Differential Expression of Gap Junction Proteins in the Canine Sinus Node

King F. Kwong, Richard B. Schuessler, Karen G. Green, James G. Laing, Eric C. Beyer, John P. Boineau, Jeffrey E. Saffitz

Abstract—Electrical coupling of pacemaker cells at gap junctions appears to play an important role in sinus node function. Although the major cardiac gap junction protein, connexin43 (Cx43), is expressed abundantly in atrial and ventricular muscle, its expression in the sinus node has been a subject of controversy. The objectives of the present study were to determine whether Cx43 is expressed by sinus node myocytes, to characterize the spectrum of connexin expression phenotypes in sinus node pacemaker cells, and to define the spatial distribution of different connexin phenotypes in the intact sinus node. To fulfill these objectives, we performed high-resolution immunohistochemical analysis of disaggregated adult canine sinus node preparations. Using enhanced tissue preservation and antigen retrieval techniques, we also performed immunohistochemical studies on sections of intact canine sinus node tissue. Analysis of disaggregated sinus node preparations revealed three populations of pacemaker cells distinguished on the basis of connexin immunohistochemical phenotype: ≈55% of cells expressed only connexin40 (Cx40); 30% to 35% of cells expressed Cx43, connexin45 (Cx45), and Cx40; and the remaining cells had no detectable connexin expression. In immunostained sections of intact sinus node, Cx43- and Cx45-positive cells were limited in their distribution and were observed in discrete bundles that appeared to abut atrial myocytes. In contrast, Cx40 immunoreactive signal was widely distributed in the sinus node region. These results indicate that subsets of pacemaker cells express distinct connexin phenotypes. Differential expression of connexins could create regions within the sinus node with different conduction properties, thereby contributing to the nonuniform conduction properties seen in this tissue. (Circ Res. 1998;82:604-612.)

Key Words: cardiac connexin ▪ gap junction ▪ sinus node ▪ immunohistochemistry

The molecular composition and spatial distribution of gap junctions are important determinants of the conduction properties of different cardiac tissues.1,2 Morphometric studies have identified small sparsely distributed gap junctions in mammalian sinus node cells3–10 consistent with the slow non-uniform conduction through this tissue.11–13 Results of both experimental and theoretical studies14–16 have also suggested the existence of a gradient in electrical coupling and the presence of preferential conduction pathways within the node that deliver the impulse to atrial muscle and, thereby, initiate the heart beat, while also protecting pacemaker cells from the strong electrotonic influences of the much larger mass of atrial muscle.14 However, the anatomic and molecular bases for the predicted inhomogeneity of electrical coupling in the sinus node are not known.

In general, individual cardiac tissues express multiple gap junction channel proteins (connexins), each of which forms channels with distinct biophysical properties.17,18 One potential determinant of heterogeneous intercellular coupling in the sinus node is differential expression of connexins. Numerous studies have focused on connexin expression phenotypes in the sinus node, but as discussed in a recent review,19 the results have been inconsistent. Some investigators have reported that Cx43, the principal cardiac connexin, is expressed in sinus node myocytes,20–23 whereas others have failed to detect Cx43 in the node and have identified Cx45 and Cx40 instead.24–26 One explanation for this discrepancy may be technical. Recognition of a specific immunohistochemical signal is more difficult in small structurally complex sinus node myocytes than in atrial or ventricular muscle. Furthermore, gap junctions in the node are considerably smaller than in other cardiac tissues,27 and the sinus node contains abundant nonmyocyte tissue elements admixed with sinus node myocytes.

