Positive Feedback Regulation of Agonist-Stimulated Endothelial Ca²⁺ Dynamics by KCa3.1 Channels in Mouse Mesenteric Arteries

Xun Qian, Michael Francis, Ralf Köhler, Viktoriya Solodushko, Mike Lin, Mark S. Taylor

Objective—Intermediate and small conductance KCa channels IK1 (KCa3.1) and SK3 (KCa2.3) are primary targets of endothelial Ca²⁺ signals in the arterial vasculature, and their ablation results in increased arterial tone and hypertension. Activation of IK1 channels by local Ca²⁺ transients from internal stores or plasma membrane channels promotes arterial hyperpolarization and vasodilation. Here, we assess arteries from genetically altered IK1 knockout mice (IK1−/−) to determine whether IK1 channels exert a positive feedback influence on endothelial Ca²⁺ dynamics.

Approach and Results—Using confocal imaging and custom data analysis software, we found that although the occurrence of basal endothelial Ca²⁺ dynamics was not different between IK1−/− and wild-type mice (P>0.05), the frequency of acetylcholine-stimulated (2 μmol/L) Ca²⁺ dynamics was greatly decreased in IK1−/− endothelium (515±153 versus 1860±319 events; P<0.01). In IK1−/−/SK3T/T mice, ancillary suppression (+Dox) or overexpression (−Dox) of SK3 channels had little additional effect on the occurrence of events under basal or acetylcholine-stimulated conditions. However, SK3 overexpression did restore the decreased event amplitudes. Removal of extracellular Ca²⁺ reduced acetylcholine-induced Ca²⁺ dynamics to the same level in wild-type and IK1−/− arteries. Blockade of IK1 and SK3 with the combination of charybdotoxin (0.1 μmol/L) and apamin (0.5 μmol/L) or transient receptor potential vanilloid 4 channels with HC-067047 (1 μmol/L) reduced acetylcholine Ca²⁺ dynamics in wild-type arteries to the level of IK1−/−/SK3T/T+Dox arteries. These drug effects were not additive.

Conclusions—IK1, and to some extent SK3, channels exert a substantial positive feedback influence on endothelial Ca²⁺ dynamics. (Arterioscler Thromb Vasc Biol. 2014;34:127-135.)

Key Words: calcium • endothelium • SK3 protein • TRPV4 protein

Endothelial Ca²⁺-activated potassium channels (KCa), including small conductance (SK3 or KCa2.3) and intermediate conductance (IK1 or KCa3.1) isoforms, are important effectors of vasodilation in the arterial circulation. These channels elicit endothelium-derived hyperpolarization (EDH) of vascular smooth muscle, and their pharmacological inhibition completely blocks nitric oxide (NO)-independent and prostacyclin-independent vasodilation in various arterial beds.1-6 Studies of IK1-deficient mice (IK1−/−) have revealed a pivotal role of this channel in hyperpolarizing the endothelial membrane, dilating resistance arteries, and modulating blood pressure.7 A combined transgenic mouse model (IK1−/−/SK3T/T), which includes conditional doxycycline (Dox)-controlled SK3 channel expression8-9 in addition to the conventional IK1 knockout genotype, has allowed for detailed elucidation of the combined and complementary roles of these channels.9 This genetic model has demonstrated the pivotal role of IK1 channels in agonist-induced (eg, acetylcholine [ACh]) dilations of conduit arteries and arterioles and has exposed the supporting role of SK3 channels, both in facilitating NO-dependent dilations and in compensating for the loss of IK1 channels. Genetic co-suppression of both channels blunts maximal ACh dilations by ≈60%.9

