Asynchronous Ca\(^{2+}\) Waves in Intact Venous Smooth Muscle

Dietrich O. Ruehlmann,* Cheng-Han Lee,* Damon Poburko, Cornelis van Breemen

Abstract—The rabbit inferior vena cava (IVC) is a large-capacitance vessel that displays typical contractile dose-response curves for caffeine and phenylephrine (PE). Using confocal microscopy on the endothelium-denuded IVC, we undertook experiments to correlate these whole-tissue contractile dose-response curves with changes in subcellular [Ca\(^{2+}\)]\(i\) signals in the in situ vascular smooth muscle cells (VSMCs). We observed that both caffeine and PE initially elicited Ca\(^{2+}\) waves in individual VSMCs. The [Ca\(^{2+}\)]\(i\), in cells challenged with caffeine subsequently returned to baseline whereas the [Ca\(^{2+}\)]\(i\) in cells challenged with PE exhibited repetitive asynchronous Ca\(^{2+}\) waves. These [Ca\(^{2+}\)]\(i\) oscillations were related to Ca\(^{2+}\) release from the sarcoplasmic reticulum as they were inhibited by ryanodine and caffeine. The lack of synchronicity of the [Ca\(^{2+}\)]\(i\), oscillations between VSMCs can explain the observed tonic contraction at the whole-tissue level. The nature of these Ca\(^{2+}\) waves was further characterized. For caffeine, the amplitude was all-or-none in nature, with individual cells differing in sensitivity, leading to their recruitment at different concentrations of the agonist. This concentration dependency of recruitment appears to form the basis for the concentration dependency of caffeine-induced contraction. Furthermore, the speed of the Ca\(^{2+}\) waves correlated positively with the concentration of caffeine. In the case of PE, we observed the same characteristics with respect to wave speed, amplitude, and recruitment. Increasing concentrations of PE also enhance the frequency of the [Ca\(^{2+}\)]\(i\) oscillations. We therefore conclude that PE stimulates whole-tissue contractility through differential recruitment of VSMCs and enhancement of the frequency of asynchronous [Ca\(^{2+}\)]\(i\) oscillations once the cells are recruited. The full text of this article is available at http://www.circresaha.org.

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Key Words: vascular smooth muscle ■ Ca\(^{2+}\) signaling ■ smooth muscle contraction ■ confocal microscopy ■ Ca\(^{2+}\) oscillation

Smooth muscle contraction is initiated by Ca\(^{2+}\)-calmodulin activation of myosin light chain kinase, resulting in crossbridge cycle interaction between actin and myosin filaments. Thus, Ca\(^{2+}\) plays a key role in controlling smooth muscle contractility. In addition, the myofilament sensitivity to Ca\(^{2+}\) is upregulated by contractile agonists and decreased by cyclic nucleotides.

Much of our understanding regarding [Ca\(^{2+}\)]\(i\) signaling in vascular smooth muscle cells (VSMCs) has been based on studies using enzymatically isolated cells and cultured cells. Isolated and cultured VSMCs, when stimulated, show cyclic elevations of [Ca\(^{2+}\)]\(i\), that progress over the entire cell length, giving it the appearance of a wave. Although these single-cell experiments have greatly improved our knowledge of the mechanisms involved in Ca\(^{2+}\) homeostasis, smooth muscle cells can present a very different phenotype once cultured. For example, cultured smooth muscle cells rapidly lose their L-type voltage-operated channels and undergo a reduction in the expression of \(\alpha\)-actin, hence changing from the contractile to the secretory or migrating phenotype. In addition, ubiquitous chemicals leaching from cell culture plasticware can potently affect the Ca\(^{2+}\) homeostasis of VSMCs, and cell culture media with high glucose concentration can reduce gap junction expression.

Even when smooth muscle cells are freshly isolated, but not cultured, it is clear that proteolysis changes cellular characteristics, which are fundamental to physiological function. The presence of gap junctions between VSMCs suggests that cells with intact intercellular communications may behave more like a syncytium and possibly very different from their behavior as isolated single cells. It therefore becomes necessary to verify mechanisms deduced from isolated or cultured cells by data obtained from smooth muscle cells in situ.

