Cellular Biology

Power-Law Behavior of Beat-Rate Variability in Monolayer Cultures of Neonatal Rat Ventricular Myocytes

Jan P. Kucera, Marc O. Heuschkel, Philippe Renaud, Stephan Rohr

Abstract—It is known that extracardiac factors (nervous, humoral, and hemodynamic) participate in the power-law behavior of heart-rate variability. To assess whether intrinsic properties of cardiac tissue might also be involved, beat-rate variability was studied in spontaneously beating cell cultures devoid of extracardiac influences. Extracellular electrograms were recorded from monolayer cultures of neonatal rat ventricular myocytes under stable incubating conditions for up to 9 hours. The beat-rate time series of these recordings were examined in terms of their Fourier spectra and their Hurst scaling exponents. A non-0 Hurst exponent was found in 21 of 22 preparations (0.29 ± 0.09; range, 0.11 to 0.45), indicating the presence of fractal self-similarity in the beat-rate time series. The same preparations exhibited power-law behavior of the power spectra with a power-law exponent of −1.36 ± 0.24 (range, −1.04 to −1.96) in the frequency range of 0.001 to 1 Hz. Furthermore, it was found that the power-law exponent was nonstationary over time. These results indicate that the power-law behavior of heart-rate variability is determined not only by extracardiac influences but also by components intrinsic to cardiac tissue. Furthermore, the presence of power-law behavior in monolayer cultures of cardiomyocytes suggests that beat-rate variability might be determined by the complex nonlinear dynamics of processes occurring at the level of the cellular network, eg, interactions among a large number of cell oscillators or metabolic regulatory systems. (Circ Res. 2000;86:1140-1145.)

Key Words: heart-rate variability ■ cardiac cell cultures ■ physiology ■ extracellular recording

Beat-to-beat variations in cardiac cycle length have received increasing attention, because their characteristics may be used for the assessment and follow-up of various cardiovascular derangements and for risk stratification in the course of cardiac disease, including prediction of arrhythmias in cardiac patients.1,2 Spectral analysis of beat-rate time series reveals periodic components in heart-rate variability (HRV).3 In humans, 2 major components are present at frequencies around 0.1 Hz (low-frequency [LF] component) and around 0.25 Hz (high-frequency [HF] component).4 It is well established that these periodic components are linked to breathing and blood pressure control and that they are mediated by the autonomic nervous system5,6 and by endocrine influences.3 However, the analysis of long-term recordings (ie, 24-hour Holter electrocardiograms) indicates that >95% of the spectral power of HRV is concentrated at frequencies below LF.1 At these very low frequencies, the spectrum follows a power-law behavior; ie, the intensity of the power spectrum is a power function of frequency. The exponent of this power-law is close to −1 in healthy human subjects.7 Several studies showed that this exponent is different after myocardial infarction8,9 or cardiac transplantation9 and that the characterization of power-law behavior can yield potent estimates for risk stratification.8,10

Although the physiological basis of HRV is established for LF and HF components, the mechanisms underlying the power-law relationship are still not known. Specifically, numerous studies focused on the characterization of HRV in vivo; however, none investigated whether HRV depends on factors intrinsic to cardiac tissue, ie, whether fluctuations in cycle length also occur in the absence of extrinsic influences (nervous, hemodynamic, or endocrine).

It was the goal of the present study to characterize beat-rate variability in spontaneously beating cultures of cardiac cells under stable experimental conditions, because these preparations are devoid of extrinsic influences and therefore permit the investigation of factors governing HRV that are intrinsic to cardiac tissue. The experiments showed that the beat-rate variability of cardiac cell monolayer cultures was characterized by fractal properties and that their power spectra exhibited power-law behavior. This suggests that complex nonlinear dynamics of processes occurring at the level of cellular oscillators are present. Because beat-rate variability found in these preparations and HRV observed in vivo shared similar characteristics, it is likely that the power-law behavior of HRV is determined not only by extracardiac influences but by factors intrinsic to cardiac tissue as well.