The objectives of the present study were as follows: (1) to determine whether Cx43 is expressed by sinus node myocytes, (2) to characterize the spectrum of connexin expression phenotypes in sinus node pacemaker cells, and (3) to compare the immunohistochemical phenotypes seen in disaggregated preparations with those in the intact sinus node tissue. To achieve these objectives, we performed high-resolution immunohistochemical analysis on isolated cells in disaggregated adult canine sinus node preparations. Previous studies of these disaggregated...
The slides were stored at 20°C. After the sinus node tissue was allowed to equilibrate in the 0.05% collagenase, 0.1% elastase (type IIA, Sigma Chemical Co), and 0.001% protease (type XIV, Sigma) for 60 minutes with constant shaking in a water bath at 37°C. The sinus node tissue was then perfused with 10% buffered formalin via the right coronary artery. The sinus node region was excised, dehydrated, and embedded in paraffin. Six-micrometer-thick sections of the sinus node were cut in a plane parallel to the epicardium and mounted on gelatin-coated slides. Immunohistochemical staining of formalin-fixed regions were deparaffinized, placed in containers of citrate buffer (10 mmol/L, pH 6.0), and heated in a microwave oven until boiling. The slides were then brought to room temperature, washed in PBS three times, and incubated with CY3-conjugated goat anti-mouse or anti-rabbit IgG antibody diluted 1:5000 (Jackson ImmunoResearch). After being washed again with 0.5% Triton in PBS, the membranes were treated with ECL detection reagent (Amersham Life Sciences) and exposed to x-ray film. Rainbow molecular weight marker standards (Amersham Life Sciences) were used to calibrate the gels.

Selected Abbreviations and Acronyms

- Cx40 = connexin40
- Cx43 = connexin43
- Cx45 = connexin45
- FITC-WGA = fluorescein isothiocyanate–conjugated wheat germ agglutinin

Materials and Methods

Isolation of Sinus Node Cells

Adult mongrel dogs were anesthetized with sodium pentobarbital (25 mg/kg IV), and a surgical plane of anesthesia was maintained with 1.5% inhalational isoflurane. Hypothermic cardioplegic arrest was induced, and the heart was excised. The right atrium was isolated, and the right coronary artery was cannulated at its ostium. The atrial preparation was perfused for 5 minutes with Tyrode's solution (mmol/L: NaCl 125, KCl 5.4, CaCl2 1.8, MgCl2 1.0, NaHCO3 24, NaHPO4 0.6, and glucose 11) saturated with 95% O2/5% CO2 at pH 7.4, followed by a 5-minute perfusion with Ca2+-free Tyrode's solution and a final perfusion for 12 minutes with Tyrode's solution containing CaCl2 (36 μmol/L), 0.02% albumin, and 0.05% collagenase (336 U/mg, type II, Worthington Biochemical Corp). After the final perfusion with collagenase was completed, the sinus node region (5×10 mm) was excised, minced into small pieces, and incubated in Tyrode's solution containing CaCl2 (36 μmol/L), 0.02% albumin, 0.05% collagenase, 0.1% elastase (type IIA, Sigma Chemical Co), and 0.001% protease (type XIV, Sigma) for 60 minutes with constant shaking in a water bath at 37°C. The sinus node tissue was then incubated in Tyrode's solution containing CaCl2 (36 μmol/L) for 2 minutes followed by a high potassium solution (mmol/L: potassium aspartic acid 100, KCl 30, K2-ATP 2, glucose 11, and 0.02% albumin; pH 7.35). After the sinus node tissue was allowed to equilibrate in the high potassium solution at 4°C for 1 hour, each piece of tissue was gently triturated using a polystyrene transfer pipette. The resulting cellular suspension was fixed in 2% parafomaldehyde, washed, resuspended in PBS, and allowed to air-dry on gelatin-coated glass slides. The slides were stored at −20°C.

Preparation of Whole Sinus Node Tissue Sections

Right atrial preparations excised from cardioplegia-arrested canine hearts were perfused with 10% buffered formalin via the right coronary artery. The sinus node region was excised, dehydrated, and embedded in paraffin. Six-micrometer-thick sections of the sinus node were cut in a plane parallel to the epicardium and mounted on gelatin–coated slides. Immunohistochemical staining of formalin-fixed paraffin-embedded sections with anti-Cx43 and anti-Cx45 antibodies was technically challenging. However, the Cx40 signal was weaker in formalin-fixed paraffin–embedded tissue than in frozen sections. Accordingly, all single- and double-label studies involving Cx40 antibody were performed in both fixed paraffin sections and unfixed frozen sections. Sinus node regions from cardioplegia-arrested canine hearts were frozen, cut with a cryostat into 10-μm-thick sections, and placed onto gelatin-coated slides.

