Molecular Mapping of Sinoatrial Node HCN Channel Expression in the Human Heart

Ning Li, MD, PhD; Thomas A. Csepe, BSc; Brian J. Hansen, BSc; Halina Dobrzynski, MD; Robert S.D. Higgins, MD; Ahmet Kilic, MD; Peter J. Mohler, PhD; Paul M.L. Janssen, PhD; Michael R. Rosen, MD; Brandon J. Biesiadecki, PhD; Vadim V. Fedorov, PhD

Background—The hyperpolarization-activated current, \( I_f \), plays an important role in sinoatrial node (SAN) pacemaking. Surprisingly, the distribution of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in human SAN has only been investigated at the mRNA level. Our aim was to define the expression pattern of HCN proteins in human SAN and different atrial regions.

Methods and Results—Entire SAN complexes were isolated from failing (n=5) and nonfailing (n=9) human hearts cardiopically arrested in the operating room. Three-dimensional intramural SAN structure was identified as the fibrotic compact region around the SAN artery with Connexin 43–negative pacemaker cardiomyocytes visualized in Masson’s trichrome and immunostained cryosections. SAN protein was precisely isolated from the adjacent frozen SAN tissue blocks using a 16G biopsy needle. The purity of the SAN protein was confirmed by Connexin 43 immunoblot. All 3 HCN isoform proteins were detected in SAN. HCN1 was predominantly distributed in the human SAN with a 125.1±40.2 (n=12) expression ratio of SAN to right atrium. HCN2 and HCN4 expression levels were higher in SAN than in atria, with SAN to right atrium ratios of 6.1±0.9 and 4.6±0.6 (n=12), respectively.

Conclusions—This is the first study to conduct precise 3D molecular mapping of the human SAN by isolating pure pacemaker SAN tissue. All 3 cardiac HCN isoforms had higher expression in the SAN than in the atria. HCN1 was almost exclusively expressed in SAN, emphasizing its utility as a new specific molecular marker of the human SAN and as a potential target of specific treatments intended to modify sinus rhythm. (Circ Arrhythm Electrophysiol. 2015;8:1219-1227. DOI: 10.1161/CIRCEP.115.003070.)

Key Words: HCN pacemaker channels • heart failure • human heart • sinoatrial node • sinus node dysfunction

The spontaneous electric activity of mammalian hearts arises from specialized cardiomyocytes of the sinoatrial node (SAN). Multiple ion channels and Ca\(^{2+}\)-handling proteins are essential for generation of the SAN action potential. Among these, the hyperpolarization-activated funny current (\( I_f \)) is a major determinant of cardiac diastolic depolarization and plays a key role in controlling heart rate. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are coded by 4 genes (HCN1-4), representing the molecular \( \alpha \)-subunits of native \( I_f \) channels.

The rarity of human SAN specimens available for research and the complex intramural structure of the human SAN have resulted, respectively, in the lack of reliable means to acquire and accurately localize SAN samples. Thus, there remain critical barriers to study the molecular profile of the SAN in normal and diseased human hearts. Studies at the mRNA level indicate that the most highly expressed HCN subtype in the mammalian SAN is HCN4, which has been considered to be crucial for the generation of the heart beat and maturation of pacemaker-type cells during embryogenesis. Species-dependent expression of HCN1 and HCN2 has also been reported in the SAN, and a recent study reports that HCN1 also contributes to stable heart rate in mouse SAN. Heart failure (HF) results in significant SAN remodeling and dysfunction. Studies on rabbit and dog suggest that HF-induced SAN dysfunction is at least in part attributable to remodeling of \( I_f \) and downregulation of both HCN4 and HCN2. However, experimental data on the molecular characteristics of human SAN is minimal. Verkerk et al showed the presence of \( I_f \) in human SAN myocytes isolated from a single human heart with history of inappropriate SAN tachycardia, and in other studies, HCN4 loss-of-function mutations have been associated with SAN dysfunction and cardiomyopathy. Although HCN1, HCN2, and HCN4 mRNAs have been...
WHAT IS KNOWN

- The hyperpolarization-activated current, $I_h$, plays an important role in sinoatrial node (SAN) pacemaking.
- The distribution of HCN1-4 isoforms in human SAN is only known at the RNA level.

