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Synchronization of sinoatrial node pacemaker cell clocks and its autonomic modulation impart complexity to heart beating intervals Short title: Beating-rate variability of sinoatrial node cells

Yael Yaniv, PhD^{1,2,*}, Ismayil Ahmet, MD PhD¹, Jie Liu, MD PhD^{1,3}, Alexey E. Lyashkov, PhD⁴, Toni-Rose Guiriba¹, Yosuke Okamoto, MD PhD¹, Bruce D. Ziman, MS¹, and Edward G. Lakatta, MD^{1,*}

¹Laboratory of Cardiovascular Science, Biomedical Research Center, Intramural Research Program, National Institute on Aging, NIH, Baltimore, Maryland, USA

²Biomedical Engineering Faculty, Technion-IIT, Haifa, Israel

³Cardiovascular Physiology Laboratory, School of Medical Sciences, F13-Anderson Stuart Building, University of Sydney, Sydney NSW 2006, Australia

⁴Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, 733 North Broadway, Baltimore, Maryland, USA

Abstract

Background—A reduction of complexity of heart-beat interval variability (BIV) that is associated with an increased morbidity and mortality in cardiovascular disease states is thought to derive from the balance of sympathetic and parasympathetic neural impulses to the heart. But rhythmic clock-like behavior intrinsic to pacemaker cells within the sinoatrial node (SAN) drives their beating, even in the absence of autonomic neural input.

Objective—To test how this rhythmic clock-like behavior intrinsic to pacemaker cells interacts with autonomic impulses to the heart-beat interval variability in vivo.

Methods—We analyzed BIV in the time and frequency domains and by fractal and entropy analyses: i) in vivo, when the brain input to the SAN is intact; ii) during autonomic denervation in vivo; iii) in isolated SAN tissue (i.e., in which the autonomic-neural input is completely absent); iv) in single pacemaker cells isolated from the SAN; and v) following autonomic receptor stimulation of these cells.

*Corresponding authors: Yael Yaniv, PhD, Biomedical Engineering Faculty, Technion-IIT, Haifa, Israel, yaely@bm.technion.ac.il, Edward G. Lakatta, MD, Laboratory of Cardiovascular Science, Biomedical Research Center, Intramural Research Program, National Institute on Aging, NIH, Baltimore, Maryland, USA, lakattae@grc.nia.nih.gov.

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Conflict of interest: None

Results—Spontaneous-beating intervals of pacemaker cells residing within the isolated SAN tissue exhibit fractal-like behavior and have lower approximate entropy than in the intact heart. Isolation of pacemaker cells from SAN tissue, however, leads to a loss in the beating-interval order and fractal-like behavior. β adrenergic receptor stimulation of isolated pacemaker cells increases intrinsic clock synchronization, decreases their action potential period and increases system complexity.

Conclusions—Both the average-beating interval in vivo and beating interval complexity are conferred by the combined effects of clock periodicity intrinsic to pacemaker cells and their response to autonomic-neural input.

Keywords

Autonomic neural impulse; Chaotic systems; Fractal behavior; Heart rate variability; Sinoatrial nodal pacemaker cells

Introduction

The heart rate never achieves a steady state because it is controlled by complex dynamic chaotic processes, oscillating at different periods over different time scales that continuously shift. Therefore, it is not surprising that the ECG in mammals, even under resting conditions, reveals complex beat-to-beat variation of heart-beat intervals.¹ Specifically, rhythmic regimes embedded within human heart-beat intervals vary from 2 to more than 25 beats. Moreover, that the heart-beat intervals obey a power law indicates that fractal-like (self-similar, scale-invariant) behavior imparts complexity to the heart rhythm.² Loss of this complexity becomes manifest as a reduction in beating interval variability (BIV), which accompanies advancing age and predicts increased morbidity and mortality in various forms of heart disease.^{3, 4}

