HCN2 Overexpression in Newborn and Adult Ventricular Myocytes
Distinct Effects on Gating and Excitability

Jihong Qu, Andrea Barbuti, Lev Protas, Bina Santoro, Ira S. Cohen, Richard B. Robinson

Abstract—Ventricular pacemaker current ($I_f$) shows distinct voltage dependence as a function of age, activating outside the physiological range in normal adult ventricle, but less negatively in neonatal ventricle. However, heterologously expressed HCN2 and HCN4, the putative molecular correlates of ventricular $I_f$, exhibit only a modest difference in activation voltage. We therefore prepared an adenoviral construct (AdHCN2) of HCN2, the dominant ventricular isoform at either age, and used it to infect neonatal and adult rat ventricular myocytes to investigate the role of maturation on current gating. The expressed current exhibited an 18-mV difference in activation ($V_{1/2} = 95.9 \pm 1.9$ in adult; $-77.6 \pm 1.6$ mV in neonate), comparable to the 22-mV difference between native $I_f$ in adult and neonatal cultures ($V_{1/2} = 98.7$ versus $-77.0$ mV). This did not result from developmental differences in basal cAMP, because saturating cAMP in the pipette caused an equivalent positive shift in both preparations. In the neonate, AdHCN2 caused a significant increase in spontaneous rate compared with control ($88 \pm 5$ versus $48 \pm 4$ bpm). In adult, where HCN2 activates more negatively, the effect was evident only during anodal excitation, requiring significantly less stimulus energy than control (2149$\pm$266 versus 3140$\pm$279 mV·ms). Thus, ventricular maturational state influences the voltage dependence of expressed HCN2, resulting in distinct physiological impact of expressed channels in neonate and adult myocytes. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001;89:e8-e14.)

Key Words: pacemaker current ■ gene expression ■ development ■ ventricle ■ HCN

The recent cloning of the HCN gene family, representing the molecular correlate of the pacemaker current $I_f$, raises the possibility of using genetic approaches for the treatment of rhythm disorders. However, $I_f$ is present in both automatic and nonautomatic$^3-6$ regions of the heart, and the threshold voltage varies widely among cardiac regions, being least negative in sinus node ($-40$ mV in rabbit$^5$) and most negative in ventricle ($-108$ mV or more negative$^6,8,9$). Interestingly, $I_f$ activates at least negative voltages in newborn ventricle ($-70$ mV in rat$^8,10$). The molecular and cellular bases for the regional variability of activation voltages in the normal adult heart and the regulation of ventricular activation voltage by development remain to be determined, but such understanding is critical to any future therapeutic application of the expressed current in myocardium.

Four members of the HCN gene family are currently known.$^{11-13}$ Three of these (HCN1, HCN2, and HCN4) are present in heart, and their relative message level varies with region and age.$^{14,15}$ Sinus node and Purkinje fibers, in which $I_f$ activates at least negative potentials, contain largely HCN1 and HCN4. Ventrikel contains HCN2 and HCN4, with the HCN2/HCN4 mRNA ratio being greater in adult than newborn. This suggests that HCN2 is inherently negatively activating, and its relative abundance determines the activation threshold in different regions of the heart or at different ages. However, when HCN2 and HCN4 were expressed in mammalian cell lines, activation voltages differed by $<10$ mV.$^{16,17}$ Thus, the intrinsic voltage dependence of different HCN isoforms is insufficient to explain the functional diversity of native cardiac $I_f$, either regionally or developmentally. Instead, it seems likely that unidentified accessory proteins or other cellular factors influence the voltage dependence of individual HCN isoforms. Thus, HCN2 activation might differ, for example, when expressed in neonatal versus adult ventricular myocytes. The present study investigated this possibility.

Materials and Methods

Cell Isolation and Culture

Adult rats were anesthetized with ketamine-xylazine before cardiectomy, and neonatal rats decapitated, in accordance with Institutional Animal Care and Use Committee protocols of Columbia University. A standard trypsin dissociation method was used to prepare newborn ventricle cell cultures.$^{11}$ The cells were preplated to reduce fibroblast...
proliferation, cultured initially in serum-containing medium, then switched to serum-free medium after 24 hours. Action potential studies were conducted on 4- to 6-day-old monolayer cultures plated directly onto fibronectin-coated 9×22-mm glass coverslips. For voltage-clamp experiments, 4- to 6-day-old monolayer cultures were resuspended by brief (2 to 3 minutes) exposure to 0.25% trypsin, then the cells replated onto fibronectin-coated coverslips and studied within 2 to 8 hours. Freshly isolated adult ventricle myocytes were prepared using the procedure described by Kaznetsov et al.19 This entailed a Langendorff perfusion of collagenase, followed by trypsinizing away of the atria. The remaining tissue was minced and dissociated in additional collagenase solution. The isolated myocytes were suspended in a serum-free medium (ACCTI20) then plated onto laminin-coated 9×22-mm glass coverslips at 0.5 to 1×10^6 cells/mm². Two to three hours later, after the myocytes had adhered to the coverslips, the adenoviral infection procedure was begun.

