Functional Tuning of Intrinsic Endothelial Ca^{2+} Dynamics in Swine Coronary Arteries

Michael Francis, Joshua R. Waldrup, Xun Qian, Viktoriya Solodushko, John Meriwether, Mark S. Taylor

Rationale: Recent data from mesenteric and cerebral beds have revealed spatially restricted Ca^{2+} transients occurring along the vascular intima that control effector recruitment and vasodilation. Although Ca^{2+} is pivotal for coronary artery endothelial function, spatial and temporal regulation of functional Ca^{2+} signals in the coronary endothelium is poorly understood.

Objective: We aimed to determine whether a discrete spatial and temporal profile of Ca^{2+} dynamics underlies endothelium-dependent relaxation of swine coronary arteries.

Methods and Results: Using confocal imaging, custom automated image analysis, and myography, we show that the swine coronary artery endothelium generates discrete basal Ca^{2+} dynamics, including isolated transients and whole-cell propagating waves. These events are suppressed by depletion of internal stores or inhibition of inositol 1,4,5-trisphosphate receptors but not by inhibition of ryanodine receptors or removal of extracellular Ca^{2+}. In vessel rings, inhibition of specific Ca^{2+}-dependent endothelial effectors, namely, small and intermediate conductance K^{+} channels (K_{Ca3.1} and K_{Ca2.3}) and endothelial nitric oxide synthase, produces additive tone, which is blunted by internal store depletion or inositol 1,4,5-trisphosphate receptor blockade. Stimulation of endothelial inositol 1,4,5-trisphosphate-dependent signaling with substance P causes idiosyncratic changes in dynamic Ca^{2+} signal parameters (active sites, event frequency, amplitude, duration, and spatial spread). Overall, substance P-induced vasorelaxation corresponded poorly with whole-field endothelial Ca^{2+} measurements but corresponded precisely with the concentration-dependent change in Ca^{2+} dynamics (linearly translated composite of dynamic parameters).

Conclusions: Our findings show that endothelium-dependent control of swine coronary artery tone is determined by spatial and temporal titration of inherent endothelial Ca^{2+} dynamics that are not represented by tissue-level averaged Ca^{2+} changes. (Circ Res. 2016;118:1078-1090. DOI: 10.1161/CIRCRESAHA.115.308141.)

Key Words: calcium signaling ■ coronary vessels ■ myography ■ potassium channels ■ substance P

The endothelium is a crucial regulator of coronary blood flow, and endothelial dysfunction is a hallmark of vasospasm and coronary artery disease. Endothelial vasoregulation occurs through graded recruitment of various Ca^{2+}-dependent cellular effectors, including endothelial nitric oxide (NO) synthase (eNOS) and small/intermediate conductance Ca^{2+}-activated K^{+} (K_{Ca3.1} and K_{Ca2.3}) channels. eNOS and K_{Ca3.1}/2.3 channels respond to Ca^{2+}-mobilizing stimuli, such as laminar shear stress and local mediators (eg, acetylcholine, bradykinin, and substance P), eliciting NO production and endothelium-derived hyperpolarization (EDH), respectively. Both NO and EDH promote relaxation of underlying vascular smooth muscle causing vasodilation and are key controllers of blood pressure and flow, including real-time regulation of coronary perfusion. Arachidonic acid metabolites, such as prostacyclin and epoxyeicosatrienoic acids, as well as hydrogen peroxide, have also been implicated in endothelial responses to local stimuli and shear stress. The endothelium plays a particularly important role in the autoregulated coronary circulation by supporting metabolic dilatation and moderating myogenic constriction to help meet the blood flow demands of the heart. This crucial role is highlighted during atherosclerosis wherein the progressive inability of the endothelium to override arterial tone exacerbates myocardial ischemia. Multiple Ca^{2+}-dependent pathways control endothelial regulation of coronary artery function at the macrovascular and microvascular levels, but few studies have directly assessed the underlying endothelial Ca^{2+} signaling in these vessels, and none have addressed the spatial and temporal regulation of these signals. Hence, our understanding of the specificity and graded recruitment of endothelial influence remains limited.

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Recent studies suggest that local transient endothelial Ca\textsuperscript{2+} changes are sufficient to direct functional responses in intact blood vessels.\textsuperscript{17,24–28} In fact, evidence from mouse mesenteric,\textsuperscript{24,26} rat cerebral,\textsuperscript{27} and hamster skeletal muscle\textsuperscript{29} arteries indicates physiological endothelial signaling involves spatially and temporally restricted Ca\textsuperscript{2+} transients that are frequency tuned by the type and level of stimulation. In mouse mesenteric arteries, basal Ca\textsuperscript{2+} events emit repetitively from distinct sites along the endothelium, originating from distinct clusters of inositol 1,4,5-trisphosphate receptors (IP\textsubscript{3}Rs) on the endoplasmic reticulum (ER) membrane.\textsuperscript{24} These spatially restricted dynamics serve as a constant vasorelaxing impetus by activating nearby K\textsubscript{Ca}3.1 channels and eliciting EDH, particularly at myoendothelial projections where portions of endothelial cell membranes protrude through holes in the internal elastic lamina to form close associations or gap junction communication with smooth muscle cells.\textsuperscript{30,31} In addition, direct endothelial stimulation can increase local Ca\textsuperscript{2+} influx events through TRPV4 or TRPA1 channels (transient receptor potential channels, subfamily V, respectively) from distinct sites along the endothelium, originating from the endoplasmic reticulum (ER) membrane.\textsuperscript{24} This Ca\textsuperscript{2+} entry also targets endothelial K\textsubscript{Ca} channels (K\textsubscript{Ca}3.1 and K\textsubscript{Ca}2.3), and our recent findings suggest that this K\textsubscript{Ca} activation may further expand Ca\textsuperscript{2+} dynamics by promoting additional influx.\textsuperscript{28} Overall, it is implied that sensitive and adjustable scenarios for vasoregulation depend on highly dynamic endothelial Ca\textsuperscript{2+} signaling patterns. The role of such dynamic Ca\textsuperscript{2+} signaling in the coronary artery endothelium remains unknown.

