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Temporal Resolution of Stimulation Threshold: A Tool for Electrophysiologic Analysis

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***Abstract*—Electrical stimulation of cardiac cultures with closed-loop control permits the determination of threshold in real time. The temporal response of stimulation threshold and underlying cell membrane excitability is valuable information for understanding the complex electrophysiologic processes within cardiac cells and can aid in understanding the mechanisms and effects of pharmaceuticals or other stimuli. This work presents the temporal response of stimulation threshold measured using HL-1 cardiac myocytes when exposed to changes in temperature and extracellular potassium concentration. These changes mimic systemic alteration of excitability and conditions that can result from ischemia in the heart. The results demonstrate the efficacy of stimulation threshold as a physiologic indicator and illustrate transient effects with both fast and slow time constants that can be resolved using a system that determines stimulation threshold in real time.**

I. INTRODUCTION

MEASUREMENT of the electrophysiological properties of cardiac cells and tissues underlies understanding of ion channel function, receptor binding and second-messenger signaling, excitation propagation, and cardiac arrhythmias. Specific ion channel or receptor blockade plays a significant role in both single-cell and whole-organ function and therapy. However, cardiac electrophysiology is a complex phenomenon relying on the interaction of intracellular signaling molecules, activity-dependent gating of ion channels, enzymatic activity of pumps and exchangers, and even channel expression in the case of electrical remodeling [1, 2].

In order to gain a comprehensive understanding of a given cell type or tissue, methods that measure whole-cell properties and their interaction in a functional tissue offer a tremendous benefit. However, it can be difficult and time-consuming to measure electrophysiologic properties in multiple cells simultaneously or to resolve changes in those properties over time in response to a given chemical or environmental stimulus.

Microelectrode arrays (MEAs) are one means of measuring the electrical activity of cardiomyocytes *in vitro* over many days non-invasively [3, 4]. Cells are cultured over metal microelectrodes that convey the changes in extracellular potential due to action potentials (APs) to signal conditioning electronics for electrical detection and measurement. MEA technology also permits the integration of electrodes for electrical stimulation on the same substrate, allowing electrical excitation of a syncitium of cardiomyocytes [5, 6].

Electrical stimulation of cardiomyocytes on MEAs reveals aspects of cardiac electrophysiology that are inaccessible when using spontaneously beating cell cultures. General membrane excitability is measured via the stimulation threshold and dependency of threshold on stimulation rate (strength-interval relationship) can be assessed. In addition, AP properties that depend on beat rate can be measured and quantified at fixed or varying rates.

Traditionally, stimulation thresholds are characterized using the strength-duration curve. Stimulation strength (pulse amplitude) required to completely capture a tissue (evoked AP with every stimulus) is determined at various pulse durations, yielding a curve with unique rheobase (b) and chronaxie (c) as described in [7]. This measurement takes time to perform and is typically measured once for a given tissue prior to an experiment. Stimulus amplitude and duration are then set at a value incorporating a safety margin in order to obtain complete capture of the tissue. In [5], we presented a new modality for cardiac stimulation that measures stimulation threshold in real time, determining the pulse amplitude at a given pulse duration that achieves complete or partial capture of a cardiac tissue. This modality permits the temporal resolution of stimulation threshold and observation of transient effects due to external stimuli that could otherwise not be measured.

In this work, we present the response of the closed-loop stimulation system and the cardiac cell line HL-1 to fundamental physiologic modulation, demonstrating its utility as a means for studying cardiac electrophysiology. The real-time response of stimulation threshold to ambient temperature changes and extracellular potassium concentration is measured as an example of the analyses

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possible using this novel method of analysis. Both transient and steady state responses are revealed, demonstrating the advantages of this method for resolving time-dependent changes in membrane excitability.

II. Methods

A. MEA and Fluidic Technology

Cardiac cells were cultured on glass MEAs fabricated in the Stanford Nanofabrication Facility. MEAs were comprised of a 6 × 6 array of platinum electrodes spaced 100 μm apart with 22 μm diameters, passivated with a silicon nitride film. Additional electrodes were included outside of the array for the purposes of stimulation (see Fig. 1). Additional details can be found in [5] and [8]. MEAs were affixed and wire bonded to a printed circuit board carrier, encapsulated using epoxy, and a polystyrene Petri dish was affixed around the MEA to provide a container for cell medium surrounding the culture chamber.

