Waves of Calcium Depletion in the Sarcoplasmic Reticulum of Vascular Smooth Muscle Cells: An Inside View of Spatiotemporal Ca\(^{2+}\) Regulation

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Abstract

Agonist-stimulated smooth muscle Ca\(^{2+}\) waves regulate blood vessel tone and vasomotion. Previous studies employing cytoplasmic Ca\(^{2+}\) indicators revealed that these Ca\(^{2+}\) waves were stimulated by a combination of inositol 1,4,5-trisphosphate- and Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the endo/sarcoplasmic reticulum. Herein, we present the first report of endothelin-1 stimulated waves of Ca\(^{2+}\) depletion from the sarcoplasmic reticulum of vascular smooth muscle cells using a calcequestrin-targeted Ca\(^{2+}\) indicator. Our findings confirm that these waves are due to regenerative Ca\(^{2+}\)-induced Ca\(^{2+}\) release by the receptors for inositol 1,4,5-trisphosphate. Our main new finding is a transient elevation in SR luminal Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{SR}\)) both at the site of wave initiation, just before regenerative Ca\(^{2+}\) release commences, and at the advancing wave front, during propagation. This strongly suggests a role for [Ca\(^{2+}\)]\(_{SR}\) in the activation of inositol 1,4,5-trisphosphate receptors during agonist-induced calcium waves. In addition, quantitative analysis of the gradual decrease in the velocity of the depletion wave, observed in the absence of external Ca\(^{2+}\), indicates continuity of the lumen of the sarcoplasmic reticulum network. Finally, our observation that the depletion wave was arrested by the nuclear envelope may have implications for selective Ca\(^{2+}\) signalling.

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Introduction

In vascular smooth muscle cells (VSMCs), fluctuations in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) selectively control multiple functions, including contraction-relaxation, energy metabolism, proliferation, migration and apoptosis, in health and disease [1,2,3,4,5]. It is generally accepted that functional selectivity of Ca\(^{2+}\) signals is encoded in their spatial and temporal characteristics [6,7,8]. In this context, we support the view that the ubiquitous asynchronous Ca\(^{2+}\) waves in VSM are optimally suited to couple agonist-mediated stimulation to vasoconstriction in healthy blood vessels, while avoiding recruitment of stress related functions [2,3,9,10]. Neylon and coworkers were the first to report that receptor stimulation of cultured human VSM did not simply elevate [Ca\(^{2+}\)]\(_{i}\), to induce activation, but in fact caused a wave of elevated [Ca\(^{2+}\)]\(_i\), to travel across the cell from an initiation site [11]. They further proposed a mechanism involving stimulation of inositol 1,4,5-trisphosphate receptors (IP3R) and Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) [11]. In 1994, Iino et al. made the next advance by recording [Ca\(^{2+}\)]\(_i\), in the intact rat tail artery smooth muscle [4]. Iino had earlier discovered that IP3Rs were sensitive to Ca\(^{2+}\), such that IP3 required Ca\(^{2+}\) as a co-activator, and that the elevation of [IP3], facilitates CICR at IP3Rs [12]. Sympathetic nerve stimulation initiated asynchronous repetitive waves of [Ca\(^{2+}\)]\(_i\) elevation, traveling in both directions along the length of the spirally arranged smooth muscle fibres. The unexpected aspect of this discovery was that the tonic increases in both the bulk smooth muscle [Ca\(^{2+}\)], and force were based on wave-like [Ca\(^{2+}\)]\(_i\) oscillations in individual VSMCs. Because of the asynchronous nature of these cellular Ca\(^{2+}\) waves, summation over thousands of cells in the vascular media results in maintained average [Ca\(^{2+}\)]\(_i\), elevation and vascular tone. Nevertheless, the repetitive Ca\(^{2+}\) waves would still confer the advantage of added informational content due to frequency encoding and prevention of potential harm due to prolonged elevated [Ca\(^{2+}\)]\(_i\).

Although a great deal of valuable information has subsequently accumulated on the mechanism of Ca\(^{2+}\) waves, previous studies were limited, to some degree, by the fact that they reported changes in cytoplasmic Ca\(^{2+}\), which represent the result of Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) rather than the process itself. In this study, in order to obtain more direct insight into regenerative Ca\(^{2+}\) release in VSMCs, we have used a specific Ca\(^{2+}\) indicator targeted to calcequestrin in the SR lumen that we refer to as D1SR. The VSMCs used in this study constituted a primary culture, which remained highly contractile during the study. Here, we report a wave of Ca\(^{2+}\) depletion from the SR itself, which in this case is activated by applying the vascular autacoid endothelin-1 (ET-1). The observed agonist-induced SR Ca\(^{2+}\)
Figure 1. Distribution of D1SR Ca^{2+} indicator in rat aortic SMCs. A) Fluorescence images of D1SR indicator distribution using CFP (440/488 nm), FRET (440/535 nm), and YFP (514/535 nm) band-pass filters (scale bars, 10 μm). B) 2-D projection of a merged fluorescence image of D1SR using CFP (440 nm excitation/488 nm emission), FRET (440 nm excitation/535 nm emission), and YFP (514 nm excitation/535 nm emission) band-pass filters, and ER-Tracker™ Red (587 nm excitation/615 nm emission) are shown. The majority of area shows overlay of D1SR and SR lumen tracker. The zoomed-in images highlight areas of SR lumen negative for D1SR signal, but positive for ER-Tracker. In all experiments, such areas were excluded from data analyses (scale bars, 10 μm unless indicated otherwise). C) SMCs are loaded with Golgi-GFP CellLight® solution for 16 hours at 37°C prior to loading with ER-Tracker™ Red dye for 15 minutes. Fluorescence images of ER-Tracker™ Red (587 nm excitation/615 nm emission) and Golgi-GFP (488 nm excitation/520 nm emission) are shown (scale bars, 10 μm).

