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Spontaneous Ca^{2+} oscillations in subcellular compartments of vascular smooth muscle cells rely on different Ca^{2+} pools

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ABSTRACT

Spontaneous Ca^{2+} oscillations in vascular smooth muscle cells have been modeled using a single Ca^{2+} pool. This report describes spontaneous Ca^{2+} oscillations dependent on two separate Ca^{2+} sources for the nuclear versus cytoplasmic compartments. Changes in free intracellular Ca^{2+} were monitored with ratiometric Ca^{2+}- fluorophores using confocal microscopy. On average, spontaneous oscillations developed in 79% of rat aortic smooth muscle cells that were synchronous between the cytoplasm and nucleus. Reduction of extracellular Ca^{2+} (< 1 μM) decreased the frequency and amplitude of the cytoplasmic oscillations with 48% of the oscillations asynchronous between the nuclear and cytoplasmic compartments. Similar results were obtained with the Ca^{2+} channel blockers, nimodipine and diltiazem. Arg-vasopressin (AVP) induced a rapid release of intracellular Ca^{2+} stores that was greater in the nuclear compartment (4.20 ± 0.23 ratio units, n = 56) than cytoplasm (2.54 ± 0.28) in cells that had spontaneously developed prior oscillations. Conversely, cells in the same conditions lacking oscillations had a greater AVP-induced Ca^{2+} transient in the cytoplasm (4.99 ± 0.66, n = 17) than in the nucleus (2.67 ± 0.29). Pre-treatment with Ca^{2+} channel blockers depressed the AVP responses in both compartments with the cytoplasmic Ca^{2+} most diminished. Depletion of internal Ca^{2+} stores prior to AVP exposure blunted the nuclear response, mimicking the response of cells that lacked prior oscillations. Spontaneous oscillating cells had a greater sarcoplasmic reticulum network than cells that did not oscillate. We propose that spontaneous nuclear oscillations rely on perinuclear sarcoplasmic reticulum stores, while the cytoplasmic oscillations rely on Ca^{2+} influx.

Keywords: oscillations, vascular smooth muscle cells, nuclear Ca^{2+}, vasopressin, thapsigargin, diltiazem, nimodipine.

INTRODUCTION

In vascular smooth muscle, as in other types of muscle, an increase in intracellular Ca^{2+} is the immediate trigger for contraction, which ultimately determines vascular tone and peripheral resistance. In the past 12 years, investigators have characterized repetitive spontaneous Ca^{2+} changes in vascular smooth muscle cells, often referred to as spikes or oscillations, depending on their frequency [1-3]. These transient elevations in intracellular Ca^{2+} correlate with oscillations in the electrical potential of the plasma membrane [3, 4], which are responsible for the rhythmic dilation and constriction noted in blood vessels [4]. The oscillatory electrical activity is not dependent on neuronal input *in vivo* [4], and can be measured in cultured arterial smooth muscle cells [5]. This spontaneous vasomotion is thought to assist in maintaining perfusion pressure and inhibiting tissue ischemia [6, 7].

Whole-cell techniques such as patch-clamp and fluorimetry have done much to characterize the spontaneous oscillations and ascribe a physiological function. Subcellular characterization of the oscillations has described the pattern of individual Ca^{2+} waves as they travel across the cell [8]. Previous reports determined that the spontaneous oscillations in vascular smooth muscle cells were dependent on extracellular Ca^{2+} [3, 9], while increasing intracellular Ca^{2+} through release of internal stores initiated oscillations or increased their frequency [2, 9]. Plasma membrane voltage-gated Ca^{2+} channels were shown to be responsible for the spontaneously oscillating free intracellular Ca^{2+}

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levels in cultured smooth muscle cells [2]. Furthermore, the oscillations were shown to be highly variable even within the same cell [10].

Confluent monolayers of the rat aortic smooth muscle cell line, A7r5, exhibit a variety of structural and functional properties of freshly dispersed smooth muscle cells including active Ca^{2+} channels [11] and pumps [12, 13]. In particular, these cells provide an excellent model for studying the spontaneous Ca^{2+} oscillations, because confluent cells electrically couple, and show evidence of spontaneous oscillations that are synchronized among cells [2, 3, 5, 14]. This accounts for the ability of single-cell oscillations to produce small, repetitive, constrictive and dilatory vasomotion in vessels, which are in contrast to other oscillations in vessels that appear to be more random and do not propagate between cells [7]. Under certain conditions 90% of all confluent cells have demonstrated spontaneous, repetitive Ca^{2+} spikes in the presence of 1.5 mM extracellular Ca^{2+} [3]. These oscillations are different events from the Ca^{2+} sparks described in vascular smooth muscle that are localized within specific areas of cells with total durations of 100 ms [15]. In contrast, spontaneous Ca^{2+} oscillations cover entire regions of cells and may have a duration of 30 s [14].

Ten years ago, we demonstrated significant non-uniformity in the distribution of Ca^{2+} in quiescent vascular smooth muscle cells using patch-clamp and microscopy techniques [16, 17]. Since then, several reports have demonstrated that non-uniform Ca^{2+} distribution exists in smooth muscle cells in a variety of conditions [1, 18, 19]. In the current report, we measured the subcellular patterns of spontaneous Ca^{2+} oscillations, using confocal microscopy and a variety of single and dual-wavelength Ca^{2+}-sensitive fluorophores. While such spontaneous oscillations have been reported in smooth muscle cells previously, this report describes nuclear oscillations dependent on intracellular Ca^{2+} stores, strikingly different from previous reports, which showed little or no difference in the oscillation pattern following depletion of intracellular Ca^{2+} stores [9, 14]. Data presented here illustrates a correlation between oscillations and subsequent agonist-induced Ca^{2+} responses. Finally, a model is proposed describing a possible mechanism for the oscillations and their effect on agonist-induced cell signaling.