Although focus has clearly shifted to elucidation of complex Ca\textsuperscript{2+} signals within the vasculature, discerning and quantifying complex dynamic Ca\textsuperscript{2+} signals in intact tissues is challenging. We recently developed and implemented a custom analysis module, LC\textsubscript{Pro},\textsuperscript{33} that allows for definitive tracking of a wide range of Ca\textsuperscript{2+} dynamics, including local transients and waves, within broad cellular fields while avoiding user error or bias. Here, we use this discriminating and comprehensive Ca\textsuperscript{2+} analysis approach to assess functional Ca\textsuperscript{2+} dynamics in the swine coronary artery (SCA) endothelium.

### Ca\textsuperscript{2+} Imaging and Analysis

Coronary artery segments were carefully opened longitudinally, pinned luminal-side-up on small silicone (Sylgard) blocks, loaded with the Ca\textsuperscript{2+} indicator (Fluo-4 AM), and placed in a HEPIES/physiological saline solution–containing chamber as previously described.\textsuperscript{28} Ca\textsuperscript{2+}-dependent fluorescence was measured with a spinning-disk laser confocal (8 frames per second). Fluorescence data were processed using the custom ImageJ plug-in, LC\textsubscript{Pro}, which is specifically designed to (1) detect and track sites of dynamic Ca\textsuperscript{2+} change above statistical noise (P<0.01), (2) define regions of interest (ROI; 5 µm in diameter) at active site centers, and (3) analyze average fluorescence intensities within ROIs. For assessments before and after drug addition, the brief period of bath change (≈2 s) was omitted from analysis to avoid movement artifact. Fluorescence data are expressed as F/F\textsubscript{0}, where F\textsubscript{0} is determined by a linear regression of base data at each ROI. Some preparations were coloaded with Fluo-4 AM and DAR-4M AM and imaged simultaneously to track both Ca\textsuperscript{2+}- and NO-dependent fluorescence.

### Immunofluorescence Imaging

Opened arteries pinned to Sylgard blocks were fixed, permeabilized, and stained as previously described.\textsuperscript{24} Data were obtained using a Nikon A1 confocal microscope (NIS-Elements and ImageJ software).

### Arterial Myography

Artery rings (=500 µm in diameter) were mounted in an isometric force myograph (Danish Myo Technology), stretched to optimal length, and allowed to equilibrate for 30 minutes before being exposed to indicated protocols. Artery viability was tested using 60 mmol/L KCl. For some experiments, 1 to 10 mmol/L of the thromboxane analog U44619 was included in the bath to establish stable tone (=2 mN). In other experiments assessing acute relaxations, vessels were precontracted with 10 to 100 mmol/L U44619. All drugs were added directly to the bath, and data were recorded using Chart software at 3 hz.

### Data Analysis

Data were expressed as mean±SEM. Group data were subjected to 1-way ANOVA, and individual comparisons were made by Tukey post-test. Parameter data were analyzed using ANOVA with linear mixed effects model. P values <0.05 were considered statistically significant. An expanded Materials and Methods section can be found in the Online Supplement.

### Results

**Localized Ca\textsuperscript{2+} Transients and Waves Occur Basally Along the SCA Endothelium**

We performed confocal imaging on opened branches of SCAs loaded with Fluo-4 to characterize basal endothelial Ca\textsuperscript{2+} dynamics. Survey of the intima layer under basal conditions (=120 endothelial cells per field assessed at 8.1 frames per second with no flow) revealed a variety of basal Ca\textsuperscript{2+} events, ranging from isolated transients (<5 µm\textsuperscript{2}) to broad cellular and multicellular waves (>30 µm\textsuperscript{2}; Figure 1). Events were detected in ≈35% of the sampled endothelial cells for a 10-minute period (Figure 1A). As depicted in Figure 1B, basal Ca\textsuperscript{2+} events were considerably variable in size and duration. Localized events tended to originate from 1 to 3 discrete sites per cell. In most cases, local signals triggered waves that propagated along the long axis of the cell, with the largest Ca\textsuperscript{2+} increase occurring at or near the nucleus. Cumulative assessment of basal Ca\textsuperscript{2+} in a single cell for 10 minutes shows discrete regional and focal heterogeneity of aggregate signal. Figure 2A shows a recording of the Ca\textsuperscript{2+} dynamics occurring within the endothelial field as depicted in Figure 1A. Notably, despite

### Methods

**Domestic male and female juvenile pigs were sedated with ketamine and xylazine and subsequently euthanized with sodium pentobarbital (58 mg/kg IV) followed by thoracotomy as approved by the University of South Alabama Institutional Animal Care and Use Committee. Branches of the left anterior descending artery were dissected from the right ventricle in cold HEPIES/bicarbonate-buffered physiological saline solution.**

### Nonstandard Abbreviations and Acronyms

- EDH: endothelium-derived hyperpolarization
- eNOS: endothelial nitric oxide synthase
- ER: endoplasmic reticulum
- IP\textsubscript{3}R: inositol 1,4,5-trisphosphate receptor
- LNNA: N\textsuperscript{-}nitro-L-arginine
- NO: nitric oxide
- ROI: region of interest
- SCA: swine coronary artery

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the disparate dynamics, the average Ca\textsuperscript{2+} signal within the whole field remained essentially constant over the full recording (Figure 2A, red line) because of the asynchronous nature of the signals. A summary of Ca\textsuperscript{2+} parameters obtained from automated analysis (LC_Pro) of 8 arteries from 8 different animals is shown in Figure 2B. On average, 10.2±1.6 events occurred at 8.9±0.6 distinct sites each minute per field. Individual event parameters (amplitude, duration, and spatial spread) all exhibited positively skewed distributions, with mean values of 1.5±0.02 (F/F\textsubscript{0}), 13.0±0.4 s, and 33.9±2.6 μm\textsuperscript{2}, respectively. Properties of basal Ca\textsuperscript{2+} dynamics are summarized in Online Table I.

