Organisation of the mouse sinoatrial node: structure and expression of HCN channels

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Abstract

Objective: To reveal the structural characteristics of the sinoatrial node (SAN) and the distribution of hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN) in the SAN in the mouse.

Methods: The structure of the SAN and the distribution of HCN channels in the SAN in the mouse were studied by histology and immunolabelling of ANP, Cx43 and HCN channels.

Results: The mouse SAN is a comma-shaped structure with a length of ∼1.5 mm parallel to the crista terminalis and is separated from atrial muscle by connective tissue at the border both with the crista terminalis and the atrial septum. A unique compact nodal structure with densely-packed nodal cells was identified at the head of the comma-shaped SAN. Cell size and fibre orientation vary regionally in the SAN: the cells in the compact node are small and are orientated perpendicular to the crista terminalis, whereas the cells in the more inferior part are larger and more loosely-packed and are orientated parallel to the crista terminalis. All SAN cells exhibited labelling of HCN4, but no cell exhibited detectable labelling of HCN1, HCN2, ANP and Cx43, while surrounding atrial cells exhibited labelling of ANP and Cx43, but not HCN1, HCN2 and HCN4. A specialised interface between the SAN and surrounding atrial muscle was also identified: strands of HCN4-positive nodal cells protrude into the atrial muscle and strands of Cx43-positive atrial cells protrude into the SAN; thus, there are interdigitations between the SAN and atrial muscle.

Conclusions: In the mouse, (i) the SAN is structurally complex with a densely-packed head and loosely-packed tail; (ii) HCN4 is the only HCN isoform detectable and is present throughout the SAN; and (iii) there is a specialised interface between the SAN and surrounding atrium that may be necessary for the SAN to drive the more hyperpolarized atrial muscle.

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1. Introduction

In order to explore the physiological functions of genes expressed in the heart, many genetically-modified mouse models have been generated during the last decade [1]. These models are also used to study heart diseases such as long QT syndrome [2] and cardiac conduction disease [3]. Some genetically-modified mouse models have sinoatrial node (SAN) dysfunction, i.e. abnormalities in SAN pacemaking and conduction [1,3]. For example, a mouse in which one copy of the SCN5A gene (responsible for the Na⁺ channel, Nav1.5) is knocked-out shows the major clinical features of sick sinus syndrome: sinus bradycardia, slow SAN conduction and SAN exit block [4]. To appreciate fully such mouse models, it is important to understand the electrophysiological properties of isolated mouse SAN cells and also the organisation (structure and ion channel expression) and function of mouse SAN tissue. This is especially important, because the mouse has a fast heart rate (350–600 beats min⁻¹). Although
several groups have studied the electrophysiological properties of isolated mouse SAN cells [5–8], there is little information on the organisation of the mouse SAN [9]. For this reason, we have investigated the structure of the mouse SAN and the distribution of the channel isoforms (HCNs), at the protein level, responsible for the hyperpolarization-activated current, $I_h$, one of the important pacemaker currents.

The HCNs in the mouse SAN have been studied at the mRNA level (but not at the protein level): in situ hybridisation shows HCN4 mRNA (abundant) > HCN2 mRNA > HCN1 mRNA > HCN3 mRNA (absent) [10], whereas quantitative PCR shows HCN4 mRNA > HCN1 mRNA > HCN2 mRNA > HCN3 mRNA [11].

2. Methods

2.1. Animals

C57BL/6J male mice weighing 20–30 g (age, 10–12 weeks; from Charles River UK Ltd., Kent, UK) were used (electrophysiology, $n=12$; histology, $n=10$; immunohistochemistry, $n=8$). Mice were killed by cervical dislocation and the heart quickly removed. All animal procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986; in addition, the investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Tissue preparation

Following Langendorff perfusion of the hearts with oxygenated Tyrode solution, the SAN region (the SAN and some surrounding atrial muscle) was dissected; a typical preparation is shown in Fig. 3A. 12 preparations (endocardial surface up) were placed in a chamber and superfused with Tyrode solution (37 °C; rate, $\sim 5$ ml/min) for electrophysiological recording. 10 preparations were embedded in OCT compound (Sakura, Netherlands) and frozen; frozen serial sections (5–10 μm thickness) were cut perpendicular to the crista terminalis from the top to the bottom of the preparations. Another 10 preparations were sectioned parallel to the endocardial and epicardial surfaces of the preparation. Sections were mounted on Super Frost Plus glass slides (BDH, Poole, UK) and stored at $-80$ °C for later use. In the case of the whole mount method [12], whole preparations ($n=5$) were used for immunohistochemistry. Single SAN cells were isolated as described previously [8].

