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The Use of SU-8 Topographically Guided Microelectrode Array in Measuring Extracellular Field Potential Propagation

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Abstract—The microelectrode array (MEA) can be used to study extracellular field potentials (exFPs) of electrogenic cells. Microcontact printing, which must be repeated after each experiment, is often used to promote accurate positioning of cells onto electrodes. The present study used MEAs with evenly spaced detection electrodes aligning along permanent SU-8 topographical guidance channels to measure propagation direction and speed. Chronotropic agents, isoproterenol (ISO, 1 nM–1 mM), and verapamil (VP, 1 nM–10 μM); and potassium channel openers (KCOs), pinacidil (PIN), and SDZ PCO400 (SDZ), were used to characterize these MEA chips. ISO (1 mM) enhanced the propagation speed from 247.25 ± 50.58 μm/ms 381.29 ± 92.01 μm/ms ($n = 9$, $p < 0.05$), whereas VP (10 μM) reduced the propagation speed completely ($n = 12$, $p < 0.001$). PIN (1 mM) significantly reduced the propagation speed from 278.6 ± 43.7 μm/ms to 49.7 ± 27.7 μm/ms ($n = 10$, $p < 0.001$), whereas SDZ (1 mM) completely stopped the propagation ($n = 9$, $p < 0.001$). Both KCOs induced conduction pattern changes similar to those observed in cardiac arrhythmia. The MEA chips with SU-8 guidance channels may be used to study cardiovascular diseases that are related to conduction disruption.

Keywords—Electrochemical measurement, Guidance, Signal conduction, Cellular disease model.

INTRODUCTION

Over the last 30 years, the microelectrode array (MEA) has been used extensively to study spatiotemporal properties of field potentials of different electrogenic cells or tissues.[22] The use of MEA to measure extracellular field potentials (exFPs) has provided us with a better understanding of neuronal excitation activities in the brain[16] and retina,[21] cardiac pacemaker functionalities,[19] and gastrointestinal activities.[17] This non-invasive way to study exFPs of a network of cells using the MEA offers a long-term platform for monitoring the development of cultured cells.[24]

In order to increase signal propagation pattern resolution, precise spatiotemporal mapping of exFPs propagation within a network of cultured cells by high-density detection electrode arrays (>200 electrodes) on a single chip has been carried out.[6] Although both cellular excitation and inhibition can be determined using these high-density arrays, it is not possible to study the unidirectional signal propagation pattern between any two cells in a culture. An aligned microcontact printing technique was developed to restrict the growth of cultured cells at a particular site of interest; i.e., on top of the detection electrodes.[9] However, microcontact printing can be tedious and time consuming to perform. The construction of permanent topographical guidance MEA chips can thus be helpful in restricting the location of a cellular network in relation to the detection electrodes. The width of the permanent topographical channels should be wide enough for individual cells to pass, and the size of the detection electrodes should be just large enough for individual cells to be immobilized on top of them, thus ensuring a good signal-to-noise ratio. Indeed, relevant studies using similar approach have been carried out in monitoring neuronal growth.[15,26]

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These MEAs with topographical guidance channels may be useful in a number of biomedical research areas where the signal propagation is of physiological significance, especially changes in cardiac rhythm and propagation direction in the presence of certain pharmacological agents. Potassium channel openers (KCOs) are such agents that are known to have a protective effect on ischemic tissues.[14] However, some KCOs also exert pro-arrhythmic effects on the heart.[5] Although the pro-arrhythmic effect of drugs can be identified using the Langendorff preparation,[18] only a single drug can be tested on each isolated heart. In contrast, not only does the MEA require only a small number of cells from a single culture, many measurements of drug actions on cardiac cells can be performed on each chip.[12]

In the present preliminary study, biocompatible SU-8 photoresist was used to construct multiple topographical guidance channels with uniformly spaced detection electrodes and cell reservoirs on a silicon wafer. SU-8 is a biocompatible epoxy negative photoresist that is suitable for biological micro-electromechanical systems due to its thick, chemically, and thermally stable characteristics.[1,23] The SU-8 can be used to construct structures with vertical sidewalls of 0.5 μm to more than 200 μm in height. The highly crosslinked epoxy material, after being exposed, developed, and cured, will stay on the device permanently. MEAs with different topographical guidance channel patterns were designed and constructed in this study, with the aim of identifying unidirectional exFPs propagation speed, and therefore the direction of propagation, of electrogenic cells. Since pro-arrhythmia may be a consequence of exFPs propagation disruption, we aimed to study the exFP propagation sequence (i.e., the pattern of conduction) using the MEA.

