Distribution of Atrial and Nodal Cells Within the Rabbit Sinoatrial Node
Models of Sinoatrial Transition

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Background—In the sinoatrial node (SAN) the course of the action potential gradually changes from the primary pacemaker region toward the atrium. It is not known whether this gradient results from different intrinsic characteristics of the nodal cells, from an increasing electrotonic interaction with the atrium, or from both. Therefore we have characterized the immunohistochemical, morphological, and electrophysiological correlates of this functional gradient.

Methods and Results—The distribution of rabbit nodal myocytes in the SAN has been studied by immunohistochemistry. After cell isolation, the electrophysiological characteristics of different nodal cell types were measured. (1) The staining pattern of a neurofilament protein coincides with the electrophysiologically mapped pacemaker region in the SAN. (2) Enzymatic digestion of the SAN reveals three morphologically different nodal cell types and one atrial type. Of each nodal cell type, neurofilament-positive as well as neurofilament-negative myocytes are found. Atrial cells are all neurofilament-negative. (3) In contrast to previous findings, we observed atrial cells in the very center of the SAN. The relative number of atrial cells gradually increases from the central pacemaker area toward the atrium. (4) Differences in electrophysiological characteristics between individual nodal cells are not associated with differences in cell type.

Conclusions—(1) The expression of neurofilaments can be used to delineate the nodal area in the intact SAN but is not sufficiently sensitive for characterizing all individual isolated nodal cells. (2) A fundamentally different organization of the SAN is presented: The gradual increase in density of atrial cells from the dominant area toward the crista terminalis in the SAN causes a gradual increase of atrial electrotonic influence that may be an important cause of the gradual transition of the nodal to the atrial type of action potential. *(Circulation. 1998;97:1623-1631.)*

Key Words: pacemakers • cells • electrophysiology • immunohistochemistry

The mammalian sinoatrial node (SAN) displays inhomogeneity in structure and function.1-4 The site of the earliest activation (ie, the primary pacemaker area) is invariably localized in an area with low myofilament density in rabbit,5 guinea pig,6 cat,7 pig,8 and monkey.9 The action potentials in the primary pacemaker area are characterized by low upstroke velocity in association with substantial diastolic depolarization.5 In several species a more or less gradual transition in action potential characteristics from a nodal type (low upstroke velocity, steep diastolic depolarization) to an atrial type (high upstroke velocity, no diastolic depolarization) has been demonstrated. The area of transition has been extensively studied in rabbit both by morphological and electrophysiological methods.5,10-12 From the earlier work the conceptual model arose of a compact center, homogeneously composed of typical nodal cells, surrounded by an area in which structure and electrophysiology of the fibers gradually changes from nodal to atrial. In accordance with this view, gap junction density was found to increase from the center of the rabbit node toward the surrounding atria.5,10 In a model study it was established that proper pacemaking in the SAN required a gradual increase in electrical coupling from the center of the SAN to the atrial myocardium.11 However, more recent data suggest a less gradual transition. Measurements of electrotonic spread of subliminal pulses demonstrated an irregular pattern consistent with an inhomogeneous distribution of nodal and atrial fibers.12 In guinea pig SAN, a combined immunohistochemical and electrophysiological study demonstrated that the center of the node is surrounded by an area in which small strands of atrial cells interdigitate with strands of nodal cells.14 On the other hand, this study also showed that the small gap junctions that have been observed with the electron microscope technique15 could not be demonstrated immunohistochemically. Thus these studies...
brought conflicting data on the question of whether the transition from the center of the node to the atrial myocardi- um is composed of gradually changing fiber types or is mainly formed by interdigitating strands of nodal and atrial cells.

The objective of the current study was to investigate the immunohistochemical, morphological, and intrinsic electrophysiological substrate of the gradual transition in action potential configuration when going from the primary pacemaker region toward the atrium. In contrast to previous findings, we observed that atrial cells can even be observed in the very center of the SAN. Furthermore, we have found that the SAN comprises three morphologically distinct nodal cell types that do not exhibit different electrophysiological characteristics.

Methods

Animals

For all experiments, New Zealand albino rabbits weighing 1.8 to 2.5 kg of either sex were used. Rabbits were anesthetized with Hypnorm (1 mL/kg, 0.32 mg/mL fentanyl/citrate, and 10 mg/mL fluanison IM. Janssen Pharmaceutica). Under artificial ventilation, the thorax was opened and 0.1 mL heparin sodium (5000 UI/mL) was injected into the left ventricle, after which the heart was excised. Animal care was in accordance with institutional guidelines.

