Store-Operated Ca\textsuperscript{2+} Influx and Expression of TRPC Genes in Mouse Sinoatrial Node

Yue-Kun Ju, Yi Chu, Herve Chaulet, Donna Lai, Othon L. Gervasio, Robert M. Graham, Mark B. Cannell, David G. Allen

Abstract—Store-operated Ca\textsuperscript{2+} entry was investigated in isolated mouse sinoatrial nodes (SAN) dissected from right atria and loaded with Ca\textsuperscript{2+} indicators. Incubation of the SAN in Ca\textsuperscript{2+}-free solution caused a substantial decrease in resting intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and stopped pacemaker activity. Reintroduction of Ca\textsuperscript{2+} in the presence of cyclopiazonic acid (CPA), a sarcoplasmic reticulum Ca\textsuperscript{2+} pump inhibitor, led to sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, a characteristic of store-operated Ca\textsuperscript{2+} channel (SOCC) activity. Two SOCC antagonists, Gd\textsuperscript{3+} and SKF-96365, inhibited 72\textpm\text{8\%} and 65\textpm\text{8\%} of this Ca\textsuperscript{2+} influx, respectively. SKF-96365 also reduced the spontaneous pacemaker rate to 27\textpm\text{4\%} of control in the presence of CPA. Because members of the transient receptor potential canonical (TRPC) gene family may encode SOCCs, we used RT-PCR to examine mRNA expression of the 7 known mammalian TRPC isoforms. Transcripts for TRPC1, 2, 3, 4, 6, and 7, but not TRPC5, were detected. Immunohistochemistry using anti-TRPC1, 3, 4, and 6 antibodies revealed positive labeling in the SAN region and single pacemaker cells. These results indicate that mouse SAN exhibits store-operated Ca\textsuperscript{2+} activity which may be attributable to TRPC expression, and suggest that SOCCs may be involved in regulating pacemaker firing rate. (Circ Res. 2007;100:1605-1614.)

Key Words: heart \* sinoatrial node \* TRPC \* store-operated Ca\textsuperscript{2+} channel
the pacemaker potential changes progressively from the periphery to the center, the latter being the leading pacemaker site. Expression of ionic channels, Ca\(^{2+}\) handling proteins, and gap junction proteins in the SAN also vary from center to periphery.

In this study, we recorded intracellular Ca\(^{2+}\) signals from intact mouse SANs, a preparation in which the structural integrity and activity of the node is preserved. SOCC activity and expression of TRPC gene and proteins were examined in this preparation. These studies indicate that TRPCs might mediate SOCC activity and as a result, regulate pacemaker firing rate.

**Materials and Methods**

For detailed Materials and Methods, please see the supplemental materials (available online at http://circres.ahajournals.org).

**SAN Preparation**

The right atrium was harvested from anesthetized (pentobarbital sodium 1 mg/kg, i.p.) male BalbC mice (7 to 10 weeks old) and opened under a dissecting microscope to expose the crista terminalis, the intercaval area, and the interatrial septum. A section of right atrial wall containing the SAN region was pinned to a thin Sylgard block and placed in a chamber on an inverted microscope stage. The epicardial surface of this SAN preparation was closest to the objective while the endocardial surface was continuously superfused with modified Tyrode’s solution. All experiments were performed at 33°C.

**Ca\(^{2+}\) Measurements**

The SAN preparation was loaded with the membrane-permeant fluorescent Ca\(^{2+}\) indicator, indo-1-AM (10 \(\mu\)mol/L), in Tyrode solution. Fluorescent signals were recorded from a restricted region of the SAN area (about 150×150 \(\mu\)m) by using a rectangular diaphragm (Figure 1A). The analog signals were digitized and the expression (R-R\(_{\text{min}}\))/(R\(_{\text{max}}\)-R) calculated, which is linearly related to \([\text{Ca}^{2+}]_i\) (for details see Kao). In some experiments, confocal microscopy was used to collect data from the SAN preparations that were loaded with fluo-4AM (10 \(\mu\)mol/L).

**mRNA for TRPC Expression**

Total RNA was isolated from 20 SANs using a RNAqueous 4PCR isolation kit (Ambion). Specific primers for mTRPC1, mTRPC2, mTRPC3, mTRPC4, mTRPC5, mTRPC6, and mTRPC7 were used for PCR amplification, respectively. To control for potential genomic DNA contamination, a cDNA reaction was performed but in the absence of reverse transcriptase and used as template for PCR reactions.

