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Dynamic Ca^{2+} signal modalities in the vascular endothelium

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Abstract

The endothelium is vital to normal vasoregulation. Although acute vasodilation associated with broad endothelial Ca^{2+} elevation is well-known, the control and targeting of Ca^{2+} dependent signals in the endothelium is poorly understood. Recent studies have revealed localized IP_3-motivated Ca^{2+} events occurring basally along the intima that may provide the fundamental basis for various endothelial influences. Here, we provide an overview of dynamic endothelial Ca^{2+} signals and discuss the potential role of these signals in constant endothelial control of arterial tone and the titration of functional responses in vivo. In particular, we focus on the functional architecture contributing to the properties and ultimate impact of these signals and explore new avenues in evaluating their prevalence and specific modalities in intact tissue. Finally, we discuss spatial and temporal effector recruitment through modification of these inherent signals. It is suggested that endothelial Ca^{2+} signaling is a continuum in which the specific framework of store-release components and cellular targets along the endothelium allows for differential modes of Ca^{2+} signal expansion and distinctive profiles of effector recruitment. The precise composition and distribution of these inherent components may underlie dynamic endothelial control and specialized functions of different vascular beds.

Keywords

Calcium; endothelium; artery; dynamic; modality; analysis

INTRODUCTION

The pivotal role of the endothelium in the regulation of various aspects of vascular function and cardiovascular homeostasis is well documented and appreciated. As the luminal interface, the endothelium is a continuous hub of signaling that regulates vascular tone and permeability as well as vascular structure [13, 24, 29, 59, 62]. Ca^{2+} signals are integral to endothelial function, and various cellular components that control Ca^{2+} concentration and associated signal transduction are linked to endothelial dysfunction and cardiovascular pathology [25, 43, 50, 56]. Despite broad acceptance of the importance of Ca^{2+} in endothelial function, a detailed understanding of its management and impact is lacking. New findings have begun to expose the true complexity of physiologic endothelial Ca^{2+} signaling [19, 30, 32, 35, 37] and suggest that our entrenched view of Ca^{2+} dependent regulation has been grossly oversimplified.

Free intracellular Ca^{2+} controls multiple endothelial targets (effectors) that promote vasodilation, including the Ca^{2+}-calmodulin-dependent proteins endothelial nitric oxide synthase (eNOS) that produces nitric oxide [5, 31] and small/intermediate conductance Ca^{2+}-activated K^+ channels, $K_{Ca}2.3$ and $K_{Ca}3.1$ [12, 18, 63], that elicit hyperpolarization of

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underlying vascular smooth muscle [4, 6, 24, 44, 58]. Additional vasoregulating factors, including eicosanoids derived from arachidonic acid metabolism [33] as well as reactive oxygen species such hydrogen peroxide, are also linked to changes in prevailing Ca^{2+} levels [22]. Precise Ca^{2+} targeting of endothelial effectors in vivo is still poorly understood although growing evidence suggests that dynamic control of Ca^{2+} signals and compartmentalized effectors may underpin true physiologic signaling. Here, we provide an overview of dynamic endothelial Ca^{2+} signals and explore implications on real-time vasoregulation.

Dynamic Endothelial Ca^{2+} signals

High-speed confocal imaging and single-excitation wavelength fluorescent indicators have dramatically improved our ability to view and record spatially and temporally discrete Ca^{2+} signals and have begun to highlight the integral role of dynamic Ca^{2+} signals in vascular biology. While spontaneous localized Ca^{2+} transients (e.g. Ca^{2+} sparks and sparklets) as well as asynchronous Ca^{2+} waves occurring in vascular smooth muscle have been strongly implicated in the level, coordination and feedback control of vasoconstriction [10, 42, 45, 55], basal Ca^{2+} transients in endothelial cells [32], including distinct spatially restricted events occurring along the intact intima [32, 37, 60], have only recently been reported and characterized. Because the endothelium is extremely fragile and is only a few microns thick, study of its function *in situ* is considerably challenging. Recent advances such as the introduction of the GCaMP2 transgenic mouse model that expresses an endothelial-specific Ca^{2+}-dependent fluorescent protein [36] as well as implementation of open-artery preparations [37] have been particularly useful for live-tissue experiments, preventing spill-over indicator loading of other cell types and making broad fields of endothelia accessible to imaging [37, 46]. Intact functional endothelium can now be evaluated in its native state within the vascular wall and under controlled conditions.

