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Coordination of Intercellular Ca^{2+} Signaling in Endothelial Cell Tubes of Mouse Resistance Arteries

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Abstract

Objective—To test the hypothesis that Ca^{2+} responses to G-protein coupled receptor (GPCR) activation are coordinated between neighboring endothelial cells of resistance arteries.

Methods—Endothelial cell tubes were freshly isolated from superior epigastric arteries of C57BL/6 mice. Intercellular coupling was tested using microinjection of propidium iodide. Following loading with fluo-4 dye, intracellular Ca^{2+} responses to ACh were imaged with confocal microscopy.

Results—Cell-to-cell transfer of propidium iodide confirmed functional gap junction channels. 1 μM ACh evoked Ca^{2+} responses [$9.8 \pm 0.8/\text{min}$, $(F/F_0) = 3.11 \pm 0.2$] which pseudo-linescan analysis revealed to be composed of Ca^{2+} waves and spatially-restricted Ca^{2+} release events. 100 nM ACh induced Ca^{2+} responses of lower frequency ($4.5 \pm 0.7/\text{min}$) and amplitude ($F/F_0 = 1.95 \pm 0.11$) composed primarily of spatially-restricted events. The interval between Ca^{2+} waves in Adjacent cells (0.79 ± 0.12 s) was shorter ($P < 0.05$) than between Nonadjacent cells (1.56 ± 0.25 s). Spatially-restricted Ca^{2+} release events had similar frequencies and latencies between Adjacent and Nonadjacent cells. Inhibiting intracellular Ca^{2+} release with 2-APB, Xestospongin C or thapsigargin eliminated Ca^{2+} responses.

Conclusions—With moderate GPCR stimulation, localized Ca^{2+} release events predominate among cells. Greater GPCR stimulation evokes coordinated intercellular Ca^{2+} waves via the endoplasmic reticulum. Calcium signaling during GPCR activation is complex among cells, varying with stimulus intensity and proximity to actively signaling cells.

Keywords

endothelium; gap junctions; G protein coupled receptors; acetylcholine

INTRODUCTION

Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is an essential component of endothelial cell (EC)-dependent regulation of vasomotor tone. Increases in EC $[\text{Ca}^{2+}]_i$ lead to production of diffusible autacoids (e.g., nitric oxide and metabolites of arachidonic acid) (10, 12, 31) and the opening of Ca^{2+} -activated K^+ channels (9, 32), both of which relax vascular smooth muscle cells (SMC) to produce vasodilation. Endothelial cell $[\text{Ca}^{2+}]_i$ is dynamically regulated by paracrine signaling pathways, including plasma membrane G protein coupled receptor (GPCR) agonists such as acetylcholine (ACh), angiotensin II, bradykinin, and ATP

(1). Each of these stimulate inositol 1,4,5 trisphosphate (IP₃) production (9) leading to intracellular release of Ca²⁺ from the endoplasmic reticulum (ER) Ca²⁺ store through IP₃ receptor (IP₃R) Ca²⁺ channels (54), thereby regulating EC [Ca²⁺]_i and vasomotor tone.

Intercellular signaling between adjacent ECs is enabled through the robust expression of gap junction channels (GJCs) (5, 23, 48), which are formed by connexin subunits arranged into hexameric complexes known as connexons. When connexons from adjacent cells dock with each other, intercellular channels are formed that directly connect the cytoplasm of neighboring cells and are permeant to ions and second messenger molecules such as Ca²⁺ and IP₃ (22, 44). Transfer of either Ca²⁺ or IP₃ between cells can result in a 'Ca²⁺ wave' that travels between coupled cells (7, 28). Local activation of muscarinic GPCRs with ACh released from a micropipette has been shown to initiate Ca²⁺ waves that travel along the endothelium of pressurized feed arteries isolated from hamster skeletal muscle (16, 55). Similar findings have been reported for cremaster muscle arterioles studied *in vivo* (52).

Endothelial cell [Ca²⁺]_i is influenced by flow (21), agonists carried in the blood stream (2) and the interplay with surrounding SMCs. Heterocellular coupling through myoendothelial GJCs allows signals originating in SMCs to evoke Ca²⁺ responses in ECs (17, 29, 33, 53, 59). The goal of the present study was to resolve mechanisms of Ca²⁺ signaling intrinsic to individual and neighboring ECs of resistance arteries through homocellular GJCs. For this purpose we studied EC tubes freshly isolated from resistance arteries of mouse skeletal muscle (4, 50). We hypothesized that during GPCR stimulation, Ca²⁺ signals originating in one EC will influence [Ca²⁺]_i in a neighboring EC. Further, we hypothesized that, independent of extracellular Ca²⁺, the release of Ca²⁺ from intracellular stores governs the coordination of Ca²⁺ responses between neighboring ECs. Calcium responses to ACh were imaged using the calcium-indicator dye fluo-4 to evaluate the temporal nature of Ca²⁺ responses in adjacent versus nonadjacent ECs. Our findings illustrate coordinated Ca²⁺ responses between adjacent ECs while Ca²⁺ responses between nonadjacent ECs in the same visual field lacked temporal correspondence. These relationships persisted in the absence of extracellular Ca²⁺ but were abolished by inhibition of ER Ca²⁺ uptake and release, indicating an integral role of the endoplasmic reticulum in coordinating intercellular Ca²⁺ signaling.

MATERIALS AND METHODS

Animal care and tissue sampling

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri-Columbia and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (8th Ed., 2011). Mice were maintained on a 12h: 12h light: dark cycle at ~23°C with standard rodent diet and water *ad libitum*. Male C57BL/6 mice bred at the University of Missouri-Columbia (3–6 mo, 25–35 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). The abdomen was shaved and a midline incision was made through the skin. The origin of each superior epigastric artery was exposed near the sternum and ligated (6-0 silk suture) to maintain a column of blood within the vessel lumen and thereby facilitate resolution of the vessel during microdissection. Abdominal muscles were excised rapidly and placed immediately into ice-cold (4°C) dissection buffer (pH 7.4, 285–290 mOsm) composed of (in mM): 137 NaCl, 5 KCl, 1 MgCl₂, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 glucose, 0.01 sodium nitroprusside (SNP), and 0.1% bovine serum albumin. The anesthetized mouse was euthanized by an intracardiac overdose of pentobarbital followed by cervical dislocation.