MATERIALS AND METHODS

Cell culture

Rat thoracic aortic smooth muscle cells (A7r5, American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin in 5% CO_2 at 37°C. Cells were plated at a density of 10^4 cells/cm^2 onto 25 mm glass cover slips in 35 mm dishes and used for experiments when the monolayers were near or at confluency (typically 2 d).

Calcium imaging with fluorescent and confocal microscopy

Dye loading and treatments

Cells were labeled with membrane-permeant, Ca^{2+}-sensitive fluorophores by incubation in 5% CO_2 at 37°C for 30 min in completed DMEM containing 1μM dye for all compounds. Experiments were replicated using more than one fluorophore to verify all results. Ratiometric Ca^{2+}-sensitive indicators used in these experiments were fluo-4 and fura-red (Molecular Probes, Eugene, OR) [20, 21]. While other indicators were tested (fluo-3, calcium orange, calcium green, calcium crimson, indo-1, and oregon Green; Molecular Probes), the ratiometric fluo-4/fura-red combination provided the most consistent results as fura-red fluorescence emission is quenched when Ca^{2+} increases, while fluo-4 fluorescence increases, thus artifacts due to movement or dye sequestration are avoided [31]. Cells were incubated in 40 μM fura red and 20 μM fluo-4 in extracellular solution for 30 min at 37°C. Then cells were rinsed in extracellular solution consisting of (in mM): 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4 and 1.4 KH_2PO_4, pH 7.4. During experiments cells were bathed in the same solution with 2 mM Ca^{2+}. Other conditions (noted in the body of the results section) consisted of one of the following: 1) 0 Ca^{2+} (1 mM EGTA); or 2) low Ca^{2+} (1 μM). The Arg^8-vasopressin (AVP; Sigma, St. Louis, MO) concentration was 10 nM, because previous dose/response studies showed that maximal Ca^{2+} responses were obtained with AVP concentrations of 10-25 nM [22]. The diltiazem (Sigma) concentration was 10 μM, nimodipine (Sigma) was 50 nM, and thapsigargin (Sigma) was 5 μM. Endoplasmic reticulum morphology was measured using ER Tracker Blue White DPX (Molecular Probes) using DAPI filters, which has been shown to be highly sensitive for ER [23]. Cells were preloaded with Ca^{2+} Crimson and monitored for Ca^{2+} oscillations. Subsequently the chamber was washed and cells were incubated in ER Tracker for 15 min followed by another wash with extracellular solution prior to the capture of ER images. Great care was taken to ensure that the cells were exposed to the same conditions when loading and capturing images with ER Tracker, as the fluorophore is known to be highly sensitive to environmental conditions.

Microscopy and image analysis

Coverslips with cells were inserted into an Attofluor cell chamber (Molecular Probes) on the stage of an inverted Nikon Eclipse TE 300/Bio-Rad MicroRadiance Plus Laser Scanning Confocal Microscope (Bio-Rad Laboratories, Germany) or an Olympus FluoView 300 Confocal Microscope [24]. Preliminary experiments were conducted on a Nikon Eclipse TE300 epi-fluorescent microscope (Nikon, Japan) attached to a SPOT32 camera (Diagnostic Instruments, Inc, Sterling Heights, MI) (results not shown). For imaging, Ca^{2+}-sensitive fluorophores were excited at the recommended wavelengths (Molecular Probes). Sampling frequency varied depending on the experimental design, but was typically 1 scan every 1–10 s. The slow sampling frequency was chosen based on the slow frequency of the Ca^{2+} oscillations of interest, and the extended length of the experiments, often more than 20 min long.

Cells were analyzed for nuclear and cytoplasmic fluorescence using methods described by Goldman et al [25]. Basically, the nucleus was identified easily by its spherical shape using simultaneously

collected transmitted light images. Background fluorescence was subtracted from all fluorescence values. Due to the fact that these cells attached well to glass coverslips, several (10-15) cells could be optically dissected in a single field. Cells without clear nuclear compartments were excluded from data analysis. Fluorescence values from regions of interest within the cells were calculated as mean pixel values ± SE, and a ratio was calculated when using the Fluo-4/Fura Red combination. The delay of an agonist-induced response was measured from the time the agonist was added to the time when the ratio exceeded the baseline by 10%. This delay represented both the time required for the agonist to reach the cell surface, and the time required for the cell to respond. Similar results were obtained in at least three separate experiments for each condition. All experiments were performed at room temperature. Nested ANOVA was used to determine significant changes among different groups.

