



Decoding dynamic Ca²⁺ signaling in the vascular endothelium

Mark S. Taylor^{1*} and Michael Francis²

¹ Department of Physiology, University of South Alabama College of Medicine, Mobile, AL, USA

² Department of Pharmacology, University of South Alabama College of Medicine, Mobile, AL, USA

Edited by:

Raymond Penn, University of Maryland Baltimore, USA

Reviewed by:

Zsolt Bagi, Georgia Regents University, USA

Thomas C. Resta, University of New Mexico Health Sciences Center, USA

*Correspondence:

Mark S. Taylor, Department of Physiology, University of South Alabama College of Medicine, 5851 USA Dr. N, MSB3074, Mobile, AL, USA
e-mail: mtaylor@southalabama.edu

Although acute and chronic vasoregulation is inherently driven by endothelial Ca²⁺, control and targeting of Ca²⁺-dependent signals are poorly understood. Recent studies have revealed localized and dynamic endothelial Ca²⁺ events comprising an intricate signaling network along the vascular intima. Discrete Ca²⁺ 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 Ca²⁺ elevation, appear to direct endothelial functions under physiologic conditions. In fact, altered patterns of dynamic Ca²⁺ 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 Ca²⁺ 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

INTRODUCTION

The endothelium plays a pivotal role in vascular function and cardiovascular homeostasis, including regulation of vascular tone, permeability, inflammation, and angiogenesis (Furchgott and Zawadzki, 1980; Vandenbroucke et al., 2008; Davis et al., 2011; Xiao et al., 2013). Endothelial function is inherently linked to cardiovascular health, and endothelial dysfunction is a hallmark of cardiovascular disease (Quyyumi, 2003; Munaron and Pla, 2009). Free intracellular Ca²⁺ concentration directs a wide range of endothelial cell responses, but our understanding of dynamic targeting and titration of Ca²⁺ signals within the intact endothelium remains surprisingly rudimentary. New findings have exposed a complex mosaic of physiologic endothelial Ca²⁺ signals (Duzza and Sarelius, 2004; Kansui et al., 2008; Ledoux et al., 2008). These spatially and temporally discrete events comprise a highly structured language along the vascular intima, allowing for selectivity and coordination of cellular responses. Here we discuss recent insights into Ca²⁺ dynamics and implications for Ca²⁺-effector coupling in the endothelium. We also address advances in signal tracking and quantification that will play a crucial role in the elucidation of endothelial Ca²⁺ signaling patterns and the development of new physiologic models.

Ca²⁺ DEPENDENT SIGNALING IN THE ENDOTHELIUM

Endothelial Ca²⁺ targets include a variety of cell effectors. Some contain intrinsic Ca²⁺-binding motifs such as C2 domains. However, most are dependent on calmodulin (CaM), a Ca²⁺-binding protein containing a high-affinity EF-hands motif that allows for cellular responses with nanomolar Ca²⁺ changes. Key

Ca²⁺-CaM dependent endothelial effectors include nitric oxide synthase (eNOS) that produces the diffusible vasorelaxing factor nitric oxide (NO) (Ignarro et al., 1987; Busse and Mulisch, 1990), as well as small/intermediate conductance Ca²⁺-activated K⁺ channels (K_{Ca}), K_{Ca2.3}, and K_{Ca3.1} (Xia et al., 1998; Crane et al., 2003; Dora et al., 2008) that elicit vasorelaxation through endothelium derived hyperpolarization (EDH) of medial vascular smooth muscle (Murphy and Brayden, 1995; Chaytor et al., 1998; Emerson and Segal, 2000; Félétou and Vanhoutte, 2000; Burnham et al., 2002; Bychkov et al., 2002; Taylor et al., 2003). Endothelial Ca²⁺ rise also contributes to phospholipase A2-mediated production of arachidonic acid metabolites, including the vasorelaxing factors prostacyclin and epoxyeicosatrienoic acids (Jaffe et al., 1987; Campbell and Fleming, 2010). The predominant factors vary considerably among vascular beds. NO exerts a major influence in large vessels whereas hyperpolarizing mechanisms predominate in small vessels and the microcirculation (de Wit and Wölflé, 2007). The primary functional consequence of endothelial Ca²⁺-effector recruitment is vasodilation. However, vasoconstricting factors such as endothelin may also be released as a result of Ca²⁺ elevation (Marsen et al., 1996), particularly under conditions of injury or disease. So, how does a seemingly ubiquitous signal, Ca²⁺, selectively recruit different endothelial effectors with diverse functional roles? A closer look at the intact endothelium has revealed a fundamental signaling paradigm involving repetitive spatially restricted Ca²⁺ transients. As discussed below, these signals are capable of exerting effector-specific influences on vascular function and likely contribute to diverse profiles of endothelial response.