Anti-Connexin Antibodies

Anti-Cx43 and anti-Cx45 antibodies used in the present study have been extensively characterized and shown previously to be monospecific. These include a mouse monoclonal anti-Cx43 IgG antibody raised against amino acids 252 to 270 of rat Cx43 (Chemicon International Inc) and rabbit antisera directed against canine Cx45 (residues 285 to 298). In previous studies, we found that this anti-Cx45 antibody did not recognize in vitro–translated Cx43 or immunostain RIN (rat insulinoma) cells transfected with a Cx43 construct. To perform the present studies, we produced new rabbit antisera directed against the rat Cx40 carboxy terminus (residues 235 to 355). There is no significant sequence homology within the peptides used to generate anti-Cx43 and anti-Cx40 antibodies. The greatest amount of sequence identity is 14.3%. Anti-Cx40 antisera were affinity-purified by peptide-agarose column chromatography (SulfoLink coupling gel, Pierce Chemical Co) using a Cx40 fusion protein generated as described below. Antisera fractions eluted from the column were collected, and maximal protein concentration was determined spectrophotometrically.

Immunoblotting of Connexin Fusion Proteins

Nucleotides encoding amino acids 237 to 384 of rat Cx43 or amino acids 235 to 355 of rat Cx40 were subcloned into the pET3A expression vector (Novagen). His6-Cx43 and His6-Cx40 fusion proteins were produced by transformed Escherichia coli and isolated with a Nickel column (Novagen). Equivalent amounts of Cx43 and Cx40 fusion proteins were loaded onto different lanes of a 12.5% polyacrylamide gel. Both of these fusion proteins migrated with apparent molecular weights of ~17 kDa. The SDS-PAGE–resolved proteins were transferred onto a nitrocellulose membrane and then blocked overnight in gelatin–Triton–PBS solution (2% gelatin and 0.5% Triton X-100 in PBS). The membrane was carefully cut into two halves; one half was incubated in mouse monoclonal anti-Cx43 antibody (diluted 1:1000), and the other half was incubated in rabbit polyclonal anti-Cx40 antibody (diluted 1:500). After being washed with 0.5% Triton in PBS, the membranes were incubated in horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG antibody diluted 1:5000 (Jackson ImmunoResearch Inc). After being washed again with 0.5% Triton in PBS, the membranes were treated with ECL detection reagent (Amersham Life Sciences) and exposed to x-ray film. Rainbow molecular weight marker standards (Amersham Life Sciences) were used to calibrate the gels.

Immunofluorescence Staining of Disaggregated Sinus Node Cells and Sinus Node Tissue Sections

Disaggregated sinus node cells on gelatin-coated slides were brought to room temperature and washed three times in PBS. Cells were simultaneously permeabilized and blocked by incubating them in PBS containing 1% Triton X-100 and 2% normal serum of the species from which the secondary antibody was derived (goat, donkey, or both in double-label experiments). Subsequently, cells were incubated in connexin-specific primary antibody (mouse monoclonal anti-Cx43 antibody diluted 1:200, rabbit polyclonal anti-Cx45 antibody diluted 1:200, rabbit polyclonal anti-Cx40 antibody diluted 1:100, or a combination of both mouse and rabbit antibodies) overnight at 4°C. The slides were then brought to room temperature, washed in PBS three times, and incubated with CY3–conjugated goat anti-mouse or anti-rabbit IgG antibody and CY2–conjugated donkey anti-rabbit IgG in double-label preparations (Jackson ImmunoResearch) for 1 hour at 25°C. Formalin-fixed paraffin-embedded sections of intact sinus node regions were deparaffinized, placed in containers of citrate buffer (10 mmol/L, pH 6.0), and heated in a microwave oven until boiling for 10 minutes. This antigen retrieval process led to excellent immunohistochemical staining by anti-Cx43 and anti-Cx45 antibodies. However, the anti-Cx40 antibody did not work as well in paraffin sections as in frozen sections. Therefore, the distribution of Cx40 was examined in both microwave-treated paraffin sections and in frozen sections of the sinus node region. All tissue sections were immunostained using the same protocols described above for disaggregated pacemaker cell preparations. Immunostained cells and tissue sections were examined by conventional epifluorescence microscopy (Carl Zeiss, Inc).
Identification of Sinus Node Myocytes in Disaggregated Preparations