**Immunohistochemistry**

Rabbit polyclonal antibodies to TRPC1, TRPC3, TRPC4, and TRPC6 (1:50, 1:100, 1:50, and 1:200 dilution, respectively; Alomone Labs) were used to label the various TRPC isoforms in the SAN whole mount preparations and single pacemaker cells. Antibodies for hyperpolarization-activated, cyclic nucleotide-gate cation channels HCN4 (1:100; Alomone Labs), and connexin-43 (3 \(\mu\)g/mL; Chemicon) were also used to identify pacemaker region within the SAN.

**Statistics**

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

**Results**

**Spontaneous Ca\(^{2+}\) Transients Recorded From the SAN Preparation**

The central pacemaker region was identified by anatomic landmarks as shown in Figure 1A. Using conventional glass microelectrodes, we were able to record spontaneous action potentials from this region (Figure 1B, upper panel) that exhibited the low upstroke velocity typical of the primary pacemaker region (Figure 1B, lower panel). The spontaneous firing rate slowed with indo-1AM loading, attributable to the increased \([\text{Ca}^{2+}]_i\), buffering. Ca\(^{2+}\) transients recorded from
the SAN node (Figure 1C) were synchronous with visible contraction of the SAN.

It is well established that β-adrenergic stimulation increases heart rate via its effect on the pacemaker cells. Therefore, the effects of isoproterenol on pacemaker action potential and Ca\(^{2+}\) transient were examined. We found that in the presence of isoproterenol, firing rate increased by 38 ± 7% (n=7, P<0.0001), and the amplitude of Ca\(^{2+}\) transients was increased by 69 ± 21% (P<0.01, Figure 1D and 1E). The observations suggest that the Ca\(^{2+}\) signals were principally from the pacemaker cells rather than from other cell types in the SAN such as endothelial cells or fibroblasts.20

To confirm this notion, confocal images of the SAN were recorded in the presence of lidocaine (100 μmol/L) to leave only the central SAN cells firing.21 Figure 2A, a & b, shows consecutive images recorded from the central SAN, and Figure 2Ac shows a ratio image, demonstrating cyclic [Ca\(^{2+}\)], changes in the central region of the SAN but not surrounding regions. A line scan image across the SAN (Figure 2B) and the time course of [Ca\(^{2+}\)], changes (Figure 2C) show that it is possible to record and characterize spontaneous Ca\(^{2+}\) signals arising from the central regions of the SAN.

**Figure 2.** Confocal images of [Ca\(^{2+}\)] in the SAN. The bathing solution contained 100 μmol/L lidocaine and 10 mmol/L 2,3-butanedione monoxime to reduce movement artifacts. A, Consecutive XY images recorded from the central SAN area with a time interval of 0.18 s between a and b. A ratio image of fluorescence intensity is showed in Panel Ac. B, XT ratio image obtained from central SAN pacemaker area. Calibration bar in B indicates the self ratio fluorescence value that is calculated from the pixel values divided by the mean values just before the transient (F/F0). The time scale is the same as in C. C, Normalized fluorescence intensity plotted as a function of time from a region near the lower part of B. (F, the intensity of region plotted; F0, the mean background intensity.) D, Control, XT image (top), and line plot of normalized fluorescence intensity against time (bottom). E, The effect of 0.5 mmol/L caffeine on [Ca\(^{2+}\)] and firing rate.