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 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; Muftic 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 Béný, 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). Termed 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 (Foskett 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 G_q protein-coupled receptor (G_qPCR) 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 K_{Ca} channels, particularly K_{Ca}3.1 channels that are highly concentrated in the plasma membrane of myoendothelial projections. Importantly, this ongoing Ca²⁺-effector 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 G_qPCR 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 K_{Ca} 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 Ca²⁺-EFFECTOR COUPLING AND FUNCTIONAL ENCODING OF Ca²⁺ DYNAMICS

Fundamental endothelial Ca²⁺ signals (pulsars and sparklets) primarily target K_{Ca}3.1 channels concentrated in densities along the endothelial basolateral membrane and myoendothelial junctions. However, related K_{Ca}2.3 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 K_{Ca}3.1 or K_{Ca}2.3 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 Ca²⁺ signal targeting of K_{Ca} isoforms. Recent findings suggest that in addition to direct EDH signaling, K_{Ca}/TRP coupling may directly influence the endothelial Ca²⁺ signals themselves. Specifically, Ca²⁺-dependent hyperpolarization may increase the driving force for further Ca²⁺ influx through TRP channels, allowing positive feedback augmentation of the original Ca²⁺ signal. In support of this scenario, ACh-induced endothelial Ca²⁺ dynamics are substantially higher in normal mesenteric arteries compared to those from mice lacking K_{Ca}3.1 and K_{Ca}2.3 channels, and this Ca²⁺-facilitating influence of K_{Ca} 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 Ca²⁺ 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 K_{Ca}2.3, TRPV4, and eNOS with caveolin suggests their 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 K_{Ca} channel activity (Sukumaran et al., 2013). Whether such scenarios involve targeting of plasma membrane eNOS, by K_{Ca}2.3-enhanced TRPV4 Ca²⁺ signals, remains to be determined.

Expansion or redirection of inherent Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ events, all of which could affect the type and extent of effector recruitment. Ultimately, definitive tracking of discrete Ca²⁺ 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 Ca²⁺ DYNAMICS

Given the inherent complexity of endothelial Ca²⁺ signals, a key challenge moving forward will lie in our ability to adequately

and comprehensively characterize Ca²⁺ activity along the intact intima. Disparate approaches have been employed to measure and analyze Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ activity typically involves hand 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 straight-forward, 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 individual cells within dense fields and to track region-specific deflections of Ca²⁺-dependent fluorescence (Mukamel et al., 2009; Wong et al., 2010; Watters et al., 2014). Separate automated analysis software has been applied to discern Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ dynamics.

Overall, automated analysis approaches have become useful for defining cell borders, discerning cellular/subcellular fluorescence signals from statistical noise, and providing comprehensive quantitation 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 ($\sim 1 \mu\text{m}$) 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 $\leq 1/2$ 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 Ca²⁺ 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 Ca²⁺ events are often represented as ratios of relative fluorescence change within an ROI (i.e., F/F_0 , where F_0 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 F_0 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/F_0 values.

PERSPECTIVES

Because Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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.

ACKNOWLEDGMENTS

This work was supported by NIH (HL085887 and S10RR027535).

REFERENCES

- Abisi, M., Burnham, M. P., Weston, A. H., Harno, E., Rogers, M., and Edwards, G. (2007). Effects of methyl beta-cyclodextrin on EDHF responses in pig and rat arteries; association between SK_{Ca} channels and caveolin-rich domains. *Br. J. Pharmacol.* 151, 332–340. doi: 10.1038/sj.bjp.0707222
- Andries, L. J., Brutsaert, D. L., and Sys, S. U. (1998). Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium. *Circ. Res.* 82, 195–203. doi: 10.1161/01.RES.82.2.195
- Bagher, P., Davis, M. J., and Segal, S. S. (2011). Visualizing calcium responses to acetylcholine convection along endothelium of arteriolar networks in

