TRP channel Ca$^{2+}$ sparklets: fundamental signals underlying endothelium-dependent hyperpolarization

Michelle N. Sullivan$^2$ and Scott Earley$^1$

$^1$Department of Pharmacology, University of Nevada School of Medicine, Reno, Nevada; and $^2$Vascular Physiology Research Group, Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado

Submitted 6 September 2013; accepted in final form 9 September 2013

Sullivan MN, Earley S. TRP channel Ca$^{2+}$ sparklets: fundamental signals underlying endothelium-dependent hyperpolarization. Am J Physiol Cell Physiol 305: C999–C1008, 2013. First published September 11, 2013; doi:10.1152/ajpcell.00273.2013.—Important functions of the vascular endothelium, including permeability, production of antithrombotic factors, and control of vascular tone, are regulated by changes in intracellular Ca$^{2+}$. The molecular identities and regulation of Ca$^{2+}$ influx channels in the endothelium are incompletely understood, in part because of experimental difficulties associated with application of patch-clamp electrophysiology to native endothelial cells. However, advances in confocal and total internal reflection fluorescence microscopy and the development of fast, high-affinity Ca$^{2+}$-binding fluorophores have recently allowed for direct visualization and characterization of single-channel transient receptor potential (TRP) channel Ca$^{2+}$ influx events in endothelial cells. These events, called “TRP channel Ca$^{2+}$ sparklets,” have been optically recorded from primary endothelial cells and the intact endothelium, and the biophysical properties and fundamental significance of these Ca$^{2+}$ signals in vasomotor regulation have been characterized. This review will first briefly discuss the role of endothelial cell TRP channel Ca$^{2+}$ influx in endothelium-dependent vasodilation, describe improved methods for recording unitary TRP channel activity using optical methods, and highlight discoveries regarding the regulation and physiological significance of TRPV4 Ca$^{2+}$ sparklets in the vascular endothelium enabled by this new technology. Perspectives on the potential use of these techniques to evaluate changes in TRP channel Ca$^{2+}$ influx activity associated with endothelial dysfunction are offered.

endothelium; TRPV4; total internal reflection fluorescence microscopy; confocal microscopy; Ca$^{2+}$ sparklet
synthase (NOS), 3
arterial smooth muscle cells, membrane depolarization causes specific subcellular domains for brief periods of time. In

Changes in Intracellular [Ca\(^{2+}\)]
channels in endothelial cells

<table>
<thead>
<tr>
<th>Channel</th>
<th>Selectivity (P_{Ca}/P_{Na})</th>
<th>Present in Endothelium(^?) (98)</th>
<th>Involved in EDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPA1</td>
<td>0.8–7.9 (79)</td>
<td>Yes</td>
<td>Yes (18)</td>
</tr>
<tr>
<td>TRPC1</td>
<td>Nonselective (108)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPC2</td>
<td>1–3 (47)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPC3</td>
<td>1.6 (30, 33)</td>
<td>Yes</td>
<td>Yes (75)</td>
</tr>
<tr>
<td>TRPC4</td>
<td>1.1 (67)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPC5</td>
<td>9 (67)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPC6</td>
<td>5 (33)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPC7</td>
<td>0.5–5.4 (63)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPM1</td>
<td>&lt;1 (62)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPM2</td>
<td>0.5–1.6 (66)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPM3</td>
<td>0.1–10 (29)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPM4</td>
<td>Not Ca(^{2+}) permeable (&lt;0.001) (43)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPM5</td>
<td>Not Ca(^{2+}) permeable (&lt;0.05) (32)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>TRPM6</td>
<td>(P_{Na}/P_{Ca}) ~6 (90)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPM7</td>
<td>0.3–3 (70)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPM8</td>
<td>1–3 (48)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPM1L</td>
<td>Nonselective (42)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TRPL2</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TRPL3</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TRPP1</td>
<td>1–5 (41)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPP2</td>
<td>4 (11)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>TRPP3</td>
<td>1–5 (92)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPV1</td>
<td>10 (7)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPV2</td>
<td>1–3 (37)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TRPV3</td>
<td>12 (101)</td>
<td>Yes (19, 50)</td>
<td>Yes (19)</td>
</tr>
<tr>
<td>TRPV4</td>
<td>6 (81)</td>
<td>Yes</td>
<td>Yes (105)</td>
</tr>
<tr>
<td>TRPV5</td>
<td>100 (61)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>TRPV6</td>
<td>100 (104)</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

Relative Ca\(^{2+}\)-to-Na\(^+\) permeability ratio \(P_{Ca}/P_{Na}\) and presence in vascular endothelial cells were detected by RT-PCR, Western blotting, or immunohistochemistry [updated from Wong and Yao (98)] for each transient receptor potential (TRP) channel. EDH, endothelium-dependent hyperpolarization; ND, not determined. Reference numbers are in parentheses.

