Marfan Syndrome Decreases Ca^{2+} Wave Frequency and Vasoconstriction in Murine Mesenteric Resistance Arteries without Changing Underlying Mechanisms

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Key Words

Calcium waves • Confocal microscopy • Isometric force • Marfan syndrome • Mesenteric artery • Vascular smooth muscle

Abstract

Background/Aims: Vascular smooth muscle in Marfan syndrome, a connective tissue disorder caused by mutations in *FBN1* encoding fibrillin-1, is associated with decreased tonic contraction. As Ca^{2+} waves are tightly associated with vasoconstriction, we hypothesized decreased tonic contraction in Marfan syndrome is due to aberrant Ca^{2+} wave signaling. **Methods:** Isometric force and intracellular Ca^{2+} were measured from second-order mesenteric arteries from mice heterozygous for the *Fbn1* allele encoding a cysteine substitution (*Fbn1*^{C1039G/+}). **Results:** Phenylephrine concentration-dependently induced tonic contraction associated with sustained repetitive oscillations in intracellular $[\text{Ca}^{2+}]$ in both control and Marfan vessels, although Marfan vessels displayed significantly decreased Ca^{2+} wave frequency and decreased number of cells exhibiting waves. Inhibition of sarcoplasmic reticulum Ca^{2+} re-uptake by cyclopiazonic acid abolished Ca^{2+} waves, dramatically decreasing tonic con-

traction. Nifedipine significantly reduced Ca^{2+} wave frequency and tonic contraction, while the nifedipine-insensitive component was abolished by SKF-96365. Ca^{2+} waves and tonic contraction were abolished by 2-aminoethoxydiphenylborate, but were unaffected by ryanodine or tetracaine. **Conclusion:** Phenylephrine-induced Ca^{2+} waves underlie tonic contraction in resistance-sized mesenteric arteries and appear to be produced by repetitive cycles of regenerative Ca^{2+} release from the sarcoplasmic reticulum. Decreased frequency of Ca^{2+} waves in Marfan syndrome appears to be responsible for reduced tonic contraction.

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Introduction

Contraction of vascular smooth muscle in response to neurotransmitters and hormones involves changes in both intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) and diverse signal transduction pathways that influence ion channels, protein kinases, phosphatases, second messengers and other enzymes [1]. The characteristics of the spatial and temporal patterns of the changes in $[\text{Ca}^{2+}]_i$ are of fundamental importance in determining their efficacy in activating

contraction, through Ca^{2+} /calmodulin-dependent myosin light chain kinase. Confocal imaging of $[\text{Ca}^{2+}]_i$ within single smooth muscle cells in the arterial wall has shown that the spatiotemporal patterns of the agonist-stimulated Ca^{2+} response are more complex than previously thought, as in many types of blood vessels activation of α_1 -adrenoceptors elicits propagating Ca^{2+} waves that occur asynchronously in the individual smooth muscle cells [2–7]. Increasing concentrations of agonist are associated with graded contraction and recruitment of cells which produce asynchronous Ca^{2+} waves of increasing frequency [5, 8, 9].

Asynchronous Ca^{2+} waves are caused by regenerative Ca^{2+} release from the sarcoplasmic reticulum (SR) and maintained by refilling of the SR by L-type Ca^{2+} channels, nonselective cation channels and reverse-mode Na^+ - Ca^{2+} exchange in specialized regions of the cell where the plasma membrane apposes the SR. In some resistance vessels, another type of Ca^{2+} oscillation, which is synchronized, signals vasomotion [6]. These waves are also paced by Ca^{2+} release from the SR, but serve to activate Ca^{2+} -dependent Cl^- channels, which depolarize the membrane and activate L-type Ca^{2+} channels. The resulting depolarization spreads through the electrically coupled smooth muscle cells, resulting in synchronized contractions [10].

Marfan syndrome is an autosomal dominant disorder caused by mutations in the gene encoding for fibrillin-1 and affects multiple systems, including cardiovascular, skeletal, ocular and pulmonary [11–13]. Although the main cause of death from Marfan syndrome is due to aortic aneurysm and rupture, Marfan syndrome is also associated with aneurysms and dysfunction of the resistance vasculature, which may have important clinical consequences [14–19]. Most recently, we have reported in our studies in a mouse model of Marfan syndrome that vasomotor function in mesenteric arteries, including maximal force development, is detrimentally affected, further suggesting that Marfan syndrome is also a disorder of the peripheral resistance vasculature [20].

