Mitochondrial Regulation of Sarcoplasmic Reticulum Ca\(^{2+}\) Content in Vascular Smooth Muscle Cells

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Abstract—Subplasmalemmal ion fluxes have global effects on Ca\(^{2+}\) signaling in vascular smooth muscle. Measuring cytoplasmic and mitochondrial [Ca\(^{2+}\)] and [Na\(^{+}\)], we previously showed that mitochondria buffer both subplasmalemmal cytosolic [Ca\(^{2+}\)] and [Na\(^{+}\)] in vascular smooth muscle cells. We have now directly measured sarcoplasmic reticulum [Ca\(^{2+}\)] in aortic smooth muscle cells, revealing that mitochondrial Na\(^{+}/Ca^{2+}\) exchanger inhibition with CGP-37157 impairs sarcoplasmic reticulum Ca\(^{2+}\) refilling during purinergic stimulation. By overexpressing hFis1 to remove mitochondria from the subplasmalemmal space, we show that the rate and extent of sarcoplasmic reticulum refilling is augmented by a subpopulation of peripheral mitochondria. In ATP-stimulated cells, hFis1–mediated relocation of mitochondria impaired the sarcoplasmic reticulum refilling process and reduced mitochondrial [Ca\(^{2+}\)] elevations, despite increased cytosolic [Ca\(^{2+}\)] elevations. Reversal of plasmalemmal Na\(^{+}/Ca^{2+}\) exchange was the primary Ca\(^{2+}\) entry mechanism following ATP stimulation, based on the effects of KB-R7943. We propose that subplasmalemmal mitochondria ensure efficient sarcoplasmic reticulum refilling by cooperating with the plasmalemmal Na\(^{+}/Ca^{2+}\) exchanger to funnel Ca\(^{2+}\) into the sarcoplasmic reticulum and minimize cytosolic [Ca\(^{2+}\)] elevations that might otherwise contribute to hypertensive or proliferative vasculopathies. (Circ Res. 2009;104:104-112.)

Key Words: hFis1 ■ mitochondria ■ fragmentation ■ DIER ■ sarcoplasmic reticulum

Mitochondria are known to modulate smooth muscle Ca\(^{2+}\) signaling,\(^1,2\) but their role in the refilling of sarcoplasmic reticulum (SR) Ca\(^{2+}\) stores has not been extensively studied in vascular smooth muscle (VSM). Refilling of intracellular Ca\(^{2+}\) stores is instrumental for prolonged activation of VSM, but the mechanisms mediating SR refilling are not completely understood. Activation of vascular contraction on stimulation of G protein–coupled receptors is almost invariably initiated by Ca\(^{2+}\) release from the SR and sustained by Ca\(^{2+}\) entry variably mediated by receptor-operated cation channels (ROCCs), store-operated cation channels (SOCCs), and voltage-gated Ca\(^{2+}\) channels.\(^3\) This classic model is based on highly reproducible observations that removal of extracellular Ca\(^{2+}\) or application of Ca\(^{2+}\)-channel blockers leaves the initial Ca\(^{2+}\) transient and most of the peak contraction intact but aborts the maintained increase in free cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and the tonic phase of vasoconstriction. Iino et al\(^4\) refined this model of VSM activation by discovering that agonists induce asynchronous Ca\(^{2+}\) waves, which, when summed over large cell populations, generate sustained plateaus of both [Ca\(^{2+}\)]\(_i\) and force development. Although these asynchronous Ca\(^{2+}\) waves in VSM are produced by regenerative inositol-1,4,5-trisphosphate receptor–mediated Ca\(^{2+}\) release, Ca\(^{2+}\) entry from the extracellular space is required to maintain excitatory Ca\(^{2+}\) waves and tonic contraction.\(^5\)

Present evidence strongly suggests that SR refilling in several forms of smooth muscle requires reversal of the Na\(^{+}/Ca^{2+}\) exchanger (NCX) coupled to Na\(^{+}\) entry.\(^6-8\) Smooth muscle ROCC and SOCC are nonselective cation channels, likely composed of TRPC proteins,\(^9,10\) mediating greater Na\(^{+}\) than Ca\(^{2+}\) influx. Na\(^{+}\) entry through these channels can cause NCX reversal at junctions of the plasmalemma (PM) and SR (PM-SR junctions), where clustering of TRPCs, NCX and Na\(^{+}/K^{+}\)-APTase-\(\alpha_{21-14}\) combined with limited ionic diffusion\(^15\) generates localized high [Na\(^{+}\)] elevations (LNats).\(^16\) Quantitative modeling suggests that extensive (~500 nm diameter) but narrow (~20 nm) cytoplasmic nanodomains within the PM-SR junctions facilitate capture of NCX-mediated Ca\(^{2+}\) entry (NCE) by the sarco-/endoplasmic reticular Ca\(^{2+}\) ATPase (SERCA) before Ca\(^{2+}\) influx diffuses into the bulk cytosol.\(^15\) Consistent with this model, separation of PM-SR junctions abolishes agonist-induced [Ca\(^{2+}\)]\(_i\), oscillations and increases global [Na\(^{+}\)] elevations.\(^7,17,18\) Hellstrand and colleagues demonstrated that mitochondrial inhibition profoundly reduces the amplitude and increases the frequency of agonist-induced Ca\(^{2+}\) oscillations, consistent with a mitochondrial role in SR refilling.
during VSM activation. We and others have proposed that a subplasmalemmal population of mitochondria facilitates the linkage between \( \text{Ca}^{2+} \) entry and SR refilling.\(^\text{16,20–22}\) In aortic smooth muscle cells, mitochondria cooperate with the SR to buffer NCE,\(^\text{16,22}\) and purinergically stimulated \( \text{Ca}^{2+} \) entry enhances mitochondrial \( \text{Ca}^{2+} \) flux and mitochondrial NCX (mNCX) activity.\(^\text{20}\) In endothelial cells, such mitochondrial \( \text{Ca}^{2+} \) “funneling” promotes endoplasmic reticulum (ER) refilling in part by preventing \( \text{Ca}^{2+} \)-dependent inactivation of store-operated channels.\(^\text{20}\) However, it is currently unclear whether a similar mechanism supports SR \( \text{Ca}^{2+} \) uptake of NCE in VSM, which is primarily driven by \( \text{Na}^{+} \)-entry through ROCC.