Microdissection of arteries and isolation of EC Tubes

Abdominal muscles were transferred to a dissection chamber containing dissection buffer at 4°C and pinned onto a transparent silicone rubber block (Sylgard 184, Dow-Corning; Midland, MI). While viewing through a stereomicroscope (MZ8, Leica Microsystems; Bannockburn, IL), an artery was dissected from the point of ligation to the first major branch point (isolated segment length, 3–3.5 mm). Cannulation pipettes were pulled (P-97, Sutter Instruments; Novato, CA) from borosilicate glass capillaries (G150T-4, Warner Instruments; Hamden, CT) broken to an outer tip diameter of 50–80 µm and heat polished. The arterial segment was cannulated at one end and the lumen flushed with chilled dissection buffer to remove residual blood. Each arterial segment was then cut into 2–3 smaller segments (each ~1–2 mm long) and transferred into 12×75 mm culture tubes on ice containing 4 mL of dissection buffer.

Intact EC tubes were isolated as recently described (4, 50). Briefly, arterial segments were transferred to a 12×75 mm culture tube containing 1 mL of preheated (37°C) dissociation buffer composed of (in mM): 137 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 10 glucose, 2 CaCl₂, and 0.1% BSA, 0.62 mg/mL papain (Sigma #P-4762), 1.5 mg/mL collagenase (Sigma #C-8051) and 1.0 mg/mL dithioerythritol (Sigma #D-8255), pH 7.4. Segments were incubated for 30 min at 37°C then transferred to enzyme-free dissociation buffer at room temperature. An individual arterial segment was transferred to a recording chamber (RC-27N, Warner) on the stage of an inverted microscope (Nikon Diaphot; Garden City, NY). During visual inspection at 40X magnification, surrounding SMCs were dissociated from the EC tube using gentle trituration through the heat-polished tip (internal diameter, ~120 µm) of a micropipette pulled (P-97, Sutter) from borosilicate glass capillary tubes (#1B100-4, World Precision Instruments; Sarasota, FL).

The freshly-isolated EC tube was positioned at the center of the recording chamber. Each end was pressed gently against the chamber bottom (a 24 mm × 50 mm coverslip) using a blunt-tip holding micropipette (heat-polished borosilicate glass; external diameter ~100 µm) secured in 3-axis micromanipulators (DT3-100, Siskiyou Design; Grants Pass, OR). The EC tube was extended to approximate *in situ* length by retracting each holding micropipette while gentle pressure was maintained on each end of the EC tube. Thus secured, the EC tube was superfused for 30 min with a physiological salt solution [PSS, 290–295 mOsm; pH 7.4, composed of (in mM): 137 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 10 glucose and 2 CaCl₂] to allow for adherence to the coverslip.

Dye coupling of endothelial cells in freshly isolated EC tubes

Control experiments were performed to confirm that ECs remained coupled to one another through functional GJCs. A microelectrode (tip resistance: 150–200 MΩ) backfilled with 0.1% propidium iodide in 2M KCl was inserted into an EC along the midline and maintained for 30 min (3, 4). Propidium iodide fluorescence (excitation: 488 nm, emission: 500–600 nm) and differential interference contrast (DIC) images were obtained from the same field of view using confocal microscopy (Leica SP5II; Mannheim, Germany) with a 40X objective (1.25 numerical aperture).

Imaging of calcium responses

Following isolation, EC tubes were loaded at room temperature with 10 µM fluo-4 AM (Invitrogen Life Sciences; Carlsbad, CA) for 10 min, followed by a 20 min wash. The chamber was transferred to the stage of an upright microscope (Olympus BX51W1; Center Valley, PA) equipped for spinning disc confocal imaging (CSU-X1; Yokogawa Corporation; Sugarland, TX) and the EC tube was imaged using a 60X objective (1.0 numerical aperture). With laser excitation at 491 nm, fluorescence emission was recorded from 500–550 nm

using an intensified charge-coupled device camera (Mega-10; Stanford Photonics Inc.; Palo Alto, CA). Image stacks were acquired at 120 frames/s for 25 s and then averaged to 40 frames/s using Piper Control Software (Stanford Photonics). Recordings were and analyzed offline using SparkAn (kindly provided by M. T. Nelson and A. Bonev, University of Vermont) or ImageJ software.

Calcium responses were elicited with ACh (100 nM or 1 μ M) delivered directly over the EC tube preparation via a gravity-fed delivery pipette (Teflon PTFE Needle, 20 Gauge) placed 300 μ m upstream in the bath. This technique for agonist delivery allowed for rapid exchange of the solution directly over the EC tube while bulk PSS superfusion through the chamber was maintained at 3–4 mL/min. In each experiment, morphological integrity and the $[Ca^{2+}]_i$ response to 1 μ M ACh was evaluated first to ascertain the viability of the preparation (50). Each experimental intervention was thereby paired to a control $[Ca^{2+}]_i$ response. In some experiments Ca^{2+} -free PSS was used to eliminate Ca^{2+} entry [0 Ca^{2+} PSS; 290–295 mOsm; pH 7.4, composition (in mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose and 1 EGTA]. Endothelial cell tubes were superfused with 0 Ca^{2+} PSS for 2 min, which was determined in control experiments (loss of excitation-contraction coupling in cardiac myocytes) to be the minimum time necessary to remove Ca^{2+} from the bath. All drug treatments were administered through the delivery pipette and maintained through the period of imaging.

Chemicals and reagents

Bovine serum albumin was obtained from US Biochemical (Cleveland, OH). Xestospongine C was obtained from Cayman Chemical Co. (Ann Arbor, MI). Chemicals and reagents were otherwise obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of the IP_3R inhibitor 2-aminoethoxydiphenyl borate (2-APB) and the SERCA inhibitor thapsigargin were prepared in dimethyl sulfoxide (DMSO), the IP_3R inhibitor Xestospongine C was dissolved in chloroform, and these reagents were diluted to final working concentrations in PSS. Final DMSO (or chloroform) concentration was < 0.5%. Following recording of control ACh responses, 2-APB, Xestospongine C or thapsigargin were applied for 20, 20 or 5 min, respectively, and ACh stimulation was repeated for paired comparisons. Vehicle controls had no effect on Ca^{2+} signaling responses to ACh. Under the same experimental conditions, Ca^{2+} responses to 1 μ M ACh stimuli are reproducible for over 3 h (50).

Analysis of Calcium Responses

Cell selection for analysis—For each analysis, a still image containing all ECs within the field-of-view ($128 \times 96 \mu$ m, ~40 cells) was presented to a naive observer who selected six triplets of cells (18 cells; i.e., approximately half of the cells in the field). Each cell triplet contained a Reference cell, an Adjacent cell in direct physical contact with the Reference cell, and a Nonadjacent cell typically 3–5 cells removed from the Reference cell (Figure 3A). Analyzing Adjacent vs. Nonadjacent cells simultaneously provides an objective reference to as to whether they differ in temporal relationships for Ca^{2+} signaling events.

