Decoding dynamic Ca\(^{2+}\) signaling in the vascular endothelium

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Although acute and chronic vasoregulation is inherently driven by endothelial Ca\(^{2+}\), control and targeting of Ca\(^{2+}\)-dependent signals are poorly understood. Recent studies have revealed localized and dynamic endothelial Ca\(^{2+}\) events comprising an intricate signaling network along the vascular intima. Discrete Ca\(^{2+}\) transients emerging from both internal stores and plasmalemmal cation channels couple to specific membrane K\(^{+}\) channels, promoting endothelial hyperpolarization and vasodilation. The spatiotemporal tuning of these signals, rather than global Ca\(^{2+}\) elevation, appear to direct endothelial functions under physiologic conditions. In fact, altered patterns of dynamic Ca\(^{2+}\) signaling may underlie essential endothelial dysfunction in a variety of cardiovascular diseases. Advances in imaging approaches and analyses in recent years have allowed for detailed detection, quantification, and evaluation of Ca\(^{2+}\) dynamics in intact endothelium. Here, we discuss recent insights into these signals, including their sources of origination and their functional encoding. We also address key aspects of data acquisition and interpretation, including broad applications of automated high-content analysis.

Keywords: Calcium dynamics, endothelium, spatiotemporal signaling, acquisition and analysis, vasodilation
**ENDOTHELIAL Ca2+ DYNAMICS**

Over the past few decades, most Ca2+ measurements have involved assessments of whole-field epifluorescence at rates slower than 1 Hz, and often at supra-physiological levels of cell/tissue stimulation. While this approach is useful for tracking global trends in Ca2+ over protracted time scales and evokes acute fluorescence signals large enough to quantify unequivocally, it does little to elucidate the spatial and temporal detail of Ca2+ dynamics. Insights into physiologic Ca2+ signaling have come largely from the use of single-excitation fluorescent Ca2+ probes in high-speed confocal imaging applications, particularly within intact tissue preparations. Evaluations of isolated arterial segments have revealed a plethora of spatially and temporally discrete intact tissue preparations. Evaluations of isolated arterial segments have revealed a plethora of spatially and temporally discrete Ca2+ signals. In arterial smooth muscle, spontaneous localized Ca2+ transients (e.g., Ca2+ sparks and sparklets) as well as asynchronous and synchronous Ca2+ waves control vascular tone through coordination of cellular activation and feedback regulation of constriction (Nelson et al., 1995; Santana et al., 2008; Mufti et al., 2010). Hints of similar dynamic signals in the endothelium were observed as Ca2+ waves coursing through isolated cells (Neylon and Irvine, 1990; Isshiki et al., 2004). Unfortunately, detailed imaging of endothelial Ca2+ activity in situ has been quite challenging because of the general inaccessibility of the vascular intima (i.e., on the internal surface and only one-cell thick). Various strategies have been employed for vascular endothelial imaging including intravital microscopy (Bagher et al., 2011), myograph-mounted arterial segments (Schuster et al., 2001), exposed endothelial tubes (Socha et al., 2012), and pinned-open artery segments (Marie and Bény, 2002). Open-artery preparations have proven quite useful. This involves cutting artery segments open longitudinally and pinning them to silicone blocks, thereby making the endothelial layer accessible to rapid indicator loading and en face confocal imaging (see Ledoux et al., 2008). In such preparations, many cells (~200 with 20X objective) can be evaluated in a single plane while preserving the native environment, including lamina attachments and cell–cell communication. Cell-permeant, single-excitation fluorescent dyes like Fluo-4 AM have enabled rapid scanning. Implementation of the transgenic GCaMP2 mouse model has also proven beneficial by providing an endothelial-expressed Ca2+-dependent fluorophore that avoids spill-over smooth muscle fluorescence and improves signal detection and quantification (Kotlikoff, 2007; Ledoux et al., 2008).