MATERIALS AND METHODS

Fabrication and Passivation of Microelectrode Array (MEA) Chip

A 4″ silicon wafer with single-sided oxide coating (1 μm) was used as the substrate of the MEA chip. Patterns of electrode arrangement were lithographically defined and developed on the substrate using AZ5200NJ photoresist (AZ Electronic Materials Co., Ltd). A layer of Ti/Au (30 nm/300 nm) was sputtered over the entire wafer, and the Ti/Au electrodes would remain after the photoresist was lifted off. A 25-μm-thick layer of biocompatible SU-8 photoresist (Microchem, SU-8 2025) was then spun onto the wafer, and the cell reservoirs and multiple guidance channels were defined by the photolithography. The SU-8 was then subsequently developed. To reduce the amount of residual chemicals that are known to be toxic to the cells, the MEA chips were hard baked in a 95 °C oven for 3 h. The Ti/Au electrode paths were thus protected under the SU-8 layer. Only 32 evenly spaced (a distance of 250 μm or 500 μm between two detection electrodes) Ti/Au detection areas (30 μm × 30 μm) along the guided channels were exposed to the cultured cells for signal detection. Individual MEA chips (0.8 cm × 0.8 cm) were cut from the 4″ silicon wafer and were then bonded to PCB boards (3.5 cm × 3.5 cm), providing 32 channels for signal sampling on each MEA chip. The bonded chip was then encapsulated with a plastic ring (diameter = 1.5 cm) that functioned as a confined culture chamber by silicon adhesive (Dow Corning, 96-083). To prevent short-circuiting, all bonding pads and wires within the culture chamber were protected by a layer of silicon adhesive.

MEA System Set-Up

A custom-made 64-channel amplifier system consisted of microelectrodes directly coupled to the inputs of high impedance operational amplifiers (OPA 627, input impedance Z_{in} = 14 TΩ, gain: 33×; Burr-Brown, Texas Instruments, Dallas, Texas 75265, USA). This was then coupled to a main amplifier (gain: 33×), providing an overall gain of 1089×. Data were sampled at 10 kHz per channel, with simultaneous acquisition across all channels (PCI 6071E, National Instruments, Hong Kong) using MED64 conductor 3.1 software (Alpha MED Sciences Co. Ltd., Japan). An extracellular Ag/AgCl electrode, which was set to ground potential, served as a reference electrode. A more detailed description of the data acquisition has been published previously.[7,10]

Characterization of MEA Chip

Animals

The Sprague-Dawley rats were outbred within the Laboratory Animal Services Centre of the Chinese University of Hong Kong and were housed at approximately 25 °C in 12 h light/dark cycles. The Animal Experimentation Ethics Committee, the Chinese University of Hong Kong, approved the experiments and protocols that were used (Project number: 10/060/MIS-1).

Solutions and Drugs

The standard culture medium was Ham's F10 medium (Sigma, N6635), containing 10% (v/v) foetal bovine serum (Gibco, 10270-106), 0.5% (v/v) insulin, transferrin, selenite solution (Sigma, I1884), 6 mM L-glutamine (Sigma, G7513), and 2% (v/v) mixture of

penicillin/streptomycin (Gibco, 15140-122) adjusted to pH 7.4 using sodium hydroxide.

In this study, a positive chronotropic drug, isoproterenol bitartrate (ISO, Sigma, I2760); and a negative chronotropic drug, verapamil (VP, Sigma, V4629) were used for characterizing the MEA chips. Two potassium channel openers (KCOs): pinacidil (PIN, a generous gift from Leo Pharmaceutical Products, Denmark) and SDZ PCO 400 (SDZ, a generous gift from Sandoz Pharmaceuticals GmbH, Germany) were used for demonstrating the importance of the unidirectional propagation direction measured by the present MEA chips. The stocks of VP (10 mM) and ISO (10 mM) were made using distilled water, while the stocks of PIN (10 mM) and SDZ (10 mM) were made using 40% (v/v) ethanol in distilled water. The working concentrations (1 nM–1 mM) of all the drugs were made using the standard culture medium (pH 7.4).

Culture of Embryonic Cardiomyocytes

Embryonic cardiomyocytes were used purely for the characterization of these MEA chips in terms of usability and sensitivity. Cardiomyocytes of Sprague-Dawley rats (E19–20 days) were prepared according to a previously published protocol.[12] In brief, hearts were removed, minced, and placed into cold Ca^{2+}/Mg^{2+}-free Hanks Balanced Salt Solution (Sigma, H6648). These tissue fragments were digested by stepwise 0.25% trypsin with EDTA (Gibco, 25200-056). After 8-min incubation with trypsin at 37 °C, the supernatant was discarded. The dissociation cycle then began with another 2 mL of trypsin for 8 min preceded by the addition of 100 μL DNase II solution (Sigma, D8764) for 1–2 min. The resulting supernatant was collected and added to the culture medium containing 20% (v/v) foetal bovine serum to stop trypsin digestion. The above dissociation cycle was repeated four to five times. This resultant cell suspension mixture was centrifuged at 2000 rpm for 5 min. The pellet was resuspended using the standard culture medium and incubated for 1 h at 37 °C for the purpose of differential adhesion. This procedure allows fibroblasts to adhere to the culture dish preferentially over cardiomyocytes and thus increases the myocyte-to-fibroblast ratio of the cell suspension.