**Store-Operated Ca\(^{2+}\) Influx in Pacemaker Cells**

To study Ca\(^{2+}\) influx through SOCCs in the SAN, preparations were first incubated in Ca\(^{2+}\)-free solution resulting in a substantial decline in resting [Ca\(^{2+}\)], and cessation of pacemaker activity (Figure 3A). Reintroduction of Ca\(^{2+}\) to the perfusate caused a small rise in [Ca\(^{2+}\)], and a recovery of pacemaker activity (Figure 3B). When SR Ca\(^{2+}\) uptake was inhibited with cyclopiazonic acid (CPA, 10 μmol/L), the resting [Ca\(^{2+}\)], increased significantly (Figure 3C). To further empty SR Ca\(^{2+}\) stores, SANs were incubated in Ca\(^{2+}\)-free solution containing CPA for 15 minutes. Reintroduction of Ca\(^{2+}\) to the perfusate in the presence of CPA evoked a marked increase in [Ca\(^{2+}\)], (Figure 3D), a characteristic of SOCC activity, as observed in other tissues.22 Ca\(^{2+}\) influx in response to reintroduction of Ca\(^{2+}\) was 7.1 ± 3.2-fold greater in the presence (Figure 3D) than in the absence of CPA (Figure 3B; P<0.03, n=11), a finding consistent with Ca\(^{2+}\) influx being activated in response to SR store depletion. Although it is not feasible to measure the SR store content in this multicellular preparation, the large rise in resting [Ca\(^{2+}\)], in the presence of CPA suggested that SR Ca\(^{2+}\) uptake was effectively
blocked and, thus, that SR store content was substantially depleted (Figure 3C).

To examine the possibility that the Ca\(^{2+}\) influx was mediated by L-type Ca\(^{2+}\) channels or Na\(^{+}/Ca\(^{2+}\) exchanger, we tested the effect of nifedipine or the Na\(^{+}/Ca\(^{2+}\) exchanger inhibitor, KBR-7943. As shown in Figure 4A, 20 \(\mu\)mol/L nifedipine had no effect on SOCC activity. In the presence of 5 \(\mu\)mol/L KBR-7943, Ca\(^{2+}\) influx was inhibited by 25±8% of the control (n=6) (Figure 4B). Therefore, even when L-type Ca\(^{2+}\) channels or Na\(^{+}/Ca\(^{2+}\) exchange are inhibited, store depletion evokes an influx of Ca\(^{2+}\) as would be expected from the presence of SOCCs.

To further confirm that this Ca\(^{2+}\) influx was mediated by SOCCs, we applied gadolinium (Gd\(^{3+}\)), a known SOCC inhibitor. We found that 100 \(\mu\)mol/L Gd\(^{3+}\) reduced the maximum [Ca\(^{2+}\)] response by 73±8% (P<0.01, n=4; Figure 4C). It has been reported that SOCCs are also sensitive to SKF-96365 in several cell types. We found that 10 \(\mu\)mol/L SKF-96365 inhibited 65±9% of Ca\(^{2+}\) influx (P<0.001, n=8; Figure 4D). A summary of these pharmacological evaluations of SOCC activity in SAN is shown in Figure 4E.

**Functional Role of SOCCs in the Mouse SAN**

Previous studies have demonstrated that in toad and rabbit pacemaker cells, pacemaker activity is modulated by SR Ca\(^{2+}\) release. To test whether spontaneous pacemaker activity of mouse SAN is also sensitive to SR Ca\(^{2+}\) release, the SR Ca\(^{2+}\) release antagonist, ryanodine (20 \(\mu\)mol/L) was applied and found to reduce firing rate to 55±11% of control (n=6, P<0.03; Figure 5A, left).

SR Ca\(^{2+}\) release can also be affected by reducing SR Ca\(^{2+}\) content. Low concentrations of caffeine reduce SR Ca\(^{2+}\) content and were found to reduce the both Ca\(^{2+}\) transient and firing rate to 87±4% and 75±3% of control respectively (n=4, P<0.01; Figure 2D and 2E). SR Ca\(^{2+}\) content can also be reduced by inhibition of SR Ca\(^{2+}\) uptake with CPA, which has been reported to slow spontaneous activity in single pacemaker cells. We found that CPA had a marginal effect on pacemaker firing rate, reducing it to 81±9% control. Both ryanodine and CPA reduced the amplitude of Ca\(^{2+}\) transients to 21±5% (n=6, P<0.0004) and 48±4.5% of control (n=7, P<0.0003), respectively. In addition, CPA increased resting [Ca\(^{2+}\)], to 167±37% of control (P<0.0006), whereas ryanodine did not alter resting [Ca\(^{2+}\)], significantly. These effects of SR store modulators on the pacemaker firing rate were confirmed by using intracellular recording of action potentials on intact SANs, which revealed qualitatively similar effects (Figure 5B).