**Basal Ca\textsuperscript{2+} Signals Result Primarily From Internal Store Release Through IP\textsubscript{3}Rs**

Next, we assessed the intracellular or extracellular source(s) of the basal endothelial Ca\textsuperscript{2+} events (Figure 3). Replacement of the chamber bath with Ca\textsuperscript{2+}-free physiological saline solution for 5 minutes had no significant effect on the occurrence of Ca\textsuperscript{2+} events, suggesting minimal contribution of extracellular Ca\textsuperscript{2+} influx (Figure 3A and 3B). However, depleting internal ER Ca\textsuperscript{2+} stores using the SERCA (sarcoendoplasmic reticulum Ca\textsuperscript{2+} ATPase) inhibitor cyclopiazonic acid (10 μmol/L for 15 minutes) suppressed basal events by >90%. Although blockade of ryanodine receptors with 10 μmol/L of ryanodine had no appreciable effect on the Ca\textsuperscript{2+} events, inhibition of IP\textsubscript{3}Rs with either 30 μmol/L of xestospongin C or 100 μmol/L of 2-aminoethoxydiphenylborane greatly attenuated their occurrence. Notably, similar blunting of Ca\textsuperscript{2+} events was observed after inhibition of phospholipase C with U73122. Immunostaining performed on open-vessel preparations revealed a distinctive distribution of IP\textsubscript{3}Rs around the nucleus and along the axis of endothelial cells (Figure 3C). Figure 3D shows an endothelial cell within an open-artery.
preparation that was probed for IP$_R$ after Ca$^{2+}$ imaging. Notably, the regional intensity of cytosolic Ca$^{2+}$ signal associated with a single wave corresponds to regional IP$_R$ density. Together, these data suggest that basal IP$_R$-induced Ca$^{2+}$ release from the ER is the predominant mechanism contributing to basal Ca$^{2+}$ dynamics in SCA endothelium.

**Basal Ca$^{2+}$ Signals Modulate Coronary Artery Tone Through Activation of eNOS, K$_{Ca}$.2,3, and K$_{Ca}$.3.1**

To assess whether the basal endothelial Ca$^{2+}$ signals along the endothelium provide an impetus for persistent modulation of coronary artery tone through activation of Ca$^{2+}$-dependent endothelial effectors, we performed isometric force measurements in isolated artery rings (Figure 4A and 4B). Although blockade of cyclooxygenase with indomethacin (10 µmol/L) or removal of hydrogen peroxide with polyethylene glycol catalase (500 U/mL) had no effect on tone (Online Figure I), inhibition of eNOS with N$\text{G}$-nitro-l-arginine (LNNA; 200 µmol/L) caused substantial contraction (16.7±1.7 mN) as did blockade of both K$_{Ca}$.2.3 and K$_{Ca}$.3.1 channels with apamin (0.5 µmol/L) and charybdotoxin (0.1 µmol/L) (3.4±0.6 and 5.1±0.7 mN, respectively). The effects of LNNA, apamin, and charybdotoxin were additive, resulting in a net increase in tone equivalent to that elicited by direct depolarization with 60 mmol/L KCl. Changing the order of application did not influence the individual effect of each drug, and the cumulative contractions could be effectively relaxed by treatment with the NO donor sodium nitroprusside (1 µmol/L), the KATP channel opener pinacidil (1 µmol/L), or the voltage-gated Ca$^{2+}$ channel blocker nifedipine (1 µmol/L; Online Figure II), indicating preservation of smooth muscle function, including direct NO- and hyperpolarization-dependent relaxation mechanisms. Importantly, we found that physical disruption of the endothelium caused an increase in resting tone (68%±6% of maximal KCl response; Online Figure III) and prevented responses to LNNA, apamin, and charybdotoxin. Although charybdotoxin can inhibit large conductance Ca$^{2+}$-activated K$^+$ (BK) channels, as well as K$_{Ca}$.3.1 channels, we found that the selective

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**Figure 2. Quantification of basal endothelial Ca$^{2+}$ signals.** A, Recording shows Ca$^{2+}$ signals autodetected during the 10-minute sample shown in Figure 1A (red line depicts average signal over the field). Insets show 3 disparate basal Ca$^{2+}$ events. B, Histograms summarizing basal Ca$^{2+}$ event parameters. Data were acquired from 8 animals.
BK channel blocker iberiotoxin (0.1 µmol/L) had no significant effect on either arterial tone or charybdotoxin-induced contractions (Online Figure IV). TRAM-34 was not used as a KCa3.1 channel inhibitor in this study because it was found to elicit relaxation of SCAs through an endothelium-independent mechanism. Immunostaining performed in open arteries revealed the expression of eNOS, KCa2.3, and KCa3.1 within the coronary artery endothelium (Figure 4C) with eNOS primarily distributing along the peripheral plasma membrane and near the nucleus in densities corresponding to the Golgi apparatus. KCa3.1 largely concentrated in focal plaques, and KCa2.3 was found primarily along the endothelial cell–endothelial cell borders. Tracking Ca²⁺ and NO-dependent fluorescence (DAR-4M AM) simultaneously revealed spatial and temporal correspondence of local Ca²⁺ elevations and NO production at both central and peripheral regions of the cell (Figure 4D).

To determine whether the persistent modulation of tone by eNOS or KCa channels is directly dependent on ongoing basal endothelial Ca²⁺ signals, we performed additional myography (Figure 5A and 5B). Although inhibition of ryanodine receptors (ryanodine, 10 µmol/L) had no significant effect on the contractions induced by apamin and charybdotoxin or LNNA, blockade of IP₃Rs (xestospongion C, 30 µmol/L, or 2-aminoethoxydiphenylborane, 100 µmol/L) or phospholipase C (U73122, 10 µmol/L) significantly reduced contractions elicited by all 3 inhibitors (P<0.05 versus control; n=6–7) compared with KCl. Overall, inhibition of IP₃-dependent signaling nearly abolished apamin/charybdotoxin-induced contractions and significantly attenuated LNNA-induced contractions. Ca²⁺-free solution could not be used for these experiments because it directly undermines smooth muscle contraction; however, inhibition of nonselective cation channels with Gd³⁺ (100 µmol/L) had little effect on apamin/charybdotoxin or LNNA-induced contractions, suggesting minimal influence of extracellular Ca²⁺ influx on KCa channel or eNOS modulation of tone under the conditions studied.