**Expression of HCN2**

For increased expression efficiency, we prepared an adenoviral construct of mouse HCN2 (mHCN2, GenBank No. AJ225122) following previously published methods.20 A DNA fragment (between EcoRI and Xhol restriction sites) that included mHCN2 DNA downstream of the cytomegalovirus promoter was obtained from plasmid pGM-TCR-mHCN220 and subcloned into the shuttle vector pDC516 (AdMax; Microbix Biosystems). The resulting pDC516-mHCN2 shuttle plasmid was cotransfected with a 35.5-kb E1-deleted adenoviral genomic plasmid pBHGrfΔE1.3FLP (AdMax) into E1-complementing HEK293 cells. The adenoviral construct AdHCN2 was subsequently harvested and purified with CsCl.

AdHCN2 infection of adult rat ventricular myocytes was carried out 2 to 3 hours after cell plating. Infection of neonatal cells was done on monolayer cultures 1 to 3 days after plating. In either case, the culture medium was removed from the 35-mm dishes and the inoculum of 0.2 to 0.3 mL/dish was added containing AdHCN2. The multiplicity of infection (MOI; ratio of viral units to cells) was 15 to 100. The inoculum was dispersed over the cells by gently tilting the dishes every 20 minutes. The dishes were kept at 37°C in a CO₂ incubator during the 2-hour adsorption period, then the inoculum was subsequently harvested and purified with CsCl.

The adenoviral infection procedure was begun.

**Electrophysiological Recording and Data Analysis**

Native If, expressed IfHCN2, and action potentials were recorded using a patch electrode in whole-cell mode on cells superfused at 35°C. Extracellular solution contained (in mmol/L) NaCl 140, NaOH 2.3, MgCl₂ 1, KCl 5.4, CaCl₂ 1.0, HEPES 5, and glucose 10 (pH 7.4). To record If or IfHCN2 (K⁺), we added 10 mmol/L, and MnCl₂ (2 mmol/L) and BaCl₂ (4 mmol/L) added to the superfusate to eliminate calcium and inward rectifier (If) currents. In some experiments, CsCl (4 mmol/L) was used extracellularly to define the Cs-sensitive pacemaker current. Pipette solution included (in mmol/L) aspartic acid 130, KOH 146, NaCl 10, CaCl₂ 2, EGTA-KOH 5, Mg-ATP 2, and HEPES-KOH 10 (pH 7.2). Where indicated, cAMP was included in the pipette solution. A fast solution–changing apparatus expedited the experimental protocols. The pipette resistance was 1 to 3 MΩ. An Axopatch-200B amplifier and pClamp8 software (Axon Instruments) were used for acquisition and analysis.

Pacemaker current (If or IfHCN2) was defined as the time-dependent component at the end of a 3- to 6-second hyperpolarizing step (range −35 to −145 mV) from a holding potential of −35 mV, unless otherwise indicated. The hyperpolarizing step was followed by an 8-second step to −125 mV to record tail current, then a 0.5-second pulse to −5 mV to ensure full deactivation. Tail currents were measured at −125 mV to avoid contamination by currents present at less negative voltages. To minimize contamination of expressed current by native If, cells from infected cultures were only included if the measured current at −125 mV was ≥200 pA. This resulted in discarding <8% of cells from AdHCN2-infected cultures at either age. In addition, all 12 noninfected adult cells and 8 of 9 noninfected neonatal cells used in the measurement of native If had current values below this criterion. Tail current, plotted against test voltage, provided maximum conductance and the activation-voltage relation; the latter was normalized by maximum conductance and fitted with the Boltzmann function [y = 1/(1 + exp[(V − V_1/2)/K])] to determine the half-maximum activation voltage (V_1/2) and slope factor (K). Kinetics of activation were determined by a single-exponential fit to the early time course of the current activated by hyperpolarization; the initial delay and any late slow activation were ignored. Kinetics of deactivation were determined by a single-exponential fit of the time course of the current trace at each test voltage after activation by a prepulse to −125 mV. For both activation and deactivation, the duration of the trace being fit was ≥3× the measured time constant to ensure accuracy. Records were not corrected for liquid junction potential (9.8 mV).