Fractal-like behavior of heart-beat intervals in vivo has mainly been attributed to the balance of sympathetic and parasympathetic neural impulses to the heart. Stimulation of autonomic receptors of pacemaker cells (i.e., β -adrenergic receptors (β -AR) or cholinergic receptors (CR)) within the sinoatrial node (SAN) couples them to G-proteins and to adenylyl cyclases (likely type 5 or 6) or to guanylyl cyclases, leading to activation or suppression of cAMP or cGMP and protein kinase activities that regulate the phosphorylation state of proteins that drive the intrinsic pacemaker cell clocks: the intracellular Ca^{2+} cycling clock and surface membrane ion channel proteins (membrane clock).^{5, 6} Specifically, these clocks intrinsic to pacemaker cells are driven by constitutive Ca^{2+} -calmodulin activation of adenylyl cyclase-dependent protein kinase A (PKA) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), that effect phosphorylation of proteins that couple the membrane and Ca^{2+} clocks.⁵ The phosphorylation states of coupled-clock proteins are the major determinant of the rate and rhythm of spontaneous action potentials (APs) generated by pacemaker cells in the sinoatrial node. Because the kinetics of each of these phosphorylation-dependent mechanisms can vary over a wide range of time scales, we hypothesized that properties intrinsic to the pacemaker cells residing in SAN tissue may contribute to BIV in vivo and its fractal-like behavior detected by ECG analysis (review in⁴ and⁷). In other terms, we hypothesized that fractal-like behavior embedded within the heart-beat intervals in vivo is

regulated by rhythmic clock-like mechanisms intrinsic to pacemaker cells and that these mechanisms are modulated by autonomic neural input.

In order to define the relative contributions of autonomic neural input to the heart and the intrinsic properties of pacemaker cells to BIV and fractal-like behavior embedded within the beating rhythm, we analyzed beating interval dynamics: i) in vivo, when the brain input to the sinoatrial node is intact; ii) during autonomic denervation in vivo; iii) in intact isolated SAN tissue (i.e., in which the autonomic neural input is absent); iv) in single pacemaker cells isolated from the SAN; and v) following autonomic receptor stimulation of these cells (see on-line methods for further details). We demonstrate that fractal-like complexity of BIV depends on rhythmic mechanisms intrinsic to the pacemaker cells embedded within the SAN.

Methods

Heart rate measurements in vivo

All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85-23, revised 1996). The experimental protocols have been approved by the Animal Care and Use Committee of the National Institutes of Health (protocol #034LCS2013). The ECG was recorded in nine male New Zealand White rabbits that were sedated with sodium phenobarbital (5mg/kg i.v. from ear vein), intubated, and ventilated at rate of 0.66 Hz (this frequency was filtered during frequency analyses by a notch filter). Light anesthesia was maintained with isoflurane (2% v/v) in oxygen. Rabbits were placed in the supine position and the front of the neck was shaved. A 3cm long skin incision was made in the center of the neck along the trachea, muscle layers were divided carefully, and vagus nerves on both sides were isolated. ECG leads were placed in the limbs. A small skin incision was made on the right thigh and the right femoral vein was isolated. A standard ECG lead II was recorded continuously, using a Power Lab system (Adinstruments) at a sampling rate of 1 KHz (Fig. S1). After a 5-min baseline ECG recording, both vagus nerves were surgically cut, and hexamethonium, a post-ganglionic blocker, was administered at a dose of 30mg/kg in 0.3cc/kg saline, via the femoral vein. The ECG was recorded for another 10 min after injection, following which, rabbits were sacrificed for sinatrial node (SAN) tissue isolation.

Beating rate measurements in SAN

The heart was quickly excised and placed into Tyrode solution ($36 \pm 0.5^{\circ}\text{C}$) of the following composition (in mM): 140 NaCl, 5.4 KCl, 1 MgCl_2 , 5 HEPES, 1.8 CaCl_2 , and 5.5 Glucose, and titrated to pH 7.4 with NaOH. A strip of tissue containing the SAN region was identified by anatomic landmarks and was dissected. The SAN preparation was fixed in a heated bath ($36 \pm 0.5^{\circ}\text{C}$) and superfused with Tyrode solution (see above) at a rate of 4 ml/min. An insulated/Teflon-coated platinum electrode with a tip of 0.25 mm diameter was placed in the center of the SAN to record extracellular signals (Fig. S1) using a Neurolog system NL900D (Digitimer, Hertfordshire, UK).