These results suggest that several mechanisms contribute to the role of SR loading in cardiac pacemaking. For instance, whereas depletion of SR stores by CPA and low dose caffeine may activate membrane channels, such as SOCCs, to generate an inward Ca\(^{2+}\) current, this would be offset by a decrease in inward Na\(^{+}/Ca\(^{2+}\) exchanger current that was caused by reduced SR Ca\(^{2+}\) release. Because high concentrations of ryanodine (>10 \(\mu\)mol/L) do not affect store content, the reduction the firing rate seen when ryanodine was applied can be explained by its effects on Ca\(^{2+}\) release and the Na\(^{+}/Ca\(^{2+}\) exchanger current without a direct effect mediated by SOCCs.

To explore the potential implications of SAN SOCC activity for pacemaker function, we examined the effect of SKF-96365 on spontaneous action potential firing rate (Figure 5B). SKF-96365 (10 \(\mu\)mol/L), which blocked SOCCs (Figure 4), slowed firing rate to 64±7% of control (P<0.005, n=5). Moreover, a further reduction in pacemaker firing to 27±4% (P<0.002, n=5) was observed when the SKF-96365 was administered together with the SR Ca\(^{2+}\) uptake blocker CPA (Figure 5B and 5C). With this combined treatment, resting [Ca\(^{2+}\)], fell to a level not different from to the control (Figure 5C and 5D). It should be noted that although this combined treatment reduced both the firing rate and Ca\(^{2+}\) transients, effects that were fully reversible, the upstroke of action potentials was unchanged (Figure 5C). Thus it seems likely that the effects of SKF on the pacemaker firing were not attributable to blockade of the L-type Ca\(^{2+}\) channels.
comparison of changes in resting and transient [Ca^{2+}]i is shown in Figure 5D.

**Expression of TRPC Gene and Proteins in SAN**

It has been suggested that SOCCs are encoded by the TRPC gene family.28 We used RT-PCR to examine mRNA expression of the 7 known mammalian TRPC isoforms in SAN preparations. PCR products were generated using primers specific for TRPC1-TRPC7 isoforms; primers that were initially validated using mouse brain RNA, a tissue known to express all TRPC isoforms. TRPC1, 2, 3, 4, 6, and 7, but not TRPC5, transcripts were detected in SAN (Figure 6Aa). Using HCN4 as a positive control, we showed both mRNA (Figure 6Ab, supplemental Figure I) and protein expression (Figure 6Ba 6Bb) of HCN4 are higher in SAN than right atria.29 HCN4 immunoreactivity was observed in the SAN region but not in the CT and surrounding atrial tissue (Figure 6Ba and 6Bb). In contrast, Cx43 (Figure 6Bc and 6Bd) is absent from the central area of the SAN but is abundant in atria and peripheral SAN as also observed by others.16 These distinctive protein expression patterns enabled us to identify the central and peripheral SAN.

Polyclonal antibodies raised against human TRPC1, TRPC3, TRPC4, and TRPC6 were used to evaluate the expression of these proteins. We confirmed that these antibodies could detect the TRPC isoforms by Western blot analyses of mouse atrial lysates (supplemental Figure II). When applied to SAN whole mount preparations, immunofluorescence was evident in both the central and peripheral...
SAN with antibodies against TRPC1, 3, 4, and 6. To further define the cell type expressing TRPCs in the central and peripheral SAN, single pacemaker cells were isolated and labeled with TRPC antibodies. The central or peripheral pacemaker cells were identified by their morphological appearance. Single pacemaker cells isolated from central (Figure 7a) and peripheral (Figure 7b) SAN all showed positive labeling with the TRPC antibodies. As observed in other cell types, labeling was predominantly cytoplasmic with the TRPC1 antibody (Figure 7A). The TRPC3 antibody produced the most intense labeling, particularly in central pacemaker cells (Figure 7Ba), and membrane staining was evident in a peripheral pacemaker cell (Figure 7Bb). The TRPC4 antibody exhibited regions of intense labeling near the ends of cells, which may indicate colocalization of TRPC4 with gap junctions (Figure 7Ca and 7Cb). TRPC6 labeling of central pacemaker cell was very weak (Figure 7Da), and in the peripheral pacemaker cells sarcomeric banding patterns were often apparent (Figure 7Db). Negative controls (Figure 7c), in which the antibodies were preincubated with their relevant peptide antigen, confirmed the specificity of TRPC isoform labeling.

