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Heterogeneity of calcium clock functions in dormant, dysrhythmically and rhythmically firing single pacemaker cells isolated from SA node

Mary S. Kim^{#a}, Alexander V. Maltsev^{#a}, Oliver Monfredi^{#a,b,c}, Larissa A. Maltseva^a, Ashley Wirth^a, Maria Cristina Florio^a, Kenta Tsutsui^a, Daniel R. Riordon^a, Sean P. Parsons^d, Syevda Tagirova^a, Bruce D. Ziman^a, Michael D. Stern^a, Edward G. Lakatta^a, and Victor A. Maltsev^{a,*}

^aLaboratory of Cardiovascular Science, National Institute on Aging, NIH, Biomedical Research Center, 251 Bayview Blvd. Suite 100, Baltimore, MD 21224-6825, USA

^bDepartment of Cardiovascular Electrophysiology, The Johns Hopkins Hospital, 1800 Orleans St, Baltimore, MD 21287, USA

^cInstitute of Cardiovascular Sciences, University of Manchester, 46 Grafton St, Manchester M13 9NT, UK

^dFarncombe Institute, McMaster University, Hamilton, ON, Canada

[#] These authors contributed equally to this work.

Abstract

Current understanding of how cardiac pacemaker cells operate is based mainly on studies in isolated single sinoatrial node cells (SANC), specifically those that rhythmically fire action potentials similar to the *in vivo* behavior of the intact sinoatrial node. However, only a small fraction of SANC exhibit rhythmic firing after isolation. Other SANC behaviors have not been studied.

Here, for the first time, we studied all single cells isolated from the sinoatrial node of the guinea pig, including traditionally studied rhythmically firing cells ('rhythmic SANC'), dysrhythmically firing cells ('dysrhythmic SANC') and cells without any apparent spontaneous firing activity ('dormant SANC'). Action potential-induced cytosolic Ca²⁺ transients and spontaneous local Ca²⁺ releases (LCRs) were measured with a 2D camera.

LCRs were present not only in rhythmically firing SANC, but also in dormant and dysrhythmic SANC. While rhythmic SANC were characterized by large LCRs synchronized in space and time towards late diastole, dys-rhythmic and dormant SANC exhibited smaller LCRs that appeared stochastically and were widely distributed in time. β -adrenergic receptor (β AR) stimulation increased LCR size and synchronized LCR occurrences in all dysrhythmic and a third of dormant

*Corresponding author. maltsevvi@mail.nih.gov (V.A. Maltsev).

Declarations of interest

None.

Appendix A. Supplementary data

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cells (25 of 75 cells tested). In response to β AR stimulation, these dormant SANC developed automaticity, and LCRs became coupled to spontaneous action potential-induced cytosolic Ca^{2+} transients. Conversely, dormant SANC that *did not* develop automaticity showed no significant change in average LCR characteristics. The majority of dysrhythmic cells became rhythmic in response to β AR stimulation, with the rate of action potential-induced cytosolic Ca^{2+} transients substantially increasing. In summary, isolated SANC can be broadly categorized into three major populations: dormant, dysrhythmic, and rhythmic. We interpret our results based on simulations of a numerical model of SANC operating as a coupled-clock system. On this basis, the two previously unstudied dysrhythmic and dormant cell populations have intrinsically partially or completely uncoupled clocks. Such cells can be recruited to fire rhythmically in response to β AR stimulation via increased rhythmic LCR activity and ameliorated coupling between the Ca^{2+} and membrane clocks.

Keywords

Sarcoplasmic reticulum; Local calcium release; Sinoatrial node; Pacemaker; Ryanodine receptor; β Adrenergic receptor

1. Introduction

Studies of isolated single sinoatrial (SA) node cells (SANC) have been fundamental in clarifying the cellular mechanisms of cardiac impulse initiation, which are both voltage-, time-, Na^+ - and Ca^{2+} -dependent. While significant progress has been achieved in understanding how pacemaker cells operate, our knowledge is limited to only those cells that behave *in vitro* in a similar way to that observed in the SA node as a whole (i.e. those that beat rhythmically). However, only 10–30% of isolated cells contracted spontaneously in the original paper describing SANC isolation by Nakayama et al. [1]. The yield of spontaneously and rhythmically contracting cells has increased over time but has never approached 100%. Isolated single SANC that do not beat rhythmically, including those exhibiting dysrhythmic firing or an absence of firing, have never been studied.

In the present study, we addressed the issue of functional heterogeneity of single isolated SANC by examining Ca^{2+} dynamics in cells isolated from guinea pig SA node. We studied, for the first time, all phenotypes of isolated single SANC, including rhythmically firing cells ('rhythmic SANC'), dysrhythmically firing cells ('dysrhythmic SANC'), and cells without any apparent rhythmic activity ('dormant SANC').

The contemporary view on cardiac pacemaker function dictates that SANC generate action potentials (AP) via a coupled clock system, involving complex interaction between electrogenic proteins of the plasma membrane (the membrane or M clock) and the Ca^{2+} pumping and release apparatus of the sarcoplasmic reticulum (SR, i.e. the Ca^{2+} clock) [2]. The Ca^{2+} clock generates spontaneous, rhythmic diastolic local Ca^{2+} releases (LCRs), which activate inward $\text{Na}^+/\text{Ca}^{2+}$ exchanger current (I_{NCX}), which in turn, accelerates diastolic depolarization, culminating in both an AP and the associated AP-induced cytosolic Ca^{2+} transient [3].