Beating rate measurements in single pacemaker cells

Spontaneously beating sinoatrial node cells (SANC) were isolated from New Zealand White rabbit hearts as previously described.⁸ The dissociated cells were stored at 4°C and were used within 10 hours of isolation. Cells were imaged with a Motik AE31 microscope using a 40x/0.9 air lens. The cell suspension was placed in a chamber on an inverted microscope and was allowed to settle for 20 min in Tyrode solution (see above) at room temperature for better attachment to the chamber. Spontaneous beats (Fig. S1) at 35±0.5°C were recorded in single pacemaker cells using a myocyte contractility recording system (IonOptix, MA). Only SANC that beat rhythmically were chosen for study. To quantify SANC beating, cell edges were detected along the long axis of the cell. Cell contraction measurements were recorded for 10 min under control conditions and 10 min after drug application. The beating rate was calculated as the time between the successive contraction periods, and the beat intervals were analyzed by a custom made program in Matlab (MathWorks). Note that the interval between successive APs is as same as the interval between successive contractions (Fig. S2A-B). Moreover, the excitation-contraction delay remains constant and independent of the beating interval (Fig. S2C) and the excitation-contraction delay is not correlated with the previous beat interval (Fig. S2D). See online supplement for description of methods to analyze beat interval variability.

Drugs

Isoproterenol, carbachol, hexamethonium and isoflurane were purchased from Sigma.

Statistical analyses

All data are presented as mean±SD. Because some measurements were repeated in the same rabbit at different functional levels (in vivo, autonomic denervation in vivo, intact SAN and single pacemaker cells), a linear mixed-effects model was employed to determine whether the four functional states differed from each other with respect to the measured variables. The Sidak multiple comparison method was employed for post-hoc comparisons to determine which of the four functional states differed from each other (Table 1). SAS p.2 (SAS Institute Inc, NC) was used to conduct the analyses. The same method was employed to test whether drug effects on measurements in isolated pacemaker cells differed from control, and to determine whether the drug responses differed from each other (Table 2). A Chi-Square test was employed to determine whether drug treatments changed the number of cells that exhibit fractal-like behavior compared to control.

Results

BIV transitions from the heart in vivo, when autonomic neural input is intact, to isolated pacemaker tissue, when neural input is absent

Fig. 1A illustrates representative examples of beating interval series in the adult rabbit in vivo, in vivo following autonomic denervation and in SAN tissue in isolation. The average beat interval increases from 192±5 to 324±11 ms in transition from the heart in vivo to SAN tissue in isolation. Fig. 1B illustrates representative examples of beating interval histograms recorded in vivo, in vivo following autonomic denervation and in SAN tissue in isolation.

Note that the variability of beating intervals also becomes reduced in transition from the heart in vivo to SAN tissue in isolation. Table 1 lists BIV time-domain parameters (SDNN, RMSSD, CV, pNN50, see on-line supplement for definitions), which tend to become reduced following autonomic denervation in vivo, and in intact SAN tissue, in which autonomic input is completely absent. The reduction in BIV following autonomic denervation can easily be appreciated in Poincaré plots, in which each beating cycle length is plotted against its predecessor to quantify the correlation between consecutive heart-beat intervals (Fig. 2). Denervation in vivo, or isolation of the SAN, decreases the scattering pattern of the points within the Poincaré plot compared to the in vivo state, and the Poincaré plots following denervation exhibit the typical cigar-shaped scatter of points (Fig. 2).⁹

Measurements of system entropy define the degree of order among its beat intervals. An increase in system entropy reflects increasing disorder, and completely random systems exhibit maximal entropy. Approximate entropy (ApEn, Table 1) indicates that upon autonomic denervation in vivo, there is a trend toward a reduction in the system entropy (for each window size), and system entropy in isolated SAN tissue is significantly reduced compared to the heart in vivo.

We employed frequency domain analysis to further examine the complexity within the brain-pacemaker system signaling cascade. Table 1 shows that denervation of the heart in vivo tends to increase the very low frequency (VLF): total power, and tends to reduce the low frequency (LF):total power, but does not alter the high frequency (HF):total power. Thus, following denervation the LF:HF power becomes reduced. These trends observed during denervation in vivo become even more marked in the completely denervated, intact, isolated SAN tissue from the heart (Table 1). Fig. S3 demonstrates that typical LF and HF peaks disappear following denervation in vivo and in intact isolated SAN.