Pacemaker cells were identified in disaggregated sinus node preparations on the basis of their characteristic morphology.28,29 These cells had an elongated spindlelike shape with a single central nucleus and two cytoplasmic projections or a spiderlike shape with a single central nucleus and multiple cytoplasmic projections. Working atrial myocytes were characteristically rod-shaped and typically larger in size than pacemaker cells. Rounded-up cells or cells that did not clearly exhibit spider or spindle cell morphology were not included in the analysis.

To distinguish pacemaker cells from nonmyocytic cells that could have a similar morphology, we performed double-label immunostaining studies as described above with a cardiac-specific anti-myosin antibody (generously provided by Dr Stacy Smith, Washington University) to identify cardiac myocytes and FITC-WGA (E-Y Laboratories Inc) to identify cells in general.

Results

Characterization of Polyclonal Anti-Cx40 Antibody

Because we used a new anti-Cx40 antibody in these studies, we first performed experiments to show that this reagent was monospecific. Immunostaining of canine atrial and ventricular myocardial tissue sections with this antibody showed staining patterns consistent with the known distribution of Cx40 in the heart. Discrete immunohistochemical signal was seen in intercalated disk regions of atrial myocytes (Fig 1A) but not ventricular myocytes (Fig 1B). Cx40 immunofluorescent signal in the ventricle was limited to vascular endothelial cells (Fig 1B). This immunohistochemical pattern in atrial and ventricular muscle is consistent with previous reports of Cx40 distribution in humans, dogs, and rats.25,26,36–39

To further exclude the possibility that the new anti-Cx40 antiserum cross-reacted with Cx43, Cx43 and Cx40 fusion proteins were resolved by SDS-PAGE and probed with both anti-Cx43 and anti-Cx40 antibodies. Antisera directed against Cx40 specifically recognized Cx40 fusion protein but not Cx43 fusion protein (Fig 1C). Anti-Cx43 antibody did not bind to Cx40 fusion protein. An immunoblot of a homogenate of rat atrial myocardium, which contains abundant Cx40,39 showed a robust signal when probed with the new anti-Cx40 antibody (Fig 1D). Thus, the new anti-Cx40 antibody was specific for Cx40 antigenic determinants and did not cross-react with Cx43.

Immunofluorescence Microscopy of Disaggregated Cells

Because isolated pacemaker cells were identified only on the basis of their characteristic spiderlike or spindlelike morphology, we performed preliminary experiments in which disaggregated sinus node cell preparations were stained with anti-cardiac-specific myosin antibodies to determine whether any nonmyocytic cells might also exhibit this type of morphology. All spider- and spindle-shaped cells stained intensely with anti-myosin antibodies (data not shown), indicating that they were cardiac myocytes. Intact cells identified with FITC-WGA that did not stain positively for cardiac myosin constituted <10% of the total cell population. The great majority of these nonmyocytic cells were clusters of small flat cells often appearing as fragments of a monolayer. These cells, presumably endothelium, were not characterized further because they were readily distinguished from pacemaker cells. Occasional myosin-negative spindle-shaped cells (presumably fibroblasts) were also seen, but they were considerably smaller than spindle-shaped pacemaker cells.

