## Physiology and Pharmacology

# Feedback via Ca$^{2+}$-Activated Ion Channels Modulates Endothelin 1 Signaling in Retinal Arteriolar Smooth Muscle

*Michael Stewart, Maurice Needham, Peter Bankhead, Tom A. Gardiner, C. Norman Scholfield, Tim M. Curtis[1] and J. Graham McGeown[1]*

**PURPOSE.** To investigate the role of feedback by Ca$^{2+}$-sensitive plasma-membrane ion channels in endothelin 1 (Et1) signaling in vitro and in vivo.

**METHODS.** Et1 responses were imaged from Fluo-4–loaded smooth muscle in isolated segments of rat retinal arteriole using two-dimensional (2-D) confocal laser microscopy. Vasoconstrictor responses to intravitreal injections of Et1 were recorded in the absence and presence of appropriate ion channel blockers using fluorescein angiograms imaged using a confocal scanning laser ophthalmoscope.

**RESULTS.** Et1 (10 nM) increased both basal [Ca$^{2+}$]$_i$ and the amplitude and frequency of Ca$^{2+}$-waves in retinal arterioles. The Ca$^{2+}$-activated Cl$^-$-channel blockers DIDS and 9-anthracene carboxylic acid (9AC) blocked Et1-induced increases in wave frequency, and 9AC also inhibited the increase in amplitude. Iberiotoxin, an inhibitor of large conductance (BK) Ca$^{2+}$-activated K$^+$-channels, increased wave amplitude in the presence of Et1 but had no effect on frequency. None of these drugs affected basal [Ca$^{2+}$]$_i$. The voltage-operated Ca$^{2+}$-channel inhibitor nimodipine inhibited wave frequency and amplitude and also lowered basal [Ca$^{2+}$]$_i$ in the presence of Et1. Intravitreal injection of Et1 caused retinal arteriolar vasoconstriction. This was inhibited by DIDS but not by iberiotoxin or penitrem A, another BK-channel inhibitor.

**CONCLUSIONS.** Et1 evokes increases in the frequency of arteriolar Ca$^{2+}$-waves in vitro, resulting in vasoconstriction in vivo. These responses, initiated by release of stored Ca$^{2+}$, also require positive feedback via Ca$^{2+}$-activated Cl$^-$-channels and L-type Ca$^{2+}$-channels. (*Invest Ophthalmol Vis Sci.* 2012;53:3059–3066) DOI:10.1167/iovs.11-9192

Signaling by the potent constrictor peptide endothelin 1 (Et1) is believed to be important in the regulation of retinal blood flow and to play a potentially pathogenic role in the development of several important ophthalmic diseases, including glaucoma and diabetic retinopathy.[1–4] Despite this physiological and pathophysiological importance, however, the cell signaling pathways through which Et1 regulates retinal blood flow have received relatively little attention. We have recently shown that Et1 increases both basal [Ca$^{2+}$]$_i$ and the frequency of phasic Ca$^{2+}$-signaling within arteriolar myocytes in isolated retinal arterioles.[5] The phasic element of the response was particularly pronounced, with both short duration Ca$^{2+}$-sparks and more prolonged events, which we termed Ca$^{2+}$-oscillations. Previous studies have shown that these "oscillations" are often associated with cell contraction, suggesting they may underpin the constrictor effects of Et1.[6] Immunostaining of porcine retinal arterioles reveals both EtA and EtB receptors in the smooth muscle layer, with EtA expression predominating at the protein level, while EtA receptor blockade inhibits most of the Et1-induced constriction in pressurized vessels.[7] Consistent with this, we found that Et1-induced increases in Ca$^{2+}$-oscillations within retinal arteriolar myocytes were also mediated via EtA receptors. Et1-induced Ca$^{2+}$-oscillations were also dependent on phospholipase-C activation, with downstream Ca$^{2+}$-release from the sarcoplasmic reticulum involving both inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) and ryanodine receptors (RyRs).[5]

We have now extended this research to look at the possible contribution of Ca$^{2+}$-activated channels in the plasma membrane to Et1 signaling. Both Ca$^{2+}$-activated Cl$^-$-currents (I$_{ClCa}$) and large conductance (BK) Ca$^{2+}$-activated K$^+$-currents (I$_{KCa}$) can be recorded from retinal arteriolar smooth muscle.[8,9] These represent potential feedback mechanisms, whereby Ca$^{2+}$-release may modulate Ca$^{2+}$-influx via voltage-operated Ca$^{2+}$-channels (VOCCs).[10] Activation of I$_{ClCa}$ would result in membrane depolarization, leading to opening of VOCCs and increased Ca$^{2+}$-influx, and so act as a positive feedback mechanism. This has been described in a variety of agonist responses in smooth muscle.[11] Ca$^{2+}$-dependent activation of BK channels, on the other hand, would favor membrane hyperpolarization and closure of VOCCs, acting as a brake or negative feedback mechanism limiting Ca$^{2+}$-influx.[12]

In the current study we used two-dimensional (2-D) Ca$^{2+}$-imaging in vitro to test for possible feedback during Et1-signaling using appropriate ion channel inhibitors of I$_{ClCa}$ and BK channels. The physiological relevance of our findings was further investigated by recording constrictor responses to intravitreal Et1 in vivo in the presence and absence of appropriate channel antagonists. Our findings indicate that positive feedback via I$_{ClCa}$ plays a crucial role both in Et1 Ca$^{2+}$-signaling and the resulting mechanical responses in retinal arterioles.

3059

## MATERIALS AND METHODS

All animal use was performed under license from the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986 and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. In vivo experiments led to terminal anesthesia without recovery.

### Ca²⁺-Imaging in Isolated Rat Retinal Arterioles

Techniques used for arteriole isolation have previously been described in detail.[6] Retinas dissected from male Sprague-Dawley rats (200–300 g) were mechanically triturated using low-$Ca^{2+}$ (100 μM) Hanks' solution. Following centrifugation, wash, and repeated centrifugation, vascular fragments were incubated for 2 hours with Fluo-4AM (10 μM) in low-$Ca^{2+}$ solution and pipetted into a glass-bottomed organ bath (0.17-mm thick base) on the stage of an inverted microscope (Nikon Eclipse TE2000U; Nikon Instruments, Kingston, UK). Arteriole segments (outer diameters of 25–50 μm) were visualized using a PlanApo, ×60, 1.4 Numerical Aperture (NA), oil-immersion objective, anchored using tungsten wire slips and superfused with normal Hanks' solution at 37°C. Two-dimensional $Ca^{2+}$-images were obtained using a rotating-disc confocal system (QLC100; Visitech International, Sunderland, UK). Fluo-4 fluorescence was excited using a 488-nm Light Emitting Diode (LED) laser (Sapphire 488-20; Coherent, Reading, United Kingdom), and emitted light was passed through a 530- to 560-nm band-pass filter. The image was captured at 20 frames per second (fps) using an electron multiplying Charge Coupled Device (CCD) camera (iXon; Andor Technology, Belfast, UK). Imaging at each site was limited to 100 seconds to minimize photodamage. Fluorescence was background corrected using the mean counts from a tissue-free image region. Background-corrected fluorescence (F) was normalized to the resting fluorescence ($F_0$) in the same cell, and increases in $F/F_0$ were interpreted as being due to changes in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$).