Materials and Methods

Microelectrode Arrays (MEAs)

Extracellular indium-tin oxide MEAs were fabricated using standard photolithographic procedures.11 The MEAs were coated with colla-
Figure 1. Monolayer culture and extracellular electrograms. A. Phase-contrast micrograph of a monolayer culture of neonatal rat ventricular myocytes grown on a transparent MEA. Square-shaped borders of the insulation layer are clearly visible. B. Extracellular unipolar electrogram (Ve) showing 3 action potentials recorded from a spontaneously active cardiac cell culture. C. Top trace shows an extracellular action potential at an expanded time scale (Ve), which displays a typical biphasic deflection (see Figure 1C). The raw traces were visually inspected to record an extracellular action potential at an expanded time scale (Ve), which displays a typical biphasic deflection (see Figure 1C). Bottom trace depicts its time derivative (dV/dt). Vertical line denotes time of occurrence of the negative minimum of dV/dt, which defines activation time.

Recording of Extracellular Electrograms
Using a reference indium-tin oxide electrode inserted into the culture medium, unipolar extracellular electrograms were recorded from up to 4 sites. The signals were amplified (gain, 1000X; bandwidth, 5 kHz) and digitized at 10 kHz with 12-bit resolution by a PC-based data-acquisition system.

Experimental Protocol
Recordings were performed on 2- to 7-day-old cultures in a water-jacketed CO2 incubator (Jouan), thus ensuring stable temperature (36°C) and CO2 concentration (0.8%) during the entire duration of the experiments. Because medium exchanges themselves tend to transiently alter spontaneous beat rates,14 they were halted 24 hours before the experiment. Spontaneous sustained activity of the cell cultures was induced by adding 0.5 to 1 μmol/L of the L-type calcium channel agonist BayK8644 from a stock solution to the culture medium (M199 with HBSS, GIBCO). An electrode yielding a high signal-to-noise ratio was selected, and data acquisition was started after an equilibration period of 15 to 20 minutes. Data acquisition was continued as long as permitted by data storage resources or until instabilities in the electrogram baseline caused missing data as a result of saturation of the amplifier.

Offline Data Analysis
Activation times were derived from extracellular action potentials as the time of occurrence of the steepest point of their downward deflection (see Figure 1C). The raw traces were visually inspected to ensure detection of all action potentials and to remove detection artifacts. To convert the resulting series of activation times into a data representation suitable for spectral analysis (beat-rate time series: frequency values spaced evenly in time), instantaneous frequencies were calculated at 0.5-second intervals (Δt) along the entire recording from the linearly interpolated successive points of the series of activation times.

Hurst Exponent
The Hurst scaling exponent (H) characterizes the shape of self-similar signals and ranges from 0 to 1. A self-similar signal with

\[ H\approx 0 \] resembles white noise with spiky oscillations. A signal with \( H = 0.5 \) shows brownian noise-like oscillations, whereas signals with \( H = 1 \) exhibit smooth oscillations. \( H \) of the beat-rate (BR) time series was calculated as the slope of linear regression of log([SD(BR(t+k-Δt)-BR(t))] versus log(k) for \( k = 1, 2, 4, 8, \ldots 2^r \) up to the maximal possible power of 2.15 Fractal self-similarity was assumed to be present if the correlation coefficient (r) was >0.85.

Spectral Analysis
The power spectral density (PSD) of beat-rate variability was computed by a discrete fast-Fourier transform with a Hann window. The exponent (β) of the power-law relationship was computed as the slope of the linear regression of log(PSD) versus log(frequency). The discrete PSD points were given a weight inversely proportional to their density on the log(frequency) abscissa. A power-law was assumed to be present if \( r > 0.85 \).

Statistics
Data are given as mean±SD. Statistical differences were assessed by the 2-tailed Student t test.

Results
The experimental data consist of 18 short recordings (23 minutes to 2 hours, 42 minutes) and 4 long recordings (4 hours, 28 minutes to 9 hours, 5 minutes).

Self-Similarity of Beat-Rate Time Series
The beat-rate variability of a cell culture is depicted in Figure 2A. The trace represents a plot of the resampled beat-rate time series as a function of time. Typically, the trace exhibits oscillations that occur in an apparently erratic manner. The self-similar property of this trace is revealed by the close-up shown in Figure 2B. The rescaled segment exhibits fluctuations that look highly similar to those on the original trace. The self-similarity of the graphical trace suggests that beat rate does not exhibit any periodic oscillations but that it comprises all frequencies such that details look similar after rescaling.

