Abstract—To study endothelial cell (EC)–specific Ca\textsuperscript{2+} signaling in vivo we engineered transgenic mice in which the Ca\textsuperscript{2+} sensor GCaMP2 is placed under control of endogenous connexin40 (Cx40) transcription regulatory elements within a bacterial artificial chromosome (BAC), resulting in high sensor expression in arterial ECs, atrial myocytes, and cardiac Purkinje fibers. High signal/noise Ca\textsuperscript{2+} signals were obtained in Cx40\textsuperscript{BAC}-GCaMP2 mice within the ventricular Purkinje cell network in vitro and in ECs of cremaster muscle arterioles in vivo. Microiontophoresis of acetylcholine (ACh) onto arterioles triggered a transient increase in EC Ca\textsuperscript{2+} fluorescence that propagated along the arteriole with an initial velocity of \(\sim 116 \text{ \mu m/s (n = 28)}\) and decayed over distances up to 974 \text{ \mu m}. The local rise in EC Ca\textsuperscript{2+} was followed (delay, 830±60 ms; n=8) by vasodilation that conducted rapidly (mm/s), bidirectionally, and into branches for distances exceeding 1 mm. At intermediate distances (300 to 600 \text{ \mu m}), rapidly-conducted vasodilation occurred without changing EC Ca\textsuperscript{2+}, and additional dilation occurred after arrival of a Ca\textsuperscript{2+} wave. In contrast, focal delivery of sodium nitroprusside evoked similar local dilations without Ca\textsuperscript{2+} signaling or conduction. We conclude that in vivo responses to ACh in arterioles consists of 2 phases: (1) a rapidly-conducted vasodilation initiated by a local rise in EC Ca\textsuperscript{2+} but independent of EC Ca\textsuperscript{2+} signaling at remote sites; and (2) a slower complementary dilation associated with a Ca\textsuperscript{2+} wave that propagates along the endothelium. (Circ Res. 2007;101:1300–1309.)

Key Words: bacterial artificial chromosome ■ calcium imaging ■ microcirculation ■ Purkinje cells
used these mice, which have endothelium lineage-specific Ca\(^{2+}\) signaling, to test the hypothesis\(^{11}\) that the conduction of vasodilation along arteriolar networks in vivo involves a wave of Ca\(^{2+}\) traveling along the endothelium.

### Materials and Methods

#### Generation of Tg(RP24–25504-GCaMP2)1Mik Mice

A BAC clone containing approximately 85 kb and 61 kb of 5′ and 3′ DNA flanking the Cx40 locus was modified by insertion of a GCaMP2-IRES-GCaMP2-pA cassette to replace 6 nucleotides at the DNA flanking the Cx40 locus was modified by insertion of a random insertion transgene.\(^{16}\) BAC targeting and founder initiation codon of Cx40 in exon2 by homologous recombination and used as a random insertion transgene.\(^{16}\) BAC targeting and founder genotyping are described in the supplemental Methods (available online at http://circres.ahajournals.org).

### Results

#### Efficient EC Targeting in Cx40\(^{BAC}\)-GCaMP2 Mice

To circumvent the problem of limited adult EC expression levels and incomplete lineage specificity associated with minimal promoter fragments such as Tie2\(^{14}\) or VE-cadherin,\(^{17}\) we chose to replicate the endogenous expression pattern of Cx40 by BAC transgenesis. A bicistronic construct (GCaMP2-IRES-GCaMP2) was targeted to the start codon in a Cx40 -spanning BAC\(^{16}\) (Figure 1A) and the recombinereaded BAC injected into fertilized oocytes, placing GCaMP2 under ECs lining cardiac arterioles (Figure 2A); no expression was observed in atrial myocytes, the Purkinje cell network, and used these mice, which have endothelium lineage-specific Ca\(^{2+}\) signaling, to test the hypothesis\(^{11}\) that the conduction of vasodilation along arteriolar networks in vivo involves a wave of Ca\(^{2+}\) traveling along the endothelium.

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### Cardiac GCaMP2 Expression and Function in Cx40\(^{BAC}\)-GCaMP2 Mice

GCaMP2 was expressed in the adult heart, where it was observed in atrial myocytes, the Purkinje cell network, and ECs lining cardiac arterioles (Figure 2A); no expression was detected in ventricular myocardium, consistent with the pattern of Cx40 expression.\(^{15}\) We confirmed the function of GCaMP2 in the Purkinje layer in cut-open, superfused and Langendorf -perfused Cx40\(^{BAC}\)-GCaMP2 hearts. Stimulation of the Purkinje network with a bipolar electrode resulted in fluorescent Ca\(^{2+}\) transients at the stimulus frequency over the entire network (Figure 2B), in the absence of equivalent signals in the ventricular myocardium. Confocal measurements in spontaneously beating hearts revealed robust fluorescent transients confined to the Purkinje network (Figure 2C).