**Substance P Causes Idiosyncratic Expansion of Endothelial Ca²⁺ Dynamics**

Substance P is a potent endothelium-dependent dilator in the coronary circulation. It acts through Gq protein–coupled receptor stimulation, which leads to phospholipase

![Figure 3. Dependence of basal endothelial Ca²⁺ events on endoplasmic reticulum inositol 1,4,5-trisphosphate receptors (IP₃Rs).](image-url)
Stimulation with substance P (10−13 to 10−6 mol/L) increased the occurrence of discrete endothelial Ca2+ dynamics in a concentration-dependent manner. At low concentrations (≤10−10 mol/L), substance P primarily increased the number of Ca2+ events while having little effect on the properties of the events themselves (ie, amplitude, duration, and spread). This increase in events was mainly because of recruitment of new active sites (ie, events firing at distinct spots or cells that were not active before substance P exposure; Figure 6B, right). Importantly, evaluation of the whole endothelial field (Figure 6B, red line) rather than individual ROIs indicated little or no net Ca2+-dependent fluorescence change at 10−10 mol/L. Substance P although relaxations were measurable. At higher substance P concentrations (>10−10 mol/L), the number of Ca2+ events saturated, whereas the magnitude of individual events increased; this included expansion of amplitude, duration, and spread (Figure 7). At the highest substance P concentration tested (≈10−6 mol/L), essentially all active Ca2+ sites generated singular biphasic responses with extended plateaus (in excess of 100 s).

**Substance P Relaxation of Coronary Arteries Corresponds to Endothelial Ca2+ Elevation**

Figure 8A (left) shows specific substance P-mediated Ca2+ parameter changes (means from Figure 7) plotted as percent change from basal. This provides a comparable linear index of change for all the dynamic parameters (sites, events per site, amplitude, duration, and spatial spread). The whole-field Ca2+ change (ie, average fluorescence change over the entire sampled field rather than within discrete ROIs) is also plotted for the same data set. The right panel of Figure 8A shows the same data wherein the dynamic Ca2+ parameters are combined into a single curve based on the average percent change in parameters at each substance P concentration. Notably, the composite Ca2+ dynamics increased by 265% over the full...
substance P concentration range, whereas the whole-field $\text{Ca}^{2+}$ only increased by 33%.

To relate functional responses directly to measured endothelial $\text{Ca}^{2+}$, we plotted concentration-dependent substance P–induced vasorelaxation with the composite change in $\text{Ca}^{2+}$ dynamics and the relative change in the whole-field $\text{Ca}^{2+}$ signal, all as percent of maximal response (Figure 8B). Comparison of normalized nonlinear regression curves shows that concentration-dependent vasorelaxation and $\text{Ca}^{2+}$ dynamics superimpose ($\log EC_{50}=−11.13±0.06$ versus $−11.31±0.09$, respectively), whereas the whole-field $\text{Ca}^{2+}$ is significantly right shifted compared with both ($\log EC_{50}=−9.75±0.11$; $P<0.01$ versus relaxation and $\text{Ca}^{2+}$ dynamics). These data indicate that changes in endothelial $\text{Ca}^{2+}$ dynamics along the coronary artery intima correspond explicitly to concentration-dependent relaxation of coronary arteries to substance P and that this functional response is poorly predicted by changes in global endothelial $\text{Ca}^{2+}$ levels.

Discussion

In this study, we provide the first characterization of intrinsic endothelial $\text{Ca}^{2+}$ signals directing basal and stimulated vasorelaxation of SCAs. Our data reveal a spatially and temporally diverse assortment of $\text{Ca}^{2+}$ dynamics occurring along the coronary artery endothelium that are highly dependent on $\text{Ca}^{2+}$ release from the ER through IP$_3$Rs. Functional findings suggest that the inherent $\text{Ca}^{2+}$ signals exert a persistent vasorelaxing influence on coronary arteries through activation of eNOS and $\text{K}_{\text{Ca}}$ channels ($\text{K}_{\text{Ca}2.3}$ and $\text{K}_{\text{Ca}3.1}$). Moreover, graded Gq-protein–coupled receptors stimulation elicits expansion of existing endothelial $\text{Ca}^{2+}$ dynamics through frequency modulation and both spatial and temporal signal amplification. Importantly, this idiosyncratic $\text{Ca}^{2+}$ signal expansion corresponds explicitly to coronary artery vasorelaxation, whereas global $\text{Ca}^{2+}$ within the same endothelial field does not. Together, our findings expose a previously unappreciated dynamic $\text{Ca}^{2+}$ signaling framework within the coronary artery intima that may underlie definitive profiles of effector recruitment and vascular function.

The endothelium of the coronary circulation is absolutely crucial for the control of cardiac blood flow and distribution. Its vital role is underscored by the predictable progression from endothelial dysfunction to ischemic heart disease. Given the accepted functional importance of $\text{Ca}^{2+}$ in the coronary vasculature, the paucity of studies directly addressing $\text{Ca}^{2+}$ signaling in the coronary endothelium is surprising. Recent studies of cultured cells and coronary artery segments have begun to expose key elements of endothelial $\text{Ca}^{2+}$ control through direct $\text{Ca}^{2+}$ measurements.22,35–37 Our goal was to apply new approaches for vascular imaging and analysis to unravel spatial and temporal detail of $\text{Ca}^{2+}$ signaling in the coronary endothelium is surprising. Recent studies of cultured cells and coronary artery segments have begun to expose key elements of endothelial $\text{Ca}^{2+}$ control through direct $\text{Ca}^{2+}$ measurements.22,35–37 Our goal was to apply new approaches for vascular imaging and analysis to unravel spatial and temporal detail of $\text{Ca}^{2+}$ signaling in the coronary endothelium.38 Assessing vessel segments rather than dispersed cells provided a comprehensive look at endothelial signaling in the context of the intact tissue. A particularly novel aspect of the study was the use of the analysis module LC_Pro to capture the full spectrum of dynamic $\text{Ca}^{2+}$ activity along the arterial endothelium and provide definitive metrics of event location, frequency, and size. We previously applied similar high-content analysis to elucidate functional $\text{Ca}^{2+}$ signaling in rodent mesenteric and...
Here, we expose robust basal Ca\textsuperscript{2+} signals in the SCA endothelium encompassing a broad range of focal transients and cellular waves. Although we focused on moderate sized vessels (≈0.5 mm), we found similar event distributions in large (≈1 mm) and small (≈0.2 mm) arteries, with smaller arteries exhibiting a somewhat higher occurrence of events (Online Figure VI). Overall, our findings suggest that the initiation and relative spread of the Ca\textsuperscript{2+} events are highly dependent on IP\textsubscript{3}R release. In fact, it seems the ongoing Ca\textsuperscript{2+} activity is largely if not entirely supported by basal IP\textsubscript{3}, similar to previously described Ca\textsuperscript{2+} pulsar activity in mouse mesenteric arteries.\textsuperscript{24} Despite clear similarities to Ca\textsuperscript{2+} pulsars, basal SCA endothelial Ca\textsuperscript{2+} events are generally lower in frequency and more variable in size. Indeed, Ca\textsuperscript{2+} pulsars mainly focus around isolated basolateral IP\textsubscript{3}R clusters, whereas SCA endothelial events are more likely to propagate as waves. This may be because of the longitudinal arrangement of ER/IP\textsubscript{3}R in the SCA endothelium that promotes directional Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release\textsuperscript{39,40} along the cell axis. The breadth of Ca\textsuperscript{2+} events predicts a distinct profile of cellular targets in the cerebral arteries.\textsuperscript{27,28}
SCA. It is also notable that quite disparate Ca\(^{2+}\) events can occur at a single site within the SCA endothelium (Online Figure VII), suggesting that specific sites do not necessarily produce 1 type of event. Hence, even basal conditions may be sufficiently flexible to shape local signals (ie, relative IP\(_3\) gradients). Interestingly, the largest and longest lasting Ca\(^{2+}\) events in the coronary endothelium occur around or within the nucleus. The potential role of these nuclear Ca\(^{2+}\) surges in directing endothelial protein expression patterns or processes, such as apoptosis, warrants further studies.41