We used power law analyses and detrended fluctuation analysis (DFA) to detect and quantify the fractal-like behavior embedded within the beating intervals. The slope of a linear function relating log frequency to log of the power spectrum density is the fractal scaling exponent, β (Fig. 3A). Fractal-like complexity among the heart-beat intervals is present in vivo (Table 1); β increases following autonomic denervation in vivo, and further increases in the intact isolated SAN tissue, in which autonomic denervation is complete (Table 1). DFA characterizes the degree of correlation among time scales embedded within the heart-beat intervals.¹⁰ The self-similarity of frequency regimes buried within the beat intervals assessed from ECG analyses by DFA are assumed to be bi-fractal, and are described by short- and long-term exponents, α_1 and α_2 , respectively.¹⁰ Bi-fractal components are not only embedded within the BIV of the heart in vivo (Fig. 3B), but are also present within completely denervated SAN tissue, albeit with altered scaling exponents (Table 1).

BIV of single pacemaker cells following their disaggregation from SAN tissue

When disaggregated from SAN tissue, the beating intervals of isolated single pacemaker cells remain rhythmic, but the average beating interval and BIV become markedly increased (Table 1) compared to the SAN tissue or the heart in vivo (Fig. 1B). Furthermore, when isolated from SAN tissue, single pacemaker cells exhibit a marked increase in ApEn (Table

1), and points in the Poincaré plot become markedly scattered (Fig. 2) around the markedly elevated mean beat interval (Table 1). Finally, the fractal-like behavior within the beating intervals observed in the heart in vivo and in the intact SAN tissue and evidenced by the β scale factor or DFA (Table 1) does not generally extend to the single isolated pacemaker cells (only 9 of 65 cells exhibited fractal-like behavior of beating intervals). Because VLF:Total power in single pacemaker cells is similar to that of the heart in vivo (Table 1), differences in distribution of the dynamic patterns within the VLF regime in pacemaker cells in isolation vs. the heart in vivo must be involved in the loss of fractal-like behavior in the majority of single pacemaker cells.

Stimulation of autonomic receptors in single isolated pacemaker cells

β -AR or CR stimulation modulates the signaling of the very same mechanisms intrinsic to pacemaker cells that control their intrinsic automaticity (Ca^{2+} -calmodulin activation of adenylyl cyclases/PKA and CaMKII). Fig. 4A illustrates representative beating interval series of pacemaker cells in control and following CR or β -AR stimulation. CR stimulation (by carbachol, 100 nM), markedly increased the average beat interval, the time-domain variability indices, and entropy of single pacemaker cells (Table 2, Fig. 4). In contrast, β -AR stimulation (by isoproterenol, 100 nM) markedly decreased the average beat interval, time-domain variability indices and entropy of single pacemaker cells (Table 2, Fig. 4). Furthermore, β -AR stimulation increased the percentage of pacemaker cells that exhibit fractal-like behavior (3 out of 9 cells vs. 9 of 65 cells, $p < 0.05$). The fractal-like behavior slope β of these cells averaged -1.2 ± 0.2 (i.e., was between β in vivo and β of SAN tissue). Furthermore, the pacemaker cells that exhibit fractal-like behavior of beating intervals during β -AR stimulation have a higher VLF:Total power (22 ± 1 vs. 9 ± 1 , $p < 0.05$) and lower entropy levels (0.7 ± 0.1 vs. 0.9 ± 0.1 , $p < 0.05$) prior to β -AR stimulation. Therefore, β -AR stimulation, per se, has the capacity to restore the fractal-like behavior and order to some isolated pacemaker cells.

The profiles of the beating intervals and beating interval coefficient of variation (CV) in vivo during autonomic denervation in vivo, within isolated SAN tissue and in single isolated pacemaker cells with and without autonomic receptor stimulation are illustrated in Fig. 5. Profiles of beating intervals, beating interval CV and entropy are illustrated in Fig. 6. Note in Fig. 6 that: (1) the average CV or ApEn decreases as average beating interval increases, in the transition from the heart in vivo to SAN tissue in isolation; (2) when single pacemaker cells are isolated from SAN tissue, the further increase in the average beating interval is accompanied by an increase in basal CV or ApEn; (3) that a reduction or increase in the beating interval of isolated cells in response to β -AR or CR, respectively, is accompanied by a respective reduction or increase in CV and ApEn. Finally, note that, in this context, the pattern of ApEn essentially reports that of CV.

