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Generality of a power-law long-term correlation in beat timings of single cardiac cells

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ABSTRACT

Statistical properties of spontaneous contractions of atrial muscle cells were examined and compared to those of ventricular muscle cells. The cells derived from atria of neonatal rats exhibit spindle morphology, and they were found to express α-smooth muscle actin and hyperpolarization-activated cation channel 4, both of which are known marker of neonatal atrial muscle cells. The short-term properties of spontaneous contractions of atrial cells, characterized by considerably large beat rate and absence of bursts, are distinct from those of ventricular muscle cells. Despite of these differences, the long-term properties of the beat-rate fluctuations exhibit a remarkable similarity to those of ventricular cells. In particular, the presence of power-law correlation characterized as $1/f^{\beta}$ noise ($\beta \approx 1$) was also confirmed for atrial cells for the first time. The observed similarity of the long-term characteristics of beat-rate fluctuation suggests the presence of a general regulatory mechanism of the cellular function.

Introduction

In recent years, increasing interests are focused on the characteristics of fluctuations of cellular functions, including gene expression, metabolism and signal transduction, of cultured mammalian cells [1–3]. Among various types of cells, neonatal cardiac muscle cells serve as an ideal model system in order to study the fluctuations of cellular functions in a quantitative manner because of several advantages. First, under appropriate conditions, cultured neonatal cardiac muscle cells exhibit spontaneous contractions, and the nature of fluctuations in this activity has been studied from various aspects so far [4–6]. Since cardiac muscle cells are terminally differentiated and do not proliferate, properties of fluctuations of cellular activities can be thoroughly examined in a stationary condition, which should shed light on the regulatory mechanism of the cellular functions. Moreover, we can utilize the vast molecular-level knowledge on the regulatory network of contraction in order to underpin the observed fluctuations in the cellular functions [7].

So far, the long-term characteristics of fluctuations in contraction timings of cultured cardiac muscle cells have been studied by several groups [8–12]. It has been reported that the timings of spontaneous contractions exhibit a long-term correlation of the power-law type. Noting that similar characteristics have been observed in the time series of intact human heartbeats [19,20], it is also interesting to closely study the nature and the mechanism of this power-law correlated fluctuations at the cellular level in order to clarify its connection to the heart rate variability. In the former studies, however, the experiments have been mainly conducted in monolayer cultures in which a number of cells form an interconnected network and exhibit complicated spatio-temporal dynamics [8–11], which makes it difficult to clarify whether the power-law correlated fluctuations arise as a result of intercellular interactions or as an intrinsic property of isolated single cells. In order to address this issue, we recently studied the statistical properties of spontaneous contractions of isolated single cardiac muscle cells over an extended period. As a consequence, it has been demonstrated that the beat timings of isolated single cells do exhibit power-law correlated fluctuations which are characterized as $1/f^{\beta}$ noise ($\beta \approx 1$). Therefore, the $1/f^{\beta}$-noise-type power-law correlation has been found to be an intrinsic property of isolated single cardiac cells [12].

In the former studies, however, all the investigations have been conducted by using neonatal ventricular muscle cells. This is partly because the cultured neonatal ventricular muscle cells have been widely utilized as a useful model system in a variety of cell-biology studies. However, cultured neonatal ventricular cells are known to possess several abnormal characteristics, e.g., autonomicity, which should be absent in ventricular cells *in vivo*, and less organized cytoskeletons compared to the cells in an intact tissue. Therefore, it is necessary to examine whether a similar tendency is found in

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other types of cells. This enables us to verify the generality of the long-term correlation of beat timings observed in single cardiac cells.

In this paper, we present our results concerning the long-term characteristics of isolated single atrial cells. For this purpose, we established a primary culture of neonatal atrial cells. We then characterized the morphological, histochemical and dynamical properties of these isolated cells. Finally, by studying the statistical properties of the beat timings, we identified power-law correlated fluctuations in the beat timings. The correlation was found to be that of $1/f^{\beta}$ noise with β close to unity. This result suggests the power-law long-term correlation is a generic property that is found independently from the types of cells.