WHAT THE STUDY ADDS

- We developed a reproducible method for pure human SAN tissue isolation and presented the first quantitative analysis of selective HCN protein distribution and prevalence in human SAN and atrial tissues.
- HCN1 protein is primarily expressed in the human SAN rather than the atria, while HCN2 and HCN4 expression is about 4–6 times higher in the SAN pacemaker tissue than surrounding right atrial myocardium, but these isoforms are less SAN-specific than that of HCN1.
- Selective expression of HCN1 in human SAN pacemaker tissue emphasizes its utility as a new specific molecular marker of the human SAN and as a potential target of specific treatments intended to modify sinus rhythm.

detected in nonfailing human SAN, no data about protein distribution of all 3 HCN isoforms in human SAN are available. In the present study, we precisely isolated protein from SAN pacemaker clusters and adjacent atrial tissues of cardiologically arrested human hearts to investigate the expression patterns of HCN1, HCN2, and HCN4. For the first time, we identified the distinct and specific expression of HCN1 protein in human SAN. Our findings suggest a potentially important contribution of HCN1 to human SAN pacemaking.

Methods

SAN and Atrial Tissue Collection

De-identified, coded human hearts were obtained from Lifeline of Ohio Organ Procurement Organization and the Division of Cardiac Surgery at The Ohio State University Wexner Medical Center (Table). This study was approved by The Ohio State University Institutional Review Board.

Explanted human hearts (n=14) were obtained in the operating room at the time of cross-clamp and immediately preserved with ice cold cardioplegic solution and stored at 4°C during transport and dissection as previously described. Hearts were transported to the experimental laboratory within 15 minutes; whole intact atria were dissected from ventricles and coronary-perfused with oxygenated cardioplegic solutions at 4°C to prevent any potential tissue degradations because of ischemia.

Twelve fresh human SAN with adjacent atrial tissues (Hearts no. 1–12) were pinned to silicone pads (Figure 1), embedded in O.C.T. Compound (Fisher Scientific), frozen in cold isopentane, and stored at −80°C until use. The pins were visible markers used to indicate the orientation of the embedded SAN during subsequent dissection. Based on anatomic and functional data, frozen SAN tissues (cryo blocks) were cut into head, center, and tail blocks (4–6 mm long), respectively, perpendicular to epicardium (Figure 1). In keeping with previous studies, we describe the most superior third of the SAN as the head, the middle third as the center, and the inferior third as the tail (Figure 1A). Cryosections were collected from both ends of the cryo blocks at 20 μm thickness. Masson’s trichrome staining and Connexin 43 (Cx43)/α-actinin double immunolabeling were performed on cryosections. Histology and immunostaining images were used to guide the SAN tissue collection from cryo block. 16G (1.3 mm ID) biopsy needles were used to

Table. Human Heart Information

<table>
<thead>
<tr>
<th>Heart No.</th>
<th>Code No.</th>
<th>Age</th>
<th>Sex</th>
<th>Source</th>
<th>Heart Failure</th>
<th>Main Diagnoses</th>
<th>Device</th>
<th>HW, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>712301</td>
<td>67</td>
<td>Male</td>
<td>LOOP</td>
<td></td>
<td>Blunt injury, HTN</td>
<td>None</td>
<td>527</td>
</tr>
<tr>
<td>2</td>
<td>809108</td>
<td>60</td>
<td>Male</td>
<td>LOOP</td>
<td></td>
<td>Cardiac arrest, HTN, DM</td>
<td>None</td>
<td>842</td>
</tr>
<tr>
<td>3</td>
<td>911614</td>
<td>65</td>
<td>Male</td>
<td>OSU</td>
<td>+</td>
<td>Non-ischemic HF (Transplant), AF</td>
<td>CRT, LVAD</td>
<td>716</td>
</tr>
<tr>
<td>4</td>
<td>774694</td>
<td>50</td>
<td>Female</td>
<td>OSU</td>
<td>+</td>
<td>Ischemic HF (Transplant), CAD</td>
<td>None</td>
<td>486</td>
</tr>
<tr>
<td>5</td>
<td>674541</td>
<td>64</td>
<td>Male</td>
<td>LOOP</td>
<td>+</td>
<td>Stroke, ischemic HF, CAD, HTN, DM</td>
<td>ICD, LVAD</td>
<td>599</td>
</tr>
<tr>
<td>6</td>
<td>600245</td>
<td>51</td>
<td>Female</td>
<td>LOOP</td>
<td>–</td>
<td>CVA/ICH</td>
<td>None</td>
<td>507</td>
</tr>
<tr>
<td>7</td>
<td>768159</td>
<td>44</td>
<td>Male</td>
<td>LOOP</td>
<td>–</td>
<td>Cardiac arrest*, VF, DM</td>
<td>None</td>
<td>279</td>
</tr>
<tr>
<td>8</td>
<td>845013</td>
<td>26</td>
<td>Male</td>
<td>LOOP</td>
<td>–</td>
<td>Cardiac arrest, VT/VF, VSD</td>
<td>PM</td>
<td>497</td>
</tr>
<tr>
<td>9</td>
<td>380071</td>
<td>43</td>
<td>Female</td>
<td>LOOP</td>
<td>–</td>
<td>Respiratory arrest*, CAD, HTN, DM, Morbid obesity</td>
<td>None</td>
<td>603</td>
</tr>
<tr>
<td>10</td>
<td>574165</td>
<td>62</td>
<td>Male</td>
<td>LOOP</td>
<td>–</td>
<td>Blunt injury, AF</td>
<td>None</td>
<td>584</td>
</tr>
<tr>
<td>11</td>
<td>724569</td>
<td>64</td>
<td>Male</td>
<td>OSU</td>
<td>+</td>
<td>Non-ischemic HF (Transplant), AF, VT, HTN, Hypothyroidism</td>
<td>PM</td>
<td>636</td>
</tr>
<tr>
<td>12</td>
<td>971258</td>
<td>57</td>
<td>Male</td>
<td>OSU</td>
<td>+</td>
<td>Ischemic HF (Transplant), CAD, MI, AF</td>
<td>ICD, LVAD</td>
<td>619</td>
</tr>
<tr>
<td>13</td>
<td>168021</td>
<td>62</td>
<td>Female</td>
<td>LOOP</td>
<td>–</td>
<td>CVA/ICH, CAD, HTN, DM</td>
<td>None</td>
<td>896</td>
</tr>
<tr>
<td>14</td>
<td>921821</td>
<td>22</td>
<td>Male</td>
<td>LOOP</td>
<td>–</td>
<td>Cardiac arrest, VT, DM</td>
<td>None</td>
<td>383</td>
</tr>
</tbody>
</table>