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depletion wave provides valuable new information about the role of SR luminal Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{SR}) in the initiation and propagation of Ca\textsuperscript{2+} waves, which was not attainable by recording fluctuations in [Ca\textsuperscript{2+}].

**Materials and Methods**

**Buffers & Reagents**

HEPES-PSS containing (in nmol·L\textsuperscript{-1}) NaCl 140, glucose 10, KCl 5, CaCl\textsubscript{2} 1.5 and MgCl\textsubscript{2} 1 (pH 7.4) was used for all calcium measurements and confocal microscopy. The nominal zero-Ca\textsuperscript{2+} PSS was prepared in the same way as normal PSS without the addition of calcium. Endothelin-1 (ET-1) was obtained from Sigma-Aldrich (ON, Canada). Thapsigargin (Tg), a cell permeable inhibitor of sarcoplasmic reticular Ca\textsuperscript{2+}-ATPase (SERCA), and xestospongin C (Xes-C), a potent membrane-permeable blocker of IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release, were purchased from EMD Chemicals (NJ, USA). Sodium orthovanadate, an inhibitor of secretory pathway Ca\textsuperscript{2+} ATPase (SPCA) pumps, was purchased from Sigma-Aldrich. Stock solutions of ET-1 and Tg were prepared in dimethyl sulfoxide (DMSO). For all experiments, vehicle-treated (0 μM) groups were incubated with equal volume of DMSO, respectively (the maximum volume of solvents used with the highest concentration of drugs). Further dilutions of reagents were made in zero-Ca\textsuperscript{2+} PSS buffer. The calcium dye Fluo-4 AM, ER-Tracker, Mito-Tracker Green FM, BODIPY TR-X thapsigargin, and CellLight\textsuperscript{TM} Golgi-GFP BacMam 2.0 were all purchased from Invitrogen (ON, Canada).

**Cell Culture**

Rat aortic SMCs were originated from the laboratories of Drs. Urs Ruegg and Nicolas Demaurex (University of Geneva, Geneva, Switzerland), and prepared from aorta of male Wistar Kyoto rats (200–300 g) as previously described [13]. Cells between passage 9 to 13 were cultured in Matrigel-coated (BD Sciences; ON, Canada) 35 mm glass bottom culture dishes (MatTek Co., MA, USA), and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated newborn calf serum, Penicillin G (100 μg.mL\textsuperscript{-1}), and streptomycin (100 μg.mL\textsuperscript{-1}) (Invitrogen, ON, Canada) at 37°C, in a humidified incubator in 5% CO\textsubscript{2}. See following sections for a detailed description of the transfection protocol and buffer/reagents used in the study.

**Transient Transfection with D1SR Constructs**

D1SR indicator, a modified variant of the D1ER cameleon [14,15,16], is kindly provided by Dr. Wayne Chen (University of Alberta, Canada). In the D1SR indicator, the original calreticulin signal sequence in D1ER has been replaced by the mutant calsequestrin sequence with a reduced binding ability to calcium (to eliminate the competition with endogenous calsequestrin in binding to calcium ion within SR). The D1SR construct consists of a truncated enhanced CFP and YFP that are joined by a linker containing modified calmodulin (CaM) and M13 (the 26-residue CaM-binding peptide of myosin light-chain kinase) sequences. The CaM-M13 modifications prevent M13 from binding endogenous calmodulin. SR retention is achieved by calsequestrin sequences on the 5’ end of CFP. Following binding to Ca\textsuperscript{2+}, conformational changes in the CaM-M13 domain increase the fluorescence resonance energy transfer (FRET) between the flanking CFP and YFP yielding a Ca\textsuperscript{2+} response. SMCs were transfected with adenoviral D1SR constructs at a multiplicity of infection of 100 (MOI = 100). Following overnight incubation at 37°C, cells were replenished with fresh medium. Fluorescence microscopy was used to assess transfection efficiency and cellular

**Figure 2. Calibration of D1SR in situ and effects of endothelin-1 and thapsigargin on SR Ca\textsuperscript{2+} signal.**

A) D1SR fluorescent ratio was calibrated in situ in semi-permeabilized SMCs (n = 12 cells from four independent culture plates, mean ± SEM) in intracellular solutions with 0.001 to 10 mM of free Ca\textsuperscript{2+}, and fitted to an exponential equation. B) Selected snapshots from a time-lapse movie (10 s intervals) of rat SMCs transfected with D1SR indicator (scale bars, 5 μm), and averaged R/R\textsubscript{0} and corresponding [Ca\textsuperscript{2+}]\textsubscript{SR} values in peripheral (red circle) and perinuclear (black circle) areas in response to ET-1 (100 μM) and Tg (2 μM) treatments in the presence of extracellular Ca\textsuperscript{2+}. As illustrated, ET-1 causes a slight change in R/R\textsubscript{0} values in the perinuclear area (ROIs = 20, n = 6 independent experiments, mean ± SEM, P < 0.005).