Recent findings have implicated IK1 channels as direct targets of dynamic repetitive and short-lived (250 ms to 3 seconds) Ca²⁺ events that occur primarily along myoendothelial junctions,10 establishing a persistent mechanism for hyperpolarizing vascular smooth muscle and modulating arterial tone. In addition to this basal activation, endothelial stimulation with agonist (eg, ACh) increases total dynamic events by increasing the number of Ca²⁺ liberating sites (often multiple sites per cell) along the intima and increasing the frequency of existing active sites.10 Although the primary Ca²⁺ events (Ca²⁺ pulsars) emit from IP3R on the endoplasmic reticulum, plasma membrane transient receptor potential (TRP) channels,11-13 including TRPA1 in rat cerebral arteries14-16 and TRPV4 in mouse mesenteric arteries,15-17 have recently been implicated as important triggers or potentiators of the endothelial Ca²⁺ dynamics.
and vasodilation. These TRP channels have been found to associate closely with KCa channels, and their direct stimulation promotes KCa-dependent EDH-mediated vasodilation in mice. In mouse mesenteric arteries, ACh has been found to evoke spatially restricted Ca\(^{2+}\) sparklets through TRPV4 channels, and these signals augment IK1 channel activation. Because Ca\(^{2+}\)-induced K\(^+\) efflux through IK1 channels and the resulting hyperpolarization might increase the electrochemical driving force for Ca\(^{2+}\) entry, a central question addressed in the current study is whether IK1 channels control endothelial Ca\(^{2+}\) dynamics as a potential positive feedback mechanism.

Quantifying endothelial Ca\(^{2+}\) dynamics is essential for understanding effector recruitment and graded vasodilation. We recently developed and implemented an autodetection and analysis algorithm that allows for comprehensive evaluation of dynamic Ca\(^{2+}\) transients and complex Ca\(^{2+}\) signal distributions in intact tissues, including endothelium. Here, we used IK1\(^{-/-}\) mice and our automated Ca\(^{2+}\) signal analysis to discern whether IK1 channels, alone or in combination with SK3 channels, enhance endothelial Ca\(^{2+}\) dynamics in mesenteric arteries.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**IK1 Channel Ablation Does Not Affect the Frequency of Basal Endothelial Ca\(^{2+}\) Dynamics**

To determine whether IK1 channels provide positive feedback regulation of ongoing Ca\(^{2+}\) dynamics in the endothelium, we evaluated fluo-4 acetoxyethyl-loaded open mesenteric arteries from wild-type (WT) and IK1-deficient (IK1\(^{-/-}\)) transgenic mice using confocal microscopy (Figure 1). The arterial endothelia of all WT, IK1\(^{-/-}\), and IK1\(^{-/-}\)/SK3\(^{T/T}\) mice produced basal Ca\(^{2+}\) transients under resting conditions (Figure 2A). In arteries

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Detection and analysis of endothelial Ca\(^{2+}\) dynamics in open mouse mesenteric arteries. Opened mesenteric arteries were mounted on silicone blocks (intima-up) and loaded with fluo-4 acetoxyethyl and the endothelium was imaged with a spinning disk confocal. Image shows mean fluorescence projection of a 2-minute recording. Image sequences were analyzed offline using LC-Pro, allowing regions of interest (ROIs) to be placed automatically at the spatial centers of detected events (20 of the 162 ROIs are shown). Inset shows recordings from 3 distinct sites (ROIs) emphasizing the variety of basal Ca\(^{2+}\) events with respect to frequency, amplitude, and duration.
Figure 2. Effect of IK1 and SK3 expression on basal endothelial Ca\textsuperscript{2+} dynamics. A, Tracings show Ca\textsuperscript{2+} transients recorded in mesenteric arteries from wild-type (WT) mice, IK1 knockout (IK1\textsuperscript{-/-}) mice, and IK1 knockout mice in which SK3 expression is suppressed (IK1\textsuperscript{-/-}/SK3\textsuperscript{TT+DOX}) or overexpressed (IK1\textsuperscript{-/-}/SK3\textsuperscript{TT-DOX}). B, The number of Ca\textsuperscript{2+} sites and events occurring per minute within sampled fields. Neither sites nor events were different among groups (P>0.05 for all comparisons; n=6). C, Individual event parameters (*P<0.01 vs WT). Dox indicates doxycycline.
Figure 3.
from WT mice, these events occurred at rate of 307±38 per minute from 112±11 sites. Although the number of events and sites trended lower in the IK1−/− mice (279±35 per minute from 96±15 sites), no significant differences were discerned (P>0.05; n=6; Figure 2B). Evaluation of IK1 knockout mice in which SK3 channel expression was either suppressed (IK1−/−/SK3T/T+Dox) or overexpressed (IK1−/−/SK3T/T−Dox) also showed no significant difference from WT or IK1−/− alone (231±12 per minute from 84±8 sites and 212±26 per minute from 95±6 sites, respectively). These data suggest no net influence of IK1/SK3 channels on the basal generation of endothelial Ca2+ dynamics, including the number of events, sites, and events per site. Assessment of specific Ca2+ event parameters (Figure 2C) revealed that event amplitudes were decreased in IK1−/− and IK1−/−/SK3T/T+Dox mice to a similar degree (P<0.01 versus WT) and were recovered to WT levels in SK3 overexpressing IK1−/−/SK3T/T−Dox mice. Also, Ca2+ event durations were significantly elevated in IK1−/−/SK3T/T−Dox mice when compared with all other genotypes, including WT.

