Distribution and Functional Role of Inositol 1,4,5-trisphosphate Receptors in Mouse Sinoatrial Node

Yue-Kun Ju, Jie Liu, Bon Hyang Lee, Donna Lai, Elizabeth A. Woodcock, Ming Lei, Mark B. Cannell, David G. Allen

Rationale: Inositol 1,4,5-trisphosphate receptors (IP_iRs) have been implicated in the generation of arrhythmias and cardiac muscle nuclear signaling. However, in the mammalian sinoatrial node (SAN), where the heart beat originates, the expression and functional activity of IP_iRs have not been investigated.

Objectives: To determine whether SAN express IP_iRs and which isoforms are present. To examine the response of the SAN to IP_iRs analogues and agonists and the potential role played by IP_iRs in cardiac pacemaking.

Methods and Results: The expression and distribution of IP_iRs, were studied by reverse-transcription polymerase chain reaction, Western blotting, and immunolabeling. IP_iRs in mucous SAN were measured with Ca^{2+}-sensitive fluorescent dyes. We found that although the entire SAN expressed three IP_iR mRNA isoforms, IP_iR2 was the predominant protein isoform detected by Western blot using the protein extracts from the SAN, AV node, and atrial tissue. Immunohistochemistry studies also showed that IP_iR2 was expressed in the central SAN region. Studies using isolated single pacemakers revealed that IP_iR2 (but not IP_iR1) was located with a similar distribution to the sarcoplasmic reticulum marker protein SERCA2a with some labeling adjacent to the surface membrane. The application of membrane-permeable IP_i3 (IP_i3-butyryloxymethyl ester) increased Ca^{2+} spark frequency and the pacemaker firing rate in single isolated pacemaker cells. In intact SAN preparations, endothelin-1-IP_iR agonist and IP_i3-butyryloxymethyl ester both increased intracellular Ca^{2+} and the pacemaker firing rate, whereas the IP_iR antagonist, 2-aminoethoxydiphenyl borate decreased Ca^{2+} and the firing rate. Both of these effects were absent in the SAN from transgenic IP_iR2 knockout mice.

Conclusions: This study provides new evidence that functional IP_iR2s are expressed in the mouse SAN and could serve as an additional Ca^{2+}-dependent mechanism in modulating cardiac pacemaker activity as well as other Ca^{2+}-dependent processes. (Circ Res. 2011;109:00:00-00.)

Key Words: pacemaker activity □ sinoatrial node

For many years, cardiac pacemaker activity was believed to be determined solely by the electric activity of voltage-dependent membrane currents, particularly the hyperpolarization-activated cation current (I_h). However, the discovery that ryanodine, an antagonist of sarcoplasmic reticulum (SR) Ca^{2+} release channels (ryanodine receptors), slows the heart rate suggested that Ca^{2+} release from the SR also plays an important role in determining the heart rate.

Although ryanodine receptors (RyRs) are the main source of SR Ca^{2+} release, another class of Ca^{2+} release channels, inositol 1,4,5-trisphosphate receptors (IP_iRs), have been found in cardiac SR membranes. Although the expression level of IP_iRs is much lower than RyRs, Ca^{2+} release from IP_iRs modulates the excitation–contraction coupling in both atrial and ventricular myocytes, and several lines of evidence suggest that arrhythmogenic activity in the atria may be associated with increased IP_i3 and IP_iR expression. In addition, new important roles for IP_iRs in controlling nuclear transcription factors are emerging.

There are three IP_iR isoforms in mammalian cells: IP_iR1, IP_iR2, and IP_iR3. Among these, IP_iR2 has the highest affinity for IP_i3 and is the predominant IP_iR isoform expressed in ventricular and atrial myocytes. Whereas IP_iR1 is the dominant isoform present in bovine Purkinje
myocytes, little IP3R1 has been found in the sinoatrial node (SAN). Thus, both the expression and possible role of IP3Rs in the SAN remain unclear. Nevertheless, it is notable that spontaneous pacemaker activity in embryonic stem cell-derived cardiomyocytes is strongly dependent on IP3R-mediated Ca2+ release. In this study, we have investigated the expression and distribution of IP3Rs in the mouse SAN at both mRNA and protein levels and their involvement in the modulation of a heart rate.