To study Ca^{2+} clock function in dormant, dysrhythmic, and rhythmic SANC, we recorded both AP-induced cytosolic Ca^{2+} transients and LCRs in a substantial number of cells ($n = 215$) using a high-resolution 2D camera. In prior studies, where AP and AP-induced cytosolic Ca^{2+} transients were measured simultaneously, we demonstrated that both measures of AP cycle length (APCL) are identical [4]. We have found that all cells, including dormant and dysrhythmic SANC, generate LCRs at baseline. β -adrenergic receptor (β AR) stimulation increased LCR size and enhanced temporal synchronization of LCR occurrences in both dormant and dysrhythmic cells. About one-third of dormant SANC developed automaticity in response to β AR stimulation, as LCRs became coupled to spontaneous AP-induced cytosolic Ca^{2+} transients. Conversely, dormant SANC that did not develop automaticity showed no significant change in average LCR characteristics. The majority of dysrhythmic cells also became rhythmic in response to β AR stimulation, with the rate of AP-induced cytosolic Ca^{2+} transients substantially increasing. Our results suggest that the enhancement and synchronization of LCRs are associated with increases in rate and rhythm of AP-induced cytosolic Ca^{2+} transients. Our numerical model simulations indicate that dysrhythmic and dormant cells have uncoupled or only partially coupled Ca^{2+} and membrane clocks, but these cells can fire rhythmically in response to β AR stimulation as the clocks become fully coupled.

2. Methods

2.1. Single cell preparation

SANC were isolated from 30 male guinea pigs in accordance with NIH guidelines for the care and use of animals, protocol # 034-LCS-2019 (as previously described for rabbits) [5]. Hartley guinea pigs (Charles River Laboratories, USA) weighing 500–650 g were anesthetized with sodium pentobarbital (50 – 90 mg/kg). The heart was removed quickly and placed in solution containing (in mM): 130 NaCl, 24 NaHCO_3 , 1.2 NaH_2PO_4 , 1.0 MgCl_2 , 1.8 CaCl_2 , 4.0 KCl, 5.6 glucose equilibrated with 95% O_2 / 5% CO_2 (pH 7.4 at 35 °C). The SA node region was cut into small strips (~1.0 mm wide) perpendicular to the crista terminalis and excised (Fig. 1A). The final SA node preparation consisted of SA node strips attached to the small portion of crista terminalis. The SA node preparation was washed twice in Ca^{2+} -free solution containing (in mM): 140 NaCl, 5.4 KCl, 0.5 MgCl_2 , 0.33 NaH_2PO_4 , 5 HEPES, 5.5 glucose, (pH = 6.9) and incubated on a shaker at 35 °C for 30 min in the same solution with the addition of elastase type IV (0.6 mg/ml; Sigma, Chemical Co.), collagenase type 2 (0.8 mg/ml; Worthington, NJ, USA), protease XIV (0.12 mg/ml; Sigma, Chemical Co.), and 0.1% bovine serum albumin (Sigma, Chemical Co.). The SA node preparation was washed in modified Kraftbruhe (KB) solution, containing (in mM): 70 potassium glutamate, 30 KCl, 10 KH_2PO_4 , 1 MgCl_2 , 20 taurine, 10 glucose, 0.3 EGTA, and 10 HEPES (titrated to pH 7.4 with KOH), and kept at 4 °C for 1 h in KB solution containing 50 mg/ml polyvinylpyrrolidone (PVP, Sigma, Chemical Co.). Finally, cells were dispersed from the SA node preparation by gentle pipetting in the KB solution and stored at 4 °C for subsequent use in our experiments for 8 h.

2.2. 2D Ca²⁺ imaging of single cells

Ca²⁺ dynamics within isolated single SANC were measured by 2D imaging of fluorescence emitted by the Ca²⁺ indicator Fluo-4 AM (Invitrogen) using a Hamamatsu C9100–12 CCD camera (100 frames/sec), with an 8.192 mm square sensor of 512 × 512 pixels resolution), as previously described [6]. The camera was mounted on a Zeiss Axiovert 100 inverted microscope (Carl Zeiss, Inc., Germany) with x63 oil immersion lens and a fluorescence excitation light source CoolLED pE-300-W (CoolLED Ltd. Andover, UK). Fluo-4 fluorescence excitation (blue light, 470/40 nm) and emission light collection (green light, 525/50 nm) were performed using the Zeiss filter set 38 HE. Cells were loaded with 5 μM Fluo-4AM (Sigma-Aldrich, USA) for 20 min at room temperature. The physiological (bathing) solution contained (in mM): 140 NaCl; 5.4 KCl; 2 MgCl₂; 5 HEPES; 1.8 CaCl₂; pH 7.3 (adjusted with NaOH). Data acquisition was performed using SimplePCI (Hamamatsu Corporation, Japan) at physiological temperature of 35 °C ± 0.1 °C during continuous perfusion of physiological solution followed by 1 μM isoproterenol. Only cells of typical SANC morphologies were recorded.

2.3. Detection and analysis of the whole ensemble of AP-induced cytosolic Ca²⁺ transients and LCRs in 2D Ca²⁺ imaging

AP-induced cytosolic Ca²⁺ transients and LCRs from the 2D Ca²⁺ recording were detected and analyzed by the method developed by Monfredi et al. [6] with our recent modification of computer-based analysis automation [7]. The program “XYT Event Detector” is freely available on NIA/NIH website <https://www.nia.nih.gov/research/labs/xyt-event-detector>.

In short, the program detects LCR birth/death events by a differential, frame-to-frame sensitivity algorithm applied to each pixel (cell location) in a series of images generated by the camera. An LCR is detected when its signal changes sufficiently quickly within a sufficiently large area. The LCR dies when its amplitude decays substantially or when it merges into a rising AP-induced cytosolic Ca²⁺ transient. LCRs and AP-induced cytosolic Ca²⁺ transients were isolated from noise by applying a series of spatial filters that set the minimum pixel size and the brightness threshold for LCRs. Cells with mechanical contractile movements were affixed by tracking points along the mid-line of the moving cell by using a computer program “SANC Analysis” described in our recent study [7]. The algorithm uses these points as a coordinate system for affine transform, producing a transformed image series that are stationary. The program is available as a free ImageJ plugin on the website <http://scepticalphysiologist.com/code/code.html> created and maintained by Dr. Parsons.