**ACh-Induced Endothelial Calcium Dynamics Are Reduced in Arteries of IK1-Deficient Mice**

ACh increases Ca2+ dynamics in mesenteric artery endothelial cells by sensitizing release from internal Ca2+ stores through IP3 receptors and by stimulating influx of extracellular Ca2+ through membrane nonselective cation channels, both of which are known to target KCa channels.10,17 We examined whether a positive influence of KCa channels on Ca2+ dynamics is unmasked under these stimulated conditions. Figure 3A shows Ca2+ recordings from the endothelia of WT, IK1−/−, and IK1−/−/SK3T/T+Dox or IK1−/−/SK3T/T−Dox mice before and after addition of 2 μmol/L ACh. ACh markedly increased Ca2+ dynamics, which included a rapid phase of multiple synchronous peaks followed by a sustained phase of heterogeneous transients. Overall, ≈70% of the endothelial cells in WT arteries responded to ACh challenge when compared with ≈25% of the endothelial cells in IK1−/−, 20% in IK1−/−/SK3T/T+Dox, and 30% in IK1−/−/SK3T/T−Dox arteries. With respect to the total number of events generated for the full 180-second time course of ACh exposure (Figure 3B), responses were blunted in IK1−/− arteries when compared with WT arteries (515±153 versus 1860±319 events; P<0.001; n=6). The number of events was seemingly further decreased in SK3-suppressed (IK1−/−/SK3T/T+Dox) arteries (409±74 events) and partially recovered in SK3-overexpressed (IK1−/−/SK3T/T−Dox) arteries (685±57 events), but neither was significantly different from IK1−/− alone, suggesting no additional net effect of SK3 channel suppression or overexpression. Notably, the frequency of events per site was similarly decreased in all IK1-deficient arteries when compared with WT arteries (WT, 4.2±0.6; IK1−/−, 2.4±0.2; IK1−/−/SK3T/T+Dox, 1.6±0.06; and IK1−/−/SK3T/T−Dox, 2.1±0.1; P<0.05 for all compared with WT), suggesting that loss of IK1 reduces both the number of active sites and the number of events occurring at each site. ACh responses may be divided into 2 phases: an initial phase primarily attributable to internal store release and a sustained phase that is highly dependent on extracellular Ca2+ entry.21 Partitioning our 3-minute ACh responses into initial (first 30 seconds) and sustained (30–180 seconds) phases
revealed that IK1 channels primarily influence the occurrence of Ca\textsuperscript{2+} events in the later phase (Figure 3B). With respect to individual event parameters (Figure 3C), event amplitudes were reduced in IK1\textsuperscript{−/−} and IK1\textsuperscript{−/−−}/SK3\textsuperscript{TT+Dox} and restored in IK1\textsuperscript{−/−SK3\textsuperscript{TT−Dox}}, similar to basal data, whereas duration and spatial spread were not different among the groups.

