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From beat rate variability in induced pluripotent stem cell-derived pacemaker cells to heart rate variability in human subjects

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Abstract

Background—We previously reported that induced Pluripotent Stem Cell-derived cardiomyocytes (iPSC-CM) manifest beat rate variability (BRV) resembling heart rate variability (HRV) in human sinoatrial node (SAN). We now hypothesized the BRV-HRV continuum originates in pacemaker cells.

Objective—To investigate whether cellular BRV is a source of HRV dynamics, we hypothesized three-levels of interaction among different cardiomyocyte entities: (1) single pacemaker cells, (2) networks of electrically coupled pacemaker cells and (3) *in situ* SAN.

Methods—We measured BRV/HRV properties in single pacemaker cells, iPSC-derived contracting embryoid bodies (EBs) and electrocardiograms from the same individual.

Results—Pronounced BRV/HRV were present at all three levels. Coefficient of variance (COV) of inter-beat intervals (IBI) and Poincaré plot SD1 and SD2 in single cells were 20x > EBs ($P < 0.05$) and *in situ* heart (the latter two were similar, $P > 0.05$). We also compared BRV magnitude among single cells, small (~5-10 cells) and larger EBs (>10 cells): BRV indices progressively increased ($P < 0.05$) as cell number decreased. Disrupting intracellular Ca^{2+} handling

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markedly augmented BRV magnitude, revealing a unique bi-modal firing pattern, suggesting intracellular mechanisms contribute to BRV/HRV and the fractal behavior of heart rhythm.

Conclusions—The decreased BRV magnitude in transitioning from single cell to EB suggests HRV of hearts *in situ* originates from summation and integration of multiple cell-based oscillators. Hence, complex interactions among multiple pacemaker cells and intracellular Ca^{2+} handling determine HRV in humans and isolated cardiomyocyte networks.

Keywords

Electrophysiology; Heart rate; Induced Pluripotent Stem Cells; Heart Rate Variability; Cardiac Myocytes

Introduction

Numerous studies have shown normal heart rate fluctuates around a mean, a phenomenon termed heart rate variability (HRV).^{1, 2} A key feature of the human heart is that HRV exhibits self-similar fractal-like oscillations contributing to its complex firing pattern.^{3, 4} The common notion is that HRV reflects the opposing effects of the autonomic nervous system branches, as well as other extra- and intra-cardiac inputs; e.g. intracellular, chemical, hormonal and metabolic. Yet, HRV persists in denervated hearts (albeit differently than innervated hearts), suggesting it derives from other extra-cardiac factors affecting heart rate dynamics, as well as from intrinsic cardiac regulatory mechanisms.⁵

We recently reported that networks (clusters) of human embryonic stem cell (hESC)-derived cardiomyocytes (hESC-CM) and induced pluripotent stem cell (iPSC)-derived cardiomyocytes (iPSCCM) completely lacking extra-cardiac inputs, exhibit beat rate variability (BRV), fractality and power-law behavior resembling HRV in human sinoatrial node (SAN).^{4, 6} This was consistent with studies of ventricular cells of newborn rat (both isolated cells and cell networks), which found fractal and power-law behavior here, as well.^{7, 8} Collectively, our findings suggest these BRV properties are intrinsic to pacemaker cells. A key question arising from the resemblance of BRV in cardiomyocyte-coupled networks to HRV *in situ* is whether BRV is a fundamental source of HRV. Hence, the sources of HRV *in situ* can be considered in terms of non-steady state firing patterns of individual cardiac pacemaker cells, interactions among neighboring pacemaker cells in a coupled network and extra-cardiac influences on these components. To investigate whether cellular BRV is a source of HRV dynamics, we hypothesized three-levels of interaction among different cardiomyocyte entities: (1) single pacemaker cell, (2) networks of electrically coupled pacemaker cells and (3) *in situ* SAN. To test this hypothesis, we analyzed HRV in electrocardiograms (ECGs) of healthy volunteers, and BRV in extracellular electrograms and action potentials (AP) recorded from spontaneously contracting embryoid bodies (EBs) and iPSC-CM, respectively, both fabricated from volunteers' keratinocytes. We also hypothesized that disrupting intracellular Ca^{2+} handling would impact on BRV magnitude. If validated, this would suggest intracellular mechanisms contribute to BRV/HRV and the fractal behavior of cardiac rhythm.