#### EC Ca\(^{2+}\) Signaling and Endothelium-Dependent Vasodilation In Vivo

To investigate the role of Ca\(^{2+}\) signaling during endothelium-dependent vasodilation in vivo, we visualized 35 arterioles in the superfused cremaster muscle of 14 adult Cx40\(^{BAC}\)-GCaMP2 mice and delivered a focal ACh stimulus using microiontophoresis (typically 1 µA; 1000 ms; 1 µm micropipette tip). Arterioles displayed a resting diameter of 30±2 µm before stimulation, dilated by 40±4% (n=33) at the site of stimulation, and recovered to resting diameter after stimulation. Acetylcholine triggered a rapid rise in fluorescence at the local site, and the Ca\(^{2+}\) signal propagated bidirectionally along the endothelium (Figure 3). The local rise in EC Ca\(^{2+}\) occurred with a half time of 250±31 ms (n=10), and the mean peak fluorescence increase was 0.39±0.04 (ΔF/F\(_{0}\);n=10). The increase in Ca\(^{2+}\) fluorescence propagated along the endothelium at a velocity of 116±6 µm/s over the first 200 µm (n=28). As the Ca\(^{2+}\) wave propagated away from the stimulus site, the rate of rise and peak ΔF/F\(_{0}\) decreased progressively, decaying to oscillations at the most remote extent of wave propagation (Figure 3B, 3D, and 3F; supplemental Movie I). Although the nonratio metric nature of GCaMP2 obviates quantitative measurements of Ca\(^{2+}\), these oscillations could be clearly observed above background levels (Figure 3B). Ca\(^{2+}\) waves also propagated into and along branches arising from the stimulated arteriole (Figure 3A, 3C, 3D, 3E; supplemental Movies I and II). Varying the ACh stimulus (100 to 1000 ms pulse) revealed a dose-dependent increase in the distance Ca\(^{2+}\) waves propagated with no change in the initial velocity (data not shown). In 14 experiments performed under identical conditions, the Ca\(^{2+}\) wave decayed by 50% in 204±17 µm. However, low level oscillations were often detected for distances up to approximately 1 mm (supplemental Movie I). These EC oscillations at remote sites have not been reported previously and may underlie changes in cell function that extend well beyond areas of EC direct activation.

After the initial local rise in EC Ca\(^{2+}\), vasodilation spread rapidly along the arteriolar network. As shown in Figure 3C and 3F, this rapidly-conducted vasodilation traveled much faster (>2 mm/s) than the Ca\(^{2+}\) wave and occurred at remote sites without a corresponding rise in Ca\(^{2+}\). The time to initiate dilation 400 µm upstream from the stimulus was 878±36 ms (n=9), which was similar to the delay recorded at the site of stimulation (831±63 ms; n=8). Remarkably, at intermediate sites the rapidly-conducted vasodilation was followed by a later phase of dilation that coincided with the arrival of the
Ca\(^{2+}\) wave (Figure 3A, compare diameters between 1.25 and 4.5 s), suggesting 2 distinct mechanisms of endothelium-dependent vasodilation (see below). ACh stimuli of shorter duration (eg, 50 or 100 ms) evoked transient, submaximal increases in local EC Ca\(^{2+}\) and diameter; rapidly conducted vasodilation occurred in all experiments in which brief delivery of ACh resulted in a submaximal increase in EC Ca\(^{2+}\) (n=17), suggesting an equivalent threshold.

In contrast to the actions of ACh, focal delivery of the nitric oxide donor SNP, which relaxes SM independent of the...
endothelium through guanylyl cyclase–dependent mechanisms,19 produced local vasodilation without increasing EC Ca\(^{2+}\)/H11001 (Figure 4; supplemental Movie III) and no vasomotor response at intermediate or remote sites, further suggesting that the local rise in EC Ca\(^{2+}\)/H11001 in response to ACh initiates rapidly-conducted vasodilation. Arteriolar fluorescence appeared to decrease during dilation to SNP (Figure 4A), but this was explained by redistribution of GCaMP2 over a larger area within the optical section (Figure 4B). Total fluorescence integrated across the entire vessel (ΣF) varied by <2.5% between rest and SNP dilation (P>0.05, n=5), and a similar redistribution effect was observed for remote dilations to ACh extending beyond the Ca\(^{2+}\)/H11001 wave (Figure 3F, site 2). Thus simple distension of the arteriolar wall does not increase EC Ca\(^{2+}\)/H11001.