**K\textsubscript{Ca} Channel Potentiation of Endothelial Ca\textsuperscript{2+} Dynamics Depends on Extracellular Ca\textsuperscript{2+} Entry Through TRPV4 Channels**

Next, we directly tested the role of extracellular Ca\textsuperscript{2+} by removing it from the bath. Ach-stimulated Ca\textsuperscript{2+} dynamics were substantially blunted in the absence of extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+}-free+1 \(\mu\text{mol/L} \) EGTA), particularly after the first 30 seconds (Figure 4A and 4B). Importantly, under Ca\textsuperscript{2+}-free conditions, Ach-stimulated Ca\textsuperscript{2+} events were not significantly different among the genotypes (WT, 256±44; IK1\textsuperscript{−/−}, 140±44; IK1\textsuperscript{−/−}/SK3\textsuperscript{TT−Dox}, 340±72; and IK1\textsuperscript{−/−}/SK3\textsuperscript{TT−Dox}, 227±96) at any phase of the response (Figure 4B), indicating that the K\textsubscript{Ca} channels primarily enhance Ca\textsuperscript{2+} influx.

TRPV4 channels are major conduits of Ca\textsuperscript{2+} entry in the mesenteric artery endothelium.\textsuperscript{23} They carry discrete Ach-stimulated Ca\textsuperscript{2+} transients at the plasma membrane that are known to elicit K\textsubscript{Ca} channel activation.\textsuperscript{12} We assessed whether the IK1 augmentation of endothelial Ca\textsuperscript{2+} dynamics in normal arteries occurs through potentiation of TRPV4 channel Ca\textsuperscript{2+} entry. Here, we used WT arteries where native feedback signaling is preserved. The TRPV4 channel blocker HC-067047 (1 \(\mu\text{mol/L} \)) had no significant effect on basal dynamics (data not shown), but it greatly impaired Ach activation of Ca\textsuperscript{2+} events (254±72 versus 1517±272; Figure 5), supporting a central role for TRPV4 in Ach-stimulated Ca\textsuperscript{2+} entry. Notably, pharmacological blockade of IK1 and SK3 channels with the combination of charybdotoxin (0.1 \(\mu\text{mol/L} \)) and apamin (0.5 \(\mu\text{mol/L} \)) decreased Ach-induced Ca\textsuperscript{2+} dynamics in WT arteries (to 364±123 events during the 30-second to 180-second phase), values comparable with those in IK1\textsuperscript{−/−}/SK3\textsuperscript{TT+Dox} arteries. The reduction of Ca\textsuperscript{2+} dynamics elicited by charybdotoxin and apamin plus HC-067047 (99±38 events) was not significantly different from that resulting from HC-067047 treatment alone (Figure 5). Notably, Ca\textsuperscript{2+} events in IK1\textsuperscript{−−}/SK3\textsuperscript{TT+Dox} arteries were not significantly altered by addition of charybdotoxin and apamin with or without HC-067047 (data not shown).

**Discussion**

Small/intermediate conductance K\textsubscript{Ca} channels have been identified as primary targets of basal and Ach-stimulated Ca\textsuperscript{2+} dynamics in arterial endothelium.\textsuperscript{10,12} Previous work has shown that genetic ablation of IK1 and SK3 channels impairs vascular hyperpolarization and Ach-induced vasodilation and promotes hypertension.\textsuperscript{5,7} Here, we used IK1-deficient mice, including those with suppressed or overexpressed SK3, to assess whether these channels exert a positive feedback influence on the endothelial Ca\textsuperscript{2+} dynamics themselves. Our data indicate that although IK1 channels do not significantly influence ongoing basal Ca\textsuperscript{2+} dynamics in mesenteric artery endothelium, they do strongly potentiate the occurrence of Ca\textsuperscript{2+} dynamics after endothelial stimulation with Ach. Increasing or decreasing SK3 expression had little additional effect on the occurrence of events but did promote increased event amplitudes and durations. We found that K\textsubscript{Ca}-promoted Ca\textsuperscript{2+} dynamics were completely dependent on extracellular Ca\textsuperscript{2+} entry through TRPV4 channels. Together, these findings suggest that IK1 channels may play an important role in amplifying vasodilation by expanding TRPV4-triggered dynamic Ca\textsuperscript{2+} signals along the intima, whereas SK3 channels may play a supporting role in adjusting event size.