Methods

ECGs were recorded from 5 healthy females, ages 25-to-54, taking no medication. We plucked 10 scalp hairs/subject from two of them and prepared iPSC-CM from keratinocytes as previously described⁹ (see on-line Supplement). The study was approved by the local Ethics Committee and all subjects gave informed consent. Our previous publication reported a normal karyotype and electrogram properties recorded from EBs generated from one volunteer (#201201) included in the present work.^{6, 9} iPSC and iPSC-CM from the second volunteer (#201202) in this study have a normal karyotype and comparable electrophysiological data (data not shown).

Data recording and processing

Extracellular electrograms were recorded for 30 minutes from spontaneously contracting 18-39 day-old EBs using the Micro-Electrode-Array (MEA) apparatus (Multi Channels Systems, Reutlingen, Germany).⁶ Recordings were at sampling frequency = 1000 Hz, down-sampled to 200 Hz, and analyzed to detect peaks of the signal from which R-R intervals were calculated using MATLAB software. Action potentials were recorded from single cardiomyocytes or small EBs containing few cells, as described below. ECGs were recorded from five healthy individuals and analyzed as described in the on-line Supplement.

Action potential recordings

For action potentials recordings, spontaneously contracting areas of EBs were mechanically dissociated and enzymatically dispersed (collagenase II 1 mg/ml; Worthington, Lakewood, New Jersey, USA, <http://www.worthington-biochem.com>). This dispersion resulted in single cells, small contracting clusters (5-10 cells) and larger contracting clusters (>10 cells). Single cardiomyocytes as well as small and large clusters were then plated on gelatin-coated glass coverslips (13 mm diameter) in 24-well plates. The coverslips were incubated at 37°C, and a recovery period of two days was allowed before the electrophysiological experiment was performed.¹⁰ In all experiments, the coverslips were perfused at 37°C with an external solution containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose and 10 HEPES titrated to pH 7.4 with NaOH (310 mOsm). The patch pipette solution contained (mM): 120 KCl, 1 MgCl₂, 3 Mg-ATP, 10 HEPES, and 10 EGTA titrated to pH 7.2 with KOH and adjusted at 290 mOsm with saccharose (all materials were purchased from Sigma-Aldrich). Axopatch 200B, Digidata 1322 and pClamp10 (Molecular Devices, Sunnyvale, CA) were used for data amplification, acquisition and analysis. Signals were digitized at 10 kHz and filtered at 2 kHz. Microelectrodes with resistances of 4-7 MΩ were pulled from borosilicate glass capillaries (Harvard Apparatus, Holliston, USA). The electrical signal obtained from the contracting cardiomyocyte was sampled at 10000 Hz and down-sampled to 200 Hz. The recordings were analyzed for detection of peaks of the recorded signal from which inter-beat-intervals were calculated using dedicated MATLAB software.

Data analysis

We used three different means to analyze BRV/HRV (see on-line Supplement): (1) The detrended fluctuation analysis (DFA) quantifies the intrinsic fractal-like behavior of non-stationary time series (Fig. 1A, on-line Supplemental).^{6, 11} (2) Poincaré plot, in which each

inter-beat interval (IBI) (IBI_{n+1}) is plotted against its predecessor (IBI_n), reflecting the dynamics of the system.¹² (3) Power-law analysis (see Supplement) which determines the scaling relationships of the process.¹³

Statistical analysis

See on-line supplement.

Results

IBI variability occurs at the three levels

Fig. 1 demonstrates the ECG of a 39-year old healthy female volunteer #201201 from whom iPSC were generated, EB electrogram and single cardiomyocyte action potentials.

Pronounced BRV/HRV at all three levels is illustrated by the IBI versus time plot, and by the IBI histograms. Similar findings from the second volunteer (#201202) are shown in Supplementary Fig. 2. That the cells within the entire EB network beat in synchrony and are electrically coupled, is shown in Supplementary Fig. 3: here, electrograms recorded from 3 sites in the cluster exhibit identical beat rate (43.5 beats/min), IBIs versus time plots, IBIs histograms and Poincaré plots.

