Endothelin 1 Stimulates Ca^{2+} -Sparks and Oscillations in Retinal Arteriolar Myocytes via IP_3R and RyR -Dependent Ca^{2+} Release

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PURPOSE. To investigate endothelin 1 (Et1)-dependent Ca^{2+} -signaling at the cellular and subcellular levels in retinal arteriolar myocytes.

METHODS. Et1 responses were imaged from Fluo-4-loaded smooth muscle in isolated segments of rat retinal arteriole using confocal laser microscopy.

RESULTS. Basal $[\text{Ca}^{2+}]_i$, subcellular Ca^{2+} -sparks, and cellular Ca^{2+} -oscillations were all increased during exposure to Et1 (10 nM). Ca^{2+} -spark frequency was also increased by 90% by 10 nM Et1. The increase in oscillation frequency was concentration dependent and was inhibited by the Et_A receptor (Et_AR) blocker BQ123 but not by the Et_B receptor antagonist BQ788. Stimulation of Ca^{2+} -oscillations by Et1 was inhibited by a phospholipase C blocker (U73122; 10 μM), two inhibitors of inositol 1,4,5-trisphosphate receptors (IP_3Rs), xestospongine C (10 μM), 2-aminoethoxydiphenyl borate (100 μM), and tetracaine (100 μM), a blocker of ryanodine receptors (RyRs).

CONCLUSIONS. Et1 stimulates Ca^{2+} -sparks and oscillations through Et_ARs . The underlying mechanism involves the activation of phospholipase C and both IP_3Rs and RyRs , suggesting crosstalk between these Ca^{2+} -release channels. These findings suggest that phasic Ca^{2+} -oscillations play an important role in the smooth muscle response to Et1 within the retinal microvasculature and support an excitatory, procontractile role for Ca^{2+} -sparks in these vessels. (*Invest Ophthalmol Vis Sci.* 2011; 52:3874–3879) DOI:10.1167/iops.10-6029

Endothelin 1 (Et1) is a peptide paracrine signaling molecule with potent vasoconstrictor¹ and mitogenic actions^{2,3} on vascular smooth muscle. As well playing an important physiological role in the homeostatic control of blood pressure and blood flow, there is growing evidence to suggest that Et1 is implicated in the pathophysiology of a range of important vascular diseases, including systemic hypertension,⁴ pulmo-

nary hypertension,⁵ and diabetic vasculopathies.⁶ In the retina, Et1 expression has been demonstrated in glial, neural, and vascular elements in the eyes of rats, pigs, and humans.^{7,8} A pathogenic role has been suggested for endothelin in glaucoma,⁹ whereas Et1 expression is increased both in animal models of diabetes and in humans with proliferative diabetic retinopathy.^{10,11} Changes in retinal expression of endothelin receptors, (Et_ARs and Et_BRs) have also been reported in various diabetic models,^{12,13} as has alteration of the hemodynamic responses to intravitreal injection of Et1.¹⁴ Despite its pathologic significance, however, the cellular signals responsible for retinal vascular responses to Et1 are not well understood. These signals will be the focus for this article.

One of the key signal transduction steps activated by Et1 in vascular smooth muscle is an increase in intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$). Early studies described an initial Et-induced Ca^{2+} -transient that was dependent on phospholipase C activity and inositol 1,4,5 trisphosphate (IP_3) production. This was followed by a sustained rise in Ca^{2+} because of the influx of extracellular Ca^{2+} .^{15–17} Use of microfluorimetry to record average changes in intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) in the smooth muscle layer of intact segments of rat retinal arterioles demonstrated that activation of the Et_ARs by Et1 resulted in both transient and sustained $[\text{Ca}^{2+}]_i$ increases, stimulating vasoconstriction.¹⁸ However, recent studies from our laboratory using high-speed Ca^{2+} -imaging have revealed faster cellular and subcellular Ca^{2+} -signaling events in retinal arteriolar myocytes that were not apparent in microfluorimetry records from arteriole segments. These are seen in both rat and pig arterioles and consist of brief localized Ca^{2+} -sparks and more global Ca^{2+} -waves and oscillations, the latter associated with cell contraction.^{19–21} Studies in other vascular smooth muscle have shown that, at the cellular level, many vascular agonists act to increase the frequency of phasic $[\text{Ca}^{2+}]_i$ -signals rather than uniformly raising mean $[\text{Ca}^{2+}]_i$.^{22–24} The experiments described here were designed to investigate Et1-evoked Ca^{2+} -signaling with high spatial and temporal resolution in retinal arteriolar myocytes for the first time and to examine the mechanisms responsible for the effects seen. They revealed dramatic Et1-induced increases in Ca^{2+} -sparks and oscillations, suggesting that regulation of constriction by Et1 relies more on frequency-modulated than amplitude-modulated signaling at the cell level.