Materials and Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Western Blot Analysis
Total protein extracts were prepared from homogenates of the SAN, AV node, atria, and brain tissue by using a mammalian cell lysis kit (Sigma). Protein concentrations were determined using a microBCA protein assay kit (Thermo Scientific). Detection of IP3R1 and IP3R2 was performed using a rabbit polyclonal antibody to IP3R1 (1:1000; ABR) or a rabbit polyclonal antibody against IP3R2 (1:500, a gift from Dr Ju Chen’s laboratory), as previously described. Immunodetection of α-actinin was performed using a mouse monoclonal antibody to sarcomeric α-actinin (1:1000; Sigma). After transfer, membranes were treated with Misher antibody extender solution NC (Pierce Biotechnology). Horseradish peroxidase–conjugated antirabbit Ig (1:16 000; Sigma) or antimouse Ig (1:3000; Invitrogen) was used as secondary antibodies, respectively. For detecting IP3R, the primary and secondary antibodies were also treated with the SignalBoost immunoreactions enhancer kit (Calbiochem).

Immunohistochemistry
Whole SAN preparations as well as isolated single pacemaker cells were labeled with primary antibodies, including IP3R1, IP3R2, connexin-43 (Cx43), HCN4, RyR2, and SR Ca2+-ATPase (SERCA2a) using established methods.

Ca2+ Measurements
Intact SANs were isolated from the right atria as described previously. The intact SANs were loaded with the membrane-permeable fluorescent Ca2+ indicator, indo-1-AM (10 μmol/L), in Tyrode solution containing 100 μmol/L Ca2+ for at least 2 hours. The SANs were then washed in Tyrode solution with 1.8 mmol/L Ca2+ containing probenecid (0.5 mmol/L) for >30 minutes at 35°C. The probenecid, an organic anion carrier inhibitor, was added to reduce leakage of the fluorescent dye. Fluorescent signals were recorded from a restricted region of the SAN area by use of a rectangular field stop. The indo-1 ratio (R - Rmin)/(Rmax - R), which is linearly related to [Ca2+2], was calculated. In some experiments, confocal microscopy was used to collect Ca2+ images of the intact SANs loaded with fluo-4AM (10 μmol/L) or isolated single pacemaker cells loaded with fluo-4AM (2 μmol/L) for 15 minutes. Single pacemaker cells were enzymatically isolated from the SAN region of the mouse heart according to the method described previously.

Statistics
Data are expressed as mean±SEM, with the number of preparations expressed as n. Statistical tests were either Student paired or unpaired t tests, and P<0.05 was taken as the level of statistical significance.

Results
Expression and Distribution of IP3R Isoforms in the Mouse SAN
We first examined mRNA expression for IP3R, Hcn4, and RyR2 across different regions of the mouse heart, including the central SAN, peripheral SAN, AV node, atria, and ventricle. All three IP3R mRNA isoforms were expressed in the SAN and other regions of the heart. HCN4 expression was highest in the central SAN and showed progressive reduction in the peripheral SAN, AV node, atria, and ventricle (Online Figure I, A), in agreement with previous studies. The mRNA expression of the 3 types of IP3Rs in pacemaker cells was also confirmed in preliminary single cell reverse-transcription polymerase chain reaction experiments using isolated pacemaker cells (data not shown).

Western blot analysis was used to examine protein expression in SAN. Protein extracts from brain tissue showed a prominent single band >250 kDa after incuba-
tion with an anti-IP3R1 antibody. However, only a weak reaction to the IP3R1 antibody was found in cardiac pacemaker tissue, including the SAN and AV node (Figure 1, top). In contrast to IP3R1, a single band migrating >250 kDa was detected in protein extracts from different regions of the pacemaker tissue, as well as atrial, but not brain tissue, when incubated with the IP3R2 antibody (Figure 1, middle). Immunodetection of α-actinin was performed on samples to normalize the protein loading of heart samples (Figure 1, lower).

These results demonstrate that mouse pacemaker tissue predominantly expresses type 2 IP3Rs, similar to the other regions of the heart. Antibodies against IP3Rs showed a single band at the predicted molecular weight for mouse tissue and also demonstrated the specificity of the antibodies (http://www.phosphosite.org/proteinAction.do?id=3973 &showAllSites=true).

To determine the distribution of IP3R protein isoforms in the SAN, whole-mount SANs were colabeled with the Cx43 antibody (green) and either IP3R1 or IP3R2 antibodies (Figure 2, red). Cx43 labeling was used to distinguish the central SAN from the peripheral SAN. We used confocal image tiling (Zeiss LSM510) to image the entire intact SAN region at high resolution (Figure 2A, B). We found weak labeling of IP3R1 in the central SAN, identified by the absence of Cx43 (Figure 2A). IP3R1 labeling appeared in the peripheral SAN, especially in the interatrial septum, which also showed strong expression of Cx43. At higher resolution, we found that some of the IP3R1 labeling in the interatrial septum appeared to be within intracardiac neurons (Online Figure II). In contrast, the entire SAN, including the central and peripheral SAN and the surrounding atrial tissue, was uniformly labeled with the IP3R2 antibody (Figure 2B, red).