“Round cells” represented ≈5% of total cells in disaggregated cell preparations. All of these appeared to be atrial myocytes that were damaged during disaggregation, and they also stained positively with antibodies against cardiac-specific myosin. Small round pacemaker cells, as described by others in rabbit preparations,30,28,40,41 were not seen in our preparations.

Immunohistochemical phenotypes of spider- and spindle-shaped pacemaker cells and atrial myocytes were analyzed in disaggregated cell preparations from four canine sinus nodes. In initial studies, isolated cell preparations were stained with a monoclonal antibody against Cx43. In each of the four preparations, all atrial myocytes showed intense Cx43 immunoreactive signal at intercalated disk regions (Fig 2A). However, only some cells having spider or spindle morphology stained positively with anti-Cx43 antibodies. Pacemaker cells that stained positively for Cx43 exhibited a distinct punctate pattern of high-intensity immunofluorescent signal that was clearly different than that of working atrial myocytes (Figs 2B and 2C).

The proportions of isolated atrial myocytes and pacemaker cells exhibiting different connexin phenotypes were determined in aliquots of cells from four different canine sinus node preparations stained with mixtures of antibodies against Cx43 and Cx45 or Cx43 and Cx40. The results are shown in the Table and in Figs 3 and 4. Disaggregated cell preparations incubated simultaneously with mouse monoclonal anti–Cx43 antibody and rabbit polyclonal anti–Cx45 antibody demonstrated two populations of spider- and spindle-shaped cells. As seen in previous studies,25,32 all atrial myocytes stained positively for both Cx43 and Cx45 (Table). However, only 38% of cells with spider or spindle morphology stained positively for both Cx43 and Cx45, whereas the remaining 62% stained neither Cx43 nor Cx45 (Table). Whenever a cell stained positively for Cx43, it also stained positively for Cx45, and these two proteins always appeared to colocalize at individual gap junctions (Fig 3).

All atrial myocytes stained for both Cx43 and Cx40 in double-labeled studies (Table). The proportion of cells with spider and spindle morphology that stained positively for Cx43 in Cx43/Cx40 double-label preparations (31%, Table) was close to the proportion of Cx43-positive cells seen in Cx43/Cx45 double-label studies (38%). Whenever Cx43 was present, Cx40 staining was always observed (Table), and these two proteins colocalized. However, 55% of the pacemaker cells in Cx43/Cx40 double-label preparations expressed only Cx40 and did not show Cx43 expression (Table and Fig 4). Thus,
86% of disaggregated pacemaker cells having spindle/spider morphology stained positively for Cx40 in Cx43/Cx40 double-label preparations, but only 31% stained positively for Cx43. The remaining 14% of spindle- or spider-shaped cells did not stain for either Cx43 or Cx40 (Table).

Immunofluorescence Microscopy of Sinus Node Tissue Sections

In whole tissue sections, the canine sinus node was invariably located between the two major branches of the sinus node artery and was composed of small cells in an abundant extracellular matrix. Because of the superior technical quality of the formalin-fixed paraffin-embedded tissue compared with the quality possible in unfixed frozen sections, the node myocytes were readily distinguished from adjacent tissues composed of atrial myocytes.

Intense punctate Cx43 immunofluorescent signal was observed unambiguously in discrete groups of cells within the intact sinus node (Fig 5). Cx43-positive sinus node cells were typically clustered in bundles that coursed through the node and were seen in some sections to abut atrial myocytes (Fig 6). Individual Cx43-positive cells in tissue sections exhibited a staining pattern identical to that seen in disaggregated cell experiments but significantly different from that of nearby atrial myocytes. Estimates of the proportion of total sinus node cells that stained positively with anti-Cx43 antibodies in tissue sections (30% to 40%) were similar to the proportion of Cx43-positive cells observed in disaggregated cell experiments. Paraffin sections of the intact sinus node stained with anti-Cx45 antibody showed a similar arrangement of positively stained cells in bundles (Fig 7), as predicted by the Cx43/Cx45 double-label studies with disaggregated cells.