Overall, 12 EBs were examined for BRV analysis: 7 of them covered 2-3 electrodes; 2 covered 8 electrodes, and 3 covered 15-16 electrodes. The EB presented in Supplemental Fig. 3 covered 16 electrodes and was beating synchronously. The electrodes selected for analysis were not adjacent to one another and had a consistent, strong signal. We accepted that cardiomyocytes within the network having identical rhythm characteristics were coupled to one another.

Collectively, these results demonstrate: (1) In addition to *in situ* heart and the network, BRV is a key feature of single cardiomyocytes, thus supporting our hypothesis. (2) As shown in Fig. 1 (and Supplementary Fig. 2, panels C, G, K), the range of IBIs is broader in single cardiomyocytes than in EB and *in situ* heart (further details regarding these observations follow). In Poincaré plots generated for volunteer #201201 (similar results are shown for volunteer #201202, Supplementary Fig. 2), horizontal and vertical dispersions are larger in single cell than EB and *in situ* heart (Figs. 1D, 1H, 1L). To better compare Poincaré plots at all levels, we superimposed their corresponding plots on the same X- and Y-axes (Fig. 1M), demonstrating that IBIs of single cell action potentials display prominent dispersion in X- and Y-axes, compared to heart and EB recordings.

Self-similarity, fractality and power law behavior of BRV/HRV at the three levels

To test for self-similarity and fractality, IBIs were analyzed using DFA, which quantifies intrinsic fractal-like correlation properties of dynamic systems.¹¹ As depicted in Supplementary Fig. 1, slope values (α) of all DFA plots were in the range of 0.9, and similar ($P > 0.05$) in the three levels, demonstrating that single pacemaker cells exhibit self-similar and fractal behavior, indicating these properties are fundamental features of a single pacemaker cell. Respecting the power law analysis of the IBIs, as shown in Supplementary Fig. 4, a similar power law behavior is seen in the three levels.

The magnitude of BRV/HRV at the three levels

The quantitative measures of BRV/HRV at the three levels provides further evidence that the IBIs dispersion is larger in single cells than EBs and hearts. The hypothesis that BRV magnitude is larger in single cells than the network and *in situ* heart derives from two assumptions: (1) the larger the number of opposing effectors of pacemaker activity, the smaller the IBIs dispersion; (2) In the single cell the number of rate generators/modulators (e.g., Ca^{2+} and voltage clocks¹⁴) is smaller than the number at the tissue level or the *in situ* heart. Supporting this hypothesis, the IBIs coefficient of variance (IBI COV) (Fig. 2B), and SD1 (Fig. 2C) and SD2 (Fig. 2D) (the Poincaré plot measures) are larger in the single cardiomyocyte than in network or the heart, suggesting the marked increase in BRV occurs in transitioning from interconnected network to single cardiomyocyte. We tested the hypothesis that the largest BRV and IBIs dispersion is at the single cell level in experiments showing the IBIs range during 1800 sec recording session is progressively smaller when shifting from single cell, through small cluster to large cluster (Figs. 3A, 3B, 3C, respectively). The hypothesis is further corroborated by comparing action potentials IBI COV (Fig. 3D), SD1 (Fig. 3E) and SD2 (Fig. 3F) of a single cell, a small cluster and a larger cluster (see Methods). These findings illustrate that as more cells are included in the network, BRV parameters decrease sharply, suggesting the higher the number of interactions among the potential pacemakers, the smaller the variability around the mean beat rate. Finally, Fig. 3G demonstrates that IBI COV is similar within the group including the heart, EB and the large cluster (similar results were obtained for SD1 and SD2, data not shown), but is significantly ($P < 0.05$) larger upon transiting to the small cluster and single cell.

Mechanisms contributing to single cell BRV and fractal-like behavior

To study intracellular mechanisms contributing to BRV, we determined the effects of RU360 (mitochondrial Ca^{2+} uptake blocker¹⁵), GCP-37157 (mitochondrial Na^{+} - Ca^{2+} exchanger antagonist¹⁶) and ryanodine (sarcoplasmic reticulum Ca^{2+} -induced Ca^{2+} -release channel blocker¹⁷), on firing pattern and BRV characteristics. As shown by the representative action potentials (Fig. 4A) and IBIs versus time plot (Fig. 4B), RU360 increased IBIs dispersion and decreased mean firing rate. This is seen in the flattening and widening of the IBIs histogram (black histogram compared to red histogram, Fig. 4C), and by the striking change in Poincaré plot pattern; in contrast to the condensed cloud during control, the post-drug cloud is markedly dispersed (Fig. 4D). The increased BRV magnitude is seen in the drug-induced increase in the IBI COV and the Poincaré plot indices SD1 and SD2 (Fig. 4E). Finally, despite the marked increase in BRV, fractality was unaltered; the slope values (α) of the DFA plot before and after RU360 were 0.974 and 0.8, respectively.