At the site of ACh delivery the initial rise in Ca\(^{2+}\)/H11001 preceded the onset of dilation (Figure 5A and 5B; supplemental Movie IV). We further resolved Ca\(^{2+}\)/H11001 signals in individual ECs by confocal imaging (n=13 cells) during 100-ms ACh stimuli.
(Figure 5C and 5D); EC Ca$^{2+}$ rose rapidly, preceding the local dilation, then oscillated asynchronously at a mean frequency of 1.2±0.1 Hz (Figure 5B and 5D; supplemental Movie V). These observations collectively indicate that endothelium-dependent vasodilation is preceded by a rise in EC Ca$^{2+}$, which triggers both the rapid conduction of vasodilation and an ensuing wave of Ca$^{2+}$ along the endothelium (see below).

Separation of Ca$^{2+}$-Dependent Vasodilation and the Vasomotor Response

As discussed above, ACh evoked a rapid dilation at remote sites that preceded arrival of a Ca$^{2+}$ wave (Figure 3C and 3F), and a slower dilation followed the arrival of the Ca$^{2+}$ wave. In 8 experiments examined 200 μm upstream from the stimulus site, rapid vasodilation preceded the Ca$^{2+}$ wave by 681±45 ms. The secondary dilation can be seen most clearly at sites 300 to 400 μm from the local stimulus when the Ca$^{2+}$ wave arrives (Figure 3A, bottom images). This secondary response was manifested as a maintained dilation during the period of increased endothelial Ca$^{2+}$ while upstream segments recovered (Figure 5E and 5F; supplemental Movie VI). As the rapid dilation has been linked to the transmission of a hyperpolarizing signal, we term this process "electrically-conducted vasodilation" and the latter process "Ca$^{2+}$ wave-dependent vasodilation".

We examined the degree to which arteriolar dilation was spatially and temporally associated with the arrival of the Ca$^{2+}$ wave in 9 experiments, by determining the dependence of peak dilation on distance from the stimulus (Figure 5G). Consistent with a Ca$^{2+}$-dependent component of dilation, the peak diameter change was greater (P<0.05) at 300 μm (56±8%) than at 1000 μm (33±7%). The respective changes (ΔΣF/ΣF$_0$) in Ca$^{2+}$ fluorescence were +40±13% and −21±15% (P<0.01). The pattern of dilation along the arteriole was also consistent with a Ca$^{2+}$-dependent component, as peak dilation did not decay monotonically, but dropped off after 300 μm, a point consistent with the decay of the Ca$^{2+}$ wave (50% decay at 204 μm). Peak dilation at the intermediate (300 μm) site followed the increase in Ca$^{2+}$, peak Ca$^{2+}$ occurring at 3.2±0.5 s and peak (second compo-
nent) dilation occurring at 10.1 ± 1.1 s after ACh stimulation. This was also reflected as sustained dilation at the intermediate arteriolar segments; between 5 and 15 s after stimulation arterioles further dilated 12.4% at 300 μm, whereas at 1000 μm arterioles were recovering tone during this period (ΔF/F₀ < 6%; P < 0.05) (Figure 5E and 5F; supplemental Movie VI). Thus for regions in which the Ca²⁺ wave propagated, arteriolar dilation was greater, occurred subsequent to the peak rise in Ca²⁺, and was sustained longer than at remote sites at which only the rapidly-conducted vasodilation occurred.

Figure 6 illustrates the time dependence of Ca²⁺ wave propagation and dilation analysis in a representative arteriole, comparing responses to ACh at the local site of stimulation (yellow bar) where both processes overlap, a remote site (blue bar) to which the propagated Ca²⁺ wave does not reach and only electrically-conducted vasodilation occurs, and an intermediate site (red bar), at which electrically conducted vasodilation occurs, followed by a secondary dilation associated with arrival of the Ca²⁺ wave (supplemental Movie VII). A virtual linescan across the vessel wall at the intermediate site (Figure 6B) reveals an initial rapid dilation that is independent of Ca²⁺ at that site and achieves a brief plateau (the apparent decrease in wall fluorescence in the linescan is explained in Figure 4B). The Ca²⁺ wave reaches the intermediate site of the linescan as shown by a rise in fluorescence and a secondary dilation, resulting in a biphasic dilation (Figure 6C), with the Ca²⁺-dependent component of dilation occurring at a slower rate. Linescans taken near ACh delivery or at the most remote site do not display a biphasic vasodilation (Figure 6D), but for different reasons. At the furthest site, conducted vasodilation occurs without a change in fluorescence because the Ca²⁺ wave does not propagate this far (or decays to ineffective oscillations) and only the rapid component is observed. By contrast, at the site of ACh exposure, there is a marked rise in Ca²⁺ that persists through the entire vasomotor response (Figure 6E), obscuring the separation of respective processes.