Iino et al were the first to examine stimulus-induced VSMC [Ca\(^{2+}\)]\(i\), signals in situ. They reported that in the intact rat tail artery, which typically responds with tonic contractions, individual smooth muscle cells oscillate asynchronously after electrical and pharmacological stimulation. This has been confirmed by Kasai et al. and similar asynchronous [Ca\(^{2+}\)]\(i\) oscillations have been observed in intact pressurized mesenteric small arteries by Miriel et al.

Because VSMCs can display considerable heterogeneity in structure and function depending on their location, the question was raised whether the observed asynchronous [Ca\(^{2+}\)]\(i\) oscillations are also characteristic for veins.

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We have previously used the rabbit vena cava to validate new roles for smooth muscle sarcoplasmic reticulum (SR), such as buffering of Ca\(^{2+}\) entry and contributing to Ca\(^{2+}\) extrusion.\(^{14,15}\) In this presentation, we report on yet another function of the SR, which is the generation of cytoplasmic Ca\(^{2+}\) oscillations. We extend the observations of Iino et al\(^{11}\) of asynchronous Ca\(^{2+}\) waves to a correlation of whole vena cava force and [Ca\(^{2+}\)], measurements with single cell [Ca\(^{2+}\)], signaling.

**Materials and Methods**

**Solutions and Chemicals**

All drugs were purchased from Sigma and were of the highest analytical grade. Normal physiological salt solution (PSS) contained (in mmol/L) NaCl 140, KCl 5, CaCl\(_2\) 1.5, MgCl\(_2\) 1, glucose 10, and HEPES 5, (pH 7.4 at 37°C). Zero Ca\(^{2+}\) PSS contains no CaCl\(_2\) but 1 mmol/L EGTA. Fura-2 AM, Fluo 3-AM, Hoechst 33342, and pluronic were purchased from Molecular Probes and dissolved in dimethyl sulfoxide (DMSO), as was ryanodine. Caffeine and phenylephrine (PE) were solubilized in normal PSS. All experiments were performed at 37°C.

**Preparation of Tissue**

Female New Zealand White rabbits (1.5 to 2.5 kg, obtained from University of British Columbia animal care) were killed by CO\(_2\) asphyxiation and then exsanguinated in accordance with local regulations. The inferior vena cava (IVC) was removed, cleaned of connective and adventitial tissues, the endothelium removed by gently swiping it with filter paper,\(^{14,15}\) and the vessel was cut into multiple ring segments that were 4 to 5 mm wide.

**Contraction Studies**

Vessel segments attached to isometric force transducers were equilibrated with 0.4 g of pretension. The solution was exchanged by rapid draining and refilling within 2 to 3 seconds. Rings failing to maintain a stable response pattern were made when necessary to exclude sampling artifacts. All data analysis was performed in ImageProPlus using customized routines written in Visual Basic. To obtain data on recruitment of cells during drug stimulation, a 3-pixel-wide scan line was drawn across multiple cells and propagated through the time stack. The resulting image (Y-t plot) revealed the number of cells responding (expressed as percentage of the cells responding to the highest drug concentration) as well as the degree of heterogeneity between and oscillation frequency within cells. Further analysis of wave parameters was performed using a 3-pixel-wide line along the longitudinal axis of a single cell. The resulting X-t plot revealed the point of origin as well as the progression speed of the apparent “wave.” All experimental traces shown represent the averaged fluorescence signals from a 3×3 pixel region (1.36 \(\mu\)m\(^2\)) in a single cell.

**Experimental Procedures**

After an initial equilibration time, the tissue was intermittently exposed to increasing concentrations of either caffeine or PE.
Between consecutive doses, ample time for recovery was allowed and several experiments were repeated to ensure that the tissue did not desensitize to either treatment.

**Statistical Analysis**

Numerical data are presented as the mean of at least 3 preparations and the corresponding SEM.

