The mechanism of phenylephrine-mediated $[Ca^{2+}]_i$ oscillations underlying tonic contraction in the rabbit inferior vena cava

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1. We characterized the mechanisms in vascular smooth muscle cells (VSMCs) that produce asynchronous, wave-like Ca^{2+} oscillations in response to phenylephrine (PE). Confocal imaging was used to observe $[Ca^{2+}]_i$ in individual VSMCs of intact inferior vena cava (IVC) from rabbits.

2. It was found that the Ca^{2+} waves were initiated by Ca^{2+} release from the sarcoplasmic reticulum (SR) via inositol 1,4,5-trisphosphate-sensitive SR Ca^{2+} release channels (IP_3R channels) and that refilling of the SR Ca^{2+} store through the sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase (SERCA) was required for maintained generation of the repetitive Ca^{2+} waves.

3. Blockade of L-type voltage-gated Ca^{2+} channels (L-type VGCCs) with nifedipine reduced the frequency of PE-stimulated $[Ca^{2+}]_i$ oscillations, while additional blockade of receptor-operated channels/store-operated channels (ROCs/SOCs) with SKF96365 abolished the remaining oscillations. Parallel force measurements showed that nifedipine inhibited PE-induced tonic contraction by 27 % while SKF96365 abolished it. This indicates that stimulated Ca^{2+} entry refills the SR to support the recurrent waves of SR Ca^{2+} release and that both L-type VGCCs and ROCs/SOCs contribute to this process.

4. Application of the Na^+–Ca^{2+} exchanger (NCX) inhibitors 2′,4′-dichlorobenzamil (forward- and reverse-mode inhibitor) and KB-R7943 (reverse-mode inhibitor) completely abolished the nifedipine-resistant component of $[Ca^{2+}]_i$ oscillations and markedly reduced PE-induced tone.

5. Thus, we conclude that each Ca^{2+} wave depends on initial SR Ca^{2+} release via IP_3R channels followed by SR Ca^{2+} refilling through SERCA. Na^+ entry through ROCs/SOCs facilitates Ca^{2+} entry through the NCX operating in the reverse mode, which refills the SR and maintains PE-induced $[Ca^{2+}]_i$ oscillations. In addition some Ca^{2+} entry through L-type VGCCs and ROCs/SOCs serves to modulate the frequency of the oscillations and the magnitude of force development.

An increase in $[Ca^{2+}]_i$ from 100 nM or less to values up to 1 μM initiates smooth muscle contraction. Conduit arteries and capacitance veins when challenged with a maintained dose of the neurotransmitter noradrenaline or other pharmacological agonists respond with a biphasic tonic contraction. These same agonists initiate a whole-tissue Ca^{2+} signal, which has a similar profile to the contraction, albeit with a relatively faster onset and lower plateau value. In addition, removal of external Ca^{2+} abolishes the plateau, but not the initial transient. These observations led to the generally accepted theory that the initial phase is initiated by Ca^{2+} release from the sarcoplasmic reticulum (SR) and the tonic phase is supported by sustained Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels (L-type VGCCs) and/or receptor-operated channels (ROCs). This view was challenged by Iino and collaborators (Iino *et al.* 1994) who first reported that noradrenaline elicits asynchronous oscillatory Ca^{2+} waves in vascular smooth muscle cells (VSMCs) within the intact wall of the rat tail artery. They postulated that agonist-induced vascular tone is maintained by asynchronous repetitive SR Ca^{2+} release rather than by sustained Ca^{2+} influx. Several subsequent reports have confirmed the presence of asynchronous Ca^{2+} waves in vascular smooth muscle fibres in isolated, intact blood vessels (Miriel *et al.* 1999; Asada *et al.* 1999; Ruehlmann *et al.* 2000). In addition, we have related these individual-cell Ca^{2+} signals quantitatively to the contractile force generated by the whole blood vessel wall (Ruehlmann *et al.* 2000). Increasing concentrations of