Materials and methods

Cell preparation and culture. The experimental procedures were approved by the Animal Use and Care Committee of the University of Fukui. Primary cultures of atrial cells were prepared from neonatal rats by modifying the method developed for ventricular cells in our previous study [12]. Briefly, right and left atria were isolated from hearts of one-day-old rats. The atria were then minced and were treated with collagenase [0.2% dissolved in phosphate buffered saline (PBS)] at 37 °C for 10 min. The treatment was repeated twice, and the supernatant from the second treatment was used in the following procedure. Next, the method of selective plating [13] was adopted in order to reduce the population of non-muscle cells; the cells were supplied with plating medium [90% Dulbecco-modified Eagle medium, 10% fatal bovine serum, 0.1 mM 5-bromo-2′-deoxyuridine (BrdU) and penicillin/streptomycin solution (P4333, Sigma–Aldrich, Tokyo)] and plated on a petri dish. Following a 90-min incubation at 37 °C and 5% CO_2, the supernatant was collected. After the cellular density being adjusted (final density, ca 60 cells/mm^2), the collected cells were plated on type-I-collagen-coated ϕ 35-mm petri dishes or collagen-coated glass-base dishes. Following 24-h incubation at 37 °C and 5% CO_2, the medium was replaced with contraction medium (90% modified Eagle medium, 10% calf serum, 0.1 mM BrdU, and penicillin–streptomycin solution). This was followed by incubation at 37 °C and 5% CO_2 up to three days.

Primary cultures of ventricular cells were prepared from the same rats by using the method described in our previous study [12]. Collected ventricular cells were plated at the density of ca 30 cells/mm^2, and were incubated in the plating medium at 37 °C and 5% CO_2 for one day. The medium was then replaced with contraction medium and the cells were further cultivated. The culture was used in the experiments within three days from preparation.

Immunohistochemistry. Cellular types were identified by immunostaining α-smooth muscle actin (α-SMA) and hyperpolarization-activated cation channel 4 (HCN4). Monoclonal anti-α-SMA developed in mouse (A 5228, Sigma–Aldrich, Tokyo) was used at 500-fold dilution with 1% bovine serum albumin dissolved in PBS. Goat anti-mouse IgG labeled with Alexa Fluor 488 (A11001, Invitrogen, Tokyo) was utilized as the second antibody. Anti-HCN4 developed in rabbit (H0284, Sigma–Aldrich, Tokyo) was used at 200-fold dilution. Goat anti-rabbit IgG labeled with Cy3 (C2306, Sigma–Aldrich, Tokyo) was used as the second antibody. For immunostaining, cells cultivated on collagen-coated glass-base dishes were used on the fourth day in culture. Upon preparation, the samples were sealed with VECTA SHIELD (Vector Laboratories, UK). Then, the samples were observed using an inverted fluorescent microscope (TE2000-U, Nikon, Tokyo) equipped with an oil-immersion objective lens (CFI PlanApo VC60×, Nikon, Tokyo). The microscopic images were recorded using an EM-CCD camera (ADT-40, Flovel, Tokyo).

Long-term recording of spontaneous contractions. Cells cultivated for two or three days were used in the measurement of spontaneous activity. First, the medium was exchanged at least 10 h before the measurement in order to avoid possible effects of the medium exchange [14]. During the measurement (10 h at maximum), the medium was not exchanged. The culture dish was then placed on a homemade on-stage incubator mounted on an inverted phase-contrast microscope (IMT-2, Olympus, Tokyo). By use of this incubator, whose interior was maintained at 37 ± 0.2 °C (range), 5% CO_2 and humidified condition, cells can be kept vital for several days with continuously observed their contractions. We chose ca 10 spontaneously contracting cells or small clusters of cells at random in a single field of view of the microscope, whose size was approximately 900 × 680 μm^2. The majority of the analyzed cells was isolated and possess no physical contact with other cells (26 cells out of 36 clusters for atrial cells, 20 cells out of 33 clusters for ventricular cells), while there were several cells that are coupled to other cells to form small clusters (up to four cells in a single cluster). We found no significant dependence of the results described below on the number of coupled cells (see also Ref. [12]). The phase-contrast microscopic images of the cells were obtained via a phase-contrast 4× objective lens. The images were recorded using a digital charge-coupled-device (CCD) camera (IPX-VGA210-LMCN, Imperx, FL) for atrial cells and an analog CCD camera (WAT-902H2, Watec, Yamagata, Japan) for ventricular cells, respectively. The data were continuously stored on a hard disk drive for later analysis.

