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Development of Semi-Separated Co-culture System of Sympathetic Neuron and Cardiomyocyte

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Abstract— **Rat superior cervical ganglion (SCG) neurons and ventricular myocytes (VMs) were co-cultured in chambers made of polydimethylsyloxane. The chambers were placed on a microelectrode-array (MEA) substrate and connected with a pathway. 24 hours after dissemination of the VMs, neurites of the SCG neurons outgrew through the pathway and reached the VMs. Spontaneous electrical activities of the SCG neurons and the VMs were observed several days after the dissemination. Constant-voltage stimualtion (1 V, 1 ms, biphasic square pulses) was applied to the SCG neurons at the frequency of 10 Hz using 32 electrodes. Contraction rate of the VMs increased by 153 ±110 % immediately after the stimulation to the SCG neurons was stopped. Then contraction rate gradually decreased and returned to almost the same rate as before the stimulation 5 minutes after the 1-min stimulation. Propranolol (beta-adrenergic receptor antagonist) prevented contraction rate of the VMs from increasing after electrical stimulation to the SCG neurons. These results suggest that neuromuscular junctions were formed between the SCG neurons and the VMs. Overall the semi-separated co-culture system in this study is available in research on changes in contraction rate of the VMs after applying electrical stimulation to the SCG neurons.**

I. Introduction

THE heart spontaneously contracts by the automaticity of the cardiomyocyte. Periodical cardiac contraction is affected by the autonomic nervous system (ANS), and its frequency and contractility are modulated in response to physiological demands of the body. The ANS is composed of the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). Heart rate increases when the SNS is active, while it decreases when the PNS is active [1], [2].

The neuromuscular junction between the sympathetic neuron and the cardiomyocyte has the beta-adrenergic receptor. Recently, the molecular mechanism of signal transmission through this receptor and the effects of neurotrophic factors have been investigated in the culture of the sympathetic neuron and the cardiomyocyte. [3]–[8] However, signal transmission is only a part of the regulating mechanism of cardiac rhythm and contractility. Kawada and co-workers [9] electrically stimulated the ANS *in vivo* to examine the regulating mechanism. However, *in vivo* experiments are affected by so many uncontrollable factors that interpretation of experimental results is difficult. Accordingly, it is necessary to reconstruct the ANS-innervated cardiomyocyte *in vitro* for understanding of the heart regulating mechanism.

The purpose of this study is to develop a semi-separated co-culture system of rat superior cervical ganglion (SCG) neurons and ventricular myocytes (VMs). by a partition with a pathway, to observe its spontaneous electrical activities by using a microelectrode array, MEA (Fig. 1) [10], [11], and to estimate change in contraction rate of the VMs after applying electrical stimulations to the SCG neurons using the same electrodes.

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II. Materials and Methods

A. Cell Culture

The SCG neurons were dissected from the SCGs in Wistar rats (2-3 day old) and were dissociated by 500 μg/ml collagenase (60 min; Invitrogen) and 15 μg/ml trypsin (15 min; Invitrogen). The VMs were obtained from the same rats and were dissociated by 50 μg/ml trypsin (16-20 hours; Worthington) and 125 μg/ml collagenase (5 min; Worthington). The base culture medium was a mixture of Neurobasal Medium (Invitrogen) and Medium 199 (Invitrogen) in the ratio of 3:2. The mixture contained 10 % fetal bovine serum and 1 % N-2 Supplement (Invitrogen), supplemented with a set of 25 ng/ml Nerve Growth Factor (Invitrogen), 10 ng/ml Glial cell-line Derived Neurotrophic Factor (Sigma-aldrich), 2 mM L-Glutamin (Invitrogen), and Penicillin-Streptmycin (Invitrogen). The culture medium was replaced every 24 hours.

B. Cell Dissemination

The culture dish 30mm in diameter consists of an MEA substrate and a polydimethylsyloxane (PDMS) structure with a partition and walls. The partition with a pathway 0.2 mm in width and 0.5 mm in length devided the dish into two chambers. Each chamber contained 32 electrodes.

The chambers were coated by type 1 collagen (Nitta Gelatin). The dissociated SCG neurons were disseminated

into one of the chambers. The cell density ranged from 6000 to 8000 cells/mm^2. 16-20 hours after dissemination of the SCG neurons, the dissociated VMs were disseminated into the other chamber using a disseminating device under a phase contrast microscope (Fig. 2).

C. Extracellular Recording

An MEA instrumentation system in a temperature-controlled box (37 ^{o}C, CO_2 5 %) was used to measure spontaneous electrical activities of the cell cultures and to electrically stimulate the cultures. The electrical signals obtained using the electrodes on the substrate were amplified (26 dB) with preamplifiers located at the electrodes. The preamplified signals were filtered (-3 dB at 10 Hz and 3 kHz) and then amplified (80 dB) with a main amplifier. The amplified signals were recorded on a hard disk drive in a PC after sampled at intervals of 40 μs by an AD converter with a resolution of 12 bits. The temperature in the box was maintained at 37 ^{o}C, and the CO_2 concentration at 5%.