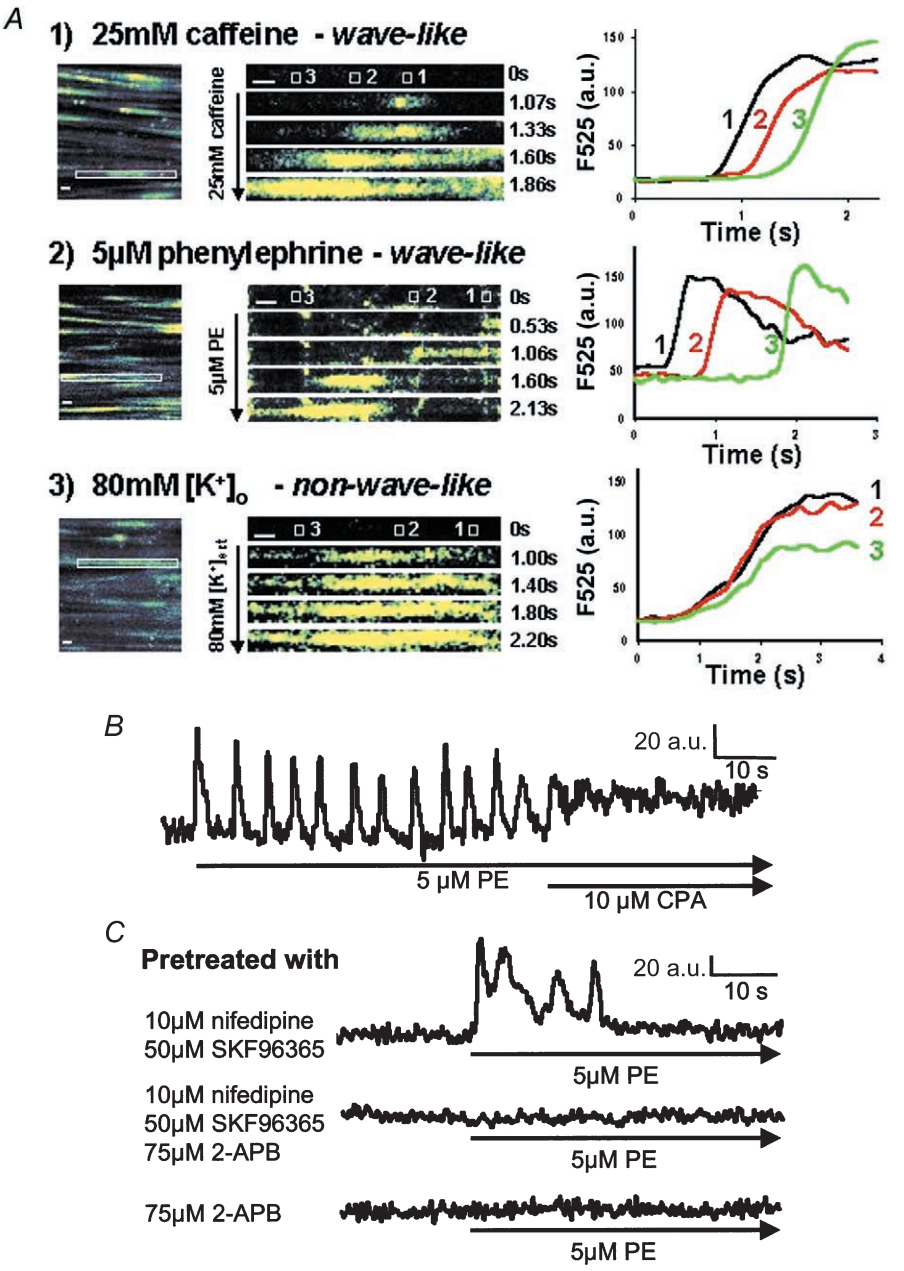

Figure 1. PE-mediated $[Ca^{2+}]_i$ oscillations result from repetitive SR Ca^{2+} release through the IP_3R channel followed by influx-dependent SR store refilling via SERCA

A, sets of time-series images (1–3) are displayed to identify the spatiotemporal patterns of $[Ca^{2+}]_i$ rise elicited by different stimuli. Still images of a field of smooth muscle cells are shown to illustrate the rectangular regions (outlined in white in the leftmost images) from which each set of time-series images was derived. The rectangular regions contain a segment of one representative ribbon-shaped SMC and are enlarged and contrast-enhanced to facilitate the visualization of the spatiotemporal patterns of $[Ca^{2+}]_i$ rise in these time-series. Variations in fluorescence intensity (F_{525}) directly reflect changes in $[Ca^{2+}]_i$. In addition, the changes in F_{525} over time from selected areas (area 1, 2 and 3 outlined in white) spaced out across the longitudinal axis of each depicted SMC are illustrated on the right in the F_{525}–time traces (a.u., arbitrary units). SR Ca^{2+} release with 25 mM caffeine caused an initial $[Ca^{2+}]_i$ elevation that was initiated at a distinctive intracellular locus (time 1.07 s) and subsequently propagated along the longitudinal axis of the SMC in a regenerative, wave-like fashion. The F_{525}–time traces (set 1) from the three intracellular areas indicate a sequential rise in $[Ca^{2+}]_i$ over time as the Ca^{2+} signal was initiated at area 1 and subsequently propagated in a wave-like fashion through area 2 and finally to area 3. Stimulation by 5 μM PE also elicited a wave-like Ca^{2+} signal as the F_{525}–time traces (set 2) demonstrated a sequential rise of $[Ca^{2+}]_i$ over time in the three intracellular areas. In contrast, Ca^{2+} influx across the plasma membrane (PM) stimulated with 80 mM extracellular K^+ PSS (80 mM $[K^+]_o$), caused an initial Ca^{2+} elevation that appears

phenylephrine (PE) applied to the rabbit inferior vena cava (IVC) resulted in the graded recruitment of responding cells, as well as an increase in the frequency of $[Ca^{2+}]_i$ oscillations. These parameters of single cell Ca^{2+} signalling were thus shown to underlie the PE dose-related tonic constriction of the IVC. During the maintained $[Ca^{2+}]_i$ oscillations, a significant amount of cytoplasmic Ca^{2+} will be extruded to the extracellular space via the plasma membrane Ca^{2+}-ATPase (PMCA) or the plasma membrane Na^+–Ca^{2+} exchanger (NCX) (Nazer & van Breemen, 1999). Therefore, stimulated Ca^{2+} entry is required to compensate for the loss of Ca^{2+} from the smooth muscle cells in order to sustain the $[Ca^{2+}]_i$ oscillations. Several modes of Ca^{2+} entry have been documented in VSMCs, including L-type VGCCs, ROCs, store-operated channels (SOCs) and the NCX operating in the reverse mode. In addition there is a significant, though poorly defined, basal Ca^{2+} leak (Khalil *et al.* 1987). The relative importance of these pathways varies with the type of blood vessel. L-type VGCCs are the principle route of Ca^{2+} entry for initiating myogenic tone in resistance arteries (Davis & Hill, 1999), while aortic smooth muscle is relatively insensitive to membrane potential and relies mainly on ROCs to maintain its tone (Cauvin *et al.* 1985; Karaki *et al.* 1997). Recently, Blaustein and collaborators (Arnon *et al.* 2000) made the intriguing proposal that the NCX operating in the reverse mode plays an important role in agonist-induced $[Ca^{2+}]_i$ elevation in vascular smooth muscle.

In our current report, we investigated the mechanism of the asynchronous $[Ca^{2+}]_i$ oscillations in the rabbit IVC, focusing on the mode(s) of Ca^{2+} entry involved in sustaining the PE-induced cyclical release of Ca^{2+} from the SR.

METHODS

Solutions and chemicals

Normal physiological salt solution (PSS), containing (mM): NaCl 140, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, glucose 10 and Hepes 5 (pH 7.4 at 37 °C), was used for all the studies. High K^+ (80 mM $[K^+]_o$) PSS was identical in composition to normal PSS with the exception of (mM): NaCl 65 and KCl 80. All the reagents were purchased from Sigma and were of the highest analytical grade. Fluo-3 AM, Pluronic F-127 and 2′,4′-dichlorobenzamil were purchased from Molecular Probes and were dissolved in dimethyl sulfoxide (DMSO). PE (Sigma), caffeine (Sigma), thapsigargin (Sigma), phentolamine (Sigma) and SKF96365 (Calbiochem) were prepared in normal PSS. Stocks of KB-R7943 (Tocris) and cyclopiazonic acid (CPA, Calbiochem) were prepared in DMSO while stocks of nifedipine (Sigma) and diphenylboric acid 2-aminoethyl ester (2-APB, Sigma) were prepared in ethanol and methanol, respectively. All experiments were performed at 37 °C.