Discussion
Endothelial cell signaling underlies an array of vascular responses that coordinates tissue blood supply. It is clear that
propagated vasomotor responses in arterioles involve signals between individual ECs, as well as between endothelial and SM cells.6,12 The study of these cell–cell interactions has been hampered by difficulties in applying techniques that monitor intercellular signaling in the intact vascular bed, which prompted us to develop a genetic strategy to direct the expression of the genetically encoded Ca²⁺ indicator GCaMP2 to the arterial endothelium. When expressed as a transgene in mice, this indicator protein has several important attributes including high brightness and dynamic range along with stable optical properties.13 Here we report the development of mice with high GCaMP2 expression in arteriolar endothelial and Purkinje cells and the use of these mice to examine Ca²⁺ signaling during endothelium-dependent vasodilation in vivo.

Rationale for the Genetic Strategy
We initially attempted to use the minimal Tie2 promoter to direct GCaMP2 expression, as this promoter has been used to drive expression of EGFP in ECs.14 Unfortunately, Tie2-GCaMP2 transgenic mice displayed little detectable indicator expression within ECs. To achieve EC expression we inserted the GCaMP2 cDNA into the initial codon of Cx40 within a

Figure 5. Temporal relationships between ACh stimulation, local rise in EC Ca²⁺, and vasodilation. A, Wide-field imaging of arteriole stimulated with ACh (1000 ms). Ca²⁺-dependent fluorescence increases rapidly and propagates beyond field of view; onset of dilation follows rise in fluorescence. B, Continuous plot of EC fluorescence and arteriolar diameter from experiment in A. Note rise in Ca²⁺-dependent fluorescence precedes onset of dilation. C, Two consecutive submaximal (100-ms) ACh stimuli during confocal imaging (arrows). Rise in Ca²⁺-dependent fluorescence is modest (ΔF/F₀ = 0.1) and isolated to individual ECs. Dilation follows rise in fluorescence, and tone recovers after fall in fluorescence. Pipette outline is shown in 1st panel adjacent to site of diameter measurement. D, Continuous plot of normalized Ca²⁺-dependent fluorescence and lumen diameter for experiment shown in C, E, Top, diameter before ACh (1000 ms) stimulation; bottom, 9 s after stimulation. F, Traces taken from intermediate (red bar) and remote sites (gray bar) in E. Note vasodilation recovered at the remote site before the intermediate site. G, Peak change in diameter with distance, indicating a nonmonotonic decline in peak dilation along the arteriolar wall (*P<0.05, n=9). Color scale in A applies throughout. Scale bars: A, 25 μm; C, 50 μm; E, 100 μm.
locus-spanning BAC, an approach that avoided limitations of Cx40 promoter constructs.\textsuperscript{15,16,20} We have found that BAC transgenesis provides an excellent recapitulation of the endogenous gene, enabling both lineage restriction and high levels of transgene expression required for detection of fluorescent protein-based genetic indicators.\textsuperscript{16}

In the mouse, Cx40 mRNAs are transcribed by alternative splicing, resulting in 3 different 5’ UTRs mRNAs.\textsuperscript{15,18} We inserted GCaMP2 into the common Cx40 start codon in exon2 of the BAC by homologous recombination, placing the sensor cDNA under the control of Cx40 transcriptional elements. Insertion of the GCaMP2 disrupts exon2 of Cx40 within the BAC, preventing Cx40 overexpression from the BAC transgene (Figure 1). Transgene expression was consistent with endogenous Cx40,\textsuperscript{15} and coating with Cx40 antibodies revealed excellent expression overlap. Although the expression of Cx40 in capillaries remains controversial,\textsuperscript{9} GCaMP2 expression in pulmonary capillaries (Figure 1) provides support for the presence of this gap junction protein in some capillary beds.