Discussion

The essence of the initiation of the heart beat is the generation of APs by pacemaker cells. The components within the signaling cascade initiated within the brain and transmitted to the pacemaker cells within the SAN comprise a complex non-linear system in which signals

can become amplified. A reductionist approach to examine only specific parts of the system, therefore, cannot reveal this complexity embedded within the complete signaling cascade from brain to SAN. In an attempt to unravel this complexity embedded within the brain-pacemaker cascade, we, therefore, segregated each component in order to discover its chaotic nature, and integrated the information obtained in individual components to their behavior back into the system.

Single isolated sinoatrial pacemaker cells in isolation

Fig. 6 shows that the relationships between average beating interval and CV or entropy are not monotonic, but conform to a U shape. The average range of basal beating intervals of single isolated pacemaker cells is well below their rate when they reside in SAN tissue. The variability of beating intervals of single isolated pacemaker cells is also higher than in cells residing in SAN tissue (Fig. 6), their entropy increases dramatically and fractal-like behavior within beating intervals is absent (Table 1). A computer-controlled version of the “coupling clamp” technique predicts that low coupling between two cells increases BIV.¹¹ Isolation of single pacemaker cells from the SAN precludes cell-to-cell interactions within the tissue (electrotonic and mechanical)^{12, 13} and intrinsic clock periods of individual cells increases.¹⁴ Loss of this property when pacemaker cells are isolated from SAN tissue leads to loss of beating interval fractal-like behavior and increase entropy. Beating intervals is observed in human embryonic-induced pluripotent stem cell-derived¹⁵ and cultured cardiomyocytes^{16–18} do obey a power-law behavior. These results, however, do not contradict the behavior of a single SAN cell in the present study because these cells are coupled within the tissue-like monolayer milieu, and therefore they behave more as a tissue than as single cells, which are both electrotonically and mechanically disconnected.¹⁹

In addition to a loss of cell-to-cell electrical and mechanical communications, autonomic receptor stimulation that is present in vivo impacts on the beating interval of pacemaker cells residing in the SAN. By isolating single pacemaker cells from SAN tissue, we could explore how autonomic receptor stimulation directly affects the pacemaker cell BIV, ApEn, and fractal-like behavior of beat intervals in the absence of cell-to-cell interactions. β -AR stimulation not only markedly decreases the average beat interval and decreases time-domain variability indices of single isolated pacemaker cells, but also markedly decreases beating interval entropy (Table 2, Fig. 6). Furthermore, β -AR stimulation increases the likelihood that pacemaker cell beating intervals exhibit fractal-like behavior. Because β -AR stimulation of isolated pacemaker cells decreases intrinsic clock period via Ca^{2+} -calmodulin activation of adenylyl cyclases/PKA and CaMKII-dependent phosphorylation of Ca^{2+} and membrane proteins,²⁰ it is reasonable to conclude that this phosphorylation effect reduces entropy. Measurements of beating interval entropy define the degree of order among its beat intervals. Because completely random systems exhibit maximal entropy, the reduction in beating interval entropy by β -AR stimulation confers beating interval complexity. Similar to these effects of autonomic receptor stimulation, mechanisms intrinsic to the pacemaker cell, e.g., phosphodiesterase and cAMP/PKA signaling can synchronize the coupled-clock period. One can assume therefore that these intrinsic mechanisms can also control the BIV complexity.

CR stimulation, in contrast to β -AR stimulation, reduces Ca^{2+} -calmodulin activation of adenylyl cyclases/PKA and CaMKII signaling and increases intrinsic clock period.²¹ CR stimulation, in contrast, not only markedly increases the average beat interval and increases time-domain variability indices of single isolated pacemaker cells, but also markedly increases their entropy (Table 2, Fig. 6). CR stimulation, in contrast, reduces Ca^{2+} -calmodulin activation of adenylyl cyclases/PKA and CaMKII signaling and increases intrinsic clock period.²¹ Thus, our analyses of the effects of autonomic receptor stimulation demonstrate that autonomic signals not only modulate the beating interval of pacemaker cells but also its complexity.