2.3. Electrophysiology

Extracellular potentials were recorded by bipolar electrodes as described by Lei et al. [8]. Intracellular action potentials were recorded with 3 M KCl-filled conventional microelectrodes (resistance, $\sim 30$ MΩ). Electrical signals were digitised at 5 kHz by a DigiData 1322A A/D converter (Axon Instruments Inc., Union City, USA) and stored on a computer for later analysis. CsCl or ZD7288 (Tocris) was added to the Tyrode solution to block $I_h$.

2.4. Histology

Sections were stained with Masson’s trichrome to show histology; with this technique, connective tissue is stained blue, cardiac myocytes are stained red and nuclei are stained dark blue. Images of tissue sections were obtained using a Leica DC camera mounted on a Leica DMIRB inverted microscope in conjunction with Leica TWAIN software.

2.5. Antibodies

Various primary antibodies (IgGs) were used for immunolabelling of sections and isolated cells: rabbit anti-ANP

Fig. 1. Effects of Cs+ and ZD7288 on the spontaneous activity of the SAN. A, continuous recording of the extracellular potential near the leading pacemaker site under control conditions, in the presence of 0.5 mM Cs+ and on wash-off of Cs+. B, fast time base recordings of the extracellular potential from each period. C, extracellular potential recordings near the leading pacemaker site under control conditions (black trace), in the presence of 3 μM ZD7288 (red trace) and after wash-off of the drug (green trace). The cycle lengths under control conditions and in the presence of ZD7288 are shown. The effect of ZD7288 was partially washed-off in this example, but in the remaining six preparations the effect was irreversible.
(atrial natriuretic peptide) IgG raised against residues 4–28 of human ANP (1:200 dilution; Biogenesis, Poole, UK), rabbit anti-Cx43 (connexin43) IgG raised against residues 363–382 of human Cx43 (1:1000 dilution; Sigma, Poole, UK; all Cx43 labelling shown was obtained using this antibody), mouse anti-Cx43 IgG raised against residues 252–270 of rat Cx43 (1:1000 dilution; Chemicon, Harrow, UK), mouse anti-neurofilament 68 IgG raised against pig neurofilament 68 (1:200 dilution; Novocastra, Newcastle, UK), rabbit anti-HCN1 IgG raised against residues 6–24 of rat HCN1 (1:20 dilution; Alomone Labs, Israel), anti-HCN2 IgG raised against residues 147–161 of human HCN2 (1:50 dilution; Alomone Labs) and anti-HCN4 IgG raised against residues 119–155 of human HCN4 (1:100 dilution; Alomone Labs).

To detect the primary IgGs, the following secondary IgGs conjugated to fluorescence markers were used: goat anti-rabbit IgG conjugated to Alexa Fluro 488 (Molecular Probes, Eugene, USA), donkey anti-mouse IgG conjugated to Cy3

Fig. 2. Immunolabelling of HCN1, HCN2 and HCN4. A, C, E, immunolabelling of HCN1, HCN2 and HCN4 in the compact part of the SAN. SA, sinus node artery (shown by dotted white lines). White dashed lines in A and E, tissue border. B, D, F, immunolabelling of HCN1, HCN2 and HCN4 in brain tissue sections. G, immunolabelling of HCN2 in a cardiac ganglion near the SAN. H, immunolabelling of HCN2 in atrial muscle. I, high magnification view of the boxed region in H (arrows, HCN2 labelling in putative nerve fibres). J, K, immunolabelling of neurofilament 68 in the SAN (J; white arrows highlight neuronal tissue) and a cardiac ganglion near the SAN (K; ganglion on the epicardial side of the same tissue section as J). L–O, immunolabelling of HCN1, HCN2 and HCN4 in isolated SAN cells. Scale bars, 200 μm (A–K) and 20 μm (L–O). Scale bar for H and I shown in G and scale bar for L–O shown in N.
(Chemicon) and donkey anti-rabbit IgG conjugated to FITC (Chemicon). The specificity of the anti-ANP [13], anti-Cx43 [14,15], anti-HCN1 [16,17], anti-HCN2 [16,18] and anti-HCN4 [14,18] IgGs has been checked previously. No labelling above background was obtained when the primary or secondary antibodies were omitted (data not shown).