Figure 6. Separation of EC Ca\textsuperscript{2+}-independent and Ca\textsuperscript{2+}-dependent vasodilation. A, Images from an arteriole stimulated locally by ACh for 1000 ms and imaged at 20 Hz. Top image shows region undergoing the local response to ACh (yellow bar), a remote region in which dilation is independent of local EC Ca\textsuperscript{2+} (blue bar), and an intermediate region (red bar) in which the Ca\textsuperscript{2+}-independent vasodilation is followed by additional dilation after arrival of the Ca\textsuperscript{2+} wave (panels 3 and 4). B, Pseudolinescan (x-t) from all images at red bar shown in A. Linescan shows rapidly-conducted (electrical) vasodilation at this intermediate site in response to ACh at local site (arrow), which occurs without a rise in Ca\textsuperscript{2+}-dependent fluorescence at intermediate site (apparent fluorescence decrease is attributable to redistribution; see B and Figure 4). Oscillations (arrows) and a sustained rise in Ca\textsuperscript{2+}-dependent fluorescence in the linescan mark arrival of the Ca\textsuperscript{2+} wave, which is followed by a further increase in vessel cross section. Diameters at end of each phase (resting tone, electrical, Ca\textsuperscript{2+} wave) are represented by lines below linescan. Sequential image numbers (from 20 Hz series) are listed at right. C, Continuous traces of normalized wall fluorescence and diameter from intermediate region (red bar) demonstrate 2 phases of dilation: a rapidly conducted response independent of EC Ca\textsuperscript{2+} and a secondary response associated with arrival of the Ca\textsuperscript{2+} wave. Note that drop in mean ROI fluorescence results from initial rapid conducted vasodilation (distribution of total fluorescence, Figure 4B), whereas the rise occurs with continued dilation associated with arrival of Ca\textsuperscript{2+} wave. Bimodal diameter trace identifies respective phases of vasodilation at intermediate site. D, Pseudolinescans from the 2 other regions shown in A demonstrate rapidly-conducted vasodilation without an increase in Ca\textsuperscript{2+} at furthest site from ACh stimulus (blue bar) and vasodilation at site of ACh stimulation (yellow bar) associated with the local rise in EC Ca\textsuperscript{2+}. E, Total fluorescence in the cross sections indicated by the colored bars in A. Data are presented as the aggregate fluorescence relative to the value before ACh exposure. Note lack of change during the electrical phase at intermediate and furthest (remote) sites, despite dilation and apparent fluorescence decrease, and increase in fluorescence during Ca\textsuperscript{2+}-dependent phase at intermediate site. Numbers above bars in E correspond to image numbers in B.
Ca$^{2+}$ Signaling in the Cardiac Conduction System
The Purkinje network coordinates ventricular excitation and Purkinje–ventricular junctional coupling is critical in the generation of ventricular arrhythmias. Activation of this system is poorly studied in situ because of the difficulty of selecting and recording optical signals from this network. Recently, intracellular Ca$^{2+}$ measurements were reported in Purkinje fibers by loading Langendorf-perfused hearts with Fluo3/AM and staining the network by acetylthiocholine iodide. Within the ventricle, Cx40$^{BAC}$-GCaMP2 mice express GCaMP2 exclusively in the Purkinje network, providing a simple and powerful method to examine Ca$^{2+}$ signaling in these myocytes and activation of the network. These studies can be combined with myocardial cell labeling, enabling the study of junctional coupling.

The Role of Ca$^{2+}$ in Endothelium-Dependent Dilation of Arterioles In Vivo
We used Cx40$^{BAC}$-GCaMP2 mice to examine the relationship between EC Ca$^{2+}$ signaling and endothelium-dependent vasodilation. Stimulation with ACh elicited an initial local rise in Ca$^{2+}$ and vasodilation, similar to observations in isolated arterioles and consistent with findings that ACh-induced vasodilation is inhibited by chelation of intracellular Ca$^{2+}$ in ECs. A local rise in EC Ca$^{2+}$ stimulates Ca$^{2+}$-activated K$^+$ channels, resulting in hyperpolarization and rapidly-conducted vasodilation. Ca$^{2+}$ release within ECs is not required for vasodilation since SNP relaxed arterioles with no change in Ca$^{2+}$ (Figure 4; supplemental Movie III) and rapidly-conducted vasodilations occurred without a change in Ca$^{2+}$ at distances $>1$ mm (Figures 3 and 6). Importantly, the propagating wave of Ca$^{2+}$ along the endothelium evoked a second phase of dilation, indicating that a rise in EC Ca$^{2+}$ is sufficient to produce vasodilation. This was most clearly seen by the separation of the electrically conducted and Ca$^{2+}$-wave dependent vasodilation in time (Figure 6). These findings are consistent with studies in isolated hamster feed arteries in which the rapidly-conducted vasodilation was blocked by inhibition of Ca$^{2+}$-activated K$^+$ channels, revealing a more slowly conducting vasodilation that was preceded by a Ca$^{2+}$ wave along the endothelium. As this slower vasomotor response was blocked by inhibiting nitric oxide synthase and cyclooxygenase, it can be explained by the release of autacoids from ECs subsequent to the rise in Ca$^{2+}$.

Our findings indicate that the endothelium-dependent arteriolar response to ACh consists of 2 distinct complementary processes in vivo. The first mechanism involves a rapidly-conducted vasodilation (ie, electromechanical) that is triggered by a local rise in Ca$^{2+}$ within ECs and extends to encompass sites beyond the propagating EC Ca$^{2+}$ wave. The second mechanism involves the slower, and more restricted, propagation of a Ca$^{2+}$ wave along the endothelium, resulting in additional vasodilation in regions to which the Ca$^{2+}$ wave spreads. As illustrated in Figure 6, these distinct mechanisms overlap, but can be temporally and spatially separated at sites of intermediate distance from the stimulus.