Figure 1. A and B, Cx40 immunofluorescence in sections of canine atrial (A) and ventricular (B) muscle. Cx40 immunoreactive signal is seen in intercellular junctions between atrial myocytes (A) but not ventricular myocytes (B). Cx40 signal in the ventricle is limited to intramural coronary vessels (arrow in panel B). C, Immunoblot showing specificity of anti-Cx40 and anti-Cx43 antibodies. The positions of molecular weight standards in lane a (not visible in autoradiograph) are denoted by the bars to the left of the blot. The molecular weights (in kilodaltons) are indicated. Anti-Cx43 antibody recognized Cx43 fusion protein (lane b) but not Cx40 fusion protein (lane c). Anti-Cx40 antibody recognized Cx40 fusion protein (lane d) but not Cx43 fusion protein (lane e). The band shown in lane d was confirmed to be a single band in other blots in which shorter exposure intervals provided finer resolution. D, Immunoblots of rat atrial myocardial homogenate probed with anti-Cx40 antibody and rat ventricular myocardial homogenate probed with a monoclonal anti-Cx43 antibody. Cx40 and Cx43 signals are located at distinct positions on the gel as indicated by the positions of molecular weight markers at 66, 46, and 30 kD. The Cx43 signal shows multiple bands as described previously by Musil and Goodenough.52
Cx40 immunoreactive signal was widely distributed in sections of the sinus node region. Intense punctate Cx40 signal was concentrated at points of apparent cellular apposition in a pattern resembling that seen in isolated cells (Fig 8). In sections of intact sinus node incubated simultaneously with anti-Cx43 and anti-Cx40 antibodies, numerous cells were observed to express Cx40 but not Cx43 (Fig 9). A smaller number of cells expressed both Cx40 and Cx43. In a few cells, neither Cx43 nor Cx40 signal was observed.

Connexin Expression Phenotypes in Isolated Canine Atrial Myocytes and Pacemaker Cells

<table>
<thead>
<tr>
<th>Connexin</th>
<th>Atrial myocytes (N=15)</th>
<th>Spider/spindle cells (N=61)</th>
<th>Atrial myocytes (N=15)</th>
<th>Spider/spindle cells (N=42)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Percentage, n/N</td>
<td>Percentage, n/N</td>
<td>Percentage, n/N</td>
<td>Percentage, n/N</td>
</tr>
<tr>
<td>Cx43/Cx45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>15</td>
<td>23</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>−/−</td>
<td>0</td>
<td>38</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>+/−</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>−/+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>

Aliquots of isolated cells prepared from four canine sinus nodes were doubly immunostained by incubating them with mixtures of antibodies against Cx43 and Cx45 (Cx43/Cx45) or mixtures of antibodies against Cx43 and Cx40 (Cx43/Cx40). Atrial myocytes and pacemaker cells having spider or spindle morphology were scored in each double-labeled preparation for the presence of both connexins (+/+), neither connexin (−/−), the presence of Cx43 but the absence of either Cx45 or Cx40 (+/−), or the absence of Cx43 but the presence of Cx45 or Cx40 (−/+). Values for n equal the total number of cells of each type analyzed in the four Cx43/Cx45 or Cx43/Cx40 double-labeled preparations; n indicates the number of cells expressing the connexin phenotype.
Discussion

Results of immunohistochemical analysis of both disaggregated sinus node preparations and intact sinus node tissue sections revealed that some but not all pacemaker cells express Cx43. Three distinct populations of pacemaker cells were defined immunohistochemically on the basis of their connexin phenotypes: cells that express Cx40 only; cells that express Cx43, Cx45, and Cx40; and cells with no detectable connexin expression. We analyzed only those cells that exhibited spider or spindle morphology, because these cells have been observed to depolarize spontaneously (phase 4 depolarization) and show slow phase 1 action potential upstrokes.\(^{28,29}\) We also demon-

Figure 5. A, A representative low-power (×50) epifluorescence image of the sinus node region from a section of formalin-fixed paraffin-embedded tissue stained with anti-Cx43 antibody. The sinus node is bounded by the two branches of the sinus node artery (a). Discrete cell bundles within the sinus node region exhibit clear Cx43 immunoreactive staining (curved arrows). Atrial myocardium can be seen flanking the sinus node. B, Higher magnification (×200) view of bundles of Cx43-positive sinus node cells (curved arrows) and adjacent Cx43-negative node cells (straight arrow).

