Abstract—The aim of the study was to identify ion channel transcripts expressed in the sinoatrial node (SAN), the pacemaker of the heart. Functionally, the SAN can be divided into central and peripheral regions (center is adapted for pacemaking only, whereas periphery is adapted to protect center and drive atrial muscle as well as pacemaking) and the aim was to study expression in both regions. In rabbit tissue, the abundance of 30 transcripts (including transcripts for connexin, Na\(^+\), Ca\(^{2+}\), hyperpolarization-activated cation and K\(^+\) channels, and related Ca\(^{2+}\) handling proteins) was measured using quantitative PCR and the distribution of selected transcripts was visualized using in situ hybridization. Quantification of individual transcripts (quantitative PCR) showed that there are significant differences in the abundance of 63% of the transcripts studied between the SAN and atrial muscle, and cluster analysis showed that the transcript profile of the SAN is significantly different from that of atrial muscle. There are apparent isoform switches on moving from atrial muscle to the SAN center: RYR2 to RYR3, Na\(_{1.5}\) to Na\(_{1.1}\), Ca\(_{1.2}\) to Ca\(_{1.3}\) and K\(_{1.4}\) to K\(_{4.2}\). The transcript profile of the SAN periphery is intermediate between that of the SAN center and atrial muscle. For example, Na\(_{1.5}\) messenger RNA is expressed in the SAN periphery (as it is in atrial muscle), but not in the SAN center, and this is probably related to the need of the SAN periphery to drive the surrounding atrial muscle. (Circ Res. 2006;99:1384-1393.)

Key Words: sinoatrial node • pacemaker • Na\(^+\) channels • Ca\(^{2+}\) channels • HCN channels • K\(^+\) channels

This is the centenary of the discovery of the sinoatrial node (SAN), the pacemaker of the heart, by Keith and Flack.\(^1\) Early intracellular recordings of pacemaker and action potentials in the SAN were made by de Carvalho et al in 1959\(^2\) and, in the \(\approx\)50 years since then, a wealth of data has been accumulated concerning the pacemaker and action potentials in the SAN and the underlying ionic currents.\(^3,4\) However, little is known about the molecular basis of ionic currents in the SAN and the aim of this study was to measure the abundance of messenger RNAs (mRNAs) coding for ion channels and related proteins in the SAN. The study was performed on rabbit, because of the existence of extensive functional data from this species: the early study of de Carvalho et al,\(^2\) as well as the majority of the studies on SAN since,\(^3,4\) have been performed on rabbit.

The SAN is a complex and heterogeneous tissue.\(^5\) The action potential is first initiated in the center of the SAN.\(^4\) It then propagates from the leading pacemaker site in the center to the periphery of the SAN (where SAN connects to atrial muscle) and then onto the atrial muscle of the crista terminalis and right atrial free wall.\(^4\) The SAN center is adapted for pacemaking: it has poor electrical coupling to protect it from the inhibitory hyperpolarizing influence of surrounding atrial muscle and it has a complement of ionic currents appropriate for pacemaking.\(^3,4,6\) The SAN periphery, although still capable of pacemaking, is adapted to drive the surrounding atrial muscle: it has (1) a large inward TTX-sensitive Na\(^+\) current (consequently, an action potential with a rapid upstroke) to generate sufficient inward (depolarizing) current to drive atrial muscle; and (2) strong electrical coupling to deliver the current to the atrial muscle.\(^4\) In this study, the abundance of mRNAs was measured in the periphery as well as the center of the rabbit SAN.

Materials and Methods
SAN tissue was sampled from male New Zealand White rabbits (1.5 to 2.5 kg; supplied by University of Leeds) for both quantitative PCR (qPCR) and in situ hybridization (ISH). Total RNA was extracted using a modified Qiagen protocol and 150 ng of total RNA from each sample was reverse transcribed; qPCR was performed using either Taqman probe or SYBR green chemistries. For ISH, digoxigenin labeled complementary RNA (cRNA) probes were used. For further details please see the online data supplement available at http://circres.ahajournals.org.

Results
Tissues Studied
Figure 1D shows a section through the rabbit SAN \(\approx\)at the level of the leading pacemaker site. The section was cut...
perpendicular to the crista terminalis and includes part of the right atrial free wall, the crista terminalis (thick bundle of atrial muscle) and the intercaval region, where the SAN center is located. Typically, the leading pacemaker site is in the SAN center, \( \approx 0.5 \) to 2 mm from the crista terminalis.\(^4\) From the leading pacemaker site, the action potential propagates through the SAN periphery (on endocardial surface of crista terminalis) to the right branch of the sinoatrial ring bundle (RSARB; vestige of embryonic venous valve) and then onto the atrial muscle of the crista terminalis etc.\(^2,4\) The action potentials in these regions are different.\(^4\) Tissue isolated from the SAN center shows spontaneous activity and the action potential has a low take-off potential, slow upstroke (\( \approx 2 \) V/s), small overshoot, small amplitude, long duration, low maximum diastolic potential (MDP), and a pacemaker potential (Figure 1C).\(^2,4\) Tissue isolated from the RSARB also shows spontaneous activity, which is paradoxically faster than that of the SAN center (Figure 1B).\(^4\) The action potential in the RSARB has a higher take-off potential, faster upstroke (\( \approx 50 \) V/s), larger overshoot, larger amplitude, shorter duration, higher MDP, and a steeper pacemaker potential than the action potential in the SAN center (Figure 1B).\(^2,4\) Similar to the RSARB, the action potential in atrial muscle has a fast upstroke (\( > 100 \) V/s), large overshoot, large amplitude, short duration and high MDP, but it does not have a pacemaker potential (Figure 1A). From 7 rabbits, tissue samples were taken from the center (in intercaval region) and periphery (on endocardial surface of crista terminalis) of the SAN, as well as the atrial muscle in the right atrial free wall. From these samples, total RNA was reverse transcribed to generate complementary DNA (cDNA). The relative abundance of cDNA from 30 different transcripts was measured