2.4. Assessing SR Ca²⁺ content by caffeine application

Cells were plated onto laminin (Sigma-Aldrich, USA; Cat. No. L2020) - coated glass bottom culture dishes (MatTek Corporation, USA; Cat. No. P35G-0–14-C) and were loaded with Fluo-4AM. Cells were superfused with a physiological solution at a physiological temperature, as described above. A brief 1 s pulse of caffeine (10 mM) was applied onto individual cells by pressure-ejection using a Picospritzer II Valve (General Valve corporation, USA) from a nearby glass pipette. The peak amplitude of caffeine-induced Ca²⁺ transient signal was measured in baseline, after 5 min of superfusion with 1 μM

isoproterenol, and after 7 min of washout. After subtracting background signal, the amplitude of caffeine-induced Ca^{2+} transient was normalized to the lowest fluorescent signal recorded before the caffeine pulse (F_0) and represented as F/F_0 .

2.5. Immunostaining Guinea pig SANC for HCN4

Isolated single SANC were plated onto laminin (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. L2020) - coated glass bottom culture dishes (MatTek Corporation, Ashland, MA, USA; Cat. No. P35G-0–14-C). Cells were incubated at room temperature for 1.5 h to allow for attachment, then fixed for 15 min at room temperature using high purity 4% formaldehyde in PBS, pH 7.3 (Image-iT® Fixation/Permeabilization Kit; ThermoFisher Scientific, Waltham, MA, USA; Cat. No. R37602), washed 3×5 min in washing solution (PBS, pH 7.4), and permeabilized with 0.5% Triton-X-100 in PBS, pH 7.4, for 15 min at room temperature. Samples were again washed 3×5 min then treated for 30 min at room temperature with Image-iT® FX Signal Enhancer solution (ThermoFisher Scientific, Waltham, MA, USA; Cat. No. i36933), washed 3×5 min and subsequently blocked overnight with 10% goat serum in PBS, pH 7.4 (ThermoFisher Scientific, Waltham, MA, USA; Cat. No. 50197Z). Cells were probed overnight with rabbit anti-HCN4 antibody (Abcam, Cambridge, MA, USA; Cat. No. ab69054) at a 1:100 dilution in blocking buffer, then washed 3×10 min followed by detection with Alexa Fluor 488 conjugated goat anti-rabbit IgG secondary antibody (ThermoFisher Scientific, Waltham, MA, USA; Cat. No. A-11008) diluted 1:1000 in blocking buffer for 1 h at room temperature. After a final series of washes (3×10 min.), plates were mounted with Vectashield antifade mounting medium containing DAPI nuclear counterstain (blue fluorescence) (Vector Laboratories, Burlingame, CA, USA; Cat. No. H-1200) and cells imaged using an EVOS FL cellimaging system (ThermoFisher Scientific, Waltham, MA, USA). Cells probed with secondary antibody only were used as a negative control.

2.6. Statistics

Data are presented as mean $\pm$ SEM. The statistical significance of the effects of β AR stimulation on LCR characteristics was evaluated by one-way ANOVA, paired *t*-test or Student *t*-test as indicated in figure legends. A value of $p < 0.05$ was considered statistically significant. Statistics in bar graphs are given in the form of box-and-whisker plots. Details of additional statistical analyses are described in respective figure legends.

3. Results

3.1. Characterization of the entire ensemble of LCRs and action potential cycle length (APCL) in isolated single guinea pig SANC using a 2D camera

Cells isolated from the entire SA node region of the guinea pig heart (Fig. 1A) exhibit marked heterogeneity in morphology as well intracellular Ca^{2+} dynamics at baseline (Fig. 1B–C). All 148 SANC generated LCRs, but only in about half were these LCRs accompanied by AP-induced cytosolic Ca^{2+} transients. One-third of SANC exhibiting AP-induced cytosolic Ca^{2+} transients showed dysrhythmic rather than rhythmic firing (Fig. 1C and Movie 1). Rhythmicity of APCL was assessed by using coefficient of variation (CV) i.e. Standard Deviation/Mean. SANC with CV in APCL $> 10\%$ were defined as dysrhythmic. In

2D Ca^{2+} recordings, LCR size is defined as the total area propagated by an LCR (Fig. 2A), and LCR period is the time from the peak of prior AP-induced cytosolic Ca^{2+} transient to LCR occurrence (Fig. 2B). We found that a higher rhythmicity in AP-induced cytosolic Ca^{2+} transients at baseline was linked to distinct LCR properties: LCRs in rhythmically firing SANC were fewer but larger and longer lasting than those in dysrhythmic SANC (Fig. 2C–E). Dormant SANC, which had no AP-induced cytosolic Ca^{2+} transients under baseline conditions, had the smallest LCR size, the shortest LCR duration, and the greatest number of LCRs (Fig. 2C–E). Histograms of LCR size and period provide additional insights into the distribution of LCR characteristics among cells (Fig. 2G–H). The histogram of LCR sizes revealed a population of large LCRs in rhythmic SANC (bins $> 100 \mu\text{m}^2$ in red). The LCR period histogram revealed a broad distribution of LCR occurrences for the dysrhythmic cell population, but a more focused distribution of LCR occurrences, i.e. more synchronized, for the rhythmic cell population (confirmed by our statistical analysis given in Fig. 2 legend).

3.2. βAR stimulation converts dysrhythmic firing to rhythmic

βAR stimulation significantly reduced the variability (CV) and the mean APCL in all 21 dysrhythmic SANC studied, and 81% of them began to fire rhythmically (i.e. developed a $\text{CV} < 10\%$; Fig. 3A–D). A typical example of the transition from irregular and infrequent firing to rhythmic and frequent firing is shown in Fig. 3E.