potential vanilloid (TRPV4) channels, termed “TRPV4 Ca\(^{2+}\) sparklets,” in vascular endothelial cells (78, 82). More interestingly, analysis of TRPV4 Ca\(^{2+}\) sparklets in the intact endothelium indicated that a small number of sparklets can evoke a near-maximal endothelium-dependent vasodilation in pressurized arteries (78). These pioneering studies indicate the essential role of unitary TRP channel Ca\(^{2+}\) microdomains in vascular function. This review discusses the role of TRP channels in endothelium-dependent dilation, methods for recording TRP channel Ca\(^{2+}\) sparklets in endothelial cells, and the significance of TRPV4 Ca\(^{2+}\) sparklets in the regulation of vascular tone.

Changes in Intracellular [Ca\(^{2+}\)] Regulate Vascular Tone

Intracellular [Ca\(^{2+}\)] is a critical determinate of vascular tone. Ca\(^{2+}\) signals can be global (i.e., continuous throughout the cytosol) and long-lasting, or they can be spatially restricted to specific subcellular domains for brief periods of time. In arterial smooth muscle cells, membrane depolarization causes Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels, resulting in elevated global cytosolic [Ca\(^{2+}\)] and subsequent contraction. Endothelium-dependent relaxation of smooth muscle is also regulated by elevations in endothelial cell [Ca\(^{2+}\)]. Three major pathways for endothelium-dependent vasodilation are widely recognized: 1) production of NO by endothelial NO synthase (NOS), 2) production of PGI\(_2\) by cyclooxygenase (COX), and 3) endothelium-derived hyperpolarizing factor (EDHF) (22) or endothelium-dependent hyperpolarization (EDH) (4, 27), which are defined as endothelium-dependent vasodilatory mechanisms that persist when NO and PGI\(_2\) synthesis is inhibited. The EDHF hypothesis follows the convention of the NO and PGI\(_2\) pathways and proposes that one or more additional endothelium-derived diffusible factor(s), such as epoxyeicosatrienoic acids (EETs) (6), K\(^{+}\) (21), C-type natriuretic peptide (9, 95), H\(_2\)S (34, 106), and/or H\(_2\)O\(_2\) (3), are responsible for smooth muscle cell hyperpolarization and subsequent vasodilation. In contrast, EDH, a “factorless” form of EDHF, is the result of endothelial cell plasma membrane hyperpolarization due to the efflux of K\(^{+}\) from small- and intermediate-conductance Ca\(^{2+}\)-activated K\(^{+}\) (K\(_{Ca}\)2.3 and K\(_{Ca}\)3.1, respectively) channels (13, 89). Endothelial cell membrane hyperpolarization rapidly spreads to underlying smooth muscle cells via myoendothelial gap junctions, directly hyperpolarizing and relaxing arterial myocytes (12, 22). EDH responses may be augmented by inwardly rectifying K\(^{+}\) (K\(_{IR}\)) channels, which conduct greater outward K\(^{+}\) currents at hyperpolarized membrane potential and amplify the original hyperpolarizing stimulus (18, 77). All these seemingly divergent pathways can be activated by increases in endothelial cell [Ca\(^{2+}\)]; production of NO by endothelial NOS is elevated by binding to Ca\(^{2+}\)-calmodulin (84), whereas liberation of arachidonic acid by Ca\(^{2+}\)-sensitive PLA2 (5) provides substrate for the biosynthesis of PGI\(_2\) (5, 17) and EETs (6). Increases in endothelial cell [Ca\(^{2+}\)] directly activate K\(_{Ca}\) channels to initiate EDH (4, 27).

Recent studies highlight the importance of myoendothelial junctions (MEJs) in EDH (18, 44, 68, 75, 78). MEJs are extensions of the endothelial cell plasma membrane that project through holes in the internal elastic lamina (IEL) and terminate proximal to the smooth muscle cell sarcolemma (51, 73). MEJs terminate in myoendothelial smooth cell communication (73). Not all membrane projections within IEL holes contain myoendothelial gap junctions (72), but the significance of these structures (called myoendothelial close contacts) is not known. Expression of components of the EDH pathway, including K\(_{Ca}\) channels, inositol 1,4,5-trisphosphate receptors, gap junctions, and TRP channels, is enriched in authentic MEJs (18, 19, 39, 44, 73, 74). Furthermore, MEJs appear to be sites of dynamic localized Ca\(^{2+}\) signaling events that mediate EDH (44, 68).

