Dominant-Negative Suppression of HCN Channels Markedly Reduces the Native Pacemaker Current $I_f$ and Undermines Spontaneous Beating of Neonatal Cardiomyocytes

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Background—The pacemaker current $I_f$ contributes to spontaneous diastolic depolarization of cardiac autonomic cells. In heterologous expression, HCN channels exhibit a hyperpolarization-activated inward current similar to $I_f$. However, the links between HCN genes and native $I_f$ are largely inferential, and it remains unknown whether $I_f$ is essential for cardiac pacing.

Methods and Results—To clarify this situation, we generated a GYG402–404 AYA pore mutation of HCN2, which rendered methods levels in different cardiac regions.8–11 Heterologous present in heart (HCN1, HCN2, and HCN4), with varying HCN gene family members have been cloned, 3 of which are identical properties compared with native $I_f$. This led to the hypothesis that HCN genes might be the molecular correlate of the native pacemaker current. By functional interference, it has been proposed that the isoforms HCN1 and HCN2 coassemble to form heteromeric channel complexes like voltage-gated $K^+$ channels.12,13 Very recently, coassembly between these 2 subunits has been shown more directly by use of the yeast 2-hybrid system14 and a dominant-negative construct.15 Detailed understanding of the molecular structure and function of the pacemaker channel is critical to any future therapeutic modulation of this current in myocardium. Because the links between HCN channels and cardiac $I_f$ are still largely inferential, we sought to confirm HCN as a critical molecular component of $I_f$ by overexpressing an HCN construct carrying a dominant-negative mutation in cardiomyocytes via viral gene transfer and looking for changes in native $I_f$. Second, we aimed to determine the functional relevance of $I_f$ in spontaneously beating neonatal cardiomyo-
cytes. We generated a GYG402-AYA pore mutation of HCN2, which not only rendered the channel nonfunctional but also suppressed wild-type HCN2 and wild-type HCN4 in a dominant-negative manner. Overexpression of this channel construct in neonatal ventriculocytes markedly reduced native \( I_f \) and undermined spontaneous beating activity. Our results demonstrate the critical role of HCN channels in \( I_f \) current and in automaticity of neonatal cardiomyocytes.

Methods

Plasmid Construction and Adenovirus Preparation

The adenovirus shuttle vectors pAdEGI, pAdCGI, and pAdCDBECR and the expression plasmid pCGI-Kv1.3AYA have been described.\(^{16,17}\) The full-length coding sequence of \( mHCN2 \) was cloned into the multiple cloning sites of pAdCGI and pAdEGI to generate pAdCGI-HCN2 and pAdEGI-HCN2, respectively. The point mutation AYA was introduced into the pore region (GYG402-AYA) of HCN2 by site-directed mutagenesis, creating the vectors pAdCGI-HCN2AYA and pAdEGI-HCN2AYA, respectively. The full-length coding sequence of \( hHCN4 \) was cloned into the multiple cloning sites of pAdCGI and pAdEGI to give pAdCGI-HCN4 and pAdEGI-HCN4, respectively. Adenovirus vectors were generated as previously described.\(^{17,19}\)

Transient Transfections

Twenty-four hours before transfection, Chinese hamster ovary (CHO)-K1 cells (ATCC CCL 61, American Type Culture Collection) were seeded at a density of \( 2.0 \times 10^5 \) per 35 mm. Cells were cotransfected with 0.5 \( \mu \)g/well plasmid DNA of mutant channels (as indicated) and 1 \( \mu \)g/well plasmid DNA of mutant channels (as indicated) with Lipofectamine Plus (Life Technologies) as directed by the manufacturer. After 4 hours, transfection media were replaced with normal growth media.