Figure 1. Preparation studied and abundance of transcripts for SAN markers, connexins, and ryanodine receptors. A through C, Typical action potentials recorded from atrial muscle, RSARB and SAN center of rabbit (32°C). Atrial action potentials recorded from intact SAN preparation, whereas action potentials from RSARB and SAN center recorded from isolated balls of tissue \( \approx 0.3 \) mm in diameter. D, Masson’s trichrome stained section through right atrial free wall, crista terminalis (CT) and intercaval region. Red, myocytes; blue, connective tissue. Dashed line in this and all similar figures represents approximate border between atrial muscle and SAN periphery. E through L, relative abundance of transcripts as measured by qPCR. Means \( \pm \) SEM (n = 7) shown. In this and other figures: *significantly different from atrial muscle or SAN periphery \( (P < 0.05) \) as determined by one-way ANOVA; †significantly different from atrial muscle \( (P < 0.05) \) as determined by paired t-test.

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with qPCR. For an assessment of the accuracy of the tissue sampling for qPCR see the online data supplement available at http://circres.ahajournals.org. The distribution of selected transcripts in sections equivalent to that in Figure 1D was visualized using ISH.

**Housekeeping Genes and SAN Markers**

In the case of qPCR, abundance of transcripts is given relative to a housekeeping gene to correct for variations in input RNA. We assessed 3 housekeeping genes: 28S, GAPDH, and Na⁺-K⁺ pump (α1 isoform). The abundance of all 3 was constant in the 3 tissues studied (supplemental Table IV and supplemental Figure I in the online data supplement); 28S was chosen as the housekeeping gene, but similar data were obtained using the other housekeeping genes.

To characterize the tissue studied (Figure 1D), 2 markers of SAN tissue,\(^3\) atrial natriuretic peptide (ANP; hormone) and middle neurofilament (cytoskeletal protein), were investigated. Figure 1 (E and F) shows abundance of mRNA for ANP and neurofilament; abundance is expressed as a percentage of abundance in atrial muscle. Figure 2 (A and B) shows localization of ANP and neurofilament mRNA in sections. ANP mRNA was abundant throughout the atrial muscle of the crista terminalis and also in the RSARB, but it was not expressed in the SAN center (Figure 2A). In the SAN periphery, many cells did not express ANP mRNA, although some cells did (examples highlighted by arrows in Figure 2A). Neurofilament mRNA was absent in the atrial muscle of the crista terminalis, but was abundant in the SAN center (Figure 2B). Neurofilament mRNA was present in many, but not all, cells in the RSARB and SAN periphery (examples of cells not expressing neurofilament mRNA highlighted by arrows in Figure 2B). Interestingly, 2 tracts of neurofilament mRNA-expressing cells penetrated into the crista terminalis (Figure 2B, *).

**Connexins**

Three connexin (Cx) transcripts (responsible for electrical coupling) were investigated. Expression of Cx40 mRNA (responsible for 200 pS gap junction channels) was significantly lower in the SAN than in atrial muscle (Figure 1G). ISH detected a low level of expression of Cx40 mRNA in atrial muscle (supplemental Figure II, red arrows) and no
detectable expression in the SAN (supplemental Figure II). Cx40 mRNA was abundant in the walls of blood vessels (supplemental Figure II, yellow arrow). Cx43 mRNA (responsible for 60 to 100 pS gap junction channels) was significantly less abundant in the SAN than in atrial muscle (Figure 1H; similar results, not shown, were also obtained using a second, independent Cx43 qPCR assay). ISH showed abundant Cx43 mRNA in the atrial muscle of the crista terminalis as well as in the RSARB, but none in the SAN center (Figure 2C). In the SAN periphery, the majority of cells did not express Cx43 mRNA, but some cells did (examples highlighted by arrows in Figure 2C). Cx45 mRNA (responsible for 20 to 40 pS gap junction channels) did not vary significantly between tissues (Figure II).

**Ca$$^2$$+ Handling Proteins**

Intracellular Ca$$^2$$+ has been suggested to play an important role in pacemaking in the rabbit SAN. M$$^3$$RNA$$^4$$s for four Ca$$^2$$+ handling proteins were investigated. RYR2 mRNA was significantly less abundant in the SAN compared with atrial muscle (Figure 1J). In contrast, RYR3 mRNA was significantly more abundant in the SAN center compared with atrial muscle (Figure 1J). Therefore, from atrial muscle to SAN center, there is an apparent isoform switch from RYR2 to RYR3. The abundance of NCX1 (Na$$^-$$Ca$$^2$$+ exchanger) mRNA was significantly lower in the SAN periphery compared with other tissues (Figure 1L). mRNA levels for SERCA2a (sarcoplasmic reticulum Ca$$^2$$+ pump) did not vary between tissues (supplemental Table IV).

**Na$$^+$$, Ca$$^2$$+ and HCN channels**

The voltage-gated Na$$^+$$ current, $I_{Na}$, is responsible for the rapid action potential upstroke in working myocardium (including atrial muscle) and in the RSARB (Figure 1, A and B). There is no measurable $I_{Na}$ in the SAN center and the L-type Ca$$^2$$+ current, $I_{Ca,L}$, is responsible for the slow action potential upstroke in this region (Figure 1C). $I_{Na,1.5}$ is the cardiac Na$$^+$$ channel responsible for the majority of $I_{Na}$. As expected, the abundance of Na$$^+,1.5$$ mRNA was significantly lower in the SAN center than in atrial muscle; in the SAN periphery, it was higher, but still significantly lower, than in atrial muscle (Figure 3B). ISH showed abundant Na$$^+,1.5$$ mRNA in atrial muscle, but no signal in the SAN center (Figure 4C). Na$$^+,1.5$$ mRNA was present in the RSARB (Figure 4B) and, although Na$$^+,1.5$$ mRNA was not present in many cells in the SAN periphery, it was present in some cells (examples highlighted by arrows in Figure 4C). For all ion channels studied with ISH, the labeling of mRNA appeared as spots at low power and intracellular rings at high power (Figure 4, A and C), which probably correspond to the rough endoplasmic reticulum (in which mRNA is translated) in the perinuclear region.