**Discussion**

**Advantages of the Intact Mouse SAN Preparation for the Study of Pacemaker Function**

Mice are of particular value for studying the mechanisms of cardiac pacemaker function because genetically modified models have been developed and are readily available. The mouse SAN is about 300×150 μm and consists of about 450 pacemaker cells, of which only a few act as leading pacemaker cells at any one time. Although studies using isolated mice pacemaker cells have provided valuable electrophysiological data, practical difficulties remain, because very few cells show spontaneous activity after enzymatic isolation. Furthermore, it is not always possible to distinguish central from peripheral pacemaker cells, although
they differ significantly in their electrophysiological and Ca\(^{2+}\) handling properties.\(^{16,21}\)

The small size of the SAN in mouse, which makes single cell isolation more difficult, is an advantage for imaging studies. As the SAN is only \(\approx 50 \mu m\) thick and is transparent, its dimensions are compatible with microscopic fields of view. In the present study we were readily able to record changes in \([\text{Ca}^{2+}]_{\text{i}}\) in intact SANs. The SAN and surrounding regions were identified by their anatomical landmarks and typical pacemaker action potentials (Figure 1). In addition, we demonstrated the expected sensitivity of spontaneous SAN activity to \(\beta\)-adrenergic stimulation. The use of a Na\(^+\) channel blocker, which inactivates peripheral SAN activity but leaves firing from the central area intact, further confirmed the distinction between peripheral and central SAN.\(^{36}\)

Moreover, the SAN preparation used in this study is relatively easy to obtain, and the pacemaker function persists for many hours. Thus it offers considerable potential for the study of mouse models including those display abnormal pacemaker function.

**Intracellular Ca\(^{2+}\) Stores and Store-Operated Ca\(^{2+}\) Entry in Pacemaker Cells**

Although there is growing evidence that diastolic depolarization can be generated by an inward Na\(^+\)/Ca\(^{2+}\) exchange current that is related to Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the SR,\(^{37}\) it remains debatable whether SR Ca\(^{2+}\) release is essential for cardiac pacemaker function.\(^{38}\) In the present study, we found that ryanodine, CPA, and low concentrations of caffeine all slowed pacemaker firing rate, supporting a role...
for SR Ca\(^{2+}\) release in pacemaker function (Figures 2E, 5A, and 5B).

Substantial Ca\(^{2+}\) influx could be induced by SR store depletion, which is a characteristic of SOCC activity (compare Figure 3B and 3D). The increase in [Ca\(^{2+}\)], induced by store depletion was not simply attributable to reduced Ca\(^{2+}\) buffering, because it was inhibited by 2 SOCC inhibitors, Gd\(^{3+}\) and SKF-96365 (Figure 4C and 4D). We also excluded the possibility that Ca\(^{2+}\) influx was attributable to L-type Ca\(^{2+}\) channels or reverse mode Na\(^{+}\)/Ca\(^{2+}\) exchanger activity, given that Ca\(^{2+}\) entry was still evident after blockade of Ca\(^{2+}\) channels with nifedipine or inhibition of the exchanger with KBR-7943 (Figure 4A and 4B). Although the specificity of Gd\(^{3+}\) and SKF-96365 remains questionable, similar effects were achieved with both SOCC blockers. This supports the notion that store depletion promotes Ca\(^{2+}\) influx mediated via SOCC. In addition, the fact that blocking L-type Ca\(^{2+}\) channels had no effect on SOCC activity in SAN strengthens our conclusion that Gd\(^{3+}\) and SKF96365 were acting mainly through their effect on SOCCs.