Myocyte Isolation and Adenovirus Infection

A standard trypsin dissociation method was used to prepare ventricular myocytes of 1- to 2-day-old neonatal rats.\(^{20}\) Action potential studies were conducted on 4- to 6-day-old monolayer cultures. For voltage-clamp experiments, 3- to 5-day-old monolayer cultures were dispersed by trypsin and replated at a low density to study isolated cells within 2 to 8 hours. Infection of neonatal cells was performed 1 to 3 days after plating at a multiplicity of infection of 15 to 100. Cells were incubated for 4 hours at 37°C, after which the infection medium was replaced with culture medium. Expression was induced by addition of ponasterone A 10 \( \mu \)mol/L (Invitrogen) for 36 to 60 hours.

Electrophysiology

Experiments were carried out with the use of standard microelectrode whole-cell patch-clamp techniques with an Axopatch 200B amplifier (Axon Instruments) while sampling at 10 kHz and filtering at 2 kHz. Current recordings were performed at room temperature (21°C to 23°C), and action potential measurements were done at 36±0.5°C. The recording bath solution contained (in mmol/L) NaCl 135, KCl 5, CaCl\(_2\) 2, glucose 10, MgCl\(_2\) 1, HEPES 10; pH was adjusted to 7.4 with NaOH. For \( I_f \) recordings of cardiomyocytes, \([K^+]_o\) was increased to 100 mmol/L, and BaCl\(_2\), 2 mmol/L, CdCl\(_2\), 200 \( \mu \)mol/L, and 4-aminopyridine 4 mmol/L were added to block \( K_0\), \( L_{Ca} \), and \( I_{Na} \), respectively. The micropipette electrode solution was composed of (in mmol/L): K-glutamate 130, KCl 15, MgCl\(_2\) 1, HEPES 10, and Mg-ATP 5; pH was adjusted to 7.3 with KOH. Borosilicate microelectrodes had tip resistances of 2 to 4 MΩ when filled with the internal recording solution.

The \( I_f \) size was measured as the difference between the instantaneous current at the beginning of a hyperpolarizing step ranging from −50 to −150 mV in 10-mV increments and the steady-state current at the end of hyperpolarization for 2.45 to 3 sec, as previously described.\(^{3}\) Fast-current inactivation was achieved by a depolarization pulse to 20 mV. For the calculation of activation curves, specific current conductances were normalized to the maximal current conductance to give \( g_f/g_{max} \). Boltzmann distributions were fitted to these normalized values: \( g_f/g_{max}=1/(1+exp(V_m-V_{1/2})/S) \), where \( V_m \) is the membrane voltage, \( V_{1/2} \) is the voltage at half-maximal activation, and \( S \) is a slope factor at \( V_m=V_{1/2} \). In HCN2-AYA-infected myocytes, action potentials were initiated by short depolarizing current pulses (2 ms, 500 to 800 pA). A xenon arc lamp was used to view enhanced green fluorescent protein (EGFP) at 488/530 nm (excitation/emission). Pooled data are presented as mean±SEM. Comparisons between groups were performed with 1-way ANOVA. Probability values of \( P<0.05 \) were deemed significant.

Results

HCN2-AYA Suppresses Wild-Type HCN2 and Wild-Type HCN4 in a Dominant-Negative Manner

Various mutant \( K^+ \) channels have been reported to exert a dominant-negative effect when coassembling with wild-type subunits.\(^{16,17}\) In analogy to the structure of \( K^+ \) channels, we speculated that the double mutation AYA in the pore region (GYG402-AYA) of HCN2 would alter channel function and would result in a dominant-negative ion channel construct. To test this hypothesis, CHO cells were cotransfected with wild-type HCN2/4 and HCN2-AYA, with wild-type HCN2/4 and a nonfunctional unrelated Kv1.3 construct carrying a similar pore mutation (Kv1.3-AYA, to exclude any unspecified effects of HCN2-AYA), or with HCN2-AYA and Kv1.3-AYA. All vectors were bicistronic, also expressing EGFP under control of a single CMV promoter for easy identification of transfected cells.

