A MICRODEVICE FOR EXAMINING CARDIAC GAP JUNCTION

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ABSTRACT

This paper reports a microengineered device capable of applying electrical stimulation to the selected cell, and examining intercellular electro-mechanical propagation through the gap junction of the stimulated and the un-stimulated cell in a longitudinally connected doublet of adult cardiac myocytes. Since the contractile performance of the un-stimulated cells due to direct electrical stimulation is eliminated, this work provides a quantitative tool for studying intercellular electro-mechanical transduction at the gap junction and is expected to develop a comprehensive understanding of the role of intercellular communication in various heart diseases.

INTRODUCTION

Intercellular electro-mechanical transduction in adult cardiac myocytes plays an important role in regulating heart function. Within heart muscle, the intercellular communication is mainly orchestrated by a thin structure located at the longitudinal end of the cardiac myocytes, named the intercalated disc. The intercalated discs connect individual cardiac myocytes into an electrochemical syncytium and are primarily responsible for electro-mechanical transmission during heart contraction [1]. Until recently, the properties of the intercalated discs have been primarily studied within the intact animal heart or using perfused tissue blocks [2, 3]. However, the intrinsic heterogeneity and complexity of the native cardiac environment, which contains multiple types of interconnected cells that are morphologically and functionally distinct, have hampered the quantitative study of cardiac intercellular communication. Cell-based studies, on the other hand, mainly use cultured embryonic and neonatal myocytes [4-6], where the changes in expression of ion channels, contractile protein isoform and contractile performance during development may make it problematic to apply the results to the fully differentiated adult myocardium [7]. In this perspective, an *ex vivo* cell-based model using adult cardiac myocytes is preferred, because it is immune to the complications arising from heterogeneity in electrical excitation and contraction in the native cardiac environment [8, 9], while the adult cardiac myocytes isolated from the hearts largely preserve the characteristics of native myocardium.

The predominant methods for investigating cardiac myocyte intercellular communications include examination of the expressions of characteristic connexin proteins at the intercalated discs using molecular biological assays [10]. To investigate the role of specific connexin proteins, transfected cells can be used to control the connexins by an artificial promoter. Transgenic animals in which one or more connexins have been totally deleted are also used (e.g. Cx43 knockout mice) [11]. Fluorescence tags can be applied to junctional proteins to allow *in-situ* evaluation of temporal and spatial arrangements of these proteins. Besides, commonly used biophysical approaches for assaying gap junctional intercellular communication, including dye transfer [12], $[Ca^{2+}]_i$ propagation [13] and dual patch clamp [14], have also been applied to study the intercellular communication of cardiac myocytes. Although these methods can provide quantitative measurements to a certain extent, the most critical function of cardiac myocytes, the mechanical contractile performance, has been largely ignored. Indeed, the studies of gap junction proteins are often based on the assumption that the quantities of gap junction proteins are correlated, to a certain

extent, to mechanical contractile performance. Similarly, $[Ca^{2+}]_i$ propagation measurement and electrophysiological studies target transport of ions, which is also believed to be correlated with mechanical properties through excitation-contraction coupling. The missing knowledge of such correlations leaves a gap between current technological approaches and investigations of cardiac function.

To address these technical challenges, we developed a microdevice for assessing intercellular cardiac mechanical performance at the gap junction using isolated adult cardiac myocytes. This device applies electrical stimulations to a selected adult cardiac myocyte in a longitudinally connected cell doublet, and quantifies the intercellular electro-mechanical transduction by comparing the contractile performance of stimulated and un-stimulated cells in the same doublet. Since the contractile performance of the un-stimulated cells due to direct electrical stimulation is eliminated, this work provides a quantitative tool for studying intercellular electro-mechanical transduction at the gap junction in adult cardiac myocytes.

Figure 1: Schematics of the microengineered device for intercellular communication studies. Left: schematics of electrical stimulation and cell contraction; Right: schematic curves showing the changes in cell length.

THEORY

To assess intercellular communication at the gap junction between adjacent cells, the contractile performances caused by external electrical stimulation and from intercellular communication have to be separated. This can be implemented by confining the electric field to one cell while shielding the adjacent cells from electric stimulation. The working principle of the microdevice is illustrated in Figure 1. A longitudinally connected cell doublet is positioned in the electrodes array where one cell resides on top of an electrode finger (myocyte B), and the other one in the same cell doublet resides between two neighboring electrodes (myocyte A). When a pulse voltage is applied to the electrodes pair, the cell sitting between the two electrodes is subjected to a local

electric field and contracts accordingly, while the cell on the electrode finger is not stimulated due to the isopotential electrode surface. The contraction of the un-stimulated cell, if any, is hence solely due to the intercellular communication with the stimulated cell through the intercalated disc. During the whole process, the cell length changes over time in both the stimulated and the un-stimulated cells provide a quantitative measure of the intercellular communication. The localized electric field was examined using finite element simulation (Figure 2). The result showed that when a voltage bias (1 V) was applied to two neighboring electrodes (200 μ m in width and 70 μ m in spacing), the electric field induced in the dielectric region between the two electrodes ($\sim 1.5 \times 10^4$ V/m) was an order of magnitude larger than that on the electrode surface ($< 10^3$ V/m).