GCP-37157 not only broadened IBIs range and increased IBI COV, SD1 and SD2 (Figs. 5A, 5C, 5E, 5G) similarly to RU360, but in 2/10 preparations it induced a bi-modal firing pattern (see change in action potential firing rhythm) (Figs. 5B, 5D, 5F), whereby the pacemaker oscillates between lower and higher firing rates. This novel observation is further illustrated by: (1) the bi-modal IBIs histograms, showing two distinct peaks; (2) four populations in the Poincaré plots, instead of one population in the regularly-firing pacemaker. We explain this phenomenon as follows. In each of the two green-circled clouds the population includes IBI_{n+1} and IBI_n points firing at the same rate, representing the low- and high- level firing

rates. For example, in Fig. 5F, the lower left population fires around $IBI=1200$ msec and the upper right population fires around $IBI=2500$ msec. Because in the Poincaré plot IBI_{n+1} is plotted against IBI_n , the two blue-circled populations represent two groups of points in which the n points are either at 2500 or 1200 msec, and the $n+1$ points are either 1200 or 2500, respectively. In contrast to RU360, the prominent increase in BRV was associated with diminution of the fractal nature of the spontaneous firing; the slope value (α) of the DFA plot was decreased from 0.83 ± 0.05 in the control to 0.69 ± 0.04 ($P < 0.05$, $n=8$) in the presence of GCP-37157.

Ryanodine increased IBIs dispersion (Fig. 6F) and converted the firing pattern from regular to bi-modal (in 3 of 4 experiments), as indicated by the two IBIs bands (Figs. 6A, 6D, 6E), the three-peaked IBIs histogram (Fig. 6B) and the 4 clouds in the Poincaré plot (Fig. 6C). Interestingly, whereas the IBIs time series show two dominant bands (e.g., Fig. 6A), the histogram shows three (black) populations (Fig. 6B). The left group represents the IBIs population immediately after ryanodine application, and the other two groups, the upper and lower distinct IBIs bands. Furthermore, with ryanodine, the Poincaré plot of the bi-modal firing pattern includes two left clouds and a right lower cloud, as previously explained. Hence (see also Figs. 6A, 6D, 6E) these results indicate the cell which alternates between the higher and the lower band of IBIs, mainly occupies the lower band (witnessed in a more condensed IBIs in the lower population). Finally, since ryanodine induced a bimodal firing pattern in 3 out of 4 cells, DFA could not be performed.

Does I_f contribute to BRV generation?—As depicted in Supplementary Fig. 5, the I_f blocker ivabradine decreased the firing rate¹⁸ but did not affect BRV properties (see Supplement).

Discussion

We investigated the origin of HRV by hypothesizing three-levels of interaction among different cardiomyocyte anatomical/functional entities: the single pacemaker cell, an EB composed of a network of electrically coupled pacemaker cells and the SAN *in situ*. To test the hypothesis we determined BRV/HRV properties in the *in situ* heart, EBs and single cardiomyocytes. The main findings were that: (1) BRV and self-similarity are shared at all levels; (2) BRV is greater in the single cell than the network and *in situ* heart; (3) Disrupting intracellular Ca^{2+} handling markedly augments BRV magnitude, suggesting that intracellular mechanisms contribute to BRV/HRV and the fractal behavior of the heart rhythm.

BRV/HRV and Poincaré plots

The basic features of BRV/HRV in single cell, network and heart are represented by the time series IBIs versus time plot and the IBIs histograms (Fig. 1). Both modes of presentation demonstrate the elementary features of BRV/HRV: variations in momentary IBI and dispersion of intervals around a mean heart rate. These novel findings demonstrate that BRV is a fundamental property of a single human cardiac pacemaker cell, and that (see below) IBIs dispersion is broader in single cells than in clusters and *in situ* heart. The validity of these conclusions is enhanced by similar observations in two volunteers. The

Poincaré plot analysis of IBIs (Figs. 1D, 1H, 1L, 1M) demonstrates a cigar-like shape of the cloud at the three levels, although at the cell level the IBIs are more dispersed (addressed below).

