A correlation between increases in the intracellular Ca$^{2+}$ concentration of vascular endothelial cells and release of endothelium-dependent relaxing factors was first reported decades ago by Lückhoff et al. In agreement with these formative observations, subsequent studies demonstrated that Ca$^{2+}$-sensitive biosynthetic pathways, such as endothelial nitric oxide synthase, as well as small and intermediate conductance Ca$^{2+}$-activated K$^+$ channels (K$_{Ca}$2.3 and K$_{Ca}$3.1) are primary drivers of endothelium-dependent vasodilation. Because endothelial dysfunction and loss of vasodilatory capacity are a common hallmark of cardiovascular diseases, the underlying Ca$^{2+}$ signaling mechanisms are of considerable interest. Technological advances, including the development of high-affinity fluorescent Ca$^{2+}$ indicator dyes, transgenic mice expressing genetically encoded Ca$^{2+}$ indicator proteins selectively in the endothelium, high-speed, high-resolution confocal Ca$^{2+}$ imaging, and total internal reflection fluorescent microscopy allow the Ca$^{2+}$ signals controlling endothelium-dependent vasodilation to be probed in ever increasing detail. Application of these methods has revealed that Ca$^{2+}$ mobilization pathways in the endothelium are unexpectedly complex, dynamic, and diverse. For example, spreading intracellular and intercellular Ca$^{2+}$ waves are stimulated by the well-characterized endothelium-dependent vasodilator acetylcholine in many vascular beds. These propagating Ca$^{2+}$ events may be critically important for conducted vasodilatory responses.

Another type of Ca$^{2+}$ signal was reported by Ledoux et al, who demonstrated that in mouse mesenteric arteries, acetylcholine enhances localized release of Ca$^{2+}$ from the endoplasmic reticulum through inositol trisphosphate receptors (IP$_3$R). These subcellular Ca$^{2+}$ signals, referred to as Ca$^{2+}$ pulsars, are localized to membrane domains that project through the internal elastic lamina separating the endothelium from underlying vascular smooth muscle cells. Ca$^{2+}$ pulsar sites colocalize with areas densely expressing K$_{Ca}$3.1 channels, suggesting that Ca$^{2+}$ pulsars may be a fundamental signal driving the activity of these channels to cause endothelium-dependent smooth muscle hyperpolarization and vasodilation. Further studies linked localized, transient influx of Ca$^{2+}$ though members of the transient receptor potential (TRP) cation channel superfamily with endothelium-dependent vasodilation. These events, called sparklets represent Ca$^{2+}$ influx through a single-TRP channel or clusters of channels present on the plasma membrane and can be detected using confocal or total internal reflection fluorescent microscopy. Sonkusare et al elegantly showed that stimulated activity of TRP vanilloid (V) TRPV4 sparklets causes K$_{Ca}$2.3- and K$_{Ca}$3.1-dependent dilation of mouse mesenteric arteries. Studies from our laboratory show that TRP ankyrin (A)-type TRPA1 sparklets promote dilation of mouse and rat cerebral pial arteries in response to electrophoretic compounds and endogenously generated reactive oxygen species, and the dietary compound carvacrol, found in oregano, stimulates TRPV3 sparklets in the endothelium of mouse cerebral parenchymal arterioles to cause endothelium-dependent smooth muscle hyperpolarization-dependent dilation. Taken together, these studies show that multiple Ca$^{2+}$-dependent pathways have significant impact on arterial tone and endothelial cell function in diverse vascular beds, but little is known about the integration and spatial and temporal regulation of Ca$^{2+}$ signaling modalities in the intact endothelial syncytium. Exciting new findings by Francis et al. published in the current issue of *Circulation Research,* boldly address this critical issue.

### Article, see p 1078

Working with coronary arteries from swine, Francis et al. showed that under basal (unstimulated) conditions, spontaneous Ca$^{2+}$ signaling in the endothelium is inherently dynamic and complex. Ca$^{2+}$ signaling events were recorded from a broad field of the endothelium (≈120 cells) of opened arteries pinned in the en face configuration. Approximately 35% of the endothelial cells in the recording field displayed spontaneous Ca$^{2+}$ signaling activity that was heterogeneous in terms of amplitude, duration, and spatial spread. However, average full field Ca$^{2+}$ fluorescence remained virtually unchanged during the entire recording period (10 minutes). A pharmacological approach was used to show that this activity was almost entirely because of release of intracellular Ca$^{2+}$ from the endoplasmic reticulum through IP$_3$R and was not affected by blockade of ryanodine receptors. Furthermore, dynamic Ca$^{2+}$ signals were almost entirely quenched under these conditions by inhibition of phospholipase C (PLC). Thus, it seems that basal endothelial cell Ca$^{2+}$ signaling in this vascular bed is dependent on tonic production of IP$_3$ by a PLC-dependent pathway (Figure). The functional consequences of basal Ca$^{2+}$ signals were explored in companion with wire myography experiments showing that blockade of endothelial nitric oxide synthase, K$_{Ca}$2.3, or K$_{Ca}$3.1 channels caused an elevation in isometric tension of endothelium-intact coronary artery rings,
and these responses were similarly linked to IP<sub>3</sub>R and PLC. These data suggest that dynamic IP<sub>3</sub>R-mediated Ca<sup>2+</sup> signals (and not global changes in intracellular Ca<sup>2+</sup> concentration) are critically important for endothelium-dependent regulation of basal tone in swine coronary arteries. This concept was extended in experiments using the potent coronary artery endothelium-dependent vasodilator substance P. Strikingly, the concentration dependence of substance P-induced relaxation of coronary artery rings was shown to be identical to that of the effect of the compound on the frequency of transient Ca<sup>2+</sup> signaling events, primarily because of increased activity of basally activity sites. Higher concentrations of substance P further elevated mean Ca<sup>2+</sup> signal amplitude, spatial spread, and event duration, but these effects seem to be unrelated to endothelium-dependent relaxation. Furthermore, the data show that changes in global intracellular Ca<sup>2+</sup> in the endothelium are not correlated with the vasorelaxant effects of substance P. This observation is consistent with a previous report from this group showing that vasodilation of cerebral arteries in response to activation of TRP1 channels with an electrophilic compound was due to elevation in local Ca<sup>2+</sup> signaling activity and not due to global changes in endothelial cell Ca<sup>2+</sup> levels. The main conclusion that can be drawn from these observations is that tissue level changes in endothelial cell Ca<sup>2+</sup> concentration do not adequately describe the Ca<sup>2+</sup> signaling pathways that underlie endothelium-dependent vasodilation and that frequency modulation of dynamic Ca<sup>2+</sup> signals is the primary driver of this response.