Data analysis. The timings of spontaneous contractions were determined from a sequence of the phase-contrast microscopic images [12]. The frame rate of the recording was set to 100 frames/s for atrial cells and 30 frames/s for ventricular cells. Because the quality as well as the noise levels was different between images for atrial cells and those for ventricular cells, the parameters for data analysis were optimized for each type of cell as follows.

First, cellular contractions were characterized as follows. Each contraction of a cell is associated with a spikewize change in the brightness of pixels, in particular those located in the vicinity of the cellular boundary. The pixel that exhibited the largest frequency of sufficiently large spikes in the time series of brightness was then automatically detected, and the time series of the brightness associated with this pixel were analyzed. By taking the stage drift or gradual deformations of the cell into account, the choice of the pixel was periodically updated (the periods were 360 s for atrial cells and 1200 s for ventricular cells).

In order to remove slow modulation of a baseline of brightness, the local averages of brightness, which were calculated over 0.5 s for atrial cells and over 10 s for ventricular cells, were subtracted from the original data. Furthermore, a moving-average filter was applied in order to reduce shot noise in the time series. The window of the filter was set five or seven frames for atrial cells, depending on the noise level of the data, and three frames for ventricular cells. Afterwards, the timings in which the time series crosses a prescribed threshold value were associated with the timings of contractions. The threshold values were manually determined for every 10 s for atrial cells and every 1200 s for ventricular cells.

Next, in order to remove fictitious spikes due to shot noise that were not removed by the abovementioned filtering procedure, spikes whose interval from the preceding spike was less than a prescribed threshold value were removed [12]. The value of this threshold was set to 0.05 s for atrial cells and 0.1 s for ventricular cells. It has been later confirmed that this procedure of spike removal results in a negligible effect on the statistical properties of the time series, in particular those in the large timescales. Afterwards, the obtained timeseries were examined by visual inspection in order to check the relevance of the adopted threshold values.

The time series of interbeat intervals (IBIs) determined in the abovementioned manner were analyzed from several aspects. In order to study the autocorrelation of the contraction timings, the detrended-fluctuation analysis (DFA) [15] was utilized in the present study. With regard to the details of this method, see Ref. [12].

In the following, statistical data are represented as average ± SEM, if not otherwise indicated. Statistical analysis was performed by use of ANOVA and Tukey HSD. Statistical differences were judged significant at $P < 0.01$.

Results

Characterization of cells

We successfully cultivated cells derived from atria of neonatal rats. We found that the atrial cells possess substantially different morphology compared with that of ventricular cells [16]. While cultured neonatal ventricular myocytes typically exhibit polygonal morphology as depicted in Fig. 1d, cultured neonatal atrial myocytes typically possess spindle morphology and their size was relatively smaller than that of ventricular cells, as exemplified in Fig. 1a. These cells were found to exhibit autonomous contractions. While the beat rate of ventricular cells was approximately 1 Hz (average ± SD was 1.1 ± 0.4 Hz, $n = 33$), that of atrial cells was approximately 3 Hz (average ± SD was 2.7 ± 0.8 Hz, $n = 36$).

There was a clear difference in the expression of specific genes. As a marker of atrial cells, we investigated the expression level of α-SMA, which has been reported to be strongly expressed in atria of neonatal rats, but only weakly expressed in ventricles of neonatal rats [17,18], by means of immunohistochemistry. Fig. 1 exemplifies the expression patterns of α-SMA for atrium-derived cells (Fig. 1b) and ventricle-derived cells (Fig. 1e). It was observed that α-SMA is strongly expressed in the atrial cell, but only weakly expressed in the ventricular cell. In order to verify this observation, we calculated average fluorescence intensity of the cells. As displayed in Fig. 1g, there was a significant difference in the expression level of α-SMA between cells derived from atria and ventricles. Clearly, the cells derived from atria express α-SMA, while the expression was weak in the cells derived from ventricles.

