UltraRapid Communication

Distribution and Prevalence of Hyperpolarization-Activated Cation Channel (HCN) mRNA Expression in Cardiac Tissues

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Abstract—HCN cation channel mRNA expression was determined in the rabbit heart and neonatal and adult rat ventricle using RNase protection assays. In the rabbit SA node, the dominant HCN transcript is HCN4, representing >81% of the total HCN message. HCN1 is also expressed, representing >18% of the total HCN mRNA. Rabbit Purkinje fibers contained almost equal amounts of HCN1 and HCN4 transcripts with low levels of HCN2, whereas rabbit ventricle contained predominantly HCN2. The SA node contained 25 times the total HCN message of Purkinje fibers and 140 times the total HCN message of ventricle. No reports of hyperpolarization-activated current (I_f) exist in rabbit Purkinje fibers, and we could not record I_f in rabbit ventricular myocytes. To investigate the possible role of isoform switching in determining the voltage dependence of I_f, we determined the prevalence of HCN isoforms in neonatal and adult rat ventricle. We had previously determined the threshold for activation of I_f to be ≈ -70 mV in neonatal rat ventricle and -113 mV in adult rat ventricle. In both neonatal and adult rat ventricle, only HCN2 and HCN4 transcripts are present. The ratio of HCN2 to HCN4 is ≈5:1 in the neonate and 13:1 in the adult. Taken together, these results suggest that different cardiac regions express different isoforms of the HCN family. The HCN1 and HCN4 isoforms are most closely associated with a depolarized threshold for I_f activation, whereas the HCN2 isoform is associated with a more negative activation curve. The full text of this article is available at http://www.circresaha.org. **(*Circ Res*. 1999;85:e1-e6.)**

Key Words: RNase protection assay ■ mRNA distribution ■ hyperpolarization-activated current

Although the heart is an electrical syncytium, the individual myocytes that comprise the whole are not homogeneous. The heart contains pacemaker regions (sinus node, atrioventricular node, and Purkinje fibers), in which the membrane potential never attains a steady value, and quiescent regions (atrial and ventricular muscle) that sit at a constant potential during diastole. Recently, a number of cardiac ion channel genes have been cloned, and the combination of molecular and biophysical measurements have resulted in the association of specific cardiac membrane currents with their individual molecular correlates.[1–3] Although this combination of biophysical and molecular approaches has had dramatic successes, until recently, the absence of a molecular basis for the hyperpolarization-activated current (I_f) has proven a roadblock for understanding the differences in pacemaker activity in different cardiac regions. Biophysical studies have demonstrated that I_f activates at dramatically different potentials in different cardiac regions with a threshold for activation of ≈ -50 mV in SA node, -85 mV in Purkinje myocytes, and -120 mV in adult ventricular myocytes.[4,5] This difference in the voltage dependence of activation is highly correlated with pacemaker capability. The most positive activation (in SA node) is associated with the highest pacing rate, whereas the most negative activation (ventricular myocytes) normally exhibits no diastolic depolarization at all. No other ion channel has demonstrated so dramatically different voltage ranges in different tissue types. Posttranslational modification via phosphorylation and direct cAMP binding can alter this voltage dependence, but this modulation is far less than the 80-mV difference observed in the different cardiac tissue types.[6,7] Thus, the basis of this wide difference in voltage dependence remains an open question. Is it due to different isoforms of the same gene family, coassembly with β subunits, or to a yet-to-be-determined form of posttranslational modification? Recently, three sets of investigators have reported the cloning of a new family of ion channel genes, which give rise to voltage-gated channels that activate on hyperpolarization and are modulated by direct cyclic nucleotide gating.[8–10] This HCN family (also known as BCNG or HAC) contains four

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members, two of which, HCN1 and HCN2, have been expressed in a heterologous expression system.[8,9] The expressed currents bear a striking resemblance to channels observed in both cardiac and nervous tissue that have been called I_f, I_h, or I_q.[11–13] Thus, with this cloning breakthrough comes the opportunity to further investigate the basis of the differing voltage dependence of I_f in the different cardiac regions. In the present study, we provide the first evidence for differences in mRNA isoform expression in cardiac tissues with differing activation thresholds for I_f.