Integrating isolated pacemaker cells into isolated intact SAN tissue

The most striking difference between pacemaker cells residing within the SAN tissue and in isolation, beyond a marked reduction in the average beating interval, is that even in the absence of autonomic neural input, beating intervals of SAN tissue exhibit a 20-fold reduction in entropy, a 3-fold increase in VLF to total power compared to isolated cells and exhibit fractal-like behavior. That fractal-like behavior of beating intervals of pacemaker cells residing in intact isolated SAN indicates that properties intrinsic to pacemaker cells residing within SAN tissue contribute an order to the in vivo heart rate variability. Note, however, system complexity of SAN tissue in which neural input is absent is lower than that of the intact heart in vivo (Table 1, Fig. 6).

The shortest beat intervals at which pacemaker cells embedded within SAN tissue can generate spontaneous action potentials are dictated by clock periodicity intrinsic to these cells. Pacemaker cells within intact SAN tissue that have the shortest clock periods entrain cells with more prolonged periods, reducing the BIV among cells within the SAN tissue. “Neighborhoods” of cells within SAN tissue differ in their average coupled-clock period, and the “neighborhood” of cells having the shortest period is the primary pacemaker area within the SAN, and has the ability to generate action potentials at shorter intervals than other SAN “neighborhoods”. Thus, the impulses that emanate from the primary SAN area excite other “neighborhoods” within the SAN tissue, and this leads to the emanation of an impulse from the SAN that excites the rest of the heart.²²

Integrating pacemaker cells in isolated SAN into heart in vivo

In the intact heart in vivo the average beating interval becomes reduced compared to that in the isolated SAN, and this is accompanied by an increase in system order (Fig. 6, Table 1) and reduced power law slope. Thus, the presence of autonomic neural impulses is the major factor that underlies the increase in entropy and CV when isolated SAN tissue is integrated into the heart in vivo (Fig. 6). Similar increases in time domain BIV parameters in vivo compared to denervated in vivo have been documented previously in dogs²³ and in transplanted hearts in humans.¹

Numerous factors present in vivo also modulate intrinsic clock mechanisms of pacemaker cell within the SAN: hormonal influences (e.g., epinephrine, atrial-natriuretic peptide, brain-natriuretic peptide) and mechanical factors (e.g., atrial stretch and arterial pressure). In addition, residual autonomic modulation due to incomplete denervation may also be present

during autonomic denervation in vivo. These factors may explain differences between the complexity of beating intervals during autonomic denervation in vivo and the intact isolated SAN tissue.

In nature, a separate activation of β -AR or CR does not usually occur; rather both are activated together to different degrees. Dominant activation of β AR of single isolated pacemaker cells decreases the average beat interval and time-domain variability indices, and also markedly decreases beating interval entropy. Under these conditions in a single pacemaker cell the probability that the beating intervals exhibit fractal-like behavior is higher. Inhibition of parasympathetic impulses to pacemaker cells within SAN tissue in vivo also decreases the slope of the line describing the fractal-like behavior (review in⁴). These results permit the conclusion that activation of sympathetic to parasympathetic receptor stimulation shifts the fractal dynamic toward more order (i.e., a Brownian noise-like component). In contrast, when activation of CR : β AR activation increases, the average beat interval and time-domain variability indices of single isolated pacemaker cells increase, and beating interval entropy also become markedly increased. When the sympathetic impulses to pacemaker cells within SAN tissue are inhibited in vivo the fractal-like behavior slope increases (review in⁴). Thus when parasympathetic to sympathetic receptor stimulation increases the beating interval fractal-like dynamic shifts toward less order (white noise). In single pacemaker cell when the log frequency var. log power spectral is already flat, i.e., the fractal-like behavior does not exist (Fig. S4), increasing parasympathetic to sympathetic receptor activation by ChR increases entropy. Our results, therefore, indicate that the sympathetic to parasympathetic balance determines fractal-like behavior, and one can assume that balance of β -AR and CR activation will bring the BIV complexity to the same level of basal state in single pacemaker cells.

