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Analysis of beat fluctuations and oxygen consumption in cardiomyocytes by scanning electrochemical microscopy

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ABSTRACT

The contractile behavior of cardiomyocytes can be monitored by measuring their action potentials, and the analysis is essential for screening the safety of potential drugs. However, immobilizing cardiac cells on a specific electrode is considerably complicated. In this study, we demonstrate that scanning electrochemical microscopy (SECM) can be used to analyze rapid topographic changes in beating cardiomyocytes in a standard culture dish. Various cardiomyocyte contraction parameters and oxygen consumption based on cell respiration could be determined from SECM data. We also confirmed that cellular changes induced by adding the cardiotonic agent digoxin were conveniently monitored by this SECM system. These results show that SECM can be a potentially powerful tool for use in drug development for cardiovascular diseases.

Analysis of cardiomyocyte contraction is currently used to evaluate the pharmacological and toxicological properties of drugs [1–3]. Measurements of action potentials and topographic changes have been developed to characterize cardiomyocyte contraction. Action potentials can be recorded by a microelectrode array (MEA) [4–6], whole-cell patch clamping [7], or voltage-sensitive dyes [8]. MEA systems detect rhythmic changes in electrical potentials that are generated by beating cells when they are immobilized on a microelectrode array. Whole-cell patch-clamp techniques are used with human ether-a-go-go-related gene-transfected HEK-293/CHO cells; however, electrical recordings are technically difficult. Although Ca^{2+}-dependent fluorescent dyes can be used to monitor contraction behaviors in a standard culture dish, fluorescent signal measurements are not suitable for long-term monitoring because of photodamage and photobleaching effects [9]. Video microscopy can be used to determine the kinetics of cardiomyocyte topographic changes, and it provides valuable information on heart contraction [10]. However, to record cell motion, isolation of a single cardiomyocyte is required [11].

Quantifying cardiomyocyte oxygen consumption is also essential for analysis of energy metabolism in the heart. Oxygen consumption can be measured using a Clark electrode [12], by myoglobin saturation [13], or by electron paramagnetic resonance oximetry [14]. These methods measure the oxygen concentration in the bulk culture medium, which provides for quantitative analysis, but it is difficult to evaluate the oxygen consumption for a single cell.

Scanning electrochemical microscopy (SECM) provides information on the local distributions of electrochemically active species by microelectrode scanning [15]. SECM has recently been used for analyzing various cellular functions [16–20]. SECM provides for noninvasive measurements of cellular topography by scanning in the vicinity of target cells adsorbed on a standard culture dish [16,17]. In addition, SECM measurements of oxygen concentrations around these cells directly reflect their respiratory activity and can be used to analyze energy metabolism in viable cells [20]. However, SECM has not yet been used for measuring the motions or oxygen consumption of beating cardiomyocytes.

In this study, we developed an SECM system for analyzing the contraction kinetics and oxygen consumption of cultured cardiomyocytes. This system enabled us to investigate changes in cardiomyocyte function that resulted from changes in temperature and after adding a cardiotonic agent. Thus, this SECM system may become a powerful tool for studying pharmacological and toxicological effects in cardiomyocytes.

Material and methods

Cell culture

Primary cardiomyocytes were prepared from 1- to 2-day-old neonatal rat ventricles (Cosmo Bio Co., Ltd., Japan) and used for SECM measurements [21]. Cardiomyocytes were seeded on

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35-mm dishes that were coated with 0.01% collagen (Koken, Japan) at a concentration of 5×10^4 cells/cm^2. These cells were then incubated in cardiomyocyte culture medium (Cosmo Bio) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. For SECM experiments, we used spontaneously beating cells after 4–10 days in a culture with serum-free medium that contained 1 mM potassium ferrocyanide ($K_4[Fe(CN)_6]$) and 2% Knockout serum replacement (Life Technologies, USA) in MCDB107 (Cosmo Bio). All other reagents were of analytical grade. Solutions were prepared with ultrapure water from a Millipore system.

Instrumentation

SECM measurements were performed using an instrument built in our laboratory as previously described [17] but with slight modifications. In brief, our SECM setup comprised a piezomotor positioning system (Physik Instrumente, Germany), an inverted microscope, a temperature-controlled plate (Tokai Hit, Japan), a data acquisition and instrument control system (National Instruments, USA), and a potentiostat (Hokuto Denko, Japan). We used a Pt microdisk electrode (5-μm radius) as the electrochemical probe for SECM measurements and a leak-free Ag/AgCl electrode (Warner Instruments, USA) as the reference and auxiliary electrode. The radius of the Pt microelectrode, including a glass sheath, was 10 μm.