Self-similarity, fractality and power law behavior

In the IBI time series, self-similarity and fractality are represented by linear slopes in the DFA plot (Supplementary Fig. 1). Finding similar linear slopes at all levels of organization signifies that the fluctuations are self-similar and this similarity is intrinsic to single cells, networks and *in situ* heart. Importantly, similar slope values have been reported for *in situ* hearts.^{2, 4, 19} Respecting power law behavior (see Results and Discussion in on-line supplement), our experimental findings suggest power-law behavior is intrinsic to pacemaker cells, being devoid of extrinsic influences, and does not necessarily stem from extra-cardiac factors, such as the autonomic nervous system.

The dependency of BRV on the organization level/structure

In addition to showing that BRV is a key feature of single pacemaker cells, we found the magnitude of the single cell IBIs dispersion is more pronounced than in EBs and the heart (Fig. 2). Moreover, because the quantitative measures of IBIs dispersion were similar in EB and *in situ* heart, we concluded that the step-wise decrease in BRV occurred between the level of single cells and EB. This concept of an abrupt decline in BRV when single cells become part of a coupled EB network is further strengthened by comparing the BRV measures at the single cell versus small (5-10 cells) and larger clusters (> 10 cells). This comparison shows the large decrease in BRV occurs between single cell and small cluster, and then BRV is further decreased as the number of cells increases in larger clusters. Finally (see Fig. 3G), BRV is similar in *in situ* heart, EB and large cluster and progressively larger in small cluster and single cell.

Why is BRV largest in single cells?—The continuum comprising pacemaker cell through multicellular interconnected network to *in situ* heart fits the definition of a biological system which is “temporally and spatially complex, built from a dynamic web of interconnected feedback loops marked by interdependence, pleiotropy and redundancy”.²⁰ While temporal complexity (i.e., BRV) is most pronounced in single cells, the reverse is true for the spatial complexity; it is largest in *in situ* heart, resulting from increasingly complex architecture and multiple inputs, compared to single cell. The following arguments led us to propose the marked differences in spatial and architectural complexity between single cells and network/*in situ* heart, can account for the noticeable decrease in BRV between single cells and progressively larger multi-cellular clusters. Our key consideration is that in the highly dynamic system which generates heart rhythm, the overall noise level - BRV magnitude - is determined by the net effect of opposing inputs. Consequently, in single pacemaker cells isolated from their neighbors and deprived of extra-cardiac influences, the number of determinants of the final firing rate is small, and therefore the noise level, expressed as BRV magnitude, is much higher (lacking other adjacent dampening factors and negative feedback processes), compared to more spatially complex levels. The precise cause for the abrupt decline in BRV beyond the single cell level is not clear. Our findings that: (1) even a small increase in number of cells in the cluster is associated with a marked decrease

in BRV (Fig. 3), and (2) BRV is not further decreased between EB and *in situ* heart, suggest the main determinant attenuating single cell BRV is coupling between oscillating pacemakers. Hence, in multicellular networks, multiple dynamic interactions between the pacemakers and the electrotonic interactions mask/dampen the single cell noise, thereby attenuating BRV.

The concept of noise reduction upon transiting from single cell to coupled network is supported by some representative reports: (1) Clay and DeHaan²¹ hypothesized the irregular firing patterns in spontaneously beating cells isolated from chick embryo heart are caused by stochastic gating properties of ion channels driving the cell to threshold potential. Pertaining to our study, they found the coefficient of the IBI variations is approximately inversely proportional to the square root of the number of interconnected cells.²¹ (2) Similarly in spontaneously-firing rat ventricular myocytes, single isolated cardiomyocytes beat slowly and irregularly, and irregularity decreases “as soon as pacemaker heart cells grow together and small groups form”.²² This decline in irregularity (i.e., decreased BRV) was explained by the large increase in the total number of ion channels in the network, which dampen the inherent variations in individual channels.²²

What are the intracellular mechanisms underlying the single cell BRV?