Preparation of Scanning Electron Microscopic Samples

MEA chips with cultured cells were rinsed with 0.1 M Sorensen's phosphate buffer before incubating with 4% glutaraldehyde fixative overnight, and this was followed by another incubation with 1% osmium tetroxide for 2 h. Fixed specimens then underwent dehydration steps with ascending concentrations of ethanol. The specimens were kept in 100% ethanol until critical point drying (Ladd) was used to remove all liquid from the samples. Specimens were then coated with a layer of gold palladium by a splutter coater (Edwards, S150B), before being examined under the scanning electron microscope (JEOL, JSM-6301F).

Pharmacological Testing

Cultured cardiomyocytes (approx. 75,000 cells per chip) were seeded on a fibronectin (Sigma, F0635)-primed MEA surface, and they would beat spontaneously after 3–4 days in culture. Each chip was re-incubated for an hour following medium change before recording. An initial recording of 30 s was taken before the standard culture medium was replaced by one containing a particular drug. Another 30-s recording was taken 1 min after the replacement of medium. The headstage was placed on a thermostatically controlled hot plate set at 37 °C to ensure a consistent and optimal experimental temperature throughout the recording period.

Data Analysis and Statistics

The exFP was recorded using the MED64 software, and the beat frequency (beats per minute, bpm) was subsequently analyzed using a program implemented in MATLAB® (Version 7.0, The MathWorks, USA). The mean of beat frequency from all available channels of each MEA chip was calculated (i.e., considered as one experiment) before obtaining the final means of all the chips within the same treatment group. The effects of drugs on the direction and speed of exFP propagation were analyzed using Origin 7 analytical software (OriginLab, USA).

The propagation speed was calculated by

$$\text{Propagation speed } (\mu\text{m/ms}) = D/T$$

where D = the distance (in μm) between the two electrodes at either end of the guidance channel, and T = the time (in ms) required for the exFPs signals to travel between these electrodes.

The actions of chronotropic agents and KCOs were expressed as pD_2 values ($-\log EC_{50}$, the concentration required to produce half of the full excitatory or inhibitory response) ± SEM. The effects of chronotropic agents and KCOs on basal beat frequency and exFP propagation speed were analyzed using Student's paired t test. Values of $p < 0.05$ were considered to indicate a statistically significant difference between measurements.

RESULTS

The sensitivity of MEA chips with different topographical guidance channel on signal detection was

characterized by applying chronotropic agents (ISO and VP) to a syncytium of spontaneously active cardiomyocytes. These cells were used as they have excellent signal-to-noise ratios, which allow a systematic and comprehensive characterization of these chips much more readily. Physiological concentration-responses of the cultured cardiomyocytes to KCOs (PIN and SDZ) were also studied and the exFP propagation direction pattern was evaluated.

The Design of MEA Chips with Different Topographical Guidance Channels Patterns

MEA chips with two different patterns of topographical guidance channels were designed and constructed for studying the concentration-related effects of drugs on electrogenic cells. The first design consisted of an MEA with four unconnected rows of eight guided electrodes (250 μm between 2 electrodes) (Fig. 1a); the second design consisted of an MEA with a continuous four rows by eight guided electrodes (500 μm between 2 electrodes). The four reservoirs (3.14 mm^2 each) fabricated in the first and the single reservoir (7.07 mm^2) fabricated in the second are for experiments involving cell migration.

In the following characterization experiments, exFPs of cultured cardiomyocytes were measured using these MEA designs. A scanning electron micrograph showing cardiomyocytes cultured on top of a detection electrode within the guidance channel of