Basal endothelial Ca^{2+} events observed in mouse mesenteric arteries have been termed Ca^{2+} pulsars. They resemble muscle cell Ca^{2+} sparks although they are typically broader in spatial range and duration [37]. Also, whereas sparks originate from endoplasmic reticulum (ER) ryanodine receptors (RyR), Ca^{2+} pulsars release intermittently from clusters of ER inositol 1,4,5-trisphosphate receptors (IP_3Rs) [37]. These endothelial events appear to be direct physiologic manifestations of previously described Ca^{2+} puffs, unitary localized release events stimulated by IP_3 in Xenopus oocytes [26, 48, 57]. Ca^{2+} puffs emit from distinct densities of IP_3Rs and their origination sites, frequencies and development into regional or cell-wide waves are all highly dependent on graded increases in IP_3, which sensitizes Ca^{2+}-induced Ca^{2+} release [26]. In this way, transients such as Ca^{2+} puffs and pulsars are intimately linked to and controlled by Gq-protein coupled receptor (GPCR) signaling. Importantly, Ca^{2+} pulsars occur basally in mesenteric arteries under resting physiological conditions, and can be blocked by inhibiting the generation of IP_3 by phospholipase C (PLC) [37], suggesting these events represent a persistent mode of Ca^{2+} signaling that can be acutely altered by local conditions and agonists. Indeed, stimulation of the mesenteric artery endothelium with acetylcholine increases the number of pulsar-emitting sites as well as the frequencies of events at pre-existing active sites along the intima [37]. A pivotal finding with respect to function is that these events occur in very close proximity to membrane K_{Ca} channels clustered at distinct myoendothelial junction (MEJ) sites in mesenteric arteries [37]. These are sites where endothelial and smooth muscle cells form close contacts (and often heterocellular gap junctions) through holes in the internal elastic lamina (IEL) [52, 54]. Altogether, this provides a steadfast and focused mechanism for soliciting hyperpolarization and relaxation of vascular smooth muscle. The physiologic relevance of this “built-in” signaling apparatus is not yet established, but is well-supported

by the known pervasive role of K_{Ca} 3.1 channels in EDH-dependent vasodilation [6, 18] and the sustained hypertension exhibited by mice lacking K_{Ca} 3.1 channels [56].

Despite advancements, analysis of Ca^{2+} dynamics has remained tedious as manual approaches are inherently time-consuming and prone to user-bias and error. Genuine characterization of diverse dynamics within vast cellular landscapes will ultimately require standard approaches for event detection and rigorous high-throughput analysis. We recently developed a custom autodetection and analysis algorithm that can be applied as a plug-in with ImageJ freeware [28]. This program distinguishes dynamic fluorescence signals from site-specific background/noise along two-dimensional image sequences, allowing rapid screening and comprehensive assessment of various event parameters (e.g. frequency, amplitude, duration, spatial spread and area under curve). Such automated analysis may define distinctive signaling modalities and submodalities among expansive Ca^{2+} event distributions. The ability to define discrete Ca^{2+} signaling profiles is particularly exciting considering the vast differences in endothelium dependent vasoregulation known to occur among different vascular beds and even along the series of a single vascular bed (i.e. predominance of EDH versus NO dependent vasodilation in smaller diameter arteries) [24]. Continued progress toward standardized comprehensive data processing and evaluation will be essential for defining and ultimately resolving physiologic modes of vascular Ca^{2+} signaling. Also, in certain vascular beds, conduits for extracellular Ca^{2+} entry such as transient receptor potential (TRP) channels and/or STIM/ORAI [15, 16] may initiate Ca^{2+} signals that superimpose on or modify existing pulsar-type signals. Future investigations should provide insight on the prevalence of basal transients and whether IP_3Rs and/or other Ca^{2+} sources contribute to basal signals in different beds.