Immunohistochemistry

Tissue and Cell Preparation

Hearts were dissected and fixed in a mixture of methanol, acetone, acetic acid, and water (35:35:5:25 vol/vol) for 48 hours, dehydrated in a graded series of ethanol, cleared in chloroform, embedded in ParaPlast Plus, and cut in a transverse plane into 5-μm-thick serial sections.

Isolated cardiomyocytes from the SAN region (see below) were collected on slides and fixed in a fixative containing methanol, acetone, acetic acid, water (35:35:5:25 vol/vol) for 20 minutes and washed with phosphate-buffered saline (PBS).

Immunohistochemical Staining

To detect the binding of the specific monoclonal antibodies with the respective antigens on the paraplast sections and on the isolated cells, the indirect un conjugated peroxidase-antiperoxidase technique (PAP technique) was applied essentially as described before. After deparaffinization, sections were treated with hydrogen peroxide (3% vol/vol in pH 7.4, PBS) for 30 minutes to reduce endogenous peroxidase activity. This procedure proved not to be necessary for isolated cells. To reduce nonspecific binding, sections and cells were preincubated with TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8.0) for 15 (cells) or 30 minutes (sections). After incubation with the monoclonal antibodies (overnight at room temperature), antibody binding was detected by subsequent incubation with goat anti-mouse, donkey anti-goat, and goat peroxidase-antiperoxidase complex diluted in PBS. This immunocomplex was visualized by incubating the sections with 0.5 mg/mL 3,3′diaminobenzidine, 0.02% hydrogen peroxide in 30 mmol/L imidazole, 1 mmol/L EDTA (pH 7.0). Tissue and cell-containing slides were mounted in Entellan (Merck).

Antibodies

The monoclonal antibody reacting with the low-molecular-weight fraction of mammalian neurofilament (68 kD), was purchased from DAKOPATTS. Anti-human desmin was purchased from Sanbio.

SAN Preparation

Electrophysiological Mapping of the SAN

Hearts were excised and immersed in a solution containing (mmol/ L): 130.6 NaCl; 5.6 KCl; 2.2 CaCl₂; 0.6 MgCl₂; 24.2 NaHCO₃; 11.1 glucose; 13.2 sucrose, saturated with 95% O₂ and 5% CO₂ at a temperature of 37±0.3°C and a pH of 7.4. The isolated right atrium preparation including the SAN and the crista terminals was mounted on a perforated silicon rubber block, endocardial side up, and perfused with the same solution. Transmembrane potentials were recorded with glass microelectrodes filled with 2.7 mol/L KCl and 2 mmol/L potassium citrate. Generally, impalements were made 0.4 mm apart and in the primary pacemaker area up to 0.2 mm apart. A unipolar surface electrogram, derived from the crista terminals, provided a time reference. The activation moment of a cell was defined as the moment that the voltage was halfway between the maximum diastolic potential (MDP) and the top of the action potential and was timed with respect to the time reference. Beat-to-beat interval and temperature were recorded continuously. Signals were stored on magnetic tape for off-line data analysis. The mapping procedure lasted 1.5 to 2 hours.

In one set of experiments the distribution of upstroke velocities of the action potentials was correlated with Neurolanfilament staining pattern. To align the electrical and immunohistochemical map, the tissue was marked with 10- to 50-μm-diameter Alcian blue dots. Therefore the electrode was backfilled with 1% Alcian blue in 0.5 mol/L sodium acetate (pH 4.0) at the end of the mapping procedure, the tip of the electrode was broken to lower resistance, and dots were obtained iontophoretically by applying rectangular 0.5-mA pulses (500 Hz; duration 30 to 300 μs) for 10 to 15 seconds. Dots were placed at an interval of 400 μm along lines parallel and perpendicular to the crista terminals. In total, about 12 dots were placed. After localization of the dots in the tissue sections, a two-dimensional reconstruction of the neurofilament labeling pattern in the SAN region was projected on the maximum upstroke velocity map.

In another set of experiments we constructed activation maps before cell isolation to estimate the distribution of morphologically different nodal cell types in functionally different areas of the SAN. Therefore, the leading pacemaker cell group, that is, the group of earliest-discharging cells (area 1 to 1.5 mm²) and an adjacent piece of tissue (2 to 3 mm²) located superior to the leading pacemaker cell group containing latent pacemaker cells, were dissected and separately dissociated (see below). After isolation, different cell types were counted and their ratio calculated.