Basal endothelial Ca2+ events were first characterized in mouse mesenteric arteries (Ledoux et al., 2008). Termined Ca2+ pulsars, these events resemble muscle cell Ca2+ sparks although somewhat broader in spatial range and duration. Unlike Ca2+ sparks that emit from ryanodine receptors (RyR), Ca2+ pulsars release intermittently from the endoplasmic reticulum through clusters of inositol 1,4,5-trisphosphate receptors (IP3Rs). Pulsars are similar to Ca2+ puffs, localized Ca2+ events previously described in Xenopus oocytes (Parker et al., 1996). Liberated from distinct IP3R clusters, Ca2+ puffs increase in frequency with increasing IP3, ultimately expanding into cell-wide waves. This transition to propagating waves occurs through IP3 sensitization of neighboring IP3R clusters, leading to a chain reaction of Ca2+-induced Ca2+ release (Fossett et al., 2007). Ca2+ pulsars occur basally in mesenteric arteries under resting conditions (at 37°C and no flow), and these ongoing events are blocked by inhibiting phospholipase C (Ledoux et al., 2008), the enzyme that produces IP3. Stimulation of the mesenteric artery endothelium with acetylcholine (ACh) increases the number of Ca2+-emitting sites along the intima and augments the frequency of events occurring at previously active sites. Thus, Ca2+ pulsars can be tuned acutely by Gq-protein-coupled receptor (GqPCR) stimulation.

Ca2+ pulsar events occur predominantly around the nucleus and at distinct myoendothelial junction (MEJ) sites where endothelial cell projections form close contacts (and often heterocellular gap junctions) with smooth muscle cells through holes in the internal elastic lamina (Sandow et al., 2002, 2009; Ledoux et al., 2008). These sites correspond with densities of IP3Rs. The primary functional target of pulsars appears to be KCa3 channels, particularly KCa3.1 channels that are highly concentrated in the plasma membrane of myoendothelial projections. Importantly, this ongoing Ca2+-effecter coupling exerts a persistent EDH influence (Ledoux et al., 2008) capable of relaxing underlying vascular smooth muscle and modulating arterial tone. The vascular smooth muscle may itself directly influence endothelial Ca2+ signals (Yashiro and Duling, 2000). In particular, smooth muscle IP3 generated by GqPCR stimulation (i.e., via sympathetic activity and circulating hormones) may be communicated across MEJs, augmenting endothelial Ca2+ dynamics (Lamboley et al., 2005). Indeed, addition of the α1-adrenergic receptor agonist phenylephrine increases endothelial Ca2+ events in mesenteric arteries (Kansui et al., 2008) and recruits new axially propagating Ca2+ wavelets in previously inactive endothelial cells of skeletal muscle feed arteries (Tran et al., 2012). This communication may allow endothelial influences to be adjusted relative to vasoconstrictor stimulation, providing real-time feedback regulation of vascular tone.

Additional players have recently been implicated in intrinsic endothelial Ca2+ signals, namely the transient receptor potential (TRP) non-selective cation channels (Di and Malik, 2010). In particular, certain vanilloid family channels (TRPV4) have been found to produce localized Ca2+ transients along the plasma membranes of mouse mesenteric artery endothelium (Sonkusare et al., 2012). Likely obscured by broader pulsar events, these small, membrane-delimited Ca2+ sparklets can be unmasked by depletion of internal stores and treatment with the TRPV4-stimulating compound GSK1016790A. Like Ca2+ pulsars, the TRPV4 sparklets couple to nearby KCa channels. Notably, when ER Ca2+ stores are not depleted, TRPV4 stimulation causes widespread whole-cell Ca2+ dynamics. Similarly, in the endothelium of rat cerebral arteries, activation of ankyrin-associated TRPA1 channels causes recruitment of discrete Ca2+ events that spread as propagating waves (Qian et al., 2013). Together, these findings suggest membrane-delimited TRP channel events may solicit broader internal Ca2+ store release events. Indeed, the interplay between external and internal Ca2+ sources may contribute to a wide spectrum of conditional Ca2+ dynamics and effector recruitment profiles.
**IDIOSYNCRATIC Ca^{2+}-EFFECtor COUPLING AND FUNCTIONAL ENCODING OF Ca^{2+} DYNAMICS**