The causes of death (transplantation) are indicated in italics. AF indicates atrial fibrillation; CAD, coronary artery disease; CRT, cardiac resynchronization therapy; CVA/ICH, cardiovascular attack/intracranial hemorrhage; DM, diabetes mellitus; HF, heart failure; HTN, hypertension; HW, heart weight; ICD, implantable cardiac defibrillator; LOOP, Lifeline of Ohio Organ Procurement Organization; LVAD, left ventricular assist device; MI, myocardial infarction; OSU, The Ohio State University Wexner Medical Center; PM, pacemaker; VT, ventricular fibrillation; VSD, ventricular septal defect; and VT, ventricular tachycardia.

*The patient had >20 min of cardiac downtime.
accurately collect SAN tissue along, but not including, the main artery within the Cx43-negative area from the SAN head, center, and tail separately. According to the size of the Cx43-negative tissue, 2 to 3 punches (10–15 mg in total) of pure SAN tissue could be collected from each cryo block. Tissue from the crista terminalis, interatrial septum, and right atrial free wall (RAFW) were also collected from the same cryo block from which the SAN center was collected using the same method. Separate samples of the atrial tissue, such as left atrial free wall, left atrial appendage, right atrial appendage, right atrioventricular ring (RR), and different locations of the RAFW (superior, middle, and inferior) were collected fresh and flash-frozen in liquid nitrogen during heart dissection. Additionally, 2 SAN preparations (Hearts no. 13 and no. 14) immediately after flash-frozen in liquid nitrogen during heart dissection. Additionally, were dewaxed and heated in citrate-based buffer for antigen retrieval staining with Masson’s trichrome (Sigma Aldrich), as described previously. Images were taken with a 20× digital slide scanner (0.5×0.5 m2 resolution, Aperio ScanScope XT, Leica).

### Histology
Cryosections were fixed in 4% paraformaldehyde for 1 hour and stained with Masson’s trichrome (Sigma Aldrich), as described previously. Images were taken with a 20x digital slide scanner (0.5x0.5 μm2 resolution, Aperio ScanScope XT, Leica).

### Immunohistochemistry
Cryosections were fixed with −20°C methanol for 5 minutes before immunostaining. Sections were permeabilized with 0.1% Triton X-100 (Sigma Aldrich), blocked with 1% bovine serum albumin, and incubated with primary antibodies overnight at 4°C. The following day, sections were incubated in secondary antibodies for 2 hours at room temperature and mounted in ProLong Gold Antifade Mountant with DAPI (Life Technologies). The primary and secondary antibodies include mouse anti-HCN1 (1:100, Abcam), rat anti-HCN4 (1:100, Abcam), rabbit anti-HCN2 (1:100, Alamone), rabbit anti-Cx43 (1:400, Sigma-Aldrich), mouse anti-α-actinin (1:200, Abcam), goat anti-rabbit Alexa Fluor 488 (1:200, Life Technologies), and goat anti-mouse Alexa Fluor 568 (1:200, Life Technologies). Paraffin sections were dewaxed and heated in citrate-based buffer for antigen retrieval before the immunostaining protocol. Sections were imaged using an Olympus FV1000 Filter confocal microscope, and fluorescence density was measured by ImageJ software. A summary of antibodies used in immunohistochemistry protocols is provided in Table I in the Data Supplement, and the specificity of antibody is discussed in the Data Supplement (Figure I in the Data Supplement).