That BRV is largest in single cells can be explained by several intracellular mechanisms influencing the firing rate, which are themselves non-linear fractal processes. These include intracellular sarcoplasmic reticulum (SR) Ca^{2+} cycling,^{23, 24} IP_3 -linked signaling,²⁵ mitochondrial function and variations in intracellular processes such as expression of circadian clock genes²⁶ and redox state.²⁷ Collectively, these can account for the fractal behavior of the single cell firing behavior.²⁸ Aon and coworkers reported that in guinea pig cardiomyocytes the mitochondrial membrane potential (Ψ_m) and reactive oxygen species (which couple individual mitochondria) exhibit fractality and power law behavior.^{29, 30} Because mitochondrial ability to accumulate and release large quantities of Ca^{2+} depends on Ψ_m (which exhibits fractal behavior), it is likely that mitochondrial Ca^{2+} extrusion also is fractal. Consequently, based on mitochondrial-SR Ca^{2+} crosstalk,^{31, 32} it is conceivable that the ‘noisy’ fractal features of the mitochondria are transmitted to the “ Ca^{2+} clock”^{23, 24} which participates in pacing, and consequently contributes to BRV. In agreement with reports on the fractal behavior of mitochondrial function and intracellular Ca^{2+} cycling, and the crosstalk between SR and mitochondria,³² we demonstrated that interfering with intracellular Ca^{2+} handling markedly augments BRV magnitude. Specifically, RU360 and GCP-37157 increased the quantitative measures of BRV: IBI COV, SD1 and SD2. Furthermore, in a sizable proportion of preparations, both GCP-37157 (20%) and ryanodine (75%) (Figs. 5 and 6, respectively) caused bimodal firing patterns. GCP-37157 also significantly decreased the linear slopes in the DFA, suggesting it had disturbed the fractal-like behavior of IPSC-CM. Finally, in contrast to our findings, in previous studies RU360 was shown to increase spontaneous firing rate in rabbit SAN cells³², HL-1 cardiomyocytes³³ and Cajal cells.³⁴ The decrease in rate in our study may imply that in iPSC-CM inhibition of mitochondrial Ca^{2+} eliminates its role in matching ATP supply to demand, which differ from that in rabbit pacemaker cells.³⁵

Two immediate questions arise from these findings:

(1) Why does blocking Ca^{2+} intracellular movements markedly augment BRV?

—There is an inverse relationship between the number of elements impacting on the pacing machinery and BRV magnitude; namely, the fewer the effectors the larger BRV magnitude.²² Consequently, eliminating a key intracellular factor such as mitochondrial Ca^{2+} cycling would augment BRV magnitude. This notion is supported by the experiments showing that blocking the pacemaker current I_f did not affect BRV (Supplementary Fig. 5); while ivabradine decreases spontaneous firing rate, it does not affect the fundamental pacing machinery driven by intracellular Ca^{2+} cycling.¹⁸ In contrast to our finding Camm et al showed that ivabradine increased HRV in patients with chronic stable angina.³⁶ However, as described herein, there are two fundamental differences which contribute to the dissimilarity between the studies. Firstly, our iPSC-CM were generated from healthy volunteers, as opposed to the angina patients studied by Camm et al. Secondly, we investigated the acute effects of ivabradine, while the angina patients received continuous ivabradine treatment for 3 months. In summary, due to the different experimental settings, these two studies cannot be considered contradictory. Given these observations, our future studies will attempt to decipher discrete mitochondrial and SR mechanisms contributing/generating BRV and hence HRV, in healthy and diseased pacemaker cells/hearts. Accordingly, it is plausible that in settings involving disturbed Ca^{2+} handling, such as heart failure, alterations in HRV properties^{37, 38} are in part due to changes in BRV properties in single cells.