In our present study, we investigated the regulation of vasoconstriction in relation to Ca^{2+} wave signaling in mouse mesenteric artery smooth muscle, in which agonist-induced Ca^{2+} waves have been reported, but the mechanism has not been studied [7, 21]. Furthermore, we hypothesize that the decreased agonist-induced tonic contraction observed in mesenteric arteries from a mouse model of Marfan syndrome is directly related to a decrease in Ca^{2+} wave frequency rather than a change in the cell Ca^{2+} signaling mechanism.

Methods

Experimental Animals and Tissue Preparation

Heterozygous ($\text{Fbn1}^{\text{C1039G/+}}$) mice were mated to C57BL/6 mice to produce equal numbers of $\text{Fbn1}^{\text{C1039G/+}}$ Marfan subjects and wild-type controls as described previously [22–26]. Both strains were housed in the institutional animal facility (Child and Family Research Institute, University of British Columbia) under standard animal room conditions (12-hour light-dark, at 25°C, 2–5 animals in a cage), and all animal procedures were approved by the institutional Animal Ethics Board. Mice at 6 months of age were anesthetized with a mixture of ketamine hydrochloride (80 $\text{mg}\cdot\text{kg}^{-1}$) and xylazine hydrochloride (12 $\text{mg}\cdot\text{kg}^{-1}$) intraperitoneally for experimentation. The mesenteric arcade was excised and placed in ice-cold oxygenated (95% O_2 -5% CO_2) HEPES-PSS solution. Second-order branches of the mesenteric artery with diameters of 130–150 μm were dissected and cut into 2-mm segments. The endothelium was removed by gently rubbing the inside of the vessel with a 40- μm tungsten wire.

Measurement of Isometric Force

Mesenteric artery segments were isometrically mounted in a small vessel wire myograph (A/S Danish Myotechnology, Aarhus, Denmark) using two 40- μm tungsten wires for measuring generated force. The chambers were kept at 37°C and bubbled continuously with 95% O_2 -5% CO_2 in HEPES-PSS. Optimal tension (the maximal force generation given in response to 60 mM KCl, which was the same for control and Marfan mouse mesenteric arteries; 4 mN) was determined in preliminary experiments by subjecting arterial segments to different resting tensions and stimulating with 60 mM KCl. The vessels were stretched to the optimal tension for 60 min and challenged twice with 60 mM KCl before experiments were continued. Tonic contraction was induced by 3 μM phenylephrine (PE) and all mechanistic studies were done at this concentration. The negative logarithm (pD_2) of the concentration of PE giving half-maximum response (EC_{50}) was assessed by linear interpolation on the semilogarithm concentration-response curve $\text{pD}_2 = -\log(\text{EC}_{50})$.

Measurement of $[\text{Ca}^{2+}]_i$

The arterial rings were loaded with Fluo-4AM (5 μM with 5 μM Pluronic F-127, 1 h at 37°C) and isometrically mounted, followed by a 30-min washout time in HEPES-PSS. Sustained Ca^{2+} waves were induced by 3 μM PE, and all mechanistic studies were done at this concentration. Images were acquired on an upright Olympus BX50WI microscope with a 40 $\times$ water-dipping objective (numeric aperture 0.9) and equipped with an Ultraview confocal imaging system (Perkin-Elmer, Waltham, Mass., USA). The rate of image acquisition was 3 frames $\cdot\text{s}^{-1}$. The tissue was illuminated using the 488-nm line of an argon-krypton laser, and a high-gain photomultiplier tube collected the emission at wavelengths between 505 and 550 nm. The scanned regions correspond to a $91.69 \times 66.68 \mu\text{m}$ area (or 248×328 pixels). The representative fluorescence traces shown reflect the averaged fluorescence signals from a region of 3×3 pixels ($1.69 \mu\text{m}^2$) of the smooth muscle cell. The frequency of Ca^{2+} waves was determined by counting the number of waves occurring within 100 s. The measured changes in Fluo-4 fluorescence level are proportional to the relative changes in $[\text{Ca}^{2+}]_i$. All parameters (laser intensity, gain and others) were maintained constant during the experiment. The confocal images

were analyzed off-line with the Ultraview 5.5 Software (Perkin-Elmer). To obtain data on recruitment of cells during PE stimulation, a fixed field of view was chosen and the number of cells responding with Ca^{2+} waves was recorded. Fluorescence traces were extracted from the movies to exclude nuclear regions and traces were normalized to initial fluorescence values.

Statistics

Values are expressed as mean $\pm$ standard error from at least 6 independent experiments. Statistical analysis and construction of concentration-response curves were performed using Graph-Pad Prism 4.0 software (San Diego, Calif., USA). Differences between control and Marfan groups were analyzed by Student's two-tailed t test. Statistical significance was defined as p values < 0.05 .