**TRPC Expression in the SAN**

Having characterized the SAN preparation and provided evidence for the involvement of SOCC activity in pacemaker cell function, we investigated whether SOCC activity could be attributable to TRPCs and, if so, which isoform is involved. We found transcripts for all TRPC isoforms, except TRPC5, in the SAN; absence of TRPC5 expression in the heart has been noted in an earlier report.\(^\text{19}\)

A recent study of adult ventricular myocytes overexpressing TRPC3 showed abundant SOCC activity that was inhibited with SKF-96365.\(^\text{5}\) We found that TRPC3 expression was
particularly prominent in central pacemaker cells and that activity in SAN was also sensitive to SKF-96365, suggesting that TRPC3 might contribute to SOCC activity in mouse pacemaker cells. A quantitative measure of mRNA abundance of TRPC3 in various mouse tissues is shown in the online data supplement (supplemental Figure II). There was no significant difference in TRPC3 expression from different regions of the mouse heart. The predominantly cytoplasmic localization of all the TRPC channels is surprising given that the functional channel should be in the membrane. However, this is not an unusual observation as it has been observed in many cell types and with 3 different antibodies to TRPC1. Given that TRPC3 has recently been shown to undergo constitutive cyclical trafficking between the plasma membrane and intracellular sites, it is possible that the failure to identify TRPCs membrane localization may be due to a short membrane retention time.

**Possible Physiological Role of SOCC in SAN**

Ca$^{2+}$ entry is critical for the cell function, and SOCCs have been suggested to mediate Ca$^{2+}$ entry in both excitable and nonexcitable cells. However, whether or not SOCCs contribute to cardiac pacemaking on a beat-to-beat basis remains unclear. We found that the SOCC blocker SKF-96563 slowed firing rate and, when SR Ca$^{2+}$ stores were depleted with CPA, pacemaker firing was further slowed (Figure 5). These observations raise the possibility that the inward current through SOCCs can influence heart rate.

SOCCs have been proposed to provide a pacemaker current in other spontaneously firing cells such as neurons and interstitial cells of Cajal. In embryonic pacemaker cells, before functional pacemaker ionic channels have developed, IP$_3$-dependent Ca$^{2+}$ influx through SOCCs has been described and under these circumstances is thought to contribute to pacemaking. Although SOCCs may generate inward currents and thereby contribute to diastolic depolarization, store content may also affect Ca$^{2+}$ handling, because release of Ca$^{2+}$ is highly dependent on store Ca$^{2+}$ content. In addition, the resting level of [Ca$^{2+}$], will also affect calcium extrusion via the Na$^+$/Ca$^{2+}$ exchanger, which can generate an inward current. Thus the exact timing of SR calcium release and its influence on pacemaker current(s) is likely to be directly related to SR content which will, in turn, reflect the balance between Ca$^{2+}$ entry and extrusion. Our data suggest that SOCCs provide an additional important Ca$^{2+}$ entry pathway to regulate SAN function.

An alternative or additional role for SOCC/TRPC in pacemaking is suggested by a recent study showing that TRPC1 also forms a stretch-activated cation channel. It has been long known that stretch of the atria increases heart rate, although the mechanism involved is unknown. If TRPC1 forms a stretch-activated channel in SAN cells, our finding of SOCCs/TRPCs in these cells raises the possibility that TRPC1 may explain the effect of SAN stretch on heart rate. In this regard, TRPC1 has been suggested to function both as a stretch-sensitive channel and a SOCC in dystrophic skeletal muscle.

In conclusion, we have demonstrated the utility of an intact murine SAN preparation that allows both structural and functional studies of the cardiac pacemaker. Our studies demonstrate SOCC activity in the mouse SAN and raise the possibility that TRPC isoforms are responsible for SOCC activity in SAN cells. This SOCC activity may contribute to the regulation of Ca$^{2+}$ entry and pacemaker firing.

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**Disclosures**

None.

**References**


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Detailed Materials and Methods

mRNA for TRPC expression

Total RNA was isolated from 20 SANs using a RNAqueous 4PCR isolation kit (Ambion, Austin, TX) following the manufacturer's instructions. Following turbo DNase I (Ambion, Austin, TX) treatment, mRNAs were reverse transcribed to synthesize first-strand cDNA by using the M-MLV Reverse Transcriptase, RNase H minus point mutant (Promega, Madison, WI) and oligo(dT) primers. For the second strand cDNA synthesis and PCR amplification we used the following specific primers:

- mTRPC1, forward 5'-GATTTTGGGAAATTCTGGG-3', reverse 5'-TGTTATCAGCTGGAAGCT-3';
- mTRPC2, forward 5'-GACATGATCCGGTTCATG-3', reverse 5'-CTGGATCTTCTGGAAGGA-3';
- mTRPC3, forward 5'-GACATATTCAAGTTGCT-3', reverse 5'-CTGGATCTCTTGGTATGA-3';
- mTRPC4, forward 5'-TGGGACATGTGGCACCCCAC-3', reverse 5'-ACGTGGAACACGCTTGTCTG-3';
- mTRPC5, forward 5'-GACTAGTCTTGATATACCAAATTTCTC-3', reverse 5'-GGGGTACCTCAGCATGATGGGCAATG-3';
- mTRPC6, forward 5'-GATATCTTCAAATTCTAGTGTC-3', reverse 5'-CTCAATTTCCTGGAATGAAC-3';
- mTRPC7, forward 5'-CTCATGGGAGGAACCTACAG-3', reverse 5'-5'CATCTTTGGTCTGTTAGGGTG-3'.
Expected lengths of amplicon were 356, 297, 309, 528, 365, 309, and 198 base pairs for mTRPC1, mTRPC2, mTRPC3, mTRPC4, mTRPC5, mTRPC6 and mTRPC7, respectively.

The amplification profile for these primer pairs was: 94°C for 15 s, 58°C for 15 s and 72°C for 1 minute, for 40 cycles. The amplified products were separated by electrophoresis in a 1.5% agarose/TAE (Tris, acetic acid, EDTA) gel, and the cDNA bands were visualized by ethidium bromide staining. For the RT control, a cDNA reaction was used as template for which the reverse transcriptase was not added, controlling for genomic DNA contamination in the source RNA (not shown).

**Western blot analysis**

The specificity of the antibodies against TRPC (1, 3, 4 and 6; Alomone Labs, Jerusalem, Israel) was evaluated by Western blot. Atrium fragments were washed in cold phosphate-buffered saline (PBS) and lysed using Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in lysis buffer (50mM Tris pH7.5, 150mM NaCl, 25mM EDTA, 25mM EGTA and 1% Triton X-100) containing protease and phosphatase inhibitors (Sigma; St Louis, MI). Hela cell cultures were lysed using the same lysis buffer. Protein concentration was measured using Bradford assay (Bio-Rad, Hercules, CA) and 20 μg of total protein per well was loaded on a 4-20 % Tris-HCl linear gradient gel (Bio-Rad). Proteins were resolved by electrophoresis and transferred to PVDF membrane. Membranes were blocked in 5% skim milk TBS-T buffer (overnight, 4 C°) and incubated with anti-TRPC antibodies diluted in blocking buffer (1:200 for TRPC1, 3, 4 & 6) for 2 hours at room temperature. The blot was then rinsed with TBS-T three times and probed with a HRP-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:4,000 in blocking buffer for 1 hour at room temperature. The blot was developed with the ECL plus Western blotting detection system (Amersham Pharmacia Biotech, UK)
according to the manufacturer’s protocol. Bands were visualised and photographed using an Alpha Innotech FluoChem SP Imaging System and analysed using AlphaEase software (Alpha Innotech, San Leandro, CA). In order to test the specificity of the antibodies, primary antibodies were pre-blocked with correspondent blocking peptides prior to incubation with the membranes.

**SAN whole mount immunostaining**

The SAN region was dissected from the right atrium and fixed in 4% paraformaldehyde for 30 min at 4°C. The SANs were permeabilised by washing for 30 min in phosphate-buffered saline (PBS) containing 1% Triton X-100. After blocking of non-specific binding by incubation for 1 h with 0.5% Triton X-100 in PBS containing 10% normal goat serum (NGS), SAN whole-mount preparations were incubated for 3 days at 4°C with the primary antibodies. They were then washed three times with PBS containing 0.1% Triton X-100, incubated for 4 h with Cy3-conjugated goat antibodies to rabbit IgG (1:200; Jackson ImmunoResearch), and washed three times with PBS containing 0.1% Triton X-100. All antibodies were diluted with 0.5% Triton X-100 in PBS containing 5% NGS. Whole-mounts were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen), the latter being a water-soluble nuclear and chromosomal counterstain. The SAN preparations were mounted with the endocardium uppermost. Immunofluorescence was examined with a confocal microscope (Leica TCS NT). DAPI, FITC and Cy3 fluorescence was excited at 364, 488 and 543 nm, respectively. Images were processed with Image J (Wayne Rasband) software.