Here we used \( \text{Ca}^{2+} \)-sensitive proteins targeted to the SR, mitochondria and cytoplasm to investigate the role of subplasmalemmal mitochondria in NCX-dependent SR refilling during agonist stimulation. To study the role of the subplasmalemmal mitochondrial subpopulation, mitochondria were drawn away from the PM by overexpressing hFis1, a protein mediating mitochondrial fission\(^\text{23}\) and causing mitochondria to migrate toward the nucleus without changing their innate ability to take up \( \text{Ca}^{2+} \).\(^\text{24}\) Based on the ultrastructural effects of hFis1 and pharmacological manipulation of ion transport, we present a novel and more complete model for SR refilling in agonist-stimulated VSM, in which subplasmalemmal mitochondria increase the efficiency of \( \text{Ca}^{2+} \) “funneling” into the SR.

**Materials and Methods**

**Reagents**

Reagents were purchased from Sigma, except KB-R7943 (Tocris, Bristol, UK), CGP-37157 and oligomycin (Calbiochem), Transfectin (Bio-Rad), pmitoDsRed (Clontech). YC3.6 (K<sub>e</sub>=250 mmol/L),\(^\text{23}\) 4mitD3-CPV (K<sub>e</sub>=1.2 mmol/L),\(^\text{26}\) and D1ER (K<sub>e</sub>=60 to 180 mmol/L)\(^\text{27}\) constructs were provided by Drs Amy Palmer and Roger Tsien (University of California, San Diego). hFis1 was provided by Dr Jean-Claude Martinou (University of Geneva, Geneva, Switzerland)\(^\text{28}\) were used for plasmid details, see the online data supplement, available at http://circres.ahajournals.org. Experiments were performed in HEPES-buffered saline solution (mmol/L): 145 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 10 glucose, 5 HEPES, 1.2 CaCl<sub>2</sub>, pH 7.6 at room temperature. N-Methyl-d-glucamine (NMDG) isoosmotically replaced \( \text{Na}^{+} \) in “0Na<sup>+</sup>” solutions.

**Cell Culture and Transfection**

Cultured rat aortic smooth muscle cells (RASMCs) from Dr Urs Ruegg (University of Geneva, Geneva, Switzerland)\(^\text{28}\) were used between passage 9 to 13\(^\text{16}\) and were grown on Matrigel-coated (BD Sciences) 25-mm glass coverslips for experiments. For morphological studies, cells were transfected with YC3.6 (2 \( \mu \)g, 4 \( \mu \)L of Transfectin) 1 day postplating and then mitoDsRed (1 \( \mu \)g) and hFis1 or pcDNA3 (2 \( \mu \)g, 6 \( \mu \)L of Transfectin) the next day. For \( \text{Ca}^{2+} \) measurements cells were transfected 2 days postplating (4 \( \mu \)L of Transfectin, 0.5 \( \mu \)g of calcium probe, 1.5 \( \mu \)g of pcDNA3 or hFis).

**Calcium Measurements**

Cytosolic, mitochondrial, and SR \( \text{Ca}^{2+} \) were measured with the FRET-based probes YC3.6, 4mitD3-CPV and D1ER, respectively. Ratiometric FRET-imaging (\( \times 40, 1.3 \text{ NA, Zeiss Axiosview s100TV} \)) was previously described (details in supplement).\(^\text{29}\) D1ER was calibrated in situ in semipermeabilized cells in intracellular solution (mmol/L: NaCl 10, KCl 135, MgCl<sub>2</sub> 1, HEPES 20, sucrose 20, digitonin 0.01, ionomycin 0.01, CCCP 0.005, pH 7.4) with \( \text{Ca}^{2+} \) from 0.003 to 10 mmol/L using HEDTA (0.4 mmol/L) to buffer 3 mmol/L and 10 mmol/L \( \text{Ca}^{2+} \) (Max Chelator v2.40, C. Patton, Stanford University, Calif). Normalized ratio values in 18 cells were fitted to:

\[
RR=\frac{R_{\text{max}}/R_{0}}{1+(y^{\log{K'}}_{d}-\log{(\text{Ca}^{2+})_{\text{free}}})^{h}}
\]

using GraphPad Prism 5.02 (GraphPad). \( R_{\text{max}} \) and \( R_{0} \) are fitted parameters, \( K'_{d} \) the apparent dissociation constant and \( h \) the Hill slope. \( \text{Ca}^{2+} \) was calculated by calibrating normalized D1ER ratios against a standard curve (Figure 1).

**Mitochondrial Localization and Fragmentation**

Detailed methods are available in the online data supplement. Briefly, mitochondria were imaged in cells expressing cytosolic YC3.6 and mitoDsRed that were cotransfected with hFis1 or pcDNA. Confocal images of fixed cells were acquired and analyzed under blinded conditions.

**Data Analysis**

Unless otherwise stated, data are means±SE. Differences was considered significant for \( P<0.05 \). Two-population comparisons were made with Student \( t \) test (with Welch correction if nonpara-
Kinetics of Agonist-Induced SR Ca\(^{2+}\) Depletion and Refilling

To quantify [Ca\(^{2+}\)\(_{\text{SR}}\)] changes in RASMCs, we used the SR-targeted ratiometric Ca\(^{2+}\) indicator D1ER because it responds to changes in [Ca\(^{2+}\)] between \(\approx 100\) mmol/L and \(\approx 1\) mmol/L and has a \(K_D\) optimally tuned for measuring [Ca\(^{2+}\)\(_{\text{SR}}\)] (see the online data supplement).\(^{27}\) D1ER exhibited the typical reticular pattern expected for a SR-resident protein (Figure 1A). For each cell, D1ER ratios were normalized to the typical reticular pattern expected for a SR-resident protein and 2C). However, steady-state [Ca\(^{2+}\)\(_{\text{SR}}\)] were largely dependent on Ca\(^{2+}\) entry. Previous experiments with targeted aequorins illustrated that the SR and mitochondria cooperatively buffer Ca\(^{2+}\) depletion. [Ca\(^{2+}\)\(_{\text{SR}}\)] increased 
\[\text{Ca}^{2+}\] transiently de-