The SAN is a multicellular tissue; therefore, to distinguish the pacemaker cells from other cell types such as endothelial cells and fibroblasts, the HCN4 antibody was used as a marker for pacemaker cells. Positive HCN4 labeling identified the central SAN as shown in Figures 2C and 2D. Very weak IP3R1 labeling was found in the HCN4-positive area (Figure 2C). In contrast, there was substantial IP3R2 labeling in this region and also some colabeling with HCN4-positive cells (Figure 2D, yellow). To further confirm that pacemaker cells express IP3R2 receptors, isolated single pacemaker cells were studied. Figure 3A shows that HCN4 positive pacemaker cells also show positive labeling for IP3R2. However, no IP3R2 immunoreaction was detected in single pacemaker cells that were isolated from IP3R2 KO mice (Figure 3B) or whole-mount SAN preparation (Supplemental Figure III), demonstrating antibody specificity. HCN4 labeling was mainly found along the surface membrane, as might be expected for a sarcolemmal membrane channel protein (Figure 3Aa, B). However, it is notable that some of the IP3R2 labeling also appeared to be located near the sarcolemmal membrane (Figure 3Ab). There was even some colocalization (Figure 3Ac, yellow) between IP3R2 and HCN4.
and HCN4, suggesting a near sarcolemmal localization for these receptors. Negative controls using second antibodies only were shown in (Online Figure IV).

**Distribution of SR and Ca\(^{2+}\) Release Channels in Isolated Pacemaker Cells**

To further explore the functional significance of the spatial distribution of IP\(_3\)Rs, we examined colocalization with the SR. SERCA2a is the primary isoform of SERCA expressed in cardiac tissue and is responsible for maintaining the Ca\(^{2+}\) gradient across the cardiac SR membrane.\(^{29}\) To establish the location of the SR in single pacemaker cells, we first used the anti-SERCA2a antibody as a SR marker. In the majority of isolated single pacemaker cells, SERCA2a staining showed clear sarcomeric labeling patterns (Figure 4A, C). Whereas pacemaker cells have a poorly developed t-tubule system, contractile machinery is organized in sarcomeres.\(^{30}\) Therefore, such sarcomeric labeling patterns suggest that SR in pacemaker cells might be closely associated with contractile proteins. Similar SR location was also found in toad pacemaker cells.\(^{31}\) This hypothesis was confirmed by using the Z-line marker α-actinin (data not shown). Some of the IP\(_3\)R2s are clearly colocalized with SERCA2a, especially close to the sarcolemma (Figure 4A, arrow, yellow). Within the cytoplasm, the distribution of IP\(_3\)R2s seemed quite scattered (Figure 4, red) but also followed the regular sarcomeric pattern of SERCA2a labeling. We found increased SERCA2a labeling was present at points of contact between two adjacent pacemaker cells (Figure 4A, green). This result is consistent with an early electron microscopy study that showed some SR was coupled to the sarcolemma in rat SAN cells.\(^{30}\)

Subsarcolemmal SR in pacemaker cells can explain why HCN4 and IP\(_3\)R2 appear to be colocalized along the sarcolemma (Figure 3Ac), but how does this relate to the distribution of RyR2 (the major Ca\(^{2+}\) release channel in cardiac cells)? Figure 4B shows a single pacemaker cell labeled with the RyR2 antibody (green). RyR2 labeling also shows a repeated sarcomeric pattern representing the distribution of SR in the cell, similar to the SERCA2a labeling pattern described (Figure 4A). IP\(_3\)R2s were frequently detected close to the sarcolemma, where some colocalization with RyR2 was also seen (Figure 4B, yellow). Again, no IP\(_3\)R1 labeling was apparent, although IP\(_3\)R1 labeling (red) was present in cell debris (Figure 4C). These immunohistochemistry data suggested that in pacemaker cells, the RyR is more or less uniformly distributed within SR with IP\(_3\)R2 having a similar distribution, includ-
ing near subsarcolemmal regions, to reports of other atrial cells.\textsuperscript{18}