Materials and Methods

Preparation of Rabbit Sinus Node Cells

Rabbits (2 to 3 kg; Charles River Laboratories, Wilmington, Mass) were injected intravenously with a euthanasia dose of sodium pentobarbitone (100 mg/kg). The hearts were quickly removed and placed in warm Tyrode solution containing heparin (4 U/mL). The ventricles were then removed from the atrium, and the remaining tissue was washed with Tyrode solution to remove the blood. The procedures to dissociate the SA node cells are similar to those described in Tromba and Cohen.[14] The SA node was quickly isolated from the right atrium and placed in a bath perfused with Tyrode solution at 37°C. The SA node was then trimmed under the microscope to remove extraneous tissue and allowed to recover for several minutes, during which spontaneous activity returned. The strips were then cut free from the crista terminalis and rinsed for 5 minutes in a Ca^{2+}-free solution. After this period, $CaCl_2$ (0.005 mmol/L), albumin (1 mg/mL, Sigma), collagenase (280 U/mL, Cls II, Worthington), and elastase (30 U/mL, type I, Sigma) were added to the Ca^{2+}-free solution, and the strips were shaken for 6 to 9 minutes. The tissue was subsequently transferred into a modified KB solution where the cells were separated and then stored for use.

Preparation of Rat Ventricular Myocytes

Adult rats were killed by intraperitoneal injection of pentobarbital (1.2 g/kg) and neonatal rats by decapitation, in accordance with protocols of the Institutional Animal Care and Use Committee. Adult rat ventricular myocytes were prepared from small pieces of epicardial tissue dissected from 9- to 12-week-old animals (300 to 350 g) using a collagenase dissociation protocol.[15] For preparing cell cultures of newborn ventricles, a standard trypsin dissociation of the ventricle was used.[15] The cells were preplated to reduce fibroblast contamination and then cultured in MEM plus 10% FCS, 0.6 μg/mL hypoxanthine, and 20 μg/mL gentamicin sulfate for 5 to 7 days. On the day of the experiment, the monolayer was resuspended by brief (2 to 3 minutes) exposure to 0.25% trypsin and replated onto fibronectin-coated 9×22-mm glass coverslips. The cells were studied 2 to 8 hours after the resuspension.

Electrophysiological Measurements

Experiments were carried out during superfusion of the isolated rat ventricular myocytes at 35°C to 36°C or SA node cells at 32°C. The external solution for SA node cells contained (mmol/L) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 5, and dextrose 10.0 (neutralized with NaOH to pH 7.35); for ventricular myocytes, the external solution contained (mmol/L) NaCl 137.7, NaOH 2.3, $MgCl_2$ 1, glucose 10, HEPES 5, KCl 5.4, $CaCl_2$ 1.8, $MnCl_2$ 2, $CdCl_2$ 0.2 (or 0 for neonatal rat ventricle), 4-aminopyridine 0 (for rabbit ventricle) or 2 (for rat ventricle), and $BaCl_2$ 8 (for rabbit ventricle) or 5 (for rat ventricle) (neutralized with NaOH to pH 7.4). The internal solution for SA node cells contained (mmol/L) K-aspartate 130, $MgCl_2$ 2, EGTA 11, Na-HEPES 10, $CaCl_2$ 5, Na_2ATP 2, Na-GTP 0.1 (pH 7.2); for ventricular myocytes, the internal solution contained (mmol/L) NaCl 6, K-aspartate 130, $MgCl_2$ 2, $CaCl_2$ 5 (or 2 in neonatal rat), EGTA 11 (or 5 in neonatal rat), Na_2-ATP 2, Na-GTP 0.1, Na-cAMP 0.2, and HEPES 10 (pH 7.2). The divalent cations Mn^{2+} and Cd^{2+} were used to reduce Ca^{2+} currents, which can overlap with and obscure I_f tail currents; Ba^{2+} was used to block the background current K^+ current (I_{K1}), which activates and inactivates in the same voltage range as I_f. The liquid junction potential (≈−10 mV) between the electrode tip and cell interior was not corrected. Currents were measured in whole-cell patch-clamp mode (for ventricular myocytes) using an Axopatch-1B amplifier or in perforated patch-clamp mode (for SA node cells) using an Axopatch-1D amplifier. The perforated patch pipettes had a resistance of 4 to 6 MΩ, and the concentration of amphotericin B was 260 μg/mL. The pipettes for ventricular myocytes had a resistance of 2 to 4 MΩ (adult and newborn culture) or 5- to 6-MΩ (newborn acute) electrodes. Data were recorded on a videocassette recorder through a digital data recorder (VR-10, Instrutech Corp) and simultaneously acquired by CLAMPEX software (pClamp, version 5.5 or 6.0.3. Axon Instrument Inc) for later analysis by CLAMPFIT (Axon Instrument Inc). Data were calculated as mean±SEM.