### In Vivo Imaging of Arteriole Diameter

Changes in retinal arteriolar diameter following intravitreal injections of Et1 with or without various ion channel blockers were recorded using fluorescein angiography with a Heidelberg Retinal Angiograph 2 (HRA2) confocal scanning laser ophthalmoscope (CSLO; Heidelberg Engineering, Heidelberg, Germany). This was modified by removing both chin and forehead rests and adding an adjustable animal stage. Pigmented male Hooded Lister rats (320–450 g) were used to maximize image contrast. Rats were anesthetized using 0.35 mL per 100 grams of a solution containing ketamine (37.5 mg/mL; Fort Dodge Animal Health, Southampton, UK) and xylazine (5 mg/mL; KVP Pharma und Veterinar-Produkte GmbH, Kiel, Germany). Depth of anesthesia, assessed using corneal and foot pinch reflexes, was maximal within 10 minutes and lasted up to 1.25 hours, after which top-up doses (0.1 mL/100 g) were introduced if needed. Rats were placed on a heat pad at 25°C (SnuggleSafe; Lenric International, Littlehampton, UK) and the rectal temperature monitored (digital thermometer model 77,020; Medisana, Dusseldorf, Germany).

Intravitreal injections (10 μL) of relevant solutions (see Results section) were administered using a 29-gauge insulin syringe (Kendall monoject; Tyco Healthcare group, Basingstoke, UK) while viewing the eye through a surgical microscope (Zeiss OPH1 1-H; Carl Zeiss, City, UK). The intraocular drug concentration was estimated assuming equilibration into a vitreal volume of 15 μL (total volume = 25 μL).[13] High intravitreal concentrations (approximately 10 times those typical in vitro) were used, since rapid clearance via the anterior segment may greatly reduce the effective concentration at the retina.[14] Viscotears (Novartis, Camberley, UK) was applied to prevent corneal drying, and 15 minutes were then allowed to pass before angiography). Ten percent sodium fluorescein (0.025 mL/100 g body weight; Martindale Pharmaceuticals, Essex, UK) dissolved in sterile water (Braun, Melsungen, Germany) was injected into the tail vein of the rat.

Fluorescence was excited at 488 nm, and emitted light was filtered using a 500-nm long-pass filter before passing through the confocal aperture and being recorded by the scanhead photodiode.

### Solutions and Drugs

The Hanks' solution used to superfuse isolated arterioles and as vehicle for intravitreal injections had the following composition (mM): NaCl, 140; KCl, 6; D-glucose, 5; $CaCl_2$, 2; $MgCl_2$, 1.3; HEPES, 10; and pH set to 7.4 with NaOH. Low-$Ca^{2+}$ solution used for vessel isolation differed only in that it contained 0.1 mM $CaCl_2$. Drugs were sourced as follows: endothelin 1 and iberiotoxin (Tocris, Bristol, UK), nimodipine (Alexis, Nottingham, UK), and DIDS (Sigma, Gillingham, UK). Stock solutions of nimodipine were prepared in dimethyl sulfoxide (DMSO) and then diluted to the final concentration using Hanks' solution, with a final DMSO concentration never more than 0.1% (vol/vol). All other drugs were dissolved directly in Hanks' solution. Drugs were applied in prewarmed bath solution to isolated arterioles, or injected intravitreally as described above.

### Data Analysis

Phasic $Ca^{2+}$-signals in the smooth muscle layer of isolated arterioles were analyzed in terms of amplitude and frequency. The relatively slow 2-D imaging rate (20 fps) made it impossible to resolve the brief, localized $Ca^{2+}$-sparks previously characterized by us in this tissue using confocal linescanning.[5,6,15] Prolonged (>500 ms duration) global rises in $[Ca^{2+}]_i$ were observed and are the focus of this article. These events are arbitrarily referred to as "oscillations" in our previous reports, since signal propagation along the length of the cell could not be detected in transverse linescans.[5,6,15,16] However, in the current study, 2-D imaging revealed both propagating $Ca^{2+}$-waves and synchronized $Ca^{2+}$-oscillations (Fig. 1). The time-course of changes in $F/F_0$, spatially averaged across the area of each cell, was plotted using ImageJ (Shareware; NIH, Bethesda, MD) and the frequency and amplitude of phasic $Ca^{2+}$-signals determined manually. Changes in the baseline $[Ca^{2+}]_i$ were assessed from the values of $F/F_0$ observed between phasic $Ca^{2+}$-signals.

Confocal retinal angiograms used for vessel diameter measurements were captured in high-resolution mode using narrow (15°) fields of view within the superior retina and centered 0.5 to 1.5 disc diameters from the edge of the optic disc. Images were analyzed using custom software written in MATLAB (Mathworks, Natick, MA), using its Image Processing Toolbox, as described in a recent article from our group.[17] Images were automatically segmented to identify vessels and arteriole diameters determined for each pixel along the centerline of the vessel. These values were combined to give an overall mean diameter along the length of all arterioles within the image. Diameter measurements at branch points or at the edge of the image were excluded. Vessel diameters were estimated using a conversion factor of 1.15 μm/pixel, based on previously published data for the use of the HRA2 CSLO with rats.[18]

Summary data have been expressed as the mean ± SEM. The distributions of frequency and amplitude for phasic $Ca^{2+}$-signals in retinal arteriolar smooth muscle are skewed,[15] so the statistical significance of apparent changes was tested using nonparametric tests (Wilcoxon signed-rank test for paired frequency and baseline $[Ca^{2+}]_i$ data, and Mann-Whitney $U$ test for unpaired amplitude data). In vivo arteriole diameters following intravitreal injections of specific agents in separate groups of animals were compared using nonparametric one-way ANOVA (Kruskal-Wallis test) with Dunn's post-hoc test to correct for multiple comparisons. In all cases, the acceptable significance level was set at 0.05.