Results

Calcium Influx Refills SR Ca\(^{2+}\) Stores During Agonist Stimulation

Rapid [Ca\(^{2+}\)\(_{\text{SR}}\)] recovery to resting levels in the presence of ATP suggested that the SR efficiently refills despite the continuous engagement of P2Y receptors by ATP. To test whether SR Ca\(^{2+}\) refilling depends on Ca\(^{2+}\) influx or internal Ca\(^{2+}\) recycling, we stimulated RASMCs with ATP in Ca\(^{2+}\)-free solution, in which ATP reduced D1ER ratios by \(0.138 \pm 0.009\) \(\Delta R/R_0\) corresponding to a [Ca\(^{2+}\)\(_{\text{SR}}\)] of 198 \(\pm 23\) mmol/L (19 cells) (Figure 2A). This degree of depletion was comparable to depletion in Ca\(^{2+}\)-containing media (Figure 1C) but less extensive than CPA-mediated depletion. [Ca\(^{2+}\)\(_{\text{SR}}\)] remained depleted until extracellular Ca\(^{2+}\) was restored, demonstrating that SR refilling required Ca\(^{2+}\) entry. Previous experiments with targeted aequorins illustrated that the SR and mitochondria cooperatively buffer NCE stimulated by extracellular Na\(^+\) removal.\(^{25}\) To delineate further the path of Ca\(^{2+}\) entry, we imaged changes in mitochondrial [Ca\(^{2+}\)]\(_{\text{mito}}\) (iCa\(^{2+}\)).\(_{\text{mito}}\) with 4mitD3-CPV.

Removing external Ca\(^{2+}\) shortly before stimulation did not reduce peak [Ca\(^{2+}\)].\(_{\text{mito}}\) elevations evoked by ATP (Figure 2B and 2C). However, steady-state [Ca\(^{2+}\)].\(_{\text{mito}}\) elevations were reduced by \(\approx 75\%\). Thus, sustained [Ca\(^{2+}\)].\(_{\text{mito}}\) elevations were largely dependent on Ca\(^{2+}\) influx and remained elevated for several minutes after [Ca\(^{2+}\)\(_{\text{SR}}\)] had returned to basal level, consistent with increased mitochondrial Ca\(^{2+}\) flux during agonist stimulation.

NCX-Mediated Ca\(^{2+}\) Entry and Transmitochondrial Ca\(^{2+}\) Flux Facilitate Rapid SR Ca\(^{2+}\) Refilling

To test whether NCE contributes to SR refilling, we monitored ATP-mediated [Ca\(^{2+}\)\(_{\text{SR}}\)] depletion in the presence of KB-R9743 (10 mmol/L). Despite several nonspecific effects reported for KB-R7943, our previous experience with this compound under similar conditions strongly indicate that its effects reported here can be attributed to selective inhibition of NCE (see the expanded Discussion section in the online data supplement and Poburko et al\(^{46}\)). KB-R9743 increased maximal [Ca\(^{2+}\)\(_{\text{SR}}\)] depletion from 202 \(\pm 31\) to 115 \(\pm 10\) mmol/L (\(-0.136 \pm 0.015\) to \(-0.178 \pm 0.008\) \(\Delta R/R_0, P=0.015\)) and delayed the time to maximal [Ca\(^{2+}\)\(_{\text{SR}}\)] depletion by \(\approx 10\) seconds (from 36.6 \(\pm 3.0\) to 47.3 \(\pm 4.0\) seconds, \(P=0.02\)) (Figure 3A). Importantly, KB-R7943 delayed the onset of [Ca\(^{2+}\)\(_{\text{SR}}\)] recovery by 35 \(\pm 5\) seconds \((P<0.001)\) and slowed the half-time of [Ca\(^{2+}\)\(_{\text{SR}}\)] recovery (24 \(\pm 4\) seconds,

Figure 2. SR refilling and sustained [Ca\(^{2+}\)\(_{\text{mito}}\)] elevations require Ca\(^{2+}\) influx. A, i, [Ca\(^{2+}\)\(_{\text{SR}}\)] response to ATP in Ca\(^{2+}\)-containing (black trace, from Figure 1C) and Ca\(^{2+}\)-free medium (gray trace, 12 cells), ii, Inset shows D1ER targeting. B, [Ca\(^{2+}\)\(_{\text{mito}}\)] responses to ATP measured in the presence (black, 16 cells) or absence (gray, 12 cells) of 1.2 mmol/L extracellular Ca\(^{2+}\). iii, Inset shows 4mitD3-CPV mitochondrial targeting. C, Statistical comparison (2-sample t test) of peak and steady-state [Ca\(^{2+}\)\(_{\text{SR}}\)].\(_{\text{mito}}\) (change in normalized ratio) responses corresponding to i and ii on the traces in B. Traces in A and B are 3-point boxcar averages rounded by mean data points.
Figure 3. Plasmalemmal and mitochondrial NCXs facilitate efficient SR refilling. A, D1ER-expressing cells were stimulated with ATP in the absence (black) or presence (gray) of KB-R7943. Double-headed arrows indicate regions compared by statistical analysis. ii, Effect of KB-R7943 on calibrated minimum [Ca\(^{2+}\)]\(_{\text{SR}}\) on ATP stimulation. B, Effects of inhibition of mitochondrial NCX with CGP-37157 (gray trace) (i) and inhibition of the mitochondrial ATP synthase with oligomycin (gray trace) (ii) on [Ca\(^{2+}\)]\(_{\text{SR}}\) refilling following ATP stimulation. The effects of CGP-37157 and oligomycin on maximal depletion and recovery were compared by ANOVA against common control cells (i and ii, black traces). The rate of recovery (\(t_{1/2}\)) between control and CGP-37157-treated cells was compared by t test. iii, Effect of CGP-37157 and oligomycin on calibrated minimum [Ca\(^{2+}\)]\(_{\text{SR}}\) on ATP stimulation. *\(P<0.05\) 2-sample t test, \(\triangle P<0.05\) Bonferroni post hoc test against control. ND indicates no difference.