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morphology at 48 h post transfection. For all experiments, a transfection efficiency of 80–90% was achieved.

SR Luminal Ca\(^{2+}\) Measurement

Ratiometric Ca\(^{2+}\) images were acquired with a 63× oil-immersion objective (Leica DM16000 inverted microscope) and a cooled Hamamatsu 91000-02 electron multiplier CCD camera. Cells were excited at 440 nm, and 513 nm; and imaged using 488 nm/535 nm (for donor and FRET channels) and 535 nm (for acceptor channel) band-pass filters (three images/time point). The acceptor channel was simultaneously recorded to monitor photobleaching. We set the intensity of light at 15% transmitted light, and excitation exposure times at 150 ms with 10 s intervals.

Cytoplasmic Ca\(^{2+}\) Measurement

During in vitro measurement of cytoplasmic Ca\(^{2+}\) signals, all parameters (laser intensity, gain, etc.) were maintained constant during the experiment. The cell culture was illuminated using an Argon-Krypton laser (488 nm) and a high-gain photomultiplier tube collected the emission (505–550 nm). The customized Hamamatsu 91000-02 electron multiplier CCD camera delivers 1000×1000 pixels, imaged to Shannon-Nyquist specifications for the 63× objectives, providing a larger field of view. The representative fluorescence traces shown reflect the averaged fluorescence signals from 15 regions of interest (ROIs) in each cell. The measured changes in Fluo-4 AM fluorescence level are proportional to the relative changes in cytoplasmic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)). The confocal images were analyzed off-line with the Improvision Volocity software (Perkin-Elmer). Fluorescence traces were extracted from the movies and were normalized to initial fluorescence values.

Image Analysis

All data used for Ca\(^{2+}\) traces were analyzed by Improvision Volocity software, using built-in regions-of-interest (ROI) function to select the areas of interest that is at least 30 pixels long in length and width. For traces involving specific sites, shape and size of ROI were adjusted to avoid artifacts and saturated areas. For distance and velocity analysis, one ROI was selected per frame (time point) based on the movement of the Ca\(^{2+}\) wave. The resulting data were formatted on Microsoft Excel 2007, and analyzed by GraphPad Prism 5.0. Pseudo-colour visualization was performed by ImageJ, using customized lookup tables to assign colour for each pixel intensity values. Line scan was performed first by analyzing pixel intensity of a series of small ROIs (1–3 pixels in length per ROI) along a line using a customized Python script to output change in intensity over time along the line. The script was confirmed to output same values when analyzing same ROI on Volocity. The resulting values were then graphed using Gnuplot 4.4 with custom lookup table.

Statistical Analysis

Data are presented as means ± SEM of at least four independent experiments. Significance was determined using Student’s t-test with two-tailed distribution using GraphPad Prism 5 (P<0.05 was considered as statistically significant).

Results

Distribution of D1SR Ca\(^{2+}\) Indicator in Rat Aortic SMCs

To monitor fluctuations in [Ca\(^{2+}\)]\(_{SR}\), we transfected rat aortic SMCs with an adenoviral vector expressing the specific FRET-based SR Ca\(^{2+}\) indicator, D1SR. Figure 1A shows the individual and merged fluorescence images of three recorded channels (CFP: 440 nm/488 nm; FRET: 440 nm/535 nm; YFP: 513 nm/535 nm) for D1SR indicator in cultured VSMCs (see Movie S1). We also compared the distribution of D1SR with that of ER-Tracker, commonly used for localization of either ER or SR. As expected, D1SR co-localizes with ER-Tracker except for a region close to the nucleus that is negative for D1SR fluorescence, but positive for ER-Tracker (Fig 1B).

D1SR Calibration in situ

The D1SR indicator was calibrated in situ in semi-permeabilized rat aortic SMCs in intracellular HEPES solution (135 mM KCl, 10 mM NaCl, 1 mM MgCl\(_2\), 20 mM HEPES, 20 mM sucrose, 0.01 mM digitonin, 0.01 mM ionomycin, 0.005 mM CCCP, PH 7.2) with 5 mM HEDTA as described before [16]. The intracellular solution was blended with the stock solution of 100 mM CaCl\(_2\) to prepare buffers with free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{free}\)) ranging from 1 μM to 10 mM (Max Chelator v2.40, C. Patton, Standford University, USA, maxchelator.stanford.edu). D1SR fluorescent ratio (R/R\(_0\)) was plotted against [Ca\(^{2+}\)]\(_{free}\) in 12 cells from four independent cultures and an exponential fit was applied to the data to determine the apparent dissociation constant K\(_d\) (197±47 μM) and the Hill slope (n = 0.87±0.19) (Fig 2A). [Ca\(^{2+}\)]\(_{SR}\) was calculated by calibrating normalized D1SR ratio against the standard calibration curve as described previously [16]. To calculate [Ca\(^{2+}\)]\(_{SR}\), normalized ratio values were fitted to:

\[
\frac{R}{R_0} = R_{\text{min}}/R_0 \times \left[ \frac{\text{Rmax} - \text{Rmin}}{(R_{\text{max}}/R_0) - (\text{Rmin}/R_0)} \right] + 1^{10\log[K_d^2 - \log(Ca^{2+} + S_{\text{R0}})]}
\]

where R\(_{\text{max}}\) and R\(_{\text{min}}\) were obtained by measuring signal intensity in cells perfused with intracellular HEPES solution containing 5 mM HEDTA (Calcium free buffer) and 10 mM free calcium (15 mM CaHEDTA), respectively.