## RESULTS

### Et1 Stimulates Ca²⁺-Waves in Retinal Arteriolar Smooth Muscle

Et1 (10 nM) elevated baseline $[Ca^{2+}]_i$ and stimulated phasic $Ca^{2+}$-signals in individual smooth muscle cells within isolated
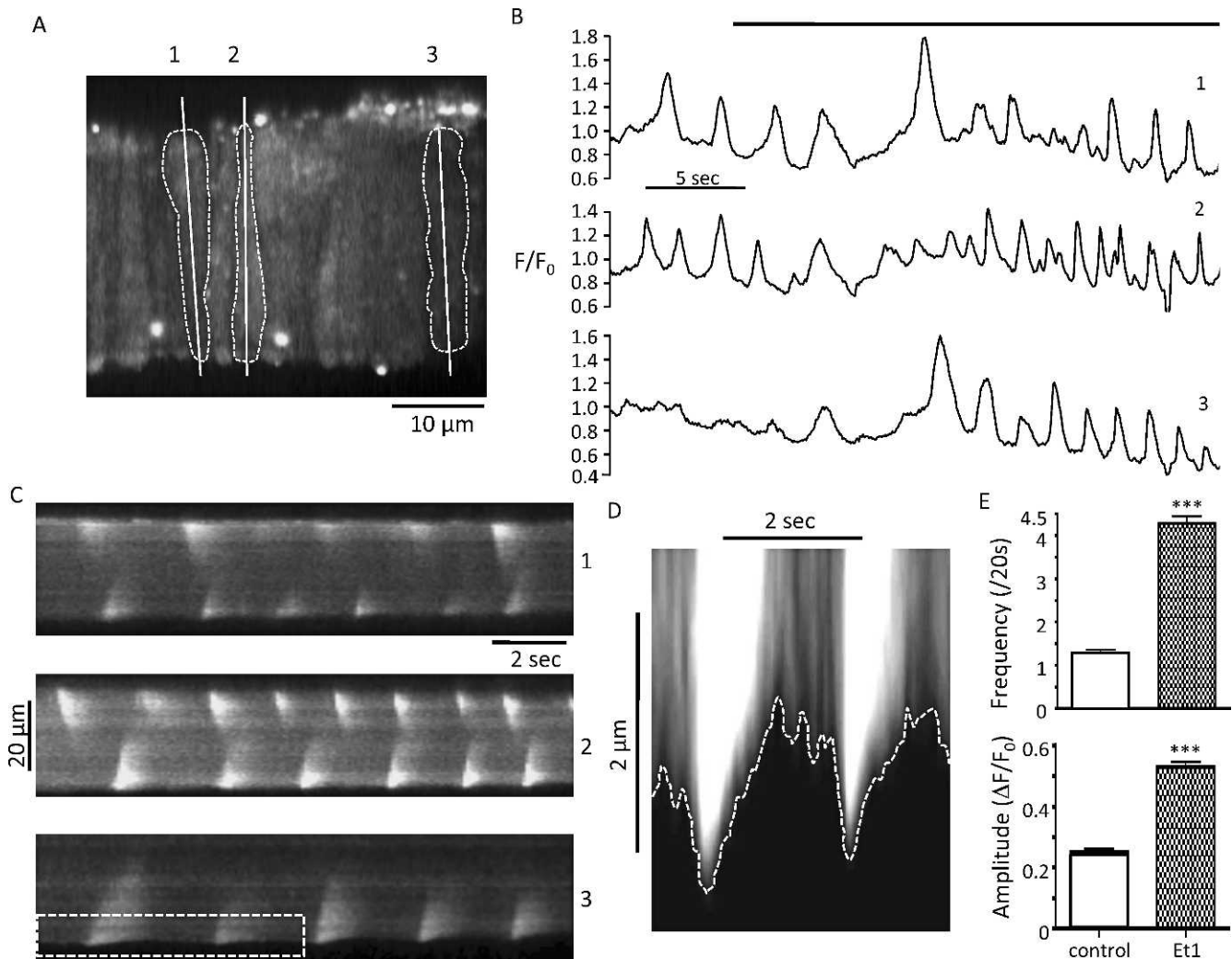
**FIGURE 1.** Et1-stimulates Ca$^{2+}$-waves in retinal arteriolar smooth muscle. (**A**) Two-dimensional confocal image of Fluo-4–loaded myocytes in the wall of a retinal arteriole. These were imaged at 20 fps before and after the addition of endothelin 1 (Et1; 10 nM). (**B**) Time series plots of the normalized fluorescence for each of the three cells outlined in **A** (*dashed line*). (**C**) The resulting stack was resampled along the long axis of the same three myocytes, (*solid lines* in **A**). The images shown were recorded in the presence of Et1 and demonstrate propagating Ca$^{2+}$-waves. In these cells, increases in [Ca$^{2+}$]$_i$ were initiated at the upper and lower ends of the cells and propagated centrally. The gradient of the Ca$^{2+}$-wavefront is a measure of the propagation velocity. (**D**) Rescaled plot of the image region outlined on cell 3 in **C**. Image contrast was manipulated to allow the cell edge to be plotted (*dashed line*), illustrating that each wave was followed by cell shortening. (**E**) Summary data (mean ± SEM) for changes in wave frequency and amplitude following addition of Et1 (284 cells in 15 arterioles). ***$P < 0.0001$ versus control.

retinal arterioles (Figs. 1A, 1B), consistent with our previous findings using one-dimensional (1-D) confocal linescanning.[5] In the current study, 2-D confocal Ca$^{2+}$-imaging provided information about the spatial patterning of signals at the cost of reduced temporal resolution. Sampling rates (20 fps) were too slow to allow the brief localized subcellular Ca$^{2+}$-sparks seen in rapid line scans to be recorded.[16] Et1 typically stimulated Ca$^{2+}$-waves, although the initiation site often varied from wave to wave in the same cell (Fig. 1C). These waves propagated along the long axis of individual myocytes with a mean velocity of 41 ± 4 μm/s ($n = 77$ events in 11 cells in 3 vessels; Fig. 1C) and were followed by myocyte contraction (Fig. 1D). This finding suggests that Ca$^{2+}$-waves represent an important cellular mechanism underlying arteriolar constriction.

For summary purposes, signals were averaged over the whole area of each cell and the phasic changes in mean [Ca$^{2+}$]$_i$ quantified. The term "Ca$^{2+}$-waves" is used to refer to these events throughout the rest of this article, but true Ca$^{2+}$-oscillations were occasionally seen, with relatively synchro-nous rises in [Ca$^{2+}$]$_i$ at different sites in the same cell. Et1 increased wave frequency from 1.26 ± 0.10/20 s under control conditions to 4.29 ± 0.16/20 s in the presence of Et1 ($P < 0.0001$; $n = 284$ cells in 15 vessels from 15 animals). Wave amplitude (ΔF/F$_0$) was also increased, from 0.25 ± 0.01 to 0.53 ± 0.02 ($P < 0.0001$; Fig. 1E). Superfusion with Et1 raised mean baseline [Ca$^{2+}$]$_i$ from 1.27 ± 0.03 to 1.70 ± 0.08 ($P < 0.0001$).