Table 1. Mitochondrial ATP-Mediated [Ca\(^{2+}\)]\(_{\text{SR}}\) depletion or the time to onset of recovery (Figure 3B). However, CGP-37157, but not oligomycin, reduced the extent and slowed the kinetics of [Ca\(^{2+}\)]\(_{\text{SR}}\) recovery (Figure 3B, i). These findings are consistent with reports that mitochondria contribute to SR/ER Ca\(^{2+}\) refilling by funneling Ca\(^{2+}\) ions extruded by the mNCX into the SR.\(^{20,31}\)

Fragmentation and Redistribution of Subplasmalemmal Mitochondria by hFis1
To determine whether mitochondrial facilitation of [Ca\(^{2+}\)]\(_{\text{SR}}\) refilling requires close proximity between mitochondria and Ca\(^{2+}\) entry pathway(s), we removed mitochondria from the subplasmalemmal space by overexpressing hFis1, a strategy previously validated in HeLa cells.\(^{24}\) Mitochondria in VSM are typically rod-like and form tubular networks, readily visible with mitochondria-targeted DsRed (Figure 4A, i). As expected, hFis1 overexpression caused mitochondrial fragmentation (Figure 4A, ii). hFis1 decreased average mitochondrial cross-sectional area by \(\approx 30\%\) and decreased mitochondrial form factor indicating a shift toward more spherical morphology (Figure 4A, iii and iv). However, the total mitochondrial area per cell area, an indirect measure of mitochondrial mass, was not affected by hFis1 (Figure 4B, iii). Consistent with our previous study of mitochondrial fragmentation,\(^{24}\) hFis1 overexpression did not reduce mitochondrial membrane potential assessed by TMRM uptake, nor did it impair cell proliferation, cell size, or indicators of apoptosis (Annexin-V labeled and nuclear condensation) (supplemental Figure I). hFis1 did, however, increase the distance from the cell perimeter to the nearest mitochondrion by \(\approx 35\%\) (control, 1.75±0.18 \(\mu\)m; hFis1, 2.37±0.19 \(\mu\)m) (Figure 4B). The percentage of pixels along the cell perimeter within 0.5, 1.0 or 1.5 \(\mu\)m of a mitochondrion was also decreased by \(\approx 30\%\) by hFis1 (supplemental Table I). Thus, hFis1 moved mitochondria away from the PM in RASMCs.

Effect of hFis1 on Agonist-Induced and NCX-Mediated [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_{\text{mito}}\) Elevations
To determine whether the separation of PM and mitochondria altered Ca\(^{2+}\) signals, we measured the effect of hFis1 on [Ca\(^{2+}\)]\(_i\) elevations induced by ATP and by extracellular sodium removal. hFis1 overexpression increased the amplitude of the ATP-induced [Ca\(^{2+}\)]\(_i\), transient, which is dependent on SR Ca\(^{2+}\) release,\(^{7}\) by \(\approx 30\%\) and increased the amplitude of the [Ca\(^{2+}\)]\(_i\) plateau, which depends on Ca\(^{2+}\) influx, by \(\approx 140\%\) (Figure 5A and 5B, i and ii). Importantly, hFis1 increased the amplitude of NCX-mediated [Ca\(^{2+}\)]\(_i\), elevations directly induced by 0Na\(^+\) by \(\approx 70\%\) (Figure 5A and 5B, iii). In contrast with its effects on [Ca\(^{2+}\)]\(_i\), hFis1 overexpression reduced the peak [Ca\(^{2+}\)]\(_{\text{mito}}\) responses (reflecting SR Ca\(^{2+}\) release) by \(\approx 25\%\) and reduced the ATP-mediated [Ca\(^{2+}\)]\(_{\text{mito}}\) plateau (reflecting Ca\(^{2+}\) influx) by \(\approx 30\%\) (Figure 5C and 5D, i and ii). Furthermore, hFis1 reduced the amplitude of 0Na\(^+\)-stimulated [Ca\(^{2+}\)]\(_{\text{mito}}\) elevations by \(\approx 50\%\), consistent with reduced mitochondrial Ca\(^{2+}\) buffering of NCE (Figure 5C and 5D, iii).
Effect of hFis1 on ATP-Mediated SR Ca\(^{2+}\) Depletion and Refilling

Finally, to test whether removal of subplasmalemmal mitochondria impaired SR refilling, we measured \([\text{Ca}^{2+}]_{\text{SR}}\) changes in cells overexpressing hFis1. hFis1 significantly enhanced ATP-mediated \([\text{Ca}^{2+}]_{\text{SR}}\) depletion (minimum \([\text{Ca}^{2+}]_{\text{SR}}\) 0.28±0.03 mmol/L versus 0.41±0.04 mmol/L for control cells, \(P=0.004\)) (Figure 6A and 6C, i). Time to maximum \([\text{Ca}^{2+}]_{\text{SR}}\) depletion was not affected (51.8±4.5 seconds, control; 53.1±4.2s hFis1, \(P=0.96\) Mann–Whitney). In contrast, hFis1 reduced the rate and extent of \([\text{Ca}^{2+}]_{\text{SR}}\) recovery (Figure 6A (inset) and 6B, ii and iii). Moreover, following ATP removal, control cells exhibited a transient \([\text{Ca}^{2+}]_{\text{SR}}\) overshoot that was absent in hFis1 overexpressing cells, further indicating that hFis1 impaired SR Ca\(^{2+}\) loading. These effects of hFis1 overexpression on the kinetics and extent of \([\text{Ca}^{2+}]_{\text{SR}}\) refilling resembled those observed on pharmacological inhibition of the mNCX with CGP-37157.

