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Genetic evidence for functional role of ryanodine receptor 1 in pulmonary artery smooth muscle cells

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Abstract

Ryanodine receptor 1 (RyR1) is well-known to be expressed in systemic and pulmonary vascular smooth muscle cells (SMCs); however, its functional roles remain largely unknown. In the present study, we attempted to determine the potential importance of RyR1 in membrane depolarization-, neurotransmitter-, and hypoxia-induced Ca^{2+} release and contraction in pulmonary artery SMCs (PASMCs) using RyR1 homozygous and heterozygous gene deletion ($\text{RyR1}^{-/-}$ and $\text{RyR1}^{+/-}$) mice. Our results indicate that spontaneous local Ca^{2+} release and caffeine-induced global Ca^{2+} release are significantly reduced in embryonic $\text{RyR1}^{-/-}$ and adult $\text{RyR}^{+/-}$ cells. An increase in $[\text{Ca}^{2+}]_i$ following membrane depolarization with high K^+ is markedly attenuated in $\text{RyR1}^{-/-}$ and $\text{RyR1}^{+/-}$ PASMCs in normal Ca^{2+} or Ca^{2+} -free extracellular solution. Similarly, muscle contraction evoked by membrane depolarization is reduced in $\text{RyR1}^{+/-}$ pulmonary arteries in the presence or absence of extracellular Ca^{2+} . Neurotransmitter receptor agonists and inositol 1,4,5-triphosphate elicit a much smaller increase in $[\text{Ca}^{2+}]_i$ in both $\text{RyR1}^{-/-}$ and $\text{RyR1}^{+/-}$ cells. We have also found that neurotransmitter-evoked muscle contraction is significantly inhibited in $\text{RyR1}^{+/-}$ pulmonary arteries. Hypoxia-induced increase in $[\text{Ca}^{2+}]_i$ and contraction are largely blocked in $\text{RyR1}^{-/-}$ and/or $\text{RyR1}^{+/-}$ PASMCs. Collectively, our findings provide genetic evidence for the functional importance of RyR1 in spontaneous local Ca^{2+} release, and membrane depolarization-,

neurotransmitter-, as well as hypoxia-induced global Ca^{2+} release and attendant contraction in PSMCs.

Keywords

Ryanodine receptor; Calcium release; Membrane depolarization; Hypoxia; Pulmonary artery smooth muscle cell

Introduction

An increase in $[\text{Ca}^{2+}]_i$ serves as an important and ubiquitous signal that mediates numerous cellular functions in systemic and pulmonary vascular smooth muscle cells (SMCs). Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptors/ Ca^{2+} release channels (RyRs) is likely to be a major route for an increase in $[\text{Ca}^{2+}]_i$, which can be manifested in local and global Ca^{2+} signaling with distinct spatiotemporal properties.

The functional importance of RyRs has been recognized in cerebral (systemic) artery SMCs where intraluminal pressure may result in extracellular Ca^{2+} influx through plasma membrane (plasmalemmal) voltage-dependent Ca^{2+} channels (VDCCs), opening of RyRs on the SR membrane, and then local Ca^{2+} release events (Ca^{2+} sparks), which in turn activates plasmalemmal Ca^{2+} -activated K^+ channels to produce spontaneous transient outward currents (STOCs), leading to membrane hyperpolarization, inhibition of Ca^{2+} influx, and cell relaxation [1]. Although all three RyR subtypes (RyR1, RyR2, and RyR3) are known to be expressed in systemic vascular SMCs, it remains unclear which of these RyR subtypes may mediate the physiological, negative regulation of systemic vascular tone. RyR3 seems unlikely to be a crucial contributor because the activity of RyR-mediated Ca^{2+} sparks and attendant STOCs are both increased in cerebral artery SMCs from RyR3 homozygous gene deletion (RyR3^{-/-}) mice [2]. Moreover, suppression of RyR3 gene expression by antisense oligonucleotides does not affect membrane depolarization-induced Ca^{2+} sparks in cultured rat portal vein myocytes [3].

We and other investigators have recently found that all three RyR subtypes are expressed in rat and mouse pulmonary artery SMCs (PSMCs) [4–6]. Pharmacological inhibition of RyRs significantly attenuates neurotransmitter- and hypoxia-induced increase in $[\text{Ca}^{2+}]_i$ and contraction in PSMCs. RyR3 homozygous gene deletion diminishes hypoxia-induced, but not neurotransmitter-induced, Ca^{2+} and contractile responses [4]. Taken together, these previous findings led us to hypothesize that RyR1 may play an important functional role in PSMCs. To test this hypothesis, we first sought to examine and compare the functional activities of RyRs, such as spontaneous local Ca^{2+} release and caffeine-induced global Ca^{2+} release, in isolated PSMCs from RyR1 homozygous and heterozygous gene deletion (RyR1^{-/-} and RyR1^{+/-}) mice as well as their corresponding wild-type (RyR1^{+/+}) animals. Because RyR1^{-/-} animals die just before and after birth, PSMCs isolated from embryonic RyR1^{-/-} mice at day 17 were used in these experiments. To determine the physiological role of RyR1 in excitation–contraction coupling, we next investigated whether an increase in $[\text{Ca}^{2+}]_i$ following membrane depolarization with high K^+ was diminished in isolated PSMCs from embryonic RyR1^{-/-} and adult RyR1^{+/-} mice in normal Ca^{2+} and Ca^{2+} -free extracellular medium. To extend these studies, we also assessed muscle contraction induced by membrane depolarization with high K^+ in isolated RyR1^{+/+} and RyR1^{+/-} pulmonary arteries. Numerous reports have shown that Ca^{2+} release through inositol 1,4,5-triphosphate receptors/ Ca^{2+} release channels (IP₃Rs) may open neighboring RyRs and then cause further Ca^{2+} release via a local Ca^{2+} -induced Ca^{2+} release (CICR) mechanism, amplifying neurotransmitter-induced Ca^{2+} and contractile responses in PSMCs [4, 7–10]. As such, we

