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Calcium and electrical signaling in arterial endothelial tubes: new insights into cellular physiology and cardiovascular function

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Abstract

The integral role of the endothelium during the coordination of blood flow throughout vascular resistance networks has been recognized for several decades now. Early examination of the distinct anatomy and physiology of the endothelium as a signaling conduit along the vascular wall has prompted development and application of an intact endothelial “tube” study model isolated from rodent skeletal muscle resistance arteries. Vasodilatory signals such as increased endothelial cell (EC) Ca^{2+} ($[\text{Ca}^{2+}]_i$) and hyperpolarization take place in single ECs while shared between electrically coupled ECs through gap junctions up to distances of millimeters (~ 2 mm). The small- and intermediate-conductance Ca^{2+} activated K^+ ($\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ or $\text{K}_{\text{Ca}2.3}/\text{K}_{\text{Ca}3.1}$) channels function at the interface of Ca^{2+} signaling and hyperpolarization; a bidirectional relationship whereby increases in $[\text{Ca}^{2+}]_i$ activate $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels to produce hyperpolarization and *vice versa*. Further, the spatial domain of hyperpolarization among electrically coupled ECs can be finely tuned via incremental modulation of $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels to balance the strength of local and conducted electrical signals underlying vasomotor activity. Multifunctional properties of the voltage-insensitive $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels of resistance artery endothelium may be employed for therapy during the aging process and development of vascular disease.

Keywords

endothelium; hyperpolarization; conduction; K_{Ca} channels; aging

Introduction

This review and opinion article introduces background on the architecture and physiology of resistance arteries as a preface to understanding the importance of endothelial function. A prominent function of endothelial cells (ECs) is to transduce increases in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) to hyperpolarization via activation of small- and intermediate-conductance Ca^{2+} -activated K^+ ($\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ or $\text{K}_{\text{Ca}2.3}/\text{K}_{\text{Ca}3.1}$) channels. As voltage-insensitive ion channels, the $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels provide an opportunity for fine external modulation of electrical conduction among ECs that are electrically coupled via gap junctions. In the context of a comprehensive analysis of the literature on vascular endothelial function, key findings from

studies performed on isolated endothelial tubes from skeletal muscle arteries are discussed. In particular, this review conveys new insights regarding the presence and potential therapeutic benefits of the endothelial “hyperpolarization-induced Ca^{2+} entry” mechanism and effects of aging and chronic disease on $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channel function to initiate and regulate the spread of hyperpolarization along endothelial cells. In light of endothelial $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channel function and oxidative signaling, the performance of studies on aging animals is an effective approach for understanding the development of impaired blood flow regulation throughout the body.

Integral role of the endothelium in vascular function

Blood flow for the perfusion of vital organs and tissues is governed by the coordination of vasodilation among arteries and parenchymal arterioles. The endothelium lining the resistance vasculature has been recognized over several decades as being integral to this task [see (1-3) for historical perspective]. Coordination of vasodilation throughout resistance networks is mediated, at least in part, via electrical signals (e.g., hyperpolarization) that are initiated by the activation of $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels and spread along ECs through gap junctions. Electrical signals that originate in the endothelium concomitantly spread along the endothelium and into surrounding smooth muscle cells (SMCs) through myoendothelial gap junctions that penetrate through holes in the internal elastic lamina (1, 4, 5). Classical stimulus of muscarinic type 3 (M_3) receptors with acetylcholine (ACh) elicits endothelium-dependent vasodilation by activating endothelial nitric oxide synthase (eNOS) and/or $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels in response to a rise in EC $[\text{Ca}^{2+}]_i$ (2, 6, 7). Diffusion of nitric oxide (NO) from ECs relaxes SMCs via phosphorylation of multiple proteins such as the sarcoplasmic reticulum Ca^{2+} -ATPases (8) and voltage-gated K^+ (K_v) channels (9) in accord with production of cyclic guanosine monophosphate (cGMP) and activation of Protein Kinase G (PKG); favoring an overall decrease in smooth muscle $[\text{Ca}^{2+}]_i$ and reduced myosin light chain (MLC) phosphorylation. A rise in EC $[\text{Ca}^{2+}]_i$ also activates $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels, triggering a process known as endothelium-derived hyperpolarization (EDH) (6, 10) described by the efflux of K^+ ions to increase negative membrane potential (V_m) across EC membranes. The resulting hyperpolarization of V_m transmits to SMCs via myoendothelial gap junctions (6, 10, 11), whereby L-type voltage-gated Ca^{2+} channels are deactivated, and smooth muscle $[\text{Ca}^{2+}]_i$ is reduced to promote vasorelaxation (12). Please note that there are other potential mechanisms underlying vasodilation that are independent of the V_m and Ca^{2+} signaling interface that may include the NO/cGMP/PKG activation of MLC phosphatase and subsequent dephosphorylation of MLC as one example (13). Indeed, PKG can directly phosphorylate precise serine and threonine residues of the myosin phosphatase targeting subunit (i.e., MYPT1) to promote vasorelaxation (14). Furthermore, there are structural and mechanical factors independent of ion channel activity that may also impede vasodilatory signals originating in the endothelium such as SMC hypertrophy (15) and increased arterial stiffness (16, 17) with aging and chronic vascular disease. Although numerous other factors of endothelium-dependent vasodilation are recognized (e.g., epoxyeicosatrienoic acids) (18), this review will primarily focus on discussion of intrinsic Ca^{2+} and electrical signaling properties of endothelium; in particular, $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channel function and the EDH signaling pathway.