Cell medium or other physiologic solution was heated while flowing through an aluminum block and passed over the cell culture through a custom-designed PDMS chamber. The flow rate was 2 mL/min and mixing time in the cell chamber was approximately 30 seconds. The temperature of the perfusate was measured using an integrated platinum RTD on the MEA, and controlled with a simple proportional controller that attained set point temperature of 37º C ±0.1º C. Cell medium (20 mL) was recirculated continuously from an airtight Teflon® cup that prevented gas exchange and maintained constant medium pH (7.4) and osmolarity (app. 300 mOsm) over the course of the experiments.

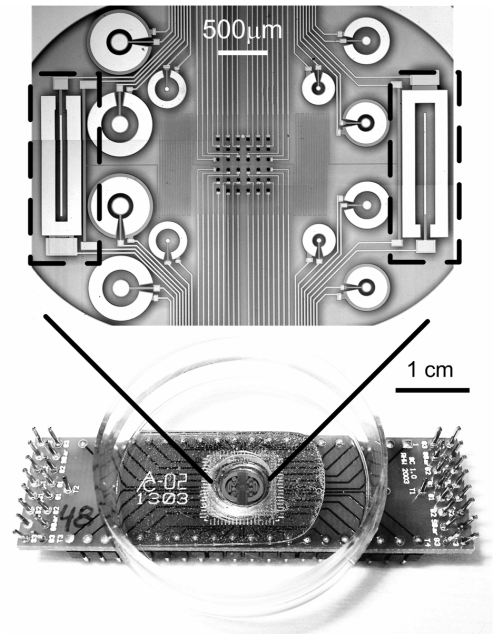

Fig. 1. **Bottom:** Photograph of assembled MEA chip. The plastic 35 mm diameter Petri dish has a hole drilled in the center to expose the MEA and form the cell culture chamber. **Top:** micrograph of the MEA showing the 6 × 6 recording array at center surrounded by the larger stimulation electrodes on each side.

Electrical signals from the MEA were amplified 1000× and bandpass filtered with a passband between 7 Hz and 3 kHz using a custom-designed 32-channel amplifier [9]; data were digitized at 12 bit resolution with a sampling frequency of 10 kHz. Data was recorded and real-time data processing and control was performed with a custom-designed graphical user interface written in Matlab™ (The MathWorks, Natick, MA).

B. HL-1 Cardiomyocytes

Immortalized HL-1 cardiomyocytes were used in this work. HL-1s were derived from the AT-1 murine atrial cardiomyocyte tumor line and maintain spontaneous beating and properties of differentiated cardiomyocytes through serial passaging [10]. Cells are cultured to confluence at 37° C, 5% CO_2, and 95% RH in T25 flasks as described in [5]. However, cells were not cultured in the presence of norepinephrine (in contrast to standard protocol) in order to suppress rapid beating, thus preventing the need to overdrive the cultures. This permitted stimulation at 2 Hz, a relatively low frequency with minimal adverse consequences for the cell culture. Flasks or microelectrode arrays were coated at least 24 hours prior to plating with fibronectin (4 μg/cm^2) and 0.02% gelatin in sterile DI water and incubated at 37° C. Culture medium was replaced every 24 - 48 hrs. Typical cell-plating density on the MEAs is 1 000 - 1 200 cells/mm^2 over the area of the culture chamber, approximately 30 mm^2.

C. Closed-Loop Stimulation System

The closed-loop stimulation system used in this work was similar to that presented in [5], and a detailed description can be found in that work. The basic concept of the system is shown in Fig. 2. Briefly, the response of the cardiac culture to a given set of stimulus pulse parameters (pulse amplitude, duration, or rate) is measured by the MEA and signal-conditioning hardware. The data recorder analyzes the data, extracts the electrical stimulation artifact, determines the efficacy of pacing (capture fraction, CF), and based on the target CF, adjusts the pulse generator's parameters (e.g., pulse amplitude) in order to achieve the target CF.

The microcontroller-based pulse generator produced current-controlled, symmetric, anodic-first, biphasic pulses in a unipolar configuration. A platinum wire with geometrical surface area of 32.7 mm^2 was used to define the reference potential.

The closed-loop controller used here was modified from that described in [5]. The discrete-time software-implemented algorithm utilized the time-history of the error between the measured and target capture fraction in order to determine the future stimulation amplitude step size and direction in the presence of CF quantization error and the low data rate associated with evoked cardiac AP data. Further details are beyond the scope of this work.