To characterize and quantify this fractal self-similarity, the Hurst exponent (H) and the corresponding correlation coefficient (r) were computed for each beat-rate time series. In the 18 short experiments, 17 were characterized by self-similar beat-rate time series \( (r = 0.95 \pm 0.04; \text{range, } 0.87 \text{ to } 0.99) \) with \( H = 0.29 \pm 0.08 \) (range, 0.11 to 0.45). The beat-rate time series of the remaining short experiment was not self-similar \( (r = 0.44) \). In the 4 long experiments, all beat-rate time series were self-similar \( (r = 0.97 \pm 0.03; \text{range, } 0.93 \text{ to } 0.99) \) with \( H = 0.29 \pm 0.12 \) (range, 0.14 to 0.42).

Spectral Analysis and Power-Law Behavior
The recording durations of the 18 short experiments permitted the examination of the power spectrum over 3 decades ranging from 0.001 to 1 Hz. The power spectra of the

Figure 2. Self-similar beat-rate time series from a 4-day-old culture. The portion marked by the gray rectangle on the trace shown in panel A was rescaled and is shown in panel B to illustrate graphically the self-similarity of the beat-rate time series.
beat-rate variations of 2 cell cultures are shown in Figure 3. The spectra did not exhibit any distinct peak and followed a descending line. This linear dependence indicates that the PSD is a power-law of frequency. In the 17 experiments in which the beat-rate time series were self-similar, the spectra of beat-rate variability were characterized by a power-law with an exponent $\beta$ (corresponding to the slope of the linear regression over the frequency range 0.001 to 1 Hz) of $-1.31 \pm 0.20$ (range, $-1.04$ to $-1.74$). In the single experiment in which the beat-rate time series was not self-similar, no power-law was present ($\beta=0.07$, $r=0.09$). As judged by visual inspection, the spectrum illustrated in Figure 3A closely follows the regression line. Such a close fit was present in 8 of the 17 short experiments ($r=0.97 \pm 0.03$, $n=8$). In the remaining cases, as illustrated by the example in Figure 3B, the spectrum displayed 2 scaling regions. Accordingly, the correlation coefficients of these data ($r=0.94 \pm 0.04$, $n=11$) were significantly lower ($P<0.05$).

The 4 long electrograms permitted the examination of the power spectrum over 4 decades, from 0.0001 to 1 Hz, as illustrated in Figure 4. When assessed over the entire frequency range (0.0001 to 1 Hz), $\beta$ amounted to $-1.48 \pm 0.38$ (range, $-1.04$ to $-1.84$). To allow comparison with short recordings and to ensure that different recording durations had no influence on the quantification of $\beta$, this exponent was also computed over the range used for short experiments (0.001 to 1 Hz). These calculations yielded an average $\beta$ of $-1.56 \pm 0.32$ (range, $-1.18$ to $-1.96$), which was not statistically different from $\beta$ of the short recordings. When assessed over frequencies ranging from 0.0001 to 0.01 (the range used in clinical studies), $\beta$ amounted to $-1.35 \pm 0.72$ (range, $-0.76$ to $-2.24$).

No correlation was found between the exponents characterizing the fractal properties of the beat-rate time series ($H$ and $\beta$) and the mean beat rate of the preparations or the age of the preparations. Also, the exponents of the experiments characterized by a close linear fit (eg, Figure 3A) and those of the experiments characterized by 2 scaling domains (eg, Figure 3B) were not significantly different.

Temporal Variations of the Power-Law Exponent

The broad distribution range of $\beta$ indicates that this exponent exhibits a large variability from preparation to preparation. Moreover, this parameter was subject to fluctuations in the course of a given experiment. To quantify these fluctuations, $\beta$ was computed in a short time window of 2048 seconds (frequency range, 0.001 to 1 Hz), which was moved along the entire beat-rate time series of a 9-hour-long recording. As illustrated in Figure 5, “local” $\beta$ calculated according to this scheme displayed temporal variations ranging from $-1.1$ to $-1.8$, whereas $\beta$ of the entire recording amounted to $-1.6$. A similar range of variability was found in the other 3 long

Figure 3. Power spectra of beat-rate variability in 2 different cultures. Double-logarithmic scale reveals the power-law relationship between PSD and frequency. The slope of the linear fit (gray) corresponds to the power-law exponent $\beta$. A, Spectrum closely follows the regression line. B, Power spectrum consists of 2 segments (dotted line fitted by eye).