Recent studies have revealed the importance of spatially and temporally distinct Ca\textsuperscript{2+} transients, rather than global Ca\textsuperscript{2+} changes, in tuning the specificity and magnitude of endothelial responses in intact arteries.\textsuperscript{10,16–18,24} These inherent Ca\textsuperscript{2+} dynamics are clearly discernible in the endothelia of both pressurized and opened mesenteric arteries.\textsuperscript{20} Confocal imaging in pressurized arteries is limited to few endothelial cells because of narrow viewable fields and movement artifact. Using open artery preparations in the current study allowed us to quantify event parameters comprehensively in broad intact endothelial fields using our custom software (LC_Pro). Designed to detect all Ca\textsuperscript{2+} deflections above noise without user bias, this approach reveals a heterogeneous assortment of intrinsic events occurring along the vascular intima. In mesenteric arteries, basal endothelial Ca\textsuperscript{2+} dynamics emit intermittently from internal stores through IP\textsubscript{3}Rs.\textsuperscript{8} These signals elicit hyperpolarization by engaging nearby IK1 channel clusters in the endothelial membrane, particularly at myoendothelial junction sites.\textsuperscript{10} At these sites, EDH is communicated to smooth muscle through gap junctions\textsuperscript{1,25–27} or via K\textsuperscript{+} activation of inward rectifier K\textsuperscript{+} channels (K\textsubscript{ir}) or Na\textsuperscript{+}/K\textsuperscript{+} ATPase.\textsuperscript{28,29} Endothelial stimulation with Gq-coupled receptor agonist (eg, Ach) amplifies this hyperpolarization and, hence, vasodilation by recruiting new Ca\textsuperscript{2+}-liberating sites along the intima and by increasing the frequency of dynamic Ca\textsuperscript{2+} events at preexisting sites.\textsuperscript{10} New data suggest that in addition to IP\textsubscript{3} elevation, this Ca\textsuperscript{2+} recruitment depends on stimulation of membrane TRPV4 channels,
which increases the occurrence of focal Ca\textsuperscript{2+} sparklet events along the plasma membrane.\textsuperscript{17} These transients are known to activate IK1 channels directly and to provoke large increases in endothelial Ca\textsuperscript{2+} dynamics. The latter effect likely involves Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from IP\textsubscript{3}-sensitized stores. In fact, isolated TRPV4 sparklets are only discernible when internal stores are depleted.\textsuperscript{17} Overall, the agonist-augmented Ca\textsuperscript{2+} dynamics support further vasodilation through expanded KCa\textsubscript{3.1}-mediated hyperpolarization, as well as endothelial NO synthesis-dependent NO production.\textsuperscript{10}

The pivotal functional role of IK1 channels has been well-demonstrated in pharmacological studies and is clearly evident in IK1-deficient mice that exhibit blunted ACh-induced hyperpolarization and dilation (in both conduit and resistance arteries), as well as hypertension.\textsuperscript{7,9} SK3 suppression further exacerbates these dilator and blood pressure changes, whereas SK3 overexpression partially recovers them.\textsuperscript{9} It should be noted that endothelial Ca\textsuperscript{2+} dynamics consistently recruit IK1 and SK3 channels in the vasculature,\textsuperscript{5,16,17} regulating membrane potential and tone (ie, via direct communication of the membrane potential to smooth muscle via gap junctions). This effect may constantly modify blood pressure with or without altering the Ca\textsuperscript{2+} signals themselves. However, the lack of voltage-gated Ca\textsuperscript{2+} channels in nonexcitable endothelial cells allows IK1 channels to act not only as Ca\textsuperscript{2+} detectors but also as Ca\textsuperscript{2+} amplifiers, whereby Ca\textsuperscript{2+}-activated K\textsuperscript{+} efflux and hyperpolarization increase the driving force for Ca\textsuperscript{2+} entry. We found that in arteries from IK1-deficient mice, basal endothelial Ca\textsuperscript{2+} dynamics are not significantly altered, whereas ACh-stimulated Ca\textsuperscript{2+} dynamics are substantially muted. This suggests that endothelial stimulation is needed to drive sufficient IK1-dependent positive feedback Ca\textsuperscript{2+} entry to enhance dynamics. The specific effect of IK1 channels on Ca\textsuperscript{2+} entry is supported by our observations that IK1 knockout decreased sustained ACh Ca\textsuperscript{2+} dynamics without affecting the initial Ca\textsuperscript{2+} release (first 30 seconds).\textsuperscript{21,31} and ACh responses in the absence of extracellular Ca\textsuperscript{2+} were indistinguishable between WT and IK1\textsuperscript{−/−} arteries.