Recently, neuronal Na$$^+$$ channels have been shown to be expressed in cardiac myocytes.$$^8$$ Figure 3A shows data for Na$$^+,1.1$$ mRNA. In contrast to Na$$^+,1.5$$ mRNA, the abundance of Na$$^+,1.1$$ mRNA was significantly greater in the SAN than in atrial muscle (Figure 3A). Therefore, from atrial muscle to the SAN center, there is an apparent isoform switch from Na$$^+,1.5$$ to Na$$^+,1.1$$.

Transcripts for 2 L-type Ca$$^2$$+ channel isoforms were investigated. Ca$$^+,1.2$$ mRNA was significantly less abundant in the SAN than in atrial muscle; the reverse was true of Ca$$^+,1.3$$ mRNA (Figure 3, C and D). In addition, Ca$$^+,1.3$$ mRNA was significantly more abundant in the center than in the periphery of the SAN. ISH showed Ca$$^+,1.2$$ mRNA to be abundant in the atrial muscle of the crista terminalis, but absent from the SAN center (Figure 5A). In the RSARB and SAN periphery (at foot of crista terminalis in this example), there was little or no Ca$$^+,1.2$$ mRNA (Figure 5A). In contrast, ISH showed Ca$$^+,1.3$$ mRNA was abundant throughout the SAN (in center and periphery and also in RSARB), with only weak labeling in atrial muscle (Figure 5B). Therefore, once again, between atrial muscle and the SAN center, there is an apparent isoform switch (from Ca$$^+,1.2$$ to Ca$$^+,1.3$$).

HCN channels are responsible for the hyperpolarization-activated current, $I_h$, that plays a key role in the pacemaker potential. The expression patterns of mRNA for 2 HCN isoforms, HCN1 and HCN4, were similar (Figure 3, E and F). HCN1 and HCN4 mRNA was significantly more abundant in the SAN center than in both the SAN periphery and atrial muscle (Figure 3, E and F). In the case of HCN4, but not HCN1, the mRNA was also significantly more abundant in the SAN periphery than in atrial muscle. ISH for HCN4 confirmed the qPCR data: it showed that HCN4 mRNA was abundant in the SAN center, but absent in the atrial muscle of the crista terminalis (Figure 6). It also showed that HCN4 mRNA was absent from the RSARB and, although present in some cells in the SAN periphery (examples highlighted by arrows in Figure 6B),...
it was absent from many (Figure 6B). This has important implications for pacemaking in the RSARB (Figure 1B).

**K⁺ Channels**

In the SAN, as in atrial muscle, there is a transient outward current, *I*_*to*. In the SAN periphery, *I*_*to* can result in an action potential with a spike and dome profile (Figure 7A). Three α subunits are responsible for *I*_*to* in the heart, Kv1.4, Kv4.2, and Kv4.3. Once again there was evidence of an apparent isoform switch: Kv1.4 mRNA was significantly less abundant in the SAN than in atrial muscle (Figure 7B), whereas the reverse was true of Kv4.2 mRNA (Figure 7C). The abundance of Kv4.3 mRNA tended to decline from atrial muscle to the SAN center, but not significantly so (Figure 7D). KChIP2 is a β-subunit for Kv4.2 and Kv4.3. The abundance of KChIP2 mRNA was significantly lower in the SAN periphery than in atrial muscle (Figure 7E). ISH showed a decrease in KChIP2 mRNA in the SAN periphery: Figure 8A shows that KChIP2 mRNA was abundant in the atrial muscle of the crista terminalis and in the SAN center, but was largely absent from the SAN periphery (see expanded view in Figure 8A).

*I*_*ur*, *I*_*r*, and *I*_*s* are the ultrarapid, rapid, and slow delayed rectifying K⁺ currents, respectively. There is a 4-AP-sensitive sustained outward current in rabbit SAN possibly corresponding to *I*_*ur*, *I*_*r*, and *I*_*s* are present in the rabbit SAN: activation during the action potential triggers repolarization and sets the MDP and slow deactivation after the action potential plays an important facilitatory role in the pacemaker potential. We investigated transcripts for Kv1.5, responsible for *I*_*ur*, ERG (Kv11.1) responsible for *I*_*r*, K,LQT1 (Kv7.1) responsible for *I*_*s*, and minK, a β subunit for K,LQT1. Whereas the abundance of Kv1.5 mRNA was similar in all tissues (Figure 7F), the abundance of K,LQT1 mRNA tended to be greatest in the SAN center (Figure 7H) and ERG mRNA was significantly more abundant in the SAN center than in atrial muscle as measured using a paired t-test (Figure 7G). Consistent with this, ISH showed that ERG mRNA was abundant in the SAN center, whereas labeling of ERG mRNA was substantially weaker in the atrial muscle of the crista terminalis (Figure 8B). No significant variation in the abundance of minK mRNA was detected (supplemental Table IV).

The background inward rectifier K⁺ current, *I*_*i*, is generated by Kir2 channels. The ACh-activated K⁺ current, *I*_*ACh*, is generated by a heterotetramer of Kir3.1 and Kir3.4. There were no significant differences in abundance of mRNAs for Kir2.1, Kir2.2 and Kir3.1 between tissues (supplemental Table IV). The ATP-sensitive K⁺ current, *I*_*ATP*, is generated by the α subunit, Kir6.2: and the β subunit, SUR2A; mRNA for both was significantly less abundant in the SAN than in atrial muscle (Figure 7, I and J).