Isolation of Partial HCN cDNA Clones From Rat and Rabbit

Because of the intrinsic specificity of the RNase protection assay, it was necessary to obtain species-specific HCN channel cDNA templates. Polymerase chain reaction (PCR) amplification was used to clone HCN channel homologues from rat and rabbit. The amplified cDNA fragments were subcloned into an appropriate vector,[16] and DNA templates were then linearized with an appropriate restriction enzyme. All constructs were confirmed by DNA sequencing.

To generate the cDNA templates from rat, degenerate oligonucleotide primers were designed to complement conserved regions of the HCN gene family. Two sets of primers were used to obtain the four different templates. The rat HCN1 cDNA was cloned using the forward primer 5′-TGYCAYTGGGAYGGNTGY-3′ directed against the amino acid sequence CHWDGC of the S5 transmembrane region and the reverse primer 5′-NACRAACATNGCRTARCA-3′ directed against the amino acid sequence CYAMFV of the S6 transmembrane region. The rat HCN2, HCN3, and HCN4 cDNAs were cloned using the forward primer 5′-ATHCAYCCNTAYWSNGAYTTY-3′ directed against the amino acid sequence IHPYSDF of the S1 transmembrane region and the reverse primer 5′-RCANCCRTCCCARTGRCA-3′ directed against the amino acid sequence CHWDGC of the S5 transmembrane region.

Four sets of primers were used to obtain the four probes from rabbit. According to the published sequences of each of the individual HCN genes from human and mouse, these four sets of primers were designed specifically for each member, but their nucleotide sequences were still conserved across human and mouse.

The rabbit HCN1 gene was identified using the forward primer 5′-TGCAGGCTTCTGGATTATCC-3′ directed against the amino acid sequence AGFWII of a region ≈8 amino acids before the S1 transmembrane region and the reverse primer 5′-CATGTGGAATATCTCTTCC-3′ directed against the amino acid sequence EEIFHM of a region ≈2 amino acids after the S4 transmembrane region.

The rabbit HCN2 gene was identified using the forward primer 5′-ACAGCGACTTCAGGTTCTAC-3′ directed against the amino acid sequence SDFRFY of the S1 transmembrane region and the reverse primer 5′-GTCTTGTAGACCTCGGAGTC-3′ directed against the amino acid sequence DSEVYK at the beginning of the S4 transmembrane region.

The rabbit HCN3 gene was identified using the forward primer 5′-GTCTTCGGCAGCCACAAAGC-3′ directed against the amino acid sequence VFGSHK of a region ≈25 amino acids before the S1 transmembrane region and the reverse primer 5′-CTGGTGTATGTAGCGGATG-3′ directed against the amino acid sequence IRYIHQ of the S4 transmembrane region.

The rabbit HCN4 gene was identified using the forward primer 5′-GGATTATCCACCCCTACAG-3′ directed against the amino acid sequence WIIHPY of a region ≈5 amino acids before the S1 transmembrane region and the reverse primer 5′-

GCGCAGGAGGCTGAGGATC-3′ directed against the amino acid sequence ILSLLR of the S4 transmembrane region.