Limitations

Electrical activity was used to quantify the beating interval under in vivo conditions and in isolated pacemaker tissue. The beating interval in isolated pacemaker cells was quantified by using contraction and not electrical activity intervals. However, electrical activity was used to quantify the beating interval under in vivo conditions and in isolated pacemaker tissue. Because contraction is a slower phenomenon compared to excitation, the analysis of the contraction patterns may possibly be prone to uncertainty in the detection of contraction times. It might be argued that there may be a higher level of noise in the time series derived from contraction recordings that can affect the BIV parameters. However, analyses of simultaneous recording of electrical activity and contraction at the same pacemaker cell provides evidence that the same interval between successive APs is the interval between successive contractions (Fig. S2).

The in vivo experiments were conducted under general anesthesia. Because it is not clear how anesthesia affects the HRV in vivo and under in vivo denervated conditions in rabbit, the anesthesia may affect the BIV parameters. Future experiments using telemetry method are needed to clarify this point.

It has been suggested previously that the oscillation period of the system always lies in the range of the intrinsic periods of the individual oscillators.²⁴ However, the average beating

interval of isolated pacemaker cells in the present study was longer than that of the cell residing in the SAN. Factors that can influence disparity of spontaneous beating intervals in isolated cells and in the intact SAN are: (1) pacemaker cells embedded within tissue contract against a load compared to unloaded isolated pacemaker cell, and therefore their beating interval decreases;²⁵ (2) the cell isolation procedure may alter the ion channel composition of the membrane and/or of the intracellular Ca^{2+} cycling mechanisms, leading to a prolongation of the intrinsic beating period. As a consequence, beating intervals in a fraction of isolated pacemaker cells may no longer exhibit power law behavior. However, because isolated SAN monolayers exhibit power law behavior, one can assume that the isolation procedure does not necessarily cause the loss of the power law behavior and the increase in the average beating interval observed in single pacemaker cells. Future experiments in which concurrent administration of ISO and CCh at different doses are required to stimulate the influence of different degrees of parasympathetic and sympathetic nervous inputs to the pacemaker cells on the beating interval and BIV.

Conclusion

By dissecting the brain-pacemaker cell signaling cascade we demonstrate that fractal-like behavior of the heart in vivo is attributable to intrinsic clock-like signaling within pacemaker cells residing in SAN tissue and its modulation by autonomic neural input from the brain. Therefore, both the average beating interval in vivo and the full complexity embodied within its beating intervals are conferred by the combined effects of clock periodicity intrinsic to pacemaker cells residing within the SAN and autonomic neural input, which modulates pacemaker cells' clock periods. When residing within the SAN tissue environment, the clock periods of individual pacemaker cells become mutually entrained, even in the absence of autonomic input, via cell-to-cell communication, in part due to electrotonic and mechanical interactions, and generate spontaneous beating intervals that exhibit fractal-like behavior. Autonomic receptor stimulation modulates the intrinsic pacemaker cell clock period, shifting the beat interval periodicity and its complexity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP	Action potential
ApEn	Approximate entropy
β-AR	β -adrenergic receptors

BIV	Beating interval variability
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CR	Cholinergic receptors
CV	Coefficient of variation
DFA	Detrended fluctuation analysis
HF	High frequency
LF	Low frequency
SAN	Sinoatrial node
SANC	Sinoatrial node cells
PKA	Protein kinase A
VLF	Very low frequency

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Figure 1.

Representative (A) beating intervals vs. time and (B) distribution of beating intervals at different levels of integration from the heart in vivo to single isolated pacemaker cells (from the same preparation).

Figure 2.

Poincaré plots of the beating interval at different levels of integration from the heart in vivo to single isolated pacemaker cells.

Figure 3.

(A) Power-law behavior (log power spectrum density-PSD vs. log frequency) of beating intervals, and (B) analysis of the self-similarity of the beat rate time series by detrended fluctuation analysis at different levels of integration from the heart in vivo to isolated pacemaker tissue at the same animal.

Figure 4.

Distributions of beating intervals in single sinoatrial node cells (A), distributions of the average beating intervals of different cells (B) and Poincaré plots of the beating interval variability (C) in single SANC under control (CON), β -adrenergic receptor stimulation (ISO) or cholinergic receptor stimulation (CCh).