### Immunoblot Analysis
For protein isolation, human SAN and atrial tissue were homogenized in 2x urea buffer (10 μL buffer per 1 mg tissue) followed by centrifugation at 14,000 rpm for 10 minutes at 10°C, and the supernatant was collected. Protein yield was quantified using RC DC Protein Assay (Bio-rad). Equal amounts (20 μg/sample) of proteins were separated by SDS-PAGE 12% (200:1) polyacrylamide gels and transferred to 0.45 μM low-fluorescence polyvinylidene fluoride membrane by methods previously described. After blocking, membranes were incubated with various primary antibodies overnight at 4°C: mouse anti-HCN1 (1:500, Abcam), rat anti-HCN4 (1:100, Abcam), rabbit anti-HCN2 (1:500, Alamone), and rabbit anti-Cx43 (1:8000, Sigma-Aldrich). Mouse anti-GAPDH (1:20000, Sigma-Aldrich) was used as reference for equal protein loading and to normalize HCN channel protein band intensity. Subsequently, 1:2000 diluted fluorescent DyLight conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were applied to membranes for 1 hour at room temperature. The specific bands were detected on a Typhoon 9410 imager (GE Healthcare) and quantified by densitometry analysis (ImageQuant, GE Healthcare).

### Statistical Analysis
Data are presented as mean±SEM. To test whether protein expression ratios of SAN to RAFW and RR to RAFW were different from 1, ratios for each heart were log10 transformed for each protein to stabilize variance, and the 2-tailed 1-sample t test was applied (Table II in the Data Supplement). The 2-tailed Satterthwaite t test was used to evaluate the difference between failing and nonfailing groups (Table III in the Data Supplement). All tests were done using R 3.1.0 for Windows. A P value of ≤0.05 was considered significant.
Localization and Extraction of Human SAN Tissue

The SAN is located at the junction of the superior vena cava and right atrium in the human heart. Figure 1A illustrates the anatomic and functional boundaries of the human SAN. The intramural SAN structure was identified in the histology images as the region of dense fibrotic matrix surrounding and isolating clusters of specialized cardiomyocytes around the SAN artery (Figure 1B, middle). Gap junction protein Cx43 is known to be absent from the SAN but abundantly expressed in the right atrium of humans and small mammals.8,21 Figure 1B shows a positive correlation between the histologically identified SAN and the SAN identified by negative Cx43 expression in the present study. SAN, crista terminalis, and RAFW tissue were collected from the locations indicated by the pink dots in Figure 1B. The average yield of total SAN protein was roughly 200 to 300 μg from each SAN cryo blocks. Immunoblots, performed with HCN4, α-actinin, and Cx43 antibodies, detected specific bands at the expected molecular weights (~150, 100, and 43 kDa, respectively). Alpha-actinin and GAPDH expression were comparable in all tested samples. Cx43 expression was markedly lower in the SAN center than in crista terminalis and RAFW tissue, whereas HCN4 expression was higher in the SAN center than in the adjacent tissues (Figure 1C). In all 12 hearts, total Cx43 expression was significantly lower in SAN tissue than in the RAFW, with GAPDH normalized band density ratio of SAN/RAFW=0.18±0.05 (P<0.01), which attests to the purity of the SAN sample.8,11

Expression Pattern of HCN Channels in Human SAN and Atria

Expression levels of the 3 HCN isoforms in human atria were determined by immunoblot and immunohistochemistry. First, the distribution of HCN isoforms within different areas of the human SAN was investigated. Figure 2 shows representative immunoblot results from Heart no. 2. The expression level of each HCN isoform was similar among the 3 SAN locations (head, center, and tail) within this heart. As such, consecutive analysis used the SAN center to represent SAN expression. We further investigated the expression pattern of HCN channels in the human SAN center and in the different atrial tissue. In all studied hearts, expressions of all 3 HCN isoforms in SAN center were always higher than the expressions in the RAFW. Figure 3 shows representative immunoblot results from Heart no. 6, and Figure 4 summarizes data from 12 individual SAN–atrial tissue preparations. All immunoblotting experiments comparing the HCN isoform expression levels between SAN and RAFW were repeated at least twice. In Figure 3A, a 90-kDa band corresponding to the mature glycosylated HCN1 protein10 can be seen in the SAN tissue, but other atrial tissues displayed bands of lesser density. The specific band density of HCN1 (GAPDH normalized) was 5.6 to 446 times greater in the SAN region than in the RAFW, with the average ratio of SAN/RAFW=125.1±40.2 (n=12, P<0.05). An 85-kDa band detected in all regions may represent a nonspecific band as has been similarly observed in mouse HCN1 immunoblots.10 Both HCN2 and HCN4 proteins were detected and quantified in the SAN and in all other atrial tissue. HCN2 protein expression was 6.1±0.9-fold (2.4–12.5 times) more abundant in the SAN than in the RAFW (n=12, P<0.05), and HCN4 protein expression was 4.6±0.6-fold (2.2–8.3 times) higher in the SAN than in the RAFW (n=12, P<0.05). We also found that HCN2 expression in the RR area was significantly higher than in the RAFW, with a band density ratio of RR/RAFW=2.4±0.4 (n=12, P<0.05). However, HCN1 and HCN4 protein expression showed no difference in the RR area compared with the RAFW (Table II in the Data Supplement).