**Results**

Isolation and preparation of the tissue have been an established technique in our laboratory, and previous experiments suggested effective concentration ranges for caffeine from 0.25 to 25 mmol/L and PE from 1.5 nmol/L to 15 μmol/L. In selected experiments, the concentration of PE was increased to as high as 1.5 mmol/L. To confirm endothelial cell removal, 3 denuded and 3 control vessels were incubated with the selective DNA stain Hoechst 33342 (300 μg/mL). Nuclei were visualized using the 360-nm laser illumination. In control vessels, a continuous layer of oblong nuclei, with their long axis oriented parallel to the direction of the flow was observed, indicating an undamaged endothelial cell layer (Figure 1A). After denudation, the same staining protocol revealed only elongated nuclei oriented transversely to the direction of the flow (Figure 1B), consistent with VSMC nuclei characterized by Daly et al.16

**Contraction and Whole-Tissue [Ca\(^{2+}\)]**

To characterize the contractile and [Ca\(^{2+}\)], responses of the vena cava to caffeine and PE, the tissues were isometrically suspended and noncumulatively exposed to increasing concentrations of either caffeine or PE. Representative traces are shown in Figure 2A (insets). PE (15 μmol/L) induced a maintained increase in contractile force of 1.79±0.42 g (mean±SEM, n=8 rings from 4 animals). Caffeine (25 mmol/L) evoked a transient contraction; 5 seconds after the onset of the response, tension increased to 0.42±0.09 g (n=11 rings from 4 animals) and subsequently relaxed completely to the basal level after 120 seconds. Increasing concentrations of the agonists enhanced the contractile force until a maximal value was reached. These typical concentration-response curves are shown in Figure 2A. The dose-response for PE fell within the concentration range of 0.0015 to 150 μmol/L, whereas caffeine increased force over a much narrower concentration range of 1 to 10 mmol/L.

We further examined how these contraction patterns were related to the whole-tissue [Ca\(^{2+}\)], signal. As shown in Figure 2B, PE (15 μmol/L) induced a rapid rise in [Ca\(^{2+}\)]. The peak value, 145±22% of basal F340/380 was reached 62±9 seconds after the onset of the response (n=4 strips from 4 animals). [Ca\(^{2+}\)] was maintained; 180 seconds after the maximal level was reached, it remained at 142±19% of the basal F340/380. A similar fast initial rise was observed with caffeine (25 mmol/L): 136±6% of basal F340/F380 was reached after 53±7 seconds (n=4 strips from 4 animals). In contrast to PE, the ratio returned to baseline after 180 seconds. Thus, the patterns of the whole-tissue [Ca\(^{2+}\)], signals

![Figure 3](http://circres.ahajournals.org/for movie file)
appear to match those observed in the contraction experiments

Confocal Experiments

Given that both PE and caffeine produce concentration-dependent, graded levels of contraction in IVC, we designed parallel experiments to correlate the macroscopic contractile concentration-response curves with the subcellular \([\text{Ca}^{2+}]_i\), signals of individual VSMCs in situ. Fast laser scanning confocal microscopy of Fluo 3-AM was used to image subcellular \([\text{Ca}^{2+}]_i\). Initially, both caffeine (25 mmol/L) and PE (1.5 \(\mu\text{mol/L}\)) elicited a rapid rise in \([\text{Ca}^{2+}]_i\), that appeared in the form of a \([\text{Ca}^{2+}]_i\) wave propagating along the longitudinal axis of the VSMCs (Figures 3A and 3B, frame 1). With time, \([\text{Ca}^{2+}]_i\), in the in situ VSMCs challenged with caffeine returned to baseline despite the continual presence of the drug (Figures 4A through 4C). The transient nature of the caffeine-induced subcellular \([\text{Ca}^{2+}]_i\) signal agrees with the transient whole-tissue \([\text{Ca}^{2+}]_i\) signal and contraction. In the same vena cava, PE stimulated repetitive smooth muscle \([\text{Ca}^{2+}]_i\) waves that were not synchronized between adjacent cells (Figure 3A, arrows to cells 1, 2, and 3). This lack of synchronicity between neighboring VSMCs explains how summation of the individual-cell \([\text{Ca}^{2+}]_i\), oscillations leads to sustained macroscopic \([\text{Ca}^{2+}]_i\), elevation and tonic contraction of the whole-tissue.

To understand the cellular basis of the concentration-response curves illustrated in Figure 2, we analyzed the nature of the agonist-induced \([\text{Ca}^{2+}]_i\) waves. Whenever a \([\text{Ca}^{2+}]_i\) wave was initiated by caffeine, the amplitude of the \([\text{Ca}^{2+}]_i\) signal was constant, irrespective of the concentrations applied (Figure 4D). This indicates an all-or-none phenomenon that excludes amplitude as a modulator of tissue contractility. However, we observed that the sensitivity of VSMCs to caffeine varies between cells, leading to an increased recruitment at higher caffeine concentrations (Figures 4C). This concentration dependence of recruitment resembles that of whole-tissue contractility (Figure 2A) and appears to be the basis for the concentration dependence of caffeine-induced contraction.

