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Mechanically induced intercellular calcium communication in confined endothelial structures

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ABSTRACT

Calcium signaling in the diverse vascular structures is regulated by a wide range of mechanical and biochemical factors to maintain essential physiological functions of the vasculature. To properly transmit information, the intercellular calcium communication mechanism must be robust against various conditions in the cellular microenvironment. Using plasma lithography geometric confinement, we investigate mechanically induced calcium wave propagation in networks of human umbilical vein endothelial cells organized. Endothelial cell networks with confined architectures were stimulated at the single cell level, including using capacitive force probes. Calcium wave propagation in the network was observed using fluorescence calcium imaging. We show that mechanically induced calcium signaling in the endothelial networks is dynamically regulated against a wide range of probing forces and repeated stimulations. The calcium wave is able to propagate consistently in various dimensions from monolayers to individual cell chains, and in different topologies from linear patterns to cell junctions. Our results reveal that calcium signaling provides a robust mechanism for cell–cell communication in networks of endothelial cells despite the diversity of the microenvironmental inputs and complexity of vascular structures.

1. Introduction

Many essential functions of the vasculature are known to be regulated by intracellular calcium signaling [1]. To allow proper physiological functions, cytosolic calcium is tightly controlled in endothelial cells by multiple intracellular and transplasmalemmal calcium regulatory mechanisms [2]. Under resting conditions, free calcium is maintained at a low concentration. The endoplasmic reticulum (ER), which contains numerous calcium-binding proteins, is a major intracellular calcium store for endothelial cells [3]. The ER accounts for ~75% of the total intracellular calcium reserve while the majority of the remaining portion is stored in the mitochondria. The release of ER calcium to the cytoplasm can be controlled by calcium release channels, such as inositol-1,4,5-triphosphate (IP_3) and ryanodine receptors, on the ER and can also be spontaneously released through luminal calcium leakage. Calcium mobilization can be triggered by agonists, e.g., IP_3 and ryanodine, which bind to their specific receptors and modulate the calcium release properties of these channels. Remarkably, calcium can trigger calcium release resulting in calcium induced calcium release in an autocatalytic manner. To avoid cytotoxicity due to high concentrations of calcium, the calcium release channels terminate after a short duration despite the presence of the agonists. At the same time, the cytosolic calcium is resequestered inside the ER as well as pumped outside of the cell through transmembrane ATPases, ATP-dependent calcium pumps, which continuously take up calcium from the cytosol. This resets the cytosolic calcium to a resting condition (~100 nM) and allows stimulation again after a refractory time period [1].

Physiologically, cells move calcium not only between cellular compartments and the exterior of a single cell, but also amongst neighboring cells [4–10]. These connections are made by gap junctions, which connect vascular as well as many other cell types and allow moving not only calcium ions but also transfer of other molecules and small proteins between cells. These junctions consist of connexin proteins which form pores between the cells allowing exchange of the various substances to pass through them. In the case of endothelial cells, several types of gap junction connexin proteins including connexin 40, 43 and 37 are relevant to calcium signaling [11]. Gap junctions directly link the cytoplasms of

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cells and allow exchange of ions and secondary messengers, including calcium and IP_3. Furthermore, many cell types are known to communicate by releasing diffusible factors into the microenvironment. As a result, once calcium release in a cell has been stimulated, the signal can be transferred to neighboring cells via gap junction intercellular communication (GJIC), and extracellular diffusion, even though they are not affected by the stimulus themselves [12,13]. The transfer of the calcium signal results in a spatiotemporal propagation of intercellular calcium wave communicating a signal between neighboring cells.

To serve as an effective cell–cell communication mechanism, intercellular calcium signaling must be robust against functional and operational conditions in vascular structures. These involve various topologies and continuous exposure to numerous biomechanical and biochemical stimuli in the cellular microenvironment. Despite the fact that extensive efforts have been devoted to elucidate the molecular mechanisms responsible for the regulation of cytosolic calcium, there is a lack of understanding in the implication of the local calcium regulation in the global characteristics of intercellular calcium communication. In particular, the functional properties of intercellular calcium signaling in the vascular systems, which have diverse dimensions (from individual cells to centimeters) and distinct architectures (e.g., linear chains and branching morphologies), are largely unknown [14–16]. Herein, we investigate the functional characteristics of intercellular calcium signaling of networks of mechanically stimulated human endothelial cells. The endothelial structures are confined using a plasma lithography cell patterning technique, which allows systematic control of the network topology and architecture [17–21]. Real-time intracellular calcium imaging is applied to observe the propagation of calcium wave in endothelial networks [22,23]. To study the probing force, comb-drive capacitive force sensing probes are also applied to stimulate cells mechanically at the single cell level [24,25].