Ca^{2+} signal tuning

The occurrence and range of ongoing endothelial Ca^{2+} transients along the intima is limited. Because most potential Ca^{2+}-liberating sites are untapped basally, a favorable backdrop exists for further expansion and amplification. For instance, elevation of IP_3 through Gq-coupled receptor stimulation triggers Ca^{2+} signals at sites that were previously inactive and can concurrently amplify specific parameters of ongoing events (e.g. frequency). This suggests that endothelial stimulation elicits both binary and analog modes of recruitment by initiating new sites of activity and by adjusting the attributes of the site-specific events themselves. Such a phenomenon would be similar to that of skeletal muscle fiber recruitment in which net muscle force is increased by activating new motor neurons (spatial summation) as well as increasing the firing frequency (temporal summation) of previously active motor neurons. This paradigm is possible for IP_3/IP_3R signaling because IP_3-sensitized Ca^{2+}-induced Ca^{2+} release (and consequent inhibition of IP_3Rs by high Ca^{2+}) favors distinct thresholds for triggering and communication between groups of IP_3Rs. Previous studies of Ca^{2+} puffs support predictable expansion of signals based on the discrete clustering of IP_3Rs and proximal levels/gradients of IP_3 [48]. Correspondingly, we anticipate that in the endothelium, IP_3R distributions and perhaps compartmentalized or graded IP_3 signals underlie incremental Ca^{2+} site recruitment and act to shape a broad range of event properties, both spatially and temporally.

Observations in our laboratory suggest that endothelial stimulation may elicit increased Ca^{2+} transient frequency in one bed and increased event duration in another [27]. In fact, in swine coronary arteries, the predominant basal events are long-lasting waves (> 8 sec vs. < 0.3 sec for pulsars) that initiate locally and spread to encompass much of the cell volume. Clearly, altering the relative persistence and/or spread of a Ca^{2+} signal from its localized source offers a tremendous opportunity to direct effector recruitment. The pre-existing GPCR- IP_3R framework may allow distinct patterning of recruitment among beds and by different

stimuli. In fact, previous findings showing that different vasodilators stimulate distinct populations of endothelial cells [39] reveal phenotypic heterogeneity along the intima and support preferential tuning of recruitment and response. More recently, the possibility has surfaced that smooth muscle itself may alter or even instigate IP_3R signals in the endothelium via direct communication of IP_3 and/or Ca^{2+} across myoendothelial junctions [35, 60], providing feedback or even feed-forward regulation of endothelial vasoregulation. This is particularly relevant in the microcirculation of various beds where myoendothelial coupling is widespread and real-time endothelial control of membrane potential is crucial for blood flow regulation [14, 49]. Finally, new findings indicate that nonselective cation channels, specifically akyrin-associated transient receptor potential (TRPA1) channels, associate closely with $K_{Ca}3.1$ channels in the MEJs of rat cerebral arteries, and may provide an additional source of Ca^{2+} that promotes endothelial K_{Ca} -dependent vasodilation [20]. Whether such signals remain separate from the inherent IP_3 signaling structure or interact with it (i.e. via enhanced Ca^{2+}-induced Ca^{2+} release) is unknown. Finally, although we focus here on spatially restricted dynamics, it should be noted that initiating sites may spread as broad cellular and multi-cellular waves. Such signals may be amplified by endothelial stimulation, including physical stimuli such as stretch and shear [15, 30], allowing certain foci to develop into periodic oscillations or directional wave fronts along the intima [3, 47]. Correspondingly, exact distributions of endothelial receptors and channels, and controlled cell-cell communication are crucial in the ultimate physiologic response.