MATERIALS AND METHODS

Ca^{2+} Imaging in Isolated Rat Retinal Arterioles

These techniques have been described in detail previously.²⁰ Briefly, rats were euthanatized in compliance with UK Home Office regulations and the standards set out in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinas were triturated in low- Ca^{2+} (100 μM) Hanks solution, centrifuged, washed, and centri-

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fused again. The resultant vascular fragments were incubated with 10 μM Fluo-4AM in low Ca^{2+} solution for 2 hours and then pipetted into an organ bath, which had a bottom formed of $\sim 0.17\text{-mm}$ -thick glass and was mounted on the stage of an inverted microscope (Eclipse TE300; Nikon Instruments, Surrey, UK). Arteriole segments (outer diameter range, 25–50 μm) were visualized using a PlanApo, $\times 60$, 1.4 NA, oil-immersion objective and anchored in position using tungsten wire slips. The bath was perfused with normal Hanks solution at 37°C , and $[\text{Ca}^{2+}]$ changes in the smooth muscle layer were imaged in line scan mode (500 s^{-1}) using a confocal scanning laser microscope (MR-A1; Bio-Rad, Hercules, CA). Fluo-4 was excited at 488 nm and emitted light band-pass filtered (530–560 nm) and was detected with a photomultiplier tube. Imaging at any single site was limited to 150 seconds to minimize photodamage. Data acquisition was controlled with software (Timecourse; Bio-Rad). Fluorescence was corrected to allow for the background count in the absence of excitation, and background-corrected fluorescence (F) was normalized to the resting fluorescence (F_0) in the same cell. Increases in F/F_0 were interpreted as increases in cell $[\text{Ca}^{2+}]$.

Solutions and Drugs

Isolated arterioles were superfused with Hanks solution of the following composition: NaCl, 140 mM; KCl, 5 mM; D-glucose, 5 mM; CaCl_2 , 2 mM; MgCl_2 , 1.3 mM; HEPES, 10 mM; and pH set to 7.4 with NaOH. The low- Ca^{2+} solution for vessel isolation differed only in that it contained 0.1 mM CaCl_2 . Drugs were obtained from (Sigma, Paisley, UK) and were applied in prewarmed bath solution at 37°C to the outside of the arterioles.

Statistical Analysis

Key parameters analyzed included basal $[\text{Ca}^{2+}]$, relatively prolonged phasic increases in $[\text{Ca}^{2+}]$ known as Ca^{2+} -oscillations, and much briefer more localized increases dubbed Ca^{2+} -sparks.^{19,20} Although the term *oscillation* has been used to describe all prolonged elevations in $[\text{Ca}^{2+}]$ (>0.5 seconds), these cannot be distinguished from propagating Ca^{2+} -waves in transverse line scan recordings. The time course of changes in F/F_0 , spatially averaged across the width of each cell, was plotted using ImageJ software (developed by Wayne Rasband, National

Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>), and the number, amplitude, and duration of Ca^{2+} -oscillations were determined manually. For Ca^{2+} -sparks, custom-designed software based on an à trous wavelet transform was used to automate detection and analysis, as previously described.²¹ Spark amplitude was defined as the maximum increase in $\Delta F/F_0$ for a 5-pixel-wide region of interest centered on the point of maximum fluorescence. The full duration at half-maximal fluorescence and the full width at half-maximal fluorescence were also measured for each event.¹⁹

For presentation, summary data have been normalized to the mean control values in the same set of experiments in the absence of any drugs (see Figs. 1, 3), or to values recorded in the presence of Et1 alone (see Fig. 4). All data have been expressed as the mean $\pm$ SEM for the data set. It should be noted that all statistical analyses were carried out on raw data before normalization. Spark and oscillation data were not normally distributed, so nonparametric tests were used.¹⁹ The Wilcoxon signed-rank test for paired data and the Mann-Whitney U test for nonpaired data were applied to two-group comparisons. The Kruskal-Wallis test (nonparametric ANOVA) was applied when three or more groups were compared with post hoc comparison using Dunn's multiple-comparison test. In all cases, the accepted significance level was set at 0.05.