Cell Distribution Without Electrophysiological Mapping

To determine local differences in cell distribution we dissected three 2.5 mm² adjacent pieces of nodal tissue, along a line perpendicular to the crista terminals: (1) the central portion of the putative node, expected to contain nodal tissue only, (2) the crista terminals part, containing nodal and some atrial tissue from the site of the crista terminals, and (3) the septal part, existing of nodal and some atrial wall tissue. These pieces were dissociated separately, after which cells were counted and rated.

Single Sinonodial Nodal Cells

Isolation Procedure

Single SAN myocytes were isolated according to the method of DiFrancesco et al., with some modifications as described previously. Single cells for immunohistochemical staining as well for experiments in which the electrical activity of the nodal cells was studied were obtained following cell isolation procedures 1 and 2 (see below). For the experiments in which cells were isolated after regional dissection of the SAN and in experiments in which the SAN was first electrophysiologically mapped, only cell isolation procedure 2 was followed (see below).

Cell Isolation Procedure 1

The heart was excised and mounted on a Langendorff perfusion system and cleared from blood for 5 minutes with a solution
containing (in mmol/L) NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1.0, HEPES 5.0, and glucose 5.5 (normal Tyrode solution). The solution was kept at 37°C; pH was adjusted to 7.4 with NaOH. The SAN region was excised and cut into small strips (width, 0.5 to 1 mm; length ≈ 2 mm) perpendicular to the crista terminalis. Strips were allowed to equilibrate for 15 minutes in normal Tyrode solution at room temperature.

Cell Isolation Procedure

Strips of nodal tissue were placed in a test tube with an oxygenated calcium-free Tyrode solution at room temperature containing (in mmol/L) NaCl 140, KCl 5.4, CaCl2 0.5, KH2PO4 1.2, HEPES 5.0, and glucose 5.5. The pH was adjusted to 6.9 with NaOH. Next, the strips were transferred to a calcium-free Tyrode solution to which collagenase B (0.28 U/mL, Boehringer Mannheim), pronase E (0.92 U/mL, Serva), elastase (12.4 U/mL, Serva), and bovine serum albumin were added (dissociation solution) and incubated at 37°C for ≈12 minutes and gently triturated through a pipette with a tip diameter of 2.0 mm. Dissociation was stopped by transferring the strips into a modified Kraft-Brithe (KB) solution containing (in mmol/L) KCl 85, K2HPO4 30, MgSO4 5.0, glucose 20, pyruvic acid 5.0, creatine 5.0, taurine 30, EGTA 0.5, β-hydroxybutyric acid 5.0, succinic acid 5.0, Na2ATP 2.0, and polyvinylpyrrolidone 50 g/L (pH adjusted with KOH to 6.9). Thereafter, strips were triturated in KB solution through a pipette (tip diameter 0.8 to 1.2 mm) for 2 minutes. Cells were placed in a recording chamber on the stage of an inverted microscope (Nikon Diaphot) and superfused with normal solution (0.6 mL/min), maintained by a translucent heating plate underneath the bottom of the recording chamber and continuously monitored.

Membrane Potential Recording and Analysis

Membrane potentials were recorded using the whole-cell technique.133 Electrodes were pulled from borosilicate glass (outer diameter, 1 mm) by use of a vertical one-stage patch-electrode puller and thereafter heat-polished and backfilled with a microprobe-filtered electrode solution containing (mmol/L): K-gluconate 120, KCl 20, HEPES 5, MgCl2 5, CaCl2 0.6, Na2ATP 5, cAMP 0.1, and EGTA 5 (pH 7.2). Electrode resistances were between 3 and 5 MΩ. Series resistance was compensated for by ≈25%. Apart from zeroing the potential before touching the surface of cell by pipette tip, no attempts were made to correct for junction potential.

Recordings were made with a custom-build current-voltage clamp amplifier. Data were sampled directly into a Macintosh Quadra 650 microcomputer (Apple Computer, Inc) with the use of custom-written data acquisition software (J. Zegers, University of Amsterdam, The Netherlands) and stored on digital audio tape using a digital tape recorder (DTR-1204, Bio-Logic Co), for off-line processing using custom-written data analysis software (A.C.G. van Ginneken, University of Amsterdam, The Netherlands).