## Role of Ca$^{2+}$-Activated Cl$^-$- and K$^+$-Currents in Cellular Ca$^{2+}$-Signaling Responses to Et1 in Vitro

The contribution of I$_{ClCa}$ to the Et1 response was tested using DIDS (1 mM; Fig. 2A). DIDS inhibits over 80% of I$_{ClCa}$ in retinal arteriolar smooth muscle at negative membrane potentials close to the resting potential.[9] Ca$^{2+}$-wave frequency was reduced from 3.40 ± 0.21/20 s in the presence of Et1 alone, to 0.62 ± 0.10/20 s when DIDS was also present ($P < 0.0001$; $n = 89$ cells in four arterioles; Fig. 2C). Wave amplitude was not

significantly altered, with mean values of $0.57 \pm 0.03$ in the presence of Et1 alone and $0.40 \pm 0.04$ when both Et1 and DIDS were present (NS). Addition of 9-anthracene-carboxylic acid (9AC, 1 mM), which also blocks $Cl^-$-currents in retinal arteriolar smooth muscle,[9] inhibited the Et1-induced $Ca^{2+}$-waves (Fig. 2D). Frequency was reduced from $3.01 \pm 0.19/20$ s in the presence of Et1, to $1.10 \pm 0.23$ when 9AC was also added ($P < 0.0001$; $n = 80$ cells in four arterioles). Wave amplitude also fell from $0.79 \pm 0.05$ with Et1 alone to $0.38 \pm 0.03$ following addition of 9AC ($P < 0.0001$). Neither DIDS nor 9AC reversed the Et1-induced elevation of baseline $[Ca^{2+}]_i$. Mean baseline $F/F_0$ was $2.05 \pm 0.14$ with Et1 alone and $2.01 \pm 0.14$ with both Et1 and DIDS present (NS). In similar experiments using 9AC, mean baseline values were $1.51 \pm 0.14$ when only Et1 was present and $1.44 \pm 0.17$ when 9AC was also added (NS).

These results suggest that positive feedback via $I_{ClCa}$ plays an important role in the stimulation of phasic $Ca^{2+}$-waves by Et1 at the cellular level. Indeed, when the $Cl^-$-channel blockers were applied to Et1-stimulated arterioles, $Ca^{2+}$-wave frequency fell back to the control levels seen in untreated vessels. Wave frequency was $0.62 \pm 0.1/20$ s in the presence of Et1 and DIDS, as compared with $1.23 \pm 0.18/20$ s under control conditions in the same cells (NS). Similarly, mean frequency was $1.10 \pm 0.23/20$ s for 9AC and Et1, compared with $0.68 \pm 0.13/20$ s during the control period (NS).

We tested for negative feedback via activation of BK current by applying iberiotoxin (100 nM), a selective BK-channel blocker, to Et1-treated vessels.[19] This increased the amplitude but not the frequency of $Ca^{2+}$-waves (Figs. 2B, 2D). Mean wave amplitude rose from $0.24 \pm 0.01$ in the presence of Et1 alone to $0.33 \pm 0.02$ in vessels superfused with Et1 and iberiotoxin ($P < 0.0001$). Mean frequency was $6.86 \pm 0.45/20$ s and $6.37 \pm 0.44/20$ s for Et1 alone and Et1 plus iberiotoxin, respectively (NS, $n = 51$ cells in four arterioles). Once again, baseline $[Ca^{2+}]_i$ wasn't affected, with mean values of $1.54 \pm 0.06$ with Et1 alone and $1.48 \pm 0.05$ when Et1 and iberiotoxin were present simultaneously (NS).

## Role of Voltage-Operated $Ca^{2+}$-Channels in Cell Signaling Responses to Et1

Alteration of membrane potential leading to downstream changes in the activation of voltage-operated $Ca^{2+}$-channels represents an important mechanism through which $Ca^{2+}$-sensitive ion channels may modulate global $Ca^{2+}$-signaling.[10] The L-type $Ca^{2+}$-channel blocker nimodipine (10 μM) inhibits stimulation of $Ca^{2+}$-waves by Et1 (Fig. 2E).[20] Frequency was reduced from $4.78 \pm 0.30/20$ s in the presence of Et1 alone to $0.93 \pm 0.14/20$ s when nimodipine was also added ($P < 0.0001$; $n = 71$ cells in four arterioles). Mean wave amplitude also decreased from $0.48 \pm 0.02$ with Et1 alone to $0.31 \pm 0.02$ in the presence of Et1 and nimodipine ($P < 0.0005$). Unlike inhibitors of $Ca^{2+}$-activated $Cl^-$- and $K^+$-currents, however, nimodipine reduced baseline $[Ca^{2+}]_i$ back to control levels seen prior to addition of Et1, from a mean value of $1.60 \pm 0.10$ in the presence of Et1 to $1.00 \pm 0.03$ with Et1 and nimodipine ($P < 0.0001$).

## Role of $Ca^{2+}$-Activated Conductances in Et1 Vasoconstrictor Responses in Vivo

The physiological significance of $Ca^{2+}$-activated $Cl^-$- and $K^+$-currents during Et1-induced vasoconstriction was tested in vivo using fluorescein angiography and confocal imaging with a scanning laser ophthalmoscope. Vessel diameters after intravitreal drug injections were compared with diameters recorded after injection of the same volume (10 μL) of vehicle

(Hanks' solution). Et1 (estimated final intraocular concentration = 100 nM) produced a marked vasoconstriction (Fig. 3). Mean arteriolar diameter was reduced from $37.3 \pm 2.0$ μm after injection of Hanks' solution to $27.7 \pm 0.8$ μm following injection of Et1 ($P < 0.01$). Arteriole diameters following injection with Hanks' solution alone were very similar to those in uninjected eyes, indicating that constrictor responses were not a mechanical artifact.

Arteriolar diameters following intravitreal injections of Et1 plus DIDS or iberiotoxin were compared with those following injection of Et1 alone. DIDS completely inhibited Et1's constrictor effect, with a mean arteriolar diameter of $40.7 \pm 2.6$ μm ($P < 0.05$ vs. Et1 alone; Fig. 3). Neither iberiotoxin nor penitrem A, both BK-channel blockers,[8,19] produced any statistically significant changes in arteriole diameter when compared with Et1 alone.