Although the endothelium-dependent vasorelaxant pathways discussed above are functionally distinct, all can be initiated by an increase in endothelial cell intracellular [Ca\(^{2+}\)]. Such increases can be accomplished by Ca\(^{2+}\) entry from the extracellular space, but our understanding of the Ca\(^{2+}\)-permeable channels directly involved in endothelium-dependent vasodilation remains incomplete. Several classes of Ca\(^{2+}\) influx channels, including the cyclic nucleotide-gated (CNG) channel A1 (99, 103), the purinergic ligand-gated P2X\(_4\) channel (102), and TRP channels (8, 18, 19, 24, 91), have been reported in the vascular endothelium. Ca\(^{2+}\) and Na\(^{+}\) influx through CNG channels in endothelial cells occurs in response to store-operated Ca\(^{2+}\) entry (99, 103). P2X\(_4\) channels are activated by extracellular ATP and conduct Ca\(^{2+}\) into endothelial cells to cause vasodilation through increased NO synthesis (99, 102, 103). Although CNG and P2X\(_4\) channels may be involved in endothelium-dependent regulation under certain conditions, there is no apparent mechanism for these channels to respond
to the multitude of stimuli presented to the intact endothelium. In contrast, Ca\(^{2+}\)-permeable members of the TRP superfamily are sensitive to physical factors [mechanical force (85) and changes in osmolality (45)], temperature (7, 48, 64, 76, 79), hypoxia/hyperoxia (1, 86, 96), and chemical substances (7, 36, 100). A number of recent studies demonstrate the importance of particular TRP channels in endothelium-dependent vasodilatory responses.

**TRP Channels in Endothelium-Dependent Vasodilation**

All members of the TRP superfamily, with the exception of TRPM4 and TRPM5, are permeable to Ca\(^{2+}\) (Table 1). Expression of \(\geq20\) Ca\(^{2+}\)-permeable TRP channels, including TRPA1 (18), TRPC1 (8, 26), TRPC3 (8, 26), TRPC4 (24, 26), TRPC5 (26), TRPC6 (26), TRPV3 (19), and TRPV4 (97), has been detected in cultured and native endothelial cells (Table 1). Freichel et al. (24), who investigated the role of TRPC4 in store-operated Ca\(^{2+}\) entry in endothelial cells, reported the first functional evidence of the involvement of a TRP channel in endothelium-dependent vasodilation. Their study demonstrates that endothelial cells isolated from TRPC4 knockout (TRPC4\(^{-/-}\)) mice did not display a store-operated Ca\(^{2+}\) entry current. More interestingly, endothelial cells isolated from TRPC4\(^{-/-}\) mice exhibited reduced agonist-induced endothelial cell Ca\(^{2+}\) entry, and aortic ring sections displayed diminished vasorelaxation in response to acetylcholine, suggesting that TRPC4 channels are vitally important for endothelial cell function (24). Subsequent studies indicate that TRPV4, TRPC3, TRPV3, and TRPA1 channels are also critically involved in endothelium-dependent vasodilation (Table 1) (18, 19, 75, 105).

The contribution of TRPV4 channels to endothelium-dependent vasodilation is well described. In heterologous expression systems and endothelial cells, TRPV4 channels are activated by EETs (94), potent vasodilators produced by endothelial cells synthesized from arachidonic acid by cytochrome P-450 (CYP) epoxygenase enzymes (10, 38). EET-induced vasodilation of isolated mesenteric arteries was absent from TRPV4\(^{-/-}\) mice, suggesting that the channel is involved in this response (20). The physiological significance of this pathway was reported by Mendoza and colleagues (49), who showed that TRPV4-mediated Ca\(^{2+}\) influx is critically important for flow-mediated dilation of human coronary arteries. Later studies demonstrate a similar role for TRPV4 in other vascular beds (31, 46). These studies imply that endothelial cell TRPV4 channels are activated by shear stress (45). However, although TRPV4 channels are activated by cell swelling, it has been reported that the channel is not inherently mechanosensitive (80). Further studies by Loot et al. (46) suggest that TRPV4 is indirectly activated by shear stress through flow-mediated production of arachidonic acid metabolites. Inhibition of CYP epoxygenase with N-(methylsulfonyl)-2-(2-propynyloxy)benzenemethamide or block of TRPV4 with ruthenium red attenuated flow-induced dilation in mouse carotid arteries (46). However, TRPV4 blockade had no effect on flow-induced dilatory responses when CYP epoxygenase was downregulated (46). These results are supported by another study showing that flow-induced dilation was impaired by PL\(A_{2}\) inhibition, TRPV4 inhibition by ruthenium red, and TRPV4 knockout (31), suggesting that flow-induced dilation involves TRPV4 and CYP epoxygenase activity. Further work (20, 71, 105) indicates that Ca\(^{2+}\) influx and vasodilation following stimulation with cholinoergic agonists in some, but not all vessels, is markedly reduced in TRPV4\(^{-/-}\) mice, clearly indicating a role for the channel in this response. Oddly, although the studies cited above and others suggest a critical role for TRPV4 in vascular regulation, global TRPV4\(^{-/-}\) mice have no obvious cardiovascular phenotype, other than slightly enhanced sensitivity to hypertensive stimuli (85). Further studies using tissue-specific and/or inducible knockout mice are warranted to resolve this apparent paradox.