All data are presented as mean ± SEM. Statistical significance was examined by t test for paired and ANOVA for multiple comparisons. Values of P<0.05 were determined to be significant.

**Results**

Comparing the characteristics of HCN2 (the major ventricular HCN isoform), at the message level, at both ages14 when expressed in adult versus neonatal ventricular myocytes required maintaining adult cells in culture for 48 hours. Because a previous report indicated that longer culture conditions resulted in a marked positive shift in activation of native current,9 we first compared native If between acutely dissociated cells and cells maintained in serum-free medium for 2 days. All cells studied were rod-shaped and quiescent. In adult cells in culture, If threshold voltage (ie, first voltage step where a time-dependent current is apparent) was relatively negative (Figure 1A) compared with that reported in neonatal cells.23 A 2-day culture period resulted in no significant difference in V_1/2 (−105.3 ± 2.6, n=12, versus −98.7 ± 1.8 mV, n=7, in acute versus cultured cells; P=0.092), slope factor (10.9 ± 1.2 versus 14.4 ± 1.9 mV), or activation kinetics (data not shown). Thus, short-term culture of adult cells can be used in studies of age-dependent differences in If or IfHCN2.

Treatment of both adult and neonatal cells in culture with the AdHCN2 construct resulted in expression of high current levels, with faster activation in neonate (Figures 1B and 1C; note different scales than in Figure 1A). Figure 2A illustrates the average activation relations in myocytes expressing HCN2 in the two preparations. It is evident that, when the same protein is expressed in newborn and adult myocytes, the resultant current activates at significantly more negative voltages in adult cells. V_1/2 for IfHCN2 in neonate and adult myocytes was −77.6 ± 1.6 (n=24) and −95.9 ± 1.9 mV (n=13), respectively (P<0.001). K also differed significantly (9.8 ± 0.6 versus 6.5 ± 0.5 mV, P<0.001), reflecting a more shallow voltage dependence in neonate. Figure 2B illustrates the activation/deactivation kinetics for IfHCN2. The data were well fit by a standard kinetic model (see legend) and exhibit little difference in the maximal value of activation time constant (τ) with age. However, the voltage dependence of the relation is shifted negatively in adult by 21 mV, comparable to the shift in the activation relation (18 mV). Moreover,
the relative peaks of the kinetic relations in the two preparations are consistent with the previously determined $V_{1/2}$ values (arrows, Figure 2B). For both expressed $I_{HCN2}$ and native $I_f$ (Figure 2C), kinetics in neonatal and adult myocytes appear related to the voltage dependence of steady-state activation.

Possible Basis for Difference Between Neonatal and Adult Myocytes Expressing HCN2

The biophysical characteristics of heterologously expressed currents can vary with current density.\textsuperscript{24–26} To determine if the difference in $V_{1/2}$ of HCN2 between neonatal and adult resulted from this phenomenon, we conducted a linear regression analysis (Figure 3). The results indicate that differences in expression level cannot explain the difference in HCN2 voltage dependence between neonatal and adult myocytes. Neonatal myocytes exhibited a wide range of current density for expressed current, with a correlation coefficient for $V_{1/2}$ of 0.51 ($P<0.01$); current density was less variable in adult, with no correlation with $V_{1/2}$ (correlation coefficient 0.043, $P=0.88$). Considering current densities common to both preparations (ie, $<60$ pA/pF, Figure 3, inset), expressed current in neonatal myocytes demonstrated a significantly less negative $V_{1/2}$ than in adult myocytes ($P<0.001$).

Both native $I_f$ and expressed HCN2 respond to cAMP by a phosphorylation-independent shift in voltage dependence of activation,\textsuperscript{27,28} although phosphorylation-dependent mechanisms also have been reported.\textsuperscript{29} To test if the neonatal/adult difference in $V_{1/2}$ of $I_{HCN2}$ simply reflected differences in basal cAMP, we repeated the determination of $V_{1/2}$ of expressed current with 10 $\mu$mol/L cAMP in the pipette solution to eliminate any differences in intracellular cAMP levels and achieve a maximal positive shift of $I_{HCN2}$ (Figure 4). $I_{HCN2}$ shifted positively by a comparable amount in both preparations and the difference in $V_{1/2}$ persisted, indicating that the
age-dependent difference in HCN2 activation does not result from a difference in basal cAMP. Results with a 10× higher cAMP concentration did not differ statistically (data not shown).