Figure 4. Power-law behavior of beat-rate variability in a long recording. A, Beat-rate time series of a 2-day-old culture. B, Power spectrum of the data shown in panel A. Linear regression over the range 0.0001 to 1 Hz yielded a power-law exponent of $-1.04$.

Figure 5. Temporal fluctuations of the power-law exponent $\beta$ during the course of a 9-hour-long recording. $\beta$ was computed over the frequency range 0.001 to 1 Hz for a “sliding window” of 2048 seconds, which was moved along a 9-hour-long beat-rate time series in steps of 2 minutes. $\beta$ exhibited variations ranging from $-1.1$ to $-1.8$ during the course of the experiment.
A Case With Different Dynamics

As mentioned above, one preparation produced a beat-rate time series that was not self-similar and that did not exhibit power-law scaling behavior. This preparation had a mean beat rate of 6.6 Hz, which was at the upper limit of the observed values (3.3 ± 1.4 Hz; range, 0.9 to 6.6 Hz). The beat-rate time series of this preparation is illustrated in Figure 6B, top trace, in comparison with another series exhibiting fractal properties (Figure 6A, top trace). Compared with the series with scaling behavior, in which no repeating pattern was present, this series exhibited repeating patterns at irregular intervals, thus indicating that this series was less complex. A simple method to untangle a deterministic behavior in a beat-rate time series consists of using phase portraits. In this data representation, a given data point is plotted as a function of the previous data point. If, in such a representation, data form an organized pattern around a so-called attractor, it is likely that the system is deterministic. As indicated by the phase portraits shown in the lower panels of Figure 6, the data points of the preparation with power-law behavior formed a normally distributed “cloud” filling the phase space. In contrast, the data points and interconnecting lines of the preparation without power-law behavior formed a cluster along an organized attractor that did not fill the phase space. This indicates determinism in the dynamics of the system and suggests that the mechanisms underlying the variations of the beat rate in this particular case were different from those present in the remaining experiments.

Discussion

It is well known that aperiodic components of human HRV in vivo exhibit a broad-band spectrum in the very low frequencies (<0.01 Hz), which is characterized by a power-law relationship between PSD and frequency.7–9 It is generally accepted that this power-law behavior of HRV in vivo depends on extracardiac influences consisting of the nonlinear interactions of the sympathetic and the parasympathetic nervous systems occurring over a broad range of time scales.16–18 The present study shows that spontaneously beating monolayer cultures of cardiomyocytes are capable of displaying similar types of beat-rate variability in vitro. Their beat rate follows a fractal pattern exhibiting a power-law behavior, whereas periodic fluctuations are absent. Because these preparations are completely devoid of neurohumoral components, this finding suggests that the power-law behavior of HRV might, in addition to extracardiac influences, be determined by factors intrinsic to cardiac tissue.

Spontaneous Activity in Monolayer Cultures of Cardiomyocytes

It is well known that cardiomyocytes in culture can form synchronously contracting cell monolayers, and it is generally assumed that these networks are driven by a focal pacemaker. Like the sinus node, the preparations consist of a network of coupled excitable elements exhibiting specific spatiotemporal activation patterns. It was shown previously on the basis of computer simulations of networks of pacemaker cells with different intrinsic cycle lengths that, once the cells are coupled, their cycle lengths synchronize, giving rise to focal excitation of the entire network.19,20 The presence of focal pacemaking regions in dense monolayer cultures of cultured cardiomyocytes has recently been verified experimentally in monolayer cultures.21 In contrast to previous studies, which found spontaneous activity to be a ubiquitous phenomenon in monolayer cultures of cardiomyocytes,14 the preparations used in the present study were generally only active after enhancing L-type Ca2+ currents with BayK8644. This is in accordance with an earlier study showing a Ca2+ current dependence of the pacemaking mechanism in these preparations.22 The failure to beat spontaneously was not due to abnormalities in the electrophysiology of the cells, as extracellular stimulation resulted in rapid activation of the entire monolayers. Rather, it might be speculated that, in the absence of BayK8644, the putative pacemaking regions within the preparations were clamped to resting potential by the surrounding well-coupled and -polarized cardiomyocytes of ventricular origin and that only an increase in L-type Ca2+ currents permitted these cells to overcome this impedance mismatch.15,23