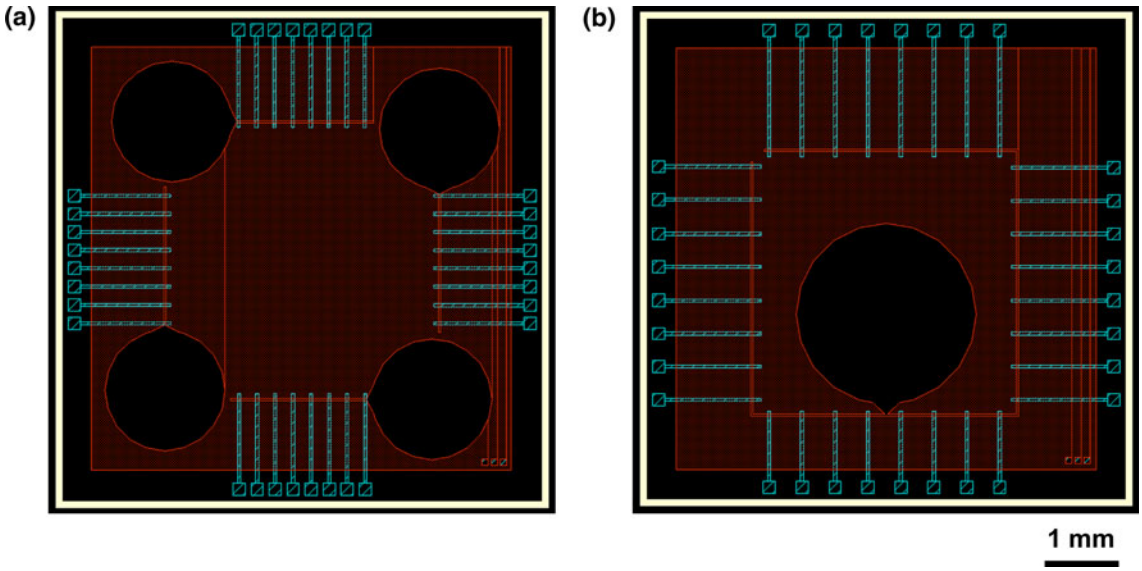

FIGURE 1. An overview showing the SU-8 topographical guidance channels of the microelectrode array (MEA) chips with different patterns. The gold detection electrodes (30 μm × 30 μm) were evenly spaced (250 μm) along the SU-8-constructed guidance channels. The descriptions of these patterns are as follows: (a) four individual cell reservoirs are aligned on an MEA chip; and (b) a design with one cell reservoir and a larger distance between two detection electrodes (500 μm).

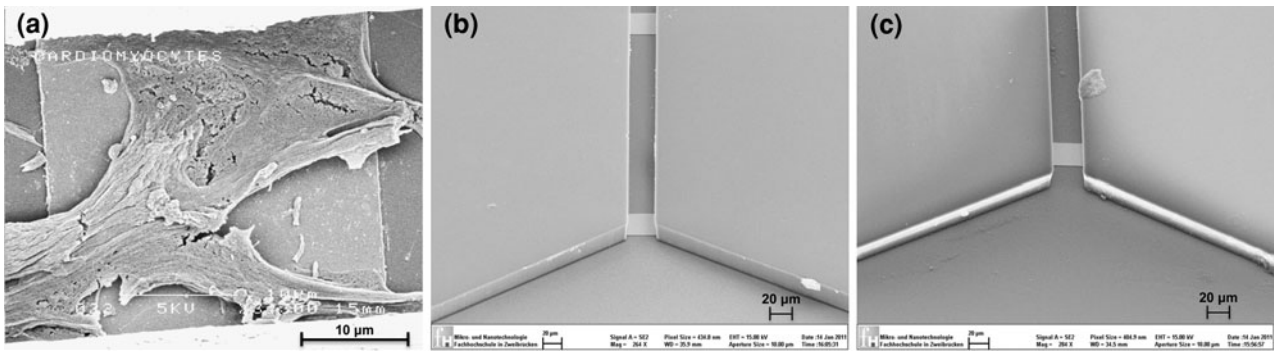

FIGURE 2. A scanning electron micrograph with cardiomyocytes cultured on top of a single detection electrode within the guidance channel of an MEA chip is shown (a). Another scanning electron micrograph was taken at a 45° tilt angle of an SU-8 constructed guidance channel on an MEA chip (b) before and (c) after being repeatedly used. These chips are highly durable as indicated by the pattern of the topological guidance remaining on the MEA and the wall of the SU-8 channels remaining vertical and smooth even after repeated use (>10 times).

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a MEA chip was shown (Fig. 2a). The fabricated MEA chips were highly resistant and appeared to be undamaged even after repetitive cell culturing and cleaning (Figs. 2b, 2c). Individual exFPs of cardiomyocytes detected by any one of the four rows of eight guided electrodes were plotted and analyzed (Fig. 3).