Discussion

The principal and novel finding of this study is that separation of mitochondria from the PM has a profound inhibitory effect on refilling of smooth muscle SR during agonist stimulation. The separation was achieved by overexpressing hFis1, a procedure that does not alter mitochondrial Ca\(^{2+}\) fluxes, and SR refilling was monitored directly using the modified cameleon D1ER. Data so obtained lead to a Ca\(^{2+}\) transport model where peripheral mitochondria funnel NCX-mediated Ca\(^{2+}\) entry into SR to support rapid store refilling, a process essential for maintenance of cytoplasmic Ca\(^{2+}\) waves and vascular tone.\(^5\)

These first quantitative measurements of \([\text{Ca}^{2+}]_{\text{SR}}\) in agonist-stimulated VSM demonstrated a relatively high resting \([\text{Ca}^{2+}]_{\text{SR}}\) in VSM. The high \([\text{Ca}^{2+}]_{\text{SR}}\) values do not reflect abnormal behavior of the genetic probe, because D1ER is well tuned to measure physiological changes in \([\text{Ca}^{2+}]_{\text{SR}}\) and our calibration curve was similar to values measured in HeLa and CHO cells that display lower resting \([\text{Ca}^{2+}]_{\text{SR}}\).\(^27\) Rather, the high \([\text{Ca}^{2+}]_{\text{SR}}\) in VSM may be a common characteristic of muscle tissue, as similarly high values were reported in cardiac muscle.\(^32,33\) Purinergic stimulation induced only partial depletion of SR Ca\(^{2+}\), half as much as was depleted by blocking Ca\(^{2+}\) uptake by SERCA.
Interestingly, contrary to common depictions of SR/ER refilling occurring after the removal of agonist, SR refilling was initiated within 20 to 30 seconds in the continued presence of ATP, such that $[Ca^{2+}]_{SR}$ returned to basal levels within ≈90s (Figure 1).

NCX-Mediated Ca$^{2+}$ Entry Enables Rapid SR Refilling

NCX is increasingly reported to mediate Ca$^{2+}$ influx in various cell types.5,6,8,18,34–36 We initially demonstrated that extracellular Ca$^{2+}$ can be routed to the SR without altering bulk $[Ca^{2+}]$, via preferential interaction of NCX with the superficial SR of smooth muscle.37 Subsequent observations by Blaustein and colleagues,38 Groschner and colleagues,13 Moore et al,14 and our group5,15 provided structural and functional evidence that reverse-mode NCX can be coupled to Na$^+$ entry through ROCCs or SOCCs to mediate SR Ca$^{2+}$ refilling. Observation that NCE sustains agonist-stimulated $[Ca^{2+}]_{i}$ oscillations driven by SR Ca$^{2+}$ release provides compelling indirect evidence for NCE-mediated SR refilling in vascular and airway smooth muscle.6,8,17 Critical support for this model came from our recent observation of the localized [Na$^+$], elevations (LNats) that were predicted to underlie NCE.$^{16}$

Here, the conclusion that SR refilling is mediated by NCE is based on the observations that KB-R7943 (1) increased $[Ca^{2+}]_{SR}$ depletion, (2) delayed the onset of refilling, and (3) slowed the rate of SR refilling. KB-R7943 has been reported to nonspecifically inhibit several ion transporters other than NCX, but such interactions are not compatible with the cumulative effects of KB-R7943 reported in smooth muscle (see the expanded Discussion section and Poburko et al$^{16}$). The increase in maximal SR depletion by KB-R7943 further illustrates that SR refilling via ROCC-NCX coupling is rapidly initiated following ATP stimulation. Although the SR eventually refilled with KB-R7943 present, the 30- to 40-second delay in the onset of refilling likely represents the time required to activate SOCC. STIM1, the ER/SR Ca$^{2+}$ sensor essential for SOCC,39 translocates to PM-SR junctions to activate SOCC 30 to 40 seconds after agonist stimulation.$^{40}$ However, $[Ca^{2+}]_{SR}$ normally fell to values reported to activate STIM1 for only a few seconds, suggesting that SOCC likely provides a backup SR-refilling mechanism during prolonged or extensive SR depletion. This underscores
the likely importance of rapidly activated NCE versus SOCC for "physiological" SR refilling in intact VSM exhibiting rapid, SR-driven Ca\(^{2+}\) oscillations.

**Subplasmalemmal Mitochondria and SR Refilling**

Mitochondria often neighbor PM-SR junctions in VSM (reviewed by Poburko et al\(^{41}\)), where they restrict the diffusion of NCX-mediated Ca\(^{2+}\) entry into the bulk cytosol.\(^{32}\) The critical mitochondrial role shown herein for efficient refilling of agonist-depleted SR may further explain how mitochondrial inhibition decreases the amplitude of SR-driven [Ca\(^{2+}\)]\(_{i}\) oscillations in VSM.\(^{19}\) The conclusion that mitochondria optimize SR refilling follows the results of inhibition of the mNCX with CGP-37157 and molecular relocation of mitochondria by hFis1. Unlike KB-R7943, neither CGP-37157 nor hFis1 altered the time to onset of refilling, consistent with SR refilling being initiated by direct uptake of NCE by SERCA molecules within the PM-SR junctions. Nonetheless, both CGP-37157 and hFis1 slowed [Ca\(^{2+}\)]\(_{SR}\) recovery and reduced the extent of refilling, demonstrating that subplasmalemmal mitochondria facilitate the transfer of Ca\(^{2+}\) ions from the plasma membrane to the SR.

In blood and endothelial cells, subplasmalemmal mitochondria sustain Ca\(^{2+}\) influx by locally buffering Ca\(^{2+}\) to prevent Ca\(^{2+}\)-dependent inactivation of store-operated CRAC channels.\(^{23}\) This mechanism is unlikely to explain our present observations because: (1) hFis1 impaired [Ca\(^{2+}\)]\(_{SR}\) recovery despite a parallel increase in the Ca\(^{2+}\) entry-dependent [Ca\(^{2+}\)]\(_{i}\), plateau (Figure 5A); (2) CGP-37157 impairs SR refilling despite enhancing global [Na\(^+\)], elevations mediated by TRPC6,\(^{16}\); and (3) ROCCs and SOCCs in VSM are likely composed of TRPC proteins exhibiting less Ca\(^{2+}\)-dependent inactivation than CRAC channels.\(^{3,9,42}\) TRPCs and NCX can physically couple and are concentrated in PM-SR junctions,\(^{11,13-15}\) and constrained Ca\(^{2+}\) diffusion within the PM-SR junction\(^{15}\) makes it unlikely that mitochondria could reduce the [Ca\(^{2+}\)] within the junction sufficiently to relieve potential Ca\(^{2+}\) inhibition of ROCCs. As in HeLa cells,\(^{24,43}\) hFis1 did not increase basal apoptosis rates, and, as discussed in supplemental text, hFis1 does not directly impair mitochondrial Ca\(^{2+}\) up-take.\(^{24}\) Therefore, the hFis1-mediated changes in Ca\(^{2+}\) signaling likely reflected the loss of local Ca\(^{2+}\) transfer between subplasmalemmal mitochondria and the SR. We propose that subplasmalemmal mitochondria in VSM sustain SR refilling by sequestering Ca\(^{2+}\) ions spilling from PM-SR junctions and transferring them to the SR (Figure 7, no. 3) before they diffuse into the bulk cytosol or are extruded by PM Ca\(^{2+}\) ATPases located outside of the junctions (reviewed by Poburko et al\(^{41}\)). In this capacity, subplasmalemmal mitochondria increase the effective SR Ca\(^{2+}\) buffering capacity and cooperate with NCE and SERCA to ensure efficient SR refilling during agonist stimulation of VSM.