To determine whether the expressed IP\textsubscript{3}R2s in pacemaker cells could function as Ca\textsuperscript{2+} release channels, we studied Ca\textsuperscript{2+} sparks, which are localized SR Ca\textsuperscript{2+} release events.\textsuperscript{32} When single pacemaker cells were loaded with the Ca\textsuperscript{2+} indicator fluo-4, Ca\textsuperscript{2+} sparks were observed in quiescent pacemaker cells, because such local Ca\textsuperscript{2+} release events do not require membrane potential depolarization.\textsuperscript{33} Ca\textsuperscript{2+} spark frequency was low under control conditions (Figure 5A). The application of a membrane-permeant IP\textsubscript{3} (IP\textsubscript{3}-butyryloxyethyl ester [IP\textsubscript{3}-BM]) increased spark frequency as shown in Figure 5B. The spark frequency increased by 2.86±1.08-times the control (n=7; P=0.01). In addition, the increased Ca\textsuperscript{2+} spark frequency induced by IP\textsubscript{3}-BM generally appeared close to the cell surface. Figure 5C shows a three-dimensional image that was derived from Figure 5B. The scan line position is indicated in the drawing on the right. There are fewer and less bright sparks in the middle of the cell, as shown in Figure 5D, when the scan line was moved in that position. The localization of Ca\textsuperscript{2+} sparks induced by the membrane permeable IP\textsubscript{3}-BM supports the immunohistochemistry described and suggests that the active IP\textsubscript{3}R sites are close to the sarcolemma. Because of this spatial distribution, Ca\textsuperscript{2+} release from IP\textsubscript{3}R2s could impact sarcolemmal ionic channels and exchangers.

\textbf{Ca\textsuperscript{2+} Release From IP\textsubscript{3}Rs Influence Pacemaker Function}

To examine whether the pacemaker activity is influenced by IP\textsubscript{3}Rs, the effect of IP\textsubscript{3}-BM on the spontaneous firing of isolated single pacemaker cells was examined. Using the Ca\textsuperscript{2+} indicator fluo-4, Ca\textsuperscript{2+} transients associated with spontaneous action potentials were recorded using confocal microscopy (Figure 6A). The spontaneous firing rate and amplitude of Ca\textsuperscript{2+} transients were determined by using XT scans in which laser scans along a fixed line were plotted against time (Figure 6). Ca\textsuperscript{2+} sparks were easily observed only when scan lines were located close to the surface membrane. When IP\textsubscript{3}-BM was added to the bath solution, we found that the firing rate was increased by
12.6% ± 5% (n = 4; P < 0.04) and the Ca²⁺ transient amplitude was increased by 15.3% (n = 4; P = 0.04). The number of Ca²⁺ sparks also appeared to increase, especially during the diastolic phase of Ca²⁺ transients (Figure 6C, arrows).

Similar firing rate changes were also observed in isolated cells that were not loaded with the fluorescent Ca²⁺ indicator (n = 6; P < 0.04). Endothelin-1 (ET-1) has been shown to increase endogenous IP₃ production through its activation of ETA receptors that are coupled to the phospholipase C–IP₃ signaling pathway. Using intact SAN loaded with the ratiometric Ca²⁺ indicator, indo-1, we recorded pacemaker activity influenced by modulation of IP₃Rs by ET-1 and phospholipase C–IP₃ signaling pathway. Although the pacemaker firing rate was significantly reduced when indo-1 was loaded into pacemaker cells, this approach has the added benefit of allowing diastolic Ca²⁺ to be measured. In the presence of ET-1 (100 nmol/L), the resting [Ca²⁺], the Ca²⁺ transient amplitude and firing rate all increased as shown in Figure 7A. The resting Ca²⁺ was increased by 36% ± 13% (n = 5; P < 0.05), the Ca²⁺ transient amplitude was increased by 10% ± 2% (P < 0.05), and the firing rate was increased by 20% ± 8% (P < 0.05). IP₃-BM (10 μmol/L) applied to intact SAN produced similar but less pronounced effects, possibly because of its slow penetration into a multicellular preparation. Figure 7C shows a summary of these data.

To further evaluate the influence of IP₃Rs on pacemaker activity, we studied intact SAN preparations from IP₃R2 KO and their wild-type (WT) counterparts. The relative mRNA expression of Ip3Rs, HCN4, and Ryr2 were analyzed in these mice and the only significant change was the absence of IP₃R2 in the KO (Supplemental Figure IB). Thus, the other IP₃R subtypes do not compensate for the loss of IP₃R2.