- Cx40BAC-GCaMP2 transgenic mice. *Am. J. Physiol. Heart Circ. Physiol.* 301, H794–H802. doi: 10.1152/ajpheart.00425
- Brähler, S., Kaistha, A., Schmidt, V. J., Wölflé, S. E., Busch, C., Kaistha, B. P., et al. (2009). Genetic deficit of SK3 and IK1 channels disrupts the endothelium-derived hyperpolarizing factor vasodilator pathway and causes hypertension. *Circulation* 119, 2323–2332. doi: 10.1161/CIRCULATIONAHA.108.846634
- Burnham, M. P., Bychkov, R., Félétou, M., Richards, G. R., Vanhoutte, P. M., Weston, A. H., et al. (2002). Characterization of an apamin-sensitive small-conductance Ca²⁺-activated K⁺ channel in porcine coronary artery endothelium: relevance to EDHF. *Br. J. Pharmacol.* 135, 1133–1143. doi: 10.1038/sj.bjpp.0704551
- Busse, R., and Mulsch, A. (1990). Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS Lett.* 265, 133–136. doi: 10.1016/0014-5793(90)80902-U
- Bychkov, R., Burnham, M. P., Richards, G. R., Edwards, G., Weston, A. H., Félétou, M., et al. (2002). Characterization of a charybdotoxin-sensitive intermediate conductance Ca²⁺-activated K⁺ channel in porcine coronary endothelium: relevance to EDHF. *Br. J. Pharmacol.* 137, 1346–1354. doi: 10.1038/sj.bjpp.0705057
- Campbell, W. B., and Fleming, I. (2010). Epoxyeicosatrienoic acids and endothelium-dependent responses. *Pflugers Arch.* 459, 881–895. doi: 10.1007/s00424-010-0804-6
- Chaytor, A., Evans, W., and Griffith, T. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. *J. Physiol.* 508, 561–573. doi: 10.1111/j.1469-7793.1998.561bq.x
- Crane, G. J., Gallagher, N., Dora, K. A., and Garland, C. J. (2003). Small- and intermediate-conductance calcium activated K⁺ channels provide different facets of endothelium-dependent hyperpolarization in rat mesenteric artery. *J. Physiol.* 553, 183–189. doi: 10.1113/jphysiol.2003.051896
- Davis, G. E., Stratman, A. N., Sacharidou, A., and Koh, W. (2011). Molecular basis for endothelial lumen formation and tubulogenesis during vasculogenesis and angiogenic sprouting. *Int. Rev. Cell Mol. Biol.* 288, 101–165. doi: 10.1016/B978-0-12-386041-5.00003-0
- de Wit, C., and Wölflé, S. E. (2007). EDHF and gap junctions: important regulators of vascular tone within the microcirculation. *Curr. Pharm. Biotechnol.* 8, 11–25. doi: 10.2174/138920107779941462
- Di, A., and Malik, A. (2010). TRP channels and the control of vascular function. *Curr. Opin. Pharmacol.* 10, 127–132. doi: 10.1016/j.coph.2009.11.010
- Dora, K. A., Gallagher, N. T., McNeish, A., and Garland, C. J. (2008). Modulation of endothelial cell K_{Ca}3.1 channels during endothelium-derived hyperpolarizing factor signaling in mesenteric resistance arteries. *Circ. Res.* 102, 1247–1255. doi: 10.1161/CIRCRESAHA.108.172379
- Duza, T., and Sarelius, I. H. (2004). Localized transient increases in endothelial cell Ca²⁺ in arterioles *in situ*: implications for coordination of vascular function. *Am. J. Physiol.* 286, H2322–H2331. doi: 10.1152/ajpheart.00006.2004
- Earley, S., Gonzales, A., and Crnich, R. (2009). Endothelium-dependent cerebral artery dilation mediated by TRPA1 and Ca²⁺-activated K⁺ channels. *Circ. Res.* 104, 987–994. doi: 10.1161/CIRCRESAHA.108.189530
- Emerson, G. G., and Segal, S. S. (2000). Electrical coupling between endothelial cells and smooth muscle cells in hamster feed arteries: role in vasomotor control. *Circ. Res.* 87, 474–479. doi: 10.1161/01.RES.87.6.474
- Félétou, M., and Vanhoutte, P. (2000). Endothelium-dependent hyperpolarization of vascular smooth muscle cells. *Acta Pharmacol. Sin.* 21, 1–18. Available online at: <http://www.chinaphar.com/1671-4083/21/1.pdf>
- Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. (2007). Inositol trisphosphate receptor Ca²⁺ release channels. *Physiol. Rev.* 87, 593–658. doi: 10.1152/physrev.00035.2006
- Francis, M., Qian, X., Charbel, C., Ledoux, J., Parker, J. C., and Taylor, M. S. (2012). Automated region of interest analysis of dynamic Ca²⁺ signals in image sequences. *Am. J. Physiol. Cell Physiol.* 303, C236–C243. doi: 10.1152/ajp-cell.00016.2012
- Francis, M., Waldrup, J., Qian, X., and Taylor, M. S. (2014). Automated analysis of dynamic Ca²⁺ signals in image sequences. *J. Vis. Exp.* 88. doi: 10.3791/51560
- Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373–376. doi: 10.1038/288373a0
- Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* 84, 9265–9269. doi: 10.1073/pnas.84.24.9265
- Isshiki, M., Mutoh, A., and Fujita, T. (2004). Subcortical Ca²⁺ waves sneaking under the plasma membrane in endothelial cells. *Circ. Res.* 95, e11–e21. doi: 10.1161/01.RES.0000138447.81133.98
- Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G., and Watanabe, K. (1987). Correlation between thrombin-induced prostacyclin production and inositol trisphosphate and cytosolic free calcium levels in cultured human endothelial cells. *J. Biol. Chem.* 262, 8557–8565.
- Janicek, R., Hotka, M., Zahradníková, A., Zahradník, I., and Zahradník, I. (2013). Quantitative analysis of calcium spikes in noisy fluorescent background. *PLoS ONE* 8:e64394. doi: 10.1371/journal.pone.0064394
- Kansui, Y., Garland, C. J., and Dora, K. A. (2008). Enhanced spontaneous Ca²⁺ events in endothelial cells reflect signalling through myoendothelial gap junctions in pressurized mesenteric arteries. *Cell Calcium* 44, 135–146. doi: 10.1016/j.ceca.2007.11.012
- Kotlikoff, M. I. (2007). Genetically encoded Ca²⁺ indicators: using genetics and molecular design to understand complex physiology. *J. Physiol.* 578, 55–67. doi: 10.1113/jphysiol.2006.120212
- Lambley, M., Pittet, P., Koenigsberger, M., Sauser, R., Beny, J. L., and Meister, J. J. (2005). Evidence for signaling via gap junctions from smooth muscle to endothelial cells in rat mesenteric arteries: possible implication of a second messenger. *Cell Calcium* 37, 311–320. doi: 10.1016/j.ceca.2004.11.004
- Ledoux, J., Taylor, M. S., Bonev, A. D., Hannah, R. M., Solodushko, V., Shui, B., et al. (2008). Functional architecture of inositol 1,4,5-trisphosphate signaling in restricted spaces of myoendothelial projections. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9627–9632. doi: 10.1073/pnas.0801963105
- Liu, J., Hughes, T. E., and Sessa, W. C. (1997). The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: a green fluorescent protein study. *J. Cell Biol.* 137, 1525–1535. doi: 10.1083/jcb.137.7.1525
- Ma, X., Du, J., Zhang, P., Deng, J., Liu, J., Lam, F. F.-Y., et al. (2013). Functional role of TRPV4-KCa2.3 signaling in vascular endothelial cells in normal and streptozotocin-induced diabetic rats. *Hypertension* 62, 134–139. doi: 10.1161/HYPERTENSIONAHA.113.01500
- Marie, I., and Bény, J. L. (2002). Calcium imaging of murine thoracic aorta endothelium by confocal microscopy reveals inhomogeneous distribution of endothelial cells responding to vasodilator agents. *J. Vasc. Res.* 39, 260–267. doi: 10.1159/000063691
- Marsen, T. A., Simonson, M. S., and Dunn, M. J. (1996). Roles of calcium and kinases in regulation of thrombin-stimulated preproendothelin-1 transcription. *Am. J. Physiol. Heart Circ. Physiol.* 40, 1918.
- Mufti, R. E., Brett, S. E., Tran, C. H. T., Abd El-Rahman, R., Anfinogenova, Y., El-Yazbi, A., et al. (2010). Intravascular pressure augments cerebral arterial constriction by inducing voltage-insensitive Ca²⁺ waves. *J. Physiol.* 588, 3983–4005. doi: 10.1113/jphysiol.2010.193300
- Mukamel, E. A., Nimmerjahn, A., and Schnitzer, M. J. (2009). Automated analysis of cellular signals from large-scale calcium imaging data. *Neuron* 63, 747–760. doi: 10.1016/j.neuron.2009.08.009
- Munaron, L., and Pla, A. F. (2009). Endothelial calcium machinery and angiogenesis: understanding physiology to interfere with pathology. *Curr. Med. Chem.* 16, 4691–4703. doi: 10.2174/092986709789878210
- Murphy, M. E., and Brayden, J. E. (1995). Apamin-sensitive K⁺ channels mediate an endothelium-dependent hyperpolarization in rabbit mesenteric arteries. *J. Physiol.* 489, 723–734.
- Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bonev, A. D., Knot, H. J., et al. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* 270, 633–637. doi: 10.1126/science.270.5236.633
- Neylon, C. B., and Irvine, R. F. (1990). Synchronized repetitive spikes in cytoplasmic calcium in confluent monolayers of human umbilical vein endothelial cells. *FEBS Lett.* 275, 173–176. doi: 10.1016/0014-5793(90)81465-Z
- Parker, I., Choi, J., and Yao, Y. (1996). Elementary events of InsP3-induced Ca²⁺ liberation in *Xenopus oocytes*: hot spots, puffs and blips. *Cell Calcium* 20, 105–121. doi: 10.1016/S0143-4160(96)90100-1
- Qian, X., Francis, M., Köhler, R., Solodushko, V., Lin, M., and Taylor, M. S. (2014). Positive feedback regulation of agonist-stimulated endothelial Ca²⁺ dynamics by KCa3.1 channels in mouse mesenteric arteries. *Arterioscler. Thromb. Vasc. Biol.* 34, 127–135. doi: 10.1161/ATVBAHA.113.302506
- Qian, X., Francis, M., Solodushko, V., Earley, S., and Taylor, M. S. (2013). Recruitment of dynamic endothelial Ca²⁺ signals by the TRPA1 channel

