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Synchronization of electrically induced calcium firings in self-assembled cardiac cells

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Abstract

We study the adaptive changes of a population of cells responding to external stimulus. Two-dimensionally distributed cardiac cells were homogeneously subjected to periodic electrical stimulus and intracellular calcium concentration ($[Ca^{2+}]_i$) changes were simultaneously observed. In the absence of stimulation, coupled cells in monolayer formed groups of several cells oscillating in similar phase, while isolated cells showed irregular periodicity. In both systems, $[Ca^{2+}]_i$ oscillations were modulated by periodic stimulation, and ascending degrees of synchronization among $[Ca^{2+}]_i$ oscillations were shown as stimulation intensity increased. In a population of coupled cells, the cells act like a single robust oscillator. These results are evaluated using statistical calculations, comparing the response manner of isolated cells.

Keywords: Biological rhythm; Calcium firing; Cardiac cell; Cellular assembly; Periodical forcing

1. Introduction

Self-sustained oscillation in cardiac cells has been widely studied as a biological oscillator spontaneously generating rhythm. A possible explanation for the intracellular calcium concentration, $[Ca^{2+}]_i$, oscillation is provided with the calcium-induced calcium release model (CICR), which is based on periodic calcium release from and uptake into the sarcoplasmic reticulum, resulting in mechanical contractions [1–3]. Isolated single cells repeat mechanical contractions or $[Ca^{2+}]_i$ firings, exhibiting irregular periodicity rather than regular. Oscillatory rhythm is modified when the cells are subjected to external perturbation. The response of the cells to perturbation depends on the phase of oscillation [4,5].

In a cultured cardiac system, a large number of cells forms a dense network, connecting electrically or chemically. Strong coupling across a network leads to a rather regular and spatially correlated beating [6,7] or propagation of spiral waves [8]; nevertheless, individual spontaneous beating activity in isolated single cells is essentially stochastic. Intercellular communication in the form of diffusive coupling of signaling molecules allows cells to coordinate the phase of oscillation rhythm. Due to this property, they can be viewed as a single oscillator. Thus, while the cells coupled in a network are expected to respond to external perturbations as a group rather than as individuals, there is little experimental verification.

The process leading to Ca^{2+} oscillation is initiated by the entry of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels into cytoplasm. Therefore, it is possible that external electrical stimulation artificially induces $[Ca^{2+}]_i$ firing [9,10]. In this paper, we investigated the response of cardiac cells, whether or not in the presence of coupling among cells, to electrical stimulation. $[Ca^{2+}]_i$ changes in the cultured monolayer on an optically transparent electrode were monitored using a fluorescent microscope in order to perform real-time recordings of spatio-temporal dynamics of Ca^{2+} activity during the application of electrical stimulation.

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2. Methods

2.1. Cell culture

We used cardiac cells from embryonic (embryonic day 18, E18) Sprague–Dawley rats. For the embryonic culture, we used a modified version of a previously described dissociation technique [11]. The hearts of about 10 embryos were removed under sterile conditions and placed in Ca/Mg-free Hanks balanced salt solution (HBSS; Gibco, Japan). After removing excess blood, the hearts were chopped into 1 mm^2 pieces, collected in a centrifuge tube, and washed three times in ice-cold Ca/Mg-free HBSS. The buffer was then replaced with 0.025% crude trypsin (Sigma-Aldrich, Japan) in versene buffer (Gibco), and after 4 min of incubation at 37 °C, the supernatant was discarded. The enzymatical dissociation was accomplished by the addition of DNAse Type II solution (10^{10} U dm^{-3}, Sigma) followed 2 min later by the addition of 0.025% crude trypsin/versene and then stirred for 8 min at 37 °C. The supernatant was then collected and added to Hepes-buffered Ham's F10 (Gibco) containing 0.5% insulin, transferrin, and selenite solution (ITS; Gibco) and 36% fetal calf serum (FCS; Gibco), centrifuged for 5 min at 1500 rpm and resuspended in ice-cold Ham's F10 containing 0.5% ITS and 10% FCS. This trypsination step was repeated until all of the remaining tissues were dissociated. The collected cell suspensions were then pooled in a tissue culture ask and incubated for 20 min at 37 °C. This differential adhesion step led to an increased proportion of myocytes in suspension. The cells were plated to fibronectin-coated devices (10^{-7} dm^3) at cell densities of 100×10^{12} cells dm^{-3}. After 1 day, the culture medium was replaced by Hepes-buffered Ham's F10 containing 0.5% IST and 3% FCS (culture medium).