Spontaneous Activity Exhibiting Power-Law Behavior

In 21 of 22 preparations, the beat-rate time series was characterized by a power-law behavior. The short recordings permitted assessment of the power-law exponent β over the range 0.001 to 1 Hz and yielded a value of −1.31 ± 0.20, whereas the long recordings, in which β could be assessed over an additional decade (0.0001 to 1 Hz), yielded a value of −1.48 ± 0.38. When examined over an identical frequency range (0.001 to 1 Hz), β of long recordings was −1.56 ± 0.32
and was not significantly different from that of short recordings. Thus, the different recording lengths had no impact on $\beta$ determined in the range of 0.001 to 1 Hz. Also, power-law behavior was not dependent on the presence of BayK8644; in 3 experiments performed without this substance, beat-rate variabilities assessed over the frequency range of 0.001 to 1 Hz exhibited a power-law with $\beta = (-1.60 \pm 0.21$; range, $-1.39$ to $-1.80$) being not significantly different from that observed in the preparations exposed to BayK8644.

In clinical studies, $\beta$ is assessed over the range 0.001 to 0.01 Hz because, at higher frequencies, the power-law is disrupted by the LF and HF components. In healthy subjects, $\beta$ is $\approx -1.0.7$ whereas it is $\approx -1.2$ after myocardial infarction$^{8–10}$ and $\approx -2$ after cardiac transplantation.$^9$ When determined over the same frequency range, $\beta$ in the present study was $-1.35 \pm 0.72$ and is therefore positioned between values after myocardial infarction and cardiac transplantation. Even though a direct comparison between $\beta$ determined in vivo and in vitro has to be made with great caution (see Limitations of the Study section), the value of $\beta$ obtained in the cultured preparations deprived of neuroendocrine influences could lend support to the hypothesis that, during cardiac disease, a partial breakdown of autonomic control may unmask components of HRV intrinsic to cardiac tissue, which display a steeper power-law relationship than that observed in healthy subjects.

**Power-Law With Multiple Scaling Domains**

In 9 of 17 experiments, the power spectrum did not closely follow the regression line but rather consisted of 2 distinct scaling domains exhibiting a crossover (see Figure 3B). This double-scaling behavior is also found in Fourier power spectra of HRV$^8$ and of blood pressure variability$^{24}$ and was assessed in more detail in human HRV time series using detrended fluctuation analysis. The double-scaling behavior was especially prominent in data from elderly subjects and from subjects with congestive heart failure. The authors suggested that the double-scaling behavior is related to the breakdown of extracardiac regulatory mechanisms during aging or disease. In the present study, in which extracardiac regulations were absent, the double-scaling behavior was observed in about half of the experiments, whereas the others exhibited only 1 scaling domain. Although this does not provide a physiological explanation for the presence or absence of multiple scaling domains, and although the caveat regarding the comparison of in vivo and in vitro results applies, this finding suggests that factors different from extracardiac influences might possibly contribute to the establishment of the true power-law behavior observed in healthy subjects.

**Fluctuations of the Power-Law Exponent**

It was observed that the power-law exponent behaved in a nonstationary and erratic manner in a given preparation over time (see Figure 5). This intrinsic nonstationarity, with $\beta$ changing drastically within hours, is most likely the main reason for the large variability of the $\beta$ and $H$ exponents observed in the short experiments. Moreover, the finding that the SD of $\beta$ in long experiments was not smaller than in the short experiments suggests that the temporal variability of $\beta$ does not follow a gaussian behavior and that the dispersion of $\beta$ values most likely would not have been decreased by performing longer (eg, 24 hours) recordings. Furthermore, the collected values of $\beta$ throughout this study exhibited a larger SD than that observed in previous clinical studies.$^9,10$ This suggests that the neuroendocrine modulation of HRV might have a stabilizing effect on $\beta$. This hypothesis is further supported by the finding of Bigger et al$^9$ that the dispersion of $\beta$ is smaller in healthy subjects than in diseased or transplanted patients.