Fundamental endothelial Ca^{2+} signals (pulsars and sparklets) primarily target KCa3.1 channels concentrated in densities along the endothelial basolateral membrane and myoendothelial junctions. However, related KCa2.3 channels are distributed quite differently, residing primarily along endothelial cell–cell borders, associated with the plasma membrane protein caveolin (Sandow et al., 2006; Absi et al., 2007). Notably, certain TRP channels (e.g., TRPV4 and TRPA1) distribute preferentially with KCa3.1 or KCa2.3 channels (Earley et al., 2009; Ma et al., 2013), perhaps due to conditional TRP association with caveolin (Rath et al., 2009). Overall, this suggests differential Ca^{2+} signal targeting of KCa isoforms. Recent findings suggest that in addition to direct EDH signaling, KCa/TRP coupling may directly influence the endothelial Ca^{2+} signals themselves. Specifically, Ca^{2+}-dependent hyperpolarization may increase the driving force for further Ca^{2+} influx through TRP channels, allowing positive feedback augmentation of the original Ca^{2+} signal. In support of this scenario, ACh-induced endothelial Ca^{2+} dynamics are substantially higher in normal mesenteric arteries compared to those from mice lacking KCa3.1 and KCa2.3 channels, and this Ca^{2+}-facilitating influence of KCa channels is blocked by inhibition of TRPV4 activity (Qian et al., 2014). Taken together, these findings imply that the specific arrangement of specific ion channels within endothelial cells is a key determinant of the prevailing Ca^{2+} signals and effector recruitment profiles.

Endothelial NOS resides in two functional pools, one associated with caveolin in the cell periphery, and the other in the membrane of the Golgi apparatus (Liu et al., 1997; Andries et al., 1998; Rath et al., 2009). The provisional association of KCa2.3, TRPV4, and eNOS with caveolin suggests possible interaction. Indeed, SK3 overexpression increases the NO contribution to ACh-induced vasodilation (Brähler et al., 2009), and relaxation of rat pulmonary arteries via TRPV4 activation is linked to both NO and KCa channel activity (Sukumaran et al., 2013). Whether such scenarios involve targeting of plasma membrane eNOS, by KCa2.3-enhanced TRPV4 Ca^{2+} signals, remains to be determined.

Expansion or redirection of inherent Ca^{2+} signals is crucial to endothelial function. Stimuli including GPCR agonists and TRP channel activators increase the occurrence of endothelial events, including recruitment of new active sites and increased firing frequency (Ledoux et al., 2008; Qian et al., 2013), and both effects are linked to proportional arterial dilation. The overarching implication is that endothelial vasoregulation is encoded by both binary and analog Ca^{2+} signaling modes. That is to say, discrete sites are either on or off (binary), and once on, the attributes of the events are tunable over some range (analog). In addition to frequency, analog signaling components include magnitude, duration, and spatial spread of Ca^{2+} events, all of which could affect the type and extent of effector recruitment. Ultimately, definitive tracking of discrete Ca^{2+} signaling patterns will be needed to reveal the nature and capacity of parameter expansion and decipher the idiosyncrasies of endothelial function and dysfunction.