Single-cell versus whole-field analysis of Ca^{2+} responses—For single-cell analyses, regions of interest (ROIs) encompassing the entire cell were placed within the borders of each EC (Figure 3A) and F/F_0 profiles were obtained using automated analysis with SparkAn. The frequency and amplitude of all whole-cell Ca^{2+} responses (above the detection threshold set at $\Delta F/F_0 > 0.5$; i.e., at least 10-fold greater than the standard deviation of baseline noise) were obtained for each experiment. For whole-field analysis, the ROI was placed over the entire image (containing all cells) and F/F_0 profiles were obtained.

Whole-Cell Latency Analysis—A latency analysis was performed to determine the temporal relationship between responses of Adjacent versus Nonadjacent cell pairs. Within each selected triplet of cells, the time course of each whole-cell Ca^{2+} response ($\Delta F/F_0 > 0.5$) was determined and two comparisons of response latency were performed: 1) Reference cell to the Adjacent cell, and 2) Reference cell to the Nonadjacent cell. The initial response to ACh was excluded from analysis as it coincided with initial exposure (i.e., initial Ca^{2+} responses were coordinated by simultaneous exposure to ACh). Therefore, only the secondary oscillations (Figure 3B) were analyzed as these represent spontaneous Ca^{2+} signaling events during sustained agonist exposure. For each analysis triplet, the cell with the lowest frequency of secondary oscillations was selected and each oscillation within that cell was matched with a temporally associated oscillation in the other 2 cells. (Figure 3B; **enlarged inset**). Because temporal association between cells may precede or follow the referenced event, absolute values of the latency times were used for data analysis to avoid negative values. An average latency time was calculated from all matched oscillations within each cell pair (Reference vs. Adjacent; Reference vs. Nonadjacent) and this value represents a single data point as shown in Figure 4A.

Pseudo Line-Scan Detection Analysis—Two Ca^{2+} signaling modalities were apparent within individual ECs: propagating Ca^{2+} waves and non-propagating spatially restricted Ca^{2+} release events. Regions of interest that encompassed the entire cell (above) were unable to resolve spatially restricted Ca^{2+} release events, limiting our ability to distinguish between respective Ca^{2+} signaling modalities. We therefore complemented the whole cell latency analysis with a pseudo line-scan latency analysis (Figure 5). This more detailed approach was applied to 3 of the 6 triplets (9 cells per EC tube) that had been originally selected from each recording for the whole-cell analyses described above. Frame-scan images were converted to pseudo line-scan images by selecting reference analysis lines using ImageJ software. A reference line was placed longitudinally within ECs (Figure 5A *i*) and corresponded to a width of 4 pixels ($0.8 \mu\text{m}$); these 4 pixels were averaged to obtain a single value at each point along the reference line. The resulting pseudo line-scan plots had distance on the X-axis and time on the Y-axis. From these plots (Figure 5A *ii*) two additional ROIs (each 4 pixels wide and separated by $10 \mu\text{m}$) were used to detect and classify Ca^{2+} responses within these respective ROIs based upon amplitude and spatial domain. A detection threshold ($\Delta F/F_0 > 0.5$) was set in ImageJ and a release event was defined as an increase in $\Delta F/F_0$ above the threshold that spread $> 0.8 \mu\text{m}$ (i.e., the dimensions of each intracellular ROI; small squares in Figure 5 *iii*). Following detection, release events were classified as a Ca^{2+} wave if release propagated without interruption between the two intracellular ROIs, or a spatially restricted release event if release did not propagate between respective ROIs.

Pseudo Line-Scan Latency Analysis—The pseudo line-scan analysis enabled comparison of Ca^{2+} release (Ca^{2+} wave or spatially restricted Ca^{2+} release event, as defined above) latency between the two ROIs within a cell, between two ROIs of Adjacent cells, and between two ROIs of Nonadjacent cells. For each analysis, the ROI with the lowest frequency of events was selected and each event within that ROI was matched with a temporally associated event in the other ROI. For intercellular comparisons (i.e., Reference Cell ROI vs. Adjacent Cell ROI or Reference Cell ROI vs. Nonadjacent Cell ROI), the ROIs within respective cells that were closest in distance were used for comparison. Because spatially restricted Ca^{2+} release events were highly variable and exhibited large frequency differences between ROIs, even within the same cell, events that did not have a temporally matched response were excluded from analysis as illustrated in Supplementary Figure 1.

Statistical Analysis

One EC tube was studied per mouse and the number of EC tubes used for each experiment in these studies was 3–6 unless stated otherwise. Data were analyzed using GraphPad Prism software (version 5.0; La Jolla, CA). The latency data were analyzed using analysis of variance (ANOVA) with a Bonferroni post hoc test. Frequency and Amplitude of Ca^{2+} Responses as well as Frequency of intracellular Ca^{2+} waves and spatially restricted Ca^{2+} events were analyzed using paired t-tests. Summary data are reported as means $\pm$ S.E. Differences were accepted as statistically significant with $P < 0.05$.

RESULTS

Dye Coupling in EC Tubes

Endothelial cells within the EC tube preparation exhibited elongated morphology and maintained physical continuity. Functional intercellular connectivity was confirmed by penetrating a single EC with a microelectrode containing propidium iodide and observing robust dye transfer into multiple neighboring ECs (Figure 1A). Thus, ECs within this preparation remain highly coupled to one another through functional GJCs.

Individual Cell vs. Integrated Calcium Responses

Application of 1 μM ACh produced a large initial elevation in EC $[\text{Ca}^{2+}]_i$ that was followed by distinct secondary oscillations (Figure 2A; Supplemental Movie 1). When the fluorescence responses of all ECs within the field of view were averaged ($128 \times 96 \mu\text{m}$, ~40 cells), the secondary phase following the initial peak exhibited a plateau (Figure 2B), with integrated responses similar to those obtained using Ca^{2+} photometry (11, 50). In response to 100 nM ACh, Ca^{2+} signaling was less robust with striking heterogeneity among individual cells. While some ECs exhibited large $[\text{Ca}^{2+}]_i$ responses similar to those observed in response to 1 μM ACh, others exhibited more gradual increases in $[\text{Ca}^{2+}]_i$ of lower amplitude (Figure 2C). When responses to 100 nM ACh were averaged across all cells, the F/F_0 recordings lacked the biphasic response seen to 1 μM ACh and were without clearly defined peak or plateau phases (Figure 2D). The frequency ($9.8 \pm 0.8/\text{min}$) and amplitude ($3.11 \pm 0.20 F/F_0$) of Ca^{2+} oscillations in response to 1 μM ACh were greater than those to 100 nM ACh ($4.5 \pm 0.7/\text{min}$; $1.95 \pm 0.11 F/F_0$, respectively, $P < 0.05$ for both) (Figures 2E and 2F).