Effects of Endothelin-1 and Thapsigargin on SR and Cytoplasmic Ca\(^{2+}\) Signals

The traces in Figure 2B illustrate the effects of receptor activation with ET-1 (100 nM) and SERCA inhibition with 2 μM thapsigargin (Tg) on [Ca\(^{2+}\)]\(_{SR}\) in the peripheral (marked with red circle) and peri-nuclear (marked with black circle) areas of SMCs.
In the cell periphery, ET-1 causes a drop in \([Ca^{2+}]_{SR}\) (from 1.08 ± 0.12 mM to 450 ± 50 mM), and subsequent SERCA inhibition results in an additional decline (to 55 ± 11 mM). In the peri-nuclear areas, however, the ET-stimulated changes in \([Ca^{2+}]_{SR}\) are moderate (from 1.05 ± 0.07 mM to 700 ± 50 mM). Although a clear loss of \([Ca^{2+}]_{SR}\) is evident following Tg application, a considerable amount of D1SR signal remains in the peri-nuclear areas (427 ± 23 μM), indicating the presence of organelles containing calsequestrin and Ca\(^{2+}\) that are not sensitive to SERCA blockade. A plausible explanation for this residual

**Figure 4. Progression of ET-1-induced regenerative Ca\(^{2+}\) depletion wave in the absence of extracellular Ca\(^{2+}\).** Average trace for \(R/R_0\) values in SMCs treated with ET-1 (100 nM) and Tg (2 μM) in the absence of extracellular Ca\(^{2+}\). ET-1 induces a delayed bi-phasic drop in \([Ca^{2+}]_{SR}\), a small initial transient phase, followed by a large precipitous drop in \([Ca^{2+}]_{SR}\) (\(n = 6\) independent experiments, mean ± SEM, \(P < 0.005\)). Representative snapshots (FRET channel) of time-lapse movie (10 s intervals) illustrates an ET-1-induced regenerative Ca\(^{2+}\) depletion wave over time in a SMC (scale bars, 4.3 μm). At 300 s, the bathing solution is replaced by nominal Ca\(^{2+}\) free HEPES. At 600 s, ET-1 (100 nM) is added, which elicits a delayed decrease in luminal Ca\(^{2+}\) around 1160 s post-treatment. Immediately prior to the initiation of the depletion wave, a rapid transient increase in Ca\(^{2+}\) is observed at the point of origin (1200 s panel, circled area), followed by a significant drop due to wave development.

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[Ca²⁺]₅ is that in these cells the SR is contiguous with membranous organelles or endosomes, which accumulate Ca²⁺ via the Tg-insensitive “secretory pathway Ca²⁺ ATPase” (SPCA). An alternative explanation is provided by a recent report from Ledeen’s group [17], which showed that NCX located in the inner membrane of the nuclear envelope is able to take up Ca²⁺ after SERCA blockade. Furthermore, since the nuclear envelope and the SR are confluent, this Ca²⁺, taken up by the NCX is capable of diffusing into the peri-nuclear SR.

In order to compare the changes in [Ca²⁺]₅ with fluctuations in [Ca²⁺]ᵢ, we used the cytoplasmic Ca²⁺ sensitive dye, fluo-4 AM in the same VSMC preparation (Fig. 3). In these cells, ET-1 (100 nM) appears to be a weak agonist eliciting only a transient increase in [Ca²⁺]ᵢ as opposed to uridine-5'-triphosphate (UTP), which stimulates a large maintained increase in [Ca²⁺]ᵢ. Subsequent inhibition of SERCA with Tg (2 μM) elicits another large [Ca²⁺], transient due to depletion of SR luminal Ca²⁺. Therefore, at least in the case of ET-1, it is clear that the luminal SR Ca²⁺ indicator shows dynamics, which are different from those observed with the cytoplasmic Ca²⁺ sensitive dye, fluo-4 AM.

ET-1-induced Regenerative SR Ca²⁺ Depletion Waves in the Absence of Extracellular Ca²⁺

In order to distinguish the effects of ET-1 on luminal SR Ca²⁺ from those related to ET-1 stimulated Ca²⁺ entry from the extracellular space, we removed Ca²⁺ from the bathing solution, but without adding a chelator. Under these conditions, which inhibit SR Ca²⁺ refilling [18], ET-1 caused a large and delayed drop in [Ca²⁺]₅ (Fig. 4). The difference between ET-induced responses in the presence and absence of external Ca²⁺ is not surprising, because in the presence of external Ca²⁺, there is rapid Ca²⁺ cycling between the SR lumen and extracellular space [19], which appears to protect the SR from excessive Ca²⁺ depletion. In contrast, in nominally Ca²⁺ free condition, which inhibits SR refilling, ET-1 causes a much larger drop in [Ca²⁺]₅.