In addition to α-SMA, we also examined the expression pattern of hyperpolarization-activated cation channel 4 (HCN4), which is predominantly expressed in sino-atrial node (SAN) and is weakly expressed in atrium, but not expressed in ventricle [21,22]. As exemplified in Fig. 1c, HCN4 was found to be expressed in atrial cells, although the expression was relatively weak. As expected, the proteins were localized at the cellular boundary. By contrast, the ventricular cells exhibited virtually no expression of HCN4 (Fig. 1f). As demonstrated in Fig. 1h, the expression of HCN4 was found to be significant in atrial cells, but not in ventricular cells.

These observations indicate that the cells derived from atria and ventricles indeed possess the expression pattern of certain marker gene close to that of each tissue in an intact heart.

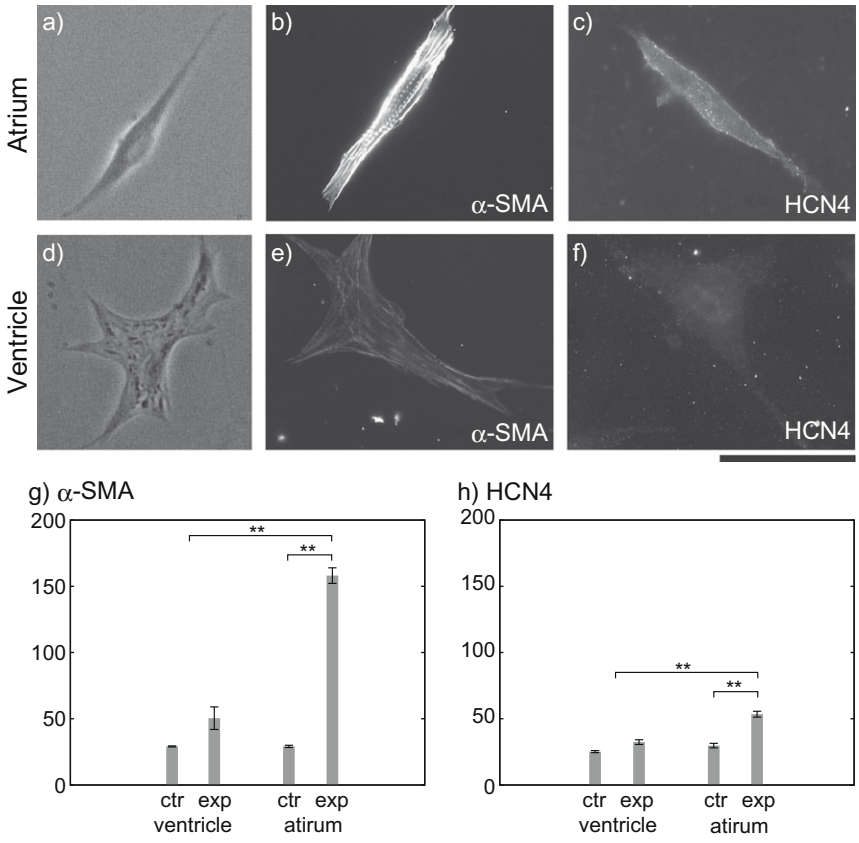

Fig. 1. Morphology and expression patterns of neonatal cardiac muscle cells. (a and d) Typical phase-contrast microscopic images of a cell derived from neonatal rat atria (a) and ventricles (d). (b and e) Immunofluorescent images against α-smooth muscle actin (α-SMA) for an atrium-derived cell (b) and for a ventricle-derived cell (e). (c and f) Immunofluorescent images against hyperpolarization-activated cation channel 4 (HCN4) for an atrium-derived cell (c) and a ventricle-derived cell (f). For (a–f), the scale bar indicates 50 μm. (g) The average fluorescence intensity of the Alexa-labeled second antibody against anti-α-SMA. 'Ctr' indicates the negative control where the first antibody is absent. 'Exp' means the experimental group where the first antibody is present. ** Indicates the significance at $P < 0.01$. (h) The average fluorescence intensity of the Cy3-labeled second antibody against anti-HCN4. The meaning of the symbols are same as those in (g).