**QUANTIFYING AND PROFILING ENDOTHELIAL Ca^{2+} DYNAMICS**

Given the inherent complexity of endothelial Ca^{2+} signals, a key challenge moving forward will lie in our ability to adequately and comprehensively characterize Ca^{2+} activity along the intact intima. Disparate approaches have been employed to measure and analyze Ca^{2+} data, often applying arbitrary, if any, selection criteria and providing little explication of spatial and temporal parameters. Regardless of experimental preparation and approach, some crucial criteria for acquisition and analysis should be considered. First, spatial or temporal under-sampling of Ca^{2+} fluorescent signals washes out discrete dynamics or misses them altogether. High-speed confocal imaging systems, particularly spinning disk platforms with high-quantum efficiency cameras, offer sub-micron spatial resolution with fast acquisition rates and high signal to noise ratios. Analysis of dynamic Ca^{2+} activity typically involves selection of an event within an image time-series and manual placement of a region of interest (ROI, often a small box or circle) around each event site for measurement of average fluorescence. Albeit straightforward, this approach is tedious, time-consuming and prone to user-bias and error. Recent efforts have produced automated detection and analysis algorithms to extract signals from continuous image sequences. Evidence suggests that in addition to saving time and resources, automated analyses can avoid inconsistencies of manual analysis and identify signaling signatures within complex fluorescence data. Multiple software applications, particularly in neurobiology, have employed independent component analysis and watershed image segmentation to define individuals within dense fields and to track region-specific deflections of Ca^{2+}-dependent fluorescence (Mukamel et al., 2009; Wong et al., 2010; Watters et al., 2014). Separate automated analysis software has been applied to discern Ca^{2+} spiking and oscillation patterns in various cell types, including plant epidermal cells (Russo et al., 2013), cardiac myocytes (Janicek et al., 2013), and T-cells (Salles et al., 2013). The algorithm LC_Pro was recently developed to track the diverse Ca^{2+} events in the vascular endothelium (Francis et al., 2012, 2014). Incorporated as a plug-in with ImageJ freeware, this statistically rigorous program distinguishes dynamic fluorescence signals from background noise, and follows the spatial profile of each Ca^{2+} event with time. It automatically assigns ROIs to event spatial centers and returns output quantifying relevant field and event parameters (e.g., sites, events, amplitude, duration, and spatial spread). The algorithm also allows “batch” analysis of multiple parallel data sets. Such analysis is particularly useful because it can generate complete parameter distributions and provide practical quantification of replicate data sets or complex signal changes following perturbation (i.e., endothelial stimulation). For instance, relative changes in binary (i.e., sites) and analog signals (i.e., amplitude, duration, spread, single-site frequency) can be automatically calculated, plotted, and statistically evaluated in a series of experiments without intermediate data processing by the user. Regardless of specific approach, stringent analysis of large data sets will be a necessary step in decoding Ca^{2+} dynamics.

Overall, automated analysis approaches have become useful for defining cell boarders, discerning cellular/subcellular fluorescence signals from statistical noise, and providing comprehensive quantification of component signal parameters. Current limitations of such approaches primarily stem from narrowly targeted applications and disparate processing algorithms that can contribute to false-negatives or false-positives when data fall outside an optimal range. As discussed below, extended initiatives should
promote more widely applicable tools capable of reducing complex and heterogeneous data sets to intuitive indices of functional signaling.

**FUTURE DIRECTIONS AND CHALLENGES**

Looking forward, careful consideration should be given to the limits and liabilities of experimental approaches. While high-speed confocal imaging is valuable for signal resolution, it imposes certain experimental restrictions, including thin-plane sampling (∼1 µm) and low tolerance for tissue movement. Wire-mounted or pressurized arteries can be studied as intact segments, but endothelial exposure is very limited. On the other hand, open vessel preparations expose vast endothelial fields in a single plane but sacrifice tubular structure and functional assessment. Imposing rigid acquisition criteria may be impractical. Rather, strategies should be implemented and optimized to ensure adequate spatial and temporal resolution and prevent image artifact. Importantly, Nyquist sampling criteria should be satisfied (i.e., sampling time and spatial intervals \(\leq\frac{1}{2}\) of smallest signal duration and size) to ensure reliable signal quantification without signal aliasing or distortion. Automated detection software such as LC_Pro may be useful for optimization by identifying which spatial and temporal acquisition conditions achieve convergence of parameter values while avoiding oversampling. Stack registration software can also be employed as a data processing step to correct for spatial drift (x-y movement) (Thévenaz et al., 1998).