(2) Why do GCP-37157 and ryanodine induce bi-modal firing patterns?—It is plausible that the bi-modal firing patterns induced by GCP-37157 and ryanodine (Figs. 5 and 6) are abnormal, distinct from the dominant pattern in which all IBIs form a single population. This bimodality reflects pacemaker alternation between two basic mean firing rates and is clearly demonstrated by the double-peaked IBIs histogram and the four Poincaré clouds (e.g., Fig. 5B, 6D-6E). Thus, time series analytical techniques (such as Poincaré plots) reveal internal events and abnormal firing patterns which are unseen when only mean heart rate is calculated. The pattern in cells most closely resembles the bi-modal firing pattern we report is Jongsma et al's demonstration in spontaneously contracting neonatal rat heart cells.²² In their Figs. 5B and 7A, the IBIs distribution displays two peaks, albeit not as distinct as those in our study. Stronger support for (pathological) bimodal firing behavior comes from an analysis of HRV properties in healthy volunteers and in patients with sick sinus syndrome (SSS).³⁹ In agreement with our findings, the authors' Fig. 3 shows a bimodal heart rate firing pattern seen in: (1) the R-R interval versus time plot demonstrating two discrete R-R intervals at ~1.0 and 1.6 sec. (2) Poincaré plots of SSS patients depict four populations. While we cannot offer a mechanistic explanation for the bimodality recorded in drug-treated iPSCCM, it is intriguing that the irregular firing pattern recorded *in situ* in SSS patients is paralleled by the *in vitro* firing behavior in iPSC-CM with disturbed Ca^{2+} handling. However, in the absence of a clear mechanism, we speculate that it may be linked with waxing and waning of intracellular Ca^{2+} due to feedback and feed-forward signaling within pacemaker cells.

The similarity between iPSC-CM and adult pacemaker cells

Regarding the cardiac phenotype of iPSC-CM, we and others have investigated mRNA and protein expression patterns of iPSC-CM. Specifically, we have shown in iPSC-CM by means of immunostaining and RT-PCR the expression of the typical cardiac markers, cardiac troponin I and T, α -sarcomeric actinin, myosin heavy chain, ryanodine receptor and calsequestrin.^{9, 40} Further, Honda et al showed in iPSC-CM the expression of additional cardiac genes such as the sodium channel ($\text{Na}_v1.5$; *SCN5A*), the α -1C and α -1D subunits of the L-type Ca^{2+} channel, the rapidly activating delayed rectifier potassium channel (hERG; *KCNH2*), the transient outward potassium channel ($\text{K}_v4.3$; *KCND3*) and the hyperpolarization-activated cyclic nucleotide gated channels (*HCN2*, *HCN4*).⁴¹ Next, the following observations support the similarity between the pacemaker mechanisms in the iPSC-CM used in the present work, and adult pacemaker cells: (1) We showed in the Supplement that ivabradine decreased the spontaneous firing rate, indicating that I_f is present and contributes to pacemaker mechanisms in iPSC-CM. (2) In preliminary experiments we found that nifedipine decreased the spontaneous firing rate of both single cardiomyocytes and networks (EBs) of cells, indicating the L type Ca^{2+} channel ($I_{\text{Ca,L}}$) contributes to automaticity. (3) We have previously demonstrated that isoproterenol and carbamylcholine increase or decrease, respectively, the spontaneous firing rate of iPSC-CM networks.⁶ In addition, Fine et al characterized in iPSC-CM several cardiac ion transporters such as Na^+/K^+ pump and NCX currents, and showed that their current densities were similar to those of adult rabbit and guinea pig cardiomyocytes.⁴² Collectively, these findings demonstrate that the pacemaker mechanisms in iPSC-CM are similar to those of the adult pacemaker cells.

Study limitation

Whereas ECG recordings were obtained from 5 healthy female volunteers, iPSC clones were generated from only two of them. Yet, these iPSC clones were sufficient to establish the association between the clinical data (ECG) and the data from the other two cellular levels (networks, single cells).

Conclusions

We have shown for the first time that BRV and power law behavior are intrinsic features of single human cardiac pacemaker cells derived from iPSC. Further, we discovered that BRV is markedly enhanced at the single cell level compared to excitable network and *in situ* heart. Our findings suggest the major increase in IBIs variability occurs in transitioning from interconnected network to single cardiomyocyte. Based on these novel findings we propose intracellular calcium cycling contributes to BRV/HRV and the fractal behavior of cardiac rhythm. Our observations support the compatibility of iPSC-CM as biological pacemakers, as they not only fire spontaneously, but also present inherent dynamic pacemaker properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

HRV	heart rate variability
hESC	human embryonic stem cell
hESC-CM	human embryonic stem cell-derived cardiomyocytes
iPSC	induced pluripotent stem cell
iPSC-CM	induced pluripotent stem cell-derived cardiomyocytes
BRV	beat rate variability
SAN	sinoatrial node
ECG	Electrocardiogram
AP	action potential
EB	embryoid body
MEA	Micro-Electrode-Array
DFA	detrended fluctuation analysis
IBI	inter-beat interval
IBI COV	inter-beat interval coefficient of variance
SR	sarcoplasmic reticulum
SSS	sick sinus syndrome

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Figure 1.