The rat and rabbit HCN clones were amplified from brain cDNA. The rat HCN1 and HCN2 clones are 100% identical to the mouse sequence at the deduced amino acid level. The rat HCN3 is 99% and the rat HCN4 is 98% identical to the mouse sequence at the deduced amino acid level. For the rabbit cDNA clones, HCN1 is 100%, HCN2 is 94%, HCN3 is 99%, and HCN4 is 95% identical to the mouse sequence at the deduced amino acid level.

All sequences have been submitted to GenBank, and the GenBank accession numbers are AF155163 through AF155170.

RNase Protection Assays

The procedures for the preparation of total RNA from rat brain and ventricle and the performance of the RNase protection assays were identical to those described previously.[17] Rabbit brain polyA$^+$ RNA was obtained commercially from Clontech, and polyA$^+$ RNA from SA node, Purkinje fibers, and left ventricle was isolated using paramagnetic poly-dT beads (Dynal Inc, Lake Success, NY). For each experiment in the rat, 5 μg of total RNA was used. For the rabbit experiments, the amount of polyA$^+$ mRNA in each sample used was different. A cyclophilin probe was used as an internal control in both rat and rabbit experiments. RNA expression was quantified directly from dried RNase protection gels using a PhosphorImager (Molecular Dynamics).

Results

Expression of HCN Isoforms in Rabbit Heart

The sinus node represents a very small fraction of total cardiac membrane, but its role as the primary pacemaker makes the prevalence of individual HCN isoforms in this tissue an important question. The expression of all four HCN isoforms in rabbit SA node was determined using an RNase protection assay (Figure 1). The SA node contains an abundance of HCN1 and HCN4 transcripts, a detectable level of HCN2, and almost no HCN3 transcripts. This result is surprising on four counts. First, a previous study using nonquantitative PCR suggested that HCN2 is the predominant channel subunit in SA node. Second, we find the isoform thought to be most prevalent in cardiac tissue,[8] HCN2, is only minimally present in SA node (0.6±0.1% of total HCN mRNA, n=4, SEM). Third, a heretofore-believed neural isoform, HCN1, is prominently expressed (18.2±5.7% of total HCN mRNA, n=4) in SA node. Finally, HCN4 is the most highly expressed (81.2±5.7% of total HCN mRNA, n=4) in the rabbit SA node.

We also examined HCN expression in rabbit Purkinje fibers and ventricular muscle. Similar to the sinus node, the Purkinje tissue expresses HCN1 (49.0±14.7% of total HCN mRNA, n=4) and HCN4 (40.1±17.1% of total HCN mRNA, n=4) with very low levels of HCN2 (10.9±5.8% of total HCN mRNA, n=4). The total expression of HCN isoforms in Purkinje fibers is only 3.9% of that expressed in SA node. Nevertheless, the Purkinje results are important because they argue against a potential artifact. The SA node has substantial neural innervation, and the finding of a prominent HCN1 expression might be attributed to contamination with neural cell bodies. Innervation is much less prevalent in the peripheral Purkinje fibers,[18] and so the finding of HCN1 in Purkinje fibers lends credence to its presence in cardiac muscle. The results from ventricular muscle are also important. Figure 1 illustrates the presence of HCN2 transcripts but at very low levels. The other isoforms are almost undetectable. The expression of HCN2 transcripts in ventricular muscle is only at 0.7% of the total HCN isoform expression in SA node.