Fast piezo focus for rapid z-axis stacking is very useful not only for acquiring depth information within the sample but also for compensating for z-axis drift. It should be noted that inclusion of z-stacks as well as increased exposure times and pixel-binning can all improve certain aspects of image quality but may lead to a loss of overall spatiotemporal resolution, and should be employed with caution.

While ROIs are convenient for assessing spatially discrete \(\text{Ca}^{2+}\) dynamics, these fixed sampling windows can be problematic when tracking widely disparate signals. For instance, a focal event occupying only a small fraction of an ROI will yield a very small average fluorescence change (amplitude) compared to a broad wave passing through the same ROI, even if both have the same absolute signal intensity. Also, a single fixed ROI may detect spill-over signal from nearby events over time, distorting quantification of site-specific activity. In addition, fixed ROI sampling can promote artifact due to x-y drift by allowing hot spots or even regions of high or low background fluorescence to move into and out of the measured region (i.e., box) over time. Such issues may be resolved by tracking each event individually in space and time, allowing a signal to define its own transient polygonal ROI without establishing a permanent sampling window. Finally, dynamic \(\text{Ca}^{2+}\) events are often represented as ratios of relative fluorescence change within an ROI (i.e., F/F₀, where F₀ is a user-defined base fluorescence value). Defining appropriate base values can be challenging, particularly when photobleaching causes signal drift or high dynamic activity obscures the background. In addition to background correction algorithms, linear regression of time-course data can be applied for F₀ designations (Francis et al., 2012). Caution is warranted when expressing data as ratios since very low or very high base values can dramatically inflate or deflate F/F₀ values.

**PERPECTIVES**

Because \(\text{Ca}^{2+}\) dynamics are complex, data are typically represented by a profile of parameters or parameter distributions rather than a single scalar value. This multidimensional description has the capacity to distinguish \(\text{Ca}^{2+}\) signaling modalities, such as responses to distinct stimuli or among different vascular beds. Notably, because perturbations can increase some parameters and decrease others, quantification, comparison, and interpretation of data can be quite complex. Future analysis and meta-analysis approaches will need to address this complexity, perhaps by tracking trends in global distribution profiles or by defining cumulative metrics that combine parameters into standard indices. Additional indices might also include site distribution, cell heterogeneity, and event synchrony. A growing number of analysis algorithms are available as open source packages and plug-ins, making them not only widely accessible but amenable to customization. The hope is that eventually, a suite of analysis modules could be employed universally for parameter compilation, data mining, and pattern recognition. This would allow a standard analysis scheme for comparison of data sets across labs and preparations. Still, the onus ultimately falls on investigators to extract data or composite parameters germane to their specific experimental questions.

**CONCLUSIONS**

New insights suggest the endothelium functions as a continuum of dynamically regulated influences that are always engaged and are constantly adjusted. The prevailing \(\text{Ca}^{2+}\) signaling modalities and effector distributions likely underlie the distinct functions of different circulations. Further dissection of this diverse activity will allow for identification of sub-modalities, and potentially distinct cell phenotypes within the intima. We submit that shifts in prevailing \(\text{Ca}^{2+}\) dynamics necessarily impact blood pressure and flow and may predict disease. Indeed, endothelial dysfunction is an overarching feature of cardiovascular pathology. It is therefore particularly imperative that future studies shift away from assumptions based on global \(\text{Ca}^{2+}\) changes and broad cellular protein concentrations and focus on spatially and temporally relevant aspects of real-time signaling. Ultimately, the development of a definitive and predictive model of endothelial function should allow for elucidation of specific control points and therapeutic targets.

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