RESULTS

Spontaneous Ca^{2+} oscillations in the nucleus

The smooth muscle cell fluorescence emission was dynamic, even in unstimulated cells. Seventy-nine percent of all cells in physiological buffer with 2 mM Ca^{2+} demonstrated repetitive Ca^{2+} oscillations (n = 105). Oscillations were defined as repetitive fluctuations in fluorescence a minimum of 20% above the basal Ca^{2+} dependent fluorescent values. The presence of spontaneous oscillations in A7r5 cells was confirmed using different Ca^{2+}-sensitive fluorophores, including Fluo-4, Fluo-3, Calcium Crimson, Calcium Orange, Calcium Green, Oregon Green, and ratiometric methods using Fluo-4 and Fura-Red [20, 21]. The oscillations were synchronized among confluent cells. The field of cells illustrated in Fig. 1A shows the synchronized oscillations between different cells. Note that at least one cell did not follow the same pattern as the Ca^{2+} dropped between images while it was rising in the majority of other cells. The Ca^{2+}-sensitive fluorescence emission was plotted in Fig. 1B. Each line represents the output obtained for a single cell, and is not normalized for different initial Ca^{2+}concentrations. On average the spontaneous oscillations occurred with a frequency of one oscillation every 1-4 min. The frequency of spontaneous oscillations reported in the literature varies widely from 12/min [3] to 0.5-3/min [14], depending on the experimental conditions.

The effects of extracellular Ca^{2+} on subcellular oscillations

To determine whether Ca^{2+} in the nuclear and cytoplasmic compartments of cells oscillated in identical manners, we analyzed Ca^{2+} activity in each subcompartment separately. Fig. 2A illustrates the time course and magnitude of the spontaneous oscillations from a single cell with synchronous cytoplasmic and nuclear Ca^{2+} oscillations. While amplitudes varied between the compartments, the general time course of the spontaneous oscillations was

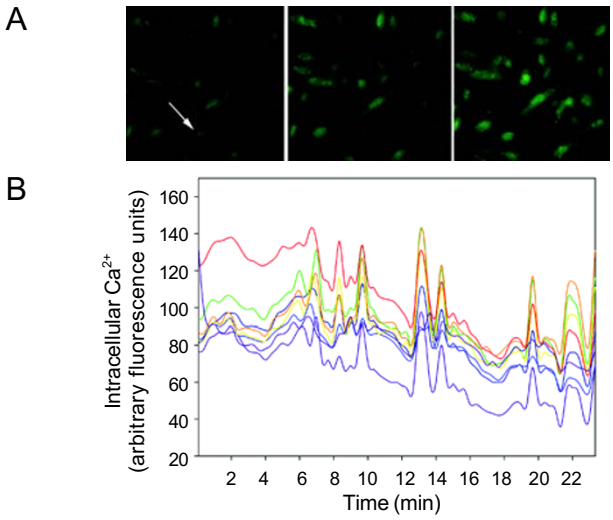
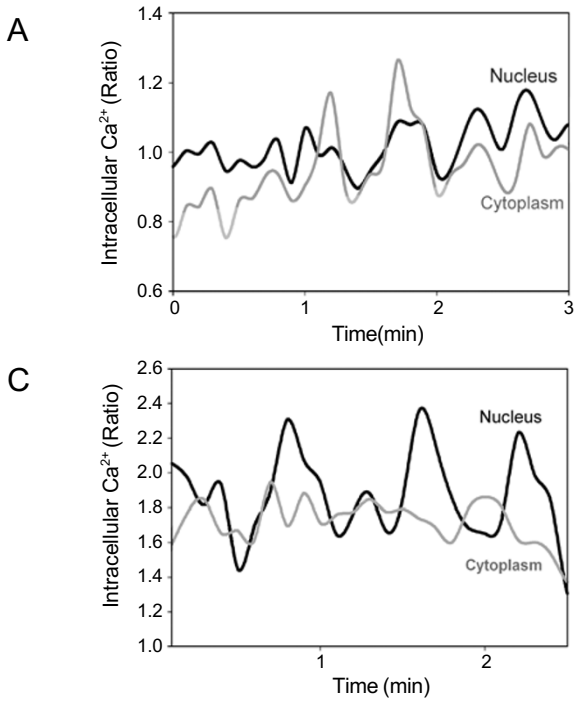


Fig. 1 Spontaneous Ca^{2+} oscillations in confluent vascular smooth muscle cells. (**A**) A7r5 cells were incubated in medium containing 2 mM Ca^{2+} and changes in Fluo-4 fluorescence were recorded within a single field. Most cells in the field showed spontaneous oscillations that were similar between cells. The three images were collected at 20 s intervals. The arrow (left panel) identifies a cell that showed decreasing fluorescence when Ca^{2+} appeared to be rising in the surrounding cells. (**B**) A graphic representation of the Ca^{2+} changes is shown over time. Each colored line indicates the whole-cell Ca^{2+} changes in an individual confluent cell.

the same. Cells bathed in 2 mM Ca^{2+} developed spontaneous intracellular Ca^{2+} oscillations that were synchronous between the nuclear and cytoplasmic compartments.