The membrane capacitance, Cm, was defined as the amplitude of a hyperpolarizing current pulse (20 to 40 pA, 200 ms) divided by the initial slope of the transmembrane voltage in response to this current pulse. The current pulse was switched on shortly after the action potential had reached its maximum diastolic potential. The current pulse was adjusted to produce a membrane hyperpolarization of ≈10 mV. Mean membrane capacitance of the nodal cells was 46±11 pF (n=21). Atrial cells were paced at 3 Hz with current pulses of 2-ms duration, 10% to 15% suprathreshold. For nodal cells the following action potential parameters were measured: cycle length, action potential overshoot, MDP, diastolic depolarization rate measured over the first 100 ms starting at the MDP (DDR), maximum upstroke velocity (dV/dtmax), and duration between 50% depolarization and 90% repolarization (APD50). For atrial cells we determined cycle length, action potential overshoot, membrane resting potential (Vrest), dV/dtmax, APD50, and duration between 50% depolarization and 90% repolarization (APD50).

Electron Microscopic Reconstruction of a Nodal Cell In Situ

Tissue sections were prepared as described by De Mazière et al.15 A series of 580 tissue sections of 50 nm was used. Every 15th section was photographed, and the cell profile was traced on a transparent sheet. For the reconstruction the successive profiles of the cell were fed, using a Summa-Sketch Plus MM 1201 (Summagraphics) digitizing tablet, into a Macintosh computer using MacStereology, written by V.A. Moss.16

Statistical Analysis

Results are presented as mean±SD. For statistical analysis of the action potential parameters we used the mean values of 15 subsequent action potentials. Statistical significance was determined by one-way or two-way ANOVA combined with a Student’s t test for paired observations with Bonferroni correction. A probability value of P≤0.05 was considered significant.

Results

Neurofilament Staining Pattern in the SAN

In the SAN, a gradual change in electrophysiological properties exists when going from the center of the node toward the atrium. Two models exist to explain this gradual transition. In one, the center of the node is surrounded by an area in which the structure and electrophysiology of the fibers gradually changes from nodal to atrial. In the other model, the center of the node is surrounded by an area in which the atrial cell density gradually increases toward the atrium. Nodal cells can specifically be stained with a neurofilament antibody,18 which enables us to discriminate between both models.

Therefore, three hearts were serially sectioned in the transverse plane to study the neurofilament distribution in the SAN and the surrounding atrium. In these sections we aimed to discriminate the border between myocardial cells and nonmyocardial cells. Myocardial cells were identified from nonmyocardial cells by an antidesmin antibody that reacted with a phosphorylated isoform of intermediate filament desmin that is expressed in the working myocardium as well as in the conduction system.19,27 Fig 1, a and b, shows that antidesmin homogeneously reacts with myocytes from the atrium and SAN region.

Fig 1c shows the neurofilament expression pattern in a section adjacent to the one used for the desmin staining. Neurofilament is not expressed in the atrial myocardium. To our surprise, the neurofilament expression in the SAN region was heterogeneous. Fig 1d shows an enlargement from the SAN area and clearly demonstrates that in the SAN, not all of the desmin-positive cells react with the antineurofilament antibody (Fig 1b and 1d). These cells are either nodal cells that do not stain with the neurofilament antibody, or, alternatively the neurofilament negative cells are atrial cells.

Neurofilament Distribution Pattern in the Electrophysiologically Mapped SAN

To correlate the immunohistochemical and electrophysiological characteristics, we studied the neurofilament distribution pattern of two right atrial preparations of which an electrophysiologically determined maximum upstroke velocity map was made. From sections cut perpendicular to the crista terminalis and spaced by 200 μm, the area of neurofilament expression was determined. Fig 2 shows a composite of the neurofilament expression (striaed area) and the electrophysiologically determined maximum upstroke velocity map within the SAN. The area in which cells show an upstroke...
Atrial and Nodal Cells in the SA Node

Figure 1. Photomicrographs of the sinoatrial node. a, Overview of the staining pattern of desmin. The box in a is shown in detail in b, c, Overview of the staining pattern of neurofilament in an adjacent section. The box in c is shown in detail in d and shows that not all cells within the nodal area react with antineurofilament antibody. The bar in a and c is 1 mm and in b and d is 100 μm. LA indicates left atrium; RA, right atrium; SAN, sinoatrial node; CT, crista terminalis; and AO, aorta.

velocity of <15 V/s coincides with the area in which cells stain with neurofilament antibody. These data clearly demonstrate that the neurofilament expression can be used to delineate the SAN from the surrounding atrium. The desmin-negative (Fig 1b) but neurofilament-negative spots in the SAN (Fig 1d) represent either atrial cells that might invaginate into the SAN region, similar to what was described previously in guinea pig,14 or nodal cells in which neurofilament is not expressed. Therefore, we questioned whether enzymatic digestion of the SAN would (1) provide us with atrial cells and (2) whether single nodal cells would all express the neurofilament.