We found that the average firing rate (at 35°C, before loading with Ca²⁺ indicator) was 206 ± 3 per minute in SANs from IP₃R2 KO mice, which was approximately 12% lower than the 235 ± 11 per minute recorded in WT mice (n = 9; P < 0.001). Isoproterenol (ISO) 100 (nmol/L) accelerated the firing rate of SANs from both WT and KO mice.
IP$_3$R2 KO mice, as shown in Figure 8B. However, the response to IP$_3$R agonists was different between WT and IP$_3$R2 KO. In the WT SAN, ET-1 increased the pacemaker firing rate and could also induce irregular firing (Figure 8C). However, no significant firing rate changes were observed in IP$_3$R KO mice when ET-1 was applied (Figure 7C, left; 185±23 per minute control vs 180±26 per minute ET-1; n=7; P>0.05). In some experiments (Figure 8C), ET-1 caused a decrease in the amplitude of the Ca$^{2+}$ transient (F/F$_0$) in KO mice. However, this change was not statistically significant (1.64±0.34 WT vs 1.40±0.33 KO; n=7; P>0.05).

2-APB is a synthetic membrane-permeable antagonist of IP$_3$R and is widely used to investigate the role of IP$_3$R, although it can have a variety of other effects.35–37 Because higher concentrations of 2-APB (>30 μmol/L) suppressed atrial EC coupling,19 a lower concentration of 2APB (< 5 μmol/L) has commonly been used to investigate the involvement of IP$_3$R in atrial arrhythmias.18,19 It was found that in isolated atrial cells, 2-APB inhibits Ca$^{2+}$ release from IP$_3$R. We found that at 5 μmol/L, 2-APB had a minimal effect on the pacemaker action potential but slowed the firing rate (data not shown). In the presence of 5 μmol/L 2-APB (Figure 8D), the firing rate of intact SANs from WT was reduced, on average, by 24%±13% (P<0.05). In contrast, the firing rate of SANs in KO mice showed no significant change (185±23 per minute control vs 174±17 per minute 2-APB; n=7; P>0.05). The absence of effects of the IP$_3$R agonist and antagonist in IP$_3$R2 KO mice is strong evidence that IP$_3$R2 can modulate heart rate.

**Discussion**

**Identification of the IP$_3$R Isoforms and IP$_3$R Ca$^{2+}$ Release Sites in Pacemaker Cells**

In mammals, the three IP$_3$R isoforms are encoded by three different genes. IP$_3$R1 is strongly expressed in Purkinje myocytes and in cardiomyocytes derived from embry-
onic stem cells, where it is involved in the generation of pacemaker activity. In the present study, we were able to detect mRNA for all three IP3R isoforms, consistent with previous studies showing that hearts express mRNA for all three IP3R isoforms.\(^\text{15,16}\) IP3R3 showed consistent with previous studies showing that hearts express mRNA for all three IP3R isoforms.\(^\text{15,16}\) IP3R3 showed consistent with previous studies showing that hearts express mRNA for all three IP3R isoforms.\(^\text{15,16}\) In the present study, we took advantage of transgenic mice in which Ip3R2 was deleted whereas the expression of Ryr2, Hcn4, Ip3R1, and Ip3R3 was essentially unaltered (Supplemental Data). The Ip3R2 immunoreactivity was also absent in single pacemaker cells isolated from Ip3R2 KO mice (Figure 3B). From these studies, we estimate that Ca\(^{2+}\) release from Ip3Rs to the normal heart rate could contribute up to 12% of the basal firing rate (the spontaneous firing rate of isolated SANs from WT vs IP3R2 KO mice). Although modest, such a contribution is also supported by the effects of the IP3R agonist ET-1 and the membrane permeable IP3 analog. Confidence of this interpretation is increased by our observation that the modulation of pacemaker firing by IP3R agonists and antagonists was abolished (Figure 8C, D) in IP3R2 KO mice.