Reports from several laboratories suggest involvement of TRPC3 in endothelium-mediated vascular responses. Gao et al. (25) provided evidence for expression of TRPC3 in the endothelial and smooth muscle layers of human internal mammary artery (IMA) and show that the TRPC3 blocker Pyr3 modestly diminished relaxation of precontracted IMA rings. Using knockout models, Kochukov et al. (40) demonstrated that TRPC1 and TRPC3 channels are involved in Ca\(^{2+}\) influx and vasorelaxation of aortic ring segments. The Sandow laboratory recently reported participation of TRPC3 channels in EDH of rat and mouse mesenteric arteries (75). This study demonstrates that TRPC3 channels are present in the mesenteric endothelium and that ~70% of endothelial TRPC3 channels are localized within holes in the IEL. In the presence of the NOS inhibitor nitro-L-arginine methyl ester (l-NAME), the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, and the COX inhibitor indomethacin, Pyr3 blunted acetylcysteine-induced vasorelaxation and endothelial cell hyperpolarization. Vasodilatory responses were further attenuated by block of K\(_{Ca}\) channels with TRAM34 and apamin. Together, these data suggest that TRPC3 channel activity can stimulate EDH in mesenteric arteries. In support of these findings, Kirby et al. (39) reported the presence of TRPC3, as well as K\(_{Ca}\)3.1 and K\(_{Ca}\)2.3, within IEL fenestrations of rat popliteal and first-order skeletal muscle arteries from the gastrocnemius muscle, suggesting that TRPC3 may contribute to EDH in this arterial bed as well, although this has not been directly demonstrated.

Our laboratory reported that TRPA1- and TRPV3-mediated Ca\(^{2+}\) influx provokes endothelium-dependent dilation of cerebral arteries (18, 19). TRPV3 channels are activated by the dietary molecule carvacrol, a substance derived from oregano (19). We found that carvacrol activates ruthenium red-sensitive cation currents and elevates intracellular Ca\(^{2+}\) levels in native cerebral artery endothelial cells (19). Carvacrol administration evoked endothelium-dependent vasodilation of cerebral arteries that was not altered by NOS and COX inhibition but was sensitive to block of K\(_{Ca}\) and K\(_{R}\) channels (19). TRPA1 channels are activated by a variety of electrophilic substances, including allin, found in garlic, and allyl isothiocyanate (AITC), derived from mustard oil (36). Allicin can dilate mesenteric arteries by activating TRPA1 channels in perivascular nerves and causing the release of calcitonin gene-related peptide (2). We found that TRPA1 channels were also present in the cerebral artery endothelium (18). AITC caused robust endothelium-dependent dilation and smooth muscle cell membrane hyperpolarization that was blocked by the selective TRPA1 inhibitor HC-030031. These responses were insensitive to NOS and COX blockade but diminished by inhibition of K\(_{Ca}\) channels with TRAM34 and apamin, as well as block of
K<sub>IR</sub> channels with BaCl<sub>2</sub> (18). We also found that that TRPA1 channels are highly concentrated within IEL fenestrations in cerebral arteries and colocalize with K<sub>Ca3.1</sub> channels in this tissue (18). In contrast, TRPV3 channels are uniformly distributed (19), suggesting that TRPA1 and TRPV3 channels may elicit vasodilation by distinct molecular pathways. Insight into TRPA1-mediated endothelium-dependent vasodilation was provided by a recent study by Qian et al. (68), who demonstrated that activation of TRPA1 channels provokes dilation by recruiting large dynamic Ca<sup>2+</sup> signals in the cerebral artery endothelium. Because food-borne molecules found in garlic and mustard can activate TRPV3 and TRPA1 channels, it is tempting to speculate that increased TRPV3 and/or TRPA1 activity in the endothelium could contribute to the putative cardioprotective benefits of certain dietary choices, but there is little direct evidence to support this idea. Further investigation into endogenous regulators of these channels is warranted.