Drugs, Solutions and Chemicals

HEPES-PSS containing (in mM) NaCl 130, HEPES 10, glucose 6, KCl 4, NaHCO_3 4, CaCl_2 1.8, MgSO_4 1.2, KH_2PO_4 1.18 and EDTA 0.03 (pH 7.4) was used for all studies. Hi- K^+ (60 mM extracellular K^+) HEPES-PSS was identical in composition to normal HEPES-PSS with the exception of (in mM) NaCl 74 and KCl 60. PE, nifedipine, cyclopiazonic acid (CPA), SKF-96365 and pluronic F-127 were obtained from Sigma-Aldrich (Oakville, Ont., Canada). Fluo-4AM was purchased from Molecular Probes (Eugene, Oreg., USA).

Results

Relation between PE-Induced Tonic Contraction and Ca^{2+} Waves

In the control mice, PE produced tonic contraction in a concentration-dependent manner, with a pEC_{50} of 5.83 ± 0.21 and maximal response of $68.8 \pm 7.3\%$ (9 animals, normalized to 60 mM KCl; fig. 1a, b). At 3 μM PE, the average contraction was $55.2 \pm 4.5\%$ (12 animals, normalized to 60 mM KCl). In parallel experiments, application of PE (3 μM) induced a large transient Ca^{2+} response which was followed by sustained repetitive oscillations in $[\text{Ca}^{2+}]_i$ which propagated along the length of the smooth muscle cells as Ca^{2+} waves (fig. 1c, 2). The frequency of PE-stimulated Ca^{2+} waves increased in a concentration-dependent manner and closely paralleled the development of force (fig. 1d; $\text{pEC}_{50} = 6.06 \pm 0.11$, maximum frequency = 0.067 ± 0.003 Hz, 97 cells from 12 animals). At 3 μM PE, the average frequency of the Ca^{2+} waves was 0.057 ± 0.005 Hz (48 cells from 8 animals). The velocity of wave propagation also shows a strong dependence on concentration of PE. At the highest concentrations, wave propagation speeds reached 60.5 ± 5.3 $\mu\text{m/s}$ (10 cells from 4 animals; fig. 1e). The number of cells displaying at least one Ca^{2+} wave was also concentration dependent; at 3 μM PE, $74.51 \pm 5.45\%$ of all cells in the field of view displayed at least one Ca^{2+} wave (57 cells from 6 animals; fig. 1f).

Frequency of Ca^{2+} Waves and Cell Recruitment Is Reduced in Marfan Syndrome

In Marfan vessels, the contraction induced by 3 μM PE was reduced by $61.5 \pm 4.1\%$ compared to control vessels (fig. 3a), although pEC_{50} was not significantly different (control: 5.83 ± 0.21 , Marfan: 5.72 ± 0.21). The average frequency of the 3 μM PE-stimulated Ca^{2+} waves was also significantly reduced by $46.2 \pm 3.5\%$ in Marfan vessels compared to control vessels (control 0.061 ± 0.005 Hz, Marfan 0.033 ± 0.004 Hz; $p < 0.05$) (fig. 3b, c). The number of cells displaying at least one Ca^{2+} wave was also significantly reduced in the Marfan vessels (control: $74.51 \pm 5.45\%$ of all cells in the field of view, 57 cells from 6 animals, Marfan: $49.71 \pm 6.72\%$ of all cells in the field of view, 63 cells from 6 animals; $p < 0.05$) (fig. 3d). However, the Ca^{2+} wave velocity was unchanged in the Marfan vessels compared to control (data not shown).

We have previously reported that in Marfan mesenteric vessels, tonic contraction in response to membrane depolarization by KCl stimulation is significantly reduced [20]. To determine whether this reduced tonic contraction was due to decreased Ca^{2+} influx from L-type Ca^{2+} channels, $[\text{Ca}^{2+}]_i$ elevation in response to KCl stimulation was measured. KCl stimulation resulted in an elevation in $[\text{Ca}^{2+}]_i$ which was not accompanied by a wave-like pattern in both control and Marfan mice, although the peak $[\text{Ca}^{2+}]_i$ elevation (as measured by F/F_0 ratio) was significantly reduced in the Marfan mesenteric vessels (1.645 ± 0.095) compared to the control vessels (2.233 ± 0.088), suggesting decreased Ca^{2+} influx from the extracellular space (fig. 4a, b).