A major question concerns the mechanism of Ca$^{2+}$ wave propagation along arteriolar ECs. In the present study, Ca$^{2+}$ waves propagated at an average initial velocity of 116 $\mu$m/s, similar to a recent report of Ca$^{2+}$ wave propagation velocity (111 $\mu$m/s) in isolated feed arteries, but in contrast to a study in isolated hamster cheek arterioles reporting that EC Ca$^{2+}$ responses evoked by ACh do not propagate. These differences may relate to loading conditions or constraints on imaging after dye loading of isolated vessels. Although Ca$^{2+}$ waves appeared to slow as they traveled (Figure 3), we suggest that their propagation along the endothelium requires an active mechanism, as the initial velocity is more than 1000-fold faster than expected for free diffusion. Nevertheless, propagation of Ca$^{2+}$ waves is markedly slower than that of hyperpolarization and rapidly-conducted vasodilation. It is possible that regenerative release of InsP$_3$, triggered by ACh, underlies Ca$^{2+}$ wave transmission along arterioles. The propagation velocity observed here is markedly faster than the rate of InsP$_3$, Ca$^{2+}$ waves traveling through cellular cytoplasm (20 $\mu$m/s), suggesting that additional coupling mechanisms between cells may be involved.

In summary, Cx40$^{BAC}$-GCaMP2 mice represent an important tool in the study of cardiovascular biology and will be useful for future studies of Ca$^{2+}$ signaling within arterial/arteriolar ECs. The present findings demonstrate the first use of these animals to resolve distinct yet complementary endothelium-dependent signaling pathways for coordinating vasodilation along branches of the arterial network in vivo during blood flow control to skeletal muscle. We show that vasodilation in response to ACh is initiated by a local rise in EC Ca$^{2+}$, which triggers a rapidly-conducted vasodilation that encompasses multiple branches and occurs independent of EC Ca$^{2+}$ at remote sites. In turn, the EC Ca$^{2+}$ wave propagates intercellularly to contribute additional vasodilation. Taken together, respective signaling pathways can coordinate the onset and magnitude of vasodilation throughout resistance networks.

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Disclosures
None.

References


Propagated Endothelial Ca\textsuperscript{2+} Waves and Arteriolar Dilation In Vivo: Measurements in Cx40BAC–GCaMP2 Transgenic Mice
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Supplemental Materials

Supplemental Movies

Supplemental Movie 1. *In vivo* imaging of Ca\(^{2+}\) wave during ACh stimulation.
Bidirectional Ca\(^{2+}\) wave within cremaster arteriolar ECs of a Cx40\(^{BAC}\)-GCaMP2 transgenic mice following local delivery of ACh (1000 ms pulse) from a micropipette. Arrow denotes pipette tip. Images acquired and played at 20 Hz.

Supplemental Movie 2. Propagation of Ca\(^{2+}\) wave into branches. ACh stimulus delivered at site indicated on left daughter branch; dilation and Ca\(^{2+}\) wave propagate into parent and other daughter branches leading to Ca\(^{2+}\) oscillations in respective ECs. Images acquired and played at 20 Hz.

Supplemental Movie 3. *In vivo* imaging during SNP stimulation. Lack of increase in Ca\(^{2+}\)-dependent fluorescence following local delivery of SNP from a micropipette (1000 ms pulse). Note local vasodilation without change in Ca\(^{2+}\)-dependent fluorescence. Arrow denotes pipette tip. Images acquired and played at 20 Hz.

Supplemental Movie 4. Local Ca\(^{2+}\)-dependent fluorescence and dilation. ACh iontophoresis (1000 ms) leads to an increase in Ca\(^{2+}\)-dependent fluorescence followed by vasodilation at site of stimulation. Images were acquired and played back at 20 Hz.

Supplemental Movie 5. Confocal imaging of calcium oscillations. Brief (100 ms) microiontophoresis of ACh results in a wave of EC Ca\(^{2+}\), followed by Ca\(^{2+}\) oscillations.
within individual ECs. There are two successive applications of ACh, each resulting in oscillations and vasodilation. Pipette tip location denoted by arrow. Images acquired and played at 10 Hz.

**Supplemental Movie 6. Maintained dilation during arrival of Ca\(^{2+}\) wave.**

Acetylcholine was delivered downstream (below observed field). Note rapidly-conducted vasodilation preceding EC Ca\(^{2+}\) wave into region of horizontal branch which maintains dilation during EC Ca\(^{2+}\) oscillations while region further upstream (to left) recovers tone. Images acquired and played at 20 Hz.