Measurement procedures for beating cardiomyocytes by SECM

We applied a 0.5-V potential or a −0.5-V potential (vs Ag/AgCl) to the probe electrode for ferrocyanide oxidation or oxygen reduction, respectively. To detect topographical changes in a beating cardiomyocyte, we positioned the microelectrode tip 9 μm away from a cell's surface by approach curve measurements (Supplementary Fig. S-1) [22]. We subsequently measured the responses of the oxidation currents of ferrocyanide with a time resolution of 10^4-points/s (Supplementary Fig. S-2). A plot of current versus time was converted to a plot of topographical change versus time using a calibration curve (Supplementary Fig. S-4). To analyze the contraction and relaxation motions of cardiomyocytes, a single average waveform was calculated from the topographical change plot using a software that we developed with LabView (National Instruments). This program identified all pulse peaks in SECM data for 30 s, which provided a peak-to-peak interval time, a peak fluctuation value, and an average waveform. In addition, the duration of contraction, the duration of relaxation, and the contraction amplitude were determined from this average waveform (Supplementary Fig. S-3). Oxygen consumption was determined from measurements of the oxygen reduction current in the vicinity of the target cells (Supplementary Fig. S-2).

We also measured the contraction behaviors and oxygen consumption of the beating cardiomyocytes by SECM at various temperatures. For these experiments, we used spontaneously beating cells 4 days after they were initially seeded. We first measured the current response at 37 °C. Further, we changed the temperature to 35 °C and waited for 7 min, after which we measured the current responses at the same position. These measurements were repeated at 33 and 31 °C.

For a representative pharmacological intervention, we measured the contraction behaviors of cardiomyocytes by SECM before and after adding digoxin. For these experiments, we used spontaneously beating heart cells after incubation for 10 days. After measuring the current response at 37 °C (0 μM), we added digoxin to a final concentration of 2.5 or 10 μM, waited for 20 min, and then measured current responses at the same position.

Results and discussion

Contraction kinetics analysis

To prevent serum proteins from being adsorbed on the electrode surface, we performed SECM measurements of the cardiomyocytes in serum-free medium that contained 1 mM potassium ferrocyanide. Cardiomyocytes exhibited continuous spontaneous beating for at least 4 h under these conditions. In addition, a leak-free reference electrode was used so as not to interfere with cardiomyocytes that were sensitive to changes in ion concentrations. The microelectrode was positioned above the central part of the contractile motion, which was identified using an optical microscope.

We measured the contraction behaviors of cardiomyocytes by our SECM system at 37 °C. A time resolution of 10^2 points/s failed to detect any current changes, whereas a resolution of 10^4-points/s obtained current pulses that were synchronized with cardiomyocyte contractions (Fig. 1, black line). A plot of current versus time could be converted to a plot of topographical change versus time (Fig. 1, gray line) using a calibration curve (Supplementary Fig. S-4). From this plot, we calculated the peak-to-peak interval time and peak fluctuation value, which showed fluctuations in the beating rhythm (Supplementary Table S-1).

To analyze the contraction and relaxation motions of cardiac cells, an average waveform for all pulse peaks is required, which can be derived from active potential measurements or video microscopic analysis [4,9,11]. Thus, we developed a software program that could convert a single average waveform from the plot of topographical changes, which resulted in generating an average waveform at 37 °C (Fig. 2A, black line). The following three parameters reflected the contraction behavior: contraction duration, relaxation duration, and contraction amplitude (Supplementary Fig. S-3). Both duration values indicated the rate of cardiac contraction, and the contraction amplitude reflected the cardiac contractility. These parameters could be determined from an average plot (Supplementary Table S-1).

To validate our analysis method, SECM measurements were performed for the same cell at 35, 33, and 31 °C (Supplementary Fig. S-5). The average waveform was determined as that at 37 °C

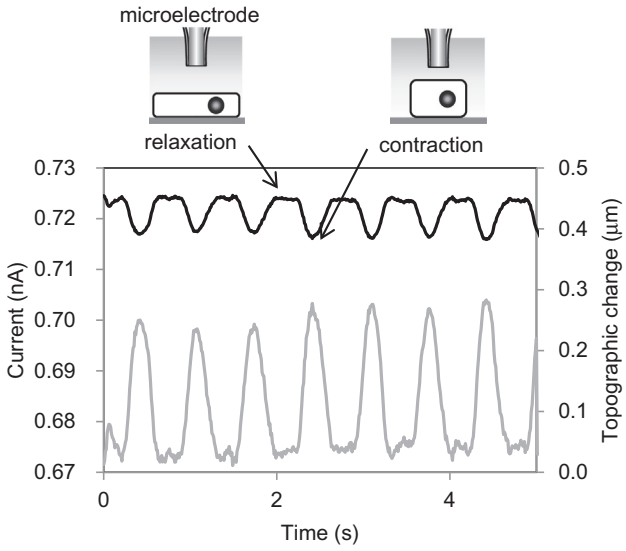

Fig.1. Current–time responses of a beating heart cell as determined by scanning electrochemical microscopy at 37 °C. The black line indicates cardiomyocyte current–time responses, and the gray line indicates topographical changes.