RESULTS

Cellular and Subcellular Ca^{2+} -Signaling Responses to Et1

Figure 1A demonstrates that superfusion with 10 nM Et1 increased $[\text{Ca}^{2+}]$ oscillations in smooth muscle cells in rat retinal arterioles. Basal $[\text{Ca}^{2+}]$ was increased in some, though not all, cells, as seen in the plots of fluorescence for cells from the same arteriole segment (Fig. 1B). The mean value of F/F_0 was increased by 1.40 ± 0.19 between the baseline at the end of the control period and that following at least 30-second superfusion with Et1 (Fig. 1B; $P < 0.0001$). Ca^{2+} -imaging at the cell level also revealed a dramatic Et1-evoked increase in the frequency and amplitude of Ca^{2+} -oscillations within the smooth

FIGURE 1. Et1 effects on Ca^{2+} -oscillations. (A) Confocal image of retinal arteriolar myocytes loaded with Fluo-4 (left). Adjacent myocytes were scanned along the marked line to generate a line scan image (right). (B) Time series data for three cells in (A) (c2, c4, c5) showing Et1-induced Ca^{2+} -oscillations. (C) Summary data for oscillation frequency and amplitude in the presence of Et1 at 1 nM (314 cells, 18 arterioles, 5 animals) or 10 nM (57 cells, 8 arterioles, 4 animals). Results have been normalized using oscillation data recorded under control conditions (362 cells in 26 arterioles from 9 animals).

FIGURE 2. Effects of Et1 on Ca²⁺-sparks. (A) 2D scan (upper) and high-speed line scan image showing a single Ca²⁺-spark (lower and graph). (B) Line scan images (upper) and time-series data (lower) during control and Et1 treatments. Asterisks: sparks sites at x. (C) Spark frequency summary data (59 spark sites, 7 arterioles, 4 animals).

muscle of retinal arterioles, a feature not previously apparent in averaged Fura-2 signals.¹⁸ Oscillation duration was not affected (data not shown). The effect on Ca²⁺-oscillations was concentration dependent. Superfusion with 1 nM Et1 produced a smaller increase in oscillation frequency than 10 nM and had no effect on amplitude (Fig. 1C). Inactive cells were included as a frequency of zero, and much of the increase in the mean oscillation frequency reflected “cell recruitment.” The proportion of cells exhibiting oscillations increased from 23% under control conditions to 41% in the presence of ET1 at 1 nM concentration and 100% at 10 nM. All subsequent experiments were carried out using 10 nM Et1 to maximize cell activity. There was no evidence of synchronization between adjacent cells in these recordings.

We have previously reported that high-speed Ca²⁺-imaging reveals brief, localized subcellular Ca²⁺-signals known as Ca²⁺-sparks in both rat and pig retinal arterioles^{20,21} (Fig. 2A). Et1 increased Ca²⁺-spark frequency in the current experiments (Fig. 2), from an average of 0.31 ± 0.04/s under control conditions to 0.59 ± 0.04/s in the presence of Et1 (10 nM). Et1 did not affect any other spark characteristic (Table 1).

Receptor Pharmacology of Et1 Responses

We tested the involvement of endothelin A receptors (Et_AR) and endothelin B receptors (Et_BR) using pharmacologic receptor blockers.²⁵ After a period of control recording, arterioles

were exposed to the relevant antagonist for at least 10 seconds. Et1 was then applied in the continued presence of the antagonist, and the effects on Ca²⁺ were recorded. The Et_AR blocker BQ123 (100 nM) completely inhibited the action of Et1 on oscillation frequency and amplitude (Fig. 3A). BQ123 (100 nM) itself had no effect on either oscillation frequency or amplitude (data not shown). BQ123 reduced, but did not completely inhibit, the effect of Et1 on baseline [Ca²⁺]. Normalized fluorescence (F/F₀) increased from 1.35 ± 0.09 in BQ123 to 1.51 ± 0.10 when Et1 was also present (*P* < 0.01).