**Intrinsic Power-Law Behavior: Possible Mechanisms**

The presence of a power-law behavior in the absence of neuroendocrine regulations opens the question of underlying physiological mechanisms. It was previously suggested that systems characterized by power-law behavior can consist of many different regulatory mechanisms operating and interacting over a broad range of temporal scales.$^{16–18}$ In cardiac tissue, the intrinsic power-law behavior might therefore be accounted for by complex interactions of a large number of such processes acting at different time scales. Possible candidates include ionic channels, in which fractal patterns of channel gating in single-channel recordings were observed and mathematical models of channel gating accounting for this property were formulated.$^{26,27}$ Moreover, electrophysiological studies showed the presence of fractal characteristics in the oscillations of the membrane potential.$^{28}$ Also, it was shown that, while isolated cardiac cells beat stochastically, fractal patterns of activity establish when a large number of cells are electrically coupled and synchronize.$^{29}$ Finally, it is feasible that other processes, such as biochemical reactions, protein synthesis, or gene expression, contribute to the observed variations in beat rate. Thus, cardiac tissue possesses a multitude of processes interacting at different time scales that might, together, be responsible for the observed tissue inherent power-law behavior.

**Spontaneous Activity Exhibiting Low-Dimensional Dynamics**

In contrast to all other preparations, a single culture failed to exhibit a power-law behavior and showed low-dimensional dynamics instead (see Figure 6). Although the shape of the extracellular action potentials and the signal amplitudes measured in this culture were not different from those measured in all other preparations, thus indicating similar basic electrophysiological properties, this culture exhibited a particularly high average beat rate (6.6 Hz versus 3.4 ± 1.4 Hz in the remaining preparations). On the basis of previous observations that preparations with very high beat rates are usually driven by functional reentrant excitation,$^{21}$ the absence of a power-law behavior in this single preparation might be explained by the presence of a pacemaking mechanism different from the remaining preparations (reentrant activation versus focal activation). This hypothesis is also supported by a theoretical study showing that phase portraits of interbeat intervals in 2-dimensional networks of cardiac cells during spiral wave reentry exhibit low-dimensional properties.$^{10}$ If indeed the occurrence of low-dimensional dynamics in cultured cardiomyocytes should be related to the presence of reentrant excitation, this experimental system might prove to be useful in screening of pharmacological
substances, as the analysis of the frequency content of signals obtained from a single point in a culture might disclose the presence of reentry.

Limitations of the Study
When extrapolating the findings of the present study to the sinus node, several caveats apply, as follows. (1) Cultured cells differ from nodal cells in situ by the type, distribution and density of ion channels and gap junctions. (2) The size of the preparations used is larger than a sinus node. (3) The dimensionality is different (2-dimensional monolayers versus 3-dimensional sinus node). (4) The tissue architecture is different (confluent monolayers versus complex nodal micro-architecture). Nevertheless, cardiomyocytes in culture represent, in a manner functionally similar to that of the sinus node, a network of coupled oscillators in which the network dynamics are essential in the generation of fluctuations in the discharge rate. However, the ultimate proof that the sinus node itself, in the absence of neurohormonal influences, is capable of displaying a power-law behavior will have to await the development of appropriate experimental models.

Conclusions
The results of the present study suggest that components intrinsic to cardiac tissue can cause spontaneous activity in cultured cardiomyocytes to display fractal properties. In this respect, the results extend earlier findings obtained in vivo that described fractal properties of HRV to be dependent on nonlinear interactions of the sympathetic and the parasympathetic nervous systems. Thus, it might be speculated that the fractal measures of HRV in vivo, which are correlated with cardiovascular health and represent an analytical tool for the clinical assessment of cardiac diseases, might ultimately be the result of an interplay of factors extrinsic and intrinsic to cardiac tissue. Obviously, further investigations will be needed to get deeper insights into the exact nature of this interplay.

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