In the case of PE, we observed similar characteristics with respect to amplitude and recruitment (Figure 5). The amplitude displayed an all-or-none character, and the concentration dependency of recruitment (Figure 5E) appeared to correlate with the concentration dependency of the PE-induced contraction (Figure 2A) at the lower concentration range of 0.015 to 1.5 \(\mu\text{mol/L}\). However, the continued oscillations of \([\text{Ca}^{2+}]_i\), allow for more elaborate modulation of contractile force. Figure 6 shows that as [PE] was increased, the frequency of the \([\text{Ca}^{2+}]_i\) waves increased. At the highest concentration used (150 \(\mu\text{mol/L}\) PE), the frequency reached 0.511 ± 0.025 Hz (n = 15 cells from 4 animals). As reflected in the representative traces from Figure 6A, the increased frequency of \([\text{Ca}^{2+}]_i\), coincides with shortened interspike intervals. At high [PE], the interspike \([\text{Ca}^{2+}]_i\) level was elevated above the basal level, but \([\text{Ca}^{2+}]_i\), peaks never fused. The concentration dependence of the frequency of PE-induced \([\text{Ca}^{2+}]_i\), oscillations appears to correlate with the concentration-response curve of PE-induced contraction at the higher concentration range of 0.15 to 150 \(\mu\text{mol/L}\).

Finally, we examined the concentration dependence of the velocity of agonist-induced \([\text{Ca}^{2+}]_i\) waves. The velocity of wave propagation illustrated in Figure 7 shows a strong dependence on the concentrations of either PE or caffeine. At the highest concentrations, wave propagation speeds reached 89.62 ± 7.36 \(\mu\text{m/s}\) (n = 15 cells from 4 animals) with PE and
126.22±7.31 μm/s with caffeine (n=15 cells from 3 animals). In addition, as illustrated in Figures 7A and 7B, both PE and caffeine commonly elicited Ca2+ waves at multiple foci within a given cell, which then eventually collided.

On α₁-adrenergic stimulation, the observed [Ca2+]i oscillations may derive their Ca2+ from the extracellular space or the SR. To determine the immediate source of Ca2+ for these PE-induced [Ca2+]i oscillations, extracellular Ca2+ was removed along with the addition of 1 mM EGTA. Under these conditions, the PE-induced [Ca2+]i oscillations persisted for 28.25±2.58 seconds (n=84 cells from 4 animals) before dissipating. Depletion of caffeine-sensitive and ryanodine-sensitive Ca2+ stores with either 25 mM caffeine (Figure 8B) or 100 μM ryanodine (Figure 8C) completely abolished PE-induced oscillations. These findings indicate that the SR is the immediate source of Ca2+, sustaining the observed PE-induced [Ca2+]i oscillations.

Discussion

Characteristics of the [Ca2+]i Signals

The vena cava is a large-capacitance vein, whose tonic contractions determine venous return to the heart and therefore modulate the stroke volume and indirectly blood pressure. Our observations on Ca2+ signaling in individual smooth muscle cells of this intact tissue shed new light on how wall tension is regulated in large veins. This response to agonists is typified by repetitive transient elevations in [Ca2+]i, which originate in certain locations and then spread as waves over the length of the cell (Figures 7A and 7B). The cells respond independently of each other in that the [Ca2+]i oscillations are not synchronized and that cells vary in their sensitivity to PE. Observing the summation of all smooth muscle cell activity in the tissue bath reveals typical dose-response curves for both force development and [Ca2+]i. Thus, the asynchronous oscillations of tens of thousands of cells blend into tonic responses as proposed earlier by Lino et al11 for the behavior of the rat tail artery. Similar oscillations were recently reported in the rat mesenteric artery by Miriel et al.13 In the present study, we have extended these observations to an actual comparison between the individual cellular responses and those of the whole vein.