In contrast to BQ123, the Et_BR inhibitor BQ788 (100 nM) did not block the action of Et1 on oscillation frequency (Fig. 3B) but did inhibit the increase in oscillation amplitude. This might have reflected the fact that BQ788 itself increased oscillation ampli-

FIGURE 3. Effects of EtR antagonists on Et1 actions. Summary data show the effects of Et1 (10 nM) on Ca²⁺-oscillations in the presence of (A) BQ123 (100 nM; 61 cells, 7 arterioles, 3 animals) and (B) BQ788 (100 nM; 200 cells, 18 arterioles, 10 animals). All data have been normalized using control data recorded before the addition of the receptor antagonists.

TABLE 1. Spark Characteristics under Control Conditions and in the Presence of Et1

Spark Characteristic	Control (<i>n</i> = 412)	Et1 (10 nM) (<i>n</i> = 1609)
Amplitude, ΔF/F ₀	1.64 ± 0.05	1.73 ± 0.03 (NS)
FDHM, ms	22.5 ± 0.9	23.1 ± 0.5 (NS)
FWHM, μM	0.53 ± 0.01	0.52 ± 0.01 (NS)

Summary data for amplitude, full duration at half-maximum (FDHM), and full width at half-maximum (FWHM). NS, no significant difference vs. control; n, number of sparks analyzed.

tude to 0.99 ± 0.11 from a control value of 0.67 ± 0.05 ($P < 0.05$). Et1 still increased basal $[\text{Ca}^{2+}]$ in the presence of BQ788, with an average rise in F/F_0 of 0.66 ± 0.13 ($P < 0.01$).

Involvement of PLC, IP_3 , and Ryanodine Receptors in Et1-Evoked Ca^{2+} Oscillations

We carried out a series of experiments designed to test for the involvement of the PLC- IP_3 signaling pathway in the generation of Et1-evoked oscillations.^{15–17} Relevant inhibitors were applied to arterioles pre-exposed to Et1 (10 nM), and the effects were observed. The PLC inhibitor U73122 (10 μM) almost completely abolished Ca^{2+} -oscillations within 10 seconds of application (Fig. 4). Two IP_3 receptor (IP_3R) blockers, 2-aminoethoxydiphenyl borate (2APB; 100 μM) and xestospongine C (10 μM),^{26,27} also reduced oscillation frequency and amplitude when added to arterioles in the presence of Et1 (Fig. 4). The effects of these IP_3R blockers on basal Ca^{2+} were less consistent; 2APB produced no significant change when added on top of Et1 (data not shown), whereas xestospongine C produced a small increase, with an average rise in basal F/F_0 of 0.08 ± 0.02 ($P < 0.005$).

The data presented here suggest that the generation of IP_3 by PLC, leading to Ca^{2+} -release through IP_3Rs , plays a role in the stimulation of Ca^{2+} -oscillations by Et1 in arteriolar smooth muscle. However, Ca^{2+} may also be released from the sarcoplasmic reticulum (SR) in smooth muscle through the activation of ryanodine receptors (RyRs), and cross-talk between IP_3Rs and RyRs has been observed.^{28–30} Tetracaine (100 μM), a RyR blocker that inhibits spontaneous Ca^{2+} -sparks and oscillations in retinal arterioles,¹⁹ also inhibited Et1-induced Ca^{2+} -

oscillations (Fig. 4). Tetracaine produced a small reduction in baseline Ca^{2+} when it was added to Et1-treated vessels, decreasing F/F_0 by 0.16 ± 0.06 ($P < 0.005$). Overall, these findings suggest that the activation of both IP_3Rs and RyRs is involved in the generation and agonist stimulation of Ca^{2+} -oscillations in retinal arteriolar myocytes.

DISCUSSION

Et1 Promotes Ca^{2+} Sparks and Oscillations in Retinal Arteriolar Myocytes

The key observation in this study was that the main action of Et1 on myocyte $[\text{Ca}^{2+}]_i$ was to increase Ca^{2+} -spark activity and stimulate phasic Ca^{2+} -oscillations (Figs. 1, 2). This contrasts with the relatively smooth, tonic rise in average $[\text{Ca}^{2+}]$ seen in microfluorometry recordings from intact arteriole segments. Averaging of asynchronous, relatively short (approximately 5-second duration) oscillations in adjacent cells could explain the much slower and more prolonged “whole-vessel” Ca^{2+} -transient, in which there is an initial Ca^{2+} -transient (>100 seconds) followed by a sustained plateau phase, associated with tonic vascular constriction.^{18,25} Tight coupling of Ca^{2+} -oscillations in a segment of arteriole would be expected to result in phasic vasomotion with the same frequency as the Ca^{2+} -signaling in the vascular smooth muscle.³¹ This was not seen under the current conditions.