We also performed double-label immunostaining (HCN1/Cx43, HCN2/Vimentin, HCN4/Cx43) to demonstrate the cell-specific distribution of the 3 HCN channel proteins in a series of paraffin slides from Heart no. 13 and no. 14 (Figure 5). The SAN area was identified by the lack of expression of Cx43, as well as a dense matrix of fibroblasts labeled by Vimentin.11 For all HCN antibodies, we achieved specific staining of cardiomyocytes in the SAN and surrounding atrial regions.

HCN Expression Changes in Heart Failure

To assess whether the HCN isoform expressions were modified by HF, we compared the protein expression level in the SAN center and RAFW between nonfailing (NF) and failing (HF) hearts (Figure 6). Exclusion criteria from the HF control group included pacemaker implantation and >20 minutes of down time (temporal duration between cardiac arrest and cardiopulmonary resuscitation or advanced cardiac life support); thus, Hearts no. 7 to 9 were omitted from this group. The SAN center showed significantly more protein expression of all 3 HCN isoforms than RAFW in both NF and HF hearts. HCN1 expression in the SAN showed no significant difference between NF and HF groups, and each group had considerable variance (GAPDH-normalized band density: NF=153.7±59.6, n=4; HF=161.8±79.8, n=5, P=0.94). Figure 6 shows that HF Hearts no. 4 and no. 5 (ischemic HF) had...
significantly lower HCN1 expression than Heart no. 3 (non-ischemic HF), whereas HCN2 and HCN4 expression was still well-preserved in Heart no. 4 and no.5. HCN1 expression (90-kDa band) in the RAFW was at a low level in all hearts. No significant changes in HCN2 and HCN4 expression were observed between NF (n=4) and HF (n=5) groups (Table III in the Data Supplement).

Discussion

Methodological Innovations: Molecular Mapping of the Human SAN Pacemaker Complex

The expression levels of HCN isoforms strongly depends on the cardiac region and varies among species.22 As we described previously,4,5 the human SAN has a complex transmural 3D structure, and the leading pacemaker sites are located at the center and close to the junction of the center and tail thirds of the SAN. Considering the variation in size and intramural location of SAN in individual heart preparations when comparing expression levels of HCN protein in SAN to those in adjacent atrial tissue, we only used SAN protein isolated from the SAN center. In each heart preparation, specificity of SAN tissue extraction was confirmed by negative Cx43 expression before and after protein isolation using immunostaining and immunoblotting, respectively. This study specifically determined HCN expression in pure SAN tissue that was not contaminated by surrounding atrial tissue; therefore, our results can be relied on to show the actual difference between SAN and the adjacent atrial tissue. We suggest that this novel
approach provides a reproducible method for specific tissue isolation to study the molecular profiles of the human SAN and other specific cardiac tissues in normal and pathological conditions.

**Potential Function of HCN in Pacemaking**

The functional relevance of If/HCN channels in the human cardiac pacemaker has been confirmed by the following: (1) specific blockade of If channels directly decreases heart rate with limited cardiovascular side effects; and (2) HCN4 gene mutations are associated with various inheritable forms of sinus arrhythmias. This in no way diminishes the relevance of other mechanisms to SAN pacing; however, our study focused on the HCN channel.

HCN4 is reported to be the most abundant HCN isoform in the mammalian SAN, suggesting that it plays a key role in the generation of primary pacemaker potentials. In mouse SAN, HCN4 mRNA represents nearly 60% of the HCN expression, followed by HCN1 (34%). The relative abundance of HCN mRNA measured in the human SAN (mRNAs HCN4/HCN1 ≈ 6.3; HCN4/HCN2 ≈ 33.8) are similar to the results in the rabbit SAN (mRNAs HCN4/HCN1=7.4 and HCN4/HCN2=46.4). Recent immunolabeling studies by Dobrzynski et al suggest that HCN4 cannot be an exclusive marker to map or identify the human SAN. These observations emphasize the differences in HCN isoform distribution between human and small mammals; in the latter, HCN4 is primarily expressed in the pacemaker conduction system. Multiple HCN isoforms may contribute to the assembly of heteromeric native cardiac If channels or to a mixed population of homomeric channels in the pacemaker cells. In agreement with these studies, we found that expression of all HCN isoforms is higher in the SAN than in the surrounding atria, especially HCN1.