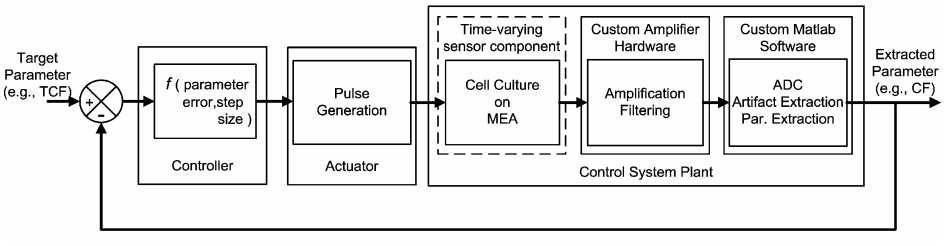

Fig. 2. Diagram of electrical stimulation closed-loop control system. Capture fraction, the fraction of stimulation pulses that evoke APs, is maintained by the controller via adjustment of stimulus pulse parameters, such as pulse amplitude. Custom software extracts stimulation artifact, determines capture fraction, and implements the real-time controller.

D. Experimental Methods

MEA chips with confluent HL-1 cultures were inserted into the signal processing unit, and preliminary stimulation was performed to assess the viability of the culture. If stimulation was successful, the PDMS cell chamber was applied and temperature was allowed to stabilize at 37º C for 20 minutes. The strength-duration (SD) curve was then measured at a pulse rate of 2 Hz, allowing acquisition of four stimuli and evoked APs. SD curves were determined at five durations (2, 4, 10, 20, and 25 ms), with an accuracy of 0.25 μA using the closed-loop algorithm (see Fig. 3). Rheobase and chronaxie were determined from the SD curve and constant monitoring of closed-loop stimulation commenced at the chronaxie pulse duration and 2 Hz, with a target CF of 1.0.

In order to demonstrate modulation of stimulation threshold by fundamental physiologic variation, the temperature of the perfusing medium was altered during real-time determination of stimulation threshold. Changes in temperature alter the reaction rates of most biochemical processes, including ion channel opening and closing rates. Thus, changes in temperature mimic systemic variations in physiological properties of the cardiac cells and demonstrate the ability of the system to resolve temporal changes in stimulation threshold.

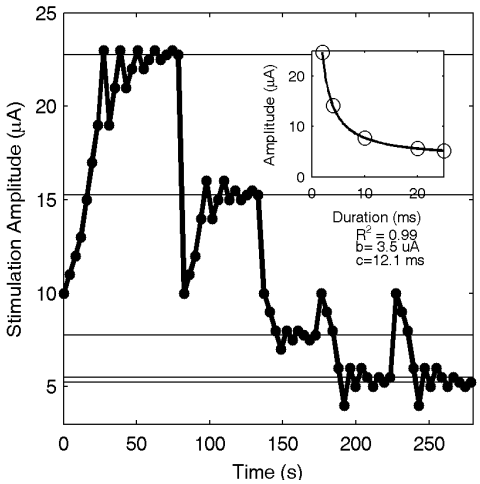

Fig. 3 Demonstration of the closed-loop stimulation system's determination of the SD curve. For each duration, stimulation is initiated with pulse amplitude 10 μA and adjusted in order to achieve the target capture fraction while reducing step size. When the step size limit of 0.25 μA is achieved, the duration is increased and the process repeats. The inset shows the SD curve obtained from this analysis by linear regression. Correlation coefficient (R^2) of the corresponding charge versus duration relationship is 0.99. Extracted rheobase (b) and chronaxie (c) parameters are shown below the inset.

Alteration of the ionic milieu surrounding the cells represents a simple yet physiologically-relevant experiment that impacts the stimulation threshold. Increase of extracellular potassium concentration ($[K^+]_o$) may occur during ischemia and lowers the resting membrane potential within the cardiac cell, leading to increasingly inactivated sodium channels in the cell membrane and increased stimulation threshold. $[K^+]_o$ was increased from 5.4 mM to 8.4 mM by addition of 3 mM potassium chloride to the recirculating media. To monitor the modulation of stimulation threshold by $[K^+]_o$, cell cultures under constant media perfusion were first stimulated at baseline conditions for ten minutes, at which time the source of the continuously-flowing medium was switched to that containing higher KCl. After twenty minutes, the medium source was switched back to wash out the KCl and return the conditions to baseline.