2. Materials and methods

2.1. Plasma lithography cell patterning

In this study, geometric confinement of cells was achieved by plasma lithography, which creates spatial templates of cell adhesive surface chemistry on polystyrene substrates [17–21]. Plasma lithography applies selective shielding of plasma via a flexible rubber polydimethylsiloxane (PDMS) mold to produce a chemical template on the substrate (additional detail of plasma lithography can be found in Supplementary information S1). The PDMS molds used to produce the selective plasma shielding were created via standard soft lithography [26], which replicated shapes by means of molding from master patterns. PDMS cast-off of photoresist structures to make shielding molds was mixed in 8:1 ratio of polymer base to curing agent and degassed before being cured for 24 h at room temperature (see also Supplementary information S2 for mold fabrication method).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (ATCC CRL-1730). HUVEC were cultured in F-12K Medium (ATCC) supplemented with 20% screened FBS (Gemini BioProducts), 0.035 mg/ml endothelial cell growth supplement (Sigma–Aldrich), 0.1 mg/ml heparin (Sigma–Aldrich), and 0.1% gentamycin (GIBCO). HUVEC were used from passages three to six in the experiments.

2.3. Real-time intracellular calcium imaging

To perform real-time imaging of intercellular wave propagation, a calcium-sensitive dye (Fluro-3AM (Invitrogen) dissolved in DMSO (Fisher)) was first introduced inside the cells with 10 mg/ml of Pluronic® F-127 (Invitrogen). The dye was incubated inside the cells for 35 min for esterase cleavage activation. The dye then became fluorescent when bound to calcium thereby allowing visualization of intracellular calcium ion concentrations and movement. For fluorescence observation, endothelial cells were maintained on a microscope stage top hotplate at 37 °C with Hank's buffered salt solution (HyClone). The buffer normally contained calcium, except for experiments exploring the signaling mechanisms without extracellular calcium which it was removed from the medium. The microscope hotplate was placed onto an epi-fluorescence microscope (Nikon TE2000-U) equipped with a CCD camera (Cooke SensiCam) for real-time fluorescence imaging. The fluorescence intensity values are reported by normalizing the initial unstimulated intensity for each individual cell.

2.4. Cell stimulation

To mechanically stimulate calcium release at the single cell level, individual endothelial cells were probed with a comb-drive (capacitive) based force probe (FemtoTools Instruments, FT-S540) or a 30-gauge syringe needle (Fisher). The comb-drive probe allows time-resolved measurement of force applied to the cell during stimulation, while the syringe needle allows improved observation of cells due to the size of the needle which is significantly smaller than the force probe. To control the location of mechanical stimulation, the probes were mounted to a custom three-axis translational stage. In our setup, two probes can be controlled simultaneously to provide mechanical stimulation to cells in the network independently. At the beginning of each experiment, the probes were brought close to the cells before stimulation and a bright field image was obtained to monitor the position of the cells. A fluorescence image was also gathered for background estimation in the image analysis. Real-time fluorescence imaging was then captured to study calcium wave propagation after mechanical stimulation. All images were captured within ~25 min of dye loading.

3. Results

3.1. Calcium wave propagation in HUVEC networks

HUVEC networks were organized via plasma lithography to create cell structures consisting of desired topologies (Fig. 1a). Individual HUVECs could then be mechanically stimulated with a force probe or a needle (Fig. 1b). Several structures, including monolayers, linear patterns, and cell junctions, were designed to explore the architecture dependence on calcium wave propagation in HUVECs (Fig. 1c–e). Upon mechanical stimulation, the cells displayed an increase in calcium in the cytoplasm and the increase in calcium was observed to pass onto neighboring cells in monolayers and networks of HUVECs (Fig. 1f; SI videos 1–3 Supplementary information). Under the gentle probing conditions normally employed, cell membranes were mildly stretched and were not permanently damaged. The damage to the cells was monitored through several observations including no visible, permanent membrane deformation or cellular detachment after probing, and the ability of cells probed in such a manner to repeatedly release calcium when subsequently probed. Alternatively, if cells were probed more forcefully, visible damage and behavioral changes were observed in the cells. Mechanical wounding of the cells was also observed to initiate the propagation of calcium wave (data not shown) [27]. Cells probed in these manners, however, were not used for analysis of calcium wave transmission in this study.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2012.11.060.

To study the nature of calcium communication in HUVEC networks, calcium release and propagation were observed under several experimental conditions (Fig. 2a). Cellular calcium release can be triggered by both mechanical probing and ATP loading consistent with previous reports [28,29]. To determine the calcium source, the endothelial cells were probed in the absence of calcium in the buffer by using a calcium-free buffer (Calcium-free HBSS (HyClone)) with additional calcium chelator, ethylene glycol tetraacetic acid (EGTA) (Boston BioProducts). Under this condition, calcium wave propagation could still be observed suggesting that extracellular calcium is not a necessary condition in the mechanotransduciton of calcium wave in HUVEC. The same observation was also reported in monolayers of bovine aortic endothelial cells [28]. The increase in calcium is, therefore, likely contributed from intracellular stores, such as from the ER. The involvement of the ER is further studied by blocking calcium uptake in the ER pharmacologically. With thapsigargin, an ER calcium pump inhibitor [30] that depletes intracellular calcium store, calcium signaling was not provoked either mechanical stimulation