Overexpression of HCN2 and HCN4 resulted in time- and voltage-dependent inward currents on hyperpolarization, consistent with previous reports of heterologous expression of these ion channel subunits (Figure 1, A and B).\(^{8,9}\) Mean current densities at −130 mV were 33.8±4.2 pA/pF (n=16) and 21.3±2.5 pA/pF (n=14) for \( I_{HCN2} \) and \( I_{HCN4} \), respectively. \( V_{1/2} \) for \( I_{HCN2} \) and \( I_{HCN4} \) was −82.6±1.0 and −98.8±1.1 mV, respectively, and the slope factor \( S \) was 10.8±0.6 and 11.5±1.0 mV, respectively. In contrast, expression of HCN2-AYA did not yield any measurable hyperpolarization-activated inward current (\( n=9 \)) (Figure 1C). This indicated that the pore mutation AYA completely undermined HCN2 channel function, which is in agreement with a very recently reported pore mutation of HCN1.\(^{15}\)

To probe the effect of HCN2-AYA on wild-type HCN2, both channel constructs were coexpressed. Representative current recordings (Figure 1D) and mean current density (3.3±0.9 pA/pF at −130 mV, n=11; Figure 1F) show that \( I_{HCN2} \) was significantly decreased by HCN2-AYA (\( P<0.001 \)), confirming the dominant-negative properties of the HCN2 pore mutation. In addition, HCN2-AYA suppressed \( I_{HCN4} \) in a dominant-negative manner when coexpressed with wild-type HCN4 (mean current density at −130 mV, 14.2±1.8 pA/pF, n=21, \( P=0.025 \)) (Figure 1, E and G). This indicated that the 2 isoforms HCN2 and HCN4 are also able to coassemble to form heteromultimeric complexes.

HCN2-AYA Suppresses Native \( I_f \) in Neonatal Cardiomyocytes

HCN genes are thought to underlie the pacemaker current \( I_f \). In neonatal rat ventriculocytes, the dominant HCN
mRNA transcripts are HCN2 and HCN4. Considering our observations in CHO cells, overexpression of HCN2 (A) and HCN4 (B) resulted in time- and voltage-dependent inward currents on hyperpolarization. Conversely, expression of HCN2-AYA did not yield any measurable hyperpolarization-activated inward current. Representative current recordings (D) and mean current densities measured at −130 mV (F) show that HCN2-AYA significantly decreased $I_{HCN2}$ in a dominant-negative manner when the mutant was coexpressed with wild-type HCN2 channels. In addition, HCN2-AYA suppressed $I_{HCN4}$ in a dominant-negative manner when coexpressed with wild-type HCN4, as illustrated by original current recordings (E) and mean current densities measured at −130 mV (G).

Figure 1. Effect of HCN2-AYA on wild-type HCN2 and wild-type HCN4 heterologously expressed in CHO cells. Overexpression of HCN2 (A) and HCN4 (B) resulted in time- and voltage-dependent inward currents on hyperpolarization. Conversely, expression of HCN2-AYA did not yield any measurable hyperpolarization-activated inward current. Representative current recordings (D) and mean current densities measured at −130 mV (F) show that HCN2-AYA significantly decreased $I_{HCN2}$ in a dominant-negative manner when the mutant was coexpressed with wild-type HCN2 channels. In addition, HCN2-AYA suppressed $I_{HCN4}$ in a dominant-negative manner when coexpressed with wild-type HCN4, as illustrated by original current recordings (E) and mean current densities measured at −130 mV (G).

Figure 2. HCN2-AYA suppressed native $I_i$ in neonatal cardiomyocytes, whereas expression of HCN2 and HCN4 resulted in a robust hyperpolarization-activated inward current. Representative original current traces of native $I_i$ recorded in a noninfected neonatal myocyte (A) and in a HCN2-AYA–infected cell (B) demonstrate dominant-negative effect of HCN2-AYA on native $I_i$. Mean $I_i$ current density measured at −130 mV was almost completely suppressed by HCN2-AYA compared with noninfected cells (C). Conversely, infection with HCN2 (D) and HCN4 (E) resulted in high current densities of $I_{HCN2}$ and $I_{HCN4}$ in neonatal cardiomyocytes. Pulse protocol identical to Figure 1.
HCN channels are a critical component of native overexpression, we observed a reduction of MDP in HCN2–3, B, C, and E). Consistent with a very recent report of HCN2 more regular rhythm and marked diastolic depolarization (Figure 3). These results confirm that HCN channels are a critical component of native If.