In dysrhythmic SANC, βAR stimulation increased LCR size and LCR duration, but decreased LCR number and shortened LCR period (Fig. 4A–D). The effect of βAR stimulation on LCRs in dysrhythmic cells is demonstrated in Movies 2 and 3. Histograms of LCR size and period distributions before and during βAR stimulation highlight shifts towards larger LCR sizes and shorter LCR periods (Fig. 4E–F). In the presence of βAR stimulation, increased rhythmicity of AP-induced cytosolic Ca^{2+} transients occurred concurrently with increased LCR synchronization. Fig. 5A–B provides an example of the effect of βAR stimulation on the LCR ensemble signal measured together with whole cell Ca^{2+} signal in the same cell. Note that the LCR ensemble becomes more synchronized towards late diastole with βAR stimulation. This point is further supported by the autocorrelation function of the LCR ensemble signal in Fig. 5C. Indeed, the autocorrelation function of the LCR ensemble signal shows distinct peaks in the presence of βAR stimulation.

3.3. βAR stimulation effects in dormant SANC

In response to βAR stimulation, one third of dormant SANC generated spontaneous, de-novo AP-induced cytosolic Ca^{2+} transients with variable APCL and CV (Fig. 6A–B). In fact, more than half of these newly activated previously dormant cells transitioned from dormancy through a dysrhythmic stage to rhythmicity. A typical example of the reversible transition from dormancy to rhythmic and frequent firing is shown in the three panels of Fig. 6C and Movies 4 and 5. An example of the transition to rhythmic firing is shown in Fig. 6D and Movie 6. Generation of spontaneous AP-induced cytosolic Ca^{2+} transients in dormant SANC was accompanied by significant changes in LCR characteristics, including increased LCR size and LCR duration (Fig. 7). Thus, LCRs transformed from small release events to large, propagating LCRs (Movies 4 and 5). The emergence of rhythmicity is linked to

increased LCR synchronization, as highlighted by distinct high-amplitude peaks in autocorrelation function of the LCR ensemble signal (Fig. 8, shown is an example of the analysis in one of 7 cells examined). In contrast to the dormant SANC that gained automaticity with β AR stimulation, nonresponsive dormant SANC showed no significant changes in average LCR size, duration, and number per second (Fig. S1A–C). Similarly, the distributions of LCR sizes and durations showed little or no shift in response to β AR stimulation (Fig. S1D,E, statistical analysis is provided in the figure legend).

3.4. Comparison of firing rhythms in rhythmic, dysrhythmic, and dormant SANC at baseline and in response to β AR stimulation

We tested whether dysrhythmic and dormant SANC were able to reach the level of physiological functionality of AP firing rates inherent to spontaneously and rhythmically firing cells. On average, the emergent spontaneous cycle length in dormant cells that began to fire AP-induced transients was notably longer than that in dysrhythmic and rhythmic cell populations in response to β AR stimulation (763 ms vs. 496 ms and 465 ms, respectively, Fig. S2). We further analyzed the data via APCL histograms before and after β AR stimulation. This allowed us to compare the distribution of APCL bin-by-bin for all cell populations in terms of their ultimate functional properties, i.e. the ability to generate AP-induced cytosolic Ca^{2+} transients at physiological rates. The distributions of APCLs in dysrhythmic and dormant SANC during β AR stimulation revealed that these cell types can fire at about the same rate as rhythmic SANC under baseline conditions (Fig. 9A). The APCL center around the 600 ms bin. Moreover, the distributions of cycle-to-cycle APCL variability in initially dormant and dysrhythmic cells in response to β AR stimulation became substantially overlapping within the range of rhythmic firing ($\text{CV} < 10\%$ shown by dashed square in Fig. 9B). This is especially evident in a closer (bin-by-bin) inspection of the histograms. For example, in the presence of β AR stimulation 36% of dormant cells and 19% of dysrhythmic cells fall within the 4-to-6% CV bin, and 16% of dormant cells and 33% of dysrhythmic cells fall within the 6-to-8% CV bin.

3.5. Effects of nonselective phosphodiesterase (PDE) inhibition in dormant cells

As we mentioned above, only a third of dormant cells became firing in the presence of β AR stimulation. This may happen if the link from the receptors to downstream signaling cascades was damaged during enzymatic isolation of individual cells from the SA node. Thus, we performed additional experiments to test the fidelity of the downstream signaling by directly activating of cAMP signaling via phosphodiesterase (PDE) inhibition. The idea underlying this test was that if all (or almost all) dormant cells become firing in the presence of PDE inhibition (i.e. increased cAMP), then this would indicate that in many cells the β ARs are not linked to the downstream signaling. Our experiments showed, however, that the percentage of cells responding to a nonspecific PDE inhibitor IBMX (12 from 29 cells tested, i.e. $\sim 41\%$) was about the same as the percentage (33%) of cells responding to β AR stimulation. Furthermore, IBMX changed LCR characteristics in a way similar to β AR stimulation. i.e. LCR size increased, duration increased, and number of events decreased (Fig. S3). Because β AR and cAMP signaling operate in sequence, these results indicate that dormant cells with efficient downstream signaling most likely also have normal functional link to β AR (and vice versa).

3.6. SR Ca^{2+} loading in rhythmic, dysrhythmic, and dormant SANC

Because SR Ca^{2+} content is an important measure of Ca^{2+} clock function, we tested and compared this in the three different cell populations (57 cells total from 7 animals) by measuring respective amplitudes of Ca^{2+} transients induced by abrupt application of caffeine (10 mM) via a pico-spritzer. Our results indicate that the SR content in dysrhythmic and dormant cells vary over a wide range that substantially overlaps with the range measured in rhythmic cells, i.e. there was no statistically significant difference in the respective mean values (Fig. S4A). This indicates that some cells within the three cell populations appear to have similar SR content. It is also possible that some cells with a high SR Ca^{2+} content can still be dormant or dysrhythmic, but some cells with a low SR Ca^{2+} content can be rhythmic. Additional LCR analyses performed in 9 dormant cells and 9 firing cells showed no correlation of the LCR parameters with the respective SR Ca^{2+} content measured in the same cells (Fig. S4B).