Whole cell latency analysis

Visual inspection of frame-scan images revealed that the secondary Ca^{2+} oscillations in response to ACh were composed of propagating Ca^{2+} waves with apparent coordination of the Ca^{2+} waves between Adjacent ECs (Supplemental Movie 1). However, interpretation of this behavior in the 2-dimensional images was difficult due to the complex and dynamic nature of Ca^{2+} signals among ECs within the observed field. To evaluate temporal relationships of these signals among ECs we utilized a whole-cell latency analysis (Figure 3, see *Methods*) to compare signaling events between adjacent ECs versus those between nonadjacent ECs during the secondary oscillation phase. Because 100 nM ACh did not typically elicit distinct secondary oscillations above the whole-cell detection threshold, responses to 1 μM ACh were analyzed. Latency times between Ca^{2+} responses in Adjacent cells were brief and clustered, while those between Nonadjacent cells exhibited a more random distribution (Figure 4A). The average latency time for Adjacent cells was significantly less than that of Nonadjacent cells ($P < 0.05$, Figure 4B). When Ca^{2+} was removed from the extracellular superfusion solution, the initial response to 1 μM ACh was diminished slightly in amplitude compared with 2 mM $[\text{Ca}^{2+}]_o$ (F/F_0 : 3.5 ± 0.2 vs. 3.7 ± 0.2 , $P < 0.05$); yet secondary Ca^{2+} response frequency ($9.7 \pm 0.7/\text{min}$ vs. $10.1 \pm 0.8/\text{min}$) and latency

times (Figure 4C and D) were not different between Ca^{2+} free and 2 mM $[\text{Ca}^{2+}]_o$ conditions. When intracellular Ca^{2+} release was inhibited with 2-APB (100 μM ; to block IP_3Rs , $n=3$ EC tubes) or ER Ca^{2+} stores were depleted with thapsigargin (5 μM , $n=3$ EC tubes), the initial Ca^{2+} response and all secondary Ca^{2+} oscillations in response to ACh stimulation were nearly eliminated (peak F/F_0 $4.8 \pm 1.3\%$ and $1.3 \pm 0.1\%$ of control responses, respectively). Treatment with Xestospongine C (5 μM) to inhibit IP_3Rs eliminated Ca^{2+} response to 1 μM ACh ($n=2$).

Pseudo Line-scan ROI analysis

The whole cell latency analysis illustrated that Ca^{2+} responses to 1 μM ACh exhibited greater coordination between Adjacent ECs than Nonadjacent ECs. However, distinct changes in the F/F_0 profiles in response to 100 nM ACh were difficult to discern when fluorescence was integrated over the entire EC. Therefore, to resolve such events, we employed a complementary pseudo line-scan analysis (Figure 5A). Signaling events in the secondary phase of ACh stimulation were classified as Ca^{2+} waves or as spatially restricted intracellular Ca^{2+} release events based on their spatiotemporal characteristics (see *Methods*). Ca^{2+} waves, defined as a continuous increase in fluorescence between two ROIs separated by 10 μm within the pseudo line-scan image (e.g., second and third events following the initial response in Figure 5A *ii*), were high amplitude events that corresponded to the secondary oscillations in F/F_0 observed in the whole cell analysis. When [ACh] increased from 100 nM to 1 μM the frequency of intracellular Ca^{2+} waves observed in the pseudo line-scan images increased correspondingly (from 3.2 ± 0.8 to 9.2 ± 1.5 waves/EC/min, $P < 0.05$, Figure 5B). These data are similar to the oscillation frequencies derived from the whole cell F/F_0 analyses (above), with modest differences attributed to detection thresholds and ROIs used for the respective analyses. Intracellular Ca^{2+} wave latency time (the interval between F/F_0 changes of the 2 ROIs within a single cell, Figure 5A *ii, iii*), was not different between 100 nM vs. 1 μM ACh (0.44 ± 0.06 s vs. 0.42 ± 0.04 s, respectively).

In contrast to Ca^{2+} waves, spatially restricted Ca^{2+} release events were of low amplitude and encompassed only a fraction of the cell interior, explaining the difficulty in detecting them using the whole cell analysis. However, our pseudo line-scan analysis revealed that spatially restricted events did not occur in the absence of ACh and were of higher frequency than Ca^{2+} waves at both 100 nM and 1 μM ACh. Moreover, spatially restricted events were of similar frequency at both concentrations of ACh (100 nM ACh: 14.9 ± 3.5 , 1 μM ACh: 17.0 ± 1.7 events/EC/min, Figure 5C). While this result may be counterintuitive, it may be explained by observing that spatially restricted events occurred concurrently with Ca^{2+} waves during ACh stimulation (Supplemental Movie 1). Thus IP_3Rs participating in Ca^{2+} waves would no longer be counted as spatially restricted events while the overall level of Ca^{2+} signaling had increased. In the absence of Ca^{2+} waves, spatially restricted Ca^{2+} release events would be expected to increase with increasing [ACh]. The latency of restricted Ca^{2+} release events was also not different between ACh concentrations (1.61 ± 0.19 s at 100 nM vs. 1.48 ± 0.19 s at 1 μM).

In response to 1 μM ACh, intercellular Ca^{2+} wave latency time (the interval between F/F_0 changes of the 2 closest ROIs within different cells) was less variable (Figure 6A) and significantly lower in Adjacent cells (0.79 ± 0.12 s) vs. Nonadjacent cells (1.56 ± 0.25 s; $P < 0.05$, Figure 6B). This difference is consistent with our results from the whole-cell latency analysis (Figure 4). Unlike the whole cell analyses, the pseudo line-scan ROI analysis enabled resolution of the latencies for spatially restricted Ca^{2+} events. As shown for 1 μM ACh (Figures 6C, 6D), spatially restricted Ca^{2+} release event latency was not significantly different between the Adjacent (2.67 ± 0.62 s) and Nonadjacent (2.67 ± 0.76 s) ECs (Figure 6D). With 100 nM ACh the frequency of Ca^{2+} waves was too low for reliable

analysis. As seen with 1 μM ACh, the spatially restricted Ca^{2+} release events were not significantly different between Adjacent and Nonadjacent cells during 100 nM ACh (data not shown). A key finding from the pseudo line-scan ROI analysis is that a shorter response latency between Adjacent cells (versus Nonadjacent cells) was observed only for Ca^{2+} waves, even with discrete release events occurring concurrently (Figure 5 *ii*). This finding illustrates that Ca^{2+} waves represent the dominant form of intercellular Ca^{2+} signal communication between neighboring ECs.