**Discussion**

Overexpression of dominant-negative constructs in native cells has been a useful tool to dissect the contributions of various ion channel genes and different native ionic currents to excitability and repolarization. In the present study, we used this strategy to confirm for the first time the identity of HCN genes as the major molecular component of native cardiac If by introducing a dominant-negative HCN construct into cardiomyocytes to cripple If. Furthermore, our results clearly indicate that HCN channels are critical for the generation of spontaneous action potentials in neonatal cardiomyocytes.

Similar to selective K⁺ channels, HCN channels contain a GYG pore motif, despite their permeability for both Na⁺ and K⁺. We mutated this pore region to generate an HCN2-AYA construct. This mutation rendered the channel completely nonfunctional, consistent with a very recent report of an HCN1 pore mutation that also disrupted channel function. This indicates that in HCN channels, the GYG pore motif has a critical role for permeation properties comparable to that in K⁺ channels. In heterologous expression, HCN2-AYA not only suppressed wild-type HCN2 current, which confirmed its dominant-negative effect in spontaneous beating activity despite a similar MDP (~59.3 ± 2.8 mV, n=11) versus control cultures (P=NS) (Figure 3, E and F).}

Figure 3. Effect of HCN overexpression and dominant-negative suppression on spontaneous activity of neonatal ventricle cultures. Representative original recordings of spontaneous action potentials (A through C) and mean data (E, F) illustrate that overexpression of HCN2 and HCN4 significantly accelerated beating rate, resulted in a more regular rhythm, and reduced MDP compared with control monolayer cultures. Conversely, HCN2-AYA infection undermined spontaneous beating activity (E) despite an unchanged MDP compared with control (F). In some HCN2-AYA-infected cells, action potentials artificially induced by a depolarizing pulse were followed by an unstable diastolic potential with minor depolarizations, which, however, were not sufficient to reach threshold and to initiate spontaneous action potentials.

DBEcR and stimulation with the ecdysone analogue ponasterone A, If density was indeed reduced markedly, from 7.8 ± 1.6 pA/pF (n=13) in control cells to 0.3 ± 0.2 pA/pF (n=11) in HCN2-AYA–infected cells, when measured at −130 mV (P<0.001) (Figure 2, A through C). These results confirm that HCN channels are the dominant molecular component of native If.

**HCN Channels Are Crucial for Spontaneous Beating of Neonatal Myocytes**

In recent years, it has been controversial whether If is the major current responsible for spontaneous diastolic depolarization of pacemaker cells and neonatal tissue. Given our results that HCN channels are the dominant molecular component of If, we therefore probed the functional effect of HCN channel overexpression and dominant-negative suppression on spontaneous beating activity of monolayer cultures of neonatal cardiomyocytes.