Anatomy and physiology of vascular endothelium versus smooth muscle

Arterioles and resistance arteries contain a single cell layer each of ECs and SMCs to effectively couple the production of vasodilatory signals with mechanical vasodilation, and thereby facilitate effective delivery of blood flow in accord with metabolic demand. This structure is conducive for rapid coordination of vasomotor activity in that there is minimal cellular surface area for charging membrane capacitance while curtailing loss of electrical charge via current “sinks” (versus conduit arteries that contain multiple SMC layers such as aortae). SMCs are circumferentially arranged and are perpendicular to the axis of the vessel wall whereas ECs are aligned parallel to the vessel axis in accord with the direction of blood flow. Taken together with approximate estimates of cellular dimensions obtained from rodent arterioles of primary to third branch orders (19, 20), one EC (diameter: $\sim 5 \mu\text{m}$, length: $\sim 100 \mu\text{m}$) can span and electrically couple to ~ 20 SMCs. Another consideration regarding cellular geometry entails the general spindle shape (+ tapered ends) of SMCs whereby ECs are considered “flat” (thickness: $\sim 0.5 \mu\text{m}$) (21). Also, ECs contain a rich expression of gap junctions as indicated by high connexin (Cx37 and Cx40) mRNA levels and protein immunofluorescence (22, 23). Altogether, arterioles and resistance arteries contain the ideal architecture for constantly altering the contractility of SMCs to control vascular resistance and timeliness of blood flow delivery to active organs and tissues. From one millisecond to the next, the contractile status of individual SMCs is governed by ions and second messengers that are axially distributed along the vascular wall via ECs while concomitantly transmitted through myoendothelial gap junctions linking SMCs to ECs.

Differentiation of the discrete coupling of Ca^{2+} and electrical signals across the vascular cell layers is optimally arranged for maintaining a narrow window of blood flow control. In general, increases in SMC $[\text{Ca}^{2+}]_i$ (e.g., via depolarization and increased L-type Ca^{2+} channel activity) effect vasoconstriction, whereas increases in EC $[\text{Ca}^{2+}]_i$ effect hyperpolarization via $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channel activation to evoke vasodilation. Such cross-talk between cell layers via myoendothelial gap junctions is useful for preventing the extremes of blood flow regulation that precede vascular rupture or ischemia. As an example, selective activation of SMC α_1 -adrenergic receptors with phenylephrine (PE) evokes production of SMC $[\text{Ca}^{2+}]_i$ and inositol 1,4,5-trisphosphate (IP_3), whereby excess Ca^{2+} ions and/or IP_3 molecules can traverse through myoendothelial gap junctions and activate EC IP_3 receptors (IP_3Rs) to thereby increase EC $[\text{Ca}^{2+}]_i$ and cease excessive vasoconstriction (24); a process known as myoendothelial “feedback” (25, 26).

With respect to how electrical signals conduct along the vascular wall, there is an additional point of differentiation between ECs and SMCs as determined by cell-type specific agonists. For example, activation of α_1 -adrenergic receptors on SMCs with PE generates depolarization (27, 28) that, at best, travels within $600 \mu\text{m}$ from the site of stimulus (27, 29). There are at least two properties of the vascular wall that act in concurrence to limit the spread of depolarization along the SMC layer: [1] depolarization of the vascular wall itself (30) activates K_v channels that rapidly counteract depolarization with hyperpolarizing charge (12, 31) and [2] SMC:SMC coupling through gap junctions [primarily composed of Cx43 subunits (20, 32)] is weak relative to EC:EC coupling (3, 33). It should also be recognized that the circumferential arrangement of SMCs along the vascular wall imposes

more points of SMC-to-SMC resistance (versus longitudinally distributed ECs) and thus, the transmission of electrical charge in general is more apt to decay per unit length of artery (29). In addition, effects of SMC G-protein stimulation mediates activation of multiple proteins (e.g., Rho kinase, guanine exchange factors, CPI-17, Protein Kinase C) that underlie Ca^{2+} “sensitization” as a more direct means for modulating MLC phosphorylation versus depolarization of V_m [consult (34) for a thorough review]. In contrast to SMCs, focal stimulation of M_3 receptors with ACh on ECs produces hyperpolarization that can transmit for several mm ($\sim 2000 \mu\text{m}$) (29, 35). In addition, the high input (and membrane) resistance of SMCs minimizes charge loss along the endothelium and thus, favors the axial passage of current flow (29). Note that such data have been obtained using microiontophoresis of respective agents (i.e., PE for SMCs & ACh for ECs) from glass pipettes, whereby the precise area of focal application of drug against flow of superfusion (“diffusion”) of physiological saline solution is unknown. The area of application appears to be within $500 \mu\text{m}$ depending on stimulus strength ($\sim 1000 \text{ nA}$) and/or duration ($\sim 1000 \text{ ms}$) during rigorous control of flow rate and fluid turbulence (36). In the examination of isolated and pressurized arteries, an especially useful method for controlling drug delivery has included the use of a dye (e.g., carboxyfluorescein, rhodamine dextran) to track the spatial application of drugs within the lumen or outside of the blood vessel without the need for pipettes (37, 38). Although “receptor-independent” and requiring mechanical stability, the most controlled form of stimulation at the present includes injection of electrical current into single cells via sharp electrodes (11, 28, 30, 39, 40) (see Figure 1). Using this approach, depolarizing current injected into a single EC or SMC of feed arteries evokes less than half of the absolute change in V_m (and alteration in diameter for vasoconstriction) compared to hyperpolarizing current of equal intensity corresponding to vasodilation (30). Altogether, the anatomical and physiological properties of endothelium support its role as an electrical conduit along the vascular wall for the spatial regulation of blood flow throughout resistance networks.