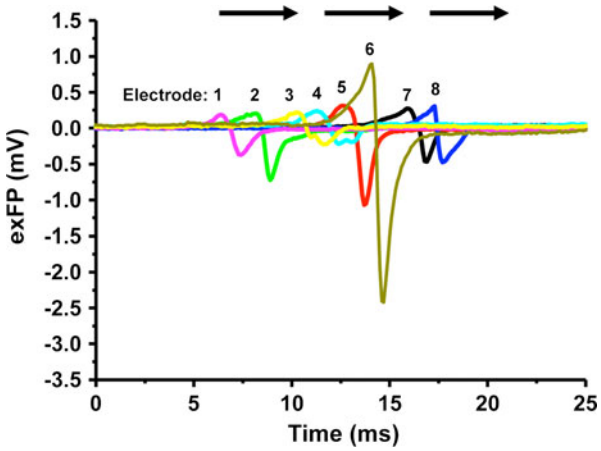

FIGURE 3. Representative traces of individual extracellular field potentials (exFPs) of cardiomyocytes detected by a row of eight electrodes. The exFPs propagated in a defined direction and sequence (arrows). Knowing the distance and the time difference between the first and the last signals detected, both exFP propagation sequence and exFP propagation speed can be determined. In this case, the propagation speed was 52.55 μm/ms.

Effects of Chronotropic Drugs on Spontaneously Active Cardiomyocytes

In the presence of ISO (1 nM–1 mM), the basal beat frequency increased concentration-dependently from 58.79 ± 14.66 bpm ($n = 9$) to 90.65 ± 17.45 bpm ($n = 9$) (Fig. 4a). The basal propagation speed (247.25 ± 50.58 μm/ms, $n = 9$) of the cultured cardiomyocytes was not affected by low concentrations (1 nM–0.1 mM) of ISO (Fig. 4c). At the highest ISO concentration (1 mM), the propagation speed of the cultured cardiomyocytes increased to 381.29 ± 92.01 μm/ms ($n = 9$). In the presence of VP (1 nM–10 μM), the basal beat frequency reduced concentration-dependently from 36.27 ± 6.26 bpm ($n = 12$) to 0 bpm ($n = 12$) (Fig. 4b). VP significantly reduced the propagation speed from 109.1 ± 14.9 μm/ms ($n = 12$) to 53.3 ± 16.2 μm/ms and 0 μm/ms in the presence of 1 μM ($n = 12$, $p < 0.05$) and 10 μM ($n = 12$, $p < 0.001$), respectively (Fig. 4d). The pD_2 values of ISO and VP on the changes in beat frequency and propagation speed were determined (Table 1).

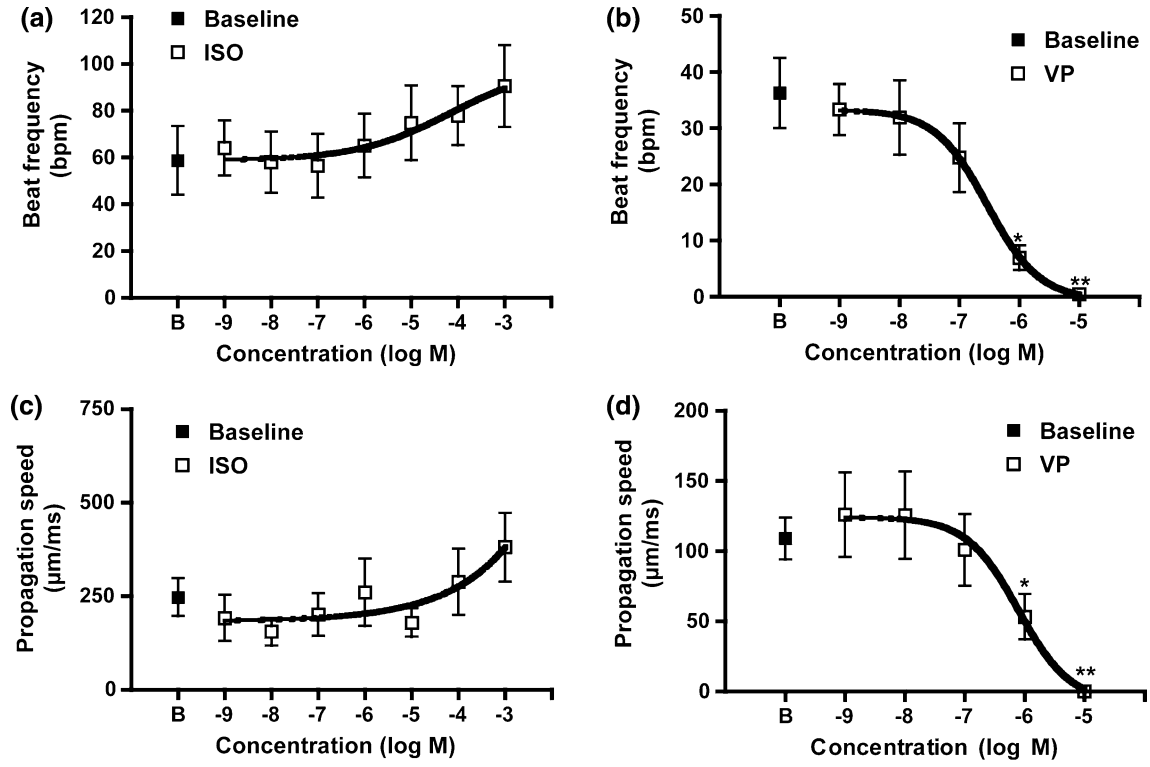

FIGURE 4. Effects of chronotropic agents, (a, c) ISO (1 nM–1 mM, $n = 9$) and (b, d) VP (1 nM–10 μM, $n = 12$), on cardiomyocytes. Both agents caused concentration-dependent changes in beat frequency (a, b) and propagation speed (c, d). The results are expressed as μm/ms ± SEM. *$p < 0.05$, **$p < 0.001$ compared with the baseline value (*B*).