Electrical activity, inter-beat intervals (IBIs) and Poincaré plots analysis at three levels: [A-D] *in situ* heart; [E-H] spontaneously contracting embryoid body (EB); [I-L] single cardiomyocyte. [A], [E], [I]: Representative electrocardiogram, extracellular electrogram and action potentials, respectively. [B], [F], [J]: IBIs time series. [C], [G], [K]: Histogram distribution of IBIs. [D], [H], [L]: Poincaré plots of IBI. [M]: Combined Poincaré plots. The minor axis (SD1) of the ellipse represents the standard deviation (SD) of short-term IBI variability; the major axis (SD2) represents the standard deviation of long-term IBI variability. Recordings from volunteer #201201.

Figure 2.

Comparisons of BRV/HRV magnitude at the three levels. Summary of Mean IBI [A], Coefficient of variance (COV) of IBIs (IBI COV) [B], SD1 [C] and SD2 [D] of Poincaré plots in heart *in situ* (n=5 individuals), contracting EBs (n=11), and single cardiomyocytes (n=22). In [A] *P < 0.05, heart versus EB and single cell. In [B-D] *P < 0.05, single cell versus EB and heart.

Figure 3.

Comparisons of BRV/HRV magnitude among single cardiomyocytes, a small (5-10 cells) and a larger cluster (>10 cells). [A-C] Representative experiments depicting action potential (AP) recordings and their corresponding IBIs versus time plot in single cardiomyocyte, small and larger cluster, respectively. [D-F] Summary of IBI COV [D], SD1 [E] and SD2 [F] of Poincaré plots in single cardiomyocytes (n=28 individuals), small cluster (n=22) and larger cluster (n=13). In [D-F], *P < 0.05, single cardiomyocytes versus small clusters versus large clusters. [G]: Summary of IBI COV in ECG (n=5), electrograms (n=11), and AP of large (n=13) and small (n=22) cardiomyocyte clusters, and single cardiomyocytes (n=28). In [G], *P < 0.05, single cardiomyocyte versus small contracting EBs versus large contracting EBs, EB and Heart.

Figure 4.

Effect of Ru360 on BRV magnitude of a small iPSC-CM cluster. Representative AP recordings in small cluster iPSC-CM, in absence (upper panel) and presence (lower panel) of 20 μ M Ru360 [A], IBI analysis [B], histogram distribution of IBIs [C], Poincaré plots of IBI [D] and summary of IBIs COV, SD1 and SD2 [E], in absence (red dotted) and presence (black dotted) of Ru360. Ru360 increased IBI COV, SD1 and SD2 compared to control (*P<0.05, n=5). Red arrow indicates drug application.

Figure 5.

Effect of GCP-37157 (1 μ M) on BRV magnitude in small iPSC-CM clusters. GCP-37157 increased BRV magnitude (n=8) and induced a bi-modal firing pattern (n=2). Representative AP recordings in absence (upper panel) and presence (middle panel) of 1 μ M GCP-37157 and corresponding IBIs time plots (lower panel) of normal firing pattern [A] and a bimodal pattern [B], histogram distributions of IBIs [C-D], and Poincaré plots [E-F]. [G] Summary of effect of GCP-37157 on IBIs COV, SD1 and SD2. *P<0.05 GCP-37157 versus control (n=8). Red arrow indicates drug application.

Figure 6.

Effect of ryanodine (1 μ M) on IBIs time series in small iPSC-CM clusters. Ryanodine induced a bimodal firing pattern ($n=3$) and increased BRV magnitude ($n=1$). [A-C] Representative experiment showing AP recording in absence (upper panel) and presence (middle panel) of ryanodine and a corresponding IBI time plot (lower panel) [A], IBIs histogram distribution [B] and Poincaré plots [C]. [D-E] Two additional experiments showing ryanodine-induced bimodality. [F] Ryanodine increased IBIs dispersion in 1 of 4 experiments. Red arrow indicates ryanodine application.