 (14.9 mV) channels.

Finally, HCN channels are characterized by weak selectivity for K\(^+\) over Na\(^+\) and the inward current is sensitive to block by application of external Cs\(^+\). Therefore, we compared the reversal potentials and the cesium sensitivity for homomeric HCN1 and HCN2 channels with heteromeric HCN1-HCN2 channels. Reversal potentials determined in ND-96 solution (2 mM K\(^+\)) and HK solution (96 mM K\(^+\)) were \(-30 \pm 1\) mV (\(n = 5\)) and \(-2 \pm 2\) mV (\(n = 6\)) for HCN1 channels, \(-29 \pm 2\) mV (\(n = 5\)) and \(0 \pm 1\) mV (\(n = 6\)) for HCN2 channels, and \(-30 \pm 2\) mV (\(n = 6\)) and \(-1 \pm 2\) mV (\(n = 6\)) for tandem HCN1-HCN2 channels. Application of external Cs\(^+\) caused a complete inhibition of the inward current through HCN channels, characterized by IC\(_{50}\) values of 124 \pm 28 \mu M (\(n = 6\)) for HCN1 channels, 63 \pm 12 (\(n = 6\)) for HCN2 channels and 79 \pm 11 (\(n = 6\)) for tandem HCN1-HCN2 channels. These results indicate that heteromeric HCN1-HCN2 channels share identical ion selectivity and cesium sensitivity with homomeric HCN1 and HCN2 channels.

Taken together, our data raise the question about the relevance to native pacemaker currents. To address this issue, it is important to note that HCN isoforms not only differ in their biophysical properties and cAMP sensitivity, but also in their distinct distribution pattern across the brain (12, 13, 30). Nevertheless, considerable overlap was shown between HCN1 and HCN2 using in situ hybridization, namely in hippocampal pyramidal cells and some brainstem nuclei (12). Moreover, the coexistence of HCN1 and HCN2 mRNA has been demonstrated at the single cell level for hippocampal CA1 neurons (13), raising the possibility that heteromeric channel complexes could form in some areas. In Table I, we have compared our data to the results obtained by Franz et al. (13) based on the similar recording conditions (room temperature, whole cell configuration) and data analysis (double exponential fits to the time course of activation). The cAMP-induced shift of the \(V_{1/2}\) value for \(I_h\) in hippocampal CA1 neurons correlates well with the shift that we observed for heteromeric HCN1-HCN2 channels. This result can be expected from a population of channels that either consists of equal amounts of homomeric HCN1 and HCN2 channels or a population of channels with a heteromeric stoichiometry, similar to the tandem-linked channels. Comparison of the kinetic properties and the voltage dependence of activation for \(I_h\) in hippocampal CA1 neurons with our results however favors the formation of heteromeric channels, because equally distributed homomeric channels cannot account for the fast gating properties of the native \(I_h\).

In conclusion, the cAMP dependence and gating properties of tandem-linked HCN1 and HCN2 subunits reveal that HCN channels can function as heteromers, most likely tetramers. Based on the resemblance of the tandem it’s distinct current phenotype with currents obtained from coexpressed HCN1 and HCN2 subunits and native pacemaker current in neurons in which different isoforms coexist, our results suggest that heteromerization of different HCN channels may further contribute to the functional heterogeneity of native pacemaker currents. Finally, the dimeric construct may also serve as a model to elucidate the physiological role of native pacemaker currents and as a relevant pharmacological tool to develop new compounds for these channels.

Acknowledgments—We are very grateful to Dr. Steven A. Siegelbaum for his generous gift of mBCNG-1 and mBCNG-2 cDNAs and for sharing his comments on our work. The μ-opioid receptor clone was kindly provided by Dr. Lei Yu. The GoQ227L clone was a gift from Dr. Yung H. Wong. The 5-HT4a receptor cDNA was kindly donated by Dr. S. Claesen and Dr. A. Dumuis.

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doi: 10.1074/jbc.C000738200 originally published online December 27, 2000

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