Constriction of vascular diameter and shunting of blood flow to metabolically active versus inactive tissues (41) may be controlled at discrete segments along the vascular wall, whereas dilation and coordinated increases in blood flow follow cell-to-cell communication over large distances (i.e., several millimeters). The biophysical phenomena underlying regulation of local versus conducted responses in *ex vivo* (i.e., isolated and pressurized blood vessels) and *in vivo* (i.e., live animal + intact vasculature and parenchyma) conditions is consistent with the integrated product of blood flow delivery that is required. As one example, such infrastructure of vascular resistance networks may be ideal for governance of “functional sympatholysis” of the skeletal muscle vasculature (42), whereby a > 100 -fold increase in blood flow can take place from inactivity to maximal aerobic exercise (43-45). This process of dynamic blood flow regulation requires that endothelial activation and production of vasodilation rapidly counteract basal activation of SMC adrenoreceptors along resistance networks in accord with metabolic demand (3, 5). Despite widespread distribution of perivascular sympathetic nerves along arteriolar networks of skeletal muscle (46, 47), vasoconstriction via norepinephrine stimulation of SMC adrenoreceptors may be restricted to distinct sites, whereas vasodilatory signals emitted by ECs require coordination over millimeter distances (3). A basis for such differential regulation of electrical signals between

SMCs (“discrete”) and ECs (“conducted”) can be inferred from the kinetics and magnitude of dilatory responses that propagate from pre-capillary arterioles to extraparenchymal feed arteries (i.e., “rapid onset vasodilation”) following contraction of mouse *gluteus maximus* skeletal muscle *in vivo* (48). Further, depending on other major vascular types of focus (e.g., mesenteric arteries), there may be additional perivascular nerve types (e.g., sensory) and respective transmitters (e.g., calcitonin gene related peptide) present to further influence the cell specific handling of electrical signals (47, 49). It is anticipated that a rigorous examination of the role of perivascular nerves in navigating cross-talk between SMCs and ECs will be accomplished using complex electrophysiology methods in the near future.

In summary for the architecture of resistance arteries, there are complementary interactions between SMCs and ECs that mediate the timely delivery of blood flow to vital tissues and organs. As the anatomy and physiology of the endothelium is suitable for effective spatial and temporal regulation of blood flow throughout vascular resistance networks (3, 11, 50), it is important that we more closely examine endothelial function independent of confounding factors such as voltage-sensitive ion regulation, myoendothelial coupling to SMCs, blood flow, and hormones.

Initial studies of endothelial function using cell culture

Endothelia compose the lining of entire networks of blood and lymphatic vessels throughout the human body. It is an organ in and of itself (51), whereby blood vessel endothelium alone encompasses up to several thousand m² of surface area (52, 53) and 100,000 km of distance (54) to interface with all other organs (e.g., brain, heart, skeletal muscle, kidneys, liver, and mesentery). Once considered a simple “cellophane” barrier (55), the endothelium is now known to engage in a wide assortment of complex moment-to-moment (e.g., blood flow regulation, vascular permeability) and chronic (e.g., angiogenesis) physiological functions (56, 57).

Provided that they are located in the intima of the vascular wall, ECs of resistance arteries have historically been difficult to isolate and examine. The advent of EC culturing approaches helped advance modern vascular biology from an accumulation of ~5,000 “endothelium” publications in the early 1990s (58) to the current standing of >140,000. Cell culture affords the amplification of cell numbers and preservation of samples for extended time periods (>1 day) and is thereby feasible for obtaining abundance of sample and flexibility for experimental coordination. This method is also amenable to gene-silencing approaches using transfection (59, 60). However, with culture, morphological changes [“cobblestone” monolayers (61, 62)] occur as well as loss of M₃ receptors (63) and connexin 40 (Cx40; composes native gap junctions) (64) while gaining voltage-sensitive transmembrane proteins such as large-conductance Ca²⁺-activated K⁺ (BK_{Ca}) channels (65). Based on this evidence of extreme alterations of fundamental EC signaling components, cell culture is not the appropriate methodology for comprehensive examination of vascular physiology and will not be discussed further here.