Clinically, cultured VSM cells from hypertensive pulmonary arteries exhibit fragmented mitochondria with perinuclear clustering, resembling hFis1-overexpressing cells.\(^{24}\) Ca\(^{2+}\)-dependent calcineurin activates Drp1, the cytosolic ligand of hFis1, and fragments mitochondria.\(^{45}\) Calcineurin inhibition can reverse the mitochondrial dysfunction that contributes to chronic [Ca\(^{2+}\)]\(_i\) elevations associated with VSM proliferation in pulmonary artery hypertension.\(^{46}\) Beyond impairing SR refilling, hFis1 caused a reciprocal increase in [Ca\(^{2+}\)]\(_i\) elevation and [Ca\(^{2+}\)]\(_{SR}\) depletion (Figures 5 and 6), indicating that mitochondrial fragmentation not only impaired SR refilling but also effectively increased SR Ca\(^{2+}\) release. Although the impact of mitochondria on SR Ca\(^{2+}\) release remains controversial,\(^{7}\) we propose that perinuclear clustering of mitochondria will reduce the surface of SR in close contact with mitochondria, thereby removing a physical barrier to diffusion of released Ca\(^{2+}\) (Figure 7, no. 6). In addition, preliminary results showed that chronic stimulation (24 hours) of RASMCs with angiotensin II (10 to 100 nmol/L), a promoter of proliferation, induces mitochondrial fragmentation (supplemental Figure II). Causal and temporal
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In conclusion, we demonstrate that NCX-mediated Ca\(^{2+}\) entry and subplasmalemmal mitochondria are both required for the rapid and efficient SR Ca\(^{2+}\) refilling in aorta smooth muscle. The coordinated interaction of subplasmalemmal mitochondria with the NCX and SR facilitates store refilling, while preventing excessive increases in bulk cytosolic [Ca\(^{2+}\)]\(_{cyt}\) during agonist activation of VSM. Demonstration that disruption of mitochondrial morphology alters VSM Ca\(^{2+}\) homeostasis represents novel avenues to explore in the etiology of proliferative vasculopathies.

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Disclosures

None.

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Figure 7. Proposed mechanism of SR refilling in VSM. A, Reverse-mode NCX is the primary source of Ca\(^{2+}\) entry mediating rapid SR refilling in agonist-stimulated aorta SMCs (1 and 2). A slower mechanism, likely SOCC, is activated if NCE is inhibited. Transmimics inhibition of transmitochondrial Ca\(^{2+}\) flux increases SR refilling efficiency and is required for complete SR refilling during stimulation (3). Mitochondria capture Ca\(^{2+}\) spilling from PM-SR junctions and funnel Ca\(^{2+}\) to the SR before it diffuses into the bulk cytosol or is extruded by the PMCA. Mitochondria adjacent to inositol trisphosphate (IP\(_3\)) receptors may reduce diffusion of released Ca\(^{2+}\) and increase feedback inhibition of IP\(_3\) receptors (4). B, Removal of subplasmalemmal mitochondria by hFis1 mimics inhibition of transmimics mitochondrial Ca\(^{2+}\) fluxes and prevent relay of Ca\(^{2+}\) to the SR (5). Perinuclear mitochondrial accumulation upon hFis1 overexpression frees much of the SR of a diffusion barrier near IP\(_3\) receptors, thus reducing Ca\(^{2+}\)-dependent inhibition of IP\(_3\) receptors and increasing SR Ca\(^{2+}\) release.\(^{6}\)

links between proproliferative/hypertensive insults, mitochondrial morphology, and VSM Ca\(^{2+}\) handling merit further investigation, and we propose a positive feedback between cumulative, chronic insults to VSM, altered mitochondrial morphology and distribution, and the exacerbated tonic [Ca\(^{2+}\)]\(_{cyt}\), elevations contributing to VSM proliferation in diseases like pulmonary artery hypertension.


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Mitochondrial Regulation of Sarcoplasmic Reticulum Ca\textsuperscript{2+} Content in Vascular Smooth Muscle Cells
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**MATERIALS & METHODS**

**Plasmids & Ca\(^{2+}\) sensor properties:** mitoDsRed was purchased from Clontech. D1ER, 4mitD3cpv and YC3.6 are based on pcDNA3 (Invitrogen). YC3.6 consists of circular permutation of Venus fluorescent protein at Asp-173 and has apparent Kd of 250 nM Ca\(^{2+}\).\(^1\) The D1ER construct consists of a truncated enhanced CFP and citrine fluorescent protein that are joined by a linker and hinge containing modified calmodulin and M13 sequences with a biphasic Ca\(^{2+}\) response measured in vitro with Kd’s (Ca\(^{2+}\)) of ~0.6µM and ~60µM.\(^2\) The CaM-M13 modifications prevent M13 from binding endogenous calmodulin. ER retention is achieved by calreticulin and KDEL retention sequences on the 5’ end of CFP and 3’ end of citrine, respectively. The high primary Kd of D1ER is well suited to measuring accurately [Ca\(^{2+}\)]\(_{SR}\), whereas probes like GCaMPs with sub-micromolar Kd values would be saturated throughout the physiological range of [Ca\(^{2+}\)]\(_{SR}\). D1ER’s high affinity Ca\(^{2+}\) binding does not appear to contribute to changes in D1ER ration over the range of [Ca\(^{2+}\)]\(_{SR}\) in situ (see below). 4mitD3cpv consists of a truncated eCFP and the Asp-173 circularly permuted Venus (cpv) joined by a linker and hinge containing a modified calmodulin and M13 pair with four tandem repeats of a 5’ mitochondrial targeting sequence of subunit VIII of Cytochrome C oxidase.\(^3\) Amplification of hFis1 by PCR from a human liver library and its cloning into a pCI (Promega) expression vector with a 5’-His tag is previously described.\(^4\)