**Summary**

Of the 30 transcripts studied by qPCR, there were significant differences between tissues in the case of 19 (63%). When grouping transcripts with similar expression profiles, cluster analysis (please see the online data supplement available at http://circres.ahajournals.org) identified 2 significantly differ-
ent clusters: transcripts that tended to increase (neurofila-
ment, Cx45, Na.1.1, Ca.1.3, HCN1, HCN4, K.4.2, ERG, K.LQT1, K.C.2.2, K.C.3.1) and those that tended to decrease
(ANP, Cx40, Cx43, RYR2, NCX1, SERCA2a, Na.1.5, Ca.1.2, K.1.4, K.4.3, KChIP2, K.C.1.5, minK, K.C.2.1, K.C.6.2, SUR2a) from atrial muscle to the SAN center. When grouping
tissues based on transcript abundance, cluster analysis
showed that the expression profiles of atrial muscle and the
SAN (center and periphery) were significantly different
(supplemental Figure III). Although overall transcript expres-
sion profiles of the center and periphery of the SAN were
similar (supplemental Figure III), there were significant
differences for individual transcripts between the center and
periphery of the SAN (RYR3, Cav1.3, HCN1 and HCN4;
Figure 1K and Figure 3, D through F). Furthermore, ISH
shows that there are differences between the center and
periphery of the SAN; Figure 8C summarizes data from ISH.
Whereas atrial muscle and the SAN center showed distinct
transcript profiles, the SAN periphery showed an intermedi-
ate profile (Figure 8C). The RSARB marks the most periph-
eral part of the SAN (Figure 1D) and it had the most
atrial-like transcript profile (Figure 8C).

Discussion
This study shows for the first time a complex variation in
expression of many ion channel transcripts from atrial muscle
to the periphery and center of the SAN.

Connexins
Cx43 forms medium conductance gap junction channels and
is the principal connexin isoform in the working myocardium.
qPCR showed that abundance of Cx43 mRNA was lower in
the SAN than atrial muscle (Figure 1H). ISH confirmed this,
but, in addition, showed that Cx43 mRNA was present in
some cells in the SAN periphery and, furthermore, Cx43
mRNA was abundant in the RSARB (Figure 2C). In rabbit,
the distribution of Cx43 protein is identical.5 Cx45 forms
small conductance gap junction channels. qPCR detected
Cx45 mRNA and showed no significant differences between
atrial muscle and the SAN (center and periphery) were significantly different
(supplemental Figure III). Although overall transcript expres-
sion profiles of the center and periphery of the SAN were
similar (supplemental Figure III), there were significant
differences for individual transcripts between the center and
periphery of the SAN (RYR3, Ca.1.3, HCN1 and HCN4;
Figure 1K and Figure 3, D through F). Furthermore, ISH
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eral part of the SAN (Figure 1D) and it had the most
atrial-like transcript profile (Figure 8C).

Discussion
This study shows for the first time a complex variation in
expression of many ion channel transcripts from atrial muscle
to the periphery and center of the SAN.
shown). Electrical coupling should be (1) weak in the SAN center to protect the SAN from the hyperpolarizing influence of atrial muscle; and (2) stronger in the SAN periphery to allow the SAN periphery to drive atrial muscle.4,6 Our findings are consistent with theoretical expectations: Cx40 mRNA was either weakly expressed or not expressed in all tissues; Cx43 mRNA was abundant in atrial muscle, present in the RSARB and SAN periphery, but absent in the SAN center; in the SAN center, Cx45 (and Cx30.2) mRNA was present.

Intracellular Ca²⁺ Handling Proteins

There was an apparent isoform switch from RYR2 to RYR3 from atrial muscle to the SAN (Figure 1, J and K). Consistent with this, we have previously shown that there is a decrease in RYR2 protein in the center of the rabbit SAN14 and, in mouse and rat heart, RYR3 mRNA has been shown to be expressed primarily in the cardiac conduction system.15 Intracellular Ca²⁺ release has been suggested to be involved in pacemaking in the rabbit SAN.7 The apparent isoform switch from RYR2 to RYR3 may, therefore, be functionally important. Differences in intracellular Ca²⁺ handling between the center and periphery of the rabbit SAN have been observed16 and this may again be related to the isoform switch. The abundance of NCX1 mRNA was significantly lower in the SAN periphery compared with the other tissue samples. This again could underlie differences in intracellular Ca²⁺ handling between the center and periphery of the rabbit SAN.16 There were no significant differences between atrial muscle and the SAN in abundance of mRNA for the Ca²⁺ handling protein, SERCA2a (supplemental Table IV).

$I_{Na}$ qPCR and ISH showed that mRNA for the cardiac Na⁺ channel isoform, Na,1.5, was abundant in atrial muscle, but absent in the SAN center (Figure 3B and Figure 4). We have shown a similar distribution of Na,1.5 protein in mouse SAN.8 ISH also showed that Na,1.5 mRNA was abundant in the RSARB and, in the SAN periphery, there were some cells with Na,1.5 mRNA (Figure 4). Functional studies are consistent with these findings: in rabbit, there is a progressive decrease in the upstroke velocity of the action potential (from \(\approx50\) to \(\approx2\) V/s) from the RSARB to the SAN center.4 TTX block of \(I_{Na}\) greatly slows the action potential upstroke in the RSARB of the rabbit to \(\approx5\) V/s; it also shortens the action potential and slows pacemaking.4 In contrast, it has no effect on the action potential in the center of the rabbit SAN.4 Furthermore, \(I_{Na}\) is recorded in putative peripheral, but not putative central, SAN cells.4 It is suggested that Na,1.5 in the SAN periphery is the important source of depolarizing current for the SAN to drive atrial muscle (see introduction).

$qPCR$ showed Na,1.1 mRNA to be present in both atrial muscle and the SAN (Figure 3A). ISH failed to generate labeling of Na,1.1 (data not shown). This suggests that the amount of Na,1.1 mRNA present is relatively low. In mouse, both Na,1.1 mRNA and protein have been shown to be present in SAN and block of Na,1.1 by nM concentrations of TTX (which have little effect on Na,1.5)
sustained outward current (possibly $I_{Kur}$) for which $I_{Kur}$ is of particular importance in the SAN - perhaps intracellular Ca$^{2+}$ is of particular importance in the RSARB.