Effector recruitment

Investigations of endothelium dependent vasodilation have clearly established the role of Ca^{2+} and Ca^{2+}-calmodulin dependent effectors. In particular, eNOS and the endothelial K_{Ca} channels, K_{Ca} 2.3 and K_{Ca} 3.1, form the primary axis for endothelium-derived relaxation and hyperpolarization of smooth muscle in a great majority of circulatory beds [21]. Nitric oxide (NO) freely diffuses to smooth muscle whereas endothelial K_{Ca} channels elicit hyperpolarization of subintimal smooth muscle [23, 64] via MEJs, either through heterocellular gap junctions [7, 8, 9, 11, 17, 40, 52] or via effluxed K^+ and activation of smooth muscle inwardly rectifying K^+ channels (K_{IR}) [21, 61]. Because supraphysiological stimulation is often studied in a laboratory setting, the nuance of Ca^{2+} mobilization and effector recruitment has remained obscured. Moreover, cursory or global evaluations of endothelial Ca^{2+} have rarely addressed exactly where signals are occurring or how long they last. Given the recent appreciation for spatially and temporally dynamic Ca^{2+} signals, attention has begun to focus more acutely on discrete expression patterns of primary functional Ca^{2+} targets. As mentioned earlier, in mesenteric arteries, basally occurring Ca^{2+} pulsar events occur at MEJ sites where $K_{Ca}3.1$ channels cluster in the plasma membrane. This provides a constant impetus for smooth muscle hyperpolarization while much of the endothelial cell, including out-of-range Ca^{2+}-dependent effectors, may remain essentially unperturbed. Moreover, stimulation increases the relative Ca^{2+} event frequency, allowing for amplification of this effect without necessarily engaging other Ca^{2+} dependent pathways cell-wide.

Distinct from $K_{Ca}3.1$ channels, $K_{Ca}2.3$ channels tend to associate with plasma membrane caveolin and distribute peripherally along endothelial cell borders [1, 53]. This general pattern suggests that whereas $K_{Ca}3.1$ channels are primary targets of isolated transients, $K_{Ca}2.3$ channels are more likely to be engaged by extended cell-wide Ca^{2+} events or by specific membrane-delimited signals. Endothelial NOS activity is regulated by phosphorylation as well as Ca^{2+}-calmodulin. The later can reduce its association with membrane caveolin and further potentiate NO production [41, 51]. Interestingly, populations of eNOS are differentially distributed between the membranes of the plasmalemma and the Golgi apparatus in endothelial cells, both of which are capable of NO production [2, 38].

This relative allocation of effector may be crucial in determining accessibility to both phosphorylation and Ca^{2+} signals [34] of different range and duration. Moreover, differences or changes in distribution could serve as an additional means of bed-specific or dynamic regulation in vivo. The implication is that the pattern and not simply the amount of effector expression is a crucial determinant of physiologic endothelial regulation and, consequently, assessing mRNA or protein levels of specific effectors alone may lead to dubious interpretations of function.