Our findings support TRPV4 channels being the primary targets of both ACh-stimulated Ca\textsuperscript{2+} influx and the IK1 Ca\textsuperscript{2+} feedback. In WT arteries, selective inhibition of TRPV4 channels greatly blunted the occurrence of sustained stimulated Ca\textsuperscript{2+} dynamics, similar to that achieved with Ca\textsuperscript{2+}-free solution (Figure 4) or general inhibition of nonselective cation channel influx with Gd\textsuperscript{3+} (data not shown). ACh-induced vasorelaxation is greatly impaired in arteries of TRPV4-deficient mice (TRPV4\textsuperscript{−/+}), including a general loss of EDH.\textsuperscript{32} However, intact ACh-induced dilation has been observed in carotid arteries of TRPV4\textsuperscript{−/+} mice, suggesting regional differences among beds.\textsuperscript{19} Notably, in the current study, specific pharmacological blockade of IK1/SK3 channels in WT arteries mimicked the effect of genetic IK1/SK3 knockdown on ACh-stimulated Ca\textsuperscript{2+} dynamics. Moreover, IK1/SK3 inhibition was not additive with TRPV4 inhibition, suggesting that this effect of KCa\textsubscript{3.1} activation requires TRPV4-mediated Ca\textsuperscript{2+} entry. Importantly, we have also found that the TRPV4 and IK1/SK3 effects on Ca\textsuperscript{2+} dynamics are preserved at 37°C (Figure I in the online-only Data Supplement), supporting their contribution under physiological conditions.

The effect of IK1 on Ca\textsuperscript{2+} dynamics seems to be multifaceted. Although they modestly augment Ca\textsuperscript{2+} event amplitude, under both basal and agonist-stimulated conditions their most notable effect is acute recruitment of new events, which involves soliciting de novo Ca\textsuperscript{2+} firing sites along the intima as well as increasing firing frequencies at preexisting sites (Figure 3). This influence is highly dependent on TRPV4, implying that IK1 channels can tune endothelial responses by critically expanding subtle TRPV4 Ca\textsuperscript{2+} entry transients into robust repetitive Ca\textsuperscript{2+} events. Physiologically, these triggered events may be further amplified by Ca\textsuperscript{2+} store release, expanding recruitment of KCa\textsubscript{3.1} channels, as well as other Ca\textsuperscript{2+}-dependent effectors. We recently reported a similar triggering role of TRP channels in rat cerebral arteries, where direct activation of ankyrin-associated TRPA1 channels initiated new wave-like endothelial cell Ca\textsuperscript{2+} events,\textsuperscript{16} and recruitment of these new Ca\textsuperscript{2+} events corresponded precisely with IK1/SK3-mediated vasodilation. Although the physiological role of TRPV4-IK1 coupling is not yet clear, recent findings from Bagher et al\textsuperscript{34} suggest that this association may effectively tune pressure-dependent responses in cremaster muscle arterioles, whereby increased endothelial TRPV4 Ca\textsuperscript{2+} dynamics at low intravascular pressure enhance EDH and contribute to autoregulation. Findings of Ma et al\textsuperscript{35} also suggest a role for TRPV4 in flow-induced endothelial Ca\textsuperscript{2+} entry. Our current findings suggest that IK1 channels may be particularly crucial in controlling the capacity of physiological responses (ie, to pressure, agonist, and shear) not only by directly evoking Ca\textsuperscript{2+}-dependent hyperpolarization but also by expanding the spatial and temporal range of the Ca\textsuperscript{2+} signals along the intima.