Development and application of endothelial “tubes”

The endothelium plays an integral role towards the production of hyperpolarization, vasodilation, and tissue perfusion (3, 6, 10, 66, 67) and thus, examination of its cellular properties in its native state is crucial. As an alternative to the study of single isolated ECs and/or cell culture, use of intact endothelial “tubes” (see Figure 1A) freshly isolated from hamster cremaster arterioles was first developed and published by the Jackson laboratory (68). The Segal laboratory applied further refinements of the endothelial tube preparation of rodent skeletal muscle arteries and arterioles by streamlining the enzyme digestion process (e.g., omitting elastase treatment step while maintaining use of a papain, dithioerythritol, and collagenase cocktail) (23), optimizing experimental temperature (69), loading of Ca^{2+} dyes and confocal microscopy applications (69, 70), and mechanical stability for intracellular V_m measurements (40). As a result, Ca^{2+} and electrical signals characteristic of the intact endothelium have been resolved in depth for the first time (39, 40, 70). In brief, there is dynamic regulation of $[\text{Ca}^{2+}]_i$ and hyperpolarization in single ECs (“discrete” or “local” signals) as well as between ECs through gap junctions (“conducted” signals) up to millimeter distances (39, 40, 70). Whether an electrical signal of a given magnitude and duration remains local or conducts to adjacent cells depends on the interplay between axial resistance defined by gap junction patency and the plasma membrane resistance defined by ion channel (e.g., $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$) activity (39, 71). For example, the distance by which a signal conducts is favored by conditions of low axial resistance (i.e., open gap junctions) and high membrane resistance (i.e., closed plasma membrane ion channels) (39, 50). Although, active and open ion channels are needed to initiate electrical signals in the first place. Thus, a delicate balance between $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channel activity and gap junction patency is required for maintaining effective coordination of blood flow and tissue perfusion (50, 71).

For translational purposes, it should be recognized that receptors and ion channels appear amenable to “fine-tuning” via pharmacology (39, 40), whereas interventions to modulate gap junctions are much more difficult to control (40, 72, 73) and may be “all or nothing” (40). Indeed, in the presence of patent gap junctions, activating $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels with the “direct” opener NS309 finely hyperpolarizes V_m while decreasing the spread of charge along ECs up to complete abolishment (39) (see Figure 1B). With matched local responses, the presence of an $\sim\text{EC}_{50}$ concentration of NS309 (1 μM) significantly reduces conducted hyperpolarization (e.g., at 500 μm , by $\sim 20\%$ & at 1,500 μm , by $\sim 50\%$) (39). The general effect of NS309 is also mimicked by treatment with exposure to hydrogen peroxide (H_2O_2 ; see Figure 1C); a relatively stable intermediate of the reactive oxygen species signaling pathway (74) that plays a prominent role in aging and development of chronic vascular disease (75-78).

In depth discussion of the biophysical determinants (e.g., axial and membrane resistances underlying the length constant of passive conduction) of the coordination of electrical signaling along endothelial tubes of skeletal muscle arteries has been covered elsewhere (50). Recent evidence of the interaction between endothelial $[\text{Ca}^{2+}]_i$ signaling and V_m regulation and the effects of aging and chronic disease on $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channel function are discussed next.

The endothelial “hyperpolarization-induced Ca^{2+} entry” controversy

A key function of the endothelium of resistance vessels is to produce increases in $[\text{Ca}^{2+}]_i$ that activate $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels and produce hyperpolarization of V_m ; a signaling pathway that governs SMC relaxation, vasodilation, and tissue blood flow (1, 2, 6, 7) (Figure 2). This is exemplified by the activation of M_3 receptors with ACh which, in turn, stimulates the production of IP_3 and diacylglycerol. The initial peak $[\text{Ca}^{2+}]_i$ response is reflected by IP_3 binding to its receptor (IP_3R) to effect Ca^{2+} release from the endoplasmic reticulum (ER) (79). A secondary “plateau” $[\text{Ca}^{2+}]_i$ response occurs during Ca^{2+} influx through an array of transient receptor potential (TRP) channels (80) following depletion of intracellular Ca^{2+} stores (e.g., ER) (68) (see Figure 2 exemplifying initial peak and then plateau). In general, the Ca^{2+} influx component can serve two purposes: [1] modulate the duration of an elevated $[\text{Ca}^{2+}]_i$ signal response initiated by Ca^{2+} release from the ER and [2] enable refilling of the ER with Ca^{2+} in order to maintain repetitive EC signaling and continued vascular function. It is well understood that increases in $[\text{Ca}^{2+}]_i$ activate $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels and thereby induce a negatively charged cell interior due to K^+ efflux during hyperpolarization (Figure 2). However, the ability of hyperpolarization to govern Ca^{2+} entry by increasing the electrical driving force of Ca^{2+} influx into ECs has been controversial (81). Also, it should be noted that the influx of Ca^{2+} is additionally influenced by a concentration gradient of ~20,000-fold from the extracellular space to the cell interior and the open probability of Ca^{2+} -permeable ion channels (82).