In about 80% of the cells, application of ET-1 after removal of external Ca²⁺ revealed a novel phenomenon that we refer to as the...
SR Ca\textsuperscript{2+} depletion wave'' (Fig. 4 and Movie S2). At 300 s, Ca\textsuperscript{2+} is removed from the bathing solution, and at 600 s, ET-1 (100 nM) is added. In the presence of ET-1, focal fluctuations in [Ca\textsuperscript{2+}]\textsubscript{SR} can be observed, and just before the onset of the Ca\textsuperscript{2+} depletion wave, [Ca\textsuperscript{2+}]\textsubscript{SR} is elevated at the site of wave initiation (Fig. 4, panel 1200 s). The SR depletion waves routinely originated close to the peri-nuclear region, rather than in the cell periphery. Measurements of changes in the \(R/R_0\) values at the point of wave initiation, confirmed a transient increase in [Ca\textsuperscript{2+}]\textsubscript{SR} to 700 ± 100 μM immediately prior to wave initiation (Fig. 5A). Subsequently a depletion of [Ca\textsuperscript{2+}]\textsubscript{SR} occurs at the site of initiation (from 700±100 μM to 60±20 μM), from which a wave of Ca\textsuperscript{2+} depletion radiates out into the surrounding SR network (Fig. 4, panels 1210–1400 s). In the absence of external Ca\textsuperscript{2+} the extent of ET-1-induced [Ca\textsuperscript{2+}]\textsubscript{SR} depletion is similar to that induced by Tg. The depletion wave progresses in a roughly circular fashion and is always surrounded by a rim of elevated [Ca\textsuperscript{2+}]\textsubscript{SR}. It thus appears that some of the Ca\textsuperscript{2+} released at the wave front is taken up by an adjacent, but not-yet-activated SR locus. Figure 5B shows that at any given time point of wave expansion, there are significant differences in [Ca\textsuperscript{2+}]\textsubscript{SR} between the depleted SR region (marked as A), the transiently refilled neighbouring SR locus (marked as B), and the surrounding SR network.

Figure 6. Refilling of SR Ca\textsuperscript{2+} and maintenance of SR structure following the depletion wave. A) The SR is refilled by subsequent re-perfusion of the cell culture with normal (1.5 mM) Ca\textsuperscript{2+} solution following depletion wave progression, confirming the reversibility of Ca\textsuperscript{2+} depletion wave. B) Live snapshots of overlay of D1SR and ER-Tracker signals in cultured SMCs showing that the SR luminal structure remains intact during and after the progression of ET-induced Ca\textsuperscript{2+} depletion wave (marked by white arrows) and in the absence of extracellular Ca\textsuperscript{2+}. (scale bars, 15 μm). doi:10.1371/journal.pone.0055333.g006
and the next SR locus further ahead of the depletion wave (marked as C). After passage of the ET-induced Ca\(^{2+}\) depletion wave, the SR was capable of refilling upon subsequent reperfusion with normal (1.5 mM) Ca\(^{2+}\) solution, confirming the reversibility of the observed SR depletion wave (Fig. 6A). Furthermore, the depletion waves recorded herein did not cause any long-lasting structural changes in SMCs, and the SR structure remained intact during the experiment (Fig. 6B).

Ca\(^{2+}\) Depletion Waves in SMCs Propagate via IP\(_3\) Receptors

A plausible explanation for the initiation of SR Ca\(^{2+}\) depletion waves is that after the IP\(_3\) concentration builds up to a critical level and the cytoplasmic and SR Ca\(^{2+}\) concentrations at the initiation site reach a certain threshold values, the IP\(_3\)Rs on the SR membrane open in a regenerative fashion; the wave is then propagated by CICR at IP\(_3\)Rs. To test this hypothesis, we blocked the IP\(_3\)Rs with a specific inhibitor xestospongin C (Xes-C) prior to the application of ET-1 (Fig. 7). Addition of Xes-C (1 \(\mu\)M) completely blocked ET-induced depletion waves, confirming that as for other VSM Ca\(^{2+}\) waves [8,18,20], the underlying mechanism is indeed mediated by CICR at IP\(_3\)R. This notion was further corroborated by the observation that our cultured SMCs failed to respond to caffeine (data not shown), confirming the lack of functional RyRs in these cells, which is in agreement with a previous report by Vallot et al [21].

Dynamics of the Ca\(^{2+}\) Depletion Waves in the SR Lumen

Another instructive way of analyzing the [Ca\(^{2+}\)]\(_{\text{SR}}\) depletion wave is to identify a line intersecting the site of wave initiation and recording the changes in \(R/R_0\) values along this line vs. time (line scan). The resulting “heat map” illustrates that initially the area of [Ca\(^{2+}\)]\(_{\text{SR}}\) depletion expands rapidly, but as time proceeds, it asymptotically approaches a final limit (Fig. 8A). Analysis of a number of such heat maps yields the average distance vs. time, as well as the velocity vs. time curves (Fig. 8B). Extrapolation of the velocity curve to the time of wave initiation (0 s), shows that the SR Ca\(^{2+}\) depletion wave has an initial velocity of about 0.7 \(\mu\)m/s, which falls below the range (2–30 \(\mu\)m/s) reported previously for intact smooth muscle Ca\(^{2+}\) waves in the presence of extracellular Ca\(^{2+}\) [22]. This could be explained by the fact that in our cultured VSMCs, ET-1 is a weak agonist, and a number of studies have shown that both the velocity and frequency of smooth muscle Ca\(^{2+}\) waves increase with the level of activation and the concentration of IP\(_3\) [9,23]. The profound decrease in velocity over time is likely related to the decline of the [Ca\(^{2+}\)]\(_{\text{SR}}\) at the rim as the wave progresses (Fig. 8C).