Figure 2: Finite element analysis of the localized electric field in the close vicinity of the excited electrodes (red). The dotted line denotes the approximate electric field threshold for inducing cell contraction.

EXPERIMENTAL

Device design and fabrication

The device was fabricated by patterning an array of interdigitated microelectrodes on a dielectric glass slide using a standard photolithographic process (Figure 3). The typical length of adult cardiac myocytes used in this study is ~ 80 -120 μ m. Accordingly, the gap between the two electrodes was designed as 70 μ m to ensure that the maximal voltage can be applied to the cell lying across the two neighbouring electrodes. The width of electrodes was designed as 200 μ m so that the second cell in the doublet can completely reside within the electrode surface. In addition, all of the electrodes pairs were numbered and corresponded to the electrode leads patterned on the both ends of the glass slide, so that the external voltage can be easily applied to any single pair of these electrodes as needed. An array of spring contact probes was made contact to the numbered electrode leads. Finally, a breadboard socket was used to control the electrical connection between a function generator (Agilent 3220A) and the contact probes for selective stimulation.

Cell isolation

Hearts were removed from anesthetized rats for left ventricular (LV) myocyte isolation during week 8. The heart was mounted on a Langendorff apparatus and was perfused with Tyrode's solution (131mM NaCl, 4mM KCl, 10mM HEPES, 1mM $MgCl_2$, 1mM $CaCl_2$ and 10mM glucose) supplemented with 10mM 2,3-butanedione monoxime (BDM), followed by perfusion buffer and then digestion with trypsin/Liberase-TH. LV myocytes were mechanically dispersed in perfusion buffer containing 12.5 μ M $CaCl_2$ and 10% FBS, filtered, and resuspended in increasing

concentrations of $CaCl_2$ to achieve a final concentration of 1mM $CaCl_2$. Isolated myocytes were incubated on laminin-coated chambers in plating media for 1 hour followed by culture media for 1 hour. This procedure yielded $>95\%$ viable cardiac myocytes, of which $\sim 3\%$ to 8% were longitudinally connected doublets.

Electrical cell stimulation

Before the experiment, the device was sterilized and coated with laminin (5 μ g/ml) overnight at room temperature. The cells were then transferred into the culture well on the device and cultured for 1-2 hours to allow cell attachment on the substrate. Prior to the electrical stimulation, the culture media was replaced by Tyrode's solution in order to facilitate cell contraction. For the blocked gap junction group, 1.08mM 1-Heptanol (Sigma-Aldrich) was added to the culture media and cells were treated for 5 minutes before Tyrode's solution was added. During the experiment, electric voltage pulses with a frequency of 1 Hz and a width of 8 ms were applied between the two neighbouring electrode fingers of a selected electrode pair where the subject myocyte doublet lay. The voltage magnitude ramped slowly from zero to an appropriate value to ensure that the selected cell was appropriately stimulated. To determine the threshold voltage for cell excitation, the cell length change under the applied voltage was monitored. When a threshold of the electric voltage optimal for cell stimulation was identified, the voltage magnitude was maintained as low as possible and kept constant during the whole stimulation process. Changes in cell lengths upon electrical stimulation were recorded at 7 frames per second (fps) with a CCD camera (Olympus DP71) attached to an upright microscope (Olympus BX51). A dual gooseneck illuminator was used as a complementary light source to obtain a high brightness.

Figure 3: The device and the testing rig for selective electrical stimulation and cell contraction assessment: (a) micropatterned interdigitated electrodes. Scale Bar=500 μ m; (b) schematics showing the device configuration; (c) the device was mounted on the stage of an upright microscope; and (d) the selective electrical stimulation system.

Quantification and data analysis

The cell length was measured using Image J (NIH), where the cell contractility was derived by dividing the cell length reduction upon contraction by the un-stimulated cell length. Cell contractility was determined from 15-20 cells with three independent experiments, and expressed as mean $\pm$ standard deviation from 6 measurements. The differences between groups were analyzed by Student's t-test, with $p < 0.05$ set as statistically significant.

RESULTS

Characterization of single cell contractile performance

Contractile performance of single myocytes was experimentally investigated by applying a local electric field to a single adult cardiac myocyte lying perpendicularly across the 70 μm wide gap between two neighbouring electrodes (Figure 4a). The cell was stimulated at three different frequencies (0.5 Hz, 1.0 Hz and 2.0 Hz). The cell length change showed that the frequency of the induced cell contraction was fairly consistent with that of the electrical stimulation (Figure 4b&c), namely, 0.5 Hz electrical stimulation resulted in 0.5 Hz cell contraction, *etc.* In contrast, the cell contractility was inversely proportional to the stimulation frequency (Figure 4d), suggesting a negative force-frequency relationship.