2.2. Electrical stimulus

An indium–tin–oxide (ITO)-coated glass plate (Kinoene, Japan) was cut into 18×18 mm and connected with Au wire to provide an electrical stimuli. The surface of the working electrode was covered with fibronectin and dissociated cells were placed on this. The counter electrode was positioned 0.1 mm above the working electrode surface with spacer. These electrodes were submerged into the culture medium. Electrical stimuli in the form of rectangular pulses with 100 ms of width were generated with a function synthesizer (Wave Factory, 1941). The cultured cells plated to the electrode were loaded with fluo-

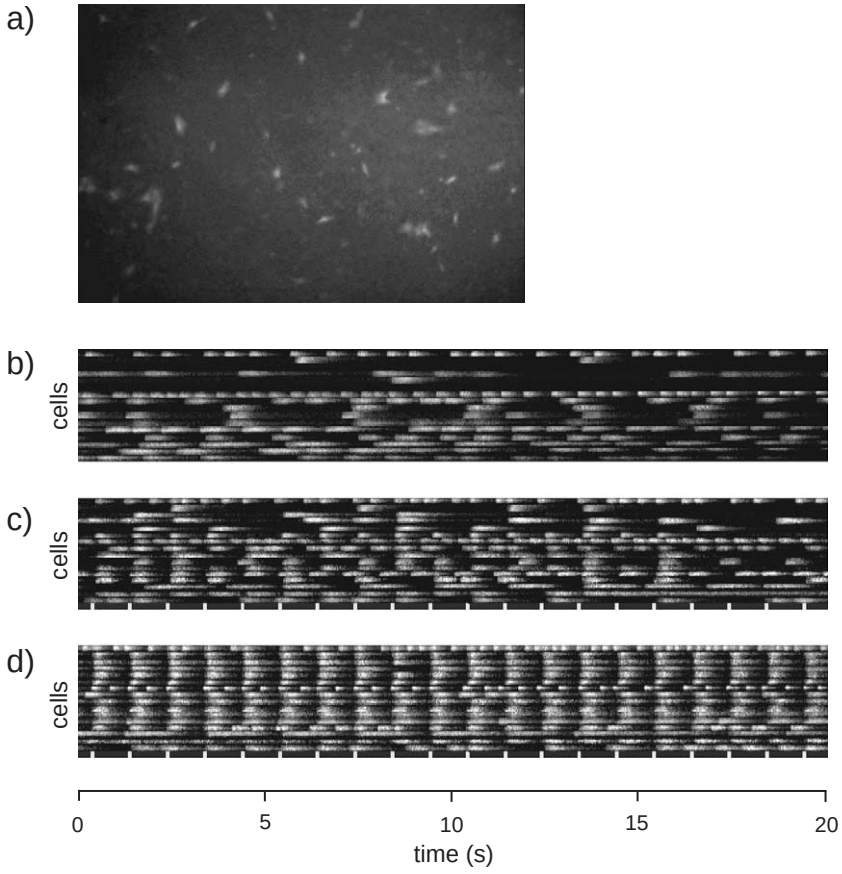

Fig. 1. Image of random Ca^{2+} firings in rat cardiac cell monolayer obtained by fluorescence measurement made visible with the calcium indicator, fluo-3 (a), and spatio-temporal plots of Ca^{2+} firings induced by periodic electrical stimulus with intensities of (b) 0 V, (c) 2.5 V, and (d) 4.0 V. White dots at the bottom of the plots in (c) and (d) indicate the timing of electrical stimulation.