Finally, we measured the SR content in dormant cells with respect to their capability of firing spontaneous AP-induced transients in response to βAR stimulation (see an example in Fig. S4C and statistical data in Figs S4D,E). While, on average, the non-responding dormant cells had a lower amplitude of caffeine-induced transient ($\Delta F/F_0$ 3.4 vs. 4.2) at base line, this difference was not statistically significant. In the presence of βAR stimulation, however, the SR Ca^{2+} content of the non-responding dormant cells showed a statistically significant decline ($\Delta F/F_0$ from 3.4 to 2.4, $p < 0.05$), whereas it remained about the same in cells responding to βAR stimulation with spontaneous firing. Thus, the SR content, *per se*, does not determine the automaticity of SANC, LCR parameters, or response to βAR stimulation.

3.7. Dormant cells are not atrial cells: immunostaining guinea pig SANC for HCN4

A small fraction of atrial cells has been reported in SA node. Verheijck et al. [8] detected 22% to 29% cells of atrial morphology in different SA node regions. Since both atrial cells and dormant cells do not exhibit automaticity in the basal state, could the dormant cells that we have studied here be those atrial cells? We have several lines of evidence that this is not the case, rather these dormant cells represent a previously unstudied SANC population, clearly distinct from atrial cells.

First, in our study the fraction of dormant cells was substantially larger ($> 50\%$, Fig. 1C) than what was found by Verheijck et al. [8]. When visually selecting cells for study, we deliberately avoided cells with typical atrial cell morphology (i.e. blocky and rectangular in shape) (Fig. 1B). Next, SA node-residing atrial cells have a resting membrane potential between -70 and -80 mV [8], whereas our pilot measurements showed that dormant cells exhibit a resting membrane potential of about -35 mV [9]. In the presence of βAR stimulation, some dormant cells gained automaticity (Fig. 6), but atrial cells under normal conditions remained silent, retaining their low resting membrane potential close to -80 mV [10]. Finally, we have performed HCN4 immunostaining of SANC isolated from guinea pig (atrial cells do not express HCN4) and found that a substantial part of all single cells that we isolated from the SA node (443 of 509, i.e. 87.03%) were positive for HCN4 (see example of staining in Fig. S5). This result lends significant evidence to our assertion that our

dormant cell population, which accounted for more than half of the surveyed cells in this study, were indeed SANC rather than atrial cells.

3.8. Numerical model simulations

Although our experiments focused on Ca^{2+} measurements, we employed numerical simulations using our model of SANC operating as a coupled-clock system [11] to glean insight into clock coupling within the 3 populations of cells identified and examined in our experiments. The clock coupling is simulated and examined by the model parametric sensitivity analysis in 2 dimensions (Fig. S6). The X axis represents g_{CaL} , the maximum conductance of L-type Ca^{2+} current (I_{CaL}), i.e. M clock function. The Y axis represents SR Ca^{2+} pumping rate (parameter P_{up}), i.e. Ca^{2+} clock function. The clocks are uncoupled in parametric space with low P_{up} and I_{CaL} (blue area) and the respective models do not fire APs. The clocks are coupled in parametric space with higher P_{up} and I_{CaL} and the respective models fire APs with increasing rates as the coupling increases (red shades coding respective firing rates). Intermediate models with partial clocks coupling generate dysrhythmic firing (red mosaic area). Representative examples of membrane potentials generated by each model type are shown in Fig. S6B. Thus, based on this model analysis, we are able to recapitulate experimentally observed SANC cohorts - dormant, dysrhythmic and rhythmic cells as cells with various degree of clocks coupling.

We simulated components of Ca^{2+} clock, M clock, and I_{NCX} that couples the clocks in representative models of different cell types (Figs S7–S9, respectively). Ca^{2+} release flux and Ca^{2+} in the submembrane space remain flat in a modeled dormant cell, whereas they both exhibit small amplitude oscillations in the intervals between the AP-induced Ca^{2+} transients in a modeled dysrhythmic cell. In contrast, a modeled rhythmic cell exhibits substantial diastolic Ca^{2+} release (before each AP-induced Ca^{2+} transient). The SR Ca^{2+} load reflected by variable Ca_{up} ($[\text{Ca}^{2+}]$ in SR uptake compartment, i.e. the free SR) is the highest in the example of the rhythmic cell model chosen here (Fig. S7C). However, in general Ca_{up} depends on P_{up} ; and according to the diagram of our parametric sensitivity analysis in Fig. S6, Ca_{up} can be higher in a dormant cell with a low g_{CaL} . For example, a dormant cell model with P_{up} of 10 mM/s and g_{CaL} of 0.3 nS/pF reaches Ca_{up} as high as 0.817 mM, whereas a rhythmic cell model with P_{up} of 3 mM/s and g_{CaL} of 0.52 nS/pF shows Ca_{up} oscillations at a much lower level of 0.45 mM (Fig. S8).

Additional simulations of I_{CaL} and I_f in representative cell models show that in rhythmic cells, the amplitudes of both currents substantially increases (Fig. S9) along with the respective increase in diastolic Ca^{2+} release (Fig. S7A). Thus, in rhythmic cells the clocks become fully operational and strongly coupled via diastolic I_{NCX} , which also substantially increases (Fig. S10).

4. Discussion

This study is the first to describe LCRs and AP-induced cytosolic Ca^{2+} transients in the full phenotypic ensemble of SANC enzymatically isolated from guinea pig SA node. The ensemble of cells is represented by three major populations: rhythmic, dysrhythmic, and dormant cells. While dormant cells show no automaticity in the basal state, they represent a

previously unstudied population of single isolated SANC, distinct from atrial cells (see Results Section 3.7 and Fig. S5).