The percentage of cells with spontaneous oscillations dropped to 61% when extracellular Ca^{2+} was lowered to 1 μM (low Ca^{2+}). The amplitudes of the cytoplasmic oscillations were dramatically diminished when extracellular Ca^{2+} was decreased or when Ca^{2+} influx was blocked by Ca^{2+} channel blockers. Nuclear oscillations were not statistically different in low or high extracellular Ca^{2+} conditions, although there was a trend towards lower peak levels in low extracellular Ca^{2+}. Interestingly, there was a disconnection in the frequency of oscillations between the cytoplasm and nucleus in low extracellular Ca^{2+} conditions. Forty-eight percent of the spontaneous oscillations that occurred in low Ca^{2+} arose in the nucleus alone (n = 192 cells). Fig. 2B illustrates a cell with asynchronous oscillations which were larger in the nuclear versus the cytoplasmic compartment. While the spontaneous oscillations in the nucleus were larger, they were also slower in frequency, as illustrated in Fig. 2C (mean nuclear oscillations = 2.2 oscillations/min, 2.8 cytoplasmic oscillations/min). Further decreasing extracellular Ca^{2+} with the addition of 1 mM EGTA suppressed the spontaneous cytoplasmic



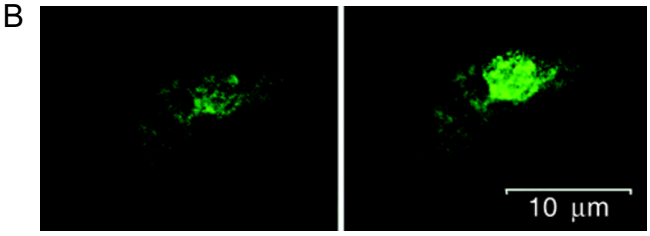


Fig. 2 Independent Ca^{2+} oscillations in the nuclear and cytoplasmic compartments. (**A**) Typical nuclear (black line) and cytoplasmic (gray line) Ca^{2+} oscillations within the same cell bathed in 2 mM Ca^{2+}. Although the amplitude of the oscillations between the compartments varied, the overall oscillatory pattern was similar. (**B**) When extracellular Ca^{2+} was lowered, nearly half of the cells developed asynchronous oscillations between the nuclear and cytoplasmic compartments as demonstrated in the representative cell with a greater nuclear oscillation. There was 20 s between the two images. (**C**) The asynchronous oscillations are illustrated with the line graph. Ca^{2+} changes in the two compartments were measured using the Fluo-4/Fura Red ratio.

oscillations, while nuclear oscillations continued in 33% of the cells. The lingering nuclear oscillations faded with time and at 8 min in 0 Ca^{2+} there were no nuclear or cytoplasmic oscillations.

Likewise the addition of the Ca^{2+} channel blocker, diltiazem, to the extracellular media halted cytoplasmic oscillations in 95% of the cells and decreased the frequency of the nuclear oscillations from 0.67 to 0.11 oscillations/min (not shown). Fig. 3 illustrates that a 5 min exposure to diltiazem reduced the peak amplitude of the spontaneous cytoplasmic oscillation from a 68 ± 6% to 12 ± 3 5% in fluorescence, while cells in low Ca^{2+} had oscillations that were 23 ± 4% above baseline ($P < 0.01$; n = 26/group). Nimodipine, another Ca^{2+} channel blocker, produced similar results (n = 20, not shown). The magnitude of the nuclear oscillations fell steadily with extended exposure to diltiazem. With 10 min of diltiazem or nimodipine application both cytoplasmic and nuclear oscillations were completely blocked.

Depolarization of the cells in the presence of Ca^{2+} (100 mM K^+, 2 mM Ca^{2+}) induced a slow rise in cytoplasmic, and a subsequent increase in nuclear fluorescence in 83% of all cells (n = 28). The time from depolarization to peak Ca^{2+} was 111 ± 12 s in the cytoplasm and 304 ± 47 s in the nucleus. Continued monitoring over the next 20 min revealed few subsequent nuclear or cytoplasmic oscillations. This result is in accord with previous reports that depolarization of the cells with high K^+ (140 mM) activated a sustained rise in Ca^{2+} and abolished subsequent Ca^{2+} oscillatory activity [2].

The effects of intracellular Ca^{2+} stores on subcellular Ca^{2+} oscillations

To examine the effect of depletion of the intracellular Ca^{2+} store on spontaneous oscillations, cells were treated with thapsigargin, a selective inhibitor of the intracellular Ca^{2+}-ATPase, which subsequently increased cytoplasmic Ca^{2+} in A7r5 cells [26]. Application of thapsigargin (5 μM) induced a relatively rapid increase in intracellular Ca^{2+} (Fig. 4). Exposure to thapsigargin halted subsequent nuclear Ca^{2+} oscillations in 54% of the cells (n = 133 cells). Likewise, cytoplasmic oscillations were halted post thapsigargin in 62% of the cells tested. Most importantly, thapsigargin blocked any subsequent asynchronous oscillations between the nucleus and cytoplasm. Thus, 100% of the nuclear oscillations measured after thapsigargin exposure were synchronous with cytoplasmic oscillations as shown in Fig. 4. These oscillations had a mean frequency of 3.2 oscillations/min. When both intracellular Ca^{2+} release and extracellular Ca^{2+} influx were prevented by the combination of EGTA (1 mM) and thapsigargin (5 mM), there was an initial increase in fluorescence, which subsided by 1 min in both compartments. Subsequent Ca^{2+} elevations were completely abolished in both the cytoplasm and nucleus (n = 25).