3 (4 mM; Molecular Probes) to allow the observation of $[Ca^{2+}]_i$. A fluorescent video imaging system (Hamamatsu, Image Intensifire Unit C8600) allowing low-light-level measurements was attached to an inverted microscope (Olympus, IX71). The timing of stimuli was recorded as a small white line on the video images by a home-made video superimposer. Images were transferred directly to a computer with 30 Hz.

3. Results and discussion

Enzymatically dissociated cells planted on the electrode surface were incubated for 72 h in a 5% CO_2 environment at 37 °C. Cells formed self-assembled monolayers and reestablished intercellular contacts, but incompletely at the outside edge of monolayers. We first examined the isolated cell system which had no cell–cell contact. Fig. 1a shows the image of isolated cells, made visible with the calcium-sensitive fluorescent dye, fluo-3. White areas correspond to Ca^{2+} localization at a fixed moment during the elevation of $[Ca^{2+}]_i$. In this measurement, individual $[Ca^{2+}]_i$, which are the result of spontaneous Ca^{2+} release from the sarcoplasmic reticulum, were observed.

The spatio-temporal plots in Fig. 1b–d show the electrical-induced transition from random to synchronous firing of $[Ca^{2+}]_i$ observed from 16 cells. This plot was constructed from gray levels along the horizontal section of 10 pixels where $[Ca^{2+}]_i$ oscillations occur in cells. The vertical axis was assigned to each cell arbitrarily, so that positional information among the cells was lost. Fig. 1b corresponds to a case of a time evolution for 20 s in the absence of electrical stimulus. We note here that there is no correlation between firing events among cells and no periodicity within each cell, and that the