Quantitative Model for Propagation of Ca\(^{2+}\) Depletion Waves

The observed transient increase in [Ca\(^{2+}\)]\(_{\text{SR}}\) at both the origin and the rim of the depletion wave (Fig. 5A and 5B) suggests an important role for local [Ca\(^{2+}\)]\(_{\text{SR}}\) elevation during initiation and propagation of regenerative IP\(_3\)R-mediated Ca\(^{2+}\) release. Since in cytoplasmic Ca\(^{2+}\) oscillations, the latency period is related to the inter-spike interval [24], which is, in turn, regulated by the SR Ca\(^{2+}\) content [24], the VSMCs activity may well be controlled by focal fluctuations in [Ca\(^{2+}\)]\(_{\text{SR}}\). In line with previous reports [25,26], we propose that these fluctuations are generated in part by a differential distribution of SR luminal Ca\(^{2+}\) sinks (clusters of IP\(_3\)Rs), and Ca\(^{2+}\) sources (SERCA) (Fig. 9A). Stochastic opening of individual IP\(_3\)Rs (which yield cytoplasmic Ca\(^{2+}\) blips) [25,26] would add variability to local [Ca\(^{2+}\)]\(_{\text{SR}}\). Whenever an increase in local [Ca\(^{2+}\)]\(_{\text{SR}}\) exceeds the threshold for regenerative opening of an IP\(_3\)R cluster, sufficient Ca\(^{2+}\) would be released to initiate a regenerative Ca\(^{2+}\) wave.

Generation of a wave according to this mechanism, and possessing the features of deceleration and decreasing intensity at the progressing rim as we observe, implies a continuity of the SR lumen. Since under control conditions the non-activated SR remains loaded with Ca\(^{2+}\) because of continual activity of SERCA, the observed decline in [Ca\(^{2+}\)]\(_{\text{SR}}\) at the rim of the depletion wave (Fig. 8C) is a strong indication that a re-equilibration of the SR Ca\(^{2+}\) content is taking place within the SR lumen. Only in this manner, when removal of extracellular Ca\(^{2+}\) blocks SR refilling, then opening of IP\(_3\)Rs at the wave front would partially deplete the SR located just ahead of the wave.

To analyze this proposed mechanism, we developed a preliminary and simplified two-dimensional quantitative model for the propagation of the observed depletion waves, which is based on previous observations and suggested models arguing for 1) a continuous SR lumen in regards to Ca\(^{2+}\) transport, 2) the appearance of functionally segregated compartments in the SR of SMCs, and 3) the existence of a luminal Ca\(^{2+}\) binding site of the IP\(_3\)R [27,28,29]. The essential steps of this model are presented in Figure 9B, and the generated trace for the velocity of the depletion wave is presented in Figure 9C.

Using the symbols and data in Table 1, our model assumes that, before any Ca\(^{2+}\) depletion wave (CDW) event, the [Ca\(^{2+}\)]\(_{\text{SR}}\) is at a normal resting level (C\(_{\text{SR}}\)) and that it needs to reach a critical level (C\(_{\text{CR}}\)) for release. Then, for example, following the depletion of a given SR compartment, for a nearest neighbouring compartment to release, its [Ca\(^{2+}\)]\(_{\text{SR}}\) needs to change by C\(_{\text{SR}}\)−C\(_{\text{SRmax}}\) = 200 \(\mu\)M, which translates to about 1500 Ca\(^{2+}\). For the sake of order-of-magnitude calculations, let us say that SERCA pumps operating at 300 s\(^{-1}\) would take a time interval \(\Delta t = 5\) s to cause a 200 \(\mu\)M [Ca\(^{2+}\)]\(_{\text{SR}}\) change. We calculate the depletion wave velocity, \(v_{\text{CDW}}\), as the inter-IP\(_3\)-R-cluster distance, \(d\), divided by the time, \(\Delta t\), taken to raise the [Ca\(^{2+}\)]\(_{\text{SR}}\) in the new compartment from normal to critical: \(v_{\text{CDW}} = d/\Delta t\). For the sake of completeness, the wave velocity should also include a component for the Ca\(^{2+}\) diffusion time from cluster to cluster. This component is however of the order of milliseconds and therefore negligible when compared to SR compartment refill times.

In this manner, for one depletion wave “step” in our model (from compartment 0 to 1 in Figure 9B), \(v_{\text{CDW}} = d/\Delta t = 0.1 \mu\text{m/s}^{-1}\).
Figure 8. Dynamics of the Ca\textsuperscript{2+} depletion waves. A) Heat map generated from the time-lapse fluorescence microscopy movie (10 s intervals) of rat SMCs treated with ET-1 (100 nM) in nominally free extracellular Ca\textsuperscript{2+}, showing that the SR depletion wave expands in a near circular fashion in both directions of the scan line (dotted white line). B) Average travel distance and velocity of ET-1-induced depletion wave vs. time. As the distance from the point of origin increases, the wave velocity decreases asymptotically to zero. C) Average values for \( R/R_0 \) and [Ca\textsuperscript{2+}]\textsubscript{SR} at the rim of the depletion wave also decreases as the wave expands over time (ROI = 5, n = 5 independent experiments, mean ± SEM, \( P < 0.005 \)).