DISCUSSION

We have investigated the nature of Ca^{2+} signaling within and between individual cells of EC tubes freshly isolated from resistance arteries of mouse skeletal muscle. This experimental approach enables resolution of intra- and intercellular signaling events intrinsic to the endothelium independent from the influence of the circulation, surrounding smooth muscle, nerves and parenchyma. Dye transfer following intracellular microinjection confirmed that intercellular coupling through GJCs is well maintained among neighboring ECs. During muscarinic receptor activation, Ca^{2+} waves within and between ECs persisted following removal of extracellular Ca^{2+} , implicating a key role of the ER for intercellular Ca^{2+} signaling. Spatially restricted Ca^{2+} release events were not correlated in time either within or between cells. With an increase in receptor activation, enhanced Ca^{2+} signaling resulted from an increase in the frequency of Ca^{2+} waves with little change in spatially restricted Ca^{2+} release events. These findings illustrate that Ca^{2+} signaling among cells during GPCR activation varies with stimulus intensity and the physical proximity between actively signaling endothelial cells.

Intercellular signaling through GJCs

Endothelial cells line the intima of the blood vessel wall. Throughout the resistance vasculature, ECs are coupled to each other through GJCs (25, 26, 36, 45, 57). The ability of small molecules (< 1kDa) to diffuse through GJCs of neighboring ECs in resistance vessels has been illustrated with intercellular dye transfer following microinjection (35, 47, 56) and this property is maintained following isolation of EC tubes (Figure 1). Intercellular electrical signaling along the endothelium has been demonstrated by simultaneously injecting current while recording membrane potential over distances exceeding 2 mm (4, 20). The orientation of ECs along the vessel axis (20, 24) further contributes to the conduction vasomotor signals along and among branches of vascular resistance networks (13, 15, 49, 57).

In the intact vessel, myoendothelial coupling through GJCs enables hyperpolarization conducted along the endothelium to coordinate SMC relaxation as the signal travels along the vessel wall (15, 19, 46). In turn, Ca^{2+} signals initiated in SMCs [e.g., pulsars (33) and wavelets (54)] spread through myoendothelial GJCs into ECs to modulate smooth muscle tone and conducted vasomotor responses (17, 29, 53, 59). Studies of arteriolar networks in the mouse cremaster muscle (52) and of isolated pressurized feed arteries of the hamster retractor muscle (16, 55) have illustrated that Ca^{2+} signals initiated by local delivery of ACh from a micropipette can propagate along the endothelium for hundreds of micrometers. The Ca^{2+} wave, which follows in the wake of the much faster conducted hyperpolarization, induces nitric oxide production that enhances the duration of vasodilation (8, 16), thereby promoting blood flow and oxygen delivery. In turn, if ACh gains access to the flow stream, entire networks of arteriolar ECs respond in concert (2). However the extent and physiological relevance of Ca^{2+} signaling within and among ECs of the resistance vasculature in the absence of SMCs, blood flow or surrounding tissue has remained unclear. Therefore, in contrast to studies that have focused on Ca^{2+} signaling in the endothelium of intact resistance vessels (2, 18, 21, 52, 55), the present experiments were designed to define

spatiotemporal relationships of Ca^{2+} signaling intrinsic to the endothelium of resistance vessels with a focus on the behavior of individual ECs in light of signaling activity in neighboring cells. Instead of initiating Ca^{2+} signaling from a discrete site to impart directionality (2, 52, 55), entire preparations were exposed to defined agonist concentrations to evaluate how the nature of Ca^{2+} signaling may be influenced by the intensity of GPCR activation. By evaluating 6 cell triplets within each field studied, the present analyses account for simultaneous behavior of multiple cells.

Whole cell analyses

Consistent with observations from single ECs during GPCR stimulation with ATP (27), intracellular Ca^{2+} responses to GPCR activation observed here in response to ACh involve multiple signaling modalities (Supplemental Movies). These modalities include spatially restricted events analogous to Ca^{2+} ‘puffs’ (6, 37, 43), propagating Ca^{2+} waves and Ca^{2+} waves that initiate but fail to propagate. A confounding factor in our 2-dimensional images is the tremendous amount of information acquired from ~40 cells in the observed field. While this strategy allows sampling of the complex signaling events occurring simultaneously within the entire field, it is difficult to quantify this information, particularly when Ca^{2+} waves and spatially restricted Ca^{2+} release events are occurring simultaneously. Thus quantitative interpretation of these data required development of new analyses.

Our whole cell latency analysis of secondary Ca^{2+} oscillations provided a basis for determining whether Ca^{2+} responses were coordinated between Adjacent vs. Nonadjacent cells. However a limitation of analyses based upon entire cells is that it can obscure all but the most robust responses. In effect, discrete localized Ca^{2+} signaling events (i.e., ‘puffs’ or ‘pulsars’) can be buffered by the lack of signal elsewhere within the cell such that the overall signal remains below the threshold for detection. Such an effect was most apparent with a moderate stimulus. Thus, when responses to 100 nM ACh were averaged across the entire field of ECs (Figure 2D), the appearance of Ca^{2+} signaling was effectively eliminated despite the occurrence of brief, low-amplitude discrete Ca^{2+} signals (Supplemental Movie 1). For larger signals (e.g., Ca^{2+} waves in response to 1 μM ACh) the secondary Ca^{2+} oscillations were of sufficient intensity (Figure 3B) to perform the latency analysis that resolved differences in the temporal nature of Ca^{2+} signaling among individual ECs. As illustrated in Figures 4A and 4C, the scatterplot of latency times between responses in Adjacent cells was clustered around an average of ~0.5 s, which we interpret as being consistent with coordinated Ca^{2+} signaling. At the same time, Nonadjacent cells in the same field exhibited a 2-fold greater range of latency times without clustering, thereby implying a lack of coordinated Ca^{2+} signaling.

Following the initial peak response reflecting all cells responding to the initial stimulus, Ca^{2+} signaling within individual ECs was comprised of distinct IP_3R -mediated signaling events in the form of Ca^{2+} waves and spatially restricted Ca^{2+} release events (6, 37, 54) (Figure 5A *ii*). However, when these individual responses are averaged the dynamic nature of the secondary oscillations within cells is lost. Indeed, when averaged across cells, the Ca^{2+} response to 1 μM ACh exhibits a “plateau-like” appearance (Figure 2B) analogous to that obtained with Ca^{2+} photometry. This behavior underscores the conclusion that increases in the ‘global’ Ca^{2+} signal observed with photometry (11, 50) cannot account for subtleties of Ca^{2+} signaling events within individual cells or how such events can change with the intensity of stimulation.