## DISCUSSION

### Endothelin 1 Stimulates Propagating $Ca^{2+}$-Waves in Retinal Arteriolar Myocytes

We have previously reported that stimulation of retinal arteriolar smooth muscle with Et1 results in an increased frequency of phasic $Ca^{2+}$-signals at the cellular level.[5] However, the use of transverse line scanning in these earlier studies meant that the spatial characteristics of these signals could not be determined, so the term $Ca^{2+}$-oscillation was arbitrarily adopted to describe these events. In the current study, 2-D confocal $Ca^{2+}$-imaging revealed that Et1 mainly stimulates propagating $Ca^{2+}$-waves in individual myocytes (Fig. 1C), although synchronized $Ca^{2+}$-oscillations were occasionally seen. These waves were associated with cell shortening, consistent with the hypothesis that they play an important role in arteriolar constriction.[6,15] Since $Ca^{2+}$-waves were asynchronous in adjacent myocytes, the phasic signaling and mechanical activity observed at the cellular level could explain the tonic constrictor responses to Et1 seen at whole vessel level.[21] Similar $Ca^{2+}$-waves have also been described in pulmonary arteriolar and bronchial smooth muscle both in response to constrictor agonists and depolarization by high $[K^+]$ solutions, suggesting that phasic $Ca^{2+}$-signaling is a common feature of excitation–contraction coupling in smooth muscle.[22,23] At 42 μm/s, the speed of wave propagation was higher in retinal arteriole myocytes than that reported for pulmonary arterioles (12 μm/s[22]) and vasopressin-induced $Ca^{2+}$-waves in cultured vascular smooth muscle (16 μm/s[24]), but was more similar to that seen in Et1-treated vena cava (29 μm/s[25]) and bronchiolar smooth muscle (36 μm/s[22]). Differences in propagation velocity presumably reflect variations in cellular architecture and the distribution of $Ca^{2+}$-signaling molecules. There was considerable variability in the frequency and amplitude of Et1-stimulated $Ca^{2+}$-waves in different groups of vessels (Fig. 2). Considerable variability in Et1 responses has previously been reported (e.g., in retinal and pulmonary arterioles.)[5,22] Although the mechanisms underlying such variability are unclear, this observation emphasizes the benefit of the "repeated-measures" experimental design to maximize statistical power in such studies. It is also worth noting that Et1 may also increase the $Ca^{2+}$-sensitivity of the contractile machinery via Rho-kinase activation.[26]

### Positive Feedback via $Ca^{2+}$-Activated $Cl^-$-Current

Several lines of evidence suggest that activation of $I_{CaCl}$ plays an important role in the vasoconstrictor response to Et1. Two $I_{CaCl}$ blockers, DIDS and 9AC, inhibited Et1 $Ca^{2+}$-signaling (Fig.
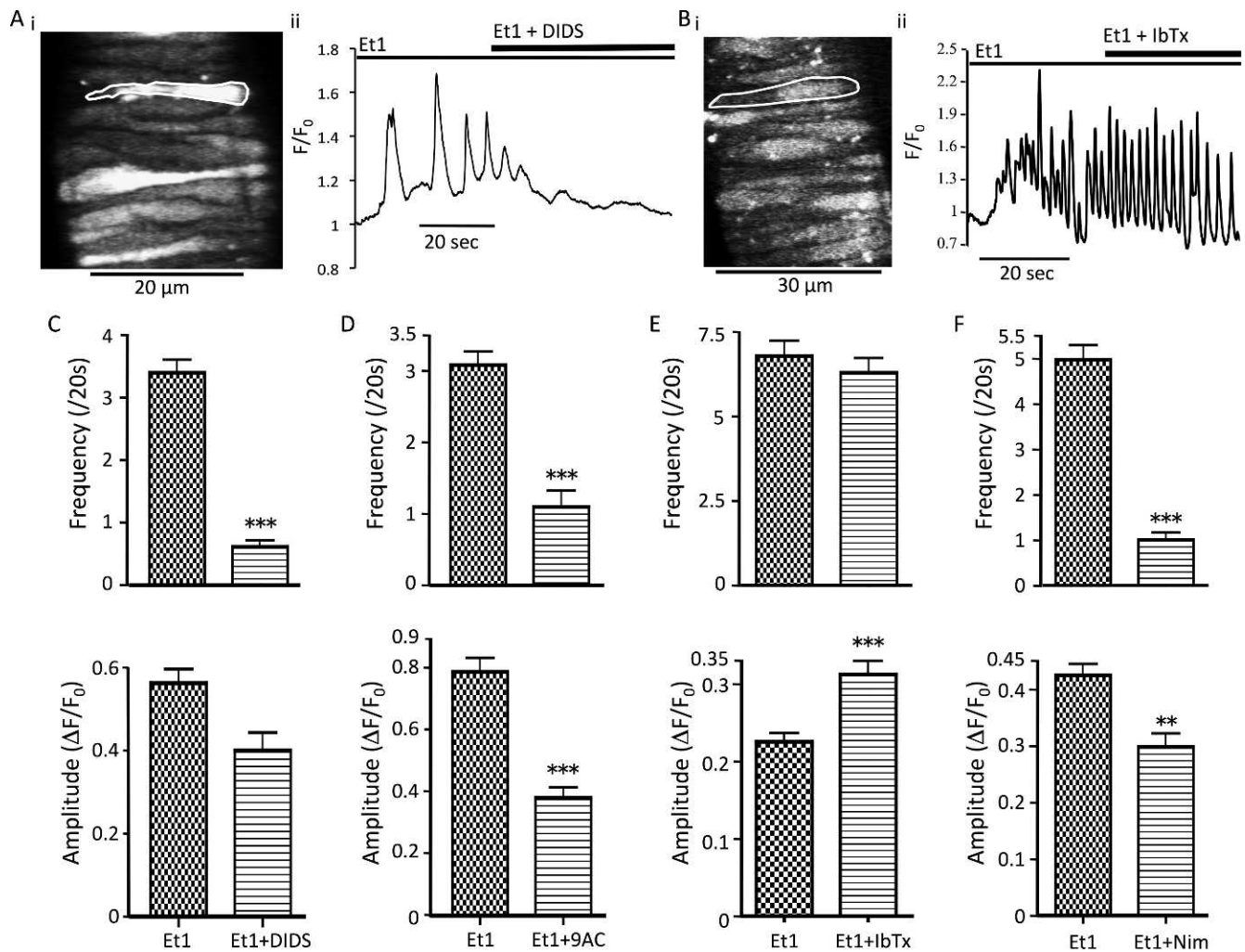
**FIGURE 2.** Effects of ion channel blockers on Et1-stimulated phasic $Ca^{2+}$-signals. (**A**) (**i**) Two-dimensional confocal image of Fluo-4–loaded myocytes in the wall of a retinal arteriole. These were imaged at 20 fps, and the change in fluorescence for each cell was used as a measure of changes in $[Ca^{2+}]_i$. (**ii**) Time series showing the normalized fluorescence ($F/F_0$) for the cell outlined in white in **i**. Addition of Et1 (10 nM) stimulated $Ca^{2+}$-waves/oscillations, but these were rapidly inhibited by addition of DIDS (1 mM). (**B**) Confocal image of arteriolar myocytes (**i**) and fluorescence time series (**ii**) for the outlined myocyte. Iberiotoxin (IbTx; 100 nM) increased the amplitude of phasic $Ca^{2+}$-signals stimulated by Et1. (**C**) Summary data showing the effects of DIDS (1 mM) on signal frequency and amplitude in presence of Et1 (89 cells in four arterioles). (**D**) Summary data showing the effects of 9-AC (1 mM) on signal frequency and amplitude (80 cells in four arterioles). (**E**) Summary data showing the effects of iberiotoxin (100 nM) on signal frequency and amplitude (51 cells in four arterioles). (**F**) Summary data showing the effects of nimodipine (10 μM) on signal frequency and amplitude (64 cells in three arterioles). **$P < 0.0005$; ***$P < 0.0001$ versus Et1 alone.