Functional Effect of Overexpression of HCN2
AdHCN2 infection resulted in expression of a large current in >90% of the cells studied. Given the activation of \( I_{\text{HCN2}} \) within the physiological voltage range in neonatal cells, we asked if HCN2 overexpression resulted in a change in spontaneous rate of these cultures. Experiments were conducted on monolayer cultures of synchronously beating cells, with a whole-cell patch electrode recording from one cell of the contiguous monolayer. Control (noninfected) cultures beat spontaneously, with a mean rate of 48.4 bpm. Data with control pipette solution (Figure 2A) are shown as dashed (neonate) and dotted (adult) lines.

As expected, given the negative activation relation of \( I_{\text{HCN2}} \) in neonatal and adult myocytes expressing HCN2 were \(-60.6 \pm 1.4\), n = 10, and \(-82.3 \pm 3.2\) mV, n = 6, respectively. Earlier data with control pipette solution (Figure 2A) are shown as dashed (neonate) and dotted (adult) lines.

Figure 4. Effect of intracellular cAMP on activation relation of \( I_{\text{HCN2}} \) in neonate and adult myocytes. Pipette solution contained 10 μmol/L cAMP. Calculated \( V_{1/2} \) values in the presence of cAMP for neonatal and adult myocytes expressing HCN2 were \(-60.6 \pm 1.4\), n = 10, and \(-82.3 \pm 3.2\) mV, n = 6, respectively. Earlier data with control pipette solution (Figure 2A) are shown as dashed (neonate) and dotted (adult) lines.

Discussion
This study investigated if the distinct activation voltage of \( I_{\text{f}} \) in neonatal and adult ventricle was the result of a difference density at the end of a 2-second voltage step to \(-125\) mV in the same cells. As predicted, infected cells more readily exhibited anode break excitation. Figure 6A illustrates representative traces of anodal stimuli and resulting action potential upstrokes from control and infected cells. The delay between the end of the anodal stimulus and action potential threshold was not statistically different between preparations (45 ± 10 versus 58 ± 9 ms, \( P > 0.05 \)). Figure 6B graphs the relation between maximal negative potential at threshold and \( I_{\text{f}} \) or \( I_{\text{HCN2}} \) density for control and infected cells. Control cells exhibited an inverse correlation (Figure 6B, inset), consistent with the prediction that native \( I_{\text{f}} \) contributes to anode break excitation. In comparison, in infected cells it was sufficient to hyperpolarize the membrane to \(-80\) mV, ie, the threshold for expressed current. Anode break threshold was independent of \( I_{\text{HCN2}} \) density, indicating the current was large enough in all infected cells to generate a sufficient overshoot for achieving excitation at \( I_{\text{HCN2}} \) threshold. Required stimulus energy (the integral of the area from stimulus start to action potential threshold) was significantly less in AdHCN2-infected cells (2149 ± 266, n = 12 versus 3140 ± 279 mV · ms, n = 10; \( P < 0.05 \)). The required stimulus energy of AdGFP-infected cells did not differ from control (data not shown). In addition, resting potential did not differ between control, AdHCN2-infected, and AdGFP-infected myocytes (data not shown).
expressing HCN2 with less negative voltage dependence. The actual $V_{1/2}$ of native $I_f$ in ventricle was $-77$ and $-99$ mV in neonate and adult, respectively, compared with values for HCN2 of $-78$ and $-96$ mV in these two preparations. Thus, HCN2 activation largely explains the voltage dependence of native ventricular $I_f$. The current referred to in this study as $I_{HCN2}$ is of course a sum of expressed and native current. However, selection criteria were established (see Materials and Methods) to minimize contamination by native current. Further, if native and expressed current differ significantly, then one would predict a marked dependence of activation voltage on current density, whereas the observed correlation was modest and did not account for the differences in newborn and adult.

The kinetic characteristics of the native current in neonate and adult ventricle also are largely, but not necessarily entirely, explainable by HCN2. Native $I_f$ in the neonate activates with kinetics close to that of $I_{HCN2}$ in these same cells. When the full activation/deactivation relation of expressed HCN2 is compared in neonate and adult, the difference is largely attributable to the difference in voltage dependence of activation. Thus, there does not appear to be an effect of maturational state of the myocyte directly on activation kinetics of expressed current, independent of the effect on voltage dependence of activation. However, native $I_f$ kinetics appear slower than expressed HCN2 kinetics, especially in adult (compare Figures 2B and 2C), and this may reflect the influence of the more slowly activating HCN4 isoform. Whether HCN2 and HCN4 can form heteromultimers is not known, but recent studies of HCN1 and HCN2 support the idea of heteromeric formation.33,34