Figure 2. Correlation of immunohistochemistry with electrophysiology in a sinoatrial node preparation. The spatial distribution of neurofilament is represented by the dashed area in the schematic representation of the sinoatrial node. In addition, the pacemaker area in which cells have an upstroke velocity of <15 V/s is enclosed by an iso-15 V/s line. It is clear that both areas coincide well. C.T. indicates crista terminalis; I.V.C., inferior vena cava; and S.V.C., superior vena cava.

Morphology of Single Nodal Cells

Cell morphology was studied on freshly isolated myocytes in storage (KB) solution and hematoxylin-eosin (H-E)-stained cells. Under both conditions, most of the cells kept their elongated appearance, although a few rounded up. From the nodal area, four types of isolated myocytes could be isolated. “Elongated spindle cells” have a slender, faintly striated cell body and contain one or two nuclei. The length of these cells ranges between 50 and 80 μm (Fig 3a). Spindle cells have a similar shape as elongated spindle cells but are considerably shorter (30 to 40 μm) with blunted ends and contain 1 nucleus only (Fig 3b). Spider cells have a varying number of irregularly shaped branches. After H-E staining, we observed that the majority of the spider cells were single cells containing one or sometimes two nuclei (Fig 3c). Only a few appeared to be composed of more than one cell. Rod cells have clear cross-striations and contain one or two nuclei (Fig 3d). In contrast to the first three cell types, these rod cells were quiescent but excitable in normal Tyrode solution. For this reason and because of their shape, which is similar to myocytes from the working atrial myocardium, these cells are considered to be atrial cells. Because elongated spindle cells, spindle cells, and spider cells all exhibit spontaneous activity in normal solution, they are all considered to be nodal pacemaking cells.

Denyer and Brown,18 who described the same types of nodal cells, assumed that spider cells are bundles of two or more closely packed spindle cells. In contrast, we found that the vast majority of spider cells have only one nucleus, indicating that they are single cells. To demonstrate that spider cells are naturally present in the SAN and not caused by the isolation procedure, we made a three-dimensional reconstruction of a cell from the central portion of the SAN on basis of electron microscopic photographs (Fig 4). This
reconstruction shows a part of a spider cell. From the central part of the cell, which contains the nucleus (striated area), three branches develop (indicated by arrows). On the basis of this reconstruction, it can be concluded that cells with a number of branches exist in the native rabbit SAN as a separate entity. In conclusion, after enzymatic dissociation of the rabbit SAN, three morphologically distinct nodal cell types as well as atrial cells can be distinguished.

**Responsiveness of Nodal Cells to Neurofilament Staining**

It was questioned whether the neurofilament negative spots in the SAN (Fig 1d) can be explained fully by the presence of atrial cells, which are all neurofilament negative. Therefore, the individual responsiveness of the three nodal cell types to neurofilament staining was tested.

All cells of the four morphologically different cell types proved positive for desmin, which indicates that the cytoskeleton is not affected by the isolation procedure. Fig 5 demonstrates the variation of neurofilament staining of the three nodal cell types. In three experiments, 58% of all tested cells responded positively to the neurofilament antibody. Although there was a tendency to a higher responsiveness in the spider cells (70%) the differences between the three cell types were not significant. All isolated atrial cells showed a negative response (not shown).

![Figure 4](image-url)

**Figure 4.** Electron microscopic reconstruction of a spider cell. Reconstruction of a part of a spider cell from the central portion of the sinoatrial node. Three cell branches develop from the cell body. The nucleus is shaded in the middle of the cell.

![Figure 5](image-url)

**Figure 5.** Neurofilament staining of morphologically different nodal cell types. Neurofilament staining of morphologically different nodal cell types, isolated from one sinoatrial node preparation. Cells can stain positive (a, b, and c) or negative (d, e, and f) for antineurofilament antibody. a and d, Elongated spindle cells; b and e, spindle cells; c and f, spider cells. Bar, 30 μm.