2.6. Immunohistochemistry and immunocytochemistry

Immunohistochemistry and immunocytochemistry were carried out using established methods as described previously [8]. Briefly, sections and cells were fixed in 10% formalin (Sigma) for 30 min and washed with 0.01 M phosphate buffer solution (PBS) three times at 10 min intervals. Sections and cells were then permeabilised by incubating them in PBS containing 0.1% Triton X-100 for 30 min, after which they were washed with PBS and then blocked in 10% normal serum in PBS for 1 h at room temperature. Sections and cells were incubated with the primary antibody (diluted in 1% bovine serum albumin, BSA) at 4 °C overnight, after which they were washed three times with PBS over 30 min. Sections and cells were incubated with secondary antibodies for 1–2 h at room temperature. After washing three times in PBS, coverslips were mounted on the microscope slides and the coverslips were sealed with nail polish. Slides were stored in the dark at 4 °C. For the whole mount method [12], tissue was fixed with 4% formalin at 4 °C for 12–24 h. After fixation, tissue was washed with PBS (with gentle rotation) three times over 1 h before it was incubated with 0.5% Triton X-100 in PBS for 48–72 h. After washing with PBS three times over 1–2 h, tissue was immersed in blocking buffer (10% normal donkey serum, NDS, 1% BSA and 0.5% Triton X-100 in PBS) overnight. The tissue was then incubated with the primary antibody (diluted in 2% NDS, 1% BSA and 0.5% Triton X-100 in PBS) overnight. The tissue was then incubated with the primary antibody (diluted in 2% NDS, 1% BSA and 0.5% Triton X-100 in PBS) overnight. The tissue was then incubated with secondary antibodies for 2–4 h at room temperature in the dark. After washing with PBS three times over 1 h, the tissue was mounted on glass slides. Immunolabelled tissue was viewed with a Leica TCS SP or Zeiss LSM 510 laser scanning confocal microscope equipped with argon and helium-neon lasers, which allowed excitation at 488 and 568 nm wavelengths for the detection of FITC/Alex 488 and Cy3, respectively. All images presented are single optical sections except in the case of whole mount preparations. In this case, 30 images were collected through the depth of tissue and then merged to produce a projection image. Images were saved and later processed using Corel Photo-Paint and Corel Draw software (Corel, Ottawa, Canada).

2.7. Statistical analysis

Data are presented as means±SEM (number of preparations). Differences were evaluated by Student’s t test and a difference was considered significant if P<0.05.

3. Results

3.1. Expression of HCN isoforms in mouse SAN

To confirm the functional role of Iₖ in mouse SAN, the effects of Cs⁺ and ZD7288, non-selective (but still informative) blockers of Iₖ [19], on the pacemaker activity of whole SAN-atrial muscle preparations were examined. Extracellular potentials were recorded from the preparations. Fig. 1 shows the effects of Cs⁺ and ZD7288 on the cycle length. 0.5 and 2 mM Cs⁺ prolonged the cycle length by 15±4% (from 164±26 ms under control conditions to 190±37 ms in the presence of 0.5 mM Cs⁺; n=6, p<0.01) and 35±15% (from 176±7 ms under control conditions to 237±15 ms in the presence of 2 mM Cs⁺, n=6, p<0.01) (Fig. 1A,B). Pacemaker activity recovered fully when Cs⁺ was washed-off for 5 to 10 min (Fig. 1B). 3 μM ZD7288 prolonged the cycle length by 48±13% from 188±7 ms to 271±35 ms (n=6, p<0.01) (Fig. 1C). Because of the irreversible binding
of ZD7288 to the open channel \([20]\), the effect of ZD7288 persisted after washing off the drug for \(>1\) h.