**Optical Methods for the Study of Ca<sup>2+</sup> Influx Channels**

Evidence cited above indicates that TRP channel activity can influence endothelium-dependent dilation, stimulating interest in the properties and regulation of TRP channels in the endothelium. Ion channels are traditionally studied using patch-clamp electrophysiology. However, small, flat cells, such as native endothelial cells, are difficult to patch successfully, and throughput using conventional methods can be painfully slow. Moreover, inherent limitations of the voltage-clamp technique can impede investigation of ion channel regulation in a physiological setting. For example, when the conventional whole cell patch-clamp configuration is employed, intracellular signaling pathways are disrupted when cells are dialyzed with the patch pipette solution. More serious disturbances in metabotropic regulatory mechanisms result from application of inside-out or outside-out patch-clamp methods. Although the intracellular environment is not disturbed when the on-cell patch-clamp configuration is employed, channel activity can only be recorded from the small area of membrane beneath the recording pipette, and voltage clamp cannot be maintained. These technical issues hamper electrophysiological investigation of Ca<sup>2+</sup> influx pathways in native endothelial cells.

Fortunately, sophisticated imaging techniques have recently been developed to surmount the limitations of patch-clamp electrophysiology and global Ca<sup>2+</sup>-imaging techniques. This methodology, pioneered by Demuro and Parker (14, 16), uses high-speed, high-resolution confocal microscopy or TIRF microscopy to record single-channel Ca<sup>2+</sup> signals in a variety of cell types. Confocal and TIRF microscopy use different approaches to achieve fluorescent excitation of a thin, isolated plane along the z-axis of a specimen (axial sectioning). High-resolution confocal microscopy employs an adjustable plane of illumination along the z-axis of a cell or tissue. This is achieved by scanning the specimen with a column of light so that only a small spot in the plane of focus is illuminated at a time. Because of light scattering, areas in out-of-focus planes are also illuminated with this setup alone. Therefore, the addition of a pinhole aperture proximal to the detector and conjugate to the in-focus plane prevents detection of light from out-of-focus planes, thus achieving a thin optical section of illumination. TIRF microscopy utilizes the total internal reflection of light that occurs when light travels through media of decreasing refractive indexes, e.g., when light travels through glass into an aqueous specimen, at an angle greater than a defined critical angle (relative to normal) (Fig. 1A). Total internal reflection of light creates a low-energy evanescent field of illumination along the surface of the aqueous specimen, generating a thin fluorescent plane of excitation that only penetrates ~100 nm into the specimen (Fig. 1B). TIRF microscopy develops a thinner optical section than does high-resolution confocal microscopy (100 nm vs. 1 μm), which is advantageous for the study of unitary Ca<sup>2+</sup> channel activity at the plasma membrane. However, this can also be a disadvantage for recording intracellular Ca<sup>2+</sup> release events. Confocal microscopy is more useful for this purpose, as the plane of excitation can be focused within the cytosol. TIRF and confocal Ca<sup>2+</sup> imaging rely on high-affinity fluorescent Ca<sup>2+</sup> reporter molecules with rapid binding kinetics to resolve Ca<sup>2+</sup> signals. The cell-permeable, nonratiometric Ca<sup>2+</sup> indicators fluo 3-AM (52) and fluo 4-AM (28) are most commonly used to record transient Ca<sup>2+</sup> signals. More recently, genetically encoded Ca<sup>2+</sup> indicator proteins have been utilized in Ca<sup>2+</sup>—imaging experiments. Most of these molecules are green fluorescent protein-calmodulin (CaM) fusion proteins, such as Pericams (54), GCaMP2 (87, 88), and Cameleon (53). Targeted expression, allowing Ca<sup>2+</sup> signals to be recorded from specific tissues without contamination from other cell types, constitutes a major advantage of genetically encoded vs. chemical Ca<sup>2+</sup> indicators. In addition, genetic expression reduces loading variability associated with chemical indicators. Thus advances in Ca<sup>2+</sup>—imaging techniques and Ca<sup>2+</sup> indicators have greatly improved the ability to optically resolve unitary Ca<sup>2+</sup> channel activity and have expanded the type of information that can be obtained from such experiments.

Optical recording of Ca<sup>2+</sup> channel activity presents distinct advantages over conventional electrophysiological methods. Spatial information regarding channel location on the plasma membrane is gained, and simultaneous channel activity can be recorded from the entire bottom surface of the plasma membrane. Moreover, optical methods are less invasive than patch-clamp techniques, allowing intracellular signaling pathways to remain intact. The main disadvantage of optical vs. patch-clamp recording is lack of membrane voltage clamp. To overcome this problem, the two techniques can be applied simultaneously. Ultimately, both methods will continue to be used to study Ca<sup>2+</sup> influx pathways.