2), reducing wave frequency to resting values seen in the absence of Et1. The physiological significance of this finding was confirmed in vivo, since intravitreal injection of DIDS along with Et1 completely inhibited the vasoconstriction seen with Et1 alone (Fig. 3). DIDS also inhibits the Et1-induced constriction of pressurized retinal arterioles ex vivo.[9] Although DIDS and 9AC are two of the most frequently used $I_{ClCa}$ blockers, they have been shown to block a variety of different $Cl^-$-channels as well as other cation channels (see Ref. 11 for review). In control experiments on retinal arteriolar myocytes, however, DIDS had no effect on any of the other currents we have previously identified in rat retinal arteriolar smooth muscle at the concentration used in this study, suggesting a specific action on $I_{ClCa}$ (data not shown).[9] The fact that two structurally distinct blockers produced similar effects on Et1-induced $Ca^{2+}$-signaling also suggests that this reflected a common action on $I_{ClCa}$, rather than disparate off-target effects. We believe, therefore, that our results constitute strong evidence that stimulation of $Ca^{2+}$-waves and contraction in

arteriolar smooth muscle by Et1 are dependent on activation of $I_{ClCa}$. These results also provide indirect evidence that the mechanical response in arteriolar smooth muscle is driven by phasic rather than tonic $Ca^{2+}$-signaling, since DIDS, which completely blocked the stimulation of $Ca^{2+}$-waves and constriction by Et1, failed to inhibit the Et1-induced increase in baseline $[Ca^{2+}]_i$. Previous reports on stimulation of bronchiolar and pulmonary arterial smooth muscle by Et1 and a range of other agonists have come to similar conclusions, again emphasizing the importance $Ca^{2+}$-waves and oscillations in stimulus–contraction coupling.[22,23,27,28]

The suggestion that $I_{ClCa}$ is involved in Et1 signaling in retinal arterioles is consistent with electrophysiological studies from our own and other laboratories. G-protein coupled agonist responses in a variety of smooth muscles are believed to be crucially dependent on activation of $I_{ClCa}$.[11,29] Et1 stimulates transient depolarizations in a range of vascular smooth muscle, including that in retinal arterioles, and these events are inhibited by $Cl^-$-channel blockade.[30,31] Et1 also
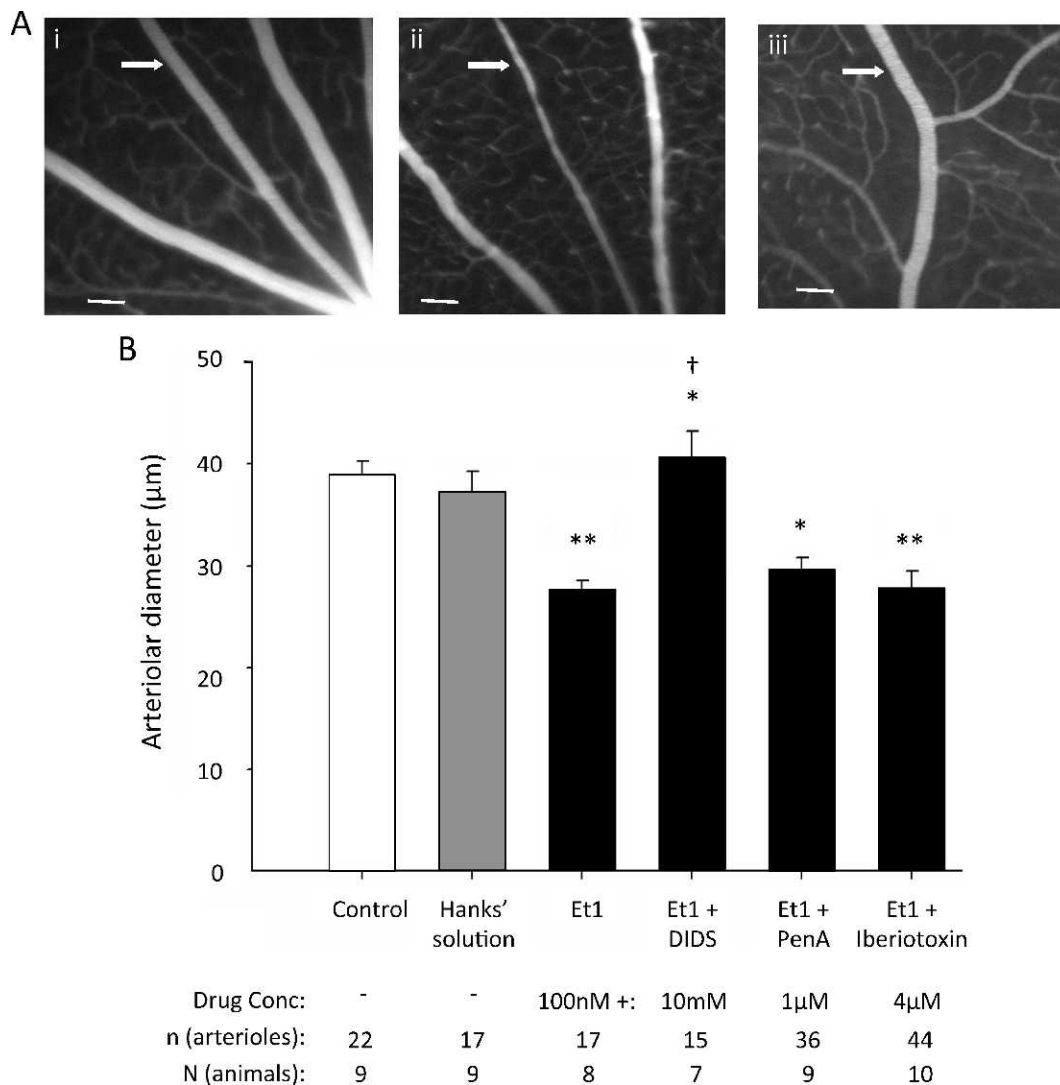
FIGURE 3.    In vivo changes in arteriolar diameter in response to Et1 and ion channel blockers. (**A**) Confocal fluorescein angiograms imaged in anesthetized rats with a 15° scan angle. (Scale bars = 100 μm.) Angiograms recorded (**i**) before and (**ii**) 15 minutes after intravitreal injection of Et1 are compared. Arterioles are indicated with an arrow. Although similar regions of the eye were imaged, the removal and replacement of the animal necessary for intravitreal injection made it difficult to identify any given arteriole before and after Et1 treatment, particularly as Et1-induced constriction changed vessel landmarks and capillary filling patterns. (**iii**) Angiogram recorded from a different animal 15 minutes after intravitreal injection of Et1 and DIDS. (**B**) Column chart plotting summary data for mean arteriolar diameter (±SEM; 15–44 arterioles in 7–10 animals per group). Measurements were made before (control) or after intravitreal injection of 10 μL Hanks' solution or the indicated drug combination. Intraocular drug concentration was estimated from the concentration of the injectate using an assumed vitreal volume of 15 μL.[13] Statistically significant differences are marked both for comparisons with injection of Hanks' solution alone (*$P < 0.05$, **$P < 0.01$), and with Et1 alone (†$P < 0.05$) .