Hyperpolarization-activated, cyclic nucleotide-gated cation channel subunits (HCN1-4) have been shown to be responsible for the current termed \(I_h\) (hyperpolarization-activated current) in the brain and \(I_f\) (funny current) in the heart \([21]\). To determine the molecular basis of \(I_f\) in mouse SAN, we examined the expression of HCN1, HCN2 and HCN4 proteins by immunolabelling using specific IgGs against these isoforms. Fig. 2A,C,E shows typical examples of immunolabelling of the different HCN isoforms in adjacent sections through the compact part of the SAN. There was no detectable labelling of HCN1 in the SAN (Fig. 2A). There was labelling of HCN2 in the SAN (Fig. 2C). However, the labelling was punctate and occurred between myocytes (Fig. 2C). It is possible that the labelling was present in neuronal tissue rather than myocytes (the SAN is well known to be highly innervated). This possibility is supported by the data in Fig. 2G–I: Fig. 2G shows that HCN2 labelling was abundant within the nerve cell bodies of cardiac ganglia close to the SAN and Fig. 2H,I shows that thread-like labelling of HCN2 (less abundant than the HCN2 labelling in the SAN) was observed in the atrial muscle. Furthermore, neurofilament 68 labelling (a neuronal cell marker) was similar to HCN2 labelling in brain tissue (Fig. 2B,D,F); the pattern of labelling in brain tissue is comparable to that described by Notomi et al. \([22]\).

Using an anti-HCN3 IgG (1:50 dilution; Alomone Labs), although we observed labelling in brain, we observed no specific labelling in the SAN (\(n=2\); data not shown).

3.2. Correlation between structure and HCN4 expression

In some of the mouse SAN-atrial muscle preparations (\(n=4\)) used for histology and immunohistochemistry, the activation sequence was first obtained by recording extracellular potentials from throughout the preparation. The activation sequence allowed the determination of the leading pacemaker site, and the position of the leading pacemaker site in the four preparations (black stars) is superimposed on a photograph of a typical preparation in Fig. 3A. Action potentials were recorded using intracellular microelectrodes: examples of action potentials from the tail and head of the
SAN are shown in Fig. 3B (recordings 1 and 2) — between the action potentials there is a pacemaker potential, whereas in the atrial muscle there is a stable resting potential (Fig. 3B, recording 4). The SAN action potentials are similar to those recorded from single SAN cells isolated from the mouse [8], but significantly different from those recorded from the rabbit SAN [23].

3.2.1. Histology

Sections were cut perpendicular to the crista terminalis (direction of the arrows in Fig. 3A). Adjacent sections were then stained with Masson’s trichrome and immunolabelled. Here, only sets of sections at 0.2, 0.3, 0.5, 0.7, 1 and 1.4 mm are shown (the levels shown by the arrows in Fig. 3A). Fig. 4 shows a set of sections at these different levels stained with Masson’s trichrome. The insets show the distribution of different cell types (nodal cells, connective tissue, atrial muscle and the sinus node artery) in the sections (the different cell types were identified by their characteristic pattern of expression of ANP, Cx43 and HCN4 described below). At 0.2 mm (close to the superior vena cava), there is a compact group of SAN cells (orange) that is separated from the atrial muscle (green) on either side by connective tissue (blue) (Fig. 4). The compact group of SAN cells is a unique structure that has not been reported in the rabbit SAN or the SAN of other species [23–26]. In the compact node, the nodal cells are tightly packed (see also Fig. 2E) with little connective tissue separating the nodal cells, unlike in the rabbit for example [24]. At other levels (more inferior; 0.3, 0.5, 0.7, 1 and 1.4 mm), the group of SAN cells is diffuse (i.e. loosely-packed) and no longer compact and, although there is still connective tissue on either side of the SAN cells, the group of SAN cells continues on either the endocardial or epicardial surface of the crista terminalis (Fig. 4).