Compared with HCN4, the available literature on HCN1 is limited and inconsistent. Despite the detection of HCN1 mRNA in the adult heart, most studies report the absence of HCN1 protein in the SAN and whole heart. A few recent studies confirmed the high expression of HCN1 protein colocalized with HCN4 in the mouse SAN and indicated the significant contribution of HCN1 to native If. A mouse HCN1 knockout study demonstrated that HCN1 could be important for stability of sinus rhythm. Our study demonstrates that HCN1 is primarily distributed in the SAN pacemaker, and its expression in the surrounding right atrium is negligible, potentially reflecting a specific contribution of HCN1 to pacemaker current in the human SAN. This finding that HCN1 is high in SAN but minimal in atrial tissue may explain why previous reports investigating HCN1 in total atrial tissue underestimate the level of HCN1 specifically in the SAN. The expression pattern of HCN1 suggests that HCN1 could be an additional selective marker for normal SAN cells. Although we have suggested multiple anatomic landmarks demarcating the SAN in previous studies, these landmarks (Cx43, fibrosis, fat) are only observed in histological or immunostaining analysis. Although 2D immunostaining of Cx43 clearly shows...
an SAN boundary from the rest of the atrial myocardium (Figure 1), immunoblot data of HCN1 levels show more significant expression differences in SAN versus atria than Cx43 or HCN4 (Figure 2).

There is evidence that HCN2 contributes to mouse SAN \( I_f \) current,\(^{29}\) although most studies report that HCN2 is the main isoform expressed in atrial and ventricular working myocytes in small mammals (rabbit, rat, and mouse).\(^{6,26}\) Consistent with these observations, we observed heterogeneous HCN2 distribution in SAN and adjacent atrial tissue, with 6x higher expression in SAN than in RAFW and 2x higher expression in latent atrial pacemakerv (RR)\(^{30}\) than in nonpacemaker regions (RAFW). Studies in animals\(^{30}\) and our preliminary optical mapping experiments\(^{31}\) revealed nodal-type action potentials and ectopic activity in the RR region that were depressed by \( I_f \) blockade.\(^{31}\) We suggest that HCN2 may be important for both primary (SAN) and latent (RR) pacemaker automaticity in the human heart.

It is well known that different isoforms of the HCN channel exhibit distinct activation kinetics and varied sensitivity to cAMP.\(^{32}\) The varied expression pattern of multiple isoforms of HCN in different pacemakers of the heart may explain their differences in intrinsic rhythm because of the wide range of \( I_f \) activation thresholds.\(^{6}\) The different ratios of HCN isoforms in SAN and latent pacemakers may also affect the regional pacemaker sensitivity to neurotransmitters and HCN channel blockers.\(^{6,33,34}\) HCN1 has faster kinetics and a more positive activation threshold than either HCN2 or HCN4; also, HCN1 is much less responsive to cAMP than either HCN2 or HCN4.\(^{32,35}\) For this reason, a greater proportion of HCN1 channels may be activated at higher membrane potentials than HCN4 channels, even though total protein expression of HCN1 is much less than HCN4. Our results demonstrated the expression of HCN1 exclusively in the SAN, which may help to protect the leading pacemaker from hyperpolarization and stabilize the leading pacemaker of the SAN during adrenergic receptor stimulation or phosphodiesterase inhibition. However, these suggestions require direct experimental confirmation.

HCN3 mRNA expression was negligible in human SAN and atrial tissues.\(^{6}\) For this reason, we did not include HCN3 in our current SAN study. However, mouse studies indicate the presence of HCN3 channel subtypes in ventricular myocytes.\(^{36-38}\) No expression of HCN3 was found in the murine cardiac conduction tissues.\(^{35}\) HCN3-deficient mice display normal cardiac pacemaker activity,\(^{37}\) but these mice have a specific defect in the repolarization of the ventricular action potential.

Role of Altered HCN Function During Cardiac Diseases

Among HCN channels, HCN4 mutations have been clearly associated with human SAN dysfunction, atrial fibrillation, and atrioventricular block.\(^{14}\) In human atria, although the mRNA level of HCN4 was significantly decreased in chronic atrial fibrillation, the protein level of HCN4 was preserved or slightly increased, causing an increase of \( I_f \).\(^{39}\) This result suggests that protein expression and function are not always correlated with mRNA because of complicated post-transcriptional modification mechanisms. Thus, it is particularly important to obtain direct evidence of HCN protein expression or function in human SAN/atria in normal and dysfunctional conditions, as was done in our study.