The repetitive [Ca2+]i waves in the intact vena cava have the following characteristics: (1) the amplitude has a constant value regardless of the [PE] (Figure 5D), (2) the frequency increases with increasing [PE] (Figure 6C), (3) the velocity increases with increasing [PE] (Figure 7C), and (4) at high [PE], the interspike [Ca2+]i becomes elevated, but the peak values remain constant (Figures 5D and 6A). In addition, the sensitivity of each cell to PE is variable, such that the recruitment of responding cells increases with increasing [PE] (Figure 5E).

The mechanism of agonist-induced Ca2+ waves has been discussed for several cell types. In endothelial cells and other nonexcitable cells, they are related to Ca2+ release and reuptake by the SR.17 This appears to be true also for the IVC, as the waves were abolished by caffeine and ryanodine but were initially not affected by removal of Ca2+ from the extracellular space. The all-or-none amplitude response indicates the involvement of Ca2+-induced Ca2+ release (CICR). At this time, we cannot distinguish between possible involvement of either or both the ryanodine receptor and the inositol 1,4,5-tris-phosphate receptor (Ins1,4,5P3R), because both receptors are sensitive to Ca2+.18 A likely scenario for the agonist-induced Ca2+ wave begins with elevation of Ins1,4,5P3

Figure 5. Fixed-amplitude Ca2+ waves and concentration-dependent recruitment in response to PE. In intact rabbit IVC preparation, PE (1.5 μmol/L) elicited [Ca2+]i oscillations in both cells 1 and 2 as shown in the propagated Y-t line scan (B) from which the respective traces in panel C were derived (images are noise-filtered and contrast-enhanced; the intensity level reflects [Ca2+]i). The traces shown represent the averaged fluorescence signals from a 3×3 pixel region in a single cell. D. Amplitude of PE-induced [Ca2+]i oscillations did not change with increasing [PE] (n=15 cells from 4 animals). No [Ca2+]i signals were observed at PE concentrations below 0.015 μmol/L. The amplitude was determined by subtracting the prestimulus baseline from the averaged peak values of all [Ca2+]i oscillations observed; the units are arbitrary on an 8-bit scale. It is important to note that the averaged peak values were used because the amplitudes of [Ca2+]i oscillations from the 3×3 pixel region in a single cell do vary with time, which is due to the variability of stochastic noise recorded from the same 3×3 pixel region over time. Averaging of the peak values thus minimizes the effects of noise. E. Greater percentage of VSMCs generated [Ca2+]i signals as the PE concentration increased. This recruitment occurred between 0.015 and 1.5 μmol/L of PE, with maximal recruitment achieved at 1.5 μmol/L of PE in all tissues examined (n=4 rings from 4 animals). *Number of cells firing expressed as a percentage of cells responding to the maximal concentration.
and [Ca\(^{2+}\)] in response to \(\alpha_1\)-adrenergic activation. Ins\(^1,4,5\)P\(_3\) sensitizes the Ins\(^1,4,5\)P\(_3\)R to Ca\(^{2+}\), and when the [Ca\(^{2+}\)] reaches a threshold concentration, the release channels open. Ca\(^{2+}\) cascades into the cytoplasm, where it promotes the opening of adjacent channels, thus starting the Ca\(^{2+}\) wave, which proceeds down the length of the smooth muscle cell.

As the [PE] is raised, so are the concentrations of Ins\(^1,4,5\)P\(_3\) and basal cytoplasmic Ca\(^{2+}\), which clearly shortens the time required for [Ca\(^{2+}\)] to reach threshold value for the initiation of the next wave. This mechanism combined with the fact that Ins\(^1,4,5\)P\(_3\) sensitizes the Ins\(^1,4,5\)P\(_3\)R to Ca\(^{2+}\) ensures that both the frequency and velocity increase with increasing [PE]. The Ca\(^{2+}\) wave may take origin at a specific site, because either the [Ins\(^1,4,5\)P\(_3\)] or the [Ca\(^{2+}\)] is elevated above the remaining cytosolic levels. This could be a consequence of inhomogeneity of receptor or channel density. However, a more puzzling aspect is that the oscillations continue even when the [Ca\(^{2+}\)] rises above the apparent threshold value. This could be explained by the fact that the Ca\(^{2+}\) release channels cycle between the closed, open, and inactivated states and that high [Ca\(^{2+}\)] has an inhibitory effect on the open probability. Thus, as the [Ca\(^{2+}\)] reaches peak value, the channels close and require some time before opening and starting another [Ca\(^{2+}\)] transient on top of the raised background level of Ca\(^{2+}\).