$I_{K,ur}$

$K_{1.4}$ mRNA was more abundant in atrial muscle than in the SAN, whereas the reverse was true of $K_{4.2}$ mRNA (Figure 7, B and C). Although $K_{1.4}$ protein expression does not necessarily follow $K_{1.4}$ mRNA expression, in this case the findings are consistent with findings of functional studies: an antisense oligodeoxynucleotide directed against $K_{1.4}$ reduced $I_{Kur}$ in atrial cells. In rabbit, recovery of $I_{Kur}$ from inactivation is slower in atrial cells (time constants, 1.5 and 6.7 s) than in SAN cells (time constant, 45 ms). This is consistent with the primary importance of $K_{1.4}$ in atrial muscle and $K_{4}$ channels in the SAN, because recovery of $K_{1.4}$ is markedly slower than that of $K_{4}$ channels.

Furthermore, the $K_{4.2}$ channel has been cloned from rabbit SAN and shows similar properties to those of $I_{Kur}$ in rabbit SAN cells. $K_{4.3}$ mRNA did not vary between tissues (Figure 7D). This is not unexpected, because antisense oligodeoxynucleotide directed against $K_{4.3}$ reduced $I_{Kur}$ in rabbit atrial cells and recovery of $K_{4.3}$ from inactivation is fast (as is recovery from inactivation in SAN). $K_{ChIP2}$ mRNA was present in the SAN center and in atrial muscle, but not in the SAN periphery (Figure 8A). $K_{ChIP2}$ closely associates with and is important for expression of $K_{4.2}$ and $K_{4.3}$, but not $K_{1.4}$. The presence of $K_{ChIP2}$ in the SAN center and atrial muscle may be important for the expression of $K_{4.2}$ and $K_{4.3}$ in the SAN center and $K_{4.3}$ in atrial muscle. The significance of the absence of $K_{ChIP2}$ mRNA from the SAN periphery is unclear. The action potential is longest at the leading pacemaker site (SAN center) and the action potential duration decreases down the conduction pathway (ie, action potential duration: SAN center $>$ SAN periphery $>$ atrial muscle). This effect can be seen in Figure 1, A through C, and it is possibly the result of $I_{Kur}$, because, in rabbit, the density of $I_{Kur}$ in atrial muscle is approximately double that in the SAN.

Delayed Rectifier $K^+$ Currents

$K_{1.5}$ mRNA was detected in atrial muscle and the SAN (Figure 7F). Previously, we have shown, in guinea-pig, the presence of $K_{1.5}$ protein in the SAN and surrounding atrial muscle and, in rabbit, the presence of 4-AP sensitive sustained outward current (possibly $I_{Kur}$ for which $K_{1.5}$ is responsible). Consistent with the presence of $I_{Kur}$ and $I_{Ks}$, in rabbit SAN, ERG, KvLQT1, and minK mRNA was de-
ected in the SAN (Figure 7, G and H, and 8B; supplemental Table IV). The abundance of both ERG and KvLQT1 mRNA tended to be higher in the SAN center than in atrial muscle (Figure 7, G and H, and 8B). Perhaps this is because, in atrial muscle, $I_{\text{K,1}}$ generates the resting potential and $I_{\text{K,r}}$ and $I_{\text{K,s}}$ control APD, whereas in the SAN $I_{\text{K,r}}$ and $I_{\text{K,s}}$ alone must generate the MDP as well as control the APD. Electrophysiological experiments suggest that the density of $I_{\text{K,r}}$ and $I_{\text{K,s}}$ is greater in the periphery than in the center of the rabbit SAN.\textsuperscript{4,28} In the present study, no evidence of such a difference was observed, but a difference in current density could be the result of control at the translational level.

**Inward Rectifier K\textsuperscript{+} Currents**

$I_{\text{K,1}}$ is responsible for the resting potential in the working myocardium. $I_{\text{K,1}}$ is absent from the SAN;\textsuperscript{1} this facilitates pacemaking and explains why the MDP in the SAN center is more positive than in atrial muscle (Figure 1, A and C). Therefore, surprisingly, the abundance of $K_{\text{r},2.1}$ and $K_{\text{r},2.2}$ mRNA was not significantly different between atrial muscle and the SAN (supplemental Table IV). This could be because $K_{\text{r},2.3}$ is a major $I_{\text{K,1}}$ isoform in atria,\textsuperscript{29} or $I_{\text{K,1}}$ is controlled at the translational level. $K_{\text{r},3.1}$ mRNA was present in atrial muscle and the SAN and there were no significant differences between tissues (supplemental Table IV). This is expected,
because ACh via \( I_{K,ACH} \) affects both atrial muscle and the rabbit SAN.\(^3\) We have previously shown \( K_{r,3.1} \) protein in both tissues in guinea-pig and rat.\(^{30}\) The abundance of both \( K_{r,6.2} \) and \( SUR2A \) mRNA was significantly lower in the SAN than in the surrounding atrial muscle (Figure 7, I and J). Again the presence of the 2 transcripts in both tissues is expected, because metabolic inhibition results in the activation of \( I_{K,ATP} \) in both atrial muscle and the rabbit SAN.\(^31\) If the lower abundance of the transcripts in the SAN is translated into a lower density of \( I_{K,ATP} \), it is possible that the density of \( I_{K,ATP} \) does not need to be as high as in atrial muscle to exert a physiological effect, because of the high input resistance of SAN cells.

For further discussion of the mechanistic implications of the results from this study see the online data supplement available at http://circres.ahajournals.org.

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Disclosures
None.