**Calcium measurements.** Ratiometric FRET images were acquired with a 40x objective (1.3 NA, Zeiss Axiovert s100TV) and a cooled CCD camera (MicroMax, Roper Scientific, USA).\(^5\) Probes were excited at 430nm (DetlaRam monochromator) and imaged with 475DF15 and 535DF25 band-pass filters (Omega Optical, USA). The range of [Ca\(^{2+}\)]\(_{free}\) used for D1ER in situ calibration was chosen to exclude the influence of D1ER’s high affinity binding site (Kd 0.58µM)\(^2\) and to simplify subsequent calibrations. D1ER ratios never fell below values corresponding to <10µM Ca\(^{2+}\) following passive SR depletion (data not shown). Therefore the high affinity binding site of D1ER is irrelevant to our study, as it was out of the physiological range of [Ca\(^{2+}\)]\(_{SR}\) in intact cells.

**Mitochondrial localization & fragmentation:** A random sample of images was analyzed, under blinded conditions, from images acquired from coverslips prepared in triplicate per condition on two separate days. One day after plating, cells on 25mm glass coverslips were transfected with cytosolic YC3.6 as a marker of cell boundaries. The next day, the same coverslips were co-transfected with mitoDsRed and hFis1 or pcDNA at a 1:3 ratio so that most cells expressing mitoDsRed should also be transfected with hFis1 or pcDNA.\(^6\) 30-36 hrs after the second transfection, nuclei were labeled with DAPI (30µM, 20-30min) in PBS containing Ca\(^{2+}\) and Mg\(^{2+}\). Cells were then rinsed with PBS (i.e. 0Ca\(^{2+}\)/0Mg\(^{2+}\)) before being fixed with paraformaldehyde (4%, 10min). Paraformaldehyde was prepared on the day of fixation to ensure optimal fixation. After three 10min washes in PBS to remove excess paraformaldehyde, coverslips were mounted on glass slides with 8-10µl Prolong Gold (Molecular Probes) and cured 24-48 hrs. Confocal images of cells expressing both YC3.6 and mitoDsRed were acquired with 40x 1.3NA Plan-Neofluar objective on a LSM510meta microscope (Carl Zeiss, Switzerland). Images were acquired sequentially with 405nm, 488nm
and 561nm excitation for DAPI (420-480nm band-pass emission filter), YC3.6 (505-550nm band-pass emission filter) and mitoDsRed (575nm long-pass emission filter). Images (0.1µm/pixel) were acquired with 4x line-averaging and analyzed with Metamorph 6.2 (Molecular Devices). Cytosolic YC3.6 images were median filtered (5 x 5), thresholded and binarized to establish a cell footprint. The footprint was eroded 10 times and subtracted from the original footprint to generate a cell perimeter that was 5-10 pixels wide. This perimeter was superimposed on a Euclidian distance map of the corresponding median-filtered (5x5), binarized mitoDsRed image to calculate the distance from each point along the perimeter to the closest mitochondrial pixel. For fragmentation analysis, cells were transfected with mitoDsRed then co-transfected with YC3.6 and hFis1 or pcDNA the following day. Image acquisition and processing was as above. Regions of interest around optically distinct mitochondria were automatically generated from binarized mitochondrial images. In some cases regions of interest included several closely clumped mitochondria. Measured morphometric parameters included mean mitochondrion area, shape factor and form factor.

**Data analysis:** Data were analyzed using Microsoft Excel, GraphPad Prism 5.01 and NCSS 2000 (NCSS Statistical and Power Analysis Software, USA). Unless otherwise stated, data show as mean ± SE. Statistical difference was considered significant when \( P<0.05 \). Two-population comparisons were made with Student’s t-test or Student’s t-test with Welch correction. Three-way comparisons were made with ANOVA and Bonferroni post-hoc comparisons.

**DISCUSSION**

**Attributing the effects of KB-R to NCX reversal:** Several reports have sited inhibitory effects of KB-R7934 on targets other than the NCX, raising reasonable concern about the selectivity of KB-R7934 for the NCX in our current study. Kraft reported that KB-R7943 inhibited TRPC6 channels over-expressed in HEK293 cells. In a previous study, we observed localized transient \([Na^+]\) elevations (LNats) at the periphery of cultured rat aorta smooth muscle cells (RASMC) in response to stimulation with ATP. LNats were inhibited by SKF-96365 and 2APB, both blockers of receptor-operated and store-operated channels, and by a dominant negative TRPC6, strongly suggesting that LNats are due to activation of TRPC6-containing non-selective cation channels. In contrast, KB-R7943 enhanced the amplitude of the LNats rather than inhibiting them. Moreover, KB-R7943 did not alter ATP-mediated global changes in \([Na^+]\), that were largely inhibited by SKF-96365, 2-APB and the dominant negative TRPC6. Thus the effects of KB-R7943 on SR \(Ca^{2+}\) refilling cannot be attributed to inhibition of TRPC6. KB-R7943 was reported to inhibit the mitochondrial uniporter in HeLa cells. We have similarly measured changes in \([Ca^{2+}]_{\text{mito}}\) in our RASMC with mitochondrial targeted aequorins and reported that KB-R7943 inhibits \([Ca^{2+}]_{\text{mito}}\) elevations in response to reversal of the plasmalemmal NCX with 0Na\(^+\) solution. In contrast, KB-R7943 (10 µM) does not alter the peak \([Ca^{2+}]_{\text{mito}}\) responses to ATP in RASMC. Thus the effects of KB-R7943 reported here in RASMC cannot be attributed to inhibition of the mitochondrial \(Ca^{2+}\) uniporter. KB-R7943 has also been reported to inhibit NMDA channels, but to the best of our knowledge NDMA
receptor channels are not involved in RASMC responses to ATP. Thus the aggregate data that we have reported on the effects of KB-R7943 on cytosolic and mitochondrial [Ca^{2+}] and [Na^{+}], strongly support the current assertion that the effects of KB-R7943 reported here can be attributed to inhibition of reverse-mode NCX.