Findings during the past 2 decades indicate that NO and EDH account for the crucial Ca\(^{2+}\)-dependent endothelial regulation of coronary flow. NO production is well recognized as a ubiquitous mechanism of coronary vasodilation, both in epicardial arteries and in the microcirculation,42–44 and its loss portends endothelial dysfunction and heart disease. The pivotal effects of KCa2.3 and KCa3.1 channels in the coronary circulation have been established more recently9,10 and include acute EDH-mediated vasorelaxation, contribution to pressure-flow autoregulation,18 and compensatory vasodilation under conditions of reduced NO bioavailability, including obesity.6,35 Our current findings indicate that basal Ca\(^{2+}\) dynamics in SCA endothelium exert a persistent relaxing influence on tone through sustained recruitment of both eNOS and KCa channels, with NO production predominating (∼70% NO; ∼30% KCa). Because these functional endothelial influences occur in the absence of shear stress or endothelial agonist, we submit that the coronary circulation is hardwired for constitutive vasoregulation, regardless of overt stimulation. Notably, this implies that compensatory EDH signaling is not an alternative to NO but rather a built-in feature of the coronary endothelium. It is important to note that because basal endothelial Ca\(^{2+}\) dynamics are asynchronous and localized in nature, functional signaling can occur, whereas average Ca\(^{2+}\) over a broad intimal field remains essentially constant. The broad implication is that important background regulation occurs (ie, modulation of myogenic constriction) even as the endothelium remains globally stable and responsive to acute stimulus.

The basal NO influence in the SCA differs from the predominant KCa3.1 channel–driven EDH in mouse mesenteric artery, implying preferential targeting in different beds and species. Such scenarios are ultimately dependent on both the specific spatiotemporal Ca\(^{2+}\) signaling profile and the existing Ca\(^{2+}\)-dependent effector distribution. Post-transcriptional
lipid modification and association with trafficking proteins, such as caveolin and AKAP150, allow different effectors to localize to discrete microdomains (ie, eNOS and Kc_2.3 to Golgi or caveolae and Kc_3.1 to basolateral myoendothelial projections), thereby determining their accessibility to certain Ca^{2+} signals. The prevalent NO signaling in swine SCA endothelium may reflect targeting of both peripheral and Golgi-associated eNOS by local near-membrane events and nuclear-centric Ca^{2+} waves. Indeed, we show NO production at discrete central and peripheral sites corresponding to basal Ca^{2+} dynamics in SCA endothelial cells. Still, ~30% of the basal endothelial modulation of tone (mainly through NO) is independent of IP_3 signaling (Figure 5B), possibly reflecting the contribution of a separate Ca^{2+} source or eNOS activation by phosphorylation, independent of Ca^{2+}.

Previous studies have shown that direct stimulation of endothelial Gq-coupled receptors increases the frequency or spatial coverage of dynamic endothelial Ca^{2+} signals by phenylephrine-stimulated retractor muscle feed arteries through myoendothelial communication of IP_3. In this study, exposure of SCAs to the potent IP_3-elevating vasodilator substance P consistently promoted distinct bursts of Ca^{2+} dynamics along the endothelium. Although multiple Ca^{2+} sources may contribute, this behavior is consistent with IP_3-sensitized Ca^{2+} store release, similar to the clustered firing of Ca^{2+} transients in Xenopus oocytes on acute elevation of intracellular IP_3 concentration. Timing of these events is likely dependent on the density, latency, and refractory nature of the IP_3Rs themselves. Here, we show that the graded expansion of discrete endothelial Ca^{2+} signals by substance P corresponds explicitly to acute vasorelaxation. Specifically, we found that the net change in dynamic Ca^{2+} signal parameters, represented as a linearly translated composite of the event number (sites and frequency) and size (amplitude, duration and spread), superimposed on concentration-dependent vasorelaxation (n=5–6 for each concentration). This functional expansion of Ca^{2+} signaling involves both spatial and temporal components, most notably, recruitment of new Ca^{2+}-liberating sites at lower concentrations and increasing event duration at higher concentrations (Figure 8). Coincidentally, assessment of Ca^{2+} event area under curve, which takes into account both event amplitude and duration (F/F_0×s; measured from half maximum to event peak), showed changes similar to duration alone, suggesting that it is also not an independent predictor of low-concentration substance P vasorelaxation. This is likely due to the fact that modest stimulation elicits both large and small events, so average event size does not necessarily change. However, when we consider not only the size but also the number of events, the relationship between Ca^{2+} dynamics and function becomes highly predictive. The sensitive correspondence of new site recruitment to vasorelaxation is similar to that observed in rat cerebral arteries after direct endothelial stimulation and may represent a conserved mode of amplifying endothelial responses across multiple circulations.