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Differential Expression of Ion Channel Transcripts in Atrial Muscle and Sinoatrial Node in Rabbit

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Ion Channel Transcripts in the Sinoatrial Node

DATA SUPPLEMENT

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R. Billeter

Methods

Tissue preparation

Intact sinoatrial node (SAN) preparations were dissected from the hearts of male New Zealand White rabbits (1.5-2.5 kg) as previously described\(^1\) for RNA isolation (n=7) or \textit{in situ} hybridisation (n=3). The rabbits were killed humanely according to the United Kingdom Animals (Scientific Procedures) Act, 1986. For RNA isolation, tissue samples (1-1.5 x 1-1.5 mm) were taken from the centre and periphery of the SAN as well as the right atrial free wall. The samples were taken approximately at the level of the leading pacemaker site in the SAN (at the level of the main branch from the crista terminalis). In the case of the sample from the periphery of the SAN, the underlying atrial muscle was removed (the periphery of the SAN lies on the endocardial surface of the crista terminalis). The samples were immersed in a drop of freezing medium (OCT, BDH) and frozen in liquid N\(_2\). The tissue was subsequently cut into 20 µm sections on a cryostat for RNA isolation. The preparations used for \textit{in situ} hybridisation were snap frozen in OCT before being immersed in isopentane cooled by liquid N\(_2\) (-162°C) and stored at -80°C. The tissue was subsequently cut into 12 µm sections on a cryostat; the sections were stored for up to several months at -80 °C.

RNA isolation and generation of cDNA

Total RNA was isolated from the frozen 20 µm sections of SAN (see above) with a modified Qiagen muscle RNA extraction procedure.\(^2\) The isolated RNA (and resulting cDNA) samples from different rabbits were kept separate, i.e. the samples were not pooled. The RNA was ethanol precipitated and the pellets dissolved in 6-20 µl (depending on the size of the samples) of RNAse free water. The RNA concentration was then estimated with the RiboGreen assay (Molecular Probes). In addition, the integrity of seven randomly picked RNA samples was confirmed on a formaldehyde-agarose gel stained with ethidium bromide. 150 ng of total RNA from each sample was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) in a 20 µl reaction according to the manufacturer’s instructions, using random hexamer priming. Aliquots of the resulting cDNA were diluted 10-fold in water for direct use in PCR.
**Quantitative PCR and primers**

The relative content of selected cDNA fragments was determined with quantitative PCR (qPCR) using either a LightCycler (Roche) and detection with SyBr green in 10 μl reactions (primer sequences and annealing temperatures are given in Table S1) or with an ABI Prism 7700 (Applied Biosystems) and detection with a TaqMan probe (6-FAM/TAMARA) in 25 μl reactions (primer and probe sequences are given in Table S2). All runs were 40 cycles in duration. For all transcripts and samples, at least three separate measurements were made with 1 μl aliquots of each cDNA sample. For quantification, we used the “fit points” method of the preinstalled software on the Roche LightCycler. An average atrial muscle CT value for each transcript was used as a reference standard. For each run, ratios relative to this reference standard were determined based on the respective delta CTs and an average efficiency (determined graphically, SD 2–3 %). These ratios were then averaged over all three runs and related to the average content of 28S cDNA in each sample to correct for variations in input RNA. The expression levels were represented graphically as a percentage of the atrial muscle expression. *In situ* hybridisation confirmed that 28S was uniformly distributed throughout the SAN (Fig. S1).

The number of mRNA molecules for smooth muscle and cardiac myosin heavy chain was estimated by performing the qPCR with a standard curve of known amounts of smooth muscle and cardiac myosin heavy chain cDNA.

**Accuracy of sampling for qPCR**

qPCR measures the abundance of transcripts in the tissue sampled and, therefore, the nature of the tissue sampled is important. It is possible that the SAN samples used for qPCR were contaminated by atrial cells. To estimate the percentage contamination of the SAN samples by atrial cells, we used ANP as a marker of atrial cells (it is assumed that no ANP is expressed in SAN cells); the maximal contamination of the peripheral and central SAN samples was estimated to be 11 and 6 %, respectively (Fig. 1E). To confirm that there was little contamination, neurofilament was used as a marker of SAN cells. There was a greater than 50× enrichment of neurofilament mRNA in the SAN samples as compared to the atrial muscle sample (Fig. 1F). It is concluded that there is little contamination of the SAN samples by atrial muscle. Another possibility is that the tissue sampled from the SAN periphery was contaminated by central SAN tissue and vice versa. *In situ* hybridisation showed KChIP2 to be absent in the SAN periphery, but present in the atrial muscle and SAN centre (Fig. 8A), and a similar pattern of expression was seen with qPCR (Fig. 7E); this...
suggests that there was minimal such contamination. To assess the level of contamination of the tissue samples by vascular tissue, we compared the expression of smooth muscle myosin heavy chain (vascular marker) and cardiac myosin heavy chain (myocyte marker). We estimated that in all samples the number of smooth muscle myosin heavy chain mRNA molecules was <1 % of the number of cardiac myosin heavy chain mRNA molecules (Fig. S4); we conclude, therefore, that vascular contamination was minimal. It cannot be ruled out that there was significant contamination of the tissue samples from other sources (e.g. neuronal tissue); however, in situ hybridisation shows unequivocally myocyte (versus non-myocyte) mRNA expression and, for all transcripts for which both qPCR and in situ hybridisation were used (with the exception of Cx40), the myocyte expression of the transcript matched the expression pattern revealed by qPCR.

**In situ hybridisation**

For in situ hybridisation, we used digoxigenin (DIG) labelled cRNA probes. The probes are given in Table S3. cDNA fragments for ion channels etc. were isolated from rabbit heart or brain cDNA by PCR and linked to SP6 or T7 promoters either by subcloning into pGEM-T Easy (Promega) or with the Lig n'Scribe system (Ambion). cRNA probes were synthesised by in vitro transcription from SP6 or T7 promoters using the Mega Script kit (Ambion), at a ratio of ~4 unlabelled UTPs (Ambion) to 1 DIG-UTP (Roche).