Dependence of PE-Induced Ca^{2+} Waves on Extracellular Ca^{2+} Influx

To define the Ca^{2+} entry pathways involved in maintaining PE-induced Ca^{2+} waves, nifedipine, an inhibitor of L-type Ca^{2+} channels, and SKF-96365, an inhibitor of receptor-operated channels, were used. In control mice, nifedipine (10 μM) reduced the frequency of PE-induced Ca^{2+} waves to $19.6 \pm 3.5\%$ of control levels, while the additional application of SKF-96365 (50 μM) completely abolished the Ca^{2+} waves ($p < 0.001$, 37 cells from 6 animals; fig. 5a, b). In parallel, tonic contraction induced by PE (3 μM) was significantly reduced by nifedipine (10 μM) to $7.31 \pm 2.57\%$ of PE-precontracted levels ($p < 0.001$), while the combined application of nifedipine and SKF-96365 (50 μM) decreased tonic contraction to $2.33 \pm 1.45\%$ of PE-precontracted levels ($p < 0.001$; $n = 6$ animals; fig. 5c, d). Similarly, in the Marfan mice, nifedipine reduced the frequency of PE-induced Ca^{2+} waves to 6.75

Fig. 1. Properties of PE-induced Ca^{2+} waves underlying tonic contraction in mouse mesenteric artery. **a** Representative trace of PE- ($3 \mu\text{M}$) induced tonic contraction. Traces are representative of results from 6 animals. **b** Concentration-response curve for PE-induced tonic contraction (12 animals). **c** Application of PE ($3 \mu\text{M}$) produced sustained Ca^{2+} waves. Experimental Ca^{2+} traces are representative of results from 58 cells from 6 animals. **d** Concentration-response curve for frequency of PE-induced Ca^{2+} waves (109

cells from 12 animals). **e** The apparent propagation speed of the Ca^{2+} waves was correlated to increasing PE concentration (87 cells from 11 animals). **f** A greater percentage of smooth muscle cells generated Ca^{2+} waves as PE concentration increased. This recruitment occurred between 100 nM and $30 \mu\text{M}$, with maximal recruitment achieved at $30 \mu\text{M}$ PE (90 cells from 10 animals). The number of cells displaying Ca^{2+} waves is expressed as a percentage of cells in the field of view responding to stimulation with PE.

$\pm 2.82\%$ of control levels, while the additional application of SKF-96365 also completely abolished the Ca^{2+} waves. Nifedipine also significantly reduced tonic contraction to $2.65 \pm 2.02\%$ of control, while the additional application of SKF-96365 reduced contraction to $0.63 \pm 0.49\%$ of control levels ($p < 0.001$; data not shown).

Dependence of PE-Induced Ca^{2+} Waves on SR Ca^{2+} Release

The addition of CPA ($10 \mu\text{M}$), an inhibitor of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase, to ongoing PE- ($3 \mu\text{M}$) induced Ca^{2+} waves in control animals resulted in a transient broadening of the Ca^{2+} waves fol-

Fig. 2. PE- ($3 \mu\text{M}$) induced Ca^{2+} waves in mouse mesenteric branch artery. **a** $[\text{Ca}^{2+}]_i$ changes in 2 intracellular regions from 2 different smooth muscle cells upon PE stimulation are depicted in the Ca^{2+} traces taken from the steady state of PE-induced Ca^{2+} waves. It should be noted that the Ca^{2+} waves occurred at different frequencies. Experimental Ca^{2+} traces are representative of results from

58 cells in 6 animals. **b** Intact vascular smooth muscle cells challenged with PE ($3 \mu\text{M}$) displayed Ca^{2+} waves which originated from distinct intracellular foci and propagated along the longitudinal axis of the smooth muscle cells, as indicated by area of interest (AOI). The AOI is 3×3 pixels ($1.69 \mu\text{m}^2$). Scale bar = $10 \mu\text{m}$.

lowed by their complete abolition, leaving a significant elevation in baseline $[\text{Ca}^{2+}]_i$ levels corresponding to $29 \pm 3\%$ of the peak $[\text{Ca}^{2+}]_i$ of the Ca^{2+} waves ($p < 0.001$, 32 cells from 5 animals; fig. 6a). In parallel experiments, CPA ($10 \mu\text{M}$) inhibited PE- ($3 \mu\text{M}$) induced tonic contraction by $82.5 \pm 4.2\%$ ($p < 0.001$, 5 animals; fig. 6b). The transient broadening of the Ca^{2+} waves and resulting significant abolition of tonic contraction were also observed in the Marfan vessels (data not shown).

To examine the role of inositol 1,4,5-triphosphate-sensitive receptors (IP_3R), 2-aminoethoxydiphenylborate (2-APB) at a concentration ($75 \mu\text{M}$) above the half maximal inhibitory concentration for inhibition of IP_3Rs [27, 28] was applied to ongoing Ca^{2+} waves in control animals, and immediately abolished them (31 cells from 5 animals; fig. 7a). In parallel, 2-APB inhibited tonic contraction to $2.49 \pm 1.18\%$ of the control level ($p < 0.001$, 5 animals;

fig. 7b). Furthermore, PE ($3 \mu\text{M}$) failed to elicit a Ca^{2+} transient or contraction in mesenteric arteries pre-incubated for 30 min with 2-APB (fig. 7c, d).