The basis for the age-dependent difference in HCN2 voltage dependence in myocytes is unclear, beyond the observation that it is not secondary to differences in basal cAMP. The range of voltage dependence reported for $I_f$ in different cardiac regions or as a function of age is pronounced and may reflect a combination of mechanisms. Studies of other channels have identified several factors that alter the biophysical properties of native or expressed current, including $\beta$ subunits,35,36 local membrane composition,37 cytoskeletal interactions,38 phosphorylation/dephosphorylation,29,39 and truncation.40 A $\beta$ subunit for the HCN gene family has recently been identified,41 but it is concentrated in the sinoatrial node of the heart and affects kinetics rather than voltage dependence. The extent to which other $\beta$ subunits or the other potential mechanisms contribute to the variation in voltage dependence of $I_f$ or $I_{HCN2}$ is unknown. In this context, it is interesting that when native $I_f$ is studied in cell-free macropatches, activation shifts markedly negatively, but treatment of the intracellular face of the patch with promiscuous activation shifts activation positively again by 56 mV.42 Further, when a large portion of the HCN2 C-terminal that includes the cyclic nucleotide-binding domain is deleted, activation shifts positively by 24 mV.43 From these results, one can speculate that interactions between cytoplasmic elements of the HCN protein contribute to more negative activation, but that these interactions are minimized in the intact cell. Although the relevance of these observations to the regional or developmental variation in activation voltage remains to be deter-

Figure 6. Effect of HCN2 overexpression in adult ventricular myocytes. A, Representative anode break excitation tracings and stimulus profile from a control myocyte (left) and an AdHCN2-infected myocyte (right). Injected current in illustrated traces: −50, −150, −250, −350, and −450 pA (left); −10, −20, and −30 pA (right). Resting potential in the two examples is −66 and −60 mV, respectively. Mean resting potential between control (−62.1±1.8 mV, n=18) and infected adult culture (−59.1±1.1 mV, n=12) did not differ statistically. B, Graph of relation between maximal negative potential achieved during anodal stimulation and $I_f$ (unfilled symbols) or $I_{HCN2}$ (filled symbols) density (measured after a 2-second step to −125 mV). Inset, Current densities of 0 to 1.2 pA/pF on an expanded time base, with calculated linear regression (line).

in the biophysical properties of the HCN2 isoform when expressed in neonatal and adult ventricular myocytes. At both ages, HCN2 is the dominant isoform based on RNase protection, although the relative ratio of HCN2/HCN4 message increases developmentally.14 The results indicate that HCN2 expression in neonatal and adult ventricular myocytes in culture results in a comparable difference (18 mV) in $V_{1/2}$ to that of native $I_f$ (22 mV). Thus, the developmental difference in pacemaker current voltage dependence under our experimental conditions is largely accounted for by an effect of the myocyte maturational state on the HCN2 isoform. Further, this difference in activation voltage results in a marked difference in the physiological impact of expressed $I_{HCN2}$ because of the relative position of the current threshold with respect to MDP as a function of age.

HCN2 in neonatal ventricle activates at less negative voltages than in other mammalian systems, with a $V_{1/2}$ of −78 mV in the present study, compared with values of −83 to −99 mV.16,17,32 Although this might suggest that neonatal myocytes provide a unique environment, relative to alternative expression systems, at least one oocyte expression study33 reported HCN2 activation equivalent to that in neonatal ventricle, suggesting other systems also are capable of...
mined, it is likely that any cellular factor(s) contributing to the less negative activation of HCN2 in the neonate are not substrate limited because we observed no negative shift in $V_{1/2}$ (and in fact a slight positive trend) at expression levels that were 2 to 3 orders of magnitude greater than that typical of native current.

Under our experimental conditions, $V_{1/2}$ of native current in newborn and adult ventricle differed by 22 mV, which is lower than the previously reported difference in threshold value of $\approx$40 mV. In part, this may result from the 48-hour culture period, because acutely isolated adult myocytes had a $V_{1/2}$ that was more negative by 6 mV. Although not a statistically significant difference, it is consistent with an earlier report that extended culture of adult myocytes under conditions that cause morphological dedifferentiation results in a marked positive shift of activation voltage. In addition, the earlier developmental study specifically used adult epicardial myocytes, whereas the present study used the whole ventricle of the adult heart to obtain a higher yield of viable cells for culture. A gradient of $I_f$ activation, with epicardium more negative than endocardium, has been observed in the canine heart. If a similar gradient exists in adult rat ventricle, then this also could contribute to the less negative adult values observed in the present study.


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