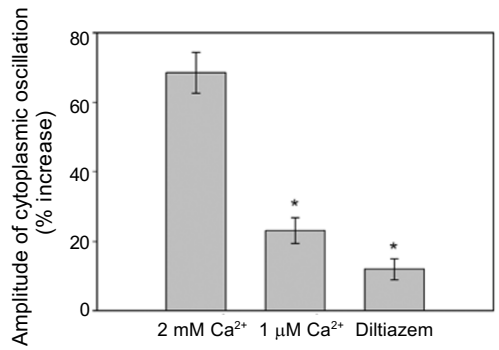


Fig. 3 Effects of extracellular Ca^{2+} on spontaneous oscillation activity. The amplitude of the spontaneous cytoplasmic oscillations was dramatically diminished by exposure to either a Ca^{2+} channel blocker (diltiazem) or to low extracellular Ca^{2+} conditions (1 μM Ca^{2+}; n = 26 cells/group). * indicates $P < 0.01$.

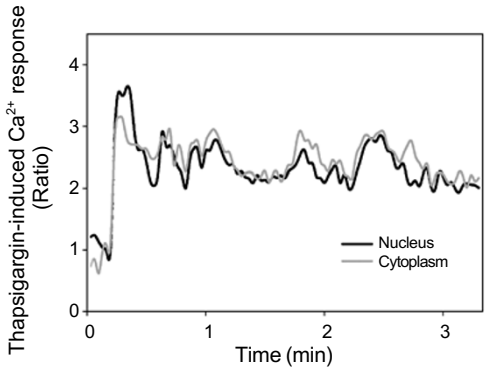


Fig. 4 Response of compartmental Ca^{2+} during SR Ca^{2+} release Stimulation of SR Ca^{2+} release with thapsigargin inhibited spontaneous oscillations in over half of all cells. In the remaining cells, all oscillations were synchronous between the nuclear and cytoplasmic compartments. The response of the two compartments from a typical cell is illustrated. Thapsigargin was added at time 0.

Spontaneous oscillations alter the Arg^8-vasopressin stimulated-response

AVP is a potent $InsP_3$-producing agonist for vascular smooth muscle cells, causing release of Ca^{2+} from intracellular stores followed by influx of Ca^{2+} from the extracellular environment [27]. Exposure to AVP (10 nM) initiated a large transient in both the nuclear and cytoplasmic compartments in over 80% of all cells tested (n = 189). In general, cytoplasmic and nuclear Ca^{2+} increased at approximately the same rate during the initiation of the AVP response (Fig. 5A). The AVP-induced responses were significantly greater than spontaneous oscillations in either the nucleus or cytoplasm, as has been reported previously [3].

As stated previously 79% of all cells in 2 mM Ca^{2+} had spontaneous oscillations. The remaining 21% failed to produce oscillations. These two populations of cells were analyzed as separate groups to decipher the characteristics of the oscillating cells. A clear difference was noted in the AVP response for cells that had prior oscillations from those that did not (Figs. 5A and 5B). The time from AVP exposure to the upstroke of the response (termed the delay) was not different in cells with or without prior oscillations (n = 50). The magnitude of the responses was significantly different, depending on whether the cells had prior spontaneous oscillations. The amplitude of the peak AVP response was greater in the nuclear compartment (4.20 ± 0.23, n = 51) than cytoplasmic (2.54 ± 0.28, $P < 0.0002$) in cells that had spontaneously developed prior oscillations (Fig. 6). Conversely, cells in the same environment that failed to produce oscillations had a greater AVP-induced Ca^{2+} transient in the cytoplasm (amplitude of transient = 4.99 ± 0.66, n = 50) than in the nucleus (2.67 ± 0.29, $P < 0.0005$). The duration of the AVP transient was longer in the nucleus than cytoplasm in both cell types (mean = 17 s. longer in nucleus; Figs. 5A and 5B). Cells without oscillations prior to the AVP exposure, often developed oscillations subsequent to the application of AVP (not shown), illustrating that the cells were in fact capable of oscillating when prompted.

The addition of AVP in the presence of the Ca^{2+} channel blocker diltiazem prevented the sustained cytoplasmic oscillations normally seen after AVP exposure. There was no statistical difference in the AVP transient between the nuclear compartment (3.33 ± 0.26, n = 18) and the cytoplasmic compartment (1.96 ± 0.36) following pre-treatment with diltiazem (Fig. 6). Pre-treatment with thapsigargin (5 min) depleted the intracellular Ca^{2+} stores and decreased the subsequent AVP-induced nuclear Ca^{2+} transient (Fig. 6). The peak AVP-induced nuclear transient was reduced in the presence of thapsigargin (2.21 ± 0.33, n = 21), while the cytoplasmic transient was not affected by the pre-treatment with thapsigargin (3.79 ± 0.37, $P < 0.005$). The duration of the AVP responses in both the nuclear and cytoplasmic compartments was significantly lengthened by exposure to thapsigargin. In the presence of thapsigargin, post-AVP oscillations appeared in the cytoplasm that were absent in nearly all of the nuclei.