The specificity of 2-APB, particularly with regard to Ca^{2+} channels and translocators such as ryanodine receptors (RyRs), sarco(endo)plasmic reticulum Ca^{2+} ATPase, store-operated channels and L-type Ca^{2+} channels, was examined in the preparation. As shown in figure 8a, pre-treatment with 2-APB ($75 \mu\text{M}$) did not significantly affect the peak amplitude of caffeine- (25 mM) induced Ca^{2+} release ($105.2 \pm 5.6\%$ of the control, $p = 0.73$, 5 animals), and therefore appears to be inactive against RyRs. Furthermore, 2-APB only marginally affected SR refilling, as the peak amplitude of the third caffeine-induced Ca^{2+} transient was decreased slightly, but not significantly by $11.2 \pm 4.1\%$ ($p = 0.41$, 6 animals).

Fig. 3. Concentration-response curves for PE-induced tonic contraction (**a**) and Ca²⁺ wave frequency (**b**) in control and Marfan mice mesenteric resistance arteries. **c** Reduced frequency of PE- (3 μM) stimulated Ca²⁺ waves in Marfan vessels compared to control vessels. **d** A decreased percentage of vascular smooth muscle cells

in Marfan vessels (63 cells from 5 animals) displayed at least one Ca²⁺ wave compared to control vessels (57 cells from 6 animals) when stimulated with PE (3 μM). The number of cells displaying Ca²⁺ waves is expressed as a percentage of cells within the field of view responding to stimulation PE (3 μM). * $p < 0.05$.

Fig. 4. Ca²⁺ response to 60 mM KCl stimulation. **a** Representative Ca²⁺ response upon KCl stimulation in control (black line) and Marfan (gray line) mice. **b** The maximal Ca²⁺ response upon KCl stimulation is significantly reduced in the Marfan group compared to the control group. * $p < 0.05$ vs. control; 8–12 animals.

Fig. 5. Effect of the L-type Ca^{2+} channel antagonist nifedipine and the receptor-operated channel antagonist SKF-96365 on PE- (3 μM) induced Ca^{2+} waves and tonic contraction. **a** The frequency of Ca^{2+} waves is significantly reduced ($9.21 \pm 2.17\%$ of control) following nifedipine (10 μM) application, but not abolished. The

nifedipine-insensitive component was completely abolished after addition of SKF-96365 (50 μM). **b** Tonic contraction is significantly reduced to $7.31 \pm 2.57\%$ by nifedipine (10 μM) and almost completely abolished ($2.33 \pm 1.45\%$) after SKF-96365 (50 μM). *** $p < 0.001$. Dashed lines indicate a 2-min interval.

Fig. 6. Effect of blockade of the sarco(endo)plasmic reticulum Ca^{2+} ATPase by CPA on PE- (3 μM) induced Ca^{2+} waves. **a** Addition of CPA (10 μM) to ongoing Ca^{2+} waves completely abolished the waves, leaving a small but significant elevation in baseline

Ca^{2+} which corresponds to $29 \pm 3\%$ of the peak $[Ca^{2+}]$ of the asynchronous Ca^{2+} waves (32 cells from 5 animals). **b** Application of CPA (10 μM) produced $82.5 \pm 4.2\%$ inhibition of the PE- (3 μM) induced tonic contraction ($p < 0.001$, 5 animals).

Fig. 7. Effect of 2-APB on PE- (3 μM) induced Ca^{2+} waves and tonic contraction. **a** The application of 2-APB (75 μM) immediately abolished PE-induced Ca^{2+} waves (31 cells from 5 animals). **b** Similarly, 2-APB (75 μM) decreased PE- (3 μM) induced tonic contraction to $2.49 \pm 1.18\%$ of maximal contraction (5 animals). **c** PE stimulation after pretreatment of vessels with 2-APB (75 μM) for 30 min failed to elicit a Ca^{2+} response (gray line, 24 cells from

3 animals). In contrast, control vessels without 2-APB pre-incubation (black line) displayed Ca^{2+} waves after PE stimulation. **d** PE (3 μM) stimulation after pretreatment of vessels with 2-APB (75 μM) for 30 min failed to induce contraction (gray line, 5 animals) compared to control vessels without 2-APB pre-incubation (black line).