D. Electrical Stimulation and Pharmacological Block

Biphasic square pulses, 1 V in amplitude, 1 ms in duration and 10 Hz in repetitive frequency, were used as the electrical stimulation. Firstly, spontaneous activities were recorded for 1 minute, and then the electrical stimulation was applied to the cultured SCG neurons for 1 minute using the 32 electrodes. After the stimulation, spontaneous activities were recorded for 7 minutes. Secondly, the same neurons were treated with 10 μM propranolol (Sigma), a beta-adrenergic receptor antagonist. The same electrical stimulation as before was applied 30 ninutes after treatment, and spontaneous activities were recorded for 7 minutes.

Contraction rate of the VMs was measured by the reciprocal of the interval between the recorded spontaneous electrical activities. The frequency change ratio was calculated by dividing contraction rate after the stimulation by that before stimulation.

III. RESULTS

A. Co-culture

The SCG neurons 48 hours after the dissemination of the VMs (Fig. 4a) proliferated in the left chamber in the co-culture system, compared with the SCG neurons immediately after the dissemination of VMs (Fig. 4b). Similarly, the VMs proliferated in the right chamber. The SCG neurons and the VMs were separated by the partition even after they were cultivated. When the VMs were disseminated 16-20 hours after the dissemination of the SCG neurons, neurites already outgrew from some of the SCG neurons, and a few glial cells grew (Fig. 4c). 24 hours after the dissemination of VMs, more neurites were observed (Fig. 4d), and some of them outgrew through the pathway in the direction of the VMs (Fig. 4e). 48 hours after the dissemination of the VMs, more neurities passed through the pathway, and also more glial cells were observed (Fig. 4f). Some of the VMs spontaneously and periodically contracted 24 hours after, and many more 48 hours after the dissemination.

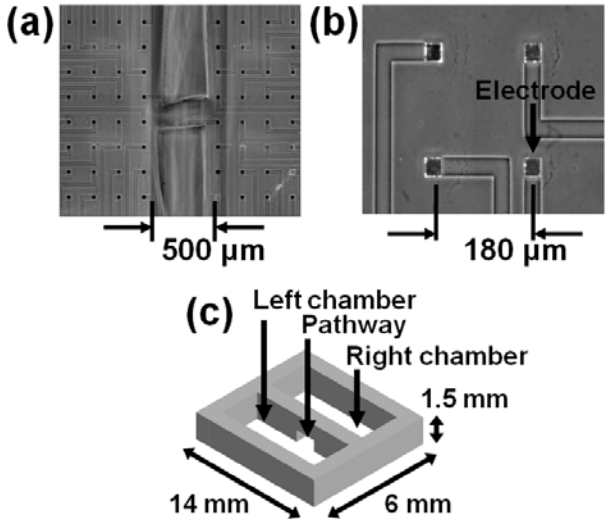

Fig. 1 A culture dish.
(a) 64 electrodes are embedded in the center of the culture dish, and are divided by partition into two groups. Each group is located at a distance 500 μm apart. (b) Each electrode covers an area of 30×30 μm square. One electrode is 180 μm apart from the other. (c) Two chambers are connected with a pathway 0.2 mm wide, 0.5 mm long and 0.5 mm high. Each chamber is 2 mm long and 6 mm wide.

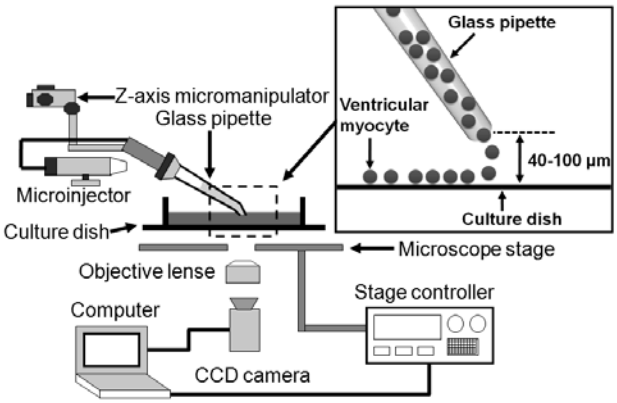

Fig. 2 Disseminating device of the VMs.
The Z-axial position of a glass pipette is controlled by a micromanipulator, and X and Y-axial position of the culture dish is regulated by computerized-stage controller connected with a phase-contrast microscope. The injection volume of a cell suspension is controlled by a microinjector.