The integrated Ca^{2+} signal obtained from a population of ECs in response to muscarinic (GPCR) receptor activation typically consists of a biphasic pattern: an initial “peak” response attributed to intracellular release followed by a “plateau” phase, with capacitive Ca^{2+} influx across the plasma membrane contributing to a significant portion of the

sustained plateau (11, 54). Whereas responses to muscarinic receptor activation can vary among ECs (38), those not responding directly to ACh (Supplemental Movie 1) while demonstrating secondary oscillations likely reflect signaling arising from neighboring cells. A distinct feature of the present study is that the secondary Ca^{2+} oscillations were sustained following removal of extracellular Ca^{2+} (Figure 4; Supplemental Movie 2), thereby indicating a lack of dependency on Ca^{2+} entry. This behavior is consistent with an integral role for the ER in sustaining Ca^{2+} signaling during receptor activation (27) and can be explained by the 25 s duration of our recordings. With functional SERCA activity and Ca^{2+} reuptake into the ER, the duration of GPCR activation studied here was less than that required to deplete ER Ca^{2+} to levels that would require store-operated Ca^{2+} entry to sustain signaling (11, 41, 42); e.g., through transient receptor potential (TRP) channels (51). In turn, this conclusion is supported by the loss of Ca^{2+} signaling following SERCA inhibition.

Pseudo Line-scan ROI analysis

To resolve discrete Ca^{2+} signals that are lost with the whole cell latency analysis, we developed the ROI latency analysis using ROIs defined for discrete areas within the interior of ECs. In contrast to assigning ROIs according to where signals were observed to originate (18), we empirically placed ROIs with reference to a line positioned along the axis of each cell selected for analysis. Due to differences in focal plane and orientation of individual ECs along an EC tube, it is difficult to obtain 3 cells (Adjacent + Nonadjacent) that are aligned and in focus along the same reference line using conventional line-scan acquisition. To circumvent this limitation we developed a post-acquisition pseudo line-scan approach that enables information obtained from 2-dimensional scanning to resolve the behavior of individual cells that were either Adjacent or Nonadjacent to each other. Given the complexity of this analysis, the cell triplets to be analyzed constituted a subset of those used for whole cell analyses from each EC tube. Thus, creating simultaneous pseudo line-scans along the axis of each of the selected cells (Figure 5) enabled quantification of spatiotemporal relationships for multiple simultaneous Ca^{2+} signaling events (spatially restricted Ca^{2+} release events, Ca^{2+} waves) simultaneously anywhere (in focus) within the observed field.

The use of ROIs imparts the ability to resolve progression of a Ca^{2+} signal throughout a cell or between Adjacent cells. While providing greater temporal and spatial resolution than the whole-cell analysis, the ROI latency analysis confirmed a key finding from the whole cell analysis (Figures 4 and 6): In response to 1 μM ACh, there was no significant difference in latency times between Ca^{2+} waves in Adjacent cells when calculated with either whole cell (0.40 ± 0.64 s) or pseudo line-scan (0.79 ± 0.12 s) analyses, nor was there a significant difference in latencies between Nonadjacent cells (1.06 ± 0.13 vs. 1.56 ± 0.25 , respectively). This consistency between respective analyses demonstrates that either approach can be used to resolve coordination of Ca^{2+} signaling between Adjacent vs. Nonadjacent cells. However the whole cell analysis could only be applied when signals were of sufficient intensity (e.g., Ca^{2+} waves during 1 μM ACh). Subtle differences between latencies calculated by the respective analyses may be attributed to differences in the size of their ROIs used for signal detection. Thus, whereas the whole cell analysis required a sufficient Ca^{2+} response throughout the cell for the signal to be detected, the pseudo line-scan analysis used ROIs of $0.8 \times 0.8 \mu\text{m}$ and thereby enabled greater spatiotemporal resolution.

The whole cell latency analysis cannot resolve the propagation of intracellular Ca^{2+} waves along individual cells. In contrast, the pseudo line-scan analysis enabled us to objectively define discrete ROIs within and between cells and thereby resolve key spatial and temporal relationships of these intracellular Ca^{2+} signals. Whether applied to the whole cell or pseudo line-scan, the analyses developed here are limited by the fact that neither approach accounts for a Ca^{2+} response originating from (or propagating to) a neighboring cell that was not

selected as the Adjacent cell. With each EC in direct contact with at least 4 neighbors, it can receive numerous inputs from different directions. Thus a key concept of the present study is that each EC has multiple inputs that can dictate its Ca^{2+} response, such that an input from one direction may determine whether a particular EC is primed or refractory to the propagation of a Ca^{2+} wave originating from an EC in another direction. This scenario differs from local stimulation within a vessel segment (2, 52, 55) or a single cell (7, 14), which imparts directionality in signal transmission. In turn, a key area for developing more complex (and automated) analyses is the ability to incorporate multiple ECs that are interconnected with each other to quantify more intricate levels of signaling within and between defined regions of the endothelium.

The fusion of elementary Ca^{2+} release events triggered by IP_3 underlies more global Ca^{2+} responses (6). In light of findings supporting such behavior within ECs (27), we hypothesized that increased activation of GPCRs would lead to a shift from spatially restricted Ca^{2+} events to intracellular Ca^{2+} waves. However the resolution afforded by the pseudo line-scan analysis resulted in the unexpected finding that raising the concentration of ACh resulted in a significant increase in Ca^{2+} waves without loss of spatially restricted events (Figure 5B and C). Thus, the higher sampling rate used in conjunction with pseudo line-scan to define objective ROIs within and between cells facilitates the resolution of spatio-temporal relationships within and between neighboring cells. In so doing, new insight is gained with respect to endothelial Ca^{2+} signaling in response to GPCR activation: With an increase in [ACh], the overall increase in $[\text{Ca}^{2+}]_i$ results primarily from Ca^{2+} waves and these are the signals that underlie communication between cells. In contrast, discrete Ca^{2+} signaling events do not propagate and are therefore not coordinated within and between ECs.