Given that the change of the firing rate induced by IP3 is small, one would not expect IP3 to have large effects on the heart rate in intact animals because any changes would be opposed by the autonomic nervous system. However, our results suggest that IP3R-mediated Ca\(^{2+}\) signaling could provide an additional pacemaker mechanism in the adult heart analogous to that which operates in embryonic stem cell-derived cardiomyocytes.\(^\text{25}\) We also previously reported that in canine toad pacemaker cells, ATP modulates intracellular Ca\(^{2+}\) and firing rate through a P2Y1 purinoceptor and phospholipase C signaling pathway.\(^\text{41}\) The fact that IP3 increases the excitability of pacemaker cells also supports previous findings that IP3 can lead to arrhythmias under some circumstances.\(^\text{18,19}\) Furthermore, under pathological conditions, the contribution of IP3R may increase and we recently reported a substantial increase in IP3 generation in atrial tissue from valvular heart disease patients, as well as in the atria of mice with dilated cardiomyopathy.\(^\text{42}\) In connection with this, increased expression of IP3R2 has been reported by others in heart failure or valve disease also.\(^\text{43,44}\) Therefore, the effect of IP3 on pacemaker electric activity could become more significant under pathological conditions. In addition, there is evidence showing that IP3R-mediated Ca\(^{2+}\) release promotes myocardial hypertrophy initiated by G-protein-coupled receptors via the Ca\(^{2+}\)-sensitive calcineurin signaling pathway that activates the nuclear factor of activated T-cell transcription factors (NFATc1).\(^\text{21,22}\) A previous study showed that this signaling pathway also modulates HCN gene expression.\(^\text{45}\) Therefore, IP3R may also influence pacemaker activity indirectly by activation of Ca\(^{2+}\)-sensitive signaling pathways and altering the expression of primary pacemaker ion channels.

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**Involvement of IP3R Ca\(^{2+}\) Signaling in the Modulation of Pacemaker Activity**

In the present study, we provide new evidence that pacemaker cells express IP3R2 in addition to RyR2. This suggests that pacemaker activity should be sensitive to modulation through G-protein/phospholipase C/IP3 signaling pathways under physiological or pathological conditions. We suggest that IP3 production increases the frequency of Ca\(^{2+}\) sparks close to the membrane (Figures 5, 6), which, in turn, activate Na/Ca exchange current (or other Ca\(^{2+}\)-dependent currents), increasing the rate of depolarization and heart rate. The involvement of SR Ca\(^{2+}\) release and NCX current is well-established,\(^\text{39}\) but Ca\(^{2+}\) release from RyR2 should predominate because there is approximately 50-times higher levels of expression of Ryr compared to Ip3Rs.\(^\text{40}\) However, the localization of IP3R2 close to the surface membrane, where IP3 is produced and where the Na/Ca exchangers are located, might offset the relative paucity of the IP3Rs and thereby allow them to contribute more effectively to changes in the firing rate. In the current study, we took advantage of transgenic mice in which Ip3R2 was deleted whereas the expression of Ryr2, Hcn4, Ip3R1, and Ip3R3 was essentially unaltered (Supplemental Data). The Ip3R2 immunoreactivity was also absent in single pacemaker cells isolated from Ip3R2 KO mice (Figure 3B). From these studies, we estimate that Ca\(^{2+}\) release from Ip3Rs to the normal heart rate could contribute up to 12% of the basal firing rate (the spontaneous firing rate of isolated SANs from WT vs IP3R2 KO mice). Although modest, such a contribution is also supported by the effects of the IP3R agonist ET-1 and the membrane permeable IP3 analog. Confidence of this interpretation is increased by our observation that the modulation of pacemaker firing by IP3R agonists and antagonists was abolished (Figure 8C, D) in IP3R2 KO mice.
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Disclosures
None.

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Novelty and Significance

What Is Known?
- Cardiac pacemaking in the sinoatrial node relies not only on voltage-dependent currents in the membrane but also on the intracellular activity of Ca^{2+}.
- Heart rate slows when Ca^{2+} release is inhibited from the major Ca^{2+} release channels (ryanodine receptors) in cardiac tissue.
- Release of Ca^{2+} from another minor group of Ca^{2+} release channels—inositol 1, 4, 5 trisphosphate receptors (IP3Rs)—has been implicated in arrhythmia and cardiac hypertrophy.

What New Information Does This Article Contribute?
- In the mouse sinoatrial node, the type II IP3Rs (IP3,R2) is the predominant IP3R isoform.
- Increasing the release of Ca^{2+} from IP3,R2s increases the heart rate, whereas inhibiting it slows the heart rate.