The most complete example of the power of these new optical approaches was reported in a sophisticated series of studies by the Santana laboratory that exploited TIRF microscopy to investigate unitary Ca<sup>2+</sup> influx events through L-type [voltage-gated Ca<sup>2+</sup>] (CaV1.2)] channels in vascular smooth muscle cells (for review see Ref. 55). These events, called “L-type Ca<sup>2+</sup> channel sparklets” (93), are recorded by imaging membrane Ca<sup>2+</sup> signals in Ca<sup>2+</sup> indicator—loaded cells with TIRF microscopy under voltage-clamp conditions while background cytosolic Ca<sup>2+</sup> is buffered with the slow chelator EGTA. L-type Ca<sup>2+</sup> channel sparklets are low-amplitude Ca<sup>2+</sup> signals (Table 2); therefore, the thin fluorescent excitation plane generated by TIRF coupled with intracellular Ca<sup>2+</sup> buffering is critical for resolution.
Ca\textsuperscript{2+} sparklets are distinct from Ca\textsuperscript{2+} sparks, another well-described Ca\textsuperscript{2+} signal in smooth muscle cells (60). Ca\textsuperscript{2+} sparklets are recordings of Ca\textsuperscript{2+} influx through individual Ca\textsuperscript{2+}-permeable ion channels on the plasma membrane and are abolished by removal of extracellular Ca\textsuperscript{2+} but unaffected by intracellular Ca\textsuperscript{2+} store depletion. Ca\textsuperscript{2+} sparks are Ca\textsuperscript{2+} release events from intracellular stores via ryanodine receptors located on the sarcoplasmic reticulum and are abolished by store depletion. Recordings of Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} sparklets (and other localized Ca\textsuperscript{2+}-signaling events) are easily distinguishable by their biophysical properties, such as amplitude and spatial spread (Table 2).

Application of the TIRF microscopy approach led to the discovery of several important aspects of L-type Ca\textsuperscript{2+} channel regulation in arterial myocytes. For example, although L-type Ca\textsuperscript{2+} channels are broadly distributed throughout the surface of smooth muscle cells, L-type Ca\textsuperscript{2+} channel sparklet activity is nonrandom; i.e., almost all channel activity occurs at a few persistently active sites on the sarcolemma (56). High-activity L-type Ca\textsuperscript{2+} channels were found to be modulated by PKA, ER, endoplasmic reticulum.

Table 2. TRPV4 Ca\textsuperscript{2+} sparklets are distinct Ca\textsuperscript{2+} microdomains

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+} Microdomain</th>
<th>Amplitude</th>
<th>Duration, ms</th>
<th>Spatial Spread, (\mu m^2)</th>
<th>Frequency, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca\textsuperscript{2+} sparks (60, 65)</td>
<td>2 ± 0.1 F/F\textsubscript{0}</td>
<td>30</td>
<td>13.6 ± 1.2</td>
<td>0.5–1</td>
</tr>
<tr>
<td>LTCC sparklets (56)</td>
<td>18–280 nM</td>
<td>30, 80</td>
<td>0.81 ± 0.01</td>
<td>N/A</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} puffs (83)</td>
<td>50–500 nM</td>
<td>1,000</td>
<td>2–4</td>
<td>N/A</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} pulsars (44)</td>
<td>1.77 ± 0.10 F/F\textsubscript{0}</td>
<td>257 ± 12</td>
<td>15.9 ± 0.6</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>TRPV4 Ca\textsuperscript{2+} sparks (fluo 4) (82)</td>
<td>0.22 ± 0.01 F/F\textsubscript{0}</td>
<td>520 ± 40</td>
<td>4.8 ± 0.8</td>
<td>0.48 ± 0.05</td>
</tr>
<tr>
<td>TRPV4 Ca\textsuperscript{2+} sparklets (GCaMP2) (78)</td>
<td>0.19 F/F\textsubscript{0}</td>
<td>37.0 ± 0.7</td>
<td>11.2 ± 0.4</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

Properties of smooth muscle cell Ca\textsuperscript{2+} sparks, L-type Ca\textsuperscript{2+} channel (LTCC) sparklets, Ca\textsuperscript{2+} puffs, Ca\textsuperscript{2+} pulsars, and TRPV4 Ca\textsuperscript{2+} sparklets were measured using fluo 4-AM, and properties of TRPV4 Ca\textsuperscript{2+} sparklets were measured using a GCaMP2 indicator. Frequency (Hz) represents number of Ca\textsuperscript{2+} events per second per cell. F/F\textsubscript{0}, relative fluorescence; N/A, not applicable. Reference numbers are in parentheses.
PKCα, and calcineurin (55, 57, 59). PKA- and PKCα-dependent changes in L-type Ca\(^{2+}\) channel activity are partly due to their interaction with protein A kinase-anchoring proteins (AKAPs), which are required for PKA-dependent modification of L-type Ca\(^{2+}\) channels (58, 107) and for intracellular targeting of PKCα (55). Taken together, the actions of PKA, PKCα, AKAPs, and calcineurin account for the variation in L-type Ca\(^{2+}\) channel activity. This body of work highlights the potential of optical recording methods to reveal fundamental aspects of Ca\(^{2+}\) influx mechanisms not detected by other means.