Fig. 1 of the Electronic Supplementary Material shows a Masson’s trichrome stained section cut parallel to the endocardial and epicardial surfaces of the preparation. The section includes the compact part of the SAN (near the
superior vena cava), SAN tissue from a more inferior position, and the atrial muscle of the crista terminalis. The cells in the compact part of the SAN are small (as judged from the density of nuclei) and perhaps orientated perpendicular to the crista terminalis, the cells in the more inferior part of the SAN are diffuse, larger and orientated parallel to the crista terminalis, and the atrial cells in the crista terminalis are large and orientated parallel to the crista terminalis. The histology of the mouse SAN was similar in all hearts studied.

**3.2.2. Distribution of ANP, Cx43 and HCN4**

The distribution of ANP and Cx43 as well as HCN4 was investigated in and around the SAN. ANP and Cx43 were used as markers to distinguish between atrial muscle and SAN tissue [24]: ANP is a hormone and Cx43 is a gap junction protein and both are known to be expressed by atrial muscle, but not by the SAN [9,27]. Fig. 5 shows immunolabelling of ANP and Cx43 at the same six levels (0.2, 0.3, 0.5, 0.7, 1 and 1.4 mm) as used for the histology (Fig. 4). Both ANP and Cx43 were present in the atrial muscle of the crista terminalis, but absent (or at least below the detection threshold for immunolabelling) from the thinner intercaval region (the tissue between the superior and inferior vena cava) adjacent to the crista terminalis. Cx43 was present in the tissue in the intercaval region towards the septum (towards the right in Fig. 5B). The tissue was, therefore, assumed to be atrial muscle. However, this tissue could lack ANP (for example, see tissue at the right of the sections at 0.3 and 0.5 mm in Fig. 5A). It is interesting to speculate that this tissue may differ from the atrial muscle of the right atrial free wall, because it originates from the sinus venosus. Similar results were obtained from a total of four preparations.

Fig. 6 shows immunolabelling of HCN4 at the 0 mm level as well as at the same levels as used for histology (Fig. 4) and ANP and Cx43 (Fig. 5). HCN4-labelling starts at the root of the superior vena cava in the company of the sinus node artery: at the 0 mm level, only a small cluster of nodal cells surrounding the sinus node artery on the epicardial side can be seen (Fig. 6). At the 0.2 mm level (more towards the inferior vena cava), the compact node abruptly appears (Fig. 6). The compact node as defined by HCN4 labelling is about 0.5 mm wide and 0.3 mm thick. In Fig. 3A, the extent of the compact node is indicated by the pink dashed line and the course of the sinus node artery is shown by the red dashed line. Further towards the inferior vena cava (0.3, 0.5, 0.7 and 1 mm levels in Fig. 6) there is a major branch of the SAN (i.e. HCN4-positive cells) on the endocardial surface of the crista terminalis, which appears to contact the atrial muscle of the crista terminalis (see also Fig. 4). Towards the inferior vena cava, the SAN tapered off — compare the extent of HCN4-positive cells at the 0.3 and 1.4 mm levels in Fig. 6. The extent of the HCN4-positive (SAN) cells is shown by the orange dashed line in Fig. 3A — it is much greater than the compact node (shown by the red dashed line in Fig. 3A). In all preparations studied, the leading pacemaker site (black stars in Fig. 3A) was within the HCN4-positive region (orange dashed line in Fig. 3A).

As already discussed, in the present study, Cx43 was absent in the SAN (although present in the surrounding atrial muscle). Using rabbit anti-Cx40 (1:1000 dilution; Chemicon) and guinea-pig anti-Cx45 (1:500 dilution; Q14/GP [28]) IgGs, we observed labelling of Cx45 but not Cx40 in
3.2.3. Fingers of HCN4 expressing cells project into the atrial muscle