**Correlation of [Ca\(^{2+}\)] Signals With the Development of Tension**

Physiologically, a blood vessel controls blood flow through its lumen by producing varying degrees of vasoconstriction. Such regulation is reflected in the classical dose-response relationship whereby increasing concentration of endogenous vasoconstrictor agents elicits increasing levels of contractions at the whole-tissue level. As demonstrated in Figure 2A, rings of rabbit IVC display concentration-dependent contractions to PE. These graded isometric contractile responses must be mediated by certain concentration-dependent signaling mechanisms over the same range of agonist concentration to PE. These graded isometric contractile responses must be mediated by certain concentration-dependent signaling mechanisms over the same range of agonist concentration. Although our current investigation focused on Ca\(^{2+}\)-dependent regulation of VSMC contractility, it is evident from previous publications that \(\alpha_1\)-adrenergic stimulation also enhances myofilament Ca\(^{2+}\) sensitivity in the vena cava and other vessels. This discrepancy is confirmed in the present study in which 0.015 \(\mu\)mol/L PE initiated low-grade contraction without the associated changes in [Ca\(^{2+}\)]. On the contrary, our laboratory has demonstrated previously that caffeine desensitizes myofilament activation to Ca\(^{2+}\) in the rabbit IVC. This discrep-
Ca\textsuperscript{2+} Waves in Intact Venous Smooth Muscle

Ruehlmann et al

Ca\textsuperscript{2+} waves are dependent on the process of CICR.\textsuperscript{29–31} The Ca\textsuperscript{2+} presence of PE (15 \textmu mol/L) is essential for the initiation of Ca\textsuperscript{2+} waves at the individual cell level to account for the graded level of contractility, we have identified distinctive, concentration-dependent signaling mechanisms both at the tissue level and contractility, which could explain the low force generation by caffeine in comparison to PE (Figure 2A).

With regard to the Ca\textsuperscript{2+}-dependent modulation of IVC contractility, we have identified distinctive, concentration-dependent signaling mechanisms both at the tissue level and at the individual cell level to account for the graded level of contraction produced. At the tissue level, a concentration-dependent recruitment of VSMC initiation of Ca\textsuperscript{2+} signals was observed in response to both PE and caffeine. The mechanism responsible for differential recruitment of cells populating the same tissue is probably based on the intrinsic heterogeneity of VSMCs.\textsuperscript{28} More specifically, our observations indicate that neighboring VSMCs from IVC differ in their sensitivity to PE and caffeine. These differences may be due to variations in ultrastructure and/or basal enzyme activities.\textsuperscript{28} Regulation of contractile strength by caffeine appears to be mainly related to the recruitment process. Clearly, the “all-or-none” nature of the cellular [Ca\textsuperscript{2+}] signal is incapable of yielding a graded dose-response curve (Figures 4D and 5D). Thus, the magnitude of the [Ca\textsuperscript{2+}] signal at the cellular level does not encode information to modulate VSMC contractility. The velocity of the caffeine-induced Ca\textsuperscript{2+} wave, however, was found to be concentration dependent, especially at the higher concentration range for caffeine between 2.5 mmol/L to 25 mol/L (Figure 7C). This observation is consistent with the concept that caffeine acts by increasing the sensitivity of ryanodine-sensitive channels to Ca\textsuperscript{2+} in the process of CICR.\textsuperscript{29–31} The Ca\textsuperscript{2+} wave observed in response to caffeine reflects regenerative release of Ca\textsuperscript{2+} through ryanodine channels downstream from the origin of the Ca\textsuperscript{2+} wave. As the concentration of caffeine applied increases, both Ca\textsuperscript{2+} sensitivity and open probability of ryanodine channels are further elevated, resulting in more rapid wave-like induction of CICR.\textsuperscript{32} However, considering that the amplitude of the Ca\textsuperscript{2+} wave is constant, it seems unlikely that wave velocity modulates VSMC contractility. Therefore, given the close resemblance of the concentration dependency relationships between tension generated (Figure 2A) and the proportion of cells recruited (Figure 4E), the magnitude of caffeine-induced contraction is mainly determined by the level of VSMC recruitment.