Effects of KCOs on Spontaneously Active Cardiomyocytes

The beat frequencies were reduced concentration-dependently in the presence of either PIN or SDZ (Figs. 5a, 5b). Pinacidil (1 mM), a cyanoguanidine derivative, significantly reduced the propagation speed of the spontaneously active cardiomyocytes from 278.6 ± 43.7 μm/ms ($n = 10$) to 49.7 ± 27.7 μm/ms ($n = 10$, $p < 0.001$) (Fig. 5c). SDZ, a benzopyran derivative, significantly reduced the propagation speed from 127.4 ± 22.0 μm/ms ($n = 9$) to 78.4 ± 29.1 μm/ms ($n = 9$, $p < 0.05$) and 0 μm/ms ($n = 9$, $p < 0.01$) when 0.1 mM and 1 mM of SDZ was added, respectively (Fig. 5d). PIN and SDZ affected the changes in beat frequency and propagation speed to a similar extent, with SDZ appeared slightly more potent than PIN (Table 1). While the order of exFP propagation direction was unchanged in most cases when PIN (10/11 chips) or SDZ (9/11 chips) was added to the culture, disruptions to the propagation patterns in the presence of these KCOs were observed in the remaining cases (Fig. 6).

TABLE 1. Effects of chronotropic agents, isoproterenol bitartrate (ISO), or verapamil (VP); and potassium channel openers, pinacidil (PIN) or SDZ PCO400 (SDZ), on beat frequency and propagation speed (n = 9–12).

	Beat frequency (pD_2)	Propagation speed (pD_2)
ISO	3.31 ± 0.63	3.61 ± 0.96
VP	6.54 ± 0.42	6.04 ± 0.18
PIN	4.46 ± 0.96	4.45 ± 0.52
SDZ	4.60 ± 0.86	4.65 ± 0.67

The pD_2 ($-\log EC_{50}$s, the concentration that elicits a 50% reduction (VP, PIN, and SDZ) or increase (ISO) in response ± SEM) values of these agents on the changes in beat frequency and propagation speed were determined. While PIN and SDZ affected the changes in propagation speed to a similar extent, SDZ appeared slightly more potent than PIN. The pD_2 values of these agents in affecting the beat frequency are similar to those found in affecting the propagation speed.

DISCUSSION

In the present preliminary study, MEA chips with topographical guidance channels were constructed using lithography techniques. Spontaneously active cardiomyocytes were cultured within the SU-8 guidance channels and their respective exFPs were detected by the gold detection electrodes underneath. Since the detection electrodes are evenly spaced along a confined track, the propagation speed and sequence of exFP from one point to the next can be easily determined.