**Supplemental Movie 7. A secondary arteriolar dilation follows the propagated Ca\(^{2+}\) wave.** The movie shows a segment of the arteriole shown in Figure 6, beginning 2.3 s after ACh stimulation (beyond observed field). At the beginning of this sequence rapidly-conducted vasodilation has already occurred and the second phase of dilation is shown as the Ca\(^{2+}\) wave travels along the endothelium. Images acquired at 20 Hz and played at 40 Hz.
Supplemental Methods

BAC Recombineering

Methods for BAC recombineering have been previously described\textsuperscript{1-3}. Specifically, BAC clone RP24-255O4 (Children’s Hospital Oakland Research Institute) was chosen based on size and extent of 5’ flanking sequence. Cx40 homologous arms were amplified and inserted upstream of GCaMP2-IRES-GCaMP2 and downstream of FRT-Neo/Kan-FRT sequence in plasmid pBS-GCaMP2-IRES-GCaMP2-FRT-Neo/Kan-FRT (Kotlikoff laboratory). Arm1 was a 415 bp fragment upstream of the AAGATG (ATG is the initiation codon of Cx40) that used as forward primer (EcoRI-P1) ‘5' - CGGAATTCAAGTGAGTGGATTGATTC-3’ and reverse primer (SmaI-P2) 5’-TCCCCCGGGGCCAAGAGTAGGAGACCGA - 3’. Arm2 was a 612bp product downstream of the AAGATG that used the forward primer (SpeI-P1) 5’-GGACTAGTGTAGGATCCGCCCGGTGCCCACGACATTCTTCTCCGGAGG - 3’, and the reverse primer (NotI-P2) 5’-ATTTGCGGCCGCGACATTCTTCTCCGGAGG - 3’. The homology arms were inserted by EcoRI/SmaI (Arm1) and SpeI/NotI (Arm 2) digestion and the targeting cassette released from the plasmid by EcoRI/NotI. EL250 cells carrying RP24-255O4 were electroporated with the targeting cassette DNA and selected with kanamycin and chloramphenical; the Neo/Kan cassette was removed in targeted clone by L-arabinose induction of flp recombinase activity. Sequencing of PCR products obtained using primers flanking the homology arms and GCaMP2-IRES-GCaMP2 verified homologous recombination and removal of the Neo/Kan cassette. Cx40\textsuperscript{BAC}-GCaMP2-IRES-GCaMP2-pA DNA was purified (Large Construct Kit, Qiagen) and microinjected into fertilized embryos in the Cornell Transgenic Mouse Core facility. Genomic DNA was purified
(Puragene, Gentra Systems, Minnesota) and mice carrying the transgene identified by PCR with a Cx40 upstream primer 5’- CAGAGCATGATGGGACCTTC - 3’ and an IRES reverse primer 5’- GGTTTCCGGGCCCTCACATT - 3’, yielding a 1.6kb PCR product, or to the BAC vector, RP24-P1 ‘5 – AATACAACGGCTATCAG - 3’ and RP24-P2 5’- AGCTTCTGGCTTTCTTTAC -3’, yielding a 595bp PCR product (Figure 1A).

Cardiac Imaging

All animal procedures were approved by the Cornell University Animal Care and Use Committee. Cardiac experiments were performed on hearts rapidly removed from adult male or female (12-16 week old) transgenic mice deeply anesthetized with pentobarbital (50 mg/kg, IP) and were placed in a custom built chamber with warmed (37±1°C), oxygenated Tyrode solution. In experiments examining spontaneous activity hearts were Langendorf - perfused. Hearts were opened and pinned to a Sylgard (Dow-Corning, Midland, MI) dish exposing the cardiac trabecula along the ventricular wall as previously described. Images were acquired at 67-128 Hz using an electron multiplied-CCD camera (iXon 860-BI, Andor Technology, Belfast, Northern Ireland) coupled to an OV100 macroimaging system (Olympus, Japan) as described or at 200 Hz using a MiCAM Ultima (SciMedia USA, Irvine CA) camera coupled to an Olympus DSU spinning disk confocal microscope (Olympus, Japan). For confocal experiments the field of view was 1 mm², providing a pixel resolution of 10 μm². Stimulation was with a bipolar electrode (15 V, 2 ms duration, and 2 Hz (Grass S48 Stimulator, Warwick, RI).
In some confocal experiments 15 mM diacetyl monoxime was used to prevent excitation-contraction coupling.

*Cremaster muscle preparation and imaging*

Adult transgenic male mice (9-12 weeks) were anesthetized with pentobarbital sodium (50 mg/kg, IP injection) and maintained with supplemental injection as needed, or with 0.5% isoflurane. Animals were transferred to the heated stage of a MVX10 (Olympus, Japan) or an E600FN (Nikon, Japan) microscope equipped with a spinning disk confocal head (Solamere Technology Group, Inc) for macro imaging and confocal imaging, respectively. MVX10 zoom imaging varied from 6x – 24x and confocal imaging was 20x or 40x; images were projected to an electron multiplied CCD camera (iXon 860-BI, Andor), yielding spatial resolutions from 2.67 to 0.4 μm/cm². GCaMP2 was excited at 472/30 nm with a mercury arc lamp for macro imaging and at 488 nm with an Argon laser for confocal imaging. Fluorescent light was collected through a 520/35 nm bandpass filter. Up to three arterioles, typically second- and third-order branches, were monitored in each cremaster muscle preparation. A total of 35 arterioles were studied in 14 mice. In several experiments, multiple responses were obtained from the same arteriole, with each response counted when referring to the ‘n’ for an experiment.