Graded expansion of Ca^{2+} signaling along the vascular intima

Fig. 1 depicts a general working model in which inherent endothelial Ca^{2+} signals constantly tune effector recruitment and vascular tone. It is suggested that the distinctive architecture of IP_3Rs and Ca^{2+}-dependent effectors within the confines of the intimal structure allow prevailing IP_3 concentrations to drive predictable profiles of endothelial vasodilating influence. Specifically, IP_3R distribution and intrinsic IP_3 production within an endothelial field establish origination sites as well as the size and frequency of ongoing events, thereby defining a base Ca^{2+} signaling modality and providing the framework for spatial and temporal Ca^{2+} signal expansion. In this way, the endothelium may shift into specific modalities of Ca^{2+} signaling and hence predictable patterns of effector recruitment in response to various conditions. For instance, effectors that are closely tethered to basal Ca^{2+} transients (e.g. $K_{Ca}3.1$ channels) would be expected to exert a consistent background influence. Subsequent stimulation of additional local foci and event frequencies would increase this effect whereas stimulation that promoted widespread, long-lasting wave events would be expected to increase the activity of more peripheral components such as $K_{Ca}2.3$. Thus, specific effector impacts may be strictly encoded by Ca^{2+} event spatial spread and duration as well as site location and frequency. It follows that adjustments in the level of endothelial stimulation titrate the level and profile of effector recruitment. Notably, fluxes of Ca^{2+} through membrane cation channels as well as communication of IP_3 through gap junction-containing MEJs may superimpose on fundamental signals to initiate new signals or further direct effector targeting. The implications of multiple convergent signals and feed-back/feed-forward regulation underscore the potential complexity of physiologic Ca^{2+} signaling and the need for caution and focus in future approaches.

Overall, it is suggested that the expanse and/or time course of Ca^{2+} signals, constantly adjust the level of vascular tone. In this respect, the endothelium may act as a constant gain control on smooth muscle contraction. For instance, by imposing a net zero gain on the rate of tone development, the endothelium might prevent progressive vasoconstriction beyond a certain point, and thereby stabilize arterial diameter over long periods of time. Clearer spatial and temporal elucidation of Ca^{2+} signaling patterns will help expose anomalous signal-effector coupling and target compensatory strategies against endothelial dysfunction and related cardiovascular disease. More selective experimental tools will be required to dissect the sources of dynamic endothelial Ca^{2+} signals as well as the relative cell-specific contributions (i.e. endothelial vs. smooth muscle). Also, detection of Ca^{2+} in restricted spaces (e.g. myoendothelial junctions) and in four dimensions (x,y,z with time), as well as the implementation of standard analysis tools, will provide a more complete picture of endothelial signaling *in situ*.

In summary, recent insights suggest the endothelium functions as a continuum of dynamically regulated influences that are always engaged and are constantly adjusted. Indeed, prevailing Ca^{2+} signaling modalities and effector distributions may underlie distinct functions of the different circulations. Further dissection of this diverse activity will allow for identification of submodalities, and potentially distinct cell phenotypes within the intima. We submit that shifts in prevailing Ca^{2+} dynamics necessarily impact blood pressure and

flow and may predict disease. Indeed, endothelial dysfunction is an overarching feature of many cardiovascular pathologies. It is therefore imperative that future studies shift away from assumptions based on global Ca^{2+} changes and broad cellular protein concentrations and focus on spatially and temporally relevant aspects of real-time signaling. Continued pursuit of a definitive and predictive model of endothelial function should allow for elucidation of specific control points and therapeutic targets.