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Figure 9. Simplified quantitative model for propagation of SR Ca\textsuperscript{2+} depletion waves. A) Schematic model of SR network depicting differential distribution of SR luminal Ca\textsuperscript{2+} sources (SERCA) and sinks (IP\textsubscript{3}Rś). B) We represent a linear section of SR as a series of compartments (labelled 0, 1, 2, etc), between which Ca\textsuperscript{2+} transport is allowed. Panels B-1, B-2, & B-3 represent the same portion of model SR at three different times, t\textsubscript{0}, t\textsubscript{1}, and t\textsubscript{2}. A wave initiating at compartment 0 in this model would move both left and right, but we focus only of the right-moving part for simplicity. Assuming that the [IP\textsubscript{3}]\textsubscript{cyt} is such that the IP\textsubscript{3}R is already Ca\textsuperscript{2+}-sensitized, a [Ca\textsuperscript{2+}]\textsubscript{i} fluctuation near the SR compartment 0 at \(t=t\textsubscript{0}\) raises the [Ca\textsuperscript{2+}]\textsubscript{SR} from a resting level to a threshold level, and thereby, causes the observed transient rise (B-1). This rise is followed by depletion of Ca\textsuperscript{2+} from compartment 0. Partial equilibration of luminal Ca\textsuperscript{2+} then takes place (blue block arrows between compartments), accompanied by an increase in [Ca\textsuperscript{2+}]\textsubscript{SR} in compartment 1 (B-2). Subsequently, [Ca\textsuperscript{2+}]\textsubscript{SR} in compartment 1 reaches a threshold value leading to the releases of (some of) its Ca\textsuperscript{2+}, which goes on to partially refill its nearest neighbouring locus (compartment 2), and so on (B-3). Ca\textsuperscript{2+} release from compartment 2 can trigger release from compartment 0, only after [Ca\textsuperscript{2+}]\textsubscript{SR} will have reached a critical level for Ca\textsuperscript{2+} release, which will take a time interval \(\Delta t\textsubscript{2}=t\textsubscript{2}-t\textsubscript{1}\). Due to the Ca\textsuperscript{2+} passage from compartment 2 to 1, \(\Delta t\textsubscript{2}\) must be greater than \(\Delta t\textsubscript{1}\), the interval it took to raise [Ca\textsuperscript{2+}]\textsubscript{SR} in 1 to the release level. Successive compartment refilling times to release level will get progressively longer because of the ability to re-equilibrate Ca\textsuperscript{2+} depletions. Assuming now that the CICR sustaining IP\textsubscript{3}R clusters are on average equidistant at a length \(d\) (500 nm) from one another, the Ca\textsuperscript{2+} depletion wave velocity (\(v\textsubscript{CDW}\)) as the wave reaches successive compartments, is given by \(v\textsubscript{CDW} = d/\Delta t\textsubscript{2} = \ldots = v\textsubscript{CDWn}\). Under the conditions of the reported experiments, disallowing Ca\textsuperscript{2+} communication between SR compartments in this model would translate in an equal interval \(\Delta t\) between the wave arrival at each compartment, and therefore in a constant wave velocity \(v\textsubscript{CDW} = d/\Delta t\). C) Ca\textsuperscript{2+} depletion wave velocity vs. time calculated from our model, considering Ca\textsuperscript{2+} communicating (solid curve) or Ca\textsuperscript{2+} tight (dashed) SR compartments.

\begin{align*}
V_{\text{CDW1}} &= \text{etc.} \\
V_{\text{CDW2}} &= \text{etc.} \\
\Delta t\textsubscript{1} &= t\textsubscript{1} - t\textsubscript{0} \\
\Delta t\textsubscript{2} &= t\textsubscript{2} - t\textsubscript{1} \\
\ldots &= \text{etc.}
\end{align*}
s. If we now assume that SR compartments 2, 3, etc lose part of their Ca\(^{2+}\) toward the depleted neighboring SR compartments by the percentages indicated in Table 1 (these values are arbitrarily chosen), their “normal” [Ca\(^{2+}\)]\(_{SR}\) will be C\(_{2,SR}\), C\(_{3,SR}\), etc., where these values are lower than CSR. The same line of reasoning then suggests that, for compartment 2 to release, its [Ca\(^{2+}\)]\(_{SR}\) needs to change by C\(^*\)\(_{SR}\) = C\(_{2,SR}\) - 200 mM, which will take a time \(\Delta t_2 >\Delta t_1\) and, in turn, yields a velocity for the second step as \(\nu_{CDW,2} = d/\Delta t_2\). To consider what would happen in a portion of SR with Ca\(^{2+}\) tight compartments, to a first degree of approximation, we can re-use the model above with 0% Ca\(^{2+}\) communication between compartments. It should be evident then that the interval needed to refill each compartment before release is the same as the putative wave progresses. Since the average inter-cluster distance is the same, the resulting wave velocity would be a constant value \(\nu_{CDW} = d/\Delta t\) at each step. The results reported in Figure 9C were obtained by following this step-by-step procedure for about 100 seconds, which confirms that introduction of such luminal Ca\(^{2+}\) flux into the model indeed caused incremental decrease of the simulated Ca\(^{2+}\) depletion wave velocity (Fig. 9C, red trace).

Our proposed model clearly depends on populations of IP\(_3\)Rs and SERCA spread over the entire SR network. This appears to be the case for the SMCs used in this study as both SERCA (Fig. 10A) and IP\(_3\)Rs (Fig. 10B) display the same diffuse distribution as the D1SR and ER-Tracker.