Tissue preparation

All experiments and procedures were carried out in accordance with the guidelines of the University of British Columbia Animal Care Committee (protocol no. A990290). Female New Zealand White rabbits (1.5–2.5 kg, obtained from Animal Care, University of British Columbia), were killed by a rising concentration of CO_2 and then exsanguinated. The IVC was removed, cleaned of surrounding connective tissues and then inverted. The endothelium was removed by gently wiping it with filter paper and the inverted vessel was cut into multiple ring segments that were 4–5 mm in width.

Whole-tissue contraction study and confocal $[Ca^{2+}]_i$ imaging

Detailed methods have previously been described for this preparation (Ruehlmann *et al.* 2000). Briefly, to study contraction the inverted rings were attached to isometric force transducers and the resting tension was set at 0.4 *g*. The bath solution was changed by rapidly draining and refilling the tissue bath. Data were acquired and analysed using Chart version 3.4.5 (ABI instruments). For confocal imaging experiments, inverted rings were loaded with Fluo-3 AM (5 μM, with 5 μM Pluronic F-127, 90 min, 25 °C) followed by a 30 min equilibration period in normal PSS. The vessel rings were then mounted isometrically on a custom-made microscope stage. $[Ca^{2+}]_i$ imaging was accomplished with the use of a Noran Oz laser-scanning confocal microscope with a 100 μm slit through an air ×20 lens (numerical aperture, 0.45) on an inverted Nikon microscope. The tissue was illuminated by the 488 nm line of an argon–krypton laser and a high-gain photomultiplier tube collected the emission after it had passed through a 525/25 bandpass filter. Such a filter will only allow light from a narrow band of 525 ± 25 nm to pass through (525 nm is the emission wavelength of Fluo-3). The scanned regions correspond to a 232 μm × 217 μm area on the tissue and yield a 512 pixel × 479 pixel image. The image acquisition was set at 133 ms per frame. All data analysis was performed in ImagePro Plus using customized routines. The representative experimental fluorescence traces shown reflect the averaged fluorescence signals from a 3 pixel × 3 pixel region (1.36 μm^2) in a single cell. Changes in this regional fluorescence level (F_{525}) directly reflect changes in the Ca^{2+} concentration in this region of the cell. Prior to stimulation, the basal fluorescence allows for delineation of the outline of the ribbon-shaped VSMC. The 3 pixel × 3 pixel region was positioned towards the midline of the ribbon-shaped smooth muscle cell. Given that the focus of this study is on cytoplasmic Ca^{2+} signals, the region was positioned away from the highly fluorescent nuclear region of the cell, as Fluo-3 is known to accumulate in this region of the cell (Perez-Terzic *et al.* 1997). All the numerical data were analysed in Excel and

to be non-wave-like in nature. Such a non-wave-like pattern is clearly demonstrated by the F_{525}–time traces (set 3), which indicate a synchronized rise in $[Ca^{2+}]_i$ from the three intracellular areas. Thus, the wave-like pattern of the $[Ca^{2+}]_i$ rise within a series of PE-induced $[Ca^{2+}]_i$ oscillations resembles that seen in response to caffeine. This suggests that individual Ca^{2+} spikes elicited by PE are the result of regenerative SR Ca^{2+} release. The scale bars shown represent 5 μm. *B*, a representative Ca^{2+} trace is shown that displays the temporal changes in $[Ca^{2+}]_i$ determined in a 1.36 μm^2 cytoplasmic region of a single SMC from the rabbit IVC. SERCA blockade with 10 μM CPA resulted in both the abolishment of PE-induced $[Ca^{2+}]_i$ oscillations and a sustained elevation in $[Ca^{2+}]_i$. *C*, PE-elicited transient $[Ca^{2+}]_i$ oscillations in tissues pretreated with 10 μM nifedipine (L-type VGCC blocker) and 50 μM SKF96365 (ROC/SOC blocker); additional pretreatment with 75 μM 2-APB (IP_3R channel blocker) abolished this transient Ca^{2+} signal. Pretreatment of the tissues with 75 μM 2-APB alone also abolished PE-mediated Ca^{2+} signals completely.

SigmaPlot. One-sample non-parametric tests (Wilcoxon signed-rank test) and two-sample non-parametric tests (Mann-Whitney U test) were used to assess statistical significance. All the fluorescence traces shown represent findings from a minimum of 60 cells in four different tissues and all the contraction traces shown represent findings from a minimum of eight different tissues. Analysis of the wave velocity and the frequency of oscillations has been described previously (Ruehlmann *et al.* 2000).

RESULTS

SR Ca^{2+} release and SERCA Ca^{2+} re-uptake

We have previously described caffeine- and PE-stimulated Ca^{2+} waves in VSMCs within the intact vessel wall of the rabbit IVC (Ruehlmann *et al.* 2000). Figure 1*A* shows a comparison of this wave-like pattern generated by caffeine and PE with the non-wave-like pattern of $[Ca^{2+}]_i$ elevation initiated by high K^+ depolarization. After addition of 25 mM caffeine to the bathing solution,

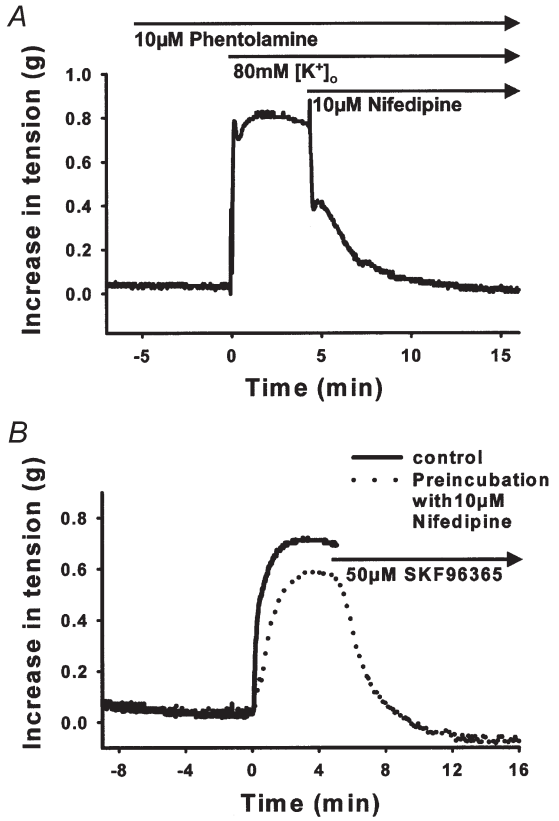

Figure 2. Effects of L-type VGCC blockade and ROC/SOC blockade on PE-mediated tonic contraction

A, a representative tension trace showing that 10 μM nifedipine completely abolished an 80 mM $[K^+]_o$-mediated tonic contraction in rings of rabbit IVC pretreated with 10 μM phentolamine. *B*, L-type VGCC blockade with 10 μM nifedipine partially inhibited a PE-mediated tonic contraction ($P < 0.05$, Wilcoxon signed-rank test), while additional ROC/SOC blockade with 50 μM SKF96365 completely abolished the remaining tonic contraction.