To test for direct effects on Ca^{2+} entry pathways, the effects of 2-APB on L-type Ca^{2+} channels and store-operated channels were examined. Although 2-APB (75 μM) reduced 60 mM KCl-induced tonic contraction by $10.8 \pm 4.3\%$ (fig. 8b; $p = 0.071$, 6 animals), it is unlikely that this slight inhibition of L-type Ca^{2+} channels could account for the complete inhibition of PE-induced tonic contraction by 2-APB, as blockade of L-type Ca^{2+} channels with nifedipine reduced force to $7.31 \pm 2.57\%$ of PE-precontracted levels.

In addition to L-type Ca^{2+} channels, store-operated channels are also important for maintaining the Ca^{2+} waves. 2-APB has been reported to have nonselective effects on store-operated channels [29]. We stimulated the vessel with PE (3 μM) to generate sustained Ca^{2+} waves and then applied CPA (10 μM) to inhibit SR Ca^{2+} re-uptake, resulting in a maintained elevation of $[\text{Ca}^{2+}]$ and depletion of the SR (fig. 8c). The application of 2-APB (75

μM) did not affect the $[\text{Ca}^{2+}]_i$ plateau, although Ca^{2+} returned to baseline upon the subsequent addition of the store-operated channel blocker SKF-96365 (50 μM), indicating that in this preparation 2-APB does not inhibit the store-operated channels directly.

Another SR Ca^{2+} release channel which may be involved in the mechanism underlying Ca^{2+} waves is the RyR. To assess the role of RyRs in PE-induced Ca^{2+} waves, high-concentration ryanodine (200 μM) and tetracaine (100 μM) were used to lock RyRs in their closed configuration. Neither ryanodine (fig. 9a, b) nor tetracaine (fig. 9c, d) had any effect on the frequency of the ongoing PE-induced Ca^{2+} waves (29 cells from 5 animals and 38 cells from 4 animals, respectively) or tonic contraction (5 and 6 animals, respectively). Ca^{2+} waves and tonic contraction were abolished in Marfan mesenteric arteries by 2-APB, but were unaffected by high-concentration ryanodine and tetracaine (data not shown).

Fig. 8. Effects of 2-APB on the ryanodine-sensitive SR Ca²⁺ release channels, sarco(endo)plasmic reticulum Ca²⁺ ATPase, L-type Ca²⁺ channels and store-operated channels in mouse mesenteric artery. **a** Left: 3 pulses of caffeine (25 mM) were applied with a 5-min interval between each pulse (dashed lines). Maximum amplitude of the caffeine-induced Ca²⁺ transient from the first pulse reflects control SR Ca²⁺ level as Ca²⁺ from the SR is released through the opened RyR channels. After the addition of 2-APB (75 μM), the second pulse of caffeine resulted in a single Ca²⁺ transient whose maximum amplitude is similar to the first pulse (105.2 ± 5.6% of control, *p* = 0.73, 6 animals). The third pulse of caffeine resulted in a Ca²⁺ transient whose maximum amplitude

was slightly, but not significantly, diminished by 11.2 ± 4.1% compared with the first pulse (*p* = 0.41, 6 animals). Right: bar graph comparing the average maximum amplitude of the second and third caffeine pulses to the third pulse (6 animals). **b** Application of 2-APB (75 μM) reduced tonic contraction induced by 60 mM KCl by 10.8 ± 4.3% (*p* = 0.071, 6 animals). **c** Application of PE (3 μM) followed by CPA (10 μM) resulted in a maintained elevation in Ca²⁺ (black line). 2-APB (75 μM) did not affect this plateau response, whereas the addition of SKF-96365 (50 μM) abolished the maintained Ca²⁺ elevation and returned to prestimulation baseline level (gray line). Representative trace shown is typical of the responses obtained in 30 cells from 4 mice.

Discussion

The concept that tonic vasoconstriction is based on asynchronous Ca²⁺ waves in individual smooth muscle cells has had a profound impact on our views on the molecular mechanisms underlying Ca²⁺ signaling in smooth muscle. The presence of agonist-induced Ca²⁺ waves in small mouse mesenteric arteries has been previously documented [7, 21], although a detailed investigation of their underlying mechanisms has not yet been conducted. In this study we used an in situ preparation of the secondary branch of the mouse mesenteric artery and systematically studied the ionic mechanisms underlying these Ca²⁺

waves, which appear to be similar to those described in vascular smooth muscle cells from other blood vessels [9, 30]. Furthermore, we also demonstrate that the decreased contraction in mesenteric arteries of a mouse model of Marfan syndrome is likely due to a decrease in Ca²⁺ wave frequency and cell recruitment, rather than changes in the underlying Ca²⁺ signaling mechanism.