Dynamical properties of spontaneous contractions

Next, we investigated the temporal patterns of spontaneous contractions of atrial and ventricular cells. As described above, single atrial cells exhibit autonomous contractions steadily as depicted in Fig. 2A. As explained above, the beat rate was considerably faster than that of single ventricular cells (Fig. 2). In contrast to the ventricular cells, for which we have previously reported the presence of bursting pattern as well as steady one (see Fig. 2A) [12], bursting pattern was not observed for atrial cells in the present condition.

We then studied the statistical properties of the time series of IBIs, particularly focusing on their autocorrelation. Fig. 3A–C display the typical result of the first-order DFA for the IBI time series. From this plot, we observe that the double-logarithmic plot of the DFA fluctuation function, $F(n)$, displays monotonic increase for every type of cell. Typically two (or three) scaling regions were identified in these plots. In the short timescale region ($n < 10^2$), the scaling exponent was close to 0.5, while in the large timescale region ($n > 10^3$), the exponent was significantly larger than 0.5. Because the exponent α is related to the scaling exponent, β, of the power spectrum density as $2\alpha = \beta + 1$ [20], this observation implies that the fluctuations of IBIs exhibit scale invariant long-term correlation in sufficiently large timescales, while they are uncorrelated in short timescales. In several cases of bursting pattern for ventricular cells, there appears another scaling region in the intermediate timescale ($10^2 < n < 10^3$) with the exponent less than a half, indicating the presence of anticorrelation (see Ref. [12]). However, this was not always the case because of large fluctuations in the length of the quiescent periods, as depicted in Fig. 3C.

Fig. 3D displays the histogram of the scaling exponents for atrial cells ($n = 36$) and ventricular cells ($n = 33$). The scaling exponents were determined by the least-square fitting of the double-logarithmic plot of $F(n)$ in the range $n > 10^{2.5}$. As seen in this figure, the scaling exponent displays a distribution centered at $\alpha \approx 1$ for both species. The mean was $\bar{\alpha} = 0.97 \pm 0.03$ for atrial cells and $\bar{\alpha} = 0.94 \pm 0.03$ for ventricular cells. Since $\alpha \approx 1$ means $\beta \approx 1$, it was concluded that the fluctuations of IBIs of single atrial cells exhibit $1/f^{\beta}$ noise in the long timescales as in the case of ventricular cells [12].