B. Extracellular Recording

The spontaneous electrical activities of the SCG neurons and the VMs were observed at sites 1 and 2, respectively (Fig. 5). The spontaneous activities of the SCG neurons were 60 μV in amplitude and 3-4 ms in duration. Single activities were observed as positive spikes once in a few minutes, and burst activities once every 10 minutes (Fig. 5b).

On the other hand, the electrical activities of the VMs had the amplitude of approximately 120 μV and width of 20 ms.

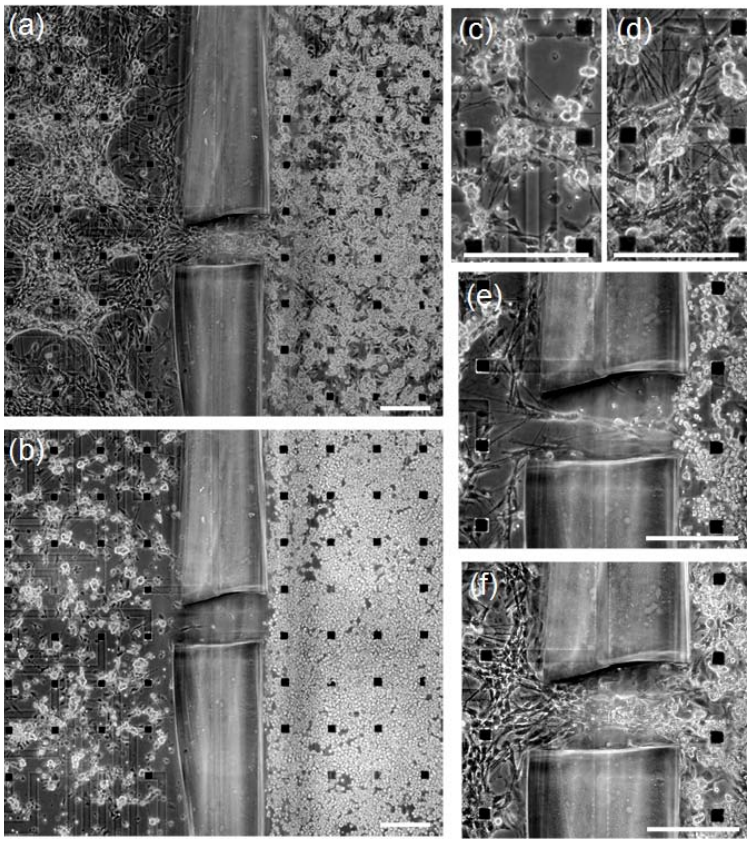

Fig. 4 Co-culture of the SCG neurons and the VMs on the MEA substrate.

(a, b) Overall view. The SCG neurons and the VMs are in the left and right chambers, respectively. (b, c) Immediately after dissemination of the VMs (the SCG neurons have been already cultivated for 12-16 hours). (a, f) 2days *in vitro*. (d, e) 1 day *in vitro*. (e, f) Close-up of the pathway. See the text for details. Scale bars indicate 200 μm.

They were negative spikes, periodically observed and synchronized with the contraction of the VMs (Fig. 5c).

C. Electrical Stimulation

Contraction rate shows no rapid increase immediately after the start of the electrical stimulation to the SCG neurons, but it gradually increased with time. When the electrical stimulation was stopped contraction rate increased by 153±110 % (Fig. 6a). After then, contraction rate gradually decreased. 5 minutes after the stimulation it returned to almost the same rate as before the stimulation (Fig. 6a). Propranolol treatment of the cell cultures prevented the increase caused by the electrical stimulation (2 dishes; Fig. 6b).

IV. DISCUSSION

A. Co-culture

The SCG neurons and the VMs were successfully cultivated and spatially separated in our co-culture system. Nelson and co-workers developed a co-culture system to investigate synaptic plasticity between SCG neurons and muscle cells. [12] Their system, based on Campenot's chamber [13], requires a complicated process for its set-up. Our system is much simpler, i.e., only needs to place the PDMS structure on the MEA substrate.

The neurites of the SCG neurons grew toward the VMs, while no VMs entered into the chamber of the SCG neurons. This result suggests that the SCG neurons and glial cells proliferating before the dissemination of the VMs might prevent the entrance of the VMs. The suggestion will be confirmed if the VMs enter into the chamber of the SCG neurons when the order of dissemination is reversed. In addition, the width and length of the pathway might have effects on the neurite outgrowth of the SCG neurons toward the VMs.

B. Electrical Stimulation

Electrical stimulation applied to the SCG neurons increased contraction rate of the VMs. This result strongly suggests that the neuromuscular junctions were made between the SCG neurons and the VMs, and that the SCG neurons maintained sympathetic effects. The stimulating conditions such as voltage and duration were fixed in the present study. It is necessary to vary the stimulating conditions in order to fully examine the effects of the SCG neurons on the VMs

Increasing ratio of contraction rate of the VMs after electrical stimulation to the SCG neurons quite differed in samples, although condition of electrical stimulation was same. To study these variations, further study needs to carry out experiments under various situations, i.e., frequency, voltage, waveform and duration of electrical stimulation.