Nuclear Ca^{2+} oscillations and sarcoplasmic reticulum morphology

Cells that demonstrated spontaneous cytoplasmic Ca^{2+} oscillations (measured with Calcium Crimson) were subsequently labeled with the fluorescent SR-specific fluorophore, ER Tracker (Fig. 7A). The presence of cellular Ca^{2+} oscillations was correlated with the level and characteristics of the SR (n = 28 cells). Cellular SR-derived fluorescence was significantly greater in the cells that had

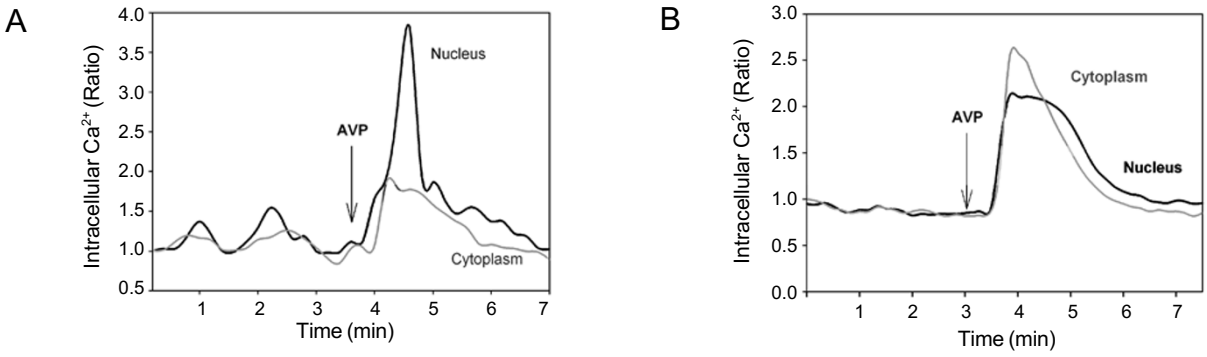


Fig. 5 Influence of spontaneous Ca^{2+} oscillations on subsequent AVP response. (**A**) In a cell with spontaneous nuclear and cytoplasmic oscillations, the addition of AVP (at the arrow) induced a greater amplitude response in the nuclear compartment (black) than in the cytoplasm (gray). (**B**) In a cell lacking prior oscillations, the cytoplasmic AVP-induced transient tended to be larger than the nuclear. In both cases the nuclear compartment Ca^{2+} took longer to recover to baseline values.

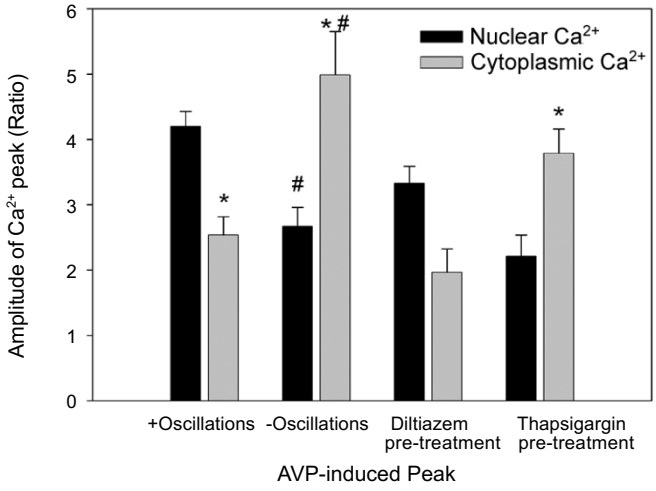


Fig. 6 Characterization of the AVP response in cellular subcompartments. Cells with spontaneous oscillations had greater AVP responses in the nuclear compartment than in the cytoplasm (n = 51, $P < 0.0002$). The reverse is true for cells that lacked spontaneous Ca^{2+} oscillations (n = 50, $P < 0.0005$). Exposure of cells to a plasma membrane Ca^{2+} channel blocker (diltiazem) reduced the amplitude of the cytoplasmic compartment Ca^{2+} response, but there was no statistical difference between the nuclear and cytoplasmic response. Pre-treatment of cells with thapsigargin resulted in an AVP response with a greater cytoplasmic than nuclear transient (n = 21, $P < 0.005$). * indicates statistical difference between nuclear and cytoplasmic compartments for each group. # indicates the difference in the nuclear and cytoplasmic Ca^{2+} peaks between cells with and without spontaneous oscillations ($P < 0.001$).

demonstrated spontaneous Ca^{2+} oscillations compared to cells in the same field that failed to elicit spontaneous oscillations (Fig. 7B). Interestingly, there was no difference in the perinuclear SR in groups of cells with or without spontaneous cytoplasmic Ca^{2+} oscillations (Fig. 7C).

DISCUSSION

Spontaneous oscillatory behavior has been noted previously in vascular smooth muscle cells, first as electrical oscillations [28, 29]. Later, spontaneous Ca^{2+} oscillations were measured in smooth muscle cells with extensive structural and electrical contact [2, 3, 5, 9, 14]. These investigators established the need for extracellular Ca^{2+}, since its removal [3], or the addition of nimodipine [14], a common L-type channel blocker, abolished the spontaneous Ca^{2+} oscillations. Prolonged incubation with thapsigargin, which depletes intracellular Ca^{2+} stores, did not block subsequent spikes in previously published reports [14]. Further, caffeine, a ryanodine receptor agonist, did not suppress the oscillations [9]. All these results indicated that Ca^{2+} entry through L-type Ca^{2+} channels was a requirement for spontaneous oscillations, and that mobilization of intracellular Ca^{2+} stores