The protocol for in situ hybridisation was adapted from the one of Braissant et al.\(^4\) the 12 µm sections (see above) were fixed in 4 % paraformaldehyde/phosphate buffer saline (PBS) for 10 min at room temperature and incubated twice for 10 min in acetic anhydride (0.25 %) in 0.1 M triethanolamine (pH 8). The sections were brought to boil in a microwave oven at 800 W in 10 mM sodium citrate (pH 6) and kept boiling for 2.5 min, before letting the solution cool at room temperature to ~70°C. This step was repeated three times. Thereafter, the sections were equilibrated in 5x saline-sodium citrate buffer (SSC) at room temperature for 15 min before a 2 h prehybridisation at 58°C in 50 % formamide, 5x SSC and 40 µg/ml herring sperm DNA. Sections were then hybridised for 60 h at 58°C, with 0.3 ng/µl of DIG-labelled cRNA probes in 50 % formamide, 5x SSC and 40 µl/ml herring sperm DNA. Some of the mRNAs were hybridised with more than one probe simultaneously (Table S3), which resulted in increased sensitivity of the procedure. In this case, each of the probes was used at 0.3 ng/µl concentration. Antisense (complementary to a mRNA fragment) and sense (identical to the mRNA fragment) cRNA probes were used. The antisense probe detected the mRNA, whereas the sense probe served as a negative control. In all cases, the sense
probes did not produce labelling. After hybridisation, the sections were washed for 30 min in 2x SSC at room temperature, followed by two 1 h washes at 65°C, the first in 2x SSC, the second in 0.1x SSC. This was followed by RNAse treatment with 0.5 µg/ml RNAse A (Sigma) for 30 min in order to degrade unbound probe. The sections were then washed twice in 2x SSC for 5 min before another stringent wash in 0.5x SSC and 20 % formamide at 60°C for 10 min. The sections were washed again twice in 2x SSC for 5 min. They were then adjusted for 5 min in DIG buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), followed by DIG buffer 2 (same as DIG buffer 1 with 1 % blocking reagent; Roche) for 1 h, before the application of anti-DIG alkaline phosphatase coupled Fab (Roche), in DIG buffer 2 at a dilution of 1:5000 for 2 h. Unbound antibody was subsequently washed away by two 15 min washes in DIG buffer 1. Before the visualisation of the bound phosphatase, the sections were adjusted for 5 min in DIG buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH 9.5). They were then incubated for 8-56 h in DIG buffer 3 supplemented with 10 % polyvinyl alcohol (PVA), 5 and 0.4 mM NBT (4-nitro-blue-tetrazolium-chloride, Roche) and 0.4 mM X-phosphate (5-bromo-4-chloro-3-indolyl-phosphate, Roche). The staining reaction was stopped with three 15 min washes in TE buffer (0.1 M Tris, 1 mM EDTA, pH 8.0). The sections were embedded in Kaiser’s gelatine.

Histology

Histology was performed using a previously described method. 6

Statistical analysis

Means ± SEM (number of rabbits) are presented. Significant differences in transcript abundance in different tissues (qPCR) were identified using one-way ANOVA and paired t-tests. Statistical comparisons for individual variables were carried out by Student-Newman-Keuls method. A difference was considered significant if $P < 0.05$.

Cluster analysis

Each of the seven rabbit samples was treated as a biological replicate, assigning a single representative expression value for each gene $i$ as the mean of all replicate measurements over each tissue $j$. Missing values were ignored (of the 630 data points only nine were missing; the missing data are unlikely to have a significant effect on the accuracy of the cluster analysis). Clusters were generated from the qPCR in two ways: (i) by clustering the three tissues (atrial muscle and periphery and centre of the SAN) based on the expression of the 30 genes, in order to cluster the tissues with similar expression patterns and (ii) by comparing the expression of the 30 genes across the three tissues and grouping genes with similar expression.
patterns. Vectors of expression values were compared and clustered using the Pearson correlation coefficient as a metric. Three different clustering algorithms were compared to gain the most information from the given data: Cluster Affinity Search Technique (CAST),\(^7\) average-linkage hierarchical (ALH)\(^8\) and fuzzy c-means (FCM).\(^9\) Random clustering (RC), in which \(k\) random clusters are obtained by placing genes randomly into separate bins,\(^10\) was used as a benchmark to compare the quality of the clusters obtained with the other methods. As a graph-based clustering algorithm, CAST intrinsically finds the “optimal” number of clusters \((n_c)\) after setting a threshold (0.8 in this analysis).\(^7\) Conversely, for both ALH and FCM (using a fuzziness degree of 1.6), the optimal partitioning of clusters had to be determined by other means. To find the best clustering of the data when using ALH and FCM, the Bayesian Information Criterion (BIC)\(^11\) was used. Allowing for a minimum of two or maximum of 10 clusters, the BIC index was calculated for each value of \(n_c\) for both the ALH and FCM methods. A plot of BIC versus \(n_c\) indicates appropriate values of \(n_c\) at a point of inflection at the lowest value of BIC. Where a clear inflection point was obtained, the corresponding \(n_c\) was used to obtain the clusters; otherwise different \(n_c\) values were compared by the quality of the resulting clusters. The quality of clustering, measured by the compactness of each cluster and its independence from other clusters, was calculated using Dunn’s validity index (DVI),\(^12\) where a larger DVI reports better quality clusters. Along with comparing different \(n_c\) for a particular clustering method, DVI was also used to compare clustering results between methods, with the quality of random clustering implied by the mean DVI after 500 simulations. Through the use of DVI, the best clustering of the data by any method was selected as the best representation of the data.