TRP Channel Ca\(^{2+}\) Sparklets

Optical techniques have recently been employed to record unitary Ca\(^{2+}\) influx events through TRP channels in vascular endothelial cells. Like L-type Ca\(^{2+}\) channel sparklets, unitary Ca\(^{2+}\) influx events through TRP channels are called “TRP channel Ca\(^{2+}\) sparklets,” and the molecular identity of the responsible TRP channel is specified (78, 82). For example, single-channel Ca\(^{2+}\) influx events through TRPV4 are called TRPV4 Ca\(^{2+}\) sparklets. TRPV4 Ca\(^{2+}\) sparklets were recently characterized in primary human microvascular endothelial cells (82). In this study, endothelial cells were loaded with the fast Ca\(^{2+}\) indicator dye fluo 4-AM, and Ca\(^{2+}\) sparklets at the plasma membrane, indicated by transient fluorescent signals, were recorded using TIRF microscopy (Fig. 2; see Supplemental Movie in Supplemental Material for this article available online at the Journal website). Ca\(^{2+}\) sparklet frequency was increased by the TRPV4 agonists 4α-phorbol 12,13-didecanoate (4α-PDD), GSK-1016790A, and 11,12-EET, inhibited by the TRPV4-selective antagonist HC-067047, and nearly abolished in the absence of extracellular Ca\(^{2+}\), demonstrating that these signals represent TRPV4-mediated Ca\(^{2+}\) influx (82). TRPV4 channel Ca\(^{2+}\) sparklets in endothelial cells are larger in amplitude than L-type Ca\(^{2+}\) channel sparklets in smooth muscle cells and can be recorded in the absence of exogenous cytosolic Ca\(^{2+}\) buffers and without voltage clamp. Custom software (LC_Pro) implemented as a plugin for ImageJ (23) was used to autodetect fluorescent signals and calculate the amplitude, duration, and spatial spread for each TRPV4 Ca\(^{2+}\) sparklet recorded. This method was used to characterize the biophysical properties of TRPV4 Ca\(^{2+}\) sparklets (Table 2). TRPV4 Ca\(^{2+}\) sparklets were rare under basal conditions but readily recruited upon stimulation of TRPV4 with an agonist (82). Additionally, even during maximal stimulation, only a few TRPV4 Ca\(^{2+}\) sparklet sites per cell (i.e., active TRPV4 channels) were detected, even though immunolabeling experiments revealed that TRPV4 protein was widely distributed (82). This is the first evidence demonstrating that most TRPV4 channels in primary endothelial cells are “silent,” suggesting a potential nonconducting role for TRPV4 protein.

The characteristics of endothelial cell TRPV4 Ca\(^{2+}\) sparklets are distinct from those of other Ca\(^{2+}\) signals recorded from vascular endothelial and smooth muscle cells (Table 2). TRPV4 Ca\(^{2+}\) sparklets recorded from endothelial cells of different vascular beds (dermal vs. mesenteric) and species (human vs. mouse) using different recording conditions (TIRF and fluo 4 vs. confocal and GCaMP2) and analysis methods displayed remarkable similarity in GSK-1016790A-stimulated frequency, amplitude, and spatial spread (Table 2). The duration of TRPV4 Ca\(^{2+}\) sparklets differed between the two studies. This discrepancy is due to differences in analysis method.

Fig. 2. Transient receptor potential vanilloid (TRPV4) Ca\(^{2+}\) sparklets in endothelial cells. A: pseudocolor time lapse of TIRF microscopy images of a primary human microvascular endothelial cell loaded with the Ca\(^{2+}\) indicator dye fluo 4-AM. Unitary TRPV4 Ca\(^{2+}\) influx events (i.e., TRPV4 Ca\(^{2+}\) sparklets) are observed as transient increases in fluorescence (green). Scale bar, 10 μm. Inset: magnifications of TRPV4 Ca\(^{2+}\) sparklet. Scale bar, 2 μm. B: pseudocolor image of a rat airway smooth muscle cell TRPV4 Ca\(^{2+}\) sparklet (top left; scale bar, 10 μm) and fluorescence signals produced from opening of 1, 2, 3, or 4 TRPV4 channels (bottom left; scale bar, 2 μm). Recording of fluorescence vs. time for region of interest (highlighted in top left) indicates opening of 1–4 TRPV4 channels (right).
(manual vs. automated) or could be due to differences in the preparation (primary endothelial cells vs. intact endothelium). However, it is clear that TRPV4 Ca\(^{2+}\) sparklets are a novel Ca\(^{2+}\) microdomain in endothelial cells with definable biophysical properties that are distinct from Ca\(^{2+}\) microdomains that have been previously described.