Stimulation of the mouse mesenteric artery with PE results in repetitive transient elevations in [Ca²⁺]_i in the smooth muscle cells, which originate in distinct intracellular foci and then spread out as waves over the length of the smooth muscle cell. The presence of Ca²⁺ waves is tightly linked with tonic contraction, and their impor-

Fig. 9. Effect of ryanodine and tetracaine on PE- ($3 \mu\text{M}$) induced Ca^{2+} waves and tonic contraction. **a** Application of a high concentration ($200 \mu\text{M}$) of ryanodine did not affect ongoing PE-induced Ca^{2+} waves (29 cells from 5 animals). **b** Ryanodine ($200 \mu\text{M}$) also did not affect PE-induced tonic contraction (5 animals). **c** High-

concentration ($100 \mu\text{M}$) tetracaine also did not affect ongoing PE-induced Ca^{2+} waves (8 cells from 5 animals). **d** Tetracaine ($100 \mu\text{M}$) also had no significant effect on PE-induced tonic contraction (6 animals).

tance is further demonstrated when their abolishment by CPA markedly reduced force, although the average $[\text{Ca}^{2+}]_i$ remains significantly elevated above baseline (fig. 6a, b). The asynchronous nature of the Ca^{2+} waves likely explains how summation of individual-cell Ca^{2+} waves can lead to tonic contraction, as the summation of Ca^{2+} signals in all the cells averages out to be a steady-state Ca^{2+} increase in whole vessels [31, 32], indicating a higher force-to- $[\text{Ca}^{2+}]_i$ ratio when smooth muscle cells are activated with Ca^{2+} waves compared to sustained elevation of $[\text{Ca}^{2+}]_i$ [5]. Furthermore, there is a strong correlation between frequency of agonist-induced Ca^{2+} waves and tonic contraction, as well as a strong relationship between the number of cells displaying Ca^{2+} waves and generation of force [5, 9, 33]. However, contraction is ultimately de-

termined by the level of phosphorylation of myosin light chain, which is both Ca^{2+} dependent and Ca^{2+} independent. The exact way in which Ca^{2+} waves might be related to this level of phosphorylation is unclear, as their role may only be conclusively determined with direct observation of myosin light-chain kinase, as has been done with carbachol-induced elevation of Ca^{2+} in mouse bladder smooth muscle cells [34].

An important finding in this study was that the reduction in frequency of PE-stimulated Ca^{2+} waves in the mesenteric arteries from a mouse model of Marfan syndrome is associated with reduced maximal contraction. As the mechanism underlying the Ca^{2+} waves in Marfan small mesenteric arteries is not different from the control arteries, the reduction in Ca^{2+} wave frequency appears to be the

major cause of decreased tonic contraction. However, at submicromolar concentrations, the frequency of the Ca^{2+} waves between control and Marfan vessels are not significantly different, although tonic contraction is considerably decreased (fig. 3). This could be explained by the following reasons: (1) Marfan syndrome is associated with decreased association between smooth muscle cells and elastic fibers, which would reduce the strain on the smooth muscle cells and blunt their response to agonist stimulation [35], (2) distention of the vessels during contraction may also disrupt the association between smooth muscle cells and extracellular matrix [20, 25], and (3) there were significantly fewer cells displaying Ca^{2+} waves in the Marfan vessels when stimulated with PE. Because contraction is also mediated by the number of cells displaying Ca^{2+} waves, a decreased number of cells displaying Ca^{2+} waves could lead to generate decreased contraction [8].

Extracellular Ca^{2+} influx through L-type Ca^{2+} channels is central in the control of vascular tone and plays a significant role in maintaining Ca^{2+} homeostasis and contraction [36, 37]. This is also observed in our studies, where inhibition of L-type Ca^{2+} channels with nifedipine resulted in significant decrease in tonic contraction. Interestingly, the Ca^{2+} response to KCl stimulation was significantly reduced in Marfan vessels compared to control (fig. 4), suggesting decreased Ca^{2+} influx through L-type Ca^{2+} channels. While the underlying reason behind this apparently decreased Ca^{2+} influx is unknown, it has been shown that the expression of the matrix metalloproteinase-2 and matrix metalloproteinase-9 enzymes is upregulated in Marfan syndrome and may inhibit Ca^{2+} influx through L-type Ca^{2+} channels, and subsequent SR refilling [25, 38, 39]. As SR luminal Ca^{2+} can regulate IP_3R channel opening probability, a reduced rate of SR Ca^{2+} refilling can lead to a decreased frequency of SR Ca^{2+} release [40, 41]. Thus, since the Ca^{2+} waves described in these experiments are dependent on regenerative Ca^{2+} release from IP_3Rs , this would result in a decreased frequency of Ca^{2+} waves.