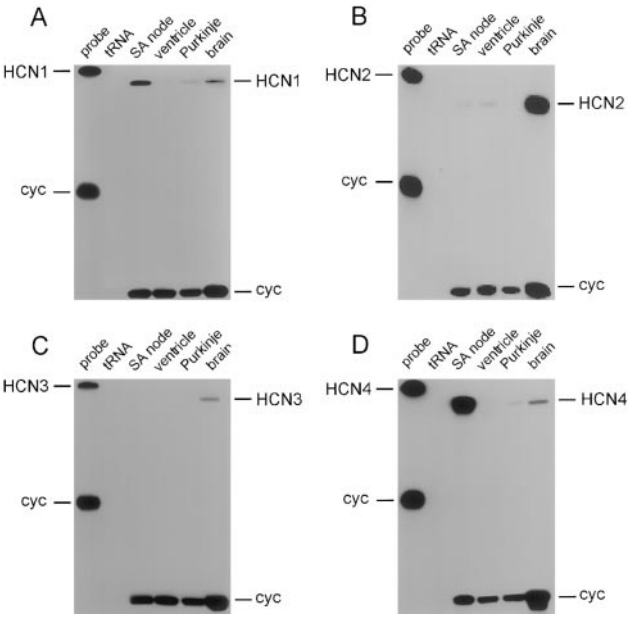

Figure 1. HCN channel mRNA expression in rabbit sinus node, left ventricle, Purkinje fibers, and brain determined by RNase protection analysis. Samples tested were sinus node, left ventricle, Purkinje fibers, and brain. There was 0.3 μg polyA$^+$ RNA in each brain sample, 0.25 μg polyA$^+$ RNA in each sinus node sample, and 0.25 μg polyA$^+$ RNA in each Purkinje fibers sample. For the left ventricle, the sample contained 0.8 μg of polyA$^+$ RNA in panels A, C, and D and 1.0 μg in panel B. A, HCN1 mRNA was expressed abundantly in sinus node and brain, and it is also expressed in Purkinje fibers. B, HCN2 mRNA was expressed at a very low level in sinus node and left ventricle, but it was expressed abundantly in brain. C, HCN3 mRNA was expressed in brain only. D, HCN4 mRNA was abundantly expressed in sinus node, more moderately expressed in brain, and expressed at a low level in Purkinje fibers. The cyclophilin (cyc) gene was used as an internal positive control in all experiments to check for sample loss and equal loading.

Given the extensive expression of HCN isoforms in rabbit SA node, it is not surprising that prominent I_f currents can be recorded from this tissue, one example of which is presented in Figure 2A. The threshold for activation in this example is −55 mV, and we recorded I_f currents even at −45 mV, with our average threshold value of −50±2 mV (n=8). Considering the low level of HCN isoform expression in rabbit Purkinje fibers, it is not surprising that previous studies of this tissue type have not reported the presence of an I_f or, indeed, substantial pacemaker activity.[19] No previous studies have investigated I_f-like currents in rabbit ventricular myocytes. Given the low level of isoform expression, we would expect little or no recordable current. Figure 2B shows membrane currents recorded from a rabbit ventricular myocyte. No I_f is present even at −150 mV (n=8). Although it is recognized that mRNA levels do not perfectly parallel those of functional channels, the large difference in total HCN message in the three regions suggests that at least some of the regulation of I_f magnitude is transcriptional.