In conclusion, all nodal cell types show an inhomogeneous response to neurofilament staining. Therefore, the neurofilament-negative spots in the SAN (Fig 1d) can be caused by either atrial cells or nodal cells that do not stain with neurofilament antibody. The large percentage of neurofilament-positive cells in all three nodal cell types still renders this technique suitable for marking the borders of the SAN in the light microscopic preparation but is not suitable to discriminate between atrial and nodal myocardium at the cellular level.

**Distribution of Nodal Cell Types Within the SAN**

In the experiments described thus far, single cells were isolated from the whole SAN region. However, in the intact SAN, a gradual transition in action potential configuration exists when going from the central area toward the atrium. It was questioned whether this gradual transition can be explained by a gradient of a specific cell type. Therefore, we established the distribution of the four morphologically different cell types within the more central area of the SAN.

In six experiments, the nodal area was divided into three zones of 2.5 mm² perpendicular to the crista terminalis, that
is, crista terminalis area, central area, and septal area (Fig 6). Zones were excised and dissociated separately. After plating, the number of each cell type per zone was counted and expressed as a fraction of the total. In every experiment, \( \approx 500 \) cells were counted. In the three zones, all cell types were present (Fig 6). Cell types were equally distributed in the central area. In the crista terminalis area, the majority of cells (63±18%) were atrial cells, whereas the elongated spindle cells were the predominant type of nodal cells (21%). In the septal area of the SAN, 88±19% of the cells were of atrial origin. In this area, nodal cells types were distributed in about equal quantities. In the central area, 41±10% were atrial cells. The density of atrial cells was not significantly between the central an crista terminalis area, whereas the difference of both areas with the septal area was significant (\( P < 0.01 \) after Bonferroni correction).

In summary, none of the cell types was exclusively present in any specific area of the SAN. In the area bordering the crista terminalis, the elongated spindle cells were the most prominent of the nodal cells. Within the other areas no difference in distribution of nodal cells was found.

To relate the distribution of cell types to the site of the dominant pacemaker, the activation pattern of the SAN was mapped electrophysiologically before cell dissociation in five experiments. Cells were isolated from a piece of tissue of less than 1 mm\(^2\) around the site of the earliest discharge and from a piece of 2 to 3 mm\(^2\) located directly superior to this area. Even in the very restricted area around the dominant pacemaker area, 22±15% of the cells were atrial cells. Between the dominant and latent pacemaker area no significant difference in distribution of any cell type was found (Table 1).

**Discussion**

**Immunohistochemical Markers in the SAN**

With the aid of immunohistochemistry it has been demonstrated that specific proteins are expressed in the conduction system of the heart of various species.\(^{28-30}\) In bovine heart it has been demonstrated that the SAN could be distinguished from the atrial myocardium by a monoclonal antibody (445–6E10) raised against the bovine conduction system.\(^{31}\) In the SAN of the guinea pig we found that the impulse originates in an α-smooth muscle actin–positive, virtually connexin-negative region. In the SAN of the adult rabbit,\(^{25}\) a specific expression of an antineurofilament antibody was described. We demonstrated that the antineurofilament monoclonal anti-

**Table 1. Cell Distribution After Electrophysiological Mapping in the Dominant and the Latent Pacemaker Areas (n=5)**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dominant, % Mean±SD</th>
<th>Latent, % Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elongated spindle</td>
<td>27±11</td>
<td>25±8</td>
</tr>
<tr>
<td>Spindle</td>
<td>21±10</td>
<td>18±9</td>
</tr>
<tr>
<td>Spider</td>
<td>30±23</td>
<td>28±15</td>
</tr>
<tr>
<td>Atrial</td>
<td>22±15</td>
<td>29±10</td>
</tr>
</tbody>
</table>

Fig 7 shows that a large variability exists with respect to the number of each cell type per zone was counted and expressed as a fraction of the total. In every experiment, \( \approx 500 \) cells were counted. In the three zones, all cell types were present (Fig 6). Cell types were equally distributed in the central area. In the crista terminalis area, the majority of cells (63±18%) were atrial cells, whereas the elongated spindle cells were the predominant type of nodal cells (21%). In the septal area of the SAN, 88±19% of the cells were of atrial origin. In this area, nodal cells types were distributed in about equal quantities. In the central area, 41±10% were atrial cells. The density of atrial cells was not significantly between the central an crista terminalis area, whereas the difference of both areas with the septal area was significant (\( P < 0.01 \) after Bonferroni correction).