When grouping transcripts with similar expression profiles, ALH and FCM identified two clusters in the qPCR data. Based on a DVI score of 0.022 (as compared to a DVI score of 0.002 obtained by random clustering), the ALH clusters were found to be of better quality. These clusters are: (i) \(\text{Na}^+\text{K}^+\) pump \(\alpha_1\) isoform, neurofilament, Cx45, Na,1.1, Ca,1.3, HCN1, HCN4, K,4.2, ERG, K\(_{\text{LQT1}}\), K\(_{\text{ir2.2}}\) and K\(_{\text{ir3.1}}\) (transcripts that tended to increase from the atrial muscle to the SAN centre) and (ii) GAPDH, ANP, Cx40, Cx43, RYR2, NCX1, SERCA2a, Na,1.5, Ca,1.2, K,1.4, K,4.3, KChIP2, K,1.5, minK, K\(_{\text{ir2.1}}\), K\(_{\text{ir6.2}}\) and SUR2a (transcripts that tended to decrease from the atrial muscle to the SAN centre). When grouping tissues based on transcript abundance, all clustering methods used (CAST, FCM and ALH) identified two clusters in the qPCR data. The DVI score for both FCM and ALH was 1.87, higher than the mean DVI score of 0.89.
obtained by random clustering. The two clusters produced are (i) atrial muscle and (ii) SAN (centre and periphery). Fig. S3 shows the expression profile of the two clusters.

**Mechanistic insights**

The results obtained provide an insight into the gene-structure-function relationships of the SAN. This is important for understanding sick sinus syndrome (dysfunction of the SAN that can necessitate the fitting of an artificial pacemaker): familial sick sinus syndrome,\textsuperscript{e.g.13} age-related sick sinus syndrome\textsuperscript{14, 15} and heart failure-related sick sinus syndrome\textsuperscript{16} have all been linked to sarcolemmal and gap junction ion channels. For example, familial sick sinus syndrome in a number of families has been linked to loss-of-function mutations in SCN5A (the gene responsible for Na\textsubscript{v}1.5) and age-related sick sinus syndrome may also be linked to Na\textsubscript{v}1.5\textsuperscript{15} and it is, therefore, paradoxical that Na\textsubscript{v}1.5 is absent from the SAN centre (Fig. 4). Na\textsubscript{v}1.5, however, is present in the SAN periphery (Fig. 4) and we suggest that the Na\textsubscript{v}1.5-related SAN dysfunction is the result of the loss of I\textsubscript{Na} from the SAN periphery and the consequent inability of the SAN to drive the atrial muscle. In an attempt to generate a biopacemaker in the working myocardium, K\textsubscript{v}2.1 has been knocked out or HCN has been overexpressed.\textsuperscript{e.g.17, 18} However, the present study shows that there are many changes in gene expression between the working myocardium (atrial muscle) and the SAN and it is possible that a successful biopacemaker may depend on the manipulation of the expression of more than one gene.

**References**


**Table and figure legends**
Table S1. Primers and PCR conditions used with the LightCycler. All primers were designed using rabbit sequences.

Table S2. Primers, TaqMan probes and PCR conditions used with the ABI Prism 7700. All primers were designed using rabbit sequences.

Table S3. Primers and PCR conditions used to generate DNA template for cRNA probe synthesis. All primers were designed using rabbit sequences.

Table S4. Abundance of various transcripts (as measured by qPCR) that were uniformly distributed. Means±SEM (n=7) given.

Figure S1. Distribution of 28S and GAPDH mRNA as detected by in situ hybridisation in atrial muscle and SAN centre. Labelling of 28S and GAPDH mRNA (abundant in all cases) appears as a dark signal.

Figure S2. Distribution of Cx40 mRNA in the atrial muscle of the crista terminalis, SAN periphery, SAN centre, and the atrial muscle towards the interatrial septum. The red arrows point to specific labelling of Cx40 mRNA in atrial myocytes and the yellow arrow points to specific labelling in endothelial cells of a blood vessel.

Figure S3. Cluster analysis. Top, transcript expression profile of the atrial muscle. Bottom, transcript expression profile of the periphery and centre of the SAN.

Figure S4. Comparison of the number of cardiac and smooth muscle myosin heavy chain mRNA molecules present in atrial muscle, SAN periphery and SAN centre tissue samples. Note log scale on the ordinate.
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<td>(C,T)GT(G,C)GAGGATGA(A,G)TTTGA(A,G)CT(A,C,G,T)T</td>
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<td>Transcript</td>
<td>% relative abundance of mRNA per 28S</td>
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<tr>
<td>--------------------------</td>
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</tr>
<tr>
<td></td>
<td>Atrial muscle</td>
<td>SAN periphery</td>
</tr>
<tr>
<td>GAPDH</td>
<td>100.0 ± 12.5</td>
<td>69.7 ± 10.1</td>
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<tr>
<td>Na-K pump (α1)</td>
<td>100.0 ± 33.8</td>
<td>101.2 ± 35.1</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>100.0 ± 25.6</td>
<td>46.7 ± 12.3</td>
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<tr>
<td>minK</td>
<td>100.0 ± 15.7</td>
<td>76.0 ± 18.7</td>
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<tr>
<td>Kir2.1</td>
<td>100.0 ± 14.7</td>
<td>103.1±30.7</td>
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<td>Kir2.2</td>
<td>100.0 ± 16.1</td>
<td>124.2 ± 23.2</td>
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<tr>
<td>Kir3.1</td>
<td>100.0 ± 25.3</td>
<td>212.4 ± 99.4</td>
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Fig. S1

28s

Right atrium

SA node centre

GAPDH

Right atrium

SA node centre

50 μm
Fig. S3

Cluster 1

- Loge(Relative abundance of mRNA)

- Transcript

Cluster 2

- Loge(Relative abundance of mRNA)

- Transcript
Fig. S4

Number of Myosin Heavy Chain Molecules

Number of mRNA Molecules

Atrial muscle  SAN periphery  SAN centre

Cardiac Myosin Heavy Chain
Smooth Muscle Myosin Heavy Chain