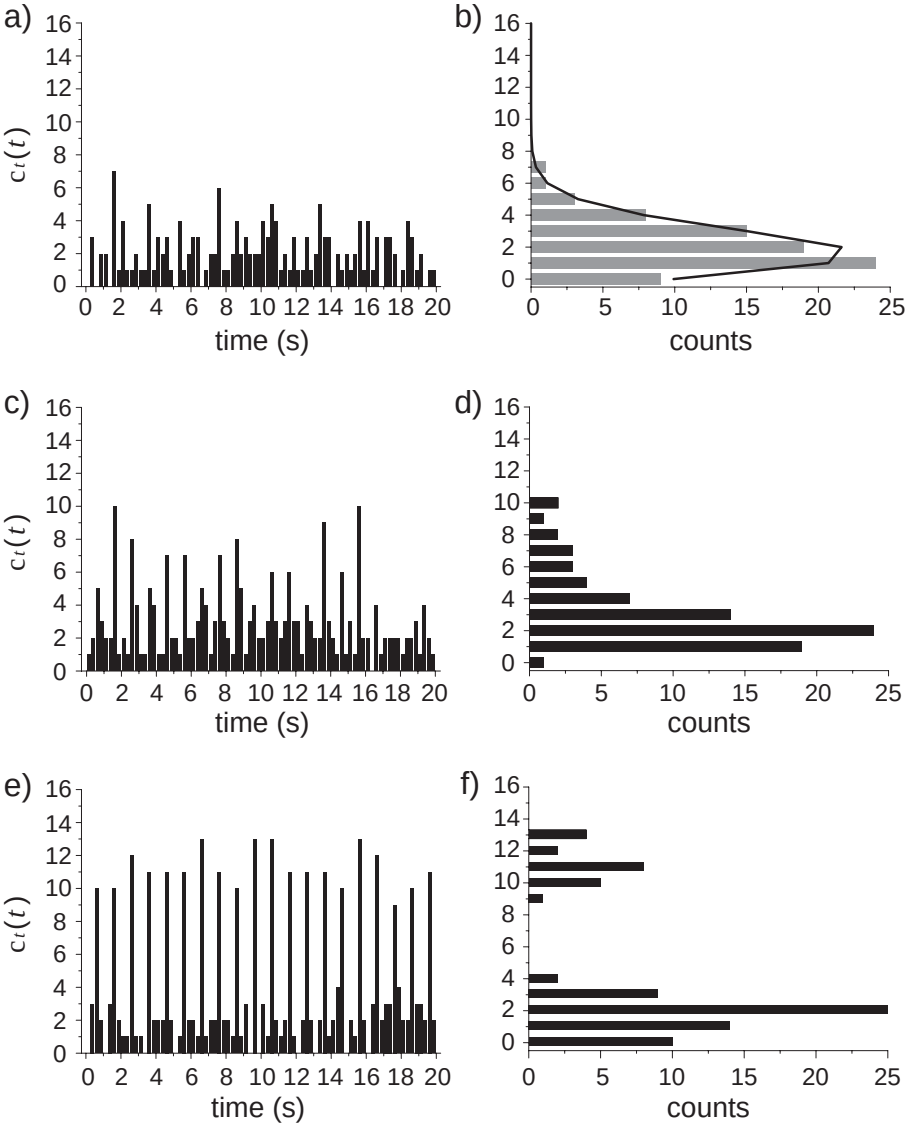

Fig. 2. Time course of $c_t(t)$ (left column) and the corresponding distribution histogram (right column), plotted from Fig. 1. The solid curve in (b) is the fitted Poisson distribution.

period of oscillations fluctuates from a subsecond order to tens of seconds.

Here, spatially homogeneous perturbations to the cardiac cells were carried out by applying the electrical stimulus with an intensity I=2.5 V and frequency f=1 Hz in Fig. 1c. White dots in the bottom layer on the plots of Fig. 1c and d indicate the timing of electrical stimulation. We can observe that $[Ca^{2+}]_i$ changes were induced by external stimulation in some, but not all cells. When the stimulus intensity was further increased to I=4.0 V, nearly all cells entrained in a 1:1 manner. Thus, as the periodic stimulus intensity is increased, $[Ca^{2+}]_i$ oscillations become more regular and synchronous.

Based on these spatio-temporal plots, time dependency of $[Ca^{2+}]_i$ changes of cells and the corresponding distribution histograms are plotted in Fig. 2. In N cells, if the ith cell fires at time t, the firing state of the ith cell is defined as $c_i(t)$=1, else $c_i(t)$=0. The total number of firings at each time step, $c_t(t)$, is thus described as:

$$c_t(t) = \sum_{i=1}^{N} c_i(t). \quad (1)$$

The timing of $[Ca^{2+}]_i$ firing was determined on the basis of the maximum intensity of fluorescence from cell. Fig. 2a represents the time dependency of $c_t(t)$, observed from the 16 cells at each 0.25 s interval, and shows that $c_t(t)$ fluctuates randomly. The height of the peak indicates the degree of synchronization (i.e., the higher the peak, the better the synchronization at each time step). This regime corresponds to the distribution histogram of $c_t(t)$ in Fig. 2b. This histogram gives the frequency of $c_t(t)$ during 20 s. The solid curve is a fitted of Poisson distribution, and it agrees well with the data. Thus, $[Ca^{2+}]_i$ firings without periodic stimulus have no significant intercellular correlation among cells.

When stimuli were applied with I=2.5 V and f=1.0 Hz, the peaks of $c_t(t)$ are higher than those in Fig. 2a, and characteristic peaks appear periodically as shown in Fig. 2c. This shows that firings were modulated by external stimulus. In Fig. 2d, expansion of the $c_t(t)$ distribution toward higher $c_t(t)$ is shown; however, there is no qualitative difference compared with Fig. 2b and d. In Fig. 2e we see that $[Ca^{2+}]_i$ oscillations are clearly induced by external stimulus with I=4.0 V. These higher peak intervals of 1 s agree with the stimulus frequency. It should be noted that the shape of the distribution undergoes an important qualitative change to a bimodal shape (see in Fig. 2f). One component of the bimodal distribution, whose center is around 11, corresponds to the electrically induced synchronous elements.