**TRPV4 Ca\(^{2+}\) Sparklets Cause Vasodilation**

An elegant study by Sonkusare et al. (78) examined the functional significance of TRPV4 Ca\(^{2+}\) sparklets in the intact endothelium of mouse mesenteric arteries. A novel feature of this study was the use of Cx40\(^{BAC}\)-GCaMP2 mice, a strain genetically modified to express a Ca\(^{2+}\)-binding biosensor protein, GCaMP2, preferentially in the vascular endothelium. GCaMP2 mice allow for Ca\(^{2+}\) imaging of endothelial cell Ca\(^{2+}\) signals without background from other cell types, such as perivascular nerves or vascular smooth muscle cells (87, 88). Mesenteric arteries from Cx40\(^{BAC}\)-GCaMP2 mice were isolated and pinned en face, and TRPV4 Ca\(^{2+}\) sparklets were recorded from the intact endothelial layer using high-speed, high-resolution confocal microscopy (78). Mesenteric artery endothelial TRPV4 Ca\(^{2+}\) sparklets were evoked by activators of TRPV4 (GSK-1016790A, 4α-PDD, and 11,12-EET), blocked by the TRPV4-specific inhibitor HC-067047, and absent from TRPV4\(^{-/-}\) mice, providing the first evidence that unitary TRPV4 activity can be optically recorded from intact mesenteric arteries (78).

Interestingly, mesenteric artery TRPV4 Ca\(^{2+}\) sparklet recordings demonstrate cooperativity in terms of channel gating. The amplitudes of a majority of the recorded signals were consistent with four simultaneous channel openings, i.e., a “four-channel meta-structure” (78). In addition, similar to responses recorded from primary human endothelial cells (82), only four to eight TRPV4 channels per cell were active during near-maximal stimulation with the potent TRPV4-selective agonist GSK-1016790A (10 nM) (78). Surprisingly, the activity of these few TRPV4 channels is sufficient to hyperpolarize the endothelial cell plasma membrane and evoke maximal endothelium-dependent dilation of isolated, pressurized mesenteric arteries (78) by an EDH mechanism involving small- and intermediate-conductance Ca\(^{2+}\)-sensitive K\(^+\) channels (Fig. 3) (78). High concentrations of GSK-1016790A (100 nM) resulted in excessive increases in global Ca\(^{2+}\) and severe vasomotion of the isolated arteries (78), suggesting that overstimulation of TRPV4 in the endothelium may have a pathophysiological role. Together, these findings indicate the significance of TRPV4 Ca\(^{2+}\) sparklets in EDH and vascular regulation in mouse mesenteric arteries.

**Perspectives**

Endothelial dysfunction is a hallmark of common cardiovascular diseases, such as atherosclerosis, stroke, and hypertension, establishing the critical nature of this fragile tissue for the maintenance of vascular health. Important functions of the endothelium are regulated by changes in intracellular Ca\(^{2+}\), emphasizing the significance of Ca\(^{2+}\) influx pathways. Recent improvements in confocal and TIRF microscopy used in conjunction with sensitive Ca\(^{2+}\) indicator molecules allow for the direct visualization of unitary TRP channel Ca\(^{2+}\) influx events, or TRP channel Ca\(^{2+}\) sparklets, in endothelial cells. Significant advantages of optical vs. traditional electrophysiological techniques of studying Ca\(^{2+}\) influx channels include higher throughput, preservation of intracellular Ca\(^{2+}\) signaling pathways, and acquisition of the number and subcellular distribution of active Ca\(^{2+}\) influx sites. Recent use of this new methodology has uncovered unexpected aspects of TRPV4 physiology in the endothelium, including evidence that gating is cooperative and that the activity of only a few channels per cell can yield maximal endothelium-dependent vasodilation. Application of this technology to the study of other Ca\(^{2+}\)-permeable TRP channels involved in endothelium-dependent dilation, such as TRPC3, TRPA1, and TRPV3, is likely to yield further novel and surprising findings. Furthermore, optical recording may prove to be particularly useful for the assessment of changes in Ca\(^{2+}\) influx pathways associated with endothelial dysfunction and the evaluation of interventions designed to resolve this pathology.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grant R01 HL-091905 (to S. Earley) and a Monfort Excellence Award from the Monfort Family Foundation (to S. Earley).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

M.N.S. and S.E. prepared the figures; M.N.S. and S.E. drafted the manuscript; M.N.S. and S.E. edited and revised the manuscript; M.N.S. and S.E. approved the final version of the manuscript.
REFERENCES