Figure 6. Cx43-positive sinus node cells apparently abutting (at arrow) atrial myocytes (a) in a section of formalin-fixed paraffin-embedded sinus node tissue (original magnification ×500).

Figure 7. Epifluorescence image of Cx45 immunoreactive signal in a discrete bundle of pacemaker cells. Cx45-negative pacemaker cells are seen above and below the bundle showing Cx45 signal (original magnification ×200).
Cx45 in Cx43/Cx45 double-label experiments. In Cx43/Cx40 experiments, every cell expressing Cx43 also expressed Cx40 (although the converse was not true—many cells expressed only Cx40). The fact that every cell expressing Cx43 also expressed Cx45 or Cx40 in separate double-label experiments established that all cells expressing Cx43 also expressed both Cx45 and Cx40. We did not explicitly prove that cells expressing only Cx40 in Cx43/Cx40 double-label experiments did not also express Cx45. No Cx45/Cx40 double-label studies were performed, because both of the primary antibodies were produced in rabbits. However, if a subset of pacemaker cells did express Cx40 and Cx45 but not Cx43, then we should have identified cells that expressed only Cx45 in Cx43/Cx45 double-label experiments, and this was not observed.

A small number of pacemaker cells did not express detectable levels of Cx43, Cx45, or Cx40. These cells could possibly have expressed another connexin that was not evaluated. However, extensive searches by us and others have failed to identify expression of other connexins by cardiac myocytes.\(^{26,42}\) Another explanation for the apparent lack of connexin expression by some pacemaker cells is that their gap junctions may be so small or sparsely distributed that they escaped immunohistochemical detection even if another connexin was expressed. Another possibility is that some pacemaker cells may not be coupled to their neighbors at gap junctions.

There has been controversy regarding Cx43 expression by sinus node myocytes. In our previous studies of the canine sinus node, we failed to detect unambiguous Cx43 expression in unfixed frozen tissue sections.\(^{25,26}\) However, Cx43 signal was clearly identified in a subset of disaggregated pacemaker cells in the present studies. Furthermore, the technical superiority of immunostained microwave-treated sections of paraffin-embedded formalin-fixed tissue provided clean high-resolution signals in the intact node tissue that demonstrated unambiguous Cx43 expression in the canine sinus node. These findings are consistent with earlier studies from independent laboratories in which Cx43 was detected in rabbit and hamster sinus nodes.\(^{20–23}\)

Our analysis of whole tissue sections showed that Cx43 expression by pacemaker cells was limited to groups of cells arranged in bundles. The distribution of Cx43 immunostaining seen in the intact sinus node correlated well with the percentage of isolated sinus node myocytes exhibiting Cx43 immunoreactive signal in the disaggregated cell preparations. The distribution of Cx45 in both disaggregated cells and the intact sinus node paralleled the distribution of Cx43, conforming to a general pattern of Cx43/Cx45 coexpression that has been observed in cardiac myocytes.\(^{25,26,30,31}\) In contrast to the limited distribution of Cx43 and Cx45 in the sinus node, Cx40 was expressed by a majority of pacemaker cells in both disaggregated cell preparations and whole tissue sections. Cx40 was also expressed abundantly by atrial myocytes, as observed previously by us and others.\(^{25,26,36–39}\) Some cells expressing Cx40 also expressed Cx43 and Cx45, but many Cx40-positive cells expressed only Cx40.
The functional significance of these complex patterns of connexin expression is not known, nor have the absolute amounts of each connexin been measured in different types of cardiac myocytes. However, results of studies in transfected oocytes or “communication-deficient” cell lines have suggested that Cx43 may form functional heterotypic channels with Cx45, and that Cx45 may form functional heterotypic channels with Cx40 but that Cx43 and Cx40 apparently cannot form functional channels. Groups of pacemaker cells expressing only Cx40 may, therefore, have a more limited range of coupling possibilities than cells expressing Cx43, Cx45, and Cx40. This could create communication boundaries or barriers of coupling resistance. Groups of pacemaker cells expressing only Cx40 could remain relatively insulated from other pacemaker cells expressing Cx43 and perhaps be protected from the hyperpolarizing influence of the larger atrial muscle.