In contrast to caffeine, a comparison between the [PE]-response curves for contraction (Figure 2A) and cell recruitment (Figure 5E) reveals a positive correlation between the degree of cell recruitment and developed force only at the lower concentrations, ranging from 0.015 to 1.5 \textmu mol/L. This implies that additional mechanism(s) must be involved in regulating contractility, especially at the higher concentration range. We have shown that both frequency of oscillation and the apparent velocity of the Ca\textsuperscript{2+} waves are dependent on [PE] (Figures 6C and 7C), whereas the amplitude of oscillations is independent of [PE] (Figure 5D). Therefore, information governing the graded level of contraction at the higher [PE] range is encoded in the frequency and possibly the velocity domains. As discussed above, it is unclear whether higher wave velocity can contribute to a greater degree of contraction. On the other hand, higher frequency of oscillations can enhance the contractile response by several possible mechanisms. First, higher frequency of fixed-amplitude Ca\textsuperscript{2+} oscillations due to shortening of the interwave periods results in higher average [Ca\textsuperscript{2+}], over time and thus enhances myofilament activation. Second, increasing frequency of oscillation may lead to activation of certain frequency-sensitive enzymes that can potentially affect the level of contraction. An example of this class of enzyme is Ca\textsuperscript{2+}-calmodulin kinase II that was found to be sensitive to the frequency of Ca\textsuperscript{2+} spikes in vitro.\textsuperscript{33} Thus, we speculate that oscillation frequency is an important regulator of contractility whereas the velocity of the Ca\textsuperscript{2+} waves remains of unknown physiological relevance.

The observations presented and discussed herein emphasize that the classical concept of an SR-Ca\textsuperscript{2+} release contribution to the initial phase of contraction followed by tonic force dependent on Ca\textsuperscript{2+} entry from the extracellular space to the myoplasm does not apply to \beta-adrenergic constriction of capacitance veins. Instead, the entire tonic contraction is mediated by the SR beginning with its initial Ca\textsuperscript{2+} release followed by repeating cycles of Ca\textsuperscript{2+} uptake and release. Because at least part of the Ca\textsuperscript{2+} released from the SR is extruded to the extracellular space,\textsuperscript{34} the maintained [Ca\textsuperscript{2+}], oscillations have to be supported by stimulated Ca\textsuperscript{2+} influx. Given that the [Ca\textsuperscript{2+}], in the myoplasm is regulated by the SR, it follows that the agonist-stimulated Ca\textsuperscript{2+} influx raises [Ca\textsuperscript{2+}], in a restricted subplasmalemmal space from where it is taken up by sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) to be subsequently directed toward the

Figure 8. PE-induced [Ca\textsuperscript{2+}] oscillations are SR dependent. A, IVC smooth muscle cells show distinct Ca\textsuperscript{2+} oscillations in the presence of PE (15 \textmu mol/L). Pretreatment with caffeine (25 \textmu mol/L, B) or ryanodine (100 \textmu mol/L, C) completely abolishes the oscillations (n=84 cells from 4 animals), suggesting that the oscillations are maintained by repetitive SR discharge.

A

Control

10 units

10 s

15 \textmu mol/L PE

B

Pre-treated with 25
\textmu mol/L caffeine

10 units

10 s

15 \textmu mol/L PE

C

Pre-treated with 100
\textmu mol/L ryanodine

10 units

10 s

15 \textmu mol/L PE

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sites of Ca\(^{2+}\) release near the myofilaments. In this regard, it is of special interest that our laboratory has presented evidence in support of redistribution of Ca\(^{2+}\) within the SR lumen.\(^3^5\) Assuming that the above sequence of Ca\(^{2+}\) movement is correct, we may speculate on the advantage that this system provides for smooth muscle cells. In essence, instead of Ca\(^{2+}\) diffusing from the plasma membrane through the cytoplasm, it is guided from the plasma membrane to the myofilaments by the SR. Because the Ca\(^{2+}\)-calmodulin sensitive myosin light chain kinase is attached to the thin filaments,\(^3^5\) the function of the SR may be to deliver the Ca\(^{2+}\) to these activating sites to enhance the speed and efficiency of the activating mechanism. These novel insights into venous [Ca\(^{2+}\)\(^{-}\)] signaling in relation to contractile modulation will undoubtedly expand the basis for future therapy of contractile malfunctions such as in venous pooling during orthostatic hypertension.

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