Integration with earlier findings

In mouse cremaster arterioles studied *in vivo* at 5 Hz, ROIs were positioned over sites within ECs that Ca^{2+} signals were apparent. In response to 100 μM adenosine, increased “synchrony” between adjacent ECs suggested enhanced Ca^{2+} signal propagation between ECs following purinergic GPCR activation (18). In these experiments, Ca^{2+} signals between respective ROIs were considered “synchronous” if they occurred within 1 s of each other. In isolated rat mesenteric (41) and cremaster muscle arteries (40) imaged at 2 Hz, increasing ACh concentration evoked progressively greater oscillations in EC Ca^{2+} signals yet synchrony between ECs was never observed. When isolated sheets of ECs derived from mesenteric arteries were studied in response to 1 μM ACh, Ca^{2+} oscillations imaged at 1 Hz were typically asynchronous but occasionally “appeared” to synchronize between adjacent cells. In light of the present analyses being performed at 40 Hz, greater temporal correspondence of Ca^{2+} signaling observed here may have been present in these earlier studies but occurred at frequencies that were not resolved at lower sampling rates.

The lack of spontaneous Ca^{2+} signaling events observed here in EC tubes differs from that reported for ECs of arterioles *in vivo* (18, 52). Flow (shear stress) has been shown to stimulate EC Ca^{2+} signaling in cannulated pressurized arterioles (21) while the elimination of flow was associated with loss of EC Ca^{2+} signaling *in vivo* (18). Further, upon dissociation of SMCs, the absence of myoendothelial coupling and signaling events originating in SMCs (29, 30, 33, 53) could further contribute to the absence of spontaneous Ca^{2+} signaling in EC tubes. Nevertheless, the absence of spontaneous Ca^{2+} signaling enabled GPCR activation to induce Ca^{2+} signaling and to evaluate how grading stimulus intensity (i.e., [ACh]) affected Ca^{2+} signaling within and between ECs. Exposure to 100 nM ACh evoked Ca^{2+} responses of lower frequency and amplitude when compared to 1 μM ACh (Figure 2, Figure 5B). Responses to ACh were abolished by depleting the ER of Ca^{2+} with the SERCA inhibitor thapsigargin, or following inhibition of the IP_3R with 2-APB or

Xestospongina C. Consistent with previous investigations (7, 27, 30, 33), these findings indicate that intra- and intercellular Ca^{2+} signaling events are mediated by IP_3 activation of Ca^{2+} release channels (IP_3R) of the ER within ECs.

Our working model is that in response to a sustained moderate concentration of GPCR agonist (100 nM ACh), production of low levels of IP_3 leads to stochastic opening of IP_3Rs and elementary Ca^{2+} release events (e.g., Ca^{2+} puffs). These spatially restricted release events occur at random within the endothelium and are asynchronous among individual ECs. In response to a sustained high concentration of a GPCR agonist (1 μM ACh), tonic production of high levels of IP_3 leads to opening of IP_3Rs and Ca^{2+} release that propagates as a Ca^{2+} wave. In turn, Ca^{2+} waves travel between ECs, thereby producing synchronized activity of adjacent ECs. These Ca^{2+} waves can propagate to all regions of the endothelium that are available to release Ca^{2+} . With multiple inputs to each EC, complex signaling patterns among ECs are subsequently created by Ca^{2+} wave annihilation and the inability of Ca^{2+} waves to propagate into regions where IP_3Rs are refractory or where the ER is depleted of Ca^{2+} .

The refractory period likely reflects a complex interplay between the levels of cytosolic IP_3 , cytosolic Ca^{2+} , and Ca^{2+} content within the ER. While we cannot rule out phasic IP_3 production during exposure to ACh, we propose that the refractory period under our experimental conditions is set primarily by the levels of cytosolic and ER Ca^{2+} . Thus with sustained exposure to 1 μM (e.g., vs. 100 nM) ACh, IP_3Rs are more likely to be uniformly bound by IP_3 . When released from an IP_3R , Ca^{2+} diffuses to a neighboring IP_3R , which will open and release Ca^{2+} until it inactivates/deactivates due to high cytosolic Ca^{2+} at the mouth of the channel (39, 58) or Ca^{2+} is depleted from within the ER. The IP_3R will then remain closed and refractory to release until Ca^{2+} is cleared from the cytosolic compartment (removing the inhibitory influence of high cytosolic Ca^{2+}) and re-sequestered back into the ER until a threshold level is restored, at which time IP_3Rs again become available to release Ca^{2+} . With these relationships described for other systems (42), defining such regulatory events in EC tubes is ripe for future studies.

Summary and Conclusions

The present findings illustrate that the endothelium of resistance arteries forms a dynamic Ca^{2+} signaling network with profound spatial and temporal variability within and between adjacent and remote ECs while remaining part of a syncytium that is functionally coupled through GJCs. Even though our analyses cannot yet quantitatively account for the complexity of Ca^{2+} signaling within EC networks, they provide new insight into the nature of coupling that allows coordination of Ca^{2+} responses between adjacent ECs in response to GPCR activation. These data illustrate the utility of the EC tube as a model system for studying EC-EC communication independent of other influences (e.g., blood or SMCs). Additionally, we demonstrate that there is a threshold level of EC stimulation required for Ca^{2+} signaling modalities to evolve from localized, discrete events to coordinated intercellular responses.

The dynamic nature of secondary responses to GPCR stimulation illustrates that Ca^{2+} signals in ECs of resistance vessels include signaling modalities that are likely to exert differential biological effects based not only on their amplitude, but on their frequency and subcellular localization. While the physiological role of the respective Ca^{2+} release events (Ca^{2+} waves versus spatially restricted Ca^{2+} release events) remain to be elucidated, accumulating evidence indicates that the frequency domain of spatially localized Ca^{2+} release events is critical to regulating Ca^{2+} -activated K^+ channel activity and vasomotor tone (33, 34, 53), particularly as electrical signals are rapidly integrated through intercellular coupling along the vessel wall. In contrast, more localized production of autacoids may rely

to a greater extent to intercellular Ca^{2+} waves (8, 52) and the influx of Ca^{2+} across the plasma membrane (31). Given the integral role of the endothelium in governing vascular resistance and tissue perfusion, resolving respective components of Ca^{2+} signaling provides a new foundation for investigating how these events are altered during endothelial dysfunction associated with aging and vascular disease.