The presence of Cx43 within discrete cell bundles in the sinus node could serve the important role of directing activation potential propagation from within the node outward to the atria. These Cx43-positive sinus node myocyte bundles appeared in some sections to abut atrial myocytes, suggesting, but certainly not proving, that Cx43-positive bundles might act as preferential conduction pathways within the node as originally hypothesized by Joyner and van Capelle. The complex connexin phenotypes in sinus node pacemaker cells may also explain, in part, the electrophysiological data of Bromberg et al., who used floating microelectrodes to record action potentials from cells within the canine sinus node while simultaneously recording from multiple extracellular sites. They showed that although the earliest intracellular activation occurred within the central portion of the sinus node, the earliest extracellular activation occurred at the superior and inferior poles of the nodal region, suggesting discrete propagation exit sites from the node. In addition, some regions in the sinus node activated much later than even the latest atrial extracellular activation. The authors hypothesized that there may be pathways within the node that connected the site of earliest nodal activation to the working atrial myocardium, leading to earlier activation of the atrium than of some regions within the sinus node. Differential spatial expression of gap junction proteins may create these putative pathways. Regulation of the expression and cellular distribution of specific gap junction proteins may, therefore, play a critical role in the sinus node pacemaking function.

As demonstrated by Jalife et al. and others, interactions among a group of pacemaker cells result in a dominant pacemaker region. These interactions are electrotonic and involve mutual alterations in diastolic or pacemaker potentials, such that slower cells modulate faster cells to decrease their diastolic potentials and decrease their firing intervals and vice versa. The overall effect of this interaction results in an intermediate rate and site of the dominant pacemaker. Spach and Heidlage have demonstrated in electrophysiological studies and models based on the geometry of intercellular junctions that both electrotonic currents and currents of wave-front propagation use the same connections. The question remains whether distinct electrophysiological functions are also related to the different connexin expression patterns within the sinus node. Perhaps certain types of intercellular channels facilitate conduction between the pacemaker and atrial myocardium and correlate with the dispersed exit sites observed in our previous studies. Other types of channels may subserve the electrotonic interactions that determine the final rate and site of the dominant pacemaker region within the sinus node and at the same time inhibit rapid impulse propagation or depolarization wave fronts within the pacemaker matrix. Answers to these questions will require more extensive electrophysiological and anatomic-biochemical studies in both dispersed cell aggregates and in vivo preparations.

Acknowledgments

This study was supported by National Institutes of Health grants HL-50598, HL-33722, and HL-07776. We are indebted to Dr Stacy Smith for providing anti-cardiac myosin antibody.

References

8. Ophof T, de Jonge B, Mackay AJC, Bleeker WK, Mason-Pevet M, Jonggra HJ, Bouman LN. Functional and morphological organization of the guinea-pig sinoatrial node compared with the rabbit sinoatrial node. J Mol Cell Cardiol. 1985;17:549–564.
Gap Junction Proteins in Canine Sinus Node


Differential Expression of Gap Junction Proteins in the Canine Sinus Node
King F. Kwong, Richard B. Schuessler, Karen G. Green, James G. Laing, Eric C. Beyer, John P. Boineau and Jeffrey E. Saffitz

Circ Res. 1998;82:604-612
doi: 10.1161/01.RES.82.5.604
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/82/5/604

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Research is online at:
http://circres.ahajournals.org/subscriptions/