- activator AITC in rat cerebral arteries. *Microcirculation* 20, 138–148. doi: 10.1111/micc.12004
- Quyyumi, A. A. (2003). Prognostic value of endothelial function. *Am. J. Cardiol.* 91, 19–24. doi: 10.1016/S0002-9149(03)00430-2
- Rath, G., Dessy, C., and Feron, O. (2009). Caveolae, caveolin and control of vascular tone: nitric oxide (NO) and endothelium derived hyperpolarizing factor (EDHF) regulation. *J. Physiol. Pharmacol.* 60, 105–109. Available online at: http://www.jpp.krakow.pl/journal/archive/10_09_s4/pdf/105_10_09_s4_article.pdf
- Russo, G., Spinella, S., Sciacca, E., Bonfante, P., and Genre, A. (2013). Automated analysis of calcium spiking profiles with CaSA software: two case studies from root-microbe symbioses. *BMC Plant Biol.* 13:224. doi: 10.1186/1471-2229-13-224
- Salles, A., Billaudeau, C., Sergé, A., Bernard, A. M., Phélipot, M. C., Bertaux, N., et al. (2013). Barcoding T cell calcium response diversity with Methods for Automated and Accurate Analysis of Cell Signals (MAAACS). *PLoS Comput. Biol.* 9:e1003245. doi: 10.1371/journal.pcbi.1003245
- Sandow, S. L., Haddock, R. E., Hill, C. E., Chadha, P. S., Kerr, P. M., Welsh, D. G., et al. (2009). What's where and why at a vascular myoendothelial microdomain signalling complex. *Clin. Exp. Pharmacol. Physiol.* 36, 67–76. doi: 10.1111/j.1440-1681.2008.05076.x
- Sandow, S. L., Neylon, C. B., Chen, M. X., and Garland, C. J. (2006). Spatial separation of endothelial small- and intermediate-conductance calcium-activated potassium channels (K(Ca)) and connexins: possible relationship to vasodilator function? *J. Anat.* 209, 689–698. doi: 10.1111/j.1469-7580.2006.00647.x
- Sandow, S. L., Tare, M., Coleman, H. A., Hill, C. E., and Parkington, H. C. (2002). Involvement of myoendothelial gap junctions in the actions of endothelium-derived hyperpolarizing factor. *Circ. Res.* 90, 1108–1113. doi: 10.1161/01.RES.0000019756.88731.83
- Santana, L. F., Navedo, M. F., Amberg, G. C., Nieves-Cintrón, M., Votaw, V. S., and Ufret-Vincenty, C. A. (2008). Calcium sparklets in arterial smooth muscle. *Clin. Exp. Pharmacol. Physiol.* 35, 1121–1126. doi: 10.1111/j.1440-1681.2007.04867.x
- Schuster, A., Oishi, H., Bény, J. L., Stergiopoulos, N., and Meister, J. J. (2001). Simultaneous arterial calcium dynamics and diameter measurements: application to myoendothelial communication. *Am. J. Physiol. Heart Circ. Physiol.* 280, H1088–H1096. Available online at: <http://ajpheart.physiology.org/content/280/3/H1088.full-text.pdf+html>
- Socha, M. J., Domeier, T. L., Behringer, E. J., and Segal, S. S. (2012). Coordination of intercellular Ca²⁺ signaling in endothelial cell tubes of mouse resistance arteries. *Microcirculation* 19, 757–770. doi: 10.1111/micc.12000
- Sonkusare, S. K., Bonev, A. D., Ledoux, J., Liedtke, W., Kotlikoff, M. I., Heppner, T. J., et al. (2012). Elementary Ca²⁺ signals through endothelial TRPV4 channels regulate vascular function. *Science* 336, 597–601. doi: 10.1126/science.1216283