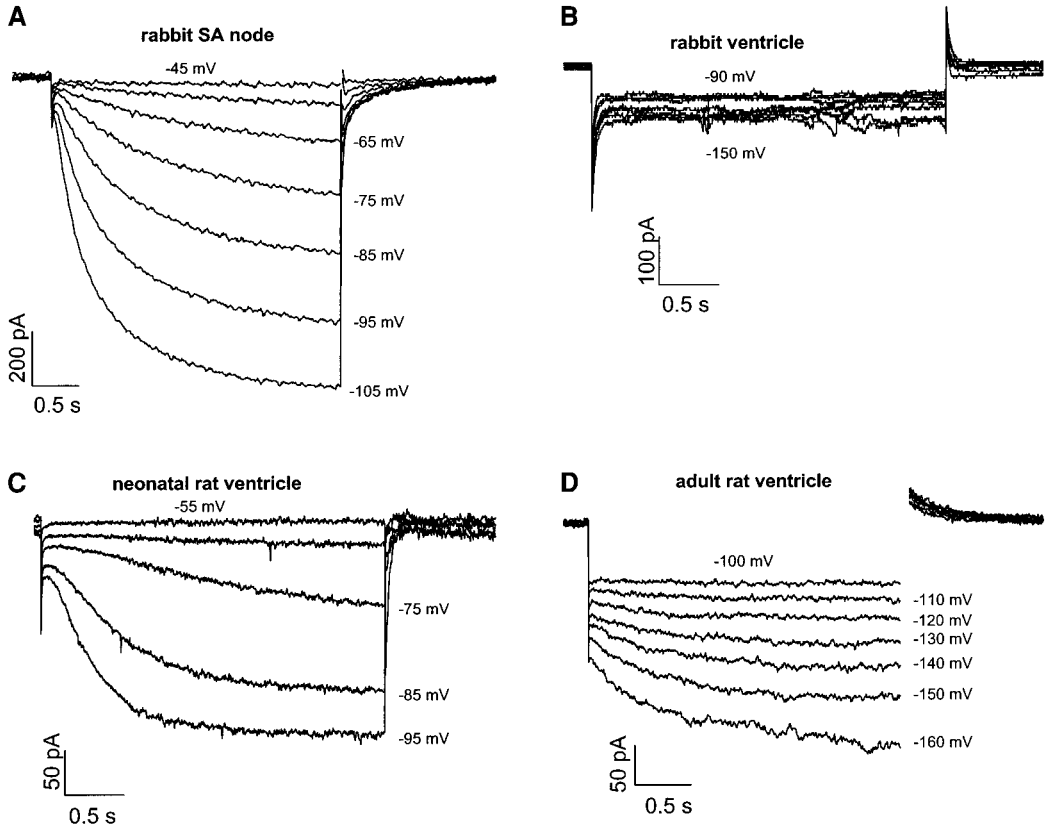

Figure 2. Voltage dependence of I_f activation. A, In a rabbit SA node cell, I_f was elicited by a hyperpolarizing pulse from −45 to −105 mV with voltage increments of 10 mV. The holding potential was −35 mV. The experiment was performed in normal Tyrode solution. B, In a rabbit ventricular myocyte, I_f was barely seen on membrane hyperpolarization down to −150 mV. The holding potential was −50 mV. The experiments were done in a modified Tyrode solution that contained 8 mmol/L Ba^{2+}, 2 mmol/L Mn^{2+}, and 0.2 mmol/L Cd^{2+}. C, In a cell-cultured neonatal (3-day-old) rat ventricular myocyte, I_f was activated by a hyperpolarizing pulse to −65 mV from a holding potential of −35 mV. Further hyperpolarization resulted in enhanced I_f activation. D, In an adult (3-month-old) rat ventricular myocyte, I_f was recorded by hyperpolarizing from a holding potential of −50 mV to potentials of −100 to −160 mV. After hyperpolarization, the cell was depolarized to +20 mV for 200 ms to accelerate the I_f recovery process. Experiments shown in panels C and D were performed in a modified Tyrode solution that contained 5 mmol/L Ba^{2+}, 2 mmol/L Mn^{2+}, and 2 mmol/L 4-aminopyridine.

Expression of HCN Isoforms in Neonatal and Adult Rat Ventricle

Because one of our aims was to examine the role that isoform "switching" could play in determining the voltage dependence of I_f in different cardiac regions, we had to examine additional cardiac tissues that expressed I_f but with differing activation ranges. We have previously demonstrated a negative shift in the activation of I_f between 3 days and 3 months of age in myocytes from the rat ventricle. Sample data from these two preparations are illustrated in Figure 2C and 2D. The threshold voltage for the I_f data recorded from a day-old rat myocyte in culture 4 days is −65 mV in Figure 2C and averaged −70±2 mV (n=9) in our previous study.[15] The threshold voltage for the I_f data recorded from a 3-month-old rat ventricular myocyte is −110 mV in Figure 2D and averaged −113±5 mV (n=12) in our previous study.[15] This difference in the activation of I_f provided a unique opportunity to examine the possible role of isoform expression in determining this difference in voltage dependence.

Figure 3 presents our data on the expression of HCN isoforms in neonatal and adult rat ventricle. RNase protection assays were performed with all four HCN isoforms on both neonatal (2 to 5 days old) and adult ventricle (3 months old). Only HCN2 and HCN4 are expressed in either tissue type. In neonatal myocytes, HCN2 represents 82.4±2.0% of total HCN mRNA (n=4), whereas HCN4 is expressed at 17.6±2.0% of the total HCN mRNA (n=4). In the adult rat ventricle, this percentage changes as a result of an increase in HCN2 expression. HCN2 transcripts now represent 93.2±1.7% of total HCN mRNA (n=3), whereas HCN4 transcripts are expressed at 6.8±1.7% of the total HCN message (n=3).