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**Electrical Activity of Morphologically Different Cell Types**

In the next series of experiments we investigated whether morphologically different cell types could be distinguished from each other electrophysiologically. In these experiments the whole SAN was used for dissociation without prior mapping.
Figure 7. Action potentials of morphologically different nodal cells. A, Action potential recordings of three different elongated spindle cells. B, Action potential recordings of three different spindle cells. C, Action potential recordings of three different spider cells. D, Action potential recording of an atrial cell paced at 3 Hz.

A antibody reacts with isolated nodal myocytes but not with atrial cells. However, this marker did not identify all isolated nodal myocytes. It is unlikely that the neurofilament-negative nodal cells were negative because of an isolation artifact because all nodal cells were desmin positive. These results demonstrate that the expression of the antineurofilament antibody cannot be used to distinguish individual nodal cells from atrial myocytes. In the intact SAN, the neurofilament-positive area coincides with the electrophysiologically defined pacemaker area, where cells had an upstroke velocity of ≥15 V/s (Fig 2). This, together with the large percentage of neurofilament-positive cells (58%), renders this technique very suitable for marking the borders of the SAN.

**Morphologically Different Nodal Cell Types**

We described three morphologically different nodal cell types, that is, elongated spindle, spindle, and spider cells as they appear after enzymatic dissociation. According to De-nyer and Brown, spider cells are actually small bundles of two or more closely packed spindle cells. However, Rossi et al demonstrated in human SAN the presence of cells with polypoid branches. After H-E and immunohistochemical

**TABLE 2. Action Potential Parameters of Morphologically Different Nodal Cells and Atrial Cells**

<table>
<thead>
<tr>
<th></th>
<th>Elongated Spindle</th>
<th>Spindle</th>
<th>Spider</th>
<th>Atrial (paced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=7)</td>
<td>(n=8)</td>
<td>(n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle length, ms</td>
<td>441±206</td>
<td>308±85</td>
<td>288±80</td>
<td>333 (paced)</td>
</tr>
<tr>
<td>APDsub, ms</td>
<td>111±22</td>
<td>102±16</td>
<td>111±36</td>
<td>32±8</td>
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<tr>
<td>APD100, ms</td>
<td>173±29</td>
<td>161±22</td>
<td>176±54</td>
<td>54±9</td>
</tr>
<tr>
<td>DDR, mV/s</td>
<td>106±54</td>
<td>134±42</td>
<td>178±63</td>
<td></td>
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<tr>
<td>dV/dtmax, V/s</td>
<td>6.4±2.9</td>
<td>7.3±5.7</td>
<td>4.8±3.5</td>
<td>160±20</td>
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<tr>
<td>MDP, mV</td>
<td>−53±5</td>
<td>−49±6</td>
<td>−46±6</td>
<td>−74±2</td>
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<tr>
<td>Overshoot, mV</td>
<td>33±7</td>
<td>32±12</td>
<td>27±9</td>
<td>22±4</td>
</tr>
<tr>
<td>Cm, pF</td>
<td>53±3f</td>
<td>32±5</td>
<td>54±4f</td>
<td>95±11</td>
</tr>
</tbody>
</table>

APD indicates action potential duration; DDR, depolarization rate measured over the first 100 ms starting at the MDP; dV/dtmax, maximum upstroke velocity; MDP, maximum diastolic potential; Cm, membrane capacitance; and Vres, membrane resting potential.

*Statistical difference between spindle and spider cells (P<.02, with Bonferroni correction).

†Statistical difference between elongated spindle and spindle cells (P<.02, with Bonferroni correction).
staining, we observed that only few apparent spider cells were composed of more than one cell (<1%). The majority were single cells that contained one or two nuclei. A three-dimensional reconstruction on the basis of electron microscopic photographs of a cell from the central portion of the intact SAN (Fig 4) demonstrates that the node contains cells with numerous branches that may run over a considerable distance between other cells. Apparently spider cells exist in the intact rabbit SAN and are not to be considered as an isolation artifact.