Any detailed model of Et1 signaling must explain the responses seen in individual cells, not just the tissue average. Et1-evoked Ca^{2+} -oscillations have previously been described in cultured A7R5 vascular smooth muscle³² and, more recently, in a number of primary vascular tissues.^{24,33} Ca^{2+} -oscillations can also be evoked in vascular smooth muscle by a range of other agonists.³⁴ We have reported similar behavior in porcine retinal arterioles exposed to arginine vasopressin (AVP),²¹ although the resultant oscillations did not have the impressive rhythmicity so obvious in Et1 responses (Fig. 1). These findings suggest an emerging model in which vascular tone is regulated by changes in oscillation frequency rather than by mean $[\text{Ca}^{2+}]$.^{24,34} The benefits of such digital control over analog regulation by steady state $[\text{Ca}^{2+}]$ are unclear, though cell exposure to potentially damaging Ca^{2+} -concentrations may be limited.³⁵

Spontaneous Ca^{2+} sparks were recorded with characteristics similar to those reported by us in earlier studies (Table 1)^{19,20} and to sparks in other smooth muscles.³⁶ These most commonly originated from sites close to the cell membrane, suggesting release from peripherally distributed SR at sites likely to have ready access to Ca^{2+} -sensitive membrane channels. Spark frequency was increased in the presence of Et1 (Fig. 2). Et1 increases Ca^{2+} -spark activity in pulmonary artery smooth muscle,^{37,38} but we believe this is the first report of such an action in systemic vascular smooth muscle. An agonist-induced increase in spark frequency in the context of increased oscillation frequency is consistent with our previous studies showing that sparks can summate to produce more prolonged oscillations and that these can activate cell contraction.^{19,20} The vasoconstrictor thromboxane analog U46619 has also been shown to stimulate Ca^{2+} -oscillations in vascular smooth muscle from porcine retinal arterioles, though only at low extracellular $[\text{Ca}^{2+}]$, suggesting other signaling mechanisms may be involved under more physiological conditions.³⁹ Pharmacologic interventions that modulate spark activity in rat retinal arteriolar smooth muscle produce positively correlated changes in Ca^{2+} -oscillations, and the stimulation of oscillations in porcine retinal arterioles with the constrictor agonist AVP was also associated with an increase in Ca^{2+} -sparks.^{19,21}

FIGURE 4. Effects of inhibitors of PLC inhibition and SR Ca^{2+} -release on Et1-induced Ca^{2+} -oscillations. Summary data showing reductions in (A) oscillation frequency and (B) oscillation amplitude when inhibitors were added to arteriole segments prestimulated with Et1 (10 nM). The drugs used were U73122 (10 μM ; 89 cells, 18 arterioles, 7 animals), xestospongine C (10 μM ; 26 cells, 4 arterioles, 3 animals), 2APB (100 μM ; 26 cells, 4 arterioles, 2 animals), and tetracaine (100 μM , 30 cells, 5 arterioles, 3 animals). Data have been normalized to observations recorded in Et1 before the addition of the relevant inhibitor. *P* values indicate statistically significant reductions from recordings made in the presence of Et1 alone.

These findings suggest that Ca^{2+} -sparks in retinal microvascular smooth muscle serve an excitatory, constrictor function. This contrasts with their inhibitory, dilator role in larger systemic arteries.⁴⁰ Activation of large-conductance Ca^{2+} -activated K^{+} -channels (BK channels) by near-membrane sparks results in myocyte hyperpolarization and deactivation of voltage-operated Ca^{2+} -channels. The resultant fall in global $[\text{Ca}^{2+}]$ leads to muscle relaxation and vessel dilatation, whereas the inhibition of either BK channels or spark activity leads to constriction.^{40,41} BK currents can be recorded as spontaneous transient outward currents in rat retinal arteriolar myocytes, and inhibition of BK channels results in the constriction of pressurized retinal vessels.⁴² It seems, however, that BK activation is not the primary function of sparks in these arterioles and that they may be better understood as fundamental building blocks for longer, more global Ca^{2+} -signals (waves and oscillations) during stimulation-contraction coupling.¹⁹