It should be noted that at low levels of stimulation, subtle changes in local Ca^{2+} dynamics (ie, recruitment of new sites)
can easily be hidden within the broad sampled field, similar to basal signals. This is apparent in Figure 6B where new Ca\textsuperscript{2+} events evoked by modest substance P stimulation contribute minimally to the average field Ca\textsuperscript{2+} because of their spatial restriction and general asynchrony over the time course. Thus, not only is global endothelial Ca\textsuperscript{2+} change a poor index of vasorelaxation in the coronary circulation, it would seem to be particularly inadequate at assessing the subtle perturbations associated with physiological stimuli. Although it is currently difficult to explicitly link individual Ca\textsuperscript{2+} parameter changes to distinct shifts in effector recruitment profiles, assessment of low- and high-concentration substance P stimulation in this study suggests that subtle Ca\textsuperscript{2+}-signal expansion (mainly new sites) at low stimulation levels may preferentially target eNOS. It will be important to determine whether impairment of such sensitive Ca\textsuperscript{2+}–eNOS coupling underlies the early and specific loss of NO signaling with progressive coronary artery disease. It is worth noting that at higher levels of acute endothelial stimulation, the overall synchrony of dynamic events tends to increase, and this corresponds to the timing and peak of arterial vasorelaxation. This relative harmony of independent signals may offer another important level of physiological tuning and also warrants further studies. One limitation of this study was that evaluations of Ca\textsuperscript{2+} signaling and functional responses had to be performed in separate vascular preparations to provide adequate endothelial access for imaging. Although this restricts direct inferences between endothelial Ca\textsuperscript{2+} and arterial tone, studies were performed in parallel using the same animals to preserve continuity of measurements.

In addition to IP\textsubscript{3}Rs, stimulation of membrane TRP (transient receptor potential) channels can also increase dynamic endothelial Ca\textsuperscript{2+} signaling.\textsuperscript{36} These channels respond to various stimuli, such as stretch, shear, temperature, and second messengers,\textsuperscript{54} to increase focal Ca\textsuperscript{2+} entry. In particular, activation of TRPV4 channels in mesenteric artery endothelium, either directly or through acetylcholine stimulation, increases the occurrence of Ca\textsuperscript{2+} transients at the plasma membrane,\textsuperscript{74} and these signals can be augmented by K\textsubscript{Ca}\textsubscript{3.1}/2.3 channel positive feedback.\textsuperscript{29} In this study, we found neither significant influence of Ca\textsuperscript{2+} entry on basal Ca\textsuperscript{2+} dynamics nor any significant effect of TRPV4 blockade on substance P relaxation (Online Figure VIII). Although this indicates minimal involvement of TRPV4 channels under the conditions studied, recent studies have implicated these channels in shear stress responses,\textsuperscript{21,55} including NO-dependent, flow-mediated dilation of human coronary arteries.\textsuperscript{36} This could have important implications in the context of this study because stimulated Ca\textsuperscript{2+} influx would superimpose on existing intrinsic Ca\textsuperscript{2+} dynamics, not only increasing membrane delimited signals but also likely enhancing Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from ER IP\textsubscript{3}Rs. Elucidating the complex effects of differential shear stress profiles on endothelial Ca\textsuperscript{2+} dynamics is a high priority for future studies. The current work provides a functional signaling framework and definitive analysis approach for discerning these effects.

Although we found no hydrogen peroxide contribution to the basal endothelial modulation of coronary artery tone, such signaling is likely enhanced under pathological conditions. In patients with coronary artery disease, Ca\textsuperscript{2+}-dependent flow-mediated dilation switches from a predominant NO-mediated mechanism to one dominated by hydrogen peroxide.\textsuperscript{37} The cause of this pathological transition is unknown but likely involves a distinct shift in endothelial Ca\textsuperscript{2+} patterning to one favoring chronic mitochondrial or nicotinamide adenine dinucleotide phosphate oxidase production of reactive oxygen species.\textsuperscript{26,57} Future studies should identify whether a distinctive pattern switch underlies endothelial dysfunction in developing coronary disease. It should also be noted that the use of juvenile pigs in this study allowed us to limit variability because of age and sex. Because endothelial dysfunction and coronary artery disease manifest differently over the life span of men and women,\textsuperscript{16} future applications of the high-content analysis described here should be particularly useful for discerning sex- and age-related disparities.

In summary, the coronary artery endothelium is an active interface, critical to cardiac health. The prodilatory, anti-inflammatory, and antiproliferative influences of the endothelium are all inherently dependent on real-time control of diverse Ca\textsuperscript{2+} signals. This study provides evidence that coronary endothelial function is fundamentally encoded by a persistent mosaic of Ca\textsuperscript{2+} transients. This intrinsic profile of Ca\textsuperscript{2+} dynamics establishes an essential active framework for directing and tuning endothelial responses in the coronary circulation. Moving forward, it will be imperative that coronary endothelial Ca\textsuperscript{2+} signaling be resolved with adequate spatial and temporal detail to characterize stimulus-specific responses and define transitions from physiological to pathological signaling patterns.

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Disclosures

None.

References


Endothelial stimulation with substance P causes vasorelaxation of swine coronary arteries through graded spatial and temporal expansion of endothelial Ca²⁺ dynamics, an effect not adequately represented by tissue-level averaged Ca²⁺ changes.
Functional Tuning of Intrinsic Endothelial $\text{Ca}^{2+}$ Dynamics in Swine Coronary Arteries
Michael Francis, Joshua R. Waldrup, Xun Qian, Viktoriya Solodushko, John Meriwether and Mark S. Taylor

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SUPPLEMENTAL MATERIAL

Expanded Materials and Methods

Animals and tissue handling. In total, 78 animals were used in the study. Equal numbers of male and female domestic juvenile pigs (~15 kg) were acquired from the Auburn University Swine Research and Education Center. All procedures were conducted in accordance with PHS Policy on Humane Care and Use of Laboratory Animals and were approved by the University of South Alabama Institutional Animal Care and Use Committee. Following sedation with ketamine (16 mg/kg) and xylazine (7 mg/kg), animals were euthanized with a lethal dose of pentobarbital via the ear vein. The right ventricle was removed, and artery segments were dissected in cold (4°C) HEPES/bicarbonate-buffered (pH 7.45) physiological saline solution (PSS) containing (mmol/L): NaCl 130.0; NaHCO3 14.9; KCl 3.7; KH2PO4 1.2; MgSO4 1.2; CaCl2 1.6; Glucose 11.0; HEPES 10.0.