Discussion

HCN Isoform Expression in the Rabbit Heart

The SA node is the primary pacemaker of the mammalian heart. I_f activates at the most positive potentials in SA node myocytes. Our results presented above indicate an average threshold for I_f activation of −50 mV in rabbit SA node. The RNase protection assays indicate the presence of a large amount of message for the HCN4 and HCN1 channel subunits and a small level of expression of HCN2. This result suggests that HCN4 may make the major contribution to I_f in SA node. Previous studies using Northern blot analysis and nonquantitative reverse transcriptase (RT)-PCR found only HCN2 and HCN4 in heart, with HCN2 being the most

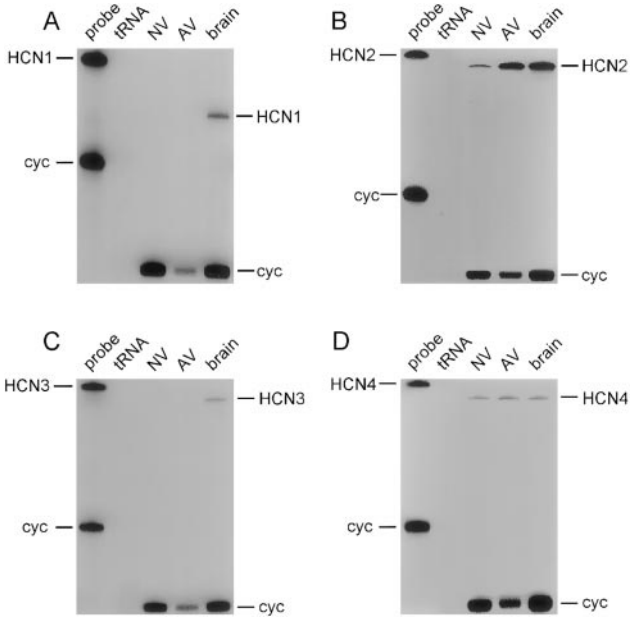

Figure 3. HCN channel mRNA expression in neonatal and adult rat ventricle determined by RNase protection analysis. Samples tested were neonatal ventricle (NV), adult ventricle (AV), and brain. All samples contained 5 μg of total RNA. The brain sample was used as a positive control for HCN1 and HCN3, and the cyclophilin (cyc) gene was used as an internal positive control in all experiments to check for sample loss and equal loading. A, HCN1 mRNA was not expressed in neonatal and adult ventricle. B, HCN2 mRNA was expressed with moderate abundance in neonatal ventricle and abundantly in adult ventricle. C, HCN3 mRNA was not expressed in neonatal and adult ventricle. D, HCN4 mRNA was expressed with moderate abundance both in neonatal and adult ventricle.

prevalent isoform.[8] The difference between previous and present results may rely on the nonquantitative nature of RT-PCR. It is also true that SA node volume is small compared with that of ventricle. This difference in overall contribution is further emphasized by the presence of only HCN2 message in rabbit ventricle. Even allowing for the low level of expression, given the differences in total volume, HCN2 would likely be the most prevalent HCN channel subunit mRNA in the whole rabbit heart.

The presence of HCN1 message in SA node comes as a surprise because it was previously undetected in heart by Northern blot analysis.[8,9] Again, this may result from the small size of the SA node. Neural innervation is most prominent in this region, and so contamination of myocyte with parasympathetic nerve cells is a significant possibility. However, it also remains possible that some of the HCN1 message is of myocyte origin because (1) mRNA for the HCN1 isoform is present in free-running Purkinje fibers, which contain few neural cell bodies, and (2) there is a higher level of HCN1 expression in SA node than in brain (Figure 1). Given the small fraction of neural tissue in our SA node sample, this would imply a much higher expression level of HCN1 in the neurons innervating the SA node than in brain itself.