Previous evaluation suggests that although SK3 channels are not as essential as IK1 channels for ACh vasodilation, their suppression augments the effects of IK1 knockout on ACh dilations, and overexpression of SK3 partially rescues these dilations.\textsuperscript{9} Overall, IK1 knockout reduces ACh dilation of pressurized arteries to ≈65% of control, which is further reduced to ≈45% by SK3 suppression and recovered to ≈80% with SK3 overexpression.\textsuperscript{9} Distinct distributions of IK1 and SK3 channels within endothelial cell plasma membranes support their differential targeting.\textsuperscript{34} In the current study, we assessed whether SK3 channels might supplement IK1 effects on endothelial Ca\textsuperscript{2+} dynamics. SK3 suppression had little additional effect on the occurrence of endothelial Ca\textsuperscript{2+} events. Moreover, SK3 overexpression failed to recover normal Ca\textsuperscript{2+} dynamics in IK1-deficient mice, indicating that SK3 channels cannot functionally replace IK1 channels. However, with respect to event parameters, SK3 overexpression tended to augment basal Ca\textsuperscript{2+} durations and effectively recovered decreased event amplitudes associated with IK1 deficiency under both basal and stimulated conditions. This suggests that SK3 channels may play a role in positive feedback Ca\textsuperscript{2+} regulation by shaping the size and time course of individual events, even under basal conditions. Protraction of Ca\textsuperscript{2+} events may be particularly important in tuning stimulation of cellular effectors, such as endothelial NO synthase,\textsuperscript{30,35–37} because increased SK3 expression was previously found to enhance NO-mediated dilation of cremaster arterioles.\textsuperscript{9} Further study is warranted to elucidate the functional implications of differential IK1 and SK3 tuning of endothelial Ca\textsuperscript{2+} dynamics.
with graded stimuli, including other receptor agonists and shear stress.

Our current findings reveal a new mechanistic role of KCa channels in expanding the very Ca²⁺ signals they detect. IK1 channels are particularly pivotal in tuning real-time endothelial Ca²⁺ signaling and physiological vasodilator responses. A limitation of the current study is that our comprehensive assessment of Ca²⁺ dynamics along the vascular intima cannot be obtained simultaneously with diameter measurements within individual pressurized arteries. However, the broad discriminating evaluation afforded by our algorithm exposes distinct profiles of physiological signaling not previously recognized. Overall, our data fit well with an emerging model of distinct profiles of physiological signaling not previously recognized. None.

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**Disclosures**
None.

**References**
The intermediate and small conductance Ca\(^{2+}\)-activated K\(^+\) channels, IK1 (K\(_{Ca3.1}\)) and SK3 (K\(_{Ca2.3}\)), have been established as key players in endothelium-dependent vasodilation, particularly through endothelium-derived hyperpolarization of vascular smooth muscle. These channels are activated by dynamic endothelial Ca\(^{2+}\) signals that increase with endothelial stimulation. Using genetically altered mouse models with differential IK1 and SK3 expression, the current study shows for the first time that these channels exert their influence not only through unidirectional signaling to smooth muscle but also by enhancing the cytosolic endothelial Ca\(^{2+}\) dynamics themselves. Dependent on augmented Ca\(^{2+}\) influx through membrane cation channels (transient receptor potential vanilloid 4), this positive feedback influence is substantial under stimulated conditions. The work suggests an expanded role of endothelial K\(_{Ca}\) channels in arterial function and heightens interest in these channels as therapeutic targets to improve endothelial function.
Positive Feedback Regulation of Agonist-Stimulated Endothelial Ca\textsuperscript{2+} Dynamics by K\textsubscript{Ca} Channels in Mouse Mesenteric Arteries

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Supplemental Figure I. Net effect of combined TRPV4 and IK1/SK3 inhibition on ACh-induced Ca\(^{2+}\) dynamics at 25°C and 37°C. Arteries from wild-type mice were treated with the combination of HC-067047 (1 µM), charbdotoxin (0.1 µM) and apamin (0.5 µM) and stimulated with 2 µM ACh (n=5). There was no statistical difference between same-phase groups at 25°C and 37°C.
Material and Methods

Animals and tissue preparation

Transgenic mice (IK1\(^{-/-}\) and IK1\(^{-/-}\)/SK3\(^{T/T}\)) and same-strain (c57BL/6) wild-type controls (WT) were bred and housed in the University of South Alabama College of Medicine vivarium. For IK1\(^{-/-}\)/SK3\(^{T/T}\) mice, the K\(_{Ca}\)2.3 gene is overexpressed (~3-fold normal) in the absence of doxycycline, and is essentially abolished by 5 days exposure to doxycycline.\(^1\) Mice (equal number male and female, 25-35 g) were euthanized with pentobarbital sodium (50 mg/kg), the abdomen was opened and small intestine collected. All animal procedures were approved by the University of South Alabama Institutional Animal Care and Use Committee, and carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Intestines were quickly removed and placed in cold (~4°C) HEPES solution.