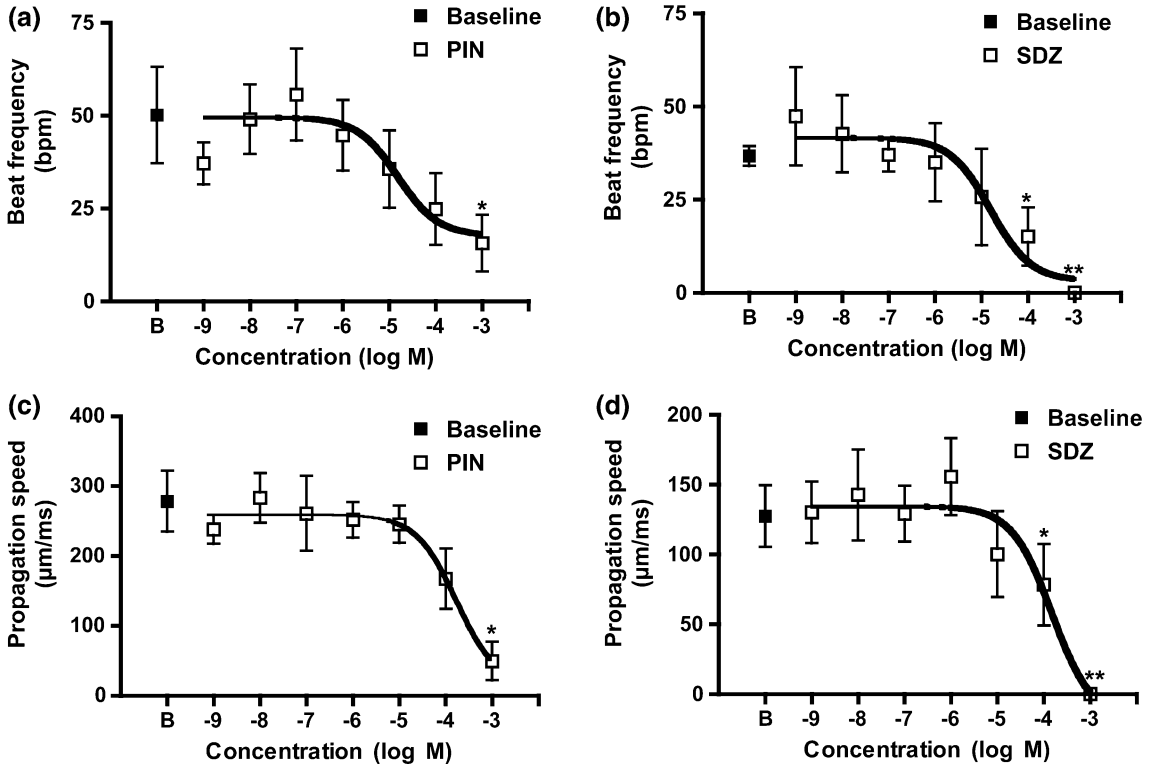

FIGURE 5. Effects of potassium channel openers, (a, c) PIN (1 nM–1 mM, n = 10) and (b, d) SDZ (1 nM–1 mM, n = 9), on cardiomyocytes. Both agents caused concentration-dependent reduction in beat frequency (a, b) and propagation speed (c, d). The results are expressed as μm/ms ± SEM. *$p < 0.05$, **$p < 0.01$ compared with the baseline value (B).

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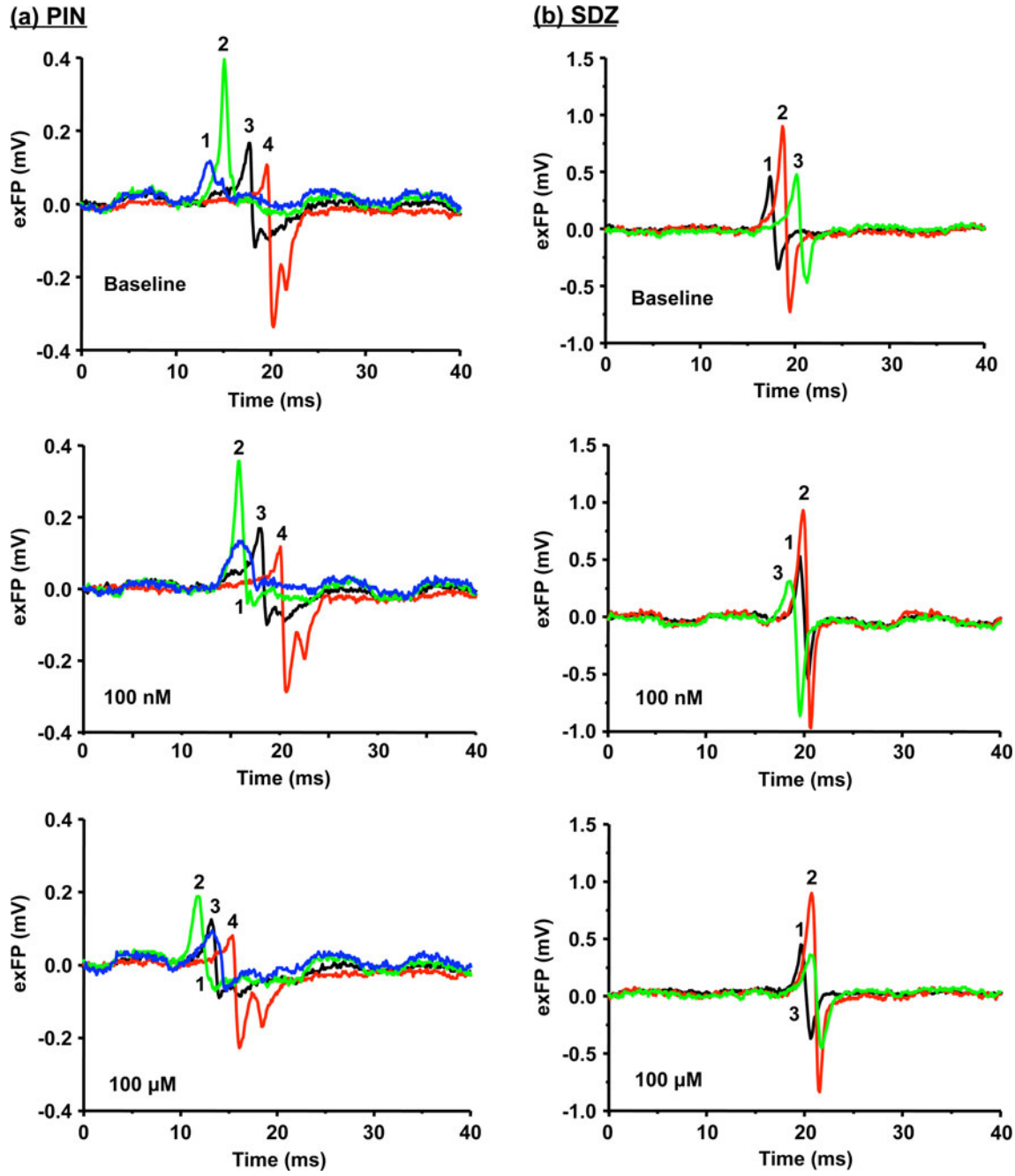