4.1. Emergence of order within and among cells with increased LCR size, duration and synchronized onset during β AR stimulation

We have demonstrated that, under baseline conditions, LCRs are generated not only by rhythmically firing SANC, but also by dys-rhythmic and even dormant SANC populations. This indicates that LCR generation is a fundamental property of SANC SR, serving as a Ca^{2+} clock [2] in all SANC. The Ca^{2+} clock's fidelity, however, is different in each cell population and manifested by different types of LCR activity. The LCRs in dormant cells and dysrhythmic cells appear irregular and small, much smaller than the large LCRs exhibited by rhythmic cells (Fig. 2C). The decrease in LCR count per unit time and larger path size in rhythmic cells are linked to LCR propagation via the Ca^{2+} -induced- Ca^{2+} release mechanism that recruits neighboring Ca^{2+} release channels (ryanodine receptors) to release Ca^{2+} .

In addition to ryanodine receptor recruitment by Ca^{2+} -induced- Ca^{2+} release, the increase in LCR size could also be linked to the ability of neighboring ryanodine receptors to be recruited at a given time, or to be spontaneously activated at a given time, characteristics that are presumably governed/modulated by β AR stimulation via phosphorylation-dependent mechanisms [16]. Indeed, small LCRs were converted to large propagating LCRs during β AR stimulation (Figs. 4 and 7) in all dysrhythmic SANC, and even in one third of dormant SANC. Importantly, the LCR period also became substantially shorter (on average by 60%) in dysrhythmic cells in response to β AR stimulation. Most importantly, the LCRs became more rhythmic and synchronized to late diastole (see autocorrelation analyses in Figs. 5 and 8). As these changes in LCR characteristics emerged in response to β AR stimulation, these cells concurrently began to fire rhythmically (exhibiting a much smaller APCL variation, Fig. 3D).

Dormant cells, by definition, do not generate AP-induced cytosolic Ca^{2+} transients; their LCRs remain uncoupled from the M clock. Additional evidence for the importance of LCRs in gaining automaticity by dormant cells in response to β AR stimulation can be drawn from our finding that nonresponsive dormant SANC show no significant changes in average LCR size, duration, and number per second (Fig. S1).

In addition to synchronizing the Ca^{2+} releases in time and space and increasing the rate and rhythm of AP-induced cytosolic Ca^{2+} transients within individual SANC, β AR stimulation also brought order to the SANC population as a whole. The distributions of APCL and cycle-to-cycle APCL variability in the entire ensemble of isolated single SANC demonstrate that the slow, dysrhythmic SANC were pulled in to join the faster, rhythmic SANC while the faster SANC changed only slightly during β AR stimulation (Fig. S2). In other words, β AR stimulation shifted the order of the SANC population from being diffuse to self-similar. This shift from disorder to order occurs at many hierarchical levels: firstly at the level of LCRs (Fig. 7) followed by AP-induced cytosolic Ca^{2+} transients (Fig. 6), and finally at the level of SANC populations (Fig. 9). Perhaps this wide degree of order present inside the cells

confers upon them the crucial characteristic of flexibility, to change their functionality in accordance to external stimuli.

4.2. Varying degrees of clock coupling account for automaticity phenotypes in numerical model simulations

While LCRs are generated by the SR, they are, in fact, a manifestation (and the essence) of the coupled clock system [2], which crucially includes surface membrane ion channels and transporters. We did not measure ionic currents in the present study, but many previous studies have reported substantial cell-to-cell variability in functional expression of ion channels in SANC [12–17]. For example, L-type Ca^{2+} current density varies several-fold on a cell-to-cell basis [13,16,17]. Cells with such different electrophysiological properties must differ in their behavior and function both *in vivo* and *in vitro*. We have demonstrated previously (via numerical model simulations) that AP firing activity in SANC is a function of both the SR Ca^{2+} pumping rate (an important component of the Ca^{2+} clock) and the L-type Ca^{2+} current density (a key component of the M clock) [11,18]. Depending on the degree of clock coupling, SANC models exhibit a bifurcation in firing activities, from quiescent to chaotic to rhythmic firing, as shown in the sensitivity analysis diagram (Fig. S6). Amplitude of I_{NCX} is both voltage and Ca^{2+} dependent. In our simulations substantially different amplitudes of diastolic I_{NCX} (Fig. S10) mediate the varying degrees of coupling between Ca^{2+} clock components (Fig. S7) and ion currents of M clock (Fig. S9). Because βAR stimulation increases both L-type Ca^{2+} current and SR Ca^{2+} pumping rate, the diagram of sensitivity analysis also predicts the key findings of the present study, i.e. transitions from dormancy to rhythmic firing and from dysrhythmic firing to rhythmic firing (green vectors in Fig. S6). βAR s in dormant cells that fire AP-induced Ca transients in response to isoproterenol are indeed coupled to cAMP signaling because the percentages of cells responding to βAR stimulation and broad-spectrum specific PDE inhibition in our experiments with IBMX were roughly the same (33% vs. 41%, results section 3.6.). cAMP signaling, in turn, regulates and couples both clocks, e.g. cAMP-dependent PKA regulates both SR Ca^{2+} pump and I_{CaL} , and cAMP directly regulates I_f .

We also used the coupled-clock model to interpret our experiments that assessed the SR Ca^{2+} content in different cell populations (using abrupt application of caffeine). The experimental results summarized in Section 3.7 and Fig. S4 indicate that the SR content, *per se*, does not determine the automaticity of SANC, LCR parameters, or response to βAR stimulation, thus indicating the importance of M clock (i.e. the Ca^{2+} clock's partner) in determining the automaticity phenotype of a cell and its response to βAR stimulation. The SR Ca^{2+} content is reflected by variable Ca_{up} in our model that depends on P_{up} , i.e. SR Ca^{2+} pumping rate. The cell automaticity, in turn, emerges as synergistic operation of Ca^{2+} clock and M clock functions that is reflected in the right upper corner of the $P_{\text{up}}/I_{\text{CaL}}$ parametric sensitivity diagram in Fig. S6. This diagram and examples of Ca_{up} simulations in different models in Figs. S7C and S8 show that the cell automaticity can still emerge at low Ca_{up} , but this will require a synergistic input of a larger I_{CaL} density. In contrast, even cells even with a high P_{up} and a respectively higher SR Ca^{2+} content can remain dormant if the I_{CaL} density is relatively small.