Arterial Myography. Artery rings (~500-700 μm diameter) were mounted on the pins of an isometric force myograph (Danish Myo Technology; DMT). Arteries were stretched to optimal length (determined previously via active length-tension relationships using 60 mmol/L KCl) in PSS at 37°C and allowed to equilibrate for 30 minutes. Artery viability was assessed with 60 mmol/L KCl challenge just before experiment; non-responsive vessels were discarded. A low concentration (1-10 nmol/L) of the thromboxane analog U44619 was included in the bath to standardize resting tone for all vessels at ~5% of maximal (60 mmol/L KCl). All drugs were added directly to the bath and data was recorded using Chart software at 3 hz. Notably, the alternative KCa3.1 inhibitor TRAM 34 was not included in these protocols as it caused acute endothelium-independent vasorelaxation of coronary arteries, possibly reflecting inhibition of smooth muscle cation channels.1

Confocal fluorescence Ca2+ imaging and analysis. Coronary artery segments (~500 μm diameter, 0.5 cm long) were opened longitudinally and pinned (10 μm tungsten micro-pins), luminal-side-up on a small silicone (Sylgard) block.2 Segments were stretched transversely to 1.5 times their original width, a change in circumference consistent with pressurization to 60-80 mmHg. Mounted vessels were loaded with Ca2+ indicator solution (containing PSS, 10 μmol/L Fluo-4 AM, 0.01% DMSO, 0.02 % Pluronic F-127) for 40 minutes at 25°C in the dark. For some experiments, DAR-4M AM (10 μmol/L) was included in loading solution. Blocks were washed and mounted inverted in a glass-bottom chamber, separated from the bottom by 100 μm diameter pins. The chamber was placed on the stage of a microscope equipped for high-speed, high-resolution imaging using a spinning-disk laser confocal microscope (PerkinElmer RS-3 Utraview); excited at 488 nm and emission filtered at 510 nm. Imaging was performed on interior regions of arterial preparations (within ~ 50 μm of central axis). Experiments were conducted at 30°C since gradual loss of fluorescent signal occurs over time at 37°C and no significant differences in Ca2+ event parameters were noted between 2-minute samples taken between 27°C and 37°C. Importantly, we have found that tracking only a handful of cells within a vascular bed gives an inadequate and often misleading impression of the inherent endothelial signaling pattern due to the heterogeneous nature of Ca2+ dynamics. Sampling spatial fields of ~150 endothelial cells (20X) provides a comprehensive valuation of Ca2+ activity as indicated by convergent parameter profiles. Images were captured at 8 frames/second and 512 x 512 pixels using Ultraview software. In simultaneous Fluo-4/DAR-4M AM (10 μmol/L) imaging experiments,
preparations were excited at 488 nm and 568 nm and emission monitored at 510 and 590 nm at 1 frame/second.

**Data acquisition and analysis with custom software LC pro.** A full description of LC_Pro development and implementation can be found in.\(^3,4\) Time lapse image sequences were obtained as 8bit grayscale .tiff stacks by confocal microscopy or rendering with Matlab. After threshold adjustment based on the standard score a background frame was rendered using the minimum intensity projection of the sequence. The background frame was then subtracted from each frame of the original image sequence. ImageJ algorithms were used to calculate the difference between the resulting image sequence and its time lapse mean intensity. This image sequence was then smoothed using a 4 pixel average function. The image sequence standard deviation was calculated, and used to generate a normalized sequence by the standard score. The ImageJ threshold function converted the image sequence to binary (black and white) using p<0.01 for a normal distribution. The ImageJ Particle Analyzer was used to assign best fit ellipses to the loci of each image sequence frame. Best fit ellipse loci were grouped into temporal “events” within a user defined spatial radius, and used to position regions of interest (ROIs) at the mean event position of each loci center.

Using a modified version Bob Daugherty’s Multimeasure plugin (ImageJ), the mean intensity time course of each ROI was measured from the original image sequence. The program approximated each ROI baseline with a horizontal line set to a weighted average intensity value (intensity values greater than the sequence mean were computed as mean). The measurements were then scaled to fold-change over baseline (\(F/F_0\)) and converted from frames to seconds with the user input frame rate. Peak event amplitudes were identified by computing global maxima between temporal event times identified by the LC_pro sorting algorithm. Maxima were checked for significance (p<0.05) using the population mean and standard deviation, and excluded if not significant. LC_pro computed t½ max values and used them to calculate the attack (1/2 max time to max), decay (1/2 max time after max), and duration. Right-side Riemann sums were used to calculate area under curve. The spatial spread is defined as the maximum best fit ellipse event area. Mean velocity vector magnitude (pixels/second) and direction (degrees) were calculated using center to center ellipse distances. For whole-field assessments, single rectangular ROIs were positioned over the captured field and average fluorescence determined relative to initial fluorescence (\(F_0\)). Potential “fluorescence-dead” area due to poor dye loading or displacement of sampled regions outside the focal plane was calculated to be 1.3 - 2.5% for the preparations employed; determined by minimal \(\Delta F\) with \(\text{Ca}^{2+}\) ionophore A23187 (1 µmol/L) or maximal stimulation with substance P (1 µmol/L).