Further Ca^{2+} -imaging experiments using pressurized arterioles will be required to explore what role sparks play in the development of myogenic, as opposed to agonist-induced, tone. Modulation of Ca^{2+} -sparks and oscillations may also play an important role in vascular responses to vasodilators. Stimulation of cortical neurones can inhibit Ca^{2+} -oscillations in cerebral arterioles, suppressing vascular vasomotion and favoring increased local blood flow during neurovascular coupling by astrocytes.⁴³ Dilators may affect sparks and oscillations differentially because the dilator nitric oxide (NO) can increase Ca^{2+} -spark activity in cerebral vascular smooth muscle, with increased BK activation and myocyte hyperpolarization.⁴⁴ Future studies to directly investigate the effects of dilators on Ca^{2+} -signaling in retinal arteriolar myocytes are required.

Receptor Pharmacology, Signal Transduction, and Ca^{2+} -Release Pathways

The data presented here suggest that Et1-induced increases in Ca^{2+} -sparks and oscillations resulted from the activation of Et_A Rs on the cell membrane and opening of IP_3 Rs, secondary to IP_3 production by phospholipase C. This signaling pathway has been well established for many years based on $[\text{Ca}^{2+}]$ microfluorometry recordings.^{15,16,25} The Et_B R blocker used, BQ788, also inhibited the action of Et1 on oscillation amplitude, though not that on frequency (Fig. 3). BQ788 itself increased oscillation amplitude in these experiments. One speculative explanation may be that BQ788 inhibits tonic activation of Et_B Rs on arteriolar endothelial cells because these are known to stimulate NO production, leading to vasodilatation.⁴⁵ Any reduction in endothelial NO may disinhibit Ca^{2+} -signaling in vascular smooth muscle, perhaps promoting oscillations. As discussed, however, the effect of NO on smooth muscle in retinal arterioles has not yet been tested.

U73122, 2APB, and xestospongine C all inhibited the effects of Et1 on Ca^{2+} -oscillations, suggesting a role for IP_3 R-mediated Ca^{2+} -release (Fig. 4). These observations must be interpreted with some caution, however, given the nonspecific character of pharmacologic agents, especially when used at relatively high concentrations as occurred here. At 100 μM , 2APB blocks IP_3 -mediated Ca^{2+} -release by 95% but also inhibits Ca^{2+} -uptake into intracellular stores by approximately 50%.⁴⁶ Inhibition of SR Ca^{2+} -uptake would be expected to raise baseline Ca^{2+} , however, and this was not seen in the current experiments. The inhibitory effects of 2APB could also be explained by reduced store-operated Ca^{2+} -influx.⁴⁷ Xestospongine C can have actions similar to those of 2APB,⁴⁸ but the PLC inhibitor U73122 has fewer reported off-target effects. Its ability to completely inhibit Et1-induced oscillations is consistent with an IP_3 -dependent Ca^{2+} -signaling pathway.

Significant involvement of RyRs, as indicated by the blockade of oscillations with tetracaine (Fig. 4), may appear to be

paradoxical given that these Ca^{2+} -release channels are not directly activated by IP_3 . Both RyRs and IP_3 Rs are sensitive to $[\text{Ca}^{2+}]$, so that local release through either class of Ca^{2+} -release channel may lead to the activation of spatially adjacent SR channels, regardless of their molecular identity.^{28,30} Cross-talk between IP_3 Rs and RyRs has also been proposed to explain the increase in spark frequency when pulmonary artery smooth muscle is exposed to Et1.²⁹ The fact that Et1 also increases Ca^{2+} -spark activity in retinal arteriolar myocytes, and that sparks result from spontaneous opening of RyRs, is also consistent with this hypothesis.¹⁹ This functional evidence suggests that structural studies looking at the cellular distribution and colocalization of different IP_3 R and RyR isoforms would be informative.

CONCLUSIONS

Increases in the frequency of Ca^{2+} -sparks and oscillations, rather than graded increases in mean $[\text{Ca}^{2+}]$, play an important but previously unreported role in the signaling response to Et1 in retinal vascular smooth muscle. This digital signal results from the activation of PLC by Et_A Rs, leading to both IP_3 R- and RyR-mediated Ca^{2+} -release. The detailed mechanisms responsible for phasic oscillations and the cellular benefits resulting from this mode of signaling remain to be determined.

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