Although there is strong evidence that intracellular Ca^{2+} plays an important role in cardiac pacemaking via the ryanodine receptor Ca^{2+} release channels, little is known about the possible functional effects of IP3Rs in the sinoatrial node. This study provides the first comprehensive evidence to our knowledge that functional IP3Rs are expressed in the adult mouse sinoatrial node. We found that in the central pacemaker region and in isolated single pacemaker cells, IP3,R2 was the predominant protein isoform expressed. IP3,R2s and IP3 induce local Ca^{2+} release events, observed as Ca^{2+} sparks, which occur near the cell membrane, where their activity might explain their modest effect on heart rate. Importantly, the modulation of pacemaker firing and intracellular Ca^{2+} by IP3 agonists and antagonists was abolished in IP3,R2 knockout (KO) mice, confirming a role for IP3,R2s in pacemaker activity. These findings suggest that Ca^{2+} release from IP3,Rs provides an additional Ca^{2+}-dependent mechanism for the modulation of heart rate, a mechanism that would depend on the activation of G-protein-coupled receptors and the phospholipase C–IP3 signaling pathway. The chemical reagents that interact with this signaling pathway may have therapeutic potential for treating cardiac arrhythmias arising from dysfunction of the sinoatrial node.
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Supplemental Material

Detailed Methods

Animals

Most experiments were carried out using BALB/c mice. The mice were deeply anaesthetised with intra peritoneal pentobarbitone (1ml/2kg). We diluted pentobarbitone 150 µl into a 5 ml heparin solution, and used 0.3 - 0.5 ml /per mice depending on the age of the mice. In some experiments, IP3-R2/- mice on a C57BL/6 background were used, a generous gift from Dr. Ju Chen (UCSD) and they were bred by crossing homozygous knockout mice. The details of gene targeting and generation of IP3-R2-deficient mice have been published previously. Wild type mice were C57BL/6 matched for age and gender. All experimental procedures on mice were performed according to the guidelines of the National Health and Medical Research Council of Australia and approved by the Institutional Committee.

The preparation of intact SANs and single pacemaker cells

Intact SANs were isolated from the right atria as described previously. The central SANs were identified from anatomical landmarks, such as the superior vena cava, the crista terminalis, the intercaval area, and the interatrial septum. For physiological experiments, intact SANs were continuously superfused with modified Tyrode’s solution. All experiments were carried out at 33°C. In some experiments, single pacemaker cells were enzymatically isolated from the SAN region of a mouse heart according to the method described previously.

CellsDirect one step qRT-PCR

Less than 1mm x 2 mm individual heart samples were collected from different regions of a mouse heart, including the central SAN (S), peripheral SAN (P), AV node (AV), right atria (A) and ventricle (V), respectively and stored in RNaLater solution (Ambion Inc.). qRT-PCR was performed using the CellsDirect one-step qRT-PCR kit (Invitrogen, Australia). To achieve complete lysis, samples were vortexed, sonicated and underwent several thermal cycles (up to 75 °C) before being treated with DNase I to degrade genomic DNA. 18s rRNA, the hypoxanthine guanine phosphoribosyl transferase (HPRT), β-actin and β2 microglobulin (B2M) mRNA expression were used as reference genes for normalising data. TaqMan gene expression assays for three Ip3r isoforms and the hyperpolarization-activated, cyclic nucleotide-gate cation channels (Hcn4) and Ryr2 are listed in the Supplementary Table 1. The heart samples from different regions of IP3-R2 KO mice and their genetic identical counterpart C57BL/6J mice were also collected and used for a comparison qPCR study. Hela cell RNA was used as a standard reference for minimizing PCR experimental system errors.

Supplementary Table I. Summary of TaqMan Gene Expression Assays

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<th>Gene</th>
<th>TaqMan Assay ID</th>
<th>RefSeq Number</th>
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Western Blot Analysis

Total protein extracts were prepared from homogenates of the SAN, AV node, atria and brain tissue by using a mammalian cell lysis kit (Sigma). Protein analysis was performed by using a microBCA protein assay kit (Thermo Scientific). 8% precast gels from Nusep were used as protein band in the gels could be visualised under UV before and after protein transfer. Immunodetection of IP<sub>3</sub>R1, IP<sub>3</sub>R2 was completed using a rabbit polyclonal antibody to IP<sub>3</sub>R1 (1:1000, ABR), or a rabbit polyclonal antibody (1:500, a gift from Dr. Ju Chen’s laboratory), as previously described. Immunodetection of α-actinin was performed on samples by using a mouse monoclonal antibody to sarcomeric α-actinin (1:1000; Sigma). After transfer, membranes were treated with Miser antibody extender solution NC (Pierce Biotechnology). Horseradish peroxidase– conjugated anti-rabbit Ig (1:16,000; Sigma) or anti-mouse Ig (1:3000; Invitrogen) were used as secondary antibodies, respectively. For detecting IP<sub>3</sub>Rs, the primary and secondary antibodies were also treated with a signalBoost immunoreactions enhancer kit (Calbiochem).