We used the rabbit heart because of its prominent application in studying pacemaker mechanisms. It is particularly unfortunate that neither the Purkinje fibers[19] nor the ventricular myocytes (the present results) exhibit any measurable I_f current. Given the very low level of expression (<4% of SA node HCN isoform expression in Purkinje fibers and <1% of SA node HCN isoform expression in ventricle), it is not entirely surprising that this is the case.

HCN Isoform Distribution in Neonatal and Adult Rat Ventricle

Our previous studies demonstrated that the activation threshold for I_f shifts to more negative potentials between 3 days and 3 months of age.[15] The 40-mV negative shift in activation threshold is accompanied by slower kinetics of I_f activation in the adult (see Figure 2 and Reference 15); however, detailed analysis of adult kinetics was not pursued because of the very negative voltages required. Our present results show that these differences in I_f properties are associated with an almost 3-fold increase in the ratio of HCN2 to HCN4 transcripts.

One additional finding is worth noting. Although HCN2 is the dominant isoform in both the rat and the rabbit ventricles, the level of HCN2 expression is much higher in the rat than the rabbit.

Isoform Expression and I_f Voltage Dependence

The sinus node expresses HCN4 most prominently with some HCN1 and only trace amounts of HCN2 and has the most positive voltage threshold for I_f. It is possible that HCN1 and HCN4 are associated with a positive voltage dependence of I_f. I_f in neonatal ventricle activates at voltages more negative than in sinus node and contains a 4.7-fold ratio of HCN2 to HCN4 whereas adult rat ventricle expresses more HCN2 (HCN2:HCN4=13.7) and has the most negative activation threshold for I_f. This suggests that HCN2 is the isoform associated with a negative activation threshold for I_f. The percentage of HCN1 and HCN4 in rabbit SA node is 99%, in neonatal rat ventricle the percentage is 18%, and in adult rat ventricle it is 7%. There is a clear monotonic relationship. Decreasing percentages of these isoforms are associated with a more negative I_f threshold.

The results suggest that "isoform switching" may contribute to the wide range of I_f activation thresholds observed in cardiac tissues. To assess our results, it is important to compare the properties of HCN1, HCN2, and HCN4 expressed in oocytes and cell lines with the native I_f of cardiac tissues. Santoro et al[8] expressed HCN1, whereas Ludwig et al[9] have expressed HCN2. Both of these isoforms activate at potentials midway between SA node and ventricle. In a recent review, Tibbs and Santoro[20] report a half-activation voltage, $V_{1/2}$, for both clones of −100 mV. The most prominent difference is a much faster activation for HCN1. No published reports of HCN4 expression exist. These results raise an obvious question. What is the origin of the relationship we observe between isoform expression and I_f activation threshold? A number of possibilities must be considered. (1) HCN4 may have a relatively positive activation curve. (2) An auxiliary subunit is missing in the heterologous expression system that can change the gating of the channel. Previous results with KCNQ1 and minK demonstrate how dramatic the effects of a β subunit on gating can be.[3] (3) Heteromultimers composed of multiple HCN isoforms may possess different

gating properties than channels composed of only one type of channel subunit. (4) The results presented above show a strong correlation, but the correlation does not imply cause and effect. That is, isoform switching is not responsible for this difference in voltage dependence. Previous studies from our laboratory[6] as well as others[7] have presented evidence for important modulations of channel gating by both phosphorylation and direct cAMP gating. However these posttranslational effects are smaller than the observed differences in I_f gating properties in the various cardiac regions. It is possible that this isoform switching, although not sufficient by itself to cause the large gating change, is necessary. For example, an auxiliary subunit may preferentially interact with HCN1 and/or HCN4 but not HCN2.

In conclusion, our studies present the first quantitative analysis of HCN isoform distribution and prevalence in cardiac tissues. We hypothesize that isoform switching may be at least partly responsible for the differing I_f gating properties observed in the various cardiac regions. Because of the cloning and expression of the relevant HCN isoforms, this hypothesis is now open to further investigation.

Acknowledgments

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