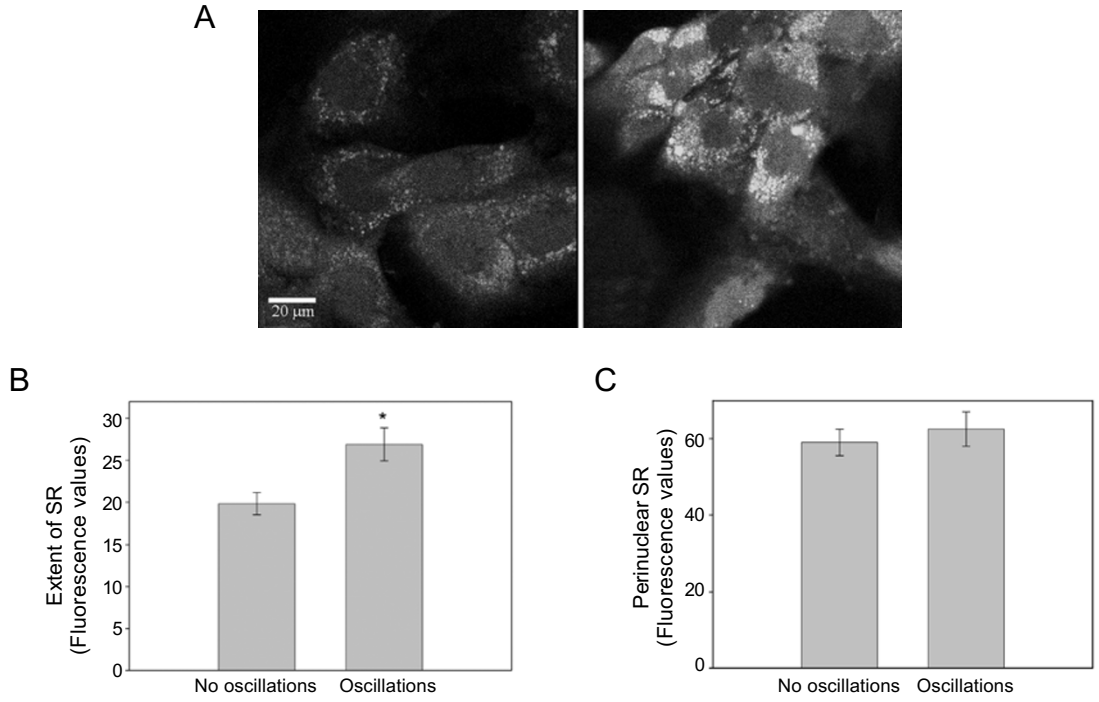


Fig. 7 Correlation of SR morphology and spontaneous Ca^{2+} oscillations. (**A**) Following the monitoring of subcellular oscillations with the Ca^{2+} fluorophore, Ca^{2+} Crimson, cells were tagged as either spontaneous oscillators or cells lacking oscillations. All cells were exposed to the SR-specific fluorescent probe, ER Tracker, which resulted in illustrations of both the distribution and amount of SR in each cell. The field of cells on the left did not demonstrate spontaneous oscillations, while the cells on the right were oscillatory. (**B**) Analysis of the levels of SR in the cells demonstrated that cells with oscillations had significantly more SR fluorescence ($P< 0.01$). (**C**) In contrast, analysis of the levels of perinuclear SR demonstrated no difference between the two groups.

was not necessary [5, 14]. However, the initial electrical event at the plasma membrane that launches the oscillation is still unknown [7]. In contrast, the data presented here identified Ca^{2+} oscillations within nuclei of smooth muscle cells that were dependent on intracellular Ca^{2+} stores. Furthermore, reduction of extracellular Ca^{2+} blocked cytoplasmic oscillations, while nuclear oscillations were maintained in nearly half of all cells. We categorized these cells as having asynchronous Ca^{2+} oscillations. Such a term may be confusing, as asynchrony commonly represents the unique Ca^{2+} behavior between cells [30] but not within cells.

The Ca^{2+} oscillations identified in this report are qualitatively different from the common Ca^{2+} oscillations measured in vascular smooth muscle cells, as these oscillations have a much lower frequency with an average of one oscillation every 0.5-4 min. In asynchronous conditions, the nuclear oscillations occurred more frequently than those in the cytoplasm. The frequency of spontaneous oscillations reported in the literature varies widely with most reports focusing on the higher frequency oscillations [28]. The low frequency oscillations have been reported previously by other groups [14], but are less well-studied, which may explain why the phenomenon of asynchronous oscillations had not been uncovered previously.

Smooth muscle cells certainly possess mechanisms for passive diffusion of Ca^{2+} across the nuclear envelope, and many signaling cascades use the passive flow of ions and small molecules across the envelope to influence nuclear events [31]. However, there is a growing appreciation for the role of the nuclear envelope and perinuclear SR in regulating nuclear Ca^{2+} levels independent of cytoplasmic concentrations [32-35]. In fact, AVP has already been reported to increase Ca^{2+} in the nuclear and cytoplasmic regions of rat aortic smooth muscle cells with the suggestion that the perinuclear region was likely an important Ca^{2+} storage site [36]. The source responsible for the independent spontaneous nuclear oscillations appears to be the perinuclear SR, which acts as a Ca^{2+} sink, initiating nuclear and perinuclear transients. In addition, the nuclear envelope itself may be involved in Ca^{2+} regulation of the nucleus as it acts as a Ca^{2+} storage site [37-39]. Multiple publications

have confirmed extensive Ca^{2+} signaling into the nucleus. Functional endothelin-1 receptors have been located on the nuclear membrane in vascular smooth muscle cells to increase nuclear Ca^{2+} when stimulated [40]. Furthermore, exposure to extracellular low-density lipoproteins induced a rise in Ca^{2+} that was localized to the nuclear region [41]. Just as researchers have ascribed unique contributions by the superficial SR, the perinuclear SR and nuclear envelope could be modeled as deep SR with its own unique functions [7].