### Arrest of the ET-induced SR Depletion Wave at the Border of the Nuclear Envelope

Close inspection of the SR depletion waves also provides novel insight into sub-cellular differential Ca\(^{2+}\) signalling. Figure 11A presents snapshots of a depletion wave, which originates very close

#### Table 1. Symbols and data for the quantitative Model.

| Quantity                           | Data                         | References |
|------------------------------------|------------------------------|------------|
| SR lumen cross-section             | 50 nm \times 50 nm           | [46,47]    |
| IP\(_3\)R cluster-cluster distance | d                            |            |
| SR compartment volume              | 50 nm \times 50 nm \times 500 nm | [46,47,48] |
| SERCA refill rate                  | 300 s\(^{-1}\)               | [46]       |
| Normal [Ca\(^{2+}\)]\(_{SR}\), C\(_{SR}\) | 500 \(\mu\)M                  | our observation |
| Critical [Ca\(^{2+}\)]\(_{SR}\), C\(^*\)\(_{SR}\) | 700 \(\mu\)M                    | our observation |
| Ca\(^{2+}\) shift from 1 to 0     | 20%                          | our choice |
| (see Fig. 9B)                      |                              |            |
| Ca\(^{2+}\) shift from 2           | 5%                           | our choice |
| Ca\(^{2+}\) shift from 3           | 1%                           | our choice |

#### Figure 10. Distribution of SERCA and IP\(_3\)Rs in cultured rat aortic SMCs. A) Live snapshots of cultured rat aortic SMCs transfected with D1SR construct and treated with 1 \(\mu\)M of BODIPY\textsuperscript{®} TR-X thapsigargin showing the distribution of SERCA in cultured SMCs. SERCA distribution follows the same pattern as SR luminal network as indicated by expression of D1SR indicator (FRET channel) within the SR lumen (scale bars, 10 \(\mu\)m). B) Receptors for IP\(_3\) (IP\(_3\)Rs) were immuno-labelled using rabbit polyclonal antibody against IP\(_3\)R type I and Alexa-594 secondary antibody (Red). Images are representative of 29 cells imaged from four independent SMC cultures, and are shown as maximal intensity projections of de-convolved image stacks. Nucleus is labeled using DAPI (blue) (scale bars, 5 \(\mu\)m).

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to the nucleus (see Movie S3). In this particular case, the shape of the wave front becomes oblong instead of circular, as it fails to involve the nuclear envelope (white arrows), and proceeds in the opposite peripheral direction. The asymmetry of this particular wave is clearly demonstrated by the heat map in Figure 11B. As shown, the distance traveled by the wave and its velocity drop dramatically when the wave collides with the nuclear envelope, without depleting its luminal Ca\textsuperscript{2+} content. In contrast, the pattern
of two neighbouring SR depletion waves colliding with each other is clearly different from the case when a depletion wave encounters the nuclear envelope. As shown in Figure 12, in some cells, multiple Ca$^{2+}$ depletion waves develop independently. In these cells, a line scan of the Ca$^{2+}$ signal through the point of origin of two neighbouring depletion waves (a & b) and the heat map of the colliding waves accentuate that the bright rim between two neighbouring waves disappears over time (white arrow), while the rims facing the plasma membrane persist (red arrows) (N, nucleus, scale bars, 5 µm). doi:10.1371/journal.pone.0055333.g012

**Discussion**

In this first report of Ca$^{2+}$ depletion waves in the smooth muscle sarcoplasmic reticulum, we describe a crucial role for luminal [Ca$^{2+}$]$_{SR}$ in the initiation and propagation of cytoplasmic Ca$^{2+}$ waves. This additional insight was gained because the dynamics of SR Ca$^{2+}$ depletion do not simply mirror [Ca$^{2+}$]$_{i}$ elevations, but display their own unique characteristics. The disparity between the [Ca$^{2+}$]$_{i}$ and [Ca$^{2+}$]$_{SR}$ transients is most likely due to significant contributions by plasma membrane Ca$^{2+}$ fluxes to smooth muscle Ca$^{2+}$ homeostasis and excitation.

Iino’s hypothesis that Ca$^{2+}$ waves in VSM are propagated by regenerative CICR at the IP$_{3}$Rs [4] was recently corroborated by a well-controlled study by McCarron and collaborators on freshly isolated SMCs, voltage clamped and incubated in a Ca$^{2+}$ free medium [22]. Progression of the wave front by CICR requires that the released Ca$^{2+}$ medium [22]. Progression of the wave front by CICR requires that the depleted areas of colliding waves coalesce (white arrow) without a dividing line of Ca$^{2+}$ depletion do not simply mirror [Ca$^{2+}$]$_{i}$ elevations, but display their own unique characteristics. The disparity between the [Ca$^{2+}$]$_{i}$ and [Ca$^{2+}$]$_{SR}$ transients is most likely due to significant contributions by plasma membrane Ca$^{2+}$ fluxes to smooth muscle Ca$^{2+}$ homeostasis and excitation.

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segregation of dynamic Ca\(^{2+}\) signalling of vasoconstriction from functions such as gene transcription.

In conclusion, the findings presented in this study provide new insight into the mechanisms whereby the ER/SR, the main Ca\(^{2+}\) regulatory organelle in living cells, determines spatial and temporal characteristics of cellular Ca\(^{2+}\) signalling, which differentially control cellular migration, growth, proliferation and apoptosis in health and disease.

Supporting Information

Movie S1 3-D structure of rat SMCs transfected with D1SR. (WMV)

Movie S2 ET-induced SR calcium depletion wave. (WMV)

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