Previous studies reported that both mRNA and protein of HCN2/4 were detected in human atria, and their expression levels were augmented in specimens from patients with HF.\(^{40}\) Our study revealed that HCN1 was predominantly expressed in all investigated SAN, although expression levels of SAN HCN1 between hearts was variable. Moreover, statistical analysis did not reveal significant differences in HCN isoform expression between NF and HF groups. This may be because of the small number of hearts analyzed, as well as specific disease variation within each group.

Study Limitations

We studied a limited number of SAN samples. To ensure the quality of the SAN protein, we did not use optically mapped SAN preparations for protein isolation, so there was no direct correlation between functional data and the HCN expression pattern. We isolated protein from the SAN tissue section instead of single SAN myocytes. Even though we avoided the main arteries when collecting SAN tissue, we could not exclude the possibility that HCN expression was contaminated by noncardiomyocytes in the SAN region. To prevent proteolysis, all heart preparations were transferred and dissected at 4°C. Although we did not observe proteolysis for the other proteins (Cx43, \( \alpha \)-actinin, and GAPDH) detected by immunoblotting in our study, it may be possible that the HCN proteins could be degraded during the procedures. The individual role of the HCN isoforms in \( I_f \) generation should be evaluated with specific blockers of individual HCN1 or HCN4 \( I_f \) channel isoforms in both tissue and single cells, which will be the focus of our future studies.

Potential Implications and Future Directions

Our study presents the first quantitative analysis of HCN protein distribution and prevalence in human SAN and atrial tissues. We hypothesize that not only HCN4 but also HCN1 may play an important role in human SAN pacing and potentially contribute to the native \( I_f \) in the SAN pacemaker cells. Although there is no HCN1 gene mutation related to arrhythmias found in humans to date, it may be considered another candidate gene for human SAN dysfunction scans. Several studies demonstrated that overexpression of the HCN genes in myocytes via gene delivery or implantation of stem cells with overexpressed HCN2 successfully induced automaticity of a biological pacemaker in different animal models.\(^{41,42}\) Because of the specificity of HCN1 to the human SAN, the role of the HCN1 isoform should be taken into consideration for gene selection when developing a biological pacemaker\(^{44,45}\) and as a potential target of specific drug treatments aimed to affect sinus rhythm.

Conclusions

Our molecular study of human SAN tissue demonstrates a distinct cardiac expression profile of individual HCN isoforms in the SAN center versus atria. We found that HCN1 is primarily
expressed in the human SAN rather than in the atria. We also observed that HCN2 and HCN4 expression is 4 to 6 times higher in the SAN than in the RAFW, but these isoforms are less SAN-specific than HCN1. Furthermore, the novel technique used to isolate SAN tissue in this study may pave the way for more comprehensive molecular and functional integrated studies of the human SAN.

Acknowledgments

We thank the Lifeline of Ohio Organ Procurement Organization and the Division of Cardiac Surgery at The OSU Wexner Medical Center for providing the explanted hearts. We thank Mr Benjamin Canan and Mr Eric Schultz (PMLJ laboratory) as well as Mr Brandon Moore (VVF laboratory) for their help with tissue processing. We also thank Dr Stanislav Zakharkin for his help in statistical data analysis and for critical review of the manuscript.

Sources of Funding

This work was supported by National Institutes of Health (NIH) HL083422, and HL114383 (Dr Mohler) and by funding from the HL115580 (Dr Fedorov), HL113084 (Dr Janssen), HL084583,.

Disclosures

None.

References


Molecular Mapping of Sinoatrial Node HCN Channel Expression in the Human Heart

Circ Arrhythm Electrophysiol. 2015;8:1219-1227; originally published online August 24, 2015; doi: 10.1161/CIRCEP.115.003070

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circep.ahajournals.org/content/8/5/1219

Data Supplement (unedited) at:
http://circep.ahajournals.org/content/suppl/2015/08/24/CIRCEP.115.003070.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Arrhythmia and Electrophysiology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Arrhythmia and Electrophysiology is online at:
http://circep.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

The specificity of the antibodies

Stillitano et al.\(^1\) used the same anti-HCN2 (Alomone) and anti-HCN4 (Abcam) antibodies as those in the current study for immunoblotting of human atrial and ventricular tissue. Their immunoblotting results supported the specificity of the antibodies for HCN2 (~100 KD) and HCN4 (~130 KD) as they showed positive control and no cross-reaction between anti-HCN2 and anti-HCN4 antibodies. Because of the different concentration of the SDS-PAGE gel used for immunoblotting, the band size of HCN2 in the blotting results of the current study was about 150 KDa. As such, the anti-HCN2 antibody was pre-incubated with the HCN2 antigen peptide and confirmed the specificity of the antibody (Supplemental Figure IA). The Stillitano et al.\(^1\) immunoblotting analysis did not detect any HCN1 protein band in either human atrial or ventricular human tissues with the HCN1 antibody (Alomone). Therefore, an antibody from Abcam (ab84816) was used for HCN1 immunoblotting. A predominant band was detected in the SAN sample at about 90-100 KDa and cross-reaction bands with HCN2/HCN4 protein at 150 KDa was not observed (Supplemental Figure IB).