The data presented here documented the presence of oscillations in the nucleus without concurrent oscillations in the cytoplasm and vice versa. The study of subcellular Ca^{2+} events during spontaneous oscillations is an important topic with few, if any, previous publications [7]. It provides compelling evidence that, under certain conditions, nuclear Ca^{2+} is independently regulated. We propose a model in which spontaneous Ca^{2+} oscillations within the cytoplasm are dependent on extracellular Ca^{2+} influx and the plasma membrane Ca^{2+} pumps. This is in agreement with earlier reports indicating that Ca^{2+} influx is needed for spontaneous smooth muscle cell oscillations [2, 3, 5, 9, 14, 42]. However, these reports monitored oscillations by means of electrophysiology or Ca^{2+} fluorescence without analysis of separate intracellular compartments. Our data showing independent nuclear oscillations in low extracellular Ca^{2+} suggest a two-pool model for Ca^{2+} oscillations for the cytoplasmic versus nuclear compartments. We propose that nuclear Ca^{2+} oscillations are governed principally by Ca^{2+} stores in the nuclear envelope and in the perinuclear SR. In this model it is possible, and quite likely, to have asynchronous nuclear and cytoplasmic oscillations. A cytoplasmic oscillation might not immediately diffuse into the nucleus through the nuclear pore complexes, because an extensive network of perinuclear SR or Ca^{2+} binding proteins would buffer the Ca^{2+} before it could near the pore. Freshly dispersed vascular smooth muscle cells have a transnuclear SR that pierces the nucleoplasm, described by Hill et al [43] and observed in our laboratory (Y Searls and L Stehno-Bittel, unpublished observations), which would act as strong nuclear Ca^{2+} buffers.

We hypothesize that cells with more extensive SR would be more capable of sustaining spontaneous nuclear Ca^{2+} oscillations. In experiments described here attempting to statistically correlate the amount of SR and the frequency of oscillations we had mixed results. We were able to statistically link spontaneous cellular oscillations with an extended cellular SR network, yet there were no differences in the perinuclear SR. We are not the first to suggest a relationship between the intracellular Ca^{2+} buffer capacity and the presence of spontaneous Ca^{2+} oscillations. Wu *et al* suggested such a correlation based on power spectrum analysis of oscillations [9]. They compared experimental data to a one pool non-oscillatory model of Ca^{2+} regenerative oscillations measured after AVP exposure, and proposed that when spontaneous oscillations occurred, the refilling of the internal Ca^{2+} stores was facilitated by the periodic change in membrane potential [9].

The mechanism responsible for the diversity of cells containing or lacking spontaneous oscillations is unknown, but could occur through changes in Ca^{2+} release channels or Ca^{2+} pumps. Both $InsP_3$ receptors and SERCA pumps have been identified previously on the nuclear envelope or in the perinuclear region of A7r5 cells [39]. We have evidence that the distribution of the SERCA2 isoform of the Ca^{2+} pump changes from a general SR pattern to a perinuclear distribution when cells are grown in high glucose. This appears to occur without a change in the ultrastructure of the SR (Y Searls and L Stehno-Bittel, unpublished observations). In the experiments described here, all cells were grown in identical conditions, yet only a certain population of cells developed spontaneous oscillations. Perhaps those cells contained a greater intracellular Ca^{2+} store that could participate and even initiate the spontaneous oscillations. Additionally, it is known that AVP-induced Ca^{2+} oscillations are dependent on the activation of protein kinase C (PKC) [44], thus Ca^{2+} oscillations and the changes in AVP-stimulated Ca^{2+} transients may be due to different activity levels of PKC. Whatever the exact mechanism is, two separate sources of Ca^{2+} appear to be responsible for the subcellular Ca^{2+} oscillations. The nuclear oscillations are dependent on a perinuclear source and cytoplasmic oscillations require the influx of Ca^{2+} from outside the cell.

Evidence for this model is provided by the following observations: 1) the magnitude of the nuclear AVP-induced peak was approximately 50% less in cells without oscillations; 2) blockers of plasma membrane Ca^{2+} channels attenuated the cytoplasmic oscillations first, followed by a slow attenuation of the nuclear oscillations; 3) depletion of the internal Ca^{2+} store halted asynchronous nuclear oscillations; 4) more extensive SR was associated with cells that exhibited spontaneous oscillations. The model refines a previously published model for Ca^{2+} oscillations in tracheal smooth muscle cells in which the oscillation amplitude was proposed to be dependent on the amount of Ca^{2+} in the SR, while the oscillation frequency and propagation velocity reflected the active state of the ryanodine receptors [45].

Ca^{2+} oscillations are a nearly universal mode of signaling in excitable and non-excitable cells [46]. Local frequency and amplitude modulation may allow for rapid adjustments in the signaling process, not available to whole-cell changes in Ca^{2+} [1]. Localized changes in Ca^{2+} regulation and release may play an important role in normal signaling

and in disease states of the vascular system [47]. It was suggested that a lack of nuclear Ca^{2+} buffering is a feature of pathological changes in vascular smooth muscle cells [43]. We are currently undertaking experiments to determine whether nuclear Ca^{2+} buffering is altered in advanced diabetes-induced vascular conditions. Identifying the mechanisms involved may lead to new approaches to pharmacological interventions for vascular disease.

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