Confocal Ca\(^{2+}\) imaging and analysis

Mesenteric artery segments (~ 300 \(\mu\)m diameter) were dissected from intestine, cut open longitudinally, and pinned down on the surface of small silicone (sylgard) blocks with the endothelium facing up\(^2\) using 14 \(\mu\)m diameter pins. Blocks were incubated at room temperature for 40 minutes in dark with Ca\(^{2+}\) indicator loading solution containing Fluo-4 AM (15 \(\mu\)M) and 0.06% Pluronic F-127 in HEPES solution (pH 7.45). After washing, blocks were placed with intima facing down in a glass-bottom chamber (separated 100 \(\mu\)m from glass by two
parallel supporting pins) containing HEPES solution. The chamber was mounted on an inverted microscope fitted with a PerkinElmer spinning disk RS-3 confocal unit. Ca\(^{2+}\)-dependent fluorescence (488 nm excitation, 510 nm emission) was measured at 8 frames/sec at 25°C (20X objective) using Ultraview software. Experiments were performed at room temperature to avoid photobleaching and acute temperature change during drug additions. Images were saved as 16-bit raw data during recording, and later converted into 8-bit TIFF format for offline processing. Only recordings with > 90% of total viewable area in focus were processed and analyzed. Data were processed using a custom algorithm LC_Pro.\(^3\) Implemented as a plug-in with ImageJ software, this software is specifically designed to: 1) detect sites of dynamic Ca\(^{2+}\) change above statistical (p < 0.01) noise, 2) define regions of interest (ROI; 5 pixel or 1.7 \(\mu\)m diameter) at active sites centers, and 3) analyze average fluorescence intensities at ROIs to determine specific event parameters. Fluorescence data are expressed as \(F/F_0\), where \(F_0\) is determined by a linear regression of base data at each ROI. Net lateral stretch placed on block-mounted vessels (~1.5 times resting width) was adequate to prevent folding of the lamina and is consistent with vascular distention by pressure based on previous experiments; a 300 \(\mu\)m diameter artery, opened and pinned at a width of 1.5 mm, is approximately equivalent to its circumference at 80 mmHg pressure. For drug addition protocols, recorded data segments corresponding to solution exchange (~ 4 seconds) were removed offline to avoid analysis artifact. Figure 1 shows auto-detection and tracking of basal endothelial Ca\(^{2+}\) events in an open mesenteric artery.
Reagents, materials and solutions

Reagents and ACh were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-4 AM and Pluronic F-127 were purchased from Invitrogen (Carlsbad, CA). Tungsten wires (for making tiny pins) were purchased from Scientific Instrument Services (Ringoes, NJ). HEPES/bicarbonate-buffered physiological saline solution (PSS) contained (mmol/L): NaCl 130.0; NaHCO₃ 14.9; KCl 3.7; KH₂PO₄ 1.2; MgSO₄ 1.2; CaCl₂ 1.6; Glucose 11.0; HEPES 10.0; pH 7.4.

Data Analysis

Data are presented as the mean ± standard errors of the mean and statistical analysis is performed with GraphPad Prism software. For multiple-set analysis, one-way ANOVA was performed followed by individual comparisons using the Tukey’s post-hoc test. P values < 0.05 were considered significant. For non-Gaussian event parameters, distributions were modeled using a generalized linear mixed model and subsequent analysis of variance was performed using R statistical analysis software. Animal genotype and experimental replicates were used as model fixed effect and random effect factors, respectively, and implemented using the lme4 package in R. Positively skewed parameter distributions were fit using an inverse Gaussian function, and fixed effects were modeled with a logarithmic link function. Analysis of variance was performed using the ANOVA function for generalized linear mixed models from the “car” package in R. Pair-wise comparisons were made using Tukey’s post-hoc test,
implemented in the “multcomp” package of R. P-values less than 0.05 were considered significant.

References