The Transitional Zone
A gradual transition from nodal to atrial tissue has been described in the rabbit SAN.\(^5\) In the present study we found that elongated spindle cells are more abundant in the area adjacent to the crista terminalis than spindle and spider cells, which suggests that they are the previously described transitional fibers. Suggestions for an intermediate class of cells in the rabbit were put forward, both on morphological and functional grounds. Fig 8 (left) shows a scheme of a central node composed of typical nodal cells surrounded by a rim of transitional cells, as was originally suggested for the rabbit SAN.\(^5\) This scheme did not prove applicable to the feline, canine, or human SAN, in which typical nodal cells were observed intermingled with and adjacent to atrial cells (Fig 8, right).\(^4,35\) However, in the rabbit we also found evidence for a less smooth transition.\(^35\) Previous studies have suggested that typical (central) nodal cells are sensitive to casum and not to TTX.\(^33\) However, our group previously challenged this concept: It was found that any nodal cell, if sufficiently hyperpolarized, will open Na\(^+\) channels.\(^36\) Preliminary results indicated larger \(I_c\) current density in larger single nodal cells.\(^37\) It was hypothesized that large nodal cells are located more to the border of the intact SAN. These cells would be more likely to overcome the hyperpolarizing load of adjacent atrium more effectively. We found that the area adjacent to the crista terminalis has indeed more elongated spindle cells than the central and septal area (Fig 6). On the other hand, elongated spindle cells are not the exclusive cell type of the crista terminalis area. In contrast with previous findings,\(^37\) we recently found the same current density for \(I_c\), \(I_{Ca}\), and \(I_{K1}\) in cells with different dimensions.\(^38\) In addition, in the current we demonstrated that the action potential configuration of small and large isolated nodal cells is similar (Fig 7, Table 2). Therefore, cell size alone cannot explain regional differences in action potential configuration, as found in the intact SAN. Unfortunately, whole-cell current clamp experiments on cells obtained after electrophysiological mapping of the SAN failed, because no calcium resistant cells could be obtained after the long time span of the whole procedure. Consequently, we were not able to relate regional differences in action potential configuration as found in the intact SAN to differences in action potential configuration found in single cells of known location within the SAN.

Geometric Considerations
A completely different explanation for the functional differentiation within the intact SAN lies in its geometric relation to the atrium. We observed in a restricted area (1 mm\(^2\)) around the electrophysiologically mapped dominant pacemaker area that 22% of the isolated cells were atrial cells. In the larger central portion of the SAN (2 to 3 mm\(^3\)), 41% of the cells were from atrial origin, and when a piece of nodal tissue bordering the crista terminalis was isolated, even a percentage of atrial cells as high as 63% was obtained. These observations show that the number of atrial cells increases progressively from the dominant pacemaker site toward the crista terminalis. These atrial cells probably originate from invaginating atrial myocardium, similar to what is suggested in bovine\(^11\) and guinea pig heart.\(^1\) These data agree with the close opposition of nodal and atrial cells in the center of the rabbit SAN, as we demonstrated previously in an electron microscopic study.\(^11\) Electrophysiological evidence for such inhomogeneity was found by measurements of space constants in different parts of the rabbit SAN. These constants appeared to vary considerably over very short distances.\(^12\) Watanabe et al.\(^39\) performed experiments in which they modulated the SAN pacemaker activity by coupling SAN cells, through an electronic artificial coupling resistance, to a resistance-capacitance circuit resembling an atrial cell. Increasing the number of "atrial" cells coupled to the SAN cell caused a gradual transition of the nodal to the atrial type of action potential.

Data on specific connexins are lacking in the rabbit at the level of contacts between atrial cells and nodal cells. Only in guinea pig labeling with connexin43 antibodies between atrial and nodal cells was found.\(^44\) Data on connexins between rabbit nodal cells, however, have been shown to consist of at least of connexin43,\(^40\) In other species, connexin43,\(^41\) connexin45\(^41\) have been described, but there is no certainty whether these connexins are located between individual nodal cells or that they may play a role in the connection between nodal and atrial cells.

We hypothesize that in the intact SAN the gradual increase in density of atrial cells from the dominant area toward the crista terminalis (Fig 8, right) causes a gradual increase of atrial electrotonic influence that may be an important cause of the gradual transition of the nodal to the atrial type of action potential. On the other hand, the variety in action potentials recorded in isolated nodal cells (Fig 7) does not rule out the possibility that differences in cellular properties of various
nodal cell types also contribute to the gradual transition of action potential configuration from the center of the node to the surrounding atrial myocardium.

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References
Distribution of Atrial and Nodal Cells Within the Rabbit Sinoatrial Node: Models of Sinoatrial Transition

E. Etienne Verheijck, Andy Wessels, Antoni C. G. van Ginneken, Jan Bourier, Marry W. M. Markman, Jacqueline L. M. Vermeulen, Jacques M. T. de Bakker, Wouter H. Lamers, Tobias Opthof and Lennart N. Bouman

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