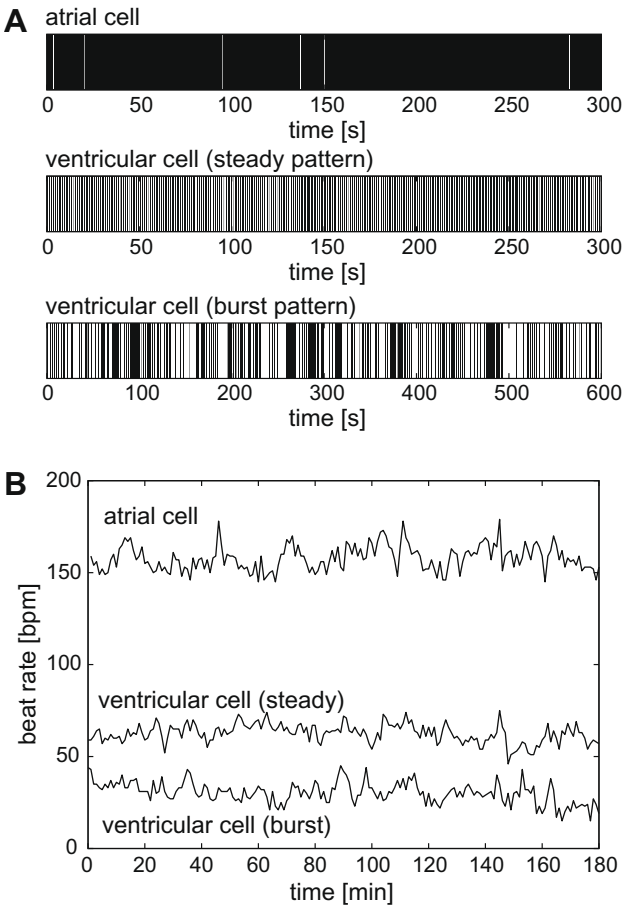

Fig. 2. Typical beating dynamics of isolated cells. (A) Raster plots for atrial and ventricular cells. Each vertical line indicates a single event of contraction. In atrial cells, fast and steady contractions continue. By contrast, in ventricular cells, typically two distinct temporal patterns, termed steady pattern and burst pattern, were identified [12]. (B) Typical time series of beat rates in units of beats per minute (bpm).

Discussion and conclusion

In the present communication, we have studied the statistical properties of the contraction timings for single atrial cells derived from newborn rats. As far as the authors are aware of, this is the first detailed study of the statistical properties of spontaneous contractions for isolated atrial cells. In our study, we first examined the morphological and histochemical properties of isolated atrial cells. The cells derived from atria typically possess spindle shape. They express α-SMA at a high level, which is a well-known marker for the neonatal atrial muscle cells [17,18]. Furthermore, it was found that HCN4 is weakly expressed in the atrial cells. This is also consistent with a previous observation regarding the distribution of this channel in the heart [22]. By contrast, these genes were not expressed in ventricular cells. On the basis of these observations, the cells derived from atria are expected to exhibit different characteristics from those of ventricular cells. A difference between atrial and ventricular muscle cells in their dynamical properties was prominent in the rate of spontaneous contractions. While ventricular cells beat typically at 1 Hz, the spontaneous beat rate of the atrial cells was around 3 Hz in the same experimental condition. One possible cause of this difference is the relatively high expression level of HCN4 in the atrial cells compared with the ventricular cells, because HCN4 is known to play a vital role in pacemaking by SAN [23]. This fact also might account for the absence of the bursting pattern in the atrial cells, which is often observed in ventricular cells [12].

In spite of the clear difference in the short-term properties of the dynamics of spontaneous contractions, we found a remarkable similarity in the long-term characteristics between the atrial and ventricular muscle cells, i.e., the presence of a power-law correlation of beat-timing fluctuations which is characterized as $1/f^{\beta}$ noise ($\beta \approx 1$). Although in several previous studies, it has been reported that the power-law correlation has been observed in the fluctuation of spontaneous beat rate of cultured cardiac muscle cells [8–12], these observations were restricted to ventricular cells and no experimental results were available on the long-term properties of the spontaneous contractions for other types of cells. In the present study, we report the presence of a similar power-law correlation in atrial cells, whose structure, gene expression pattern and the short-time properties are distinct from ventricular cells. This suggests that some of the long-term properties of the beat-timing fluctuations and the underlying intracellular processes are universal over different species of cells. This type of universality, if it presents, is of particular importance, because it enables us to access the generic properties of the regulatory mechanism of cellular functions through investigation of their long-term dynamics. Toward this goal, it is necessary to clarify the molecular mechanism responsible to the power-law correlated long-term fluctuations of spontaneous contractions of cardiac muscle cells.