Ca^{2+} waves originate from distinct intracellular loci and travel along the longitudinal axis of the long ribbon-shaped vascular smooth muscle cells, probably as a consequence of regenerative SR Ca^{2+} release. This wave-like pattern is very different from the non-wave-like rise in $[Ca^{2+}]_i$ along the length of the cells when the L-type VGCCs are activated by elevating $[K^+]_o$ to 80 mM. The difference between these spatiotemporal patterns of the increments in $[Ca^{2+}]_i$ strongly suggest that when the IVC is stimulated by PE, the initial event is intracellular Ca^{2+} release, rather than Ca^{2+} influx from the extracellular space.

Figure 1*B* explores the source of the PE-induced Ca^{2+} oscillations. SERCA blockade with 10 μM CPA or 2 μM thapsigargin (data not shown) practically abolished the waves while the $[Ca^{2+}]_i$ remained elevated just below wave peak value, confirming the critical role of the SR in the generation of the repetitive Ca^{2+} waves. Pretreatment of the IVC with a combination of nifedipine to block the L-type VGCCs and SKF96365 to block ROCs/SOCs caused a delayed inhibition of the $[Ca^{2+}]_i$ oscillations and maintained the $[Ca^{2+}]_i$ near the basal value (Fig. 1*C*). This result indicates that stimulated Ca^{2+} entry is required to refill the SR in order to maintain the periodic Ca^{2+} release, which is responsible for each up-stroke of the PE-induced $[Ca^{2+}]_i$ oscillations. Thus, in the absence of stimulated Ca^{2+} entry, PE-induced $[Ca^{2+}]_i$ oscillations can only persist for a few cycles during which Ca^{2+} is lost from the SR to the extracellular space. To test the hypothesis that the Ca^{2+} waves originate from the opening of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} release channels (IP_3R channels), 2-APB (a selective IP_3R channel inhibitor; Ascher-Landsberg *et al.* 1999; Ma *et al.* 2000) was added to the pre-incubation solution before stimulating with 5 μM PE. Figure 1*C* shows that blockade of the IP_3R channel prevented all PE-induced Ca^{2+} waves. Control experiments performed on the IVC confirmed that 75 μM 2-APB did not abolish the caffeine-induced $[Ca^{2+}]_i$ transient (data not shown). These results indicate that PE-induced $[Ca^{2+}]_i$ oscillations are a direct consequence of Ca^{2+} release from the SR via IP_3R channels and, in addition, rely on stimulated Ca^{2+} entry for refilling of the SR. Interestingly, 2-APB pretreatment alone also prevented any change in $[Ca^{2+}]_i$, induced by PE, indicating that activation of IP_3R channels is the very first and requisite event for PE-mediated $[Ca^{2+}]_i$ signalling in VSMCs from the rabbit IVC.

Relative contributions by the L-type VGCCs and ROCs/SOCs

Nifedipine (L-type VGCC blocker) and SKF96365 (ROC/SOC and L-type VGCC blocker) were employed to investigate the routes of Ca^{2+} entry. However, due to the non-selective actions of SKF96365, its effects on PE-induced $[Ca^{2+}]_i$ oscillations and tonic contraction were only assessed in tissues pretreated with 10 μM nifedipine. As shown in Fig. 2*A*, 10 μM nifedipine abolished 80 mM

$[K^+]_o$-induced tonic contraction ($n = 8$ rings) in rabbit IVC pretreated with 10 μM phentolamine (an α-adrenergic receptor antagonist used to inhibit the actions of any noradrenaline released from the nerve endings). However, as shown in Fig. 3, 10 μM nifedipine pretreatment failed to abolish the asynchronous oscillatory Ca^{2+} waves elicited by 5 μM PE. This is similar to what has been reported by Miriel *et al.* (1999) in the rat mesenteric artery. Furthermore, in a detailed analysis of the spatiotemporal characteristics of the oscillations before and after addition of nifedipine, we found that nifedipine pretreatment significantly reduced the frequency of the oscillations from 0.44 ± 0.02 Hz to 0.28 ± 0.02 Hz (mean ± S.E.M., $n = 86$ cells). In contrast, nifedipine had no significant effect on the amplitude of the oscillations and the velocity of the Ca^{2+} waves. These results suggest that there is a relatively large nifedipine-insensitive component of Ca^{2+} entry that is capable of refilling the SR and maintaining PE-induced $[Ca^{2+}]_i$ oscillations. It is clear from Fig. 3 that blockade of ROCs/SOCs with SKF96365 (50 μM) abolished the nifedipine-insensitive component of the PE-induced Ca^{2+} signal.

In a parallel contraction study using identical protocols, we found that nifedipine pretreatment significantly ($P < 0.05$) reduced PE-induced tonic contraction by 27 ± 6 % (mean ± S.E.M., $n = 15$ rings; Fig. 2*B*) while addition of SKF96365 completely abolished the remaining contraction. This observation again supports the existence of a nifedipine-insensitive, SKF96365-sensitive component