Besides the stimulation frequency, effect of stimulation intensity on cell contractile performance was also investigated (Figure 4e). An optimal “window” of electrical voltage was found that can induce cell contraction. Given the electrode configuration and the gap distance between the neighbouring electrodes, most cells contracted under a voltage between 1.0 V and 1.3 V. A lower voltage could not induce cell contraction while a higher voltage led to immediate cell death. Within the voltage “window”, there was no significant correlation between the cell contractility and the voltage magnitude (Figure 4f). This result was in good agreement with the “all-or-none” law for electrical stimulation of cardiac myocytes, namely, the cell responds to the best of its ability if the stimulus is above the threshold [15].

Figure 4: Electrical stimulation of single cardiac myocytes. (a) A cardiac myocyte lying across the gap between the neighboring electrodes. (b) Contractile performance of the cell responding to electrical stimuli at different frequencies. Contraction frequency (c) and cell contractility (d) both depend on the stimulation frequency. (e) Cell contractility as a function of the stimulation intensity. Each curve represents the data recorded from one cell. (f) Statistics showing the effect of stimulation intensity on the cell contractility.

Figure 5: Selective electrical stimulation was validated by comparing the contractile performance of a cell doublet before (a) and after (b) a gap junction blocker was applied. Statistic result was shown in (c).

Assessment of intercellular electro-mechanical transduction

To validate the selective electrical stimulation, cell doublets were positioned in the electrode array where one cell resided on top of an electrode finger, and the other cell resided between two adjacent electrodes. The cell contractions under electric stimulation were measured by tracking the changes in cell length. In a doublet with intact gap junction, synchronized contractions of the two cells were observed (Figure 5a). However, once the intercellular communication through the gap junction was blocked using a gap junction blocker (1-Heptanol), the un-stimulated cell on the electrode surface no longer exhibited periodic contraction, with the length kept approximately constant, while the stimulated cell between the two electrodes was still in active contraction (Figure 5b). This was confirmed by the repeating measurements from a number of cell doublets (Figure 5c). While both cells of the doublets with intact gap junctions contracted at a comparable magnitude (~ 0.053 to ~ 0.047), the contractility of the stimulated cell when the gap junction was blocked (~ 0.078) was an order of magnitude larger than that of the un-stimulated one (~ 0.007). The results suggested that the contraction of the un-stimulated cell was solely attributed to the electro-mechanical transduction from the stimulated cell through the gap junction.

Finally, a proof-of-concept study was conducted to examine the efficacy of the device in assessing the intercellular electro-mechanical transduction within adult cardiac myocytes doublets. The first cell doublet was isolated following the regular protocol, while the second cell doublet was prepared with the reduced calcium concentration in the digestion buffer (14.5 μM CaCl_2). In the first cell doublet (Figure 6a&b), the two cells contracted at the same frequency and at the same phase, which were in register with the applied electrical stimulation. The result suggested that the gap junction in the first pair was well-preserved and was able to synchronize the contractile functions in the two coupled cells in the doublet. This is similar to that in a healthy heart where all cells are contracting synchronously so that the ventricle can exhibit a maximal pumping performance. In the second cell doublet (Figure 6c&d), the un-stimulated cell exhibited an irregular

contractile performance with a discernible phase delay comparing to the stimulated cell. Meanwhile, there was a greater difference in cell contractility between the stimulated cell and the un-stimulated cell. The unsynchronized contractions indicated an impaired conductance in the gap junction or an altered electro-mechanical coupling mechanism, which may represent the conditions of myocardium in the cardiac fibrillation.

Besides the capability of examining gap junction change due to varying calcium concentration, the reported device is also able to examine alterations in the intercellular performance due to other extrinsic and intrinsic factors that may cause asynchronous contraction and the contractility degradation between the two cells in a longitudinally connected doublet. In this perspective, this work not only develops a quantitative platform for studying the role of intercellular electro-mechanical transduction under various heart diseases, but also provides a powerful tool to assess the possibilities of pharmacological approaches towards efficient clinical therapies.

Figure 6: Contractile performance of the cell doublets with regular (a) and impaired (c) gap junctions. (b) The two cells recorded in (a) showing a synchronous contraction; (d) while the two cells recorded in (c) showing an asynchronous contraction accompanied with a reduced contractility in the un-stimulated cell.

CONCLUSIONS

This study presents a microengineered approach to study intercellular communication in cardiac cells by measuring the contractile performance of isolated doublets of adult cardiac myocytes. The work provides a quantitative tool for studying intercellular mechanotransduction at the gap junction and is expected to establish a comprehensive understanding of intercellular communication in various heart diseases.

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