For stimulation at a discrete site along arterioles exhibiting spontaneous vasomotor tone, acetylcholine (1 M) was delivered using microiontophoresis (1 μA) and sodium nitroprusside (1 mM) by pressure ejection (20-35 kPa) from glass micropipettes having tip internal diameters of 1 μm and 3-4 μm, respectively⁶. The tips of micropipettes were positioned adjacent to an arteriole and individual pulses from 50 to
1000 ms in duration were used to produce a transient vasodilation at the ‘local’ site of stimulus delivery. At defined sites, arteriolar diameter was measured as the distance across the fluorescent boundary, which corresponded to the width of the endothelial ‘tube’ forming the intima. Following each stimulus, arteriolar diameter was allowed to recover to baseline and rest for at least 1 min before proceeding.

**Immunohistochemistry**

GCaMP2 immunolabeling was performed as previously described\(^3\)\(^5\). Co-labeling of α-actin and GCaMP2 was performed by microwaving slides in 0.01M citrate buffer pH 6.0 for 20 minutes, and blocking with mouse IgG from a Mouse on Mouse (MOM) labeling kit (Vector Laboratories, Burlingame, CA) followed by 10% swine/10% goat/2X casein for 20 minutes at room temperature. Rabbit anti-GFP 1:5 (Chemicon International Inc., Temecula, CA) and mouse anti-Smooth Muscle Actin 1:20 (Dako Cytomation, Carpenteria, CA) were combined in TBS/MOM and applied for 20 minutes at 37°C followed by 40 minutes at room temperature. Slides were washed and incubated with biotinylated swine anti-rabbit immunoglobulin 1:50 (Dako Cytomation), Streptavidin Alexa Fluor 488 1:100 (Invitrogen/Molecular Probes, Carlsbad, CA), and Texas Red goat anti-mouse 1:100 (Vector Laboratories). Stained sections were mounted with Vectashield (Vector Laboratories) DAPI. Negative controls were nontransgenic tissue and normal mouse IgG and normal rabbit IgG substituted for primary antibodies.

Co-labeling for Cx40 and GCaMP2 was performed in formalin-fixed slides microwaved for 25 minutes in citrate buffer pH 6.9, blocked with 10% goat serum/10% nonfat dry milk, and incubated with anti-Cx40 antibody (Zymed Laboratories) diluted
1:15 in TBS for 2 hours at 37 °C, then washed in TBS and incubated with biotinylated goat anti-rabbit IgG (1:200; Vector laboratories) followed by Streptavidin Alexa Fluor 488 (1:100; Invitrogen/Molecular Probes). Slides were stored overnight in distilled water at 4 °C and on day 2 were blocked with normal rabbit IgG (Vector Laboratories) 1:400, washed in TBS, and incubated with unlabeled Fab fragment goat anti-rabbit IgG 1:50 (Jackson Immunoresearch, West Grove, PA) for 45 minutes at 37 °C. Rabbit anti-GFP antibody 1:30 in TBS with 1X casein was applied for 2 hours at 37 °C, washed, and incubated with Texas Red goat anti-rabbit IgG 1:50 (Vector Laboratories) for 10 minutes, washed again, and mounted with Vectashield DAPI (Vector Laboratories). Immunohistochemistry images were captured either with a Zeiss 510 Meta Scanning Confocal Microscope or LEICA DMLB microscope with an Olympus DP70 camera. For clarity of presentation purposes colors were reversed in Figure 1 (anti-GFP antibody staining presented as green).

**Data Analysis**

Image analysis was performed in ImageJ (NIH) using standard and custom written macros and all images within a series are presented with the same contrast and brightness. Fluorescence increases are reported as \((F-F_0)/F_0\) where \(F\) is the time variant and \(F_0\) basal fluorescence. Normalized diameter change was determined by edge detection from fluorescence values along a line perpendicular to the vessel using the equation \([ (D_r-D_b)/D_m ] \times 100\), where \(D_r\) is the response diameter at each time point after stimulus (ACh or SNP) delivery, \(D_b\) is the pre-stimulus arteriolar diameter, and \(D_m\) is the maximum observed diameter. Percent change in diameter was reported as \(\Delta D/D_0\) where
ΔD is diameter at specified time (in seconds) and location minus D₀ and D₀ is nominal diameter. The initial velocity of Ca^{2+} wave propagation was obtained by determining the time elapsed between the first detectable increase in F at the pipette tip and at 200 µm upstream. Data were analyzed with SigmaPlot/SigmaStat software using one-way Analysis of Variance with Tukey’s post hoc comparisons. Summary values are mean ± SE. Differences were accepted as statistically significant with P<0.05.

References