wondered whether it might require the involvement of RyR1 by testing the effect of RyR1 gene deletion on neurotransmitter-induced increase in $[Ca^{2+}]_i$ in PSMCs and muscle contraction in pulmonary arteries. Our previous study has revealed that RyR antagonists can further inhibit hypoxic responses in RyR3^{-/-} PSMCs, suggesting the potential role of RyR1 in the hypoxic response [4]. Thus, we finally examined and compared hypoxic increase in $[Ca^{2+}]_i$ in RyR1^{-/-} and RyR1^{+/-} PSMCs, as well as muscle contraction in RyR1^{+/-} and RyR1^{+/+} pulmonary arteries.

Materials and methods

Isolated cell preparation

All animal experiments were performed in accordance with the approved Protocol of Animal Use and Care of Albany Medical College. RyR1^{-/-} mice were generated as reported previously [11] and maintained as heterozygotes. To obtain RyR1^{-/-} mice and their matching RyR1^{+/-} controls, RyR1^{+/-} animals were bred as timed pregnancy. Mice at 17 days of gestation were euthanized by an intraperitoneal injection of sodium pentobarbital (150 mg/kg). RyR1^{-/-}, RyR1^{+/-}, and RyR1^{+/+} mice were identified using tail DNA genotyping [11]. Pulmonary arteries were removed from embryonic RyR1^{+/-} and RyR1^{-/-} mice and placed in ice-cold physiological saline solution (PSS) containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 10 HEPES, 1.8 CaCl₂, and 10 glucose (pH 7.4). Blood vessels were cut into small pieces (1 × 10 mm), and then digested using the two-step enzymatic method [4]. Arteries were initially incubated in low (100 μM) Ca²⁺ PSS containing 0.5 mg/ml papain at 37°C for 10–15 min, followed by incubation in low Ca²⁺ PSS containing 0.4 mg/ml type F and 0.5 mg/ml type II collagenase for 10–15 min. Single cells from fully digested arteries were harvested by gentle trituration, placed in DMEM containing 1% fetal bovine serum albumin (BSA) and 100 μM bromodeoxyuridine to inhibit fibroblast growth in a humidified incubator with 5% CO₂ at 37°C, and used for experiments within 2 days.

In some experiments, RyR1^{+/-} and matching RyR1^{+/+} mice at age of 8–10 weeks (adult) were used. The third and smaller branches of the pulmonary arteries were taken from these mice and then digested using the same procedure as described above, except that the concentration of papain, type F collagenase, and type II collagenase were increased to 1.0, 0.8, and 1.0 mg/ml, respectively. Freshly isolated PSMCs were kept on ice for daily use.

Immunofluorescence staining

Immunofluorescence staining was performed, as we described before [4]. Cells were fixed in 4% paraformaldehyde in PSS for 20 min at room temperature, incubated with 0.2% Triton X-100 in PSS for 30 min, and then blocked for 1 h with 2.5% BSA PSS. After that, cells were incubated with monoclonal anti-RyR1 antibody (1:100 dilution; Upstate Biotechnology, Lake Placid, NY, USA) overnight at 4°C, followed by Alexa 488-conjugated antibody (1:500; Molecular Probes, Eugene, OR, USA) for 2 h. Immunofluorescence staining was examined using a Zeiss LSM510 laser-scanning confocal microscope.

The confocal pinhole was set to provide a spatial resolution of 1 μm in the *x-y* axis. The *z* interval was adjusted to 1 μm to obtain sufficient fluorescence signals. Alexa Fluor 488 was excited at 488 nm using a krypton–argon laser, and the emitted fluorescence at 510 nm was detected. To minimize the potential effects of the variations in sample processing and image acquisition on the recorded fluorescent signals, we carefully and concurrently prepared RyR1^{-/-}, RyR1^{+/-}, and their matching RyR1^{+/+} mouse PSMCs and took fluorescence images.

Measurement of Ca^{2+} sparks

Single PSMCs were loaded with 5 μM fluo-4/AM in normal Ca^{2+} (1.8 mM) PSS for 30 min and then were superfused for 10 min. The dye was excited with 488 nm, and the emitted fluorescence was measured at