**hFis1, mitochondrial shape, Ca^{2+} handling & cell viability:** Changes in mitochondrial shape might be expected to influence the ability of mitochondria to retain calcium, either by altering the activity of Ca^{2+} transporters or by increasing the surface-to-volume ratio of mitochondria and thus their Ca^{2+} buffering capacity (i.e. a sphere’s surface-to-volume ratio exceeds that of a rod). It is for this reason that we used hFis1 to remodel mitochondria, because we previously validated in HeLa cells that hFis1 remodeling: 1) did not alter the amplitudes and kinetics of the physiological [Ca^{2+}]_{mit} signals evoked by histamine, and 2) did not alter the membrane potential or the resting pH of mitochondria. Thus, although hFis1 can increase mitochondrial matrix volume and open the curvature of cristae, hFis1-fragmented mitochondria take up and release Ca^{2+} normally, suggesting that the changes in mitochondrial shape did not affect mitochondria bioenergetics or the activity of mitochondrial Ca^{2+} handling proteins. Similarly, in RASMC the rate and extent of recovery of [Ca^{2+}]_{mit} following removal of ATP was not impaired by hFis1. We can therefore conclude that the reduced [Ca^{2+}]_{mit} responses to ATP and 0Na^{+} were not due to enhanced Ca^{2+} mitochondrial extrusion in fragmented mitochondria. Rather, the reduced [Ca^{2+}]_{mit} signals are most likely due to the relocation of mitochondria away from the plasma membrane as demonstrated in Figure 4, and in earlier studies from HeLa cells.

**RASMC culture:** The cultured rat aorta smooth muscle cells used in this study, although clearly of a proliferative phenotype, retain important characteristics of intact VSM Ca^{2+} handling: They express L-type voltage-gated channels, have a TRPC expression profile similar to intact aorta with the exception of TRPC3 and TRPC5 and, like intact conduit smooth muscle (aorta, vena cava) exhibit vectorial Ca^{2+} extrusion. NCX reversal has been well characterized in these RASMC, and operates in a fashion very similar to the reversal that we have described in vena cava and airway smooth muscle. These cultured cells readily express exogenous proteins, and we typically achieve 30-40% transfection efficiency in RASMC, much better than typically observed in primary cultured smooth muscle cells. The loss in physiological relevance by using late passage cultures rather than freshly isolated cells is thus outweighed by the accommodation of molecular techniques that enable to measure compartmentalized Ca^{2+} signals and to manipulate the distribution of mitochondria.

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**SUPPLEMENTAL DATA**

**Online Table 1. hFis1 reduces PM-mitochondria proximity.**

<table>
<thead>
<tr>
<th>PM-Mito distance (µm)</th>
<th>Control % of perimeter pixels</th>
<th>hFis1 % of perimeter pixels</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>3.7 ± 0.4</td>
<td>2.5 ± 0.4*</td>
</tr>
<tr>
<td>1.0</td>
<td>7.9 ± 0.8</td>
<td>5.4 ± 0.8*</td>
</tr>
<tr>
<td>1.5</td>
<td>13.0 ± 1.2</td>
<td>9.5 ± 1.4*</td>
</tr>
</tbody>
</table>

*n= 21 cells/condition. * p<0.05 hFis1 vs. control.*
Online Fig 1. Effect of hFis1 over-expression on cell health. A. Cell proliferation. 12,000 cells/well were seed in 96-well plates and transfected with 0.5µg 4mitD3 and 1.5µg pcDNA3 or hFis1. 48hours later, cell proliferation was assessed using an ImageXpress automated microscope (Molecular Devices) to image DAPI labeled nuclei in 12 images per well, and 4 well per condition. Cell counts are normalized by image area showing mean ± SE for for wells. B. Total cells area taken from images of YC3.6_cytO expressing cells used to generate cell outlines for measurement of mito-PM separation (20 cells with pcDNA3, 22 with hFis1). C. Basal apoptosis. Using the 96-well plates as in A, cells were double stained with Alexa-568-conjugated Annexin-V to labeled externalized phosphotydylerion as an early indicator of apoptosis and DAPI to identify cells. Images were acquired with the ImageXpress microscope and scored manually. hFis1 over-expression did not increase the % of 4mitD3 labelled cells that were positive for Annexin-V. Average of 8 wells per treatment on two independent plates, combining data from 5-9 images per well. D. The size of DAPI labeled nuclei was assessed as an indicator of nuclear condensation assessed with later stages of apoptosis. pcDNA cells and hFis1 over-expression cells show identical over-lapping nuclear size distributions. No side population of smaller nuclei occurred in hFis1 over-expressing cells. Total of 7515 cells for pcDNA3 and 7600 for hFis1. E. Mitochondrial membrane potential assessed by TMRM uptake. Cells were incubated with 200 nM (non-quenching) for 30 min then TMRM was washed away. Average TMRM intensity was assess cell-wise in arbitry fluorescence units from images captured using laser-guided autofocus (ImageXpress) to avoid light-induced mitochondrial depolarization during manual focus. Histograms show cell-wise distribution of TMRM intensity. Data points show mean ± SD.
Online Fig II. Mitochondrial fragmentation by Angiotensin-II. 3 days post-plating, RASMC were treated with ATII (10-100nM) or distilled water (control) for 24 hrs. Cells were then labeled with MitoTracker CMXroX (25nM, 45min, 37C) and DAPI (blue, 5µM, 15min) and immediately imaged without fixation. Images are maximal intensity projections of 3 confocal images at 0.6µm separation (0.13µm/pixel) and are representative of ~40 regions imaged on 4 coverslips in 2 independent experiments per condition. Scale bars are 20µm at left and 10µm in the zoomed regions at right.