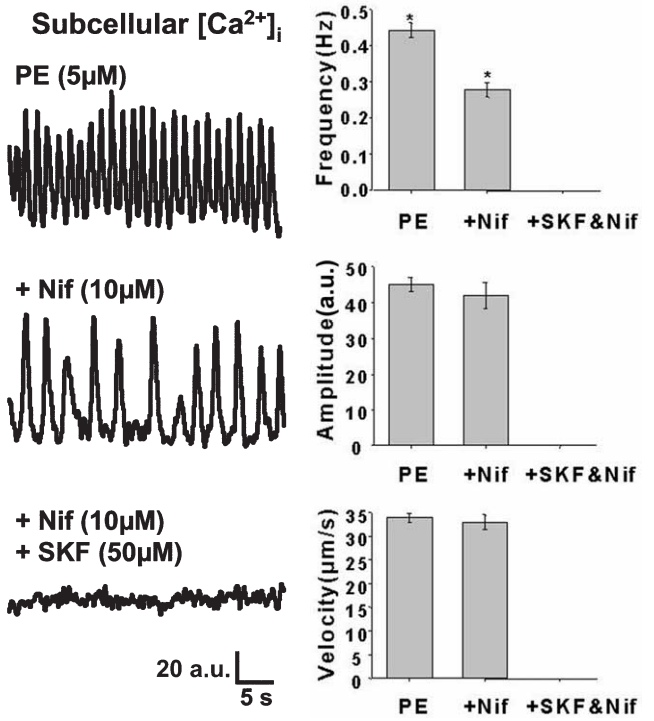

Figure 3. Effects of L-type VGCC blockade and ROC/SOC blockade on PE-mediated $[Ca^{2+}]_i$ oscillations

As shown in the representative traces, which indicate temporal changes in $[Ca^{2+}]_i$ from a 1.36 μm^2 subcellular region, L-type VGCC blockade with 10 μM nifedipine (Nif) did not abolish PE-induced $[Ca^{2+}]_i$ oscillations. It reduced the frequency of PE-induced $[Ca^{2+}]_i$ oscillations, but did not affect the amplitude or the apparent velocity of the oscillatory Ca^{2+} waves. ROC/SOC blockade with 50 μM SKF96365 (SKF) in addition to L-type VGCC blockade abolished PE-induced $[Ca^{2+}]_i$ oscillations completely. The frequency of the $[Ca^{2+}]_i$ oscillations was derived by counting the number of Ca^{2+} waves generated in a single SMC over a time period of 30–60 s. For estimation of the apparent velocity of the Ca^{2+} wave, two subcellular 1.36 μm^2 regions separated by distance x (Δx) were selected and the time lag (Δt) in the onset of Ca^{2+} rise between the two regions was determined. The fraction of Δx over Δt yields the apparent velocity of the Ca^{2+} wave. (* Statistical significance with Mann-Whitney U test.)

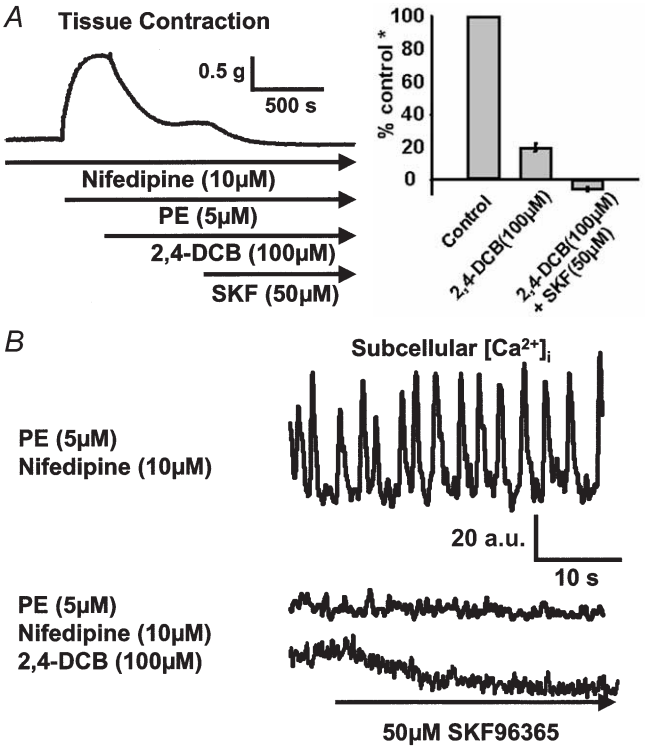

Figure 4. Effects of NCX inhibitor 2′,4′-dichlorobenzamil on the nifedipine-resistant component of PE-mediated $[Ca^{2+}]_i$ oscillations and tonic contraction

Blockade of Ca^{2+} entry through NCX with 100 μM 2′,4′-dichlorobenzamil (2,4-DCB) led to a large inhibition of PE-induced tonic contraction (*A*) and a complete inhibition of PE-induced $[Ca^{2+}]_i$ oscillations (*B*) in rings pretreated with 10 μM nifedipine. The remaining contraction was abolished by application of 50 μM SKF96365 (SKF) while the non-oscillating $[Ca^{2+}]_i$ was correspondingly reduced. (* Control refers to the amplitude of PE-mediated tonic contraction in rings pretreated with 10 μM nifedipine.)

of Ca^{2+} entry, which is responsible for 73 ± 6 % (mean ± S.E.M., $n = 15$ rings) of tonic contraction.

Calcium entry through reverse-mode NCX

Arnon *et al.* (2000) have recently shown that in the mesenteric artery agonists stimulate Ca^{2+} entry via reverse-mode NCX as a consequence of Na^+ entry through SOCs (see Discussion). We therefore tested the possibility that at least part of the SKF96365-sensitive component of stimulated Ca^{2+} entry in the IVC is mediated by the NCX. Two distinct inhibitors of NCX, 2′,4′-dichlorobenzamil (2,4-DCB, forward- and reverse-mode inhibitor; Blaustein & Lederer, 1999) and KB-R7943 (selective reverse-mode inhibitor; Ladilov *et al.* 1999) were employed in parallel $[Ca^{2+}]_i$ and contraction studies. For this series of studies all tissues were pretreated with 10 μM nifedipine to eliminate Ca^{2+} entry through L-type VGCCs.

In a series of contraction studies, it was found that 2,4-DCB at both 10 μM (data not shown) and 100 μM (Fig. 4*A*) significantly ($P < 0.05$) reduced PE-induced tonic contraction in tissues pretreated with nifedipine by 81 ± 6 % ($n = 8$ rings) and 80 ± 2 % ($n = 16$ rings), respectively. The remaining 20 % of contraction was completely inhibited with 50 μM SKF96365. In a parallel $[Ca^{2+}]_i$ study, it was found that 2,4-DCB (100 μM) abolished PE-induced $[Ca^{2+}]_i$ oscillations in tissues pretreated with nifedipine (Fig. 4*B*), but did not reduce the steady-state $[Ca^{2+}]_i$ to the baseline level. However, subsequent addition of SKF96365 did reduce the level of non-oscillating $[Ca^{2+}]_i$ to the baseline level. These results indicate that the $[Ca^{2+}]_i$ oscillations are, in large part, dependent on Ca^{2+} entry via the NCX.