A defining strength of this study lies in the use of a custom-made plug-in for the National Institutes of Health ImageJ image analysis software suite called LC_Pro. This plug-in was originally developed by the authors and allows automated analysis of dynamic Ca<sup>2+</sup> signaling events recorded using fluorescent microscopy. The algorithm is designed to detect and track sites of dynamic changes in Ca<sup>2+</sup> that rise above statistical noise and automatically define regions of interest. Amplitude (F<sub>max</sub>/F<sub>0</sub>), kinetics, and spatial spread are determined for all events occurring within each regions of interest, rapidly providing a biophysical fingerprint of discrete Ca<sup>2+</sup> signals. In addition to the removal of potential investigator bias, LC_Pro allows rapid high-throughput analysis of complex dynamic Ca<sup>2+</sup> signaling events occurring within all endothelial cells in a broad imaging field, allowing the large-scale statistical evaluation of these events. Findings enabled by this powerful tool presented in this study and recent reports from other investigators strongly argue that Ca<sup>2+</sup> signaling in the endothelium could be more accurately described as definitive Ca<sup>2+</sup> patterning set by multiple interacting parts. Efforts directed at defining the principal components involved in shaping these patterns will help to build useful bed-specific vascular models to predict the overall influence of the endothelium. One hope is that distinctive pattern shifts will predict specific endothelial functions and dysfunctions.

As with all groundbreaking studies, the novel insights provided by Francis et al. raise many new questions. For example, the findings indicate that under basal conditions the majority of Ca<sup>2+</sup> signaling events result from Ca<sup>2+</sup> release from the endoplasmic reticulum through IP<sub>3</sub>R that is dependent on PLC activity. However, the physiological basis for IP<sub>3</sub> generation and spontaneous Ca<sup>2+</sup> signaling activity under these conditions and the particular PLC isoform(s) responsible are not known. In addition, all the Ca<sup>2+</sup> imaging experiments were performed in the absence of physiological levels of flow and shear stress, factors that strongly stimulate endothelium-dependent vasodilation. Studies to determine the effects of shear stress on endothelial cell Ca<sup>2+</sup> dynamics may require the use of advanced endothelial cell–specific genetically encoded Ca<sup>2+</sup> indicator mice, such as Cx40-GCaMP5-mCherry mice created by Dr Michael Kotlikoff and colleagues as part of the CHROMus resource development program (http://chromus.vet.cornell.edu/). These mice will allow high spatial and temporal resolution of discreet signal components under the most physiological conditions possible. It is also critically important to have a better understanding of the intracellular organization of IP<sub>3</sub>R clusters along the endothelial cell axis that likely promote the propagation of Ca<sup>2+</sup> waves in the endothelium. The organization of TRP channel domains that regulate Ca<sup>2+</sup> influx in the endothelium is also unresolved. It is possible that the distribution and organization of these structures differs between vascular beds with important functional consequences. Complete description of this architecture in the intact endothelium will require the use of advanced imaging techniques, such as super-resolution microscopy. Finally, it remains undetermined how chronic pathological conditions alter Ca<sup>2+</sup> dynamics in
endothelial cells. Expanding Ca\(^{2+}\) events that occur during supraphysiological levels of stimulation (ie, high concentrations of substance P) may underlie a switch to a more pathological, or refractory, signaling pattern. In addition, current evidence suggests that hypoxia increases expression of IP\(_3\)R in cerebellar neurons.\(^{15}\) Thus, it is possible that chronic hypoxia caused by coronary artery disease may increase expression or disrupt the architecture of IP\(_3\)R in coronary arteries, leading to alteration in intracellular Ca\(^{2+}\) dynamics in endothelial cells and dysfunction. The work by Francis et al\(^{11}\) provides an excellent starting point for new studies using advanced analytic techniques to address these important issues.

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**Disclosures**

None.

**References**


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Paulo W. Pires and Scott Earley

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