References


Supplemental Table I: Primary antibodies used in the study

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Company and Catalogue Number</th>
<th>Dilution for Immunostaining</th>
<th>Dilution for Immunoblotting</th>
<th>Band Size (KDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx43</td>
<td>Sigma (C6219)</td>
<td>1:400</td>
<td>1:8,000</td>
<td>37-50</td>
</tr>
<tr>
<td>HCN1</td>
<td>Abcam (ab84816)</td>
<td>1:100</td>
<td>1:500</td>
<td>90-100</td>
</tr>
<tr>
<td>HCN2</td>
<td>Alamone (APC030)</td>
<td>1:100</td>
<td>1:500</td>
<td>~150</td>
</tr>
<tr>
<td>HCN4</td>
<td>Abcam (ab32675)</td>
<td>1:100</td>
<td>1:100</td>
<td>~150</td>
</tr>
<tr>
<td>α-Actinin</td>
<td>Sigma (A7811)</td>
<td>1:200</td>
<td>1:10,000</td>
<td>~100</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sigma (G8795)</td>
<td>----</td>
<td>1:20,000</td>
<td>~37</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Sigma (V6389)</td>
<td>1:400</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>
**Supplemental Table II:** Cx43 and HCN expression ratio of SAN/RAFW and RR/RAFW

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sample</th>
<th>Mean Ratio to RAFW</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx43</td>
<td>SAN</td>
<td>0.184</td>
<td>1.653E-06</td>
</tr>
<tr>
<td>HCN1</td>
<td>SAN</td>
<td>125.065</td>
<td>3.463E-06</td>
</tr>
<tr>
<td>HCN1</td>
<td>RR</td>
<td>1.424</td>
<td>0.4219</td>
</tr>
<tr>
<td>HCN2</td>
<td>SAN</td>
<td>6.128</td>
<td>3.908E-07</td>
</tr>
<tr>
<td>HCN2</td>
<td>RR</td>
<td>2.427</td>
<td>6.549E-03</td>
</tr>
<tr>
<td>HCN4</td>
<td>SAN</td>
<td>4.638</td>
<td>6.979E-07</td>
</tr>
<tr>
<td>HCN4</td>
<td>RR</td>
<td>1.032</td>
<td>0.9966</td>
</tr>
</tbody>
</table>

To test if protein expression ratios of SAN to RAFW and RR to RAFW were different from 1, ratios for each heart were log10 transformed for each protein to stabilize variance and the two-tailed one-sample t-test was applied.

**Abbreviations:** SAN=Sinoatrial Node; RAFW=Right Atrial Free Wall; RR=Right Atrioventricular Ring

**Supplemental Table III:** HCN expression in HF and NF group

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sample</th>
<th>Mean HF</th>
<th>Mean NF</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCN1</td>
<td>SAN</td>
<td>161.834</td>
<td>153.673</td>
<td>0.9370</td>
</tr>
<tr>
<td>HCN2</td>
<td>SAN</td>
<td>10.123</td>
<td>8.356</td>
<td>0.6020</td>
</tr>
<tr>
<td>HCN4</td>
<td>SAN</td>
<td>6.287</td>
<td>4.701</td>
<td>0.4865</td>
</tr>
<tr>
<td>HCN1</td>
<td>RAFW</td>
<td>1.276</td>
<td>0.901</td>
<td>0.1761</td>
</tr>
<tr>
<td>HCN2</td>
<td>RAFW</td>
<td>1.285</td>
<td>1.475</td>
<td>0.7276</td>
</tr>
<tr>
<td>HCN4</td>
<td>RAFW</td>
<td>1.581</td>
<td>1.137</td>
<td>0.2235</td>
</tr>
</tbody>
</table>

The two-tailed Satterthwaite t-test was used to evaluate the difference between failing and non-failing groups.

**Abbreviations:** HF=Heart Failure; NF=Non Failure; SAN=Sinoatrial Node; RAFW=Right Atrial Free Wall
Supplemental Figure I. Immunoblot for HCN2 and HCN1 protein in human atrial and SAN tissue (Heart #6). (A) Immunoblot for HCN2 protein in human atrial tissue. The effect of pre-incubation with control antigen peptide is shown on the right panel. (B) Immunoblot for HCN1 and Cx43 protein in human SAN and atrial tissue. GAPDH was used as a loading reference in both panel A and B.