4.3. Relevance to SA node function

Using small tissue pieces dissected from rabbit SA node, Opthof et al. confirmed that different regions of the tissue generate different frequencies of APs, with some areas being significantly slower than the intact SA node taken as a whole [19]. They discovered that peripheral small pieces of SA node from regions bordering the crista terminalis had faster firing rates than pieces dissected from the “primary pacemaker site”. SA node pieces bordering the left atrium were quiescent when dissected, but became activated to fire APs in the presence of adrenaline or acetylcholine. The small slices of the central node region of rabbit SA node prepared by Brown et al. [20] were similarly quiescent. In a recent study by Torrente et al. [21], SANC of sodium-calcium exchanger knockout mice became quiescent when isolated, whereas the intact SA node had pacemaking activity.

Furthermore, the primary pacemaker shift phenomenon that occurs within the SA node during autonomic nervous system signaling and in response to a variety of local mediators may indicate that different firing rates are driven by different cells within the SA node [22]. Alternative pacemakers and “sinoatrial conduction pathways” within the SA node can function as subsidiary sites of automaticity, guaranteeing ongoing pacemaking in times of physiological crisis when the primary pacemaker becomes preferentially ‘inhibited’ by an external stimulus or alternatively the subsidiary areas become ‘activated’ [23]. Our finding of different functional cell populations isolated from SA node may corroborate, at least in part, these prior findings concerning different firing rate of parts (pieces or slices) of the SA node [19,20]. Recent recording of Ca^{2+} in the intact mouse SA node using high-resolution cameras also indicate that some HCN4 positive cells within the intact SA node do generate LCRs, but do not generate APs, with the SAN generating normal physiological rhythm at its exits [24]. Those cells in the intact SA node are similar to the isolated dormant cells described in the present study (many of them also HCN4-positive, see Fig. S5).

The results of our study, however, cannot be directly extrapolated to the tissue level. Indeed, the heterogeneity measured in isolated single SANC may not occur identically *in vivo* because we cannot exclude the possibility that the cells of different types exist as a consequence of the isolation procedure. Furthermore, SANC exhibit markedly different behavior when they are interacting with each other at the tissue level. As a matter of fact, even two interacting cells under “coupling clamp” show new emergent properties as a system of two cells [25], not to mention even more complex behaviors that are likely to arise when numerous cells interact within the intricate network of SA node tissue. Mutual pacemaker synchronization of coupled cells results in part from the phase-resetting effects of the AP of one cell on the depolarization phase of the other [25]. According to the “democratic” model of pacemaking suggested by Michaels et al. [26], cells that differ in firing rate all contribute to pacemaking via electrical coupling. Finally, the SA node is comprised of many cell types that interact via different mechanisms, including electrical, chemical, mechanical. For example, intertwined with SANC are fibroblasts that express either Connexin40 or Connexin45 depending on whether they are surrounded by other fibroblasts or myocytes [27]. Also, a dense and complex ganglionated neural network has been reported in the SA node [28].

Following the logic above, when cells interact, their behaviors will likely deviate substantially from those described here for single cells isolated from their natural environment. But, the same logic applied in reverse could be relevant to our study; cell behaviors, like those reported here in isolated cells, might be expected to occur in intact SA node when cells are (or are becoming) less communicative whether it is due to a normal, pathological, or aging process. Furthermore, even if some of the dormant cells, such as those that failed to respond to β AR stimulation (or PDE inhibition) or had very low SR Ca^{2+} content were indeed damaged (e.g. due to cell isolation procedure), their behaviors could be still of interest in future studies with respect to pathological conditions in the SA node.

5. Conclusions

Isolated single SANC fall into three major populations that include, in addition to the well-described rhythmically firing cells, dormant and dysrhythmically firing SANC. These latter two previously unstudied populations of cells can be reversibly recruited to fire rhythmic AP-induced Ca^{2+} transients during β AR stimulation. We interpret our results of experimental measurements of Ca^{2+} based on numerical model simulations which showed that 1) dysrhythmic and dormant cells can originate from cells with intrinsically uncoupled or only partially coupled Ca^{2+} and membrane clocks and 2) such cells can fire rhythmically in response to β AR stimulation as the coupling between the clocks becomes enhanced.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

SA node	Sinoatrial node (the primary pacemaker of the heart)
SANC	SA node cell
AP	action potential
Cell automaticity	ability of a cell to generate spontaneous, rhythmic APs
APCL	AP cycle length
SR	sarcoplasmic reticulum
AP	induced cytosolic Ca^{2+} transient, whole-cell cytosolic Ca^{2+} spike triggered by AP via L-type Ca^{2+} channel activation and its attendant Ca^{2+} -induced Ca^{2+} release from SR

LCR	local Ca^{2+} release from SR during diastolic depolarization
LCR period	the time period between the peak of the prior AP-induced cytosolic Ca^{2+} transient and subsequent LCR onset
Ca^{2+} clock	SR with Ca^{2+} pump and Ca^{2+} release channels generating rhythmic LCRs
M clock	ensemble of membrane electrogenic proteins (ion channels and ion exchangers) generating AP
Coupled clock	a contemporary concept of cardiac pacemaker cell function, i.e. Ca^{2+} clock generates diastolic rhythmic LCRs and respective $\text{Na}^+/\text{Ca}^{2+}$ exchanger current accelerating diastolic depolarization, but M clock generates both APs and Ca^{2+} influx providing Ca^{2+} to Ca^{2+} clock
βAR	β -adrenergic receptor; Rhythmic
SANC	SANC rhythmically firing AP-induced Ca^{2+} transients
Dysrhythmic SANC	SANC dysrhythmically firing AP-induced Ca^{2+} transients
Dormant SANC	SANC generating no AP-induced Ca^{2+} transients
CV	coefficient of variation (i.e. Standard Deviation/Mean)

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Fig. 1.