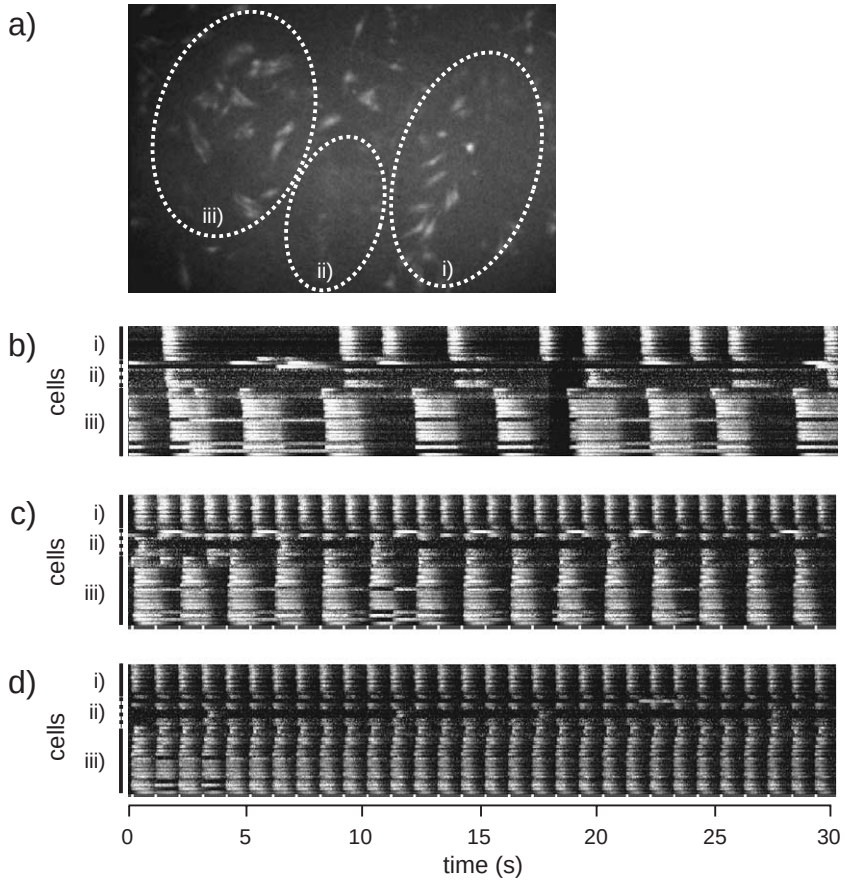

Fig. 3. (a) A fluorescence image of rat cardiac cell monolayer, showing correlated Ca^{2+} firings. Spatio-temporal plots for stimulus intensities of (b) 0 V, (c) 2.0 V, and (d) 3.0 V. White dots at the bottom of the plots in (c) and (d) indicate the timing of electrical stimulation.

We next examined $[Ca^{2+}]_i$ activities of coupled cells in network. Optical microscope observation showed that the cells was of high density, connected near the center of assembly. The 32 firing cells within the field of view were divided into three groups, (i), (ii), and (iii), as shown in Fig. 3a. Each of the groups was comprised of 5–17 cells and located close to the others. Fig. 3b is the spatio-temporal plot in the absence of periodic stimulus. Here, three distinct firing groups are found, which correspond to the three regions within dashed lines in Fig. 3a. Even though there is no stimulus, the cells within groups were synchronous $[Ca^{2+}]_i$, but asynchronous among groups.

When a periodic stimulus of I=2.0 V and f=1.0 Hz was applied, these groups responded in distinctive manners (i.e., the cells in group (i) and (iii) synchronized with periodic electrical stimulus with the 1:1 and 2:1 locking in Fig. 3c). In spite of spatially homogeneous stimulations being applied, a mixed locking manner appeared within the field of view. Upon increasing the stimulus intensity to 3.0 V, synchronous oscillations were observed, as seen in Fig. 3d.