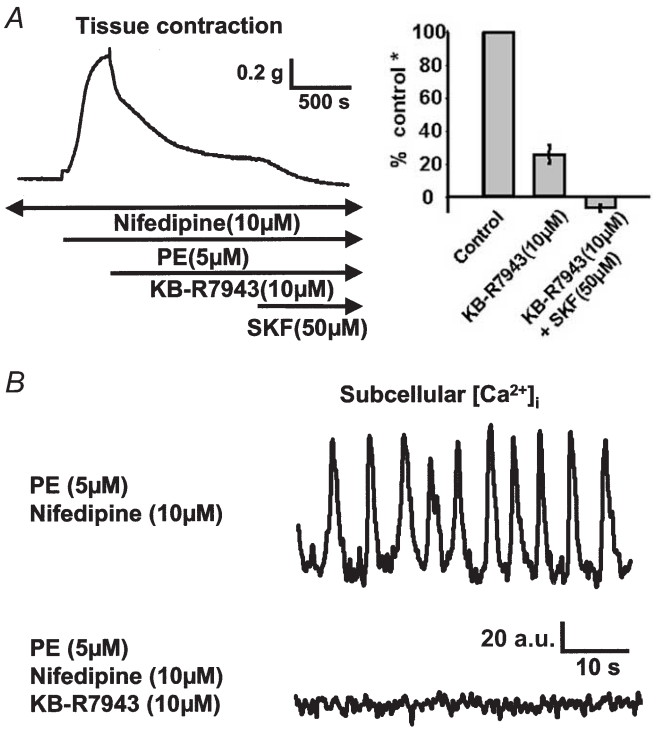

Figure 5. Effects of the selective reverse-mode NCX inhibitor KB-R7943 on the nifedipine-resistant component of PE-mediated $[Ca^{2+}]_i$ oscillations and tonic contraction

Blockade of the NCX operating in the reverse with 10 μM KB-R7943 led to a large inhibition of PE-induced tonic contraction (*A*) in rings pretreated with 10 μM nifedipine and complete inhibition of PE-induced $[Ca^{2+}]_i$ oscillations (*B*). The remaining contraction was abolished with 50 μM SKF96365 (SKF). (* Control refers to the amplitude of PE-mediated tonic contraction in rings pretreated with 10 μM nifedipine.)

To test whether the NCX operating in the reverse mode provides Ca^{2+} to sustain PE-mediated $[Ca^{2+}]_i$ oscillations and tonic contraction in IVC pretreated with nifedipine, we used KB-R7943, which specifically blocks this mode of Na^+–Ca^{2+} exchange. As shown in Fig. 5*A*, 10 μM KB-R7943 also significantly reduced PE-induced tonic contraction in vessels pretreated with nifedipine by 75 ± 5 % (mean ± S.E.M., $n = 9$ rings) while the remaining 25 % of contraction was completely inhibited by 50 μM SKF96365. In a parallel $[Ca^{2+}]_i$ study (Fig. 5*B*) the same concentration of KB-R7943 also abolished PE-induced $[Ca^{2+}]_i$ oscillations. Thus, the fact that two structurally unrelated inhibitors of NCX similarly abolished the PE-induced $[Ca^{2+}]_i$ oscillations indicates that there is a large reverse-mode Na^+–Ca^{2+} exchange component of Ca^{2+} entry, which is required for refilling the SR and allowing the $[Ca^{2+}]_i$ oscillations to persist. Interestingly, there is a relatively smaller 2,4-DCB-insensitive and KB-R7943-insensitive component within the SKF96365-sensitive component, which may reflect Ca^{2+} entry through ROCs/SOCs.

DISCUSSION

The concept that tonic vasoconstriction is based on asynchronous Ca^{2+} waves in individual smooth muscle cells has a profound impact on our views on the molecular mechanisms underlying Ca^{2+} signalling in smooth muscle. Iino and collaborators (Iino *et al.* 1994) proposed that agonist-induced Ca^{2+} waves are propagated by regenerative Ca^{2+} release from the SR network. This hypothesis is supported by our data on the inhibitory effect of 2-APB, which shows that the obligatory first step in generating an agonist-induced Ca^{2+} wave is the opening of IP_3-sensitive channels. In smooth muscle, the release of Ca^{2+} from the SR is always accompanied by Ca^{2+} extrusion from the cells and in a Ca^{2+}-free medium all Ca^{2+} released from the SR is irreversibly lost to the extracellular space (Leijten & van Breemen, 1986). Thus, it follows that refilling of smooth muscle SR involves Ca^{2+} entry across the cell membrane. In this paper we present evidence to support the view that agonist-induced $[Ca^{2+}]_i$ oscillations are generated by cyclical movements of Ca^{2+}. This Ca^{2+} cycle, depicted in Fig. 6, starts with Ca^{2+} release from the

SR through IP_3R channels into the myoplasm. A large fraction of the elevated myoplasmic Ca^{2+} is then extruded via the PMCA to the extracellular space, while the remaining fraction of the Ca^{2+} is taken up by the SERCA. Ca^{2+} entry via the NCX operating in the reverse mode and Ca^{2+} channels subsequently supplies Ca^{2+} to a sub-plasmalemmal, cytoplasmic microdomain from where it is pumped back into the SR via the SERCA. The mechanisms contributing to the individual components of this cyclical movement of Ca^{2+} are discussed below.