promotes repetitive inward $I_{ClCa}$ currents in rabbit choroidal smooth muscle. Depletion of intracellular $Ca^{2+}$-stores blocks these currents, suggesting they are triggered by $Ca^{2+}$-release.[32] Transient depolarizations resulting from repetitive activation of $I_{ClCa}$ would be expected to promote $Ca^{2+}$-influx via activation of voltage-operated $Ca^{2+}$-channels, amplifying $Ca^{2+}$-release to generate phasic waves or oscillations.[10,11]

### Negative Feedback via $Ca^{2+}$-Activated $K^+$-Current

It is well established that activation of large conductance $Ca^{2+}$-activated $K^+$-currents (BK currents) can inhibit contractility in vascular and other smooth muscle through membrane hyperpolarization and deactivation of L-type $Ca^{2+}$-channels.[33–35] We obtained some evidence that BK channels exerted negative

feedback on Et1-induced $Ca^{2+}$-waves in retinal arteriolar smooth muscle (Fig. 2). Only wave amplitude was affected by the BK-selective inhibitor iberiotoxin, however, with no change in either wave frequency or baseline $[Ca^{2+}]_i$. Intravitreal injection of either iberiotoxin or penitrem A, another BK-blocker, along with Et1 produced no measurable increase in the Et1-induced vasoconstriction (Fig. 3). This is consistent with the hypothesis that Et1-induced vascular constriction is primarily regulated by the frequency, rather than the amplitude, of phasic $Ca^{2+}$-signals.[5,22] It should be noted, however, that the estimated Et1 concentration within the vitreous may have approached 100 nM, which can generate maximal vascular constriction in vitro.[36] The functional relevance of BK-mediated negative feedback cannot, therefore, be entirely dismissed on the basis of these experiments.

## Role of Other Ion Channels in Et1-Mediated Signaling

It is well established that $Ca^{2+}$-influx via L-type channels plays an important role in agonist stimulation of vascular smooth muscle.[11-37] Nimodipine reduced both the amplitude and frequency of Et1-induced $Ca^{2+}$-waves in retinal arterioles (Fig. 2), entirely consistent with the hypothesis that membrane depolarization secondary to activation of $I_{ClCa}$ is an important mechanism in phasic $Ca^{2+}$-signaling. Unlike $I_{ClCa}$ inhibitors, however, nimodipine also blocked the Et1-induced elevation in baseline $[Ca^{2+}]_i$. This suggests that depolarization by a mechanism independent of $I_{ClCa}$ is responsible. Electrophysiological studies also show that Et1 produces a tonic decrease in resting membrane potential in retinal arteriolar smooth muscle and that this is not affected by inhibition of $I_{ClCa}$.[31] One may speculate that Et1 activates a nonspecific cation current, possibly conducted via Transient Receptor Potential Canonical (TRPC) channels, as observed in coronary myocytes.[38]

## CONCLUSION

We propose that activation of $I_{ClCa}$ represents an important positive feedback mechanism during Et1 signaling in retinal arteriolar myocytes. Stimulation of $Et_A$ receptors by Et1 promotes $Ca^{2+}$-release from the sarcoplasmic reticulum (SR) via both $IP_3$ and ryanodine gated receptors ($IP_3Rs$ and RyRs).[5] This triggers activation of $Ca^{2+}$-sensitive $Cl^-$-channels, with the resulting depolarization opening L-type voltage-operated $Ca^{2+}$-channels. Previous studies on smooth muscle have suggested that positive feedback via calcium-induced calcium release from the intracellular store provides an adequate mechanistic explanation for agonist-stimulated $Ca^{2+}$-waves.[24] In retinal arterioles, however, additional $Ca^{2+}$-influx seems to be required.

## References

1. Clermont AC, Bursell SE. Retinal blood flow in diabetes. *Microcirculation*. 2007;14:49–61.

2. Salvatore S, Vingolo EM. Endothelin-1 role in human eye: a review. *J Ophthalmol*. 2010:354645.

3. Resch H, Karl K, Weigert G, et al. Effect of dual endothelin receptor blockade on ocular blood flow in patients with glaucoma and healthy subjects. *Invest Ophthalmol Vis Sci*. 2009;50:358–363.

4. Venkataraman ST, Flanagan JG, Hudson C. Vascular Reactivity of Optic Nerve Head and Retinal Blood Vessels in Glaucoma—A Review. *Microcirculation*. 17:568–581.

5. Tumelty J, Hinds K, Bankhead P, et al. Endothelin 1 stimulates Ca2+-sparks and oscillations in retinal arteriolar myocytes via IP3R and RyR-dependent Ca2+ release. *Invest Ophthalmol Vis Sci*. 2011;52:3874–3879.

6. Curtis TM, Tumelty J, Dawicki J, Scholfield CN, McGeown JG. Identification and spatiotemporal characterization of spontaneous Ca2+ sparks and global Ca2+ oscillations in retinal arteriolar smooth muscle cells. *Invest Ophthalmol Vis Sci*. 2004;45:4409–4414.

7. Hein TW, Ren Y, Yuan Z, et al. Functional and molecular characterization of the endothelin system in retinal arterioles. *Invest Ophthalmol Visual Sci*. 2009;50:3329–3336.