**Immunofluorescence.** Artery segments were opened longitudinally and pinned before fixing with 4 % formaldehyde for 15 minutes. Arteries were permeabilized with 0.5 % Triton X, blocked with 1 % bovine serum albumin and exposed to primary antibodies (\(\text{KCa}_{3.1}\); \(\text{KCa}_{2.3}\), Alomone; eNOS and Golgi, Abcam; \(\text{IP}_3\)R, Chemicon) overnight at 4°C. Arteries were subsequently treated with fluorescent secondary goat anti-rabbit antibodies (Alexafluor 488, 568; Molecular Probes) and nuclear counterstain (Hoechst; Molecular Probes). Z-sections were acquired at 0.25 or 0.5 µm increments. Any signal from secondary antibody alone was used to adjust background in primary-exposed groups. All primary antibodies were screened by western analysis as previously described.\(^2,5\) Data was obtained using a Nikon A1 confocal microscope (20X or 60X plan apo objectives; excitation 405, 488, and 568 nm) and compiled/processed with Nikon Elements and ImageJ software. Data are presented as composite stacks of 5 to 20 images.
Reagents and solutions. Unless otherwise noted, substances and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-4 AM and Pluronic F-127 were purchased from Invitrogen (Carlsbad, CA) and DAR-4M AM from Santa Cruz Biotechnology (Dallas, TX). Tungsten wires (for making micro-pins) were purchased from Scientific Instrument Services (Ringoes, NJ).

References


Online Movie I. Representative image sequence showing dynamic Ca\textsuperscript{2+} activity in swine coronary artery endothelium before and after addition of 10\textsuperscript{-11} mol/L substance P. Video is displayed at 7X real-time. Substance P is added at the 25 second mark.

Online Movie II. Representative image sequence showing dynamic Ca\textsuperscript{2+} activity in swine coronary artery endothelium before and after addition of 10\textsuperscript{-9} mol/L substance P. Video is displayed at 7X real-time. Substance P is added at the 25 second mark.

Online Movie III. Representative image sequence showing dynamic Ca\textsuperscript{2+} activity in swine coronary artery endothelium before and after addition of 10\textsuperscript{-6} mol/L substance P. Video is displayed at 7X real-time. Substance P is added at the 25 second mark.
Online Table I. Properties of basal Ca\(^{2+}\) events occurring in SCA endothelial cells (3.6×10\(^4\) \(\mu\)m\(^2\) field). Data are means ± SEM from eight animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field frequency (Events/min)</td>
<td>10.2 ± 1.6</td>
</tr>
<tr>
<td>Distinct sites (#/min)</td>
<td>8.9 ± 0.6</td>
</tr>
<tr>
<td>Amplitude (F/F(_0))</td>
<td>1.5 ± 0.02</td>
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<tr>
<td>Duration (s)</td>
<td>13.0 ± 0.4</td>
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<tr>
<td>Attack (s)</td>
<td>5.4 ± 0.2</td>
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<tr>
<td>Decay (s)</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>Area under curve (F/F(_0)*s)</td>
<td>304.0 ± 23.8</td>
</tr>
<tr>
<td>Spread ((\mu)m(^2))</td>
<td>33.9 ± 2.6</td>
</tr>
</tbody>
</table>
Online Figure I. Coronary artery contraction in response to eNOS inhibition but not to cycloxygenase inhibition or H$_2$O$_2$ removal. Representative myograph recordings from three artery rings from the same animal show no substantial effect of indomethacin (Indo; 10 µmol/L) or PEG catalase (500 U/ml) on tone whereas LNNA (200 µmol/L) elicited substantial contraction.
Online Figure II. Coronary artery tone in response to combined inhibition of endothelial K\textsubscript{Ca} channels and nitric oxide can be reversed by directly inhibiting smooth muscle voltage-gated Ca\textsuperscript{2+} channels (VGCCs), activating smooth muscle K\textsuperscript{+} (ATP-sensitive K\textsubscript{ATP}) channels, or providing exogenous nitric oxide (NO). The combined contraction to apamin (Apa; 0.5 µmol/L), charybdotoxin (Chtx; 0.1 µmol/L) or nitro-L-arginine (LNNA; 200 µmol/L) was relaxed by treatment with the L-type VGCC inhibitor nifedipine (NIF; 1 µmol/L), the K\textsubscript{ATP} channel opener pinacidil (PINA; 1 µmol/L) or the NO donor sodium nitroprusside (SNP; 1 µmol/L).
Online Figure III. Effect of endothelial removal on swine coronary artery rings. Representative myograph recordings show responses in two artery rings from the same animal, one intact and the other subjected to physical endothelial denudation. Following endothelial denudation, resting tone is unstable and exhibits a net increase within ~10 minutes. After contracting the rings with thromboxane analog U46619 (10 nmol/L), endothelial denudation is confirmed by lack of response to bradykinin (BK; 1 µmol/L); KCl is 60 mmol/L and w indicates wash with PSS.
Online Figure IV. Charybdotoxin-induced contraction of swine coronary arteries is not altered by pre-block of large-conductance Ca$^{2+}$-activated (KCa1.1) channels. Myograph recordings show charybdotoxin (Chtx; 0.1 µmol/L) contractions in the absence or presence of iberiotoxin (Ibtx; 0.1 µmol/L). Iberiotoxin treatment neither mimicked nor prevented charybdotoxin contraction.
Online Figure V. Contributions of eNOS, K<sub>Ca</sub>3.1 and K<sub>Ca</sub>2.3 to SCA relaxations evoked by high or low concentrations of substance P. Bars show relative block of 0.03 nmol/L and 30 nmol/L substance P-induced relaxation by pretreatment with LNNA (L), charybdotoxin and apamin (CA), or the combination of all three. * indicates p<0.001 vs control.
Online Figure VI. Comparison of dynamic endothelial Ca²⁺ signal parameters in large and small swine coronary arteries. Panels show mean value distributions for the indicated parameters recorded from 0.8-1 mm diameter (Large) or 0.2-0.4 mm diameter (Small) coronary artery segments under unstimulated (basal) conditions. * indicates p<0.05 compared to Large.
Online Figure VII. Properties of Ca\textsuperscript{2+} dynamics occurring at repetitively discharging sites within the SCA endothelium. Composite image of confocal SCA endothelial Ca\textsuperscript{2+} (Fluo4-AM fluorescence) shows distinct regions of interest (ROIs; red circles) where more than one event occurred over the 10-minute measurement. Panels depict specific parameters of events occurring at the individual ROIs.
Online Figure VIII. Effect of TRPV4 inhibition on substance P-induced SCA relaxation. Myograph recordings show 3 nmol/L substance P relaxations in U46619-contracted swine coronary arteries (from the same animal) treated with the TRPV4 inhibitor HC-067047 (0.5 μmol/L) or vehicle (0.01% DMSO). Graph shows summary data from three experiments.