Several investigators initially suggested that the SR could be directly filled by ROCs (Fasolato *et al.* 1994), capacitative Ca^{2+} entry (Casteels & Droogmans, 1981; Putney, 1986) or VGCCs (Gagov *et al.* 1994). However, the advent of SERCA blockers, like thapsigargin and CPA, provided evidence that refilling requires SERCA activity (Deng & Kwan, 1991). It was found that refilling of smooth muscle SR is not accompanied by the expected rise in global $[Ca^{2+}]_i$ and myofilament activation (Aaronson & van Breemen, 1981; Casteels & Droogmans, 1981). The results reported herein indicate that each upstroke of the $[Ca^{2+}]_i$ oscillations is associated with release through IP_3R channels rather than with the influx of Ca^{2+}, again suggesting that refilling does not proceed via the bulk of the cytoplasm. This and other functional evidence has led to the hypothesis that refilling of smooth muscle SR takes place in part across a restricted cytoplasmic space, or micro-domain, located between the cell membrane and the peripheral SR (for reviews see van Breemen *et al.* 1995 and Karaki *et al.* 1997). Although much of the evidence for cytoplasmic micro-domains is indirect and deduced from functional data, narrow spaces of approximately 15 nm in width between the plasma membrane (PM) and the SR have been visualized by electron microscopy (Somlyo, 1985). In addition, ion transport proteins residing in the PM have been co-localized with the superficial SR using deconvolution and confocal microscopy of fluorescent antibodies (Moore, 1993; Juhaszova *et al.* 1994, 1997).

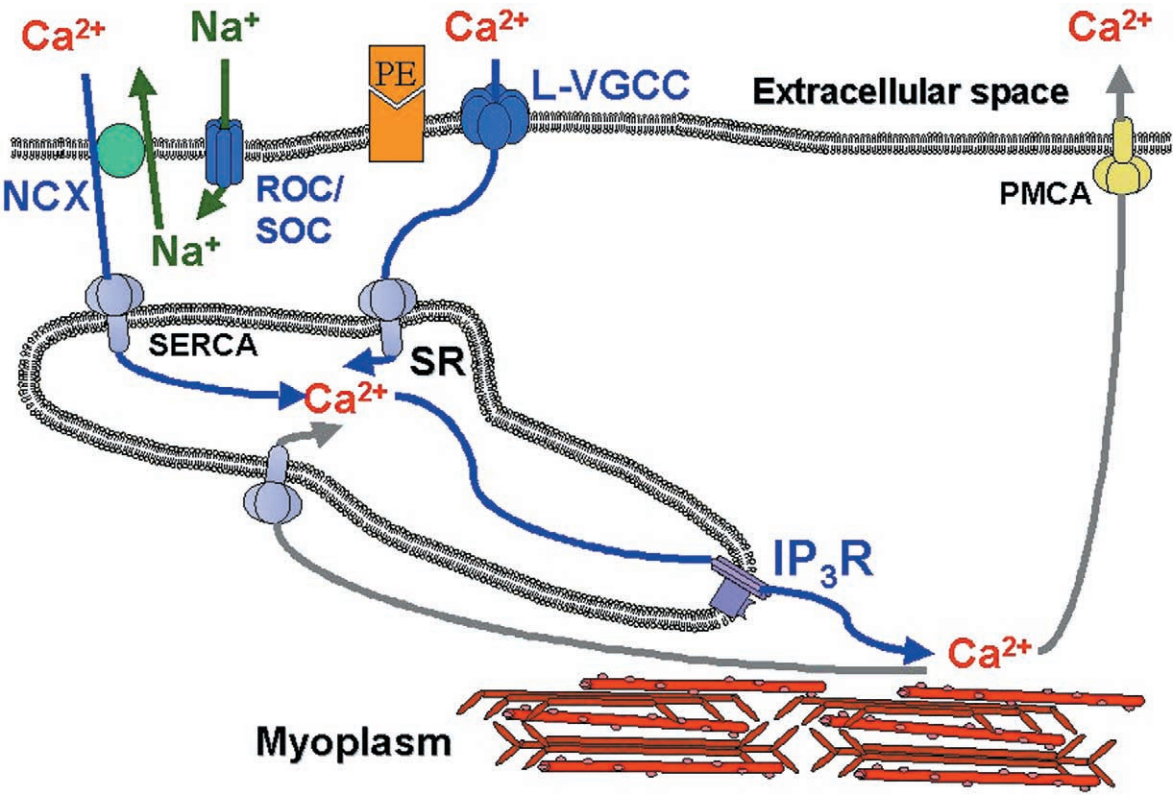

Figure 6. Model for Ca^{2+} movements during PE-induced $[Ca^{2+}]_i$ oscillations in VSMCs from rabbit IVC

IP_3 generated as a consequence of α-adrenergic activation releases Ca^{2+} towards the myoplasm to activate the myofilaments. Subsequent Ca^{2+} removal occurs partially through the PMCA, while the remainder is taken up into the SR by the SERCA. ROCs/SOCs, activated by receptor activation and SR depletion, allow entry of mainly Na^+ and some Ca^{2+}. This Na^+ entry raises $[Na^+]_i$ in a restricted space between the plasma membrane and the SR membrane and drives NCX in the reverse mode to supply Ca^{2+} to SERCA to refill the SR lumen, thereby completing the cycle. PE, phenylephrine; NCX, sodium/calcium exchanger; ROC/SOC, receptor-operated channel/store-operated channel; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase; L-type VGCC, L-type voltage-gated Ca^{2+} channel; PMCA, plasma membrane Ca^{2+}-ATPase; IP_3R, IP_3-sensitive SR Ca^{2+} release channel; SR, sarcoplasmic reticulum.

A major finding in this report is that blocking the ROCs/SOCs with SKF96365 prevented the maintenance of repetitive Ca^{2+} waves. Unfortunately, the identity of the ROCs/SOCs has not been resolved at this time. α-Adrenergic receptor activation has been reported to open ROCs, which are cation channels permeable to both Ca^{2+} and Na^+ (Fasolato *et al.* 1994). Since the α-adrenergic receptor is in itself not a channel, or channel activator, intermediate signalling possibly involving a second messenger (G_α, IP_3, inositol tetrakisphosphate (IP_4) or Ca^{2+}) is required (Fasolato *et al.* 1994; Barritt, 1999). Another hypothetical mechanism, which links emptying of the SR store to the opening of SOCs, has been described for smooth muscle and non-excitable cells (Casteels & Droogmans, 1981; Cauvin *et al.* 1984; Putney, 1986). However, the molecular mechanism, which may involve either a calcium-influx factor (Trepakova *et al.* 2000) or direct mechano-coupling with the IP_3R channel (Putney, 1999; Ma *et al.* 2000), remains to be determined. It has also been reported that different modes of activation function in parallel to open a common channel identified as either ROC or SOC (Wang & van Breemen, 1997). In any case the influx of Na^+ and Ca^{2+} through these non-selective cation channels depolarizes the plasma membrane potential sufficiently to open L-type VGCCs. This was confirmed by the inhibitory effect of nifedipine on the PE-induced contraction.