Another finding in this study was that the nifedipine-insensitive component of the Ca^{2+} waves was only abolished with the receptor-operated channel antagonist SKF-96365, suggesting a role for these channels in the refilling of the SR. In vascular smooth muscle cells, Na^+ influx through receptor-operated nonselective cation channels, particularly those from the canonical transient receptor potential protein family, changes the reversal potential of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to which receptor-operated channels are functionally coupled and allows it to operate in the Ca^{2+} influx mode [42–45]. This method

of SR refilling is essential for sustaining the regenerative Ca^{2+} release upon which the Ca^{2+} waves are based and has been demonstrated in a variety of vascular smooth muscle preparations [5, 9]. However, the use of SKF-96365 is complicated by its nonselective effects, such as blockade of L-type Ca^{2+} channels, inhibition of SERCA and emptying of SR Ca^{2+} stores [46, 47]. In our preparation, we have only used SKF-96365 after the application of nifedipine, in order to control for the possibility of its blockade of L-type Ca^{2+} channels. Furthermore, as we did not see an elevation of cytoplasmic Ca^{2+} following SKF-96365 application, it is unlikely that SKF-96365 inhibited SERCA or released Ca^{2+} from the SR stores in our preparation.

Although the fact that adrenergic agonists induce asynchronous Ca^{2+} waves in small-resistance arteries has been established, the identity of the SR Ca^{2+} release channels involved is still open to debate. In rat mesenteric arteries, PE-induced Ca^{2+} waves were abolished by blockade of IP_3R with 2-APB, a small-molecular-weight membrane-permeable modulator of the IP_3R [29, 48]. Furthermore, acetylcholine-induced Ca^{2+} waves in rat portal vein myocytes are also dependent on activation of IP_3Rs , mostly through the IP_3R_2 subtype [49, 50]. However, its use to block IP_3Rs has been criticized for its nonspecific effects on other ion transport mechanisms, notably its inhibition of store-operated channels [51–53]. In our preparation, 2-APB immediately abolished ongoing Ca^{2+} waves and tonic contraction, and did not affect caffeine-releasable Ca^{2+} stores, which is consistent with a specific action of 2-APB to block IP_3Rs (fig. 8a). Furthermore, stimulation with PE after pre-incubation with 2-APB did not elicit a Ca^{2+} response or contraction. In addition, 2-APB also had only a minor insignificant effect on SR Ca^{2+} re-uptake and had no significant inhibition on store-operated channels, and although it does affect L-type Ca^{2+} channels, the slight inhibition observed could not have accounted for the abolishment of Ca^{2+} waves (fig. 8b, c). Our findings support the notion that opening of IP_3R channels is required for subsequent regenerative release of Ca^{2+} underlying the propagation of the Ca^{2+} waves.

However, this does not exclude involvement of RyRs. In particular, it is unclear whether Ca^{2+} release from the IP_3Rs activates RyRs to generate further release by Ca^{2+} -induced Ca^{2+} release during wave propagation, or whether the entire release process arises from IP_3Rs without significant RyR involvement [54]. Indeed, drugs which block RyRs also abolished Ca^{2+} waves initiated by $\text{Ins}(1,4,5)\text{P}_3$ -generating agonists [6, 55–57]. However, the use of pharmacological tools to inhibit RyRs, particularly the plant alkaloid ryanodine, is complicated by its con-

centration-dependent effects in different tissues. For example, low concentrations (<100 μM) of ryanodine cause persistent opening of the channels which may lead to store depletion [58–60], while higher concentrations are reported to lock RyRs in a closed state to inhibit Ca^{2+} release [61]. Therefore, we used high-dose ryanodine (200 μM) to lock the RyRs in the closed configuration and found that neither PE-induced Ca^{2+} waves nor tonic contraction were affected (fig. 9a, b). To further verify the absence of a role of RyRs, we also used tetracaine, the use of which is not dependant on the opening of RyRs [62], and observed the same lack of effect (fig. 9c, d). This suggests that SR Ca^{2+} release from RyRs does not participate in PE-induced Ca^{2+} waves in this preparation. It was particularly relevant that none of the mechanistic characteristics of the Ca^{2+} waves were different in the Marfan mice.

In summary, the data presented in this study show that multiple Ca^{2+} translocating proteins are involved in the

generation of Ca^{2+} waves in PE-stimulated smooth muscle cells of second-order branch mouse mesenteric artery. These Ca^{2+} waves appear to underlie tonic contraction, and their decreased frequency, possibly through reduced SR refilling resulting from reduced Ca^{2+} influx from the extracellular space, is linked to decreased maximal contraction in vessels from a mouse model of Marfan syndrome.

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