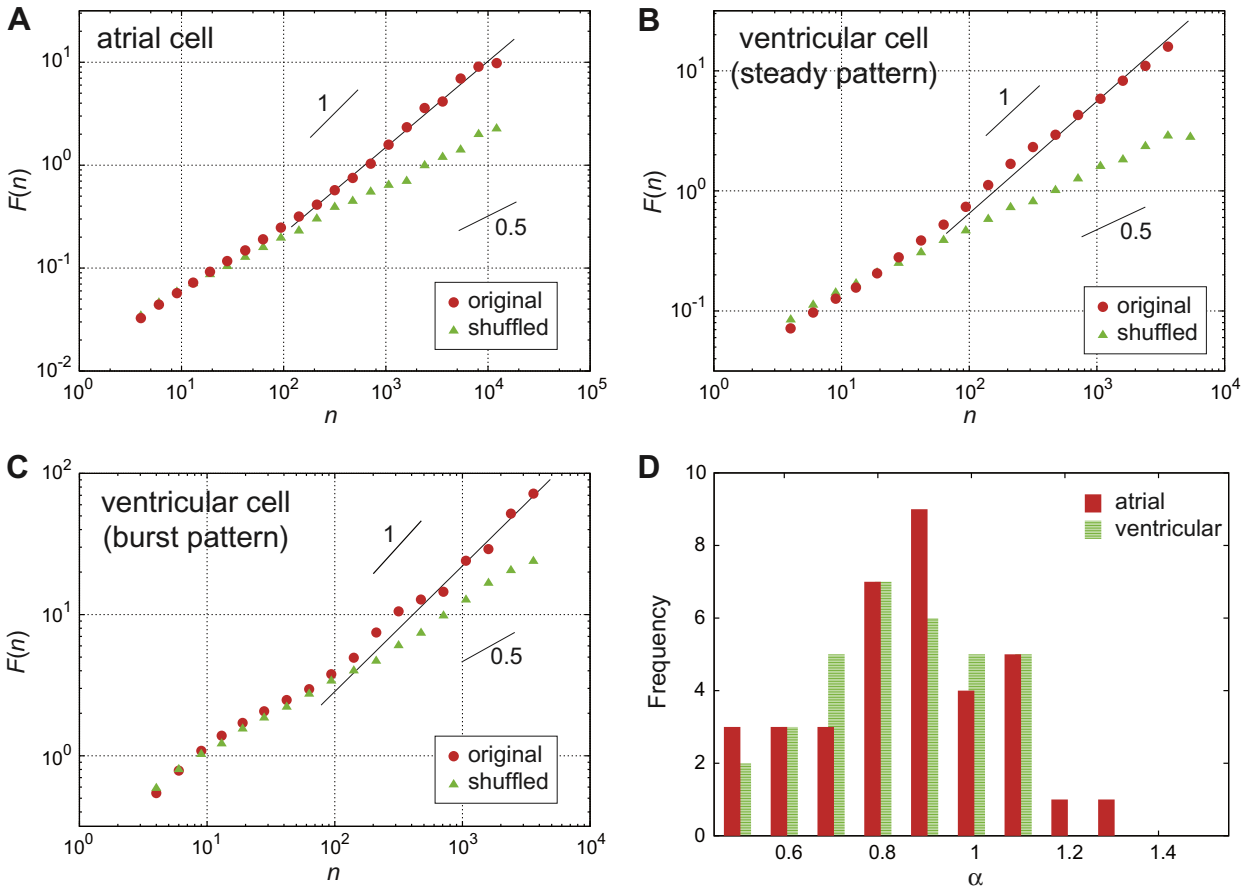

Fig. 3. The result of DFA applied to the time series of IBIs, revealing the long-term correlation in IBIs. Typical results for (A) atrial cells, (B) ventricular cells in the steady pattern, and (C) ventricular cells in the burst pattern are displayed. In each plot from (A) to (C), the filled circles represent the original experimental data, while the filled triangles represent the results of DFA applied to the time series whose sequential order was randomly shuffled, consisting the null hypothesis. The solid lines are the least-square fit to the scaling region in the large timescales ($n > 10^{2.5}$), yielding the exponents $\alpha = 0.83 \pm 0.04$ (A), $\alpha = 0.93 \pm 0.07$ (B) and $\alpha = 0.89 \pm 0.07$ (C), respectively. (D) The histogram of the scaling exponent α in the large timescales. The solid bars are the data for the atrial cells ($n = 36$), and the shaded bars represent the data for ventricular cells ($n = 33$).

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