Emergence of the coordinated $[Ca^{2+}]_i$ oscillations among the coupled cells within groups reflects the time dependency of $c_t(t)$. In Fig. 4a, we see that oscillation manner is different from the random activities in Fig. 2a. This disagreement is enhanced in the shape of the distribution of $c_t(t)$, as shown in Fig. 4b. The deviation from a Poisson distribution was caused by the collective behaviors. When periodic stimulus of I=2.0 V was applied to this coordinated state, as shown, each of the groups responds differently to stimulus, as reflected by higher and lower peaks. The distribution histogram in Fig. 4b is divided into three fractions and shows that qualitative change occurs (shown in Fig. 4d). Increasing the stimulus intensity led to complete synchronization in a 1:1 manner, as shown in Fig. 4e. There are two

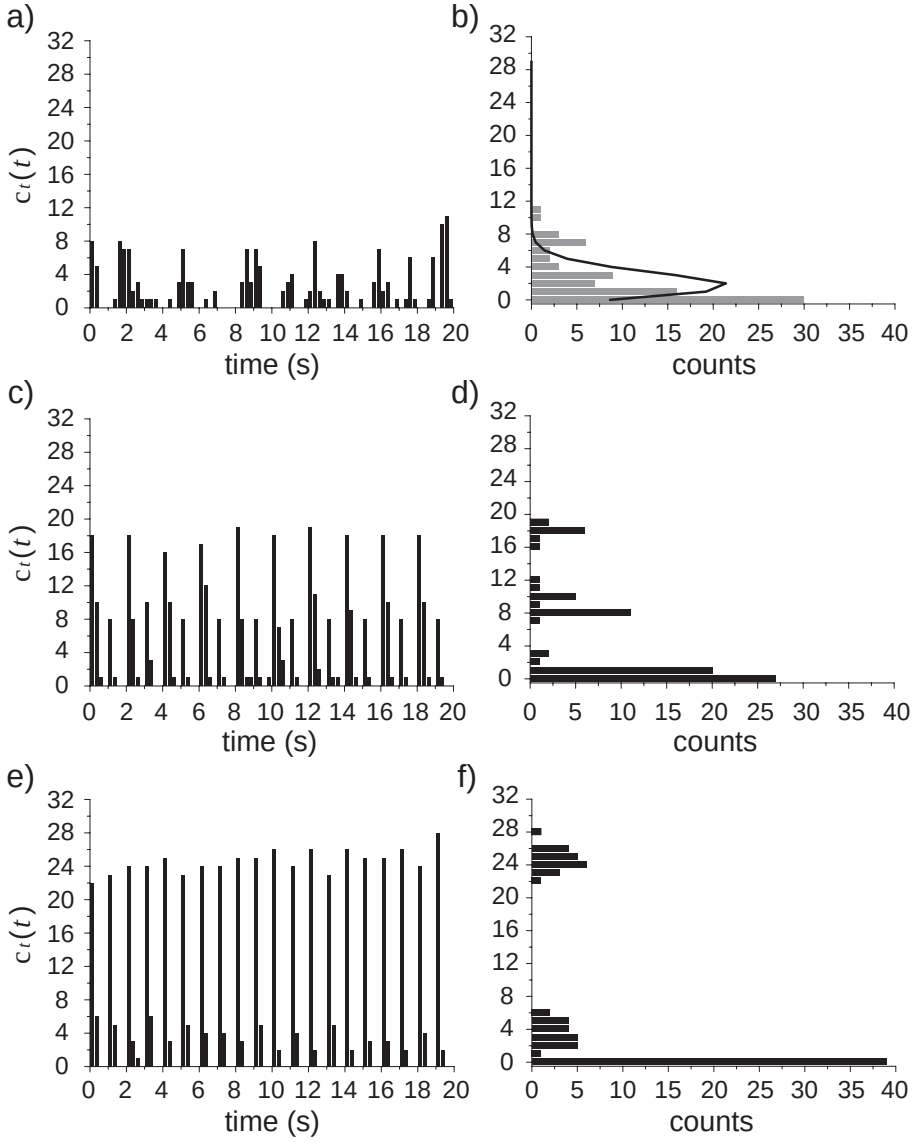

Fig. 4. Time dependency of $c_t(t)$ (left column) and the corresponding distribution histogram (right column), plotted from Fig. 3. The solid curve in (b) is the fitted Poisson distribution.