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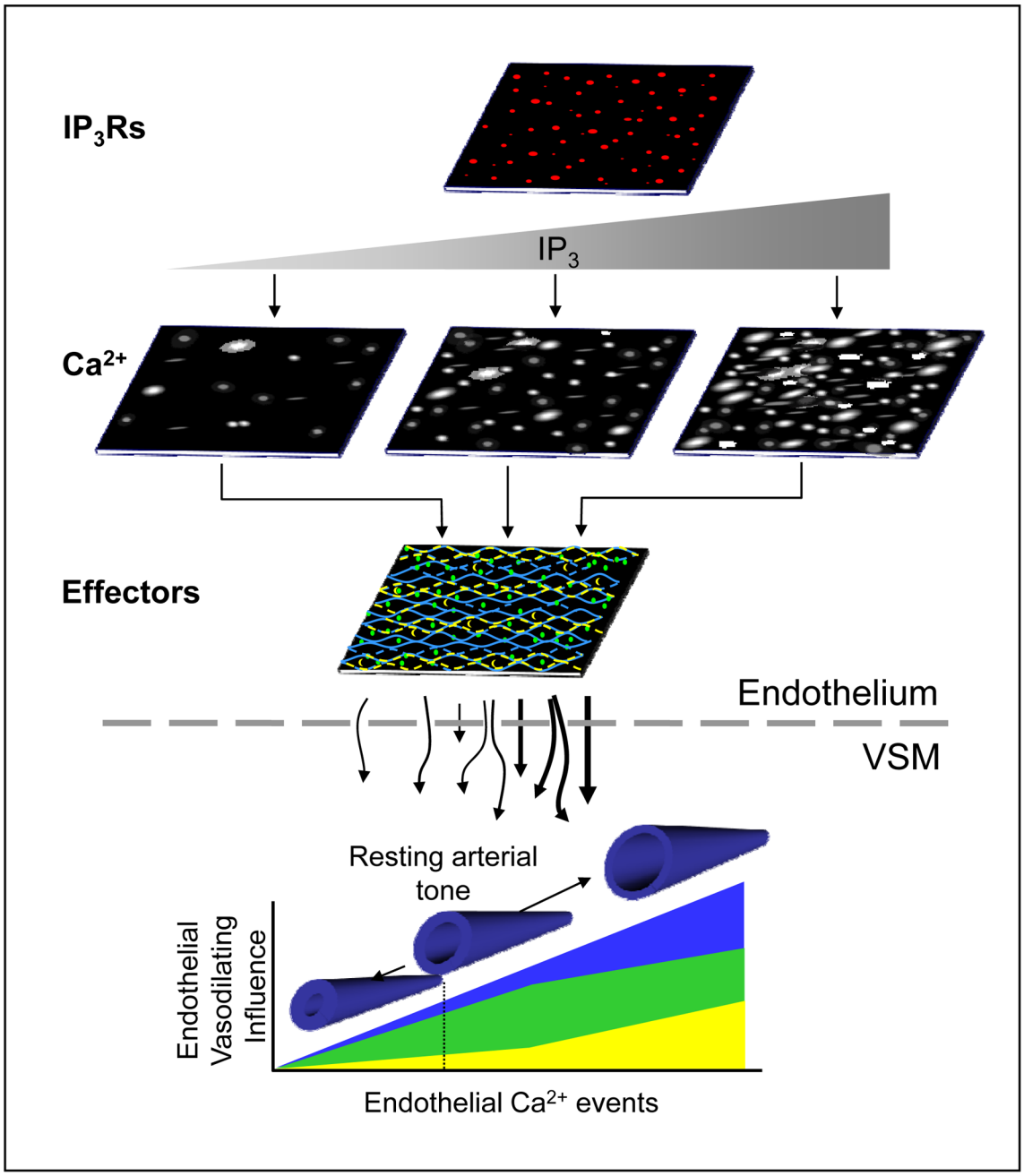

Figure 1.

Conceptual model of dynamic Ca^{2+}-effector coupling in the endothelium and its real-time regulation of arterial tone. It is proposed that a discrete scaffold of IP_3Rs within the endoplasmic reticulum of the vascular intima establishes an intrinsic mode of dynamic Ca^{2+} signals. Relative shifts in IP_3 production define new profiles of dynamic signaling with respect to the number of sites as well as event amplitude, frequency, duration and spatial spread. The distribution and density of specific Ca^{2+}-dependent effectors within the endothelium, including those that are tightly coupled to local Ca^{2+} origination sites (blue) such as $K_{Ca}3.1$ channels, and those that are more peripherally or widely distributed (yellow and green) such as $K_{Ca}2.3$ channels and eNOS, determine the ultimate level of vasodilating

influence communicated across the internal elastic lamina (IEL) to the vascular smooth muscle (VSM) as well as the predominating mechanism solicited (i.e. NO vs. hyperpolarization). This suggests a basal dynamic Ca^{2+} signaling modality exerts a steadfast and predictable endothelial influence on arterial tone (dotted line) that is constantly tuned by prevailing conditions.