Single SANC isolated from guinea pig heart exhibit a wide variety of morphologies and are heterogeneous in their intracellular Ca^{2+} dynamics at baseline. A) Location of guinea pig SA node in relation to the whole heart during our isolation procedure. LA = left atrium; LV = left ventricle; RA = right atrium; RV = right ventricle; TCV = tricuspid valve; CT = cristae terminalis; SAN = sinoatrial node. B) Typical morphologies of the three cell populations (dormant, dysrhythmic, and rhythmic SANC). The morphologies were similar in all three cell populations. C) Population survey of single SANC with respect to their three distinct patterns of Ca^{2+} dynamics (i.e. dormant, dysrhythmic, and rhythmic SANC).

Fig. 2.

Heterogeneous LCR properties in the three SANC types: Rhythmic SANC have fewer but larger LCRs with more synchronized periods than dysrhythmic dormant SANC. A) LCR size was defined as total propagation area of an LCR. LCR dynamics are demonstrated in four consecutive images acquired with 10 sampling interval. B) Illustration of the LCR period, defined as the time period between the peak of the prior AP-induced cytosolic Ca^{2+} transient and subsequent onset. Inset shows region of interest (in green) used to calculate the local Ca^{2+} signal. C-F) Comparisons of LCR characteristics among different cell populations ($n = 7$ for dormant and dysrhythmic SANC and $n = 5$ rhythmic SANC). * $P < 0.05$ via ANOVA and Student t -test. G-H) The histograms of LCR size and period distributions in dormant, dysrhythmic, and rhythmic guinea pig SANC at baseline (based on measurements of 2617, 2956, and 488 LCRs in each cell type, respectively). Please note: Panel G has two different Y scales separated by a white band to clearly show infrequent,

larger size events. For data in panel H we used modified, robust Brown-Forsythe Levene-type test based on the absolute deviations from the median. Test Statistic was 56.334 and p-value = 7.019×10^{-14} . Thus, two variances are not the same and the distributions for rhythmic and dysrhythmic SANC are statistically different. This analysis indicates that the distribution LCRs in rhythmic cells is more focused than that in other cells (For interpretation of the references to colour in this figure legend, the reader is referred to the version of this article.).

Fig. 3. β AR stimulation shortens AP-induced cytosolic Ca^{2+} transient cycle length and decreases cycle length variability in all dysrhythmic SANC. A–D) Effect of β AR stimulation (1 μM isoproterenol) on the mean value and variability about the mean value (reported as coefficient of variation) of time series of AP-induced cytosolic Ca^{2+} transient cycles in 21 dysrhythmic SANC. * $P < 0.05$ via paired t -test, $n = 21$. Horizontal dashed line in B indicates a border between rhythmic and dys-rhythmic firing. E) An example of the transition from dysrhythmic and rare firing to rhythmic and frequent firing with β AR stimulation.

Fig. 4. β AR stimulation alters major LCR characteristics in dysrhythmic cells. A–D) Average LCR characteristics before and during β AR stimulation (1 μM isoproterenol). * $P < 0.05$ via paired t -test, $n = 7$ cells. E–F) Histograms of LCR size and period distributions before (2956 total LCRs) and during β AR stimulation (1080 total LCRs) demonstrate shifts towards larger LCR sizes and shorter LCR periods.

Fig. 5.

Increased rhythmicity and periodicity of AP-induced cytosolic Ca^{2+} transients cycle lengths and synchronization of LCR ensemble signal, in response to β AR stimulation demonstrated by autocorrelation function in a representative dysrhythmic SANC. A–B) whole-cell cytosolic Ca^{2+} signal shown concurrently with individual LCRs (different colors) and LCR ensemble signal (red) before (panel A) and during of β AR stimulation (panel B) in the same cell. C) Autocorrelation function of LCR ensemble signal before and during β AR stimulation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Fig. 6.

Dormant SAN generated spontaneous de-novo AP-induced cytosolic Ca^{2+} transients in the presence of β AR stimulation. A–B) Distribution, in a time series, of de-novo AP-induced cytosolic Ca^{2+} transient cycle lengths and their coefficient of variation in 25 cells. C) Changes in the whole-cell cytosolic Ca^{2+} signal in a dormant SANC during baseline, β AR stimulation, and wash-out. Reversible rhythmic AP-induced cytosolic Ca^{2+} transients emerge from the noise in the presence of β AR stimulation. D) An example of transition of a dormant cell to rhythmic firing in the presence of β AR stimulation. See also Movie 6.

Fig. 7.

β AR stimulation induces an increase in LCR size and duration in dormant SANC. A–C) Average LCR characteristics in dormant SANC prior to and during β AR stimulation. * $P < 0.05$ via paired t -test, $n = 7$ cells. D) Histogram of LCR sizes shows a substantial shift towards larger sizes during β AR stimulation (2617 total LCRs at baseline; 1563 total LCRs during β AR stimulation).

Fig. 8.

β AR stimulation induces de-novo AP-induced cytosolic Ca^{2+} transients and increases LCR synchronization in dormant SANC. A–B) Whole-cell cytosolic Ca^{2+} signal is shown concurrently with individual LCRs (different colors) and LCR ensemble signal (red) in a typical dormant SANC before and during β AR stimulation. The growth of LCR signal in panel B is represented by red line. LCRs became synchronized to late diastole, prior to the rapid upstroke of the AP-induced cytosolic Ca^{2+} transient. C) Autocorrelation functions of LCR ensemble signal in a dormant SANC before and during β AR stimulation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Fig. 9.

Dormant and dysrhythmic SANC can achieve a similar firing rate as rhythmic SANC in the presence of β AR stimulation. Histograms of AP-induced cytosolic Ca^{2+} transient cycle lengths (A) and coefficients of variation (B) in SANC that differ in AP firing phenotypes. Both the nonparametric Kruskal Wallis test and the parametric one-way ANOVA indicated that the medians/means of the CVs in all four data groups in this panel statistically differ.