III. Results

The electrophysiologic response of the cell culture to an increase in temperature is shown in Fig. 4. The black line with round markers shows the stimulation threshold (left y-axis) determined in real time during a stepwise increase in temperature (right y-axis) over the course of approximately 90 minutes. The gray line shows the temperature measured using the integrated RTD on the MEA, and is plotted on an inverse y-axis (values increasing downwards) to show the inverse relationship of stimulation threshold with temperature. The control algorithm tracks the modulation of threshold using the minimum step size possible while temperature is adjusted from 31º to 34º and 37º C. Notice that the system resolves not only the steady-state threshold at a given temperature, but also the transient changes while the threshold stabilizes. The decreasing steps in temperature induce an immediate and rapid decrease in threshold, followed by an increasing rebound and decay to the final steady-state value.

The effects of increasing $[K^+]_o$ on stimulation threshold are shown in Fig. 5. The addition of 3 mM KCl results in an immediate and rapid increase of stimulation threshold, followed by a slight rebound and then a slow rise of threshold with a time constant on the order of 5-7 minutes.

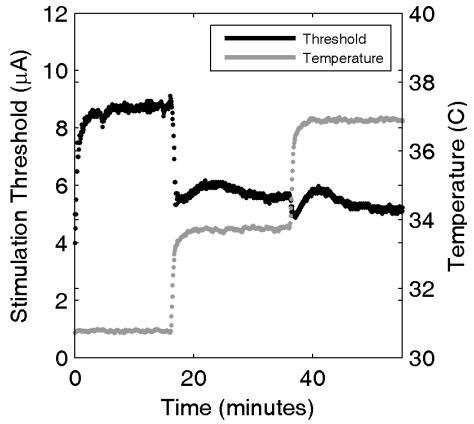

Fig. 4 Temporal response of stimulation threshold (left axes, black) to changes in temperature of the media perfusate (right axes, gray). The stimulation threshold that achieves CF = 0.50 is maintained in real time while temperature is increased stepwise from 31º to 37º C.

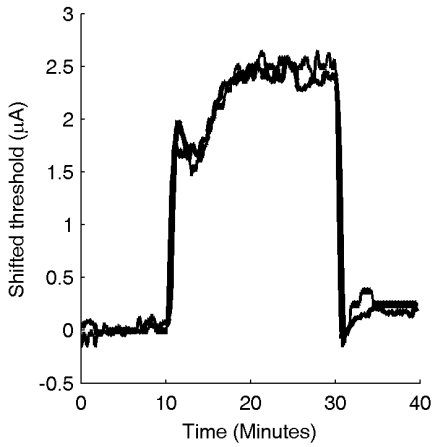

Fig. 5: Temporal response of stimulation threshold to addition and washout of 3 mM KCl. Responses of two different cell cultures are shown, demonstrating the repeatability of the measurement. Thresholds at 9 min. (8.3 μA and 4.8 μA) were subtracted from each trace to overlay the responses.

The rapid adjustment of stimulation threshold is likely due to the immediate depolarization of the membrane. Considering the Nernst potential for K^+ alone, a depolarization of approximately 10 mV could be expected from the increase of $[K^+]_o$, and would lead to sustained inactivation of sodium channels and a corresponding higher stimulation threshold. The slower rise in stimulation threshold could be due to several processes, including the action of Na^+/K^+ pumps and Na^+/Ca^{++} exchangers responding to the disruption of ionic equilibrium.

The washout of KCl produces an effect similar to that observed with KCl addition. The primary rapid response returns stimulation threshold nearly to its original value, but following this decrease are transient changes of threshold with a net increase from baseline of about 0.2 μA. This is another example of the advantage of temporal resolution of stimulation threshold, these subtle effects can be observed and quantified easily in a non-invasive manner. Subsequent analysis could include other, more invasive biological techniques to answer specific questions regarding the mechanisms of these observations.

IV. Conclusion

Temporal resolution of stimulation threshold realized using real-time control represents a new modality for electrophysiologic analysis. This method permits the observation of the time course of changes in cell membrane excitability that were not previously observable using other methods. While the system presented here is based upon cardiac cultures on MEAs, it is not limited to such application and could be used in whole heart preparations or other cell cultures in order to elucidate the impact of pharmacological, environmental, or other stimuli on stimulation threshold. However, when used in concert with MEAs, other electrophysiologic parameters can be monitored simultaneously and non-invasively as well, including extracellular AP parameters like amplitude and duration, conduction velocity, and propagation dynamics.

These properties make the temporal resolution of stimulation threshold a very powerful tool applicable to basic research in cardiac electrophysiology as well as pharmacology of cardiac tissues. The technique is also quite applicable to the design of clinical pacing products, as transient modulation of membrane excitability due to drug exposure, drug interactions, or other pathologies could lead to loss of capture in implanted pacemakers.

V. References

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