Previous endeavors for addressing the “hyperpolarization-induced Ca^{2+} entry” hypothesis in native ECs have included maximal stimulation of muscarinic receptors (e.g., with agonists such as ACh and methacholine), manipulating extracellular K^+ concentration, activating or inhibiting K^+ channels, and electrically clamping V_m (68, 81). In addition, with the exception of one study using endothelial tubes (68), all studies were performed using intact vessels that include SMCs that express voltage-gated ion channels (7, 31) and are coupled to ECs via myoendothelial junctions (25, 81, 83, 84). Thus, the presence of SMCs in intact vessels may indirectly affect EC V_m and $[\text{Ca}^{2+}]_i$. One to all of such experimental complications may have led to the overall conclusion that changes in endothelial $[\text{Ca}^{2+}]_i$ are independent of changes in V_m (68, 85-87). In particular, maximal stimulation of muscarinic receptors could evoke a supraphysiological level of $[\text{Ca}^{2+}]_i$ that would supersede any observable effect of Ca^{2+} influx due to hyperpolarization of V_m alone. An unavoidable layer of experimental complexity involves the integration of Ca^{2+} release from the ER with Ca^{2+} influx through the plasma membrane following muscarinic receptor stimulation. Unfortunately, omitting Ca^{2+} release from the ER precludes the key stimulus (i.e., “capacitative Ca^{2+} -entry”) needed for appreciable Ca^{2+} influx to occur (88, 89).

The author has proposed a set of guidelines in order to resolve the relationship of V_m and Ca^{2+} entry strictly in accord with the electrochemical driving force: [1] only native endothelium should be present; [2] the experimental approach should not involve voltage-gated ion channels [e.g., as intact vessels with SMCs (7, 90) or isolated endothelium in culture (65)]; [3] V_m should be altered independent of $[\text{Ca}^{2+}]_i$ while both variables are evaluated simultaneously; and [4] experimental stimulations should be of sufficient intensity

and duration to evoke definitive responses without saturating the variables under investigation (91).

The author's approach for addressing the “hyperpolarization-induced Ca^{2+} entry” hypothesis entailed Fura-2 photometry to monitor EC $[\text{Ca}^{2+}]_i$ and intracellular electrodes to control V_m in intact endothelial tubes isolated from male mouse superior epigastric arteries (91). It is important to note that skeletal muscle arterial endothelial tubes do not demonstrate voltage-gated ion channel activity or a “threshold” V_m indicative of excitatory cell types (e.g., cardiac myocytes and neurons) as demonstrated by strict linear voltage responses in accord with a V_m range of -80 mV to $+10$ mV (39, 40, 91). During resting conditions (i.e., no agonist present), a V_m range from ~ -80 mV to $\sim +10$ mV did not influence EC $[\text{Ca}^{2+}]_i$ (91) (Figures 3A and 3C). Despite a robust driving force for Ca^{2+} entry [$\sim 20,000$ -fold concentration gradient in the direction from extracellular to intracellular compartments & electronegativity of cell interior (82)], EC membranes must be permeable to Ca^{2+} in order for Ca^{2+} influx to occur. During M_3 receptor stimulus using a half-maximal (~ 100 nM) concentration of ACh, $[\text{Ca}^{2+}]_i$ increased as V_m hyperpolarized below -40 mV and $[\text{Ca}^{2+}]_i$ decreased as V_m depolarized above -40 mV (Figures 3B and 3D) (91). It is important to note that the dynamic range of $[\text{Ca}^{2+}]_i$ was observed between ~ -60 mV and ~ -25 mV (Figures 3B and 3D) (91), an EC V_m range that contains the greatest physiological relevance for vasodilation and promotion of blood flow (11, 28, 92). These data can also explain how and why past endeavors using maximal muscarinic receptor stimulation (whereby V_m approximates $E_K \approx -80$ mV to -90 mV) can mask the effect of membrane hyperpolarization alone on Ca^{2+} influx.

Whether it is believed that the hyperpolarization-induced Ca^{2+} entry mechanism in ECs appreciably underlies regulation of blood flow or not (81, 93), a strict regulation of Ca^{2+} entry and regulation of $[\text{Ca}^{2+}]_i$ needs to be maintained as Ca^{2+} (vs. Na^+ , K^+ , and Cl^-) also serves as a second messenger to rapidly mediate signaling underlying cell life or death (94). Regardless of V_m alterations, it is clear that Ca^{2+} influx is not significant under resting conditions where Ca^{2+} -permeable “gateways” (e.g., TRP channels) are relatively deactivated or blocked (91, 95) (Figures 3A and 3C). Several TRPs have been recognized for mediating EC Ca^{2+} entry such as the canonical (TRPC; isoforms 1, 3, 4, 5 & 6), vanilloid (TRPV; isoforms 1, 3 & 4), and ankyrin [TRPA; isoform 1 (96)] families (80). Furthermore, such isoforms may assemble into homomeric or heteromeric channels (97-101) and additionally incorporate the stromal interaction molecule (STIM) and Orai channel signaling axis that underlies storage-operated Ca^{2+} entry following depletion of ER Ca^{2+} (89). Due to a powerful combination of pharmacological and transgenic animal tools (102), studies of the TRPV4 channel in particular have been the most productive for locating the precise source of Ca^{2+} entry in ECs (4, 83, 91, 95, 103, 104). Elimination of TRPV4 function [via use of TRPV4 knock out ($\text{TRPV4}^{-/-}$) animals] reduces Ca^{2+} signaling and vasodilation in response to ACh by 70% (83, 95, 104). Experiments using endothelial tubes isolated from $\text{TRPV4}^{-/-}$ animals indicate that TRPV4 accounts for approximately half of the capacity to increase $[\text{Ca}^{2+}]_i$ during $V_m > -40$ mV (91). However, such effects are most apparent during $V_m \sim -60$ mV (91) which coincides with an excessive degree of K_{Ca} activation (39, 71) and loss of myogenic tone (4); conditions that may reflect severe pathology (e.g., septic shock) and significant impairment of blood flow control.