For controls, pre-incubation of the primary antibodies with their peptide antigen, or incubation of SAN whole mounts with only the secondary antibody,
reduced fluorescence to non-detectable levels. The lack of detectable fluorescence confirmed the specificity of the antibody binding.

**Single cell immunostaining**

Single pacemaker cells were enzymatically isolated from SAN region of mouse heart. The SANs were dissected free and placed into calcium–free Tyrode solution contained (mM): NaCl 133; KCl, 4.0; NaH2PO4, 1.2; MgCl2, 1.2; glucose, 10; HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid), 10 for 5 min. Intact SAN tissue was then transferred into 25 µmol/L calcium-Tyrode solution containing 1mg/ml collagenase (Worthington Type II) at 35°C for 60 min before it was transferred in to fresh 25 µmol/L calcium-Tyrode solution containing 0.5 mg/ml elastase (Sigma) for further 50 min. The SAN was then place in KB solution containing (mM) KCl, 50; KATP, 5; glucose, 10; taurine, 20; creatine, 5; glutamine acid, 5; succinic acid, 5; Hepes, 5; KH2PO4, 20; MgSO2, 5; pyruvic acid, 5; K-EGTA, 0.04. pH adjusted to 7.2 with KOH. SANs were kept in KB solution for at least 4h at 4°C, before being triturated gently to dissociate the cells. The cells were then placed onto glass coverslips and maintained at room temperature for about 1h in order to allow cells to adhere to the glass.

Isolated single pacemaker cells were fixed in 4%paraformaldehyde for 1 min. They were permeabilised by incubating for 5 min in phosphate-buffered saline (PBS) containing 2% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.1%Triton X-100. After blocking of nonspecific binding sites by incubation for 30 min with 0.01%BSA in PBS containing 10% NGS, single pacemaker cells were exposed to primary antibodies.
Supplementary Figure legends:

Online Figure 1. Relative quantities of TRPC3 and HCN4 gene expression in mouse tissues. The samples were collected from brain, aorta and different regions of the mouse heart, including: left ventricle (LV), the atria (AT), sino-atrial (SAN), and atrioventricular node (AVN) according to their anatomic landmarks. The hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used for normalizing the data. The expression of HCN4 was relatively high in the SAN tissues, compared to the other tissue. There was no significant difference in TRPC3 expression from different regions of the mouse heart.

Online Figure 2: Immunoblot analysis of TRPC proteins in HeLa cell (H) and mouse atrial tissue lysate (M) with rabbit anti TRPC 1 (A), TRPC3 (B), TRPC4 (C), and TRPC(6) polyclonal IgG (1:200, Alomone Lab), respectively. A: a single immunoreactive band of ~72KDa protein (H, lane 1) and additional ~81k Da (M, lane 2) were detected by TRPC1 antibody. The molecular mass of TRPC1 was consistent with literature. B: Multiple bands ~59, 80, 91 103 kDa protein bands were found with TRPC3 antibody (lane 1 & 2), probably in corresponding to protein isoforms 6430519I19, PA160542.1, PA160552.11 (http://locate.imb.uq.edu.au/). The low molecular band of ~ 59kDa was also observed previously. C: Multiple protein bands were detected by TRPC4 only in atrial lysates (lane 2). Similar molecular mass of TRPC4 mouse isoforms have been reported (http://locate.imb.uq.edu.au/), probably in corresponding to protein AAC05187 isoforms: PA 71882.1; PA71882.2, PA72882.9, and PA72238.23. D: A molecule of ~81kDa was recognised by TRPC6 antibody (M...
lane 2) although two bands of ∼103kDa and ∼90 KDa were weakly detected (H lane 1). The results are largely consistent with the previous observations \(^1,4\). The nature of these multiple bands, which have been suggested as splice variants \(^4\) or due to protein glycosylation. \(^5\) In a parallel experiment, all the bands mentioned above were abolished or largely diminished by using preabsorbed TRPC antibodies with corresponding specific peptide as shown in (A-D) line 3 & 4.

Reference List


Figure 1: Graph showing relative mRNA content compared to HPRT X100.

- **TRPC3**
- **HCN4**

Categories: Brain, LV, AT, SAN, AVN, Aorta.
Online Fig. 2