C. Pharmacological Block

Contraction rate of the VMs after electrical stimulation to the SCG neurons showed no change in the presence of propranolol (beta-adrenoceptor antagonist). Therefore, it is strongly suggests that propranolol blocked beta-adrenoceptors on the VMs and hence negated trans-synaptic effects of electrical stimulation. This result reconfirms that the neuromuscular junctions were formed between the SCG neurons and the VMs.

Weber and co-workers [14], [15] reported that the SCG neurons cultured together with the cardiomyocytes turned cholinergic. The reason why the SCG neurons remained adrenergic in our experiments might be the difference in the culture medium. Further study is necessary to confirm the reason.

V. CONCLUSION

A semi-separated co-culture system of the SCG neurons and the VMs were developed, and spontaneous electrical activities were obtained from both the SCG neurons and the

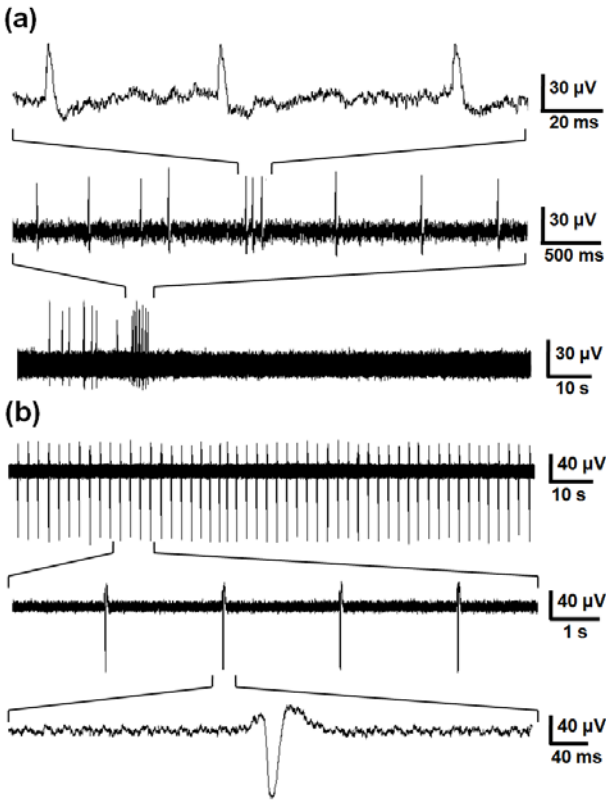

Fig. 5 Spontaneous electrical activities of the SCG neurons and the VMs (6 days *in vitro*).
Electrical activities are observed simultaneously from two sites on a same sample. One electrode at the chamber of the SCG neurons recorded the electrical activities shown in (a), and the other electrode at the chamber of the VMs recorded those shown in (b).

VMs by using the MEA substrate. Furthermore, contraction rate of the VMs was increased after applying electrical stimulation to the SCG neurons.

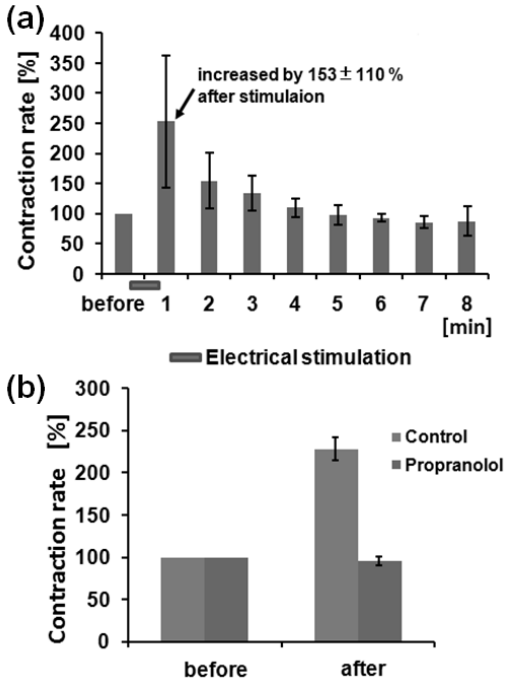

Fig. 6 Changes in contraction rate of the VMs after electrical stimulation to the SCG neurons.
(a) Change in contraction rate after stimulation (71 experiments on 10 samples (4-6 days *in vitro*)) (b) Effect of the propranolol block (Control: 14 experiments on 2 samples. Propranolol: 4 experiments on the same samples (4 days *in vitro*)). Error bars indicate standard deviations.

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