Modulation of EC V_m to regulate EC $[Ca^{2+}]_i$ (91) may be used to treat conditions of impaired vasodilation during aging (5, 105) or loss of vascular reactivity during conditions such as septic shock (106, 107). At the present, the most effective approach for advancing this mechanism is to use synthetic compounds that can finely modulate (e.g., SKA-31, NS309) or block (e.g., UCL 1684, TRAM-34) SK_{Ca}/IK_{Ca} channel activity to influence vasodilation and vasoconstriction respectively as needed. Most studies have focused on promoting vasodilation, whereby K^+ channel activation with SKA-31 may ameliorate hypertension (108, 109) or enhance coronary blood flow in the heart during diabetes (110). With the hyperpolarization-induced Ca^{2+} entry mechanism in ECs intact, the benefits underlying mild SK_{Ca}/IK_{Ca} channel activation would not only include enhanced Ca^{2+} entry to further amplify SK_{Ca}/IK_{Ca} channel activity but boosted production of NO as well. However, activating SK_{Ca}/IK_{Ca} channels to the extent where V_m exceeds -60 mV results in current leak that reduces the spread of hyperpolarization among ECs by more than half (39, 71). Significant charge loss through electrically leaky membranes may impair conducted vasodilation and thereby, the coordination of blood flow among vascular resistance networks (5). Thus, as with any important physiological mechanism, it is recommended that modulating $[Ca^{2+}]_i$ and/or V_m via activation or blockade of ion channels should be treated with caution.

Effects of aging and chronic vascular disease on endothelium-derived hyperpolarization

Gerontology experts have projected that individuals of old age (> 65 years) will compose at least 25% of the United States population by 2030 (111). Aging is the key risk factor for chronic conditions such as cardiovascular disease (112, 113), whereby endothelial “dysfunction” is a prime marker (114, 115). This disorder is characterized by impaired vasodilation in response to ACh (116-118) or physical activity (117, 119). In general, such observations are corroborated by findings obtained from *in vivo* (e.g., intravital microscopy) and *ex vivo* (e.g., pressure myography) studies of experimental rodents that are “old” (i.e., 20 months of age) (48, 120-124). In particular, conducted vasodilation in response to ACh (120) and ascending vasodilation in response to skeletal muscle contraction (48, 122, 124) are reduced in old mice in comparison to animals 3 to 4 months of age (“young”). It is well known that NO bioavailability decreases in old age and that molecules of oxidative signaling [e.g., hydrogen peroxide (H_2O_2)] may play a compensatory role for maintaining vasodilation and tissue perfusion (75, 125-128).

In comparison to findings for actions of NO, how aging and conditions of high risk during old age (e.g., diabetes, hypertension) impact EDH through SK_{Ca}/IK_{Ca} channel activation is less clear. Some studies indicate a loss of endothelial SK_{Ca}/IK_{Ca} channel-dependent vasodilation (129-133), whereas others have found an enhancement in SK_{Ca}/IK_{Ca} channel function of intact vessels (128, 134-139) and isolated endothelial tubes (71). This discrepancy in conclusions can be attributed to differences in species of experimental animals (e.g., mouse vs. rat), vascular type [(regional differences: e.g., skeletal muscle vs. gut) & (size: aorta & large arteries vs. feed arteries & arterioles)], assigned definitions of “aged” or “old” animals ($< 2 >$ years of age) and/or extent of severity in vascular pathology. Perhaps most importantly, studies of intact vessels encounter confounding factors that interfere with testing EC function such as the presence of perivascular nerves, SMCs, blood

flow and hormones. The presence of SMCs presents activity of high conductance BK_{Ca} channels which may mask analysis of the more modest conductance of SK_{Ca}/IK_{Ca} channels in ECs (7). Also, signaling pathways complementary to EDH such as the $[Ca^{2+}]_i$ activation of eNOS and BK_{Ca} on SMCs hyperpolarize ECs through myoendothelial coupling. These vasodilatory processes are additionally modulated by endothelial mitochondria and reactive oxygen species production (140, 141). Indeed, BK_{Ca} channel function has been demonstrated to increase in smooth muscle cells of mouse superior epigastric arteries of old mice (24 to 26 months) (15). Finally, conclusions between studies can differ based on a lack of awareness for structural changes of the vasculature (e.g., arterial stiffening, smooth muscle hypertrophy) that occur alongside ion channel activity during aging (15-17, 131). At least in skeletal muscle feed arteries, the underlying components of EDH signaling (i.e., increased $[Ca^{2+}]_i$ and SK_{Ca}/IK_{Ca} channel function) are enhanced during old age (24 months) due to increased levels and activity of H_2O_2 (71, 142). This increase in EDH signaling in old age may be viewed as a compensatory mechanism (67) in response to a decreased NO bioavailability for vasodilation (67, 75). Although, as a trade-off, the consequence of enhanced SK_{Ca}/IK_{Ca} channel signaling is membrane “leak” of electrical charge and thereby, a restricted spatial domain for blood flow control along vascular resistance networks (3, 50, 71). See Figure 4 for a proposed working model on the effects of aging and chronic vascular disease on endothelial SK_{Ca}/IK_{Ca} channel function.