Fig. 7 shows Cx43 or HCN4 labelling in whole SA node-atrial muscle preparations. The boxed regions in Fig. 3 show the approximate location from which the images in Fig. 7 were taken. The images in Fig. 7 were taken at the interface of the SAN (Cx43-negative/HCN4-positive) with the atrial muscle of the atrial septum (Cx43-positive/HCN4-negative). The interface between the SAN and atrial muscle at the crista terminalis could not be studied in a whole SAN preparation, because of the thickness of the tissue (the crista terminalis is a thick muscle bundle). Fig. 7 shows an interdigitation of the two tissue types at the interface: Fig. 7A shows that fingers of Cx43-positive atrial cells project into the SAN and, conversely, Fig. 7B,C (see also Fig. 6, 0.5 mm level, arrow) shows that fingers of HCN4-positive SAN cells project into the atrial muscle. The SAN fingers are about 1–3 cells long (100–200 μm) and 1–2 cells wide (10–20 μm). Fig. 7D shows a high magnification image of one finger of HCN4-positive SAN cells — two individual HCN4-positive SAN cells with nuclei (outlined in red) can be identified.

Fig. 2 in the Electronic Supplementary Material shows two other features at the border of the SAN with the atrial muscle. Electronic Supplementary Material Fig. 2A,B shows labelling of HCN4 and Cx43 in adjacent sections — the boxed region in Fig. 5B (0.5 mm level) shows the approximate location from which the images were taken. Electronic Supplementary Material Fig. 2A,B shows a bundle of Cx43-positive/HCN4-negative atrial cells protruding into the Cx43-negative/HCN4-positive SAN. Electronic Supplementary Material Fig. 2C shows labelling of HCN4 (green) and Cx43 (red) in a double labelled section — the boxed region in Fig. 6 (0.2 mm level) shows the approximate location from which the image was taken. Electronic Supplementary Material Fig. 2C shows a group of cells expressing both Cx43 and HCN4 at the border of the SAN with the atrial muscle; such Cx43-positive/HCN4-positive cells were rare.

4. Discussion

In the present study, the structure of the SAN and the distribution of HCNs in the SAN in the mouse were studied by histology and immunolabelling of ANP, Cx43 and HCNs. The study has revealed the complex make-up of the mouse SAN.

4.1. Structure of the mouse SAN

Based on immunolabelling of ANP, Cx43 and HCN4, different cell types were identified in and around the mouse SAN. Comparison of Figs. 5 and 6 shows that myocytes that expressed Cx43 expressed ANP (except towards the atrial septum) and did not express HCN4, whereas the myocytes that did not express Cx43 and ANP did express HCN4. The ANP-positive/Cx43-positive/HCN4-negative tissue is assumed to be atrial muscle and is shown in green in Fig. 4. The ANP-negative/Cx43-negative/HCN4-positive tissue is assumed to be SAN and is shown in orange in Fig. 4 and is outlined by a dashed orange line in Fig. 3A. Fig. 3A shows that the leading pacemaker site (black stars) is within the ANP-negative/Cx43-negative/HCN4-positive region.
The mouse SAN as revealed by the immunolabelling is a comma-shaped structure located in the intercaval region adjacent to the crista terminalis (Fig. 3A) and is in part separated from the surrounding atrial muscle by connective tissue at the border of the crista terminalis and at the atrial septum (connective tissue is shown in blue in Fig. 4). This connective tissue perhaps shields the SAN from the hyperpolarizing influence of the atrial muscle [23], although electrical coupling of cardiac myocytes via fibroblasts has recently been demonstrated [29]. A characteristic feature of the SAN in many species is abundant connective tissue (varying from 50% in the rabbit, guinea-pig and rat to 75–90% in the cat [23]). For example, in the centre of the rabbit SAN, clusters of nodal cells are separated by connective tissue [25]. This is not the case in the compact part of the mouse SAN (a compact nodal structure that forms the head of the comma, in which cells are tightly packed with little separating connective tissue; Figs. 2E, 4 and 6). This unique compact node structure has not been reported previously in other species [23–26]. The functional significance of this structure is not known. Cell size and orientation vary between the compact node and the rest of the SAN (Fig. 4, Electronic Supplementary Material Fig. 1). The mouse SAN has a complex interface with the surrounding atrial muscle: there are interdigitations between the SAN and atrial cells (Fig. 7 and Electronic Supplementary Material Fig. 2). Such interdigitations have been reported in several species (including human) [9,24,30–32] and they may be important for conduction of the action potential from the SAN to the surrounding more hyperpolarized atrial muscle [23]. In the future, it will be of interest to investigate the electrical properties of the head and tail of the SAN in the mouse.