Figure 5. Distribution of (A) beating intervals (B) coefficients of variation at different levels of integration from the heart in vivo to single pacemaker cells in isolation in all experiments.

Figure 6.

The relationship between beating intervals and (A) coefficients of variation, (B) entropy and (C) power law analyses slope at different levels of integration from the heart in vivo to single pacemaker cells in isolation.

Table 1

Measures of beating interval dynamics.

Parameter	Heart in vivo (n=9)	Heart in vivo, denervation (n=9)	Intact isolated sinoatrial node tissue (n=8)	Single, isolated sinoatrial node cells (n=64)
Time domain parameters				
Beat interval (ms)	192±5	202±4 [*]	324±11 ^{*,§}	390±9 ^{*,\$,#}
SDNN (ms)	10±3	7±1	7±1	28±3 ^{*,\$,#}
RMSSD (ms)	14±4	9±2	8±2	45±4 ^{*,\$,#}
CV (%)	5.5±1.5	3.7±0.5	2.8±0.6 [*]	8.9±0.5 ^{*,\$,#}
pNN50 (%)	1±0.3	0.3±0.1	0.3±0.1	4±2 ^{*,\$,#}
ApEn	0.17±0.07	0.12±0.07	0.06±0.03 [*]	0.7±0.03 ^{*,\$,#}
Frequency domain parameters				
VLF/Total (%)	10±2	19±7	34±9 [*]	20±1 [#]
LF/Total (%)	32±6	25±2	18±3 [*]	30±1 [#]
HF/Total (%)	58±6	56±6	48±6	50±1
LF/HF	0.8±0.3	0.5±0.1	0.3±0.1 [*]	0.5±0.1 [*]
Fractal analyses				
β	-1±0.2	-1.2±0.2	-1.6±0.2 [*]	N.A.
α_1	0.7±0.2	0.75±0.06	1±0.14 ^{*,§}	N.A.
α_2	0.6±0.05	0.7±0.05	0.85±0.15 [*]	N.A.

SDNN, standard deviation of the beats; RMSSD, root mean square of the successive differences; CV, coefficient of variation; pNN50, percentage of adjoin beating intervals differing by more than 50 ms; VLF, very-low frequency power; LF, low frequency power; HF, high frequency power; Total, total spectrum power; ApEn, approximate entropy; β , slope of the power-law relationship; α_1 short-term scaling exponent; and α_2 long-term scaling exponent.

^{*} p<0.05 vs. in vivo,

[§] p<0.05 vs. in vivo denervation,

[#] p<0.05 vs. intact SAN. N.A. indicates that fractal-like complexity is not present.

Table 2

Measures of beating interval dynamics of single isolated pacemaker cells in response to autonomic receptor stimulation.

Parameter	Isoproterenol (n=9)	Carbachol (n=6)
Time domain parameters		
Beat interval (ms)	356±10 % -20±2 *	499±18 % 24±10 ^{*^}
SDNN (ms)	23±8 % -37±9 *	62±19 % 100±35 ^{*^}
RMSSD (ms)	35±9 % -34±7 *	83±24 % 79±35 ^{*^}
CV (%)	6±2 % -32±6 *	23±6 % 97±32 ^{*^}
pNN50 (%)	2±2 % -40±9 *	10±8 % 89±20 ^{*^}
ApEn	0.6±0.1 % -10±4 *	0.88±0.2 % 20±12 [^]
Frequency domain parameters		
VLF/Total (%)	15±3 % -10±12	25±3 % 10±18
LF/Total (%)	30±2 % 5±5	25±3 % -5±8
HF/Total (%)	55±8 % 5±7	50±7 % -5±5
LF/HF (%)	0.55±0.1 % 14±14	0.37±0.06 % -10±2 [^]

SDNN, indicates standard deviation of the beats; RMSSD indicates root mean square of the successive differences; CV indicates coefficient of variation; pNN50 percentage of adjoin beating intervals differing by more than 50 ms; VLF, very-low frequency power; LF, low frequency power; HF, high frequency power; Total, total spectrum power; ApEn, approximate entropy.

*
p<0.05 vs. control.

[^]
p<0.05 vs. isoproterenol.