PERSPECTIVE

The present findings illustrate that the endothelial cell layer forms a dynamic, interconnected Ca^{2+} signaling network with spatially and temporally distinct modes of Ca^{2+} signaling events. Discerning the physiological roles of respective signaling events and how Ca^{2+} release is altered during cardiovascular disease will reveal cellular targets to advance more selective therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ACh	acetylcholine
$[\text{Ca}^{2+}]_i$	intracellular free calcium concentration
$[\text{Ca}^{2+}]_o$	extracellular free calcium concentration
2-APB	2-aminoethoxydiphenyl borate
DMSO	dimethyl sulfoxide
EC	endothelial cell
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
GPCR	G protein coupled receptor
GJC	gap junction channel
HEPES	N-2-Hydroxyethyl piperazine-N'-2-ethanesulfonic acid
DMSO	dimethyl sulfoxide
IP_3	Inositol 1,4,5 trisphosphate
IP_3R	inositol 1,4,5 trisphosphate receptor
PSS	physiological salt solution
ROI	region of interest

SERCA	sarco-endoplasmic reticulum Ca^{2+} ATPase
SMC	smooth muscle cell

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Figure 1. Dye transfer illustrates functional coupling along endothelial cell tubes

A. Representative image of intercellular dye transfer in an EC tube confirming functional coupling among cells through GJCs. The central EC (*arrow*) was impaled with a microelectrode filled with 0.1% propidium iodide in 2 M KCl and dye transfer was recorded after 30 min. Image is representative of $n=3$. **B.** Differential Interference Contrast image of EC tube shown in **A**. Scale bars = 40 μm .

Figure 2. Complex nature of EC Ca²⁺ responses to ACh

A. Representative fluo-4 fluorescence (F/F₀) profiles of three individual ECs (*Black, Red, and Blue* traces) within an EC tube (shown in Figure 3) in response to 1 μM ACh. The ACh solution was introduced just prior to the onset of recordings and the response delay reflects the time required for the ACh to reach the EC tube. **B.** F/F₀ profiles from same response as in **A** with fluorescence obtained from all the cells within the field of view (ROI=128 × 96 μm, ~40 cells). When the fluo-4 fluorescence response is obtained from all cells the amplitude is reduced and oscillations within individual cells are dampened. **C.** As in **A** with 100 nM ACh. Note reduced amplitude and frequency of oscillations compared to **A**. **D.** F/F₀ profiles from response in **C** with fluorescence obtained from all cells within the field of view. Note loss of individual events with averaging. **E.** Summary data (means ± S.E.; n=15 cells from 5 EC tubes) showing frequency of Ca²⁺ responses per cell per min for 1 μM and 100 nM ACh. **F.** Amplitude (F/F₀) of Ca²⁺ responses. Summary data (means ± S.E.; n=15 cells from 5 EC tubes). * *P* < 0.05, 1 μM vs. 100 nM ACh.

Figure 3. Whole cell latency analysis of Ca^{2+} responses between Adjacent and Nonadjacent cells
A. Representative image of fluo-4 fluorescence showing an example of an EC triplet: Reference cell (black outline), Adjacent cell (blue outline) and Nonadjacent cell (red outline). Scale bar = 40 μm . **B.** Representative fluorescence (F/F_0) profiles from an EC triplet in response to superfusion with 1 μM ACh initiated just prior to recording onset from a stable baseline. *Vertical arrow*: onset of initial Ca^{2+} response; *horizontal bar*: secondary oscillations used for analyses. *Box*: Enlarged profile of secondary oscillations illustrating latency calculation: Latency = time between onset of F/F_0 increase in Reference EC and Adjacent (0.4 s) and Nonadjacent (1.7 s) ECs measured as intervals between respective vertical lines.

Figure 4. Coordinated Ca^{2+} responses between Adjacent ECs independent of extracellular Ca^{2+}

A. Scatter plot of latency times calculated using the whole cell analysis for 1 μM ACh-induced Ca^{2+} oscillations between Adjacent and Nonadjacent ECs in the presence of 2 mM $[\text{Ca}^{2+}]_o$. Individual data points indicate mean time between secondary Ca^{2+} oscillations for each triplet set of cells analyzed. **B.** Summary data (means $\pm$ S.E.; $n=28$ events from 5 EC tubes) illustrate shorter latency for Ca^{2+} oscillations between Adjacent vs. Nonadjacent cells. * $P < 0.05$ vs. Adjacent. **C.** As in panel **A** but in the absence of $[\text{Ca}^{2+}]_o$. Each EC tube was stimulated after 2 min superfusion with solution containing 0 Ca^{2+} and 1 mM EGTA. **D.** Summary data from paired experiments (means $\pm$ S.E.; $n=28$ events from 5 EC tubes). * $P < 0.05$ vs. Adjacent.

Figure 5. Pseudo line-scan ROI latency analysis of Ca^{2+} responses

A. *Top:* Representative image of fluo-4 loaded EC (red outline) showing *i*) an example of an ROI (orange rectangle; vertical width, $0.8\ \mu\text{m}$) positioned within the longitudinal axis of a single cell used to construct the pseudo line-scan. Fluorescence intensity within this ROI was acquired from each frame of the recording. *ii*) Resulting pseudo line-scan profile (X axis: distance along scan line, scale bar = $10\ \mu\text{m}$; Y-axis: time, scale bar = 5 sec) taken from cell in *i*). Two secondary analysis ROIs ($0.8\ \mu\text{m}$ wide, orange boxes) placed $5\ \mu\text{m}$ on each side of the center line of the pseudo line-scan image (i.e., $10\ \mu\text{m}$ apart) were used to classify Ca^{2+} release events based on their amplitude and spatial spread. *iii*) Same cell image from *i*)

illustrating that resulting F/F_0 profiles vs. time are equivalent between **ii** and **iii**; pseudo line-scan plots retain spatial information for event detection and classification. **B.** Frequency of intracellular Ca^{2+} waves in response to 100 nM and 1 μM ACh as detected in pseudo line-scan analysis. * $P < 0.05$, 1 μM vs. 100 nM. **C.** Frequency of spatially restricted Ca^{2+} events. There was no significant difference in response to 100 nM vs. 1 μM ACh. Summary data in **B** and **C** are means $\pm$ S.E.; n=15 from 5 EC tubes.

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Figure 6. Intercellular coordination of Ca^{2+} waves but not spatially restricted events
A. Scatter plot of latency times calculated using the pseudo line-scan analysis for Ca^{2+} waves between Adjacent and Nonadjacent ECs in response to $1 \mu\text{M}$ ACh. **B.** Summary data (means $\pm$ S.E.; $n=28$ events from 5 EC tubes) illustrate shorter latency for Ca^{2+} waves between Adjacent vs. Nonadjacent cells. * $P < 0.05$ vs. Adjacent. **C.** Scatter plot of latency times calculated using the pseudo line-scan analysis for $1 \mu\text{M}$ ACh-induced spatially restricted Ca^{2+} release events between Adjacent and Nonadjacent. Note different scales between A and C. **D.** Summary data (means $\pm$ S.E.; $n=18$ events from 5 EC tubes) illustrate no difference in latency for spatially restricted Ca^{2+} release events between Adjacent vs. Nonadjacent cells.