Summary, Conclusions, and Future Directions

The endothelium of blood vessels is an organ distributed throughout the body to help coordinate the perfusion of all other key organs and tissues. Aside from its well-known barrier function, endothelial cells have recently been recognized for exciting and unique manifestations of intracellular Ca^{2+} and electrical signaling versus “excitable cells” such as neurons and cardiac myocytes. Endothelial tubes have been developed as an ideal study model for resolving physiological integration at the level of the endothelium alone (e.g., local and conducted Ca^{2+} and electrical signals) while eliminating confounding influences (e.g., perivascular nerves, SMCs, myoendothelial coupling, transmural pressure, and blood flow). Although developed from rodent skeletal muscle arteries, this approach will likely emerge and reveal new arrangements of integration between Ca^{2+} , oxidative, and V_m signaling in many other vascular types recognized for the local and remote coordination of vasoreactivity such as those in the brain (143, 144), retina (145), heart (146), lung (147), mesentery (37, 86, 148) and kidney (149, 150). With experimental advantages discussed in this review, it should also be recognized that endothelial tubes lack myoendothelial projections to SMCs, and thus, key microdomain Ca^{2+} signaling events relayed between TRP channels, IP_3Rs , and SK_{Ca}/IK_{Ca} channels (4, 25, 83, 151, 152) may be disrupted. In addition, the lack of coupling to SMCs and loss of physiological input by high conductance voltage-gated channels (e.g., K_v and BK_{Ca} channels) may alter the otherwise typical resting V_m and electrical activity of endothelial cells in intact vessels (40, 153, 154). With respective experimental limitations in mind, developing a dynamic bidirectional basis of study between isolated endothelium and complex *ex vivo* (i.e., isolated and pressurized blood vessels) and/or *in vivo* (i.e., live animal + intact vasculature and parenchyma) systems will advance our knowledge of vascular physiology and development of disease.

Endothelial “dysfunction” has been primarily attributed to a decreased bioavailability of nitric oxide. With recognition of the spatial regulation of blood flow, SK_{Ca}/IK_{Ca} channel function may sustain local vasodilation with aging, whereas conducted vasodilation may be restricted due to appreciable membrane current “leak”. Effective translational approaches could include the fine pharmacological tuning of the activity of plasma membrane channels such as the SK_{Ca}/IK_{Ca} in order to restore effective vascular reactivity and blood flow control in the face of chronic alterations in vascular function. For completeness, we should also consider structural changes in the vascular wall (e.g., arterial stiffening, smooth muscle hypertrophy) that collectively serve as an additional source of impediment to effective blood flow regulation. Whether studies are centered on ion channel function and/or structural alterations, a primary goal is to locate and understand the subtle transitions from points of physiology to pathology. An effective method of addressing this is to test animal models of healthy aging alongside others that display a shortened or extended lifespan and quality of life. Also, consideration for gender should be incorporated as male animals have comprised the majority of studies performed thus far (~80%) (155, 156), whereby females (~50% of the population) have been effectively neglected. Altogether, the integration of oxidative, Ca^{2+} , and electrical signaling during aging and cardiovascular disease remains a strong avenue of research to be elucidated and employed for therapeutic interventions.

PERSPECTIVES

As an organ distributed throughout the entire body, the primary function of blood vessel endothelium is to coordinate delivery of blood flow to all other organs and tissues in accord with metabolic demand. Experimental use of isolated resistance artery endothelial “tubes” has revealed the dynamic regulation of vasodilatory Ca^{2+} and electrical signals along electrically coupled endothelial cells by Ca^{2+} activated K^+ (SK_{Ca}/IK_{Ca} or $K_{Ca2.3}/K_{Ca3.1}$) channels. As nitric oxide signaling decreases during aging, the resilient activity of endothelial SK_{Ca}/IK_{Ca} channels may be calibrated as needed to fulfill a healthy integration of oxidative, Ca^{2+} , and electrical signals and thereby, prevent cardiovascular disease.