Control cultures beat spontaneously with a mean rate of 83.4 ± 4.5 bpm (n=13). Cycle length tended to vary from beat to beat (Figure 3A). Maximal diastolic potential (MDP) was −63.6 ± 3.2 mV. Infection of neonatal cells with the adenoviral vectors AdEGI-HCN2 and AdEGI-HCN4 resulted in high HCN current levels (Figure 2, D and E). Mean current densities of IfCN2 and IfCN4 at −130 mV were 32.9 ± 5.1 pA/pF (n=11) and 25.1 ± 4.5 pA/pF (n=8), respectively. Enhanced expression of HCN2 and HCN4 accelerated beating frequency to 230.5 ± 8.6 bpm (n=12, P<0.001 versus control) and 223.5 ± 12.3 bpm (n=10, P<0.001 versus control), respectively, and resulted in a more regular rhythm and marked diastolic depolarization (Figure 3, B, C, and E). Consistent with a very recent report of HCN2 overexpression, we observed a reduction of MDP in HCN2-infected (~45.4 ± 2.2 mV) and HCN4-infected (~48.7 ± 1.6 mV) cells compared with controls (P<0.01) (Figure 3F). Conversely, HCN2-AYA–expressing cultures did not generate any spontaneous beating activity despite a similar MDP (~59.3 ± 2.8 mV, n=11) versus control cultures (P=NS) (Figure 3, E and F). Figure 3D illustrates a representative recording of an action potential that was induced artificially by a short depolarizing current pulse in an HCN2-AYA–infected myocyte. Diastolic potentials in some of these cells were unstable, exhibiting irregular minor depolarizations, which, however, were not sufficient to reach threshold and initiate spontaneous action potentials (Figure 3D). These observations indicate a critical role of HCN channels for automaticity of neonatal cardiomyocytes.
homomeric complexes but also reduced wild-type HCN4 current in a dominant-negative manner. These results indicate that HCN2 and HCN4 can coassemble to form heteromeric complexes. Thus far, heteromultimerization has been proposed only for the isoforms HCN1 and HCN2. Tandem heterodimers and coexpression of HCN1 and HCN2 yielded intermediate electrophysiological phenotypes that cannot be explained by a simple addition of individual isoform properties at any population proportional ratio.12,13 More recently, interaction of these 2 subunits has been suggested in use of a dominant-negative construct and the 2-yeast hybrid system.14,15 Our observations now extend evidence that different HCN isoforms can coassemble and may suffice to explain the regional diversity of pacemaker current in cardiac tissue.

In neonatal ventricle, HCN2 and HCN4 are the dominant HCN isoforms on the basis of RNase protection.10 Because in heterologous expression, our HCN2-AYA vector was able to suppress both HCN2 and HCN4 wild-type channels in a dominant-negative manner, this channel construct was considered useful to test the effect of HCN suppression on native I\textsubscript{f} current in neonatal cardiomyocytes. Indeed, we could demonstrate almost complete reduction of native I\textsubscript{f} after infection with HCN2-AYA, indicating a dominant-negative suppression of native HCN channels in neonatal cells. This demonstrates that HCN subunits are the major determinants of cardiac I\textsubscript{f}. The remaining minor I\textsubscript{f} current recorded in HCN2-AYA–expressing cells can be attributed to incomplete elimination of I\textsubscript{f} because of some functional homotetramers that are made up of only wild-type subunits. Alternatively, incomplete elimination may result from competition between expression of suppressive gene products and turnover of preexisting functional channel proteins.

There has been a great deal of controversy about the functional contribution of I\textsubscript{f} to cardiac pacing. Some authors speculate that I\textsubscript{f} is essential for spontaneous diastolic depolarization, whereas others postulate that I\textsubscript{f} only modulates the beating frequency of autonomic cardiac cells.4–7,23 Overexpression of HCN2 and HCN4 markedly accelerated the spontaneous beating rate of neonatal cardiomyocyte monolayers, demonstrating the ability of HCN channels to modify pacing frequency. More importantly, however, HCN channels and native I\textsubscript{f} were found to be critical for spontaneous action potential generation revealed by HCN\textsubscript{II}, “knockout” in HCN2-AYA–infected cultures. The diastolic potential of some HCN2-AYA–expressing cells was unstable, exhibiting minor depolarizations. These irregular depolarizations might have been a result of the small remaining I\textsubscript{f} current, which was not sufficient to drive the potential toward threshold or might reflect the balance of other ionic currents (ie, I\textsubscript{K1}, I\textsubscript{K2}) involved in pacemaker activity.4,5,23

Given the obvious importance of HCN channels in cardiac pacing, it seems plausible that mutations in HCN genes might cause bradycardia or sick sinus syndrome in patients, although our observations in neonatal cardiomyocytes cannot readily be generalized to sinus node cells. In any event, our HCN constructs may prove useful to further determine the physiological role of I\textsubscript{f} and HCN channels for cardiac pacing in the sinoatrial node and for arrhythmogenesis in diseased adult ventricular myocardium.1–3

Detailed molecular insight into the contribution of this diastolic current to cardiac pacing might facilitate the rational design of blockers or activator drugs and of gene therapy strategies to modify cardiac automaticity.

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