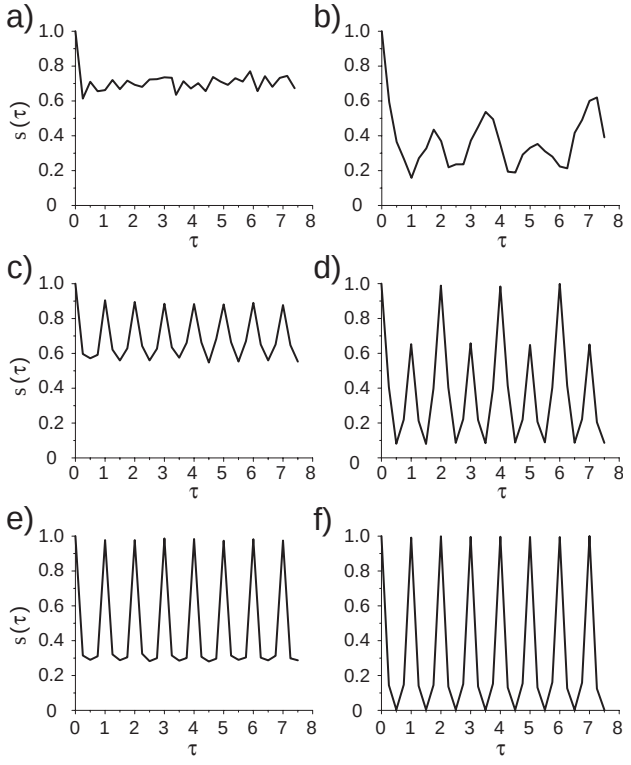

Fig. 5. Temporal correlations of Ca^{2+} firings among cells during periodic stimulus, corresponding to experiments shown in Fig. 1 (left column) and Fig. 3 (right column). These traces were calculated using Eq.(2).

notable distinctions: the spontaneous correlation of firings in the absence of periodic stimulus and the different responses of each groups to periodic stimulus with I=2.0 V.

As the stimulus intensity was increased from zero, an improvement in the synchrony both in isolated and coupled cell systems was observed. To estimate the degree of synchronization of firing activities induced by external stimulation, temporal correlations among cells are calculated using:

$$s(\tau) = \sum_{t=1}^{T-1} (c_t(t+\tau) - \bar{c}_t)(c_t(t) - \bar{c}_t), \qquad (2)$$

where $\bar{c}_t$ is the time average of $c_t(t)$ during T and τ is the time lag. In the case of the isolated cell system, asynchronous $[Ca^{2+}]_i$ oscillation among cells is related to the fluctuation of trace $s(\tau)$ in Fig. 5a. We can see a monotonic increment in the degree of synchronization of firing activities with increasing stimulus intensity, as shown in Fig. 5c and e. In contrast to this, there are some peaks in Fig. 5b in coupled cell system even without external stimulation. Furthermore, the improvement in the degree of synchronization is no longer monotonic due to the coexistence of 1:1 and 2:1 locking at the intermediate stimulus intensity, as shown in Fig. 5d.

There is a qualitative difference in the manner of responses. In the case of coupled cell system, as shown in Fig. 3b, cells form robust oscillators and exhibit the coordinated $[Ca^{2+}]_i$ firing activities. To coordinate the activity, cells require the intercellular communication. How is synchrony achieved? It has been well known that gap junctions, localized on cellular membrane, allow cells to communicate directly with nearest-neighbor coupling [12]. It is therefore possible that cells can interact with any neighboring cells in coupled system.

4. Conclusions

We studied the electrically induced synchronization of calcium firings with attention on the adaptive changes of cardiac populations: isolated and coupled cell systems. We showed that, in the absence of stimulation, coupled cells exhibited the coordination of $[Ca^{2+}]_i$ firings, while isolated cells repeated $[Ca^{2+}]_i$ firings with irregular periodicity characterized by Poisson distribution. The difference of manners of $[Ca^{2+}]_i$ is enhanced by the application of external stimulation. Since the cells in coupled system act like single robust oscillator against external stimulus, the evolution of the correlation function for increasing intensities of stimulus is different from the isolated system. Thus, it is expected that applying a homogeneous electrical stimulation is a way to detect the existence of populations of coupled cells. More qualitative details along this line are now in progress and will provide us to understand the spatio-temporal dynamics of biological systems [13,14].

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