FIGURE 6. Representative traces of individual extracellular field potentials (exFPs) of cardiomyocytes in the absence or the presence of pinacidil (PIN, a) or SDZ PCO400 (SDZ, b). The recorded signal sequences in the presence of PIN (Channel 1 to Channel 4) and SDZ (Channel 1 to Channel 3) were disrupted.

Since cardiomyocytes have an average cell diameter of 20–30 μm,[4,13] the present SU-8 topographical guidance channels with a width of 30 μm and a depth of 25 μm help in restricting the number of possible connections between cells. The pre-determined channel patterns serve the same purpose as performing micro-contact patterning, but the SU-8-constructed channels remain permanent on the chips and can be reused for more than 10 times (Fig. 2).

Using some well-established pharmacological agents, such as ISO, VP, PIN, and SDZ, the changes in rate of contraction, speed of propagation, and conduction sequence were used as the key parameters to characterize and validate the feasibility and applicability of the present MEA chips with guided electrodes. The spontaneously active cardiomyocyte culture with large measurable exFPs signals is a useful cellular model for characterizing the present MEAs with

guidance channel designs (Fig. 1). ISO and VP are well-established positive and negative chronotropic agents, respectively, which affect the heart rate when an optimal concentration is added.[25] However, the unidirectional propagation speed, which can be used as a parameter in reflecting the electrical coupling among cells,[20] between any two points within the same culture has not been elucidated. In the present study, the cardiomyocytes were aligned and connected within the guided channels, and the unidirectional propagation speed of cardiomyocytes was successfully measured in the presence of ISO or VP (Figs. 4c and 4d, respectively). The topographical guidance chips offer a platform for visualizing the changes in exFP propagation direction as well as for calculating the exFP propagation speed in relation to the treatment given.

We have previously demonstrated that both PIN and SDZ could decrease the basal beat frequency of the cultured cardiomyocyte cell line HL-1, and yet PIN might have advantages over SDZ in acute metabolically compromised conditions.[11] These KCOs, however, can also induce arrhythmia in the heart.[8] These results, albeit in only a small number of cases, illustrated this arrhythmia-inducing property of KCOs as these drugs altered the cardiac electrophysiological rhythm, in terms of beat frequency, as well as exFP propagation speed and direction, concentration-dependently (Fig. 5). The relative potency of PIN and SDZ in reducing the beat frequency is similar to that found in reducing the propagation speed (Table 1). Since pro-arrhythmia could induce ventricular arrhythmia, atrial arrhythmia, and abnormal impulse formation and conduction,[2] the propagation speed changes could thus be used as one of the parameters in cardiac research. One of the advantages of these topographical guidance channel chips is that the order of the propagating exFPs can be determined. Visualization of the propagation of exFPs showed that the propagation sequence was disrupted in the presence of PIN or SDZ (Fig. 6). This disruption of order may be due to abnormal impulse formation and uncoupling conduction by the pro-arrhythmic effect of KCOs. It is believed that the efflux of K^+ reduces action potential duration, leading to dispersion of repolarization and refractory period. The dispersion of refractory period can then induce some common symptoms associated with arrhythmia, such as irregular reentrant pathways and ventricular arrhythmias.[3]

CONCLUSION

The present MEA chips with permanent SU-8 topographical guidance channels offer an alternative tool to microcontact printing for restricting electrogenic cells on a desired area. These chips can thus be used to study unidirectional changes in exFP propagation of electrogenic cells and to identify pharmacological agents that may have the potential to modify the inherent propagation sequence. The MEA chips with the SU-8 constructs, which are easy to handle and durable, may also be used to study a variety of clinically significant areas in addition to cardiac ailments, such as neurological network formation and maturation.

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