4.2. Expression of HCN channels in the mouse SAN

$I_f$ has been recorded from isolated mouse SAN cells [5]. In the present study, block of $I_f$ by 3 μM ZD7288 slowed pacemaker activity of whole SAN-atrial muscle preparations by 48±13%. These results confirm the role of $I_f$ in mouse SAN pacemaking. Doses of ZD7288 and Cs+ that are likely to block $I_f$ almost completely [19] did not stop the pacemaker activity of the mouse SAN and this demonstrates that other currents are also involved in pacemaking in the mouse SAN. SAN pacemaking is the result of the decay of outward delayed rectifier K+ currents ($I_{K_D}$) on the one hand and activation of $I_f$, Na+-dependent background ($I_{K_Na}$), T- and L-type Ca2+ ($I_{Ca,T}$, $I_{Ca,L}$) and sustained inward ($I_{Na}$) currents on the other [23,33,34]. Na+-K+ pump ($I_{NaK}$) and Na+-Ca2+ exchanger ($I_{NaCa}$) currents and intracellular Ca2+ handling also contribute [23,33,35]. Most of these contributing mechanisms have been identified in mouse SAN [5–7,36]. In addition, a neuronal (possibly Nav.1.1) Na+ channel isoform has been recently identified in the mouse SAN and shown to be involved in SAN pacemaking [8].

To determine the molecular basis of $I_f$ in mouse SAN, the expression of HCN proteins was examined: anti-HCN1 IgG did not produce a signal above the background fluorescence (Fig. 2A). However, a low level of expression of HCN1 mRNA has been detected in mouse SAN [10,11]. In the present study, the expression level of the HCN1 protein may have been below the detection threshold of immunolabelling. In the present study, there was labelling of HCN2 in the SAN (Fig. 2C). HCN2 mRNA has also been detected in the mouse SAN at a moderate level [10]. However, in the present study, the immunolabelling of HCN2 was punctate and occurred between myocytes (Fig. 2C). A similar pattern of labelling was obtained using anti-neurofilament 68 IgG. Anti-neurofilament 68 IgG, in the mouse, labels neuronal tissue and this suggests that, in the mouse, the HCN2 is present in nerve fibres in the SAN (the SAN is well known to be highly innervated). However, in a recent study of the mouse, knockout of HCN2 caused sinus dysrhythmia and a reduction of $I_f$ in SAN cells by ∼30% [37]. Therefore, HCN2 protein may be present in mouse SAN cells (but once again below the detection threshold of immunolabelling). In the present study, no evidence of HCN3 protein in the SAN was observed. HCN3 mRNA has also been reported to be absent from the mouse SAN [10]. In the present study, HCN4 protein was found to be abundant in the SAN (Fig. 6) and we suggest that HCN4 is the major HCN isoform responsible for $I_f$ in the mouse SAN. In the rabbit SAN, HCN4 mRNA constitutes >80% of the total HCN mRNA [38]. In the dog SAN, both HCN4 and HCN2 proteins (detected using the same IgGs as in the present study) are expressed, but again HCN4 mRNA constitutes ∼80% of the total HCN mRNA [39]. Although HCN4 is the major isoform, the sinus dysrhythmia and reduction of $I_f$ caused by knockout of HCN2 [37] demonstrates the involvement of other HCN protein in $I_f$ in the mouse. Furthermore, the functional properties and kinetics of $I_f$ in mouse SAN cells do not match those of any one HCN channel [5] and, therefore, the native channel may be a heteromer formed by the coassembly of different HCN isoforms.

In summary, the mouse SAN is a structurally complex tissue, HCN4 is the only HCN isoform detectable at the protein level, and there is a specialised interface between the SAN and surrounding atrium that may be necessary for the SAN to drive the more hyperpolarized atrial muscle; this constitutes a new element in our understanding of the mouse SAN.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2006.11.016.
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