The effect of nifedipine was interesting in that it did not affect either the amplitude or velocity of the repetitive Ca^{2+} waves, but did reduce their frequency. There are two plausible mechanisms through which the frequency could be decreased. Firstly, the absence of stimulated Ca^{2+} influx through L-type VGCCs may reduce the rate of refilling of the SR Ca^{2+} store. Given that the SR luminal $[Ca^{2+}]$ can regulate IP_3R channel opening probability (Meldolesi & Pozzan, 1998), a reduced rate of SR Ca^{2+} refilling can result in a decreased frequency of SR Ca^{2+} release at the wave initiation site. Secondly, Ca^{2+} influx through L-type Ca^{2+} channels can elevate $[Ca^{2+}]_i$ in regions adjacent to IP_3Rs, which facilitates calcium-induced calcium release (CICR) mediated by both types of SR Ca^{2+} release channel. Thus, removal of Ca^{2+} entry through L-type VGCCs can result in a delay of regenerative CICR and thereby reduce the frequency of the $[Ca^{2+}]_i$ oscillations.

The nifedipine-insensitive component was not only blocked by SKF96365, but was also greatly inhibited by blockers of the NCX. This implicates reverse-mode NCX as a physiological route of Ca^{2+} entry in the VSMC. Since the NCX-mediated Ca^{2+} entry is also sensitive to SKF96365, it follows that the reverse-mode NCX Ca^{2+} entry is dependent on the stimulation of ROCs/SOCs. While evidence indicates that at a resting $[Na^+]_i$ of ~10 mM the NCX typically operates in the forward Ca^{2+} extrusion mode, when $[Na^+]_i$ is elevated above 20 mM, the energetics favour the reverse-mode activity. In order to significantly raise $[Na^+]_i$, Na^+ must enter the cell, putatively, via the non-selective cation-permeable ROCs/SOCs into a restricted space, which is considerably smaller than the bulk cytoplasm. In contrast to L-type VGCCs (permeability ratio of Ca^{2+} to Na^+, $P_{Ca}/P_{Na} > 1000$; Bean, 1989), ROCs/SOCs are believed to be much more permeable to Na^+ with a $P_{Ca}/P_{Na} < 10$ (Philipp *et al.* 1996). With 140 mM extracellular Na^+ as compared to 1.5 mM extracellular Ca^{2+}, opening of ROCs/SOCs should result in a large Na^+ influx in addition to the Ca^{2+} influx. It has previously been proposed that Na^+ entering the cardiac myocyte accumulates in a restricted sub-plasmalemmal space (fuzzy space) formed by the close apposition between the superficial SR and the PM (Philipson & Nicoll, 2000), similar to the one discussed above for smooth muscle. Thus, conditions may exist to temporarily reverse the balance between the Na^+ and Ca^{2+} gradients to promote Ca^{2+} entry via the NCX. In fact our findings support a mechanism, similar to that previously proposed by Blaustein and colleagues (Arnon *et al.* 2000), whereby Ca^{2+} entry through reverse-mode NCX is serially coupled to Na^+ entry through the activated ROCs/SOCs. Therefore, either blockade of the ROCs/SOCs or blockade of the NCX (reverse mode) abolishes the $[Ca^{2+}]_i$ oscillations. This model is further supported by the structural findings that the plasmalemmal NCX and the high ouabain affinity isoform (α3) of the Na^+–K^+-ATPase appear to colocalize with the superficial SR and SERCA in smooth muscles (Moore *et al.* 1993; Juhaszova *et al.* 1994; Juhaszova *et al.* 1997). This close spatial arrangement would allow Ca^{2+} entry through reverse-mode NCX to refill the superficial SR via its SERCA pumps. Since the SR in SMCs is believed to be an interconnecting tubular network, refilling of the deeper SR would occur through SR Ca^{2+} redistribution from the superficial SR. We have previously shown that at rest the close association between the superficial SR and NCX functions in the forward mode to unload the SR (Nazer & van Breemen, 1998). These studies also provided evidence in support of Ca^{2+} redistribution within the SR. The mechanisms described above may apply to other cell types, as Graier and coworkers recently reported that reverse-mode NCX-dependent endoplasmic reticulum Ca^{2+} refilling supports agonist-mediated $[Ca^{2+}]_i$ oscillations in endothelial cell lines (Paltauf-Doburzynska *et al.* 2000).

Ca^{2+} entry through ROC/SOC-coupled reverse-mode NCX, L-type VGCCs and ROCs/SOCs all contribute to force generation. However, Ca^{2+} entry through the ROCs/SOCs alone is incapable of supporting the recurrent Ca^{2+} waves. This may be due to the relatively slow rate of Ca^{2+} entry through these non-selective cation channels, which is not able to refill the SR Ca^{2+} store to a sufficiently high level to maintain the repetitive firing of Ca^{2+} waves. However, the slow Ca^{2+} entry through the ROCs/SOCs does elevate steady-state $[Ca^{2+}]_i$ to support sub-maximal force development as shown in Fig. 4.

Although the mechanisms underlying the PE-mediated $[Ca^{2+}]_i$ oscillations are now being resolved, the physiological advantage of repetitive waves of SR Ca^{2+} release over steady-state Ca^{2+} elevation is rather poorly understood. In the temporal domain, oscillations of varying frequencies can regulate frequency-sensitive enzyme(s) that serve to amplify downstream signals (De Koninck & Schulman, 1998). It is also possible that recurrent Ca^{2+} waves, given their transient nature, may result in organellar Ca^{2+} concentrations (e.g. nuclear $[Ca^{2+}]$) that are different from those that would result if $[Ca^{2+}]_i$ were held at a steady-state level. Thus, Ca^{2+} oscillations may provide a means to selectively activate the contractile machinery in the myoplasm without disrupting Ca^{2+}-sensitive processes that occur in the nuclear compartment.

In summary, our data show that the tonic contraction of a large vein in response to α-adrenergic stimulation is maintained by repetitive, asynchronous Ca^{2+} waves, which are initiated by the opening of IP_3-sensitive SR channels. This oscillating SR Ca^{2+} release is maintained by stimulated Ca^{2+} entry through ROC/SOC-coupled reverse-mode NCX, L-type VGCCs and ROCs/SOCs, which in the IVC are responsible for 59, 27 and 14% of the tonic contraction, respectively.

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