Immunohistochemistry

Whole-mount SANs and isolated single pacemaker cells were labeled with ranges primary antibodies according to established methods. Rabbit polyclonal antibodies to IP<sub>3</sub>R1, IP<sub>3</sub>R2 were used to label the IP<sub>3</sub>R isoforms in 1:200, 1:100 respectively, (gifts from Dr. Ju Chen’s laboratory as well as commercially available ones from Affinity Bioreagents, Santa Cruz and Alomone lab). Antibodies for connexin-43 (Cx43) (1:100, Chemicon) and the antibody for HCN<sub>4</sub> (1:1000, abcam) were used to identify the pacemaker region within the SANs. A mouse monoclonal RyRs antibody (1:100, Affinity Bioreagents,) and SERCA2a (1:500, abcam), were also used to identify the distribution of SR in pacemaker cells. Anti-mouse or anti-rabbit secondary antibodies (Alex-488 anti-mouse and Alex-594 anti-rabbit) (Molecular Probes) were used as secondary antibodies, respectively. Prolong gold antifade reagent with DAPI (Molecular Probes) was used as the mounting media and for nuclear staining.

Ca<sup>2+</sup> measurements

The isolated SANs were loaded with the membrane-permeant fluorescent Ca<sup>2+</sup> indicator, indo-1-AM (10 µmol/L), in Tyrode’s solution containing 100 µmol/L Ca<sup>2+</sup> for at least 2h. The SANs were then washed in Tyrode’s Solution containing probenecid (0.5 mM) for more than 30 min at 35°C. The probenecid, a specific anion carrier inhibitor, was added to prevent leakage of the fluorescent dye. Fluorescent signals were recorded from a restricted region of the SAN area (about 150 X 150 µm) by using a rectangular diaphragm. The analog signals were digitized and the expression \((R-R_{min})/(R_{max}-R)\) calculated, which is linearly related to [Ca<sup>2+</sup>]. Confocal microscopy was used to collect Ca<sup>2+</sup> images of the SANs loaded with fluo-4AM (10 µmol/L) or isolated single pacemaker cells loaded with fluo-4AM (2 µmol/L) for 15 min.

Statistics

Data are expressed as mean ± SEM, with the number of preparations as n. Statistical tests were either Student’s paired or unpaired t-test, and \(P < 0.05\) was taken as the level of significance. The relative expression software tool (REST) was used to calculate the changes in mRNA expression and significance tests. Samples from the central SAN of BALB/c mice (n = 6) were used as the control to calculate the relative mRNA expression in different regions of the heart. To evaluate the changes in relative mRNA expression in IP<sub>3</sub>R2 knock out mice (n = 5), the samples collected from the same region as their genetically identical counterpart C57BL/6J mice (WT) (n = 5) were used as the control. The relative mRNA expression was calculated and displayed as \(\log_2\) scale.


Figure I *Ip*<sub>r</sub>*s* mRNA expression in mouse sino-atrial node tissue. The one-step quantitative PCR analysis of mRNA expressions of mouse sino-atrial node. A, Relative mRNA gene expression to the central SAN, including *Ip*r<sub>1</sub>, *Ip*r<sub>2</sub>, *Ip*r<sub>3</sub>, *Ryr*, and *Hcn4* in different regions of the heart (n = 6). Data are presented as mean ± SEM, n = 6. B, Relative mRNA expression of genes in IP3R2 knock out mice to identical heart regions of wild type mice, n = 5. Statistics analysis was performed using Relative Expression Software Tool (REST MCS, Corbett Life Science). *P* < 0.05, **P* < 0.01.
Figure II: A Confocal immunofluorescence images of interatrial septum (IAS) region of mouse sino-atrial node labelled with anti-Cx43 in green, anti-IP,R1 in red, DAPI in blue. B. In same preparation, there is no positive labelling of anti-IP,R1 in both of crista terminalis (CT) and central SAN region that is indicated by lack of Cx43 labelling in green.
Figure III  IP₃R2 is absent in IP₃R2 KO mouse SAN 
Confocal immunofluorescent tiling image of IP₃R2 KO mouse SAN. The whole mounted IP₃R2 KO mouse SAN was labelled with anti-HCN₄ in green reveals the central sino-atrial node. Anti-IP₃R2 (in red) staining is absent in SAN and surrounding atrial tissue.
Figure IV  Negative control when primary antibodies were omitted
Confocal immunofluorescent images were collected using Alex-488 anti-mouse (A) and Alex-594 anti-rabbit (B) (Molecular Probes) alone from cell shown in C & D. C: Transmission light images of a mouse pacemaker cell. D: the merging image of transmission light and confocal scanning.