- Sukumaran, S. V., Singh, T. U., Parida, S., Narasimha Reddy, C., Thangamalai, R., Kandasamy, K., et al. (2013). TRPV4 channel activation leads to endothelium-dependent relaxation mediated by nitric oxide and endothelium-derived hyperpolarizing factor in rat pulmonary artery. *Pharm. Res.* 78, 18–27. doi: 10.1016/j.phrs.2013.09.005
- Taylor, M. S., Bonev, A. D., Gross, T. P., Eckman, D. M., Brayden, J. E., Bond, C. T., et al. (2003). Altered expression of small-conductance Ca²⁺-activated K⁺ (SK3) channels modulates arterial tone and blood pressure. *Circ. Res.* 93, 124–131. doi: 10.1161/01.RES.0000081980.63146.69
- Thévenaz, P., Ruttimann, U. E., and Unser, M. (1998). A pyramid approach to sub-pixel registration based on intensity. *IEEE Trans. Image Process.* 7, 27–41. doi: 10.1109/83.650848
- Tran, C. H., Taylor, M. S., Plane, F., Nagaraja, S., Tsoukias, N. M., Solodushko, V., et al. (2012). Endothelial Ca²⁺ wavelets and the induction of myoendothelial feedback. *Am. J. Physiol. Cell Physiol.* 302, C1226–C1242. doi: 10.1152/ajpcell.00418.2011
- Vandenbroucke, E., Mehta, D., Minshall, R., and Malik, A. B. (2008). Regulation of endothelial junctional permeability. *Ann. N. Y. Acad. Sci.* 1123, 134–145. doi: 10.1196/annals.1420.016
- Watters, O., Pickering, M., Murphy, R., Murphy, K. J., and O'Connor, J. J. (2014). Automated analysis of intracellular calcium fluorescence in rat organotypic hippocampal cultures: comparison to a manual, observer based method. *J. Neurosci. Methods* 223, 20–29. doi: 10.1016/j.jneumeth.2013.11.021
- Wong, L. C., Lu, B., Tan, K. W., and Fivaz, M. (2010). Fully-automated image processing software to analyze calcium traces in populations of single cells. *Cell Calcium* 48, 270–274. doi: 10.1016/j.ceca.2010.09.008
- Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., et al. (1998). Mechanism of calcium gating in small-conductance calcium-activated potassium channels. *Nature* 395, 503–507. doi: 10.1038/26758
- Xiao, L., Liu, Y., and Wang, N. (2013). New paradigms in inflammatory signaling in vascular endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* 306, H317–H325. doi: 10.1152/ajpheart.00182.2013
- Yashiro, Y., and Duling, B. R. (2000). Integrated Ca²⁺ signaling between smooth muscle and endothelium of resistance vessels. *Circ. Res.* 87, 1048–1054. doi: 10.1161/01.RES.87.11.1048

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 August 2014; paper pending published: 22 September 2014; accepted: 31 October 2014; published online: 17 November 2014.

Citation: Taylor MS and Francis M (2014) Decoding dynamic Ca²⁺ signaling in the vascular endothelium. *Front. Physiol.* 5:447. doi: 10.3389/fphys.2014.00447

This article was submitted to *Vascular Physiology*, a section of the journal *Frontiers in Physiology*.

Copyright © 2014 Taylor and Francis. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.