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Abbreviations

ACh	acetylcholine
BK_{Ca} channel	large-conductance Ca^{2+} -activated K^+ channel
cGMP	cyclic guanosine monophosphate
$[Ca^{2+}]_i$	intracellular Ca^{2+}
EC	endothelial cell
EDH	endothelium-derived hyperpolarization
E_K	equilibrium potential of K^+

ER	endoplasmic reticulum
eNOS	endothelial nitric oxide synthase
IP₃	inositol trisphosphate
IP₃R	inositol trisphosphate receptor
K_V channel	voltage-gated K ⁺ channel
M₃ receptor	Muscarinic type 3 receptor
MLC	myosin light chain
NO	nitric oxide
PE	phenylephrine
PKG	Protein Kinase G
SMC	smooth muscle cell
SK_{Ca}/IK_{Ca} channels	small- and intermediate-conductance Ca ²⁺ -activated K ⁺ channels
TRP channel	transient receptor potential channel
V_m	membrane potential

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Figure 1.

Sharp electrode recordings of V_m and electrical conduction along endothelium of skeletal muscle resistance arteries: Central role of SK_{Ca}/IK_{Ca} channels. (A) Brightfield image of an endothelial tube isolated from a male mouse superior epigastric artery with dual intracellular electrodes positioned for current injection (Site 1, *left* red oval) and respective V_m measurements (Site 2, *right* orange oval). (B) Representative V_m recording at Site 2 during ± 0.1 to 3 nA injected at site 1 (distance = 500 μ m) before and during a treatment with a maximal concentration of the SK_{Ca}/IK_{Ca} channel opener NS309 (10 μ M) and (C) hydrogen peroxide (H_2O_2 ; 200 μ M). Note progressive hyperpolarization to -90 mV ($\sim E_K$) and elimination of conducted V_m2 responses. Please note that NS309 responses are not significantly dependent on $[Ca^{2+}]_i$; increases (91). A robust increase of $[Ca^{2+}]_i$ underlies hyperpolarization in response to H_2O_2 (142). Hyperpolarization via H_2O_2 treatment is sensitive to SK_{Ca}/IK_{Ca} channel block by apamin and charybdotoxin (71). Illustrated endothelial tube and data are from (39, 71) with permission.

Figure 2.

Intimate cooperation of $[\text{Ca}^{2+}]_i$ and hyperpolarization of V_m during treatment with the M_3 receptor agonist acetylcholine in resistance artery endothelium: classic representation of the endothelium-derived hyperpolarization (EDH) pathway. Traces indicate simultaneous F_{340}/F_{380} (top) and V_m (bottom) recordings during treatment with a maximal concentration of ACh ($3 \mu\text{M}$). Arrowheads indicate Rest, Peak and Plateau (90 s after Peak) phases of the integrated EDH response respectively. The initial Peak phase represents Ca^{2+} release from the endoplasmic reticulum whereas the sustained Plateau phase indicates Ca^{2+} influx into the cell interior across the plasma membrane (68, 91). F_{340}/F_{380} data (top) were obtained from ~50 cells in a Fura-2 loaded endothelial tube isolated from a male mouse superior epigastric artery using an IonOptix system equipped with a camera and photomultiplier tube.

Figure 3.

Demonstration of endothelial “hyperpolarization-induced Ca^{2+} entry” in resistance artery endothelium during submaximal stimulation of M_3 receptors with 100 nM ACh. (A) Simultaneous V_m (top) and F_{340}/F_{380} (bottom) recordings during control conditions (i.e., no ACh present) and (B) during treatment with ACh (100 nM). Current injection was applied from -5 to $+5$ nA in 20 s pulses (arrowheads). (C) Summary data illustrating stability of F_{340}/F_{380} throughout range of V_m (~ -80 mV to $\sim +10$ mV) during respective levels of current injection ($n = 7$ endothelial tubes from 6 mice). (D) Summary data illustrating a sigmoidal response of F_{340}/F_{380} values throughout range of V_m . Note increase in F_{340}/F_{380} with the increasing magnitude of hyperpolarization (maximum: ~ -60 mV to ~ -70 mV) and a decrease in F_{340}/F_{380} to baseline levels as V_m approximates 0 mV ($n = 30$ endothelial tubes from 25 mice). $*P < 0.05$; F_{340}/F_{380} during current injection versus F_{340}/F_{380} without current injection (grey point). Data were obtained in Fura-2 loaded endothelial tubes isolated from male mouse superior epigastric arteries. Data are from (91) with permission.

Figure 4.

General working model of the effects of aging and chronic vascular disease on endothelial SK_{Ca}/IK_{Ca} channel function. In old age and chronic disease, oxidative signaling increases via superoxide (O₂^{•-}) production from a variety of intracellular sources (NADPH oxidases, mitochondria, uncoupled nitric oxide synthase, and xanthine oxidase) which inactivates nitric oxide to peroxynitrite (75, 77). Via superoxide dismutase, O₂^{•-} is rapidly converted to the relatively stable intermediate H₂O₂ [spontaneous breakdown product: hydroxyl radical (OH[•])] (74, 78) which increases SK_{Ca}/IK_{Ca} (primarily IK_{Ca}) channel function (71, 128). While upregulation of SK_{Ca}/IK_{Ca} channel function may be viewed as a compensatory mechanism for sustaining local vasodilation (67), this may be at the expense of a restricted spatial domain of the spread of hyperpolarization and vasodilation via appreciable loss of charge via “leaky” endothelial plasma membranes (3, 50, 71).