8. McGahon MK, Dash DP, Arora A, et al. Diabetes downregulates large-conductance Ca2+-activated potassium beta 1 channel subunit in retinal arteriolar smooth muscle. *Circ Res*. 2007;100:703–711.

9. McGahon MK, Needham MA, Scholfield CN, McGeown JG, Curtis TM. Ca2+-activated Cl-current in retinal arteriolar smooth muscle. *Invest Ophthalmol Vis Sci*. 2009;50:364–371.

10. McGeown JG. Seeing is believing! Imaging Ca2+-signalling events in living cells. *Exp Physiol*. 2010;95:1049–1060.

11. Large WA, Wang Q. Characteristics and physiological role of the Ca(2+)-activated Cl- conductance in smooth muscle. *Am J Physiol*. 1996;271:C435–454.

12. Yang Y, Murphy TV, Ella SR, et al. Heterogeneity in function of small artery smooth muscle BKCa: involvement of the Œ≤1-subunit. *J Physiol*. 2009;587:3025–3044.

13. Dureau P, Bonnel S, Menasche M, Dufier JL, Abitbol M. Quantitative analysis of intravitreal injections in the rat. *Curr Eye Research*. 2001;22:74–77.

14. del Amo EM, Urtti A. Current and future ophthalmic drug delivery systems: a shift to the posterior segment. *Drug Discov Today*. 2008;13:135–143.

15. Tumelty J, Scholfield N, Stewart M, Curtis T, McGeown G. Ca2+-sparks constitute elementary building blocks for global Ca2+-signals in myocytes of retinal arterioles. *Cell Calcium*. 2007;41:451–466.

16. Jeffries O, McGahon MK, Bankhead P, et al. cAMP/PKA-dependent increases in Ca sparks, oscillations and SR Ca stores in retinal arteriolar myocytes after exposure to vasopressin. *Invest Ophthalmol Vis Sci*. 2010;51:1591–1598.

17. Bankhead P, Scholfield CN, McGeown JG, Curtis TM. Fast retinal vessel detection and measurement using wavelets and edge location refinement. *PLoS ONE*. In press.

18. Maass A, von Leithner PL, Luong V, et al. Assessment of rat and mouse RGC apoptosis imaging in vivo with different scanning laser ophthalmoscopes. *Curr Eye Res*. 2007;32:851–861.

19. Shao Y, Liew R, Guan B, et al. Different expression of large-conductance calcium-activated K+ channels in human internal mammary and radial arteries. *Cardiovasc Res*. 2011;89:329–335.

20. Du H, He J, Wang S, He L. Investigation of calcium antagonist-L-type calcium channel interactions by a vascular smooth muscle cell membrane chromatography method. *Anal Bioanal Chem*. 2010;397:1947–1953.

21. Curtis TM, Scholfield C, McGeown DJ. Calcium signaling in ocular arterioles. *Crit Rev Eukaryot Gene Expr*. 2007;17:1–12.

22. Perez-Zoghbi JF, Sanderson MJ. Endothelin-induced contraction of bronchiole and pulmonary arteriole smooth muscle cells is regulated by intracellular Ca2+ oscillations and Ca2+ sensitization. *Am J Physiol Lung Cell Mol Physiol*. 2007;293:L1000–1011.

23. Sanderson MJ, Bai Y, Perez-Zoghbi J. Ca(2+) oscillations regulate contraction of intrapulmonary smooth muscle cells. *Adv Exp Med Biol*. 2010;661:77–96.

24. Blatter LA, Wier WG. Agonist-induced [Ca2+]i waves and Ca(2+)-induced Ca2+ release in mammalian vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol*. 1992;263:H576–H586.

25. Dai J, Lee CH, Poburko D, Szado T, Kuo KH, van Breemen C. Endothelin-1-mediated wave-like [Ca2+]i oscillations in intact rabbit inferior vena cava. *J Vasc Res*. 2007;44:495–503.

26. Hein TW, Rosa RH, Yuan Z, Roberts E, Kuo L. Divergent roles of nitric oxide and Rho kinase in vasomotor regulation of human retinal arterioles. *Invest Ophthalmol Vis Sci*. 2010;51:1583–1590.

27. Perez JF, Sanderson MJ. The frequency of calcium oscillations induced by 5-HT, ACH, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. *J Gen Physiol*. 2005;125:535–553.

28. Perez JF, Sanderson MJ. The contraction of smooth muscle cells of intrapulmonary arterioles is determined by the frequency of Ca2+ oscillations induced by 5-HT and KCl. *J Gen Physiol*. 2005;125:555–567.

29. Large WA, Greenwood IA, Piper AS. Recent advances on the properties and role of Ca2+-activated chloride currents in

smooth muscle. In: Fuller C, ed. *Calcium-Activated Chloride Channels*. San Diego, CA: Academic Press; 2002;99–118.

30. Salter KJ, Kozlowski RZ. Differential Electrophysiological Actions of Endothelin-1 on Cl- and K+ Currents in Myocytes Isolated from Aorta, Basilar and Pulmonary Artery. *J Pharmacol Exp Ther*. 1998;284:1122–1131.

31. Scholfield CN, McGeown JG, Curtis TM. Cellular physiology of retinal and choroidal arteriolar smooth muscle cells. *Microcirculation*. 2007;14:11–24.

32. Curtis TM, Scholfield CN. Transient Ca2+-activated Cl-currents with endothelin in isolated arteriolar smooth muscle cells of the choroid. *Invest Ophthalmol Vis Sci*. 2000;41:2279–2285.

33. Nelson MT, Cheng H, Rubart M, et al. Relaxation of arterial smooth muscle by calcium sparks. *Science*. 1995;270:633–637.

34. Burdyga T, Wray S. Action potential refractory period in ureter smooth muscle is set by Ca sparks and BK channels. *Nature*. 2005;436:559–562.

35. Hill MA, Yang Y, Ella SR, Davis MJ, Braun AP. Large conductance, Ca2+-activated K+ channels (BKCa) and arteriolar myogenic signaling. *FEBS Lett*. 2010;584:2033–2042.

36. Ohlstein EH, Horohonich S, Hay DW. Cellular mechanisms of endothelin in rabbit aorta. *J Pharmacol Exp Ther*. 1989;250:548–555.

37. Byron KL. Vasopressin stimulates Ca2+ spiking activity in A7r5 vascular smooth muscle cells via activation of phospholipase A2. *Circ Res*. 1996;78:813–820.

38. Peppiatt-Wildman CM, Albert AP, Saleh SN, Large WA. Endothelin-1 activates a Ca2+-permeable cation channel with TRPC3 and TRPC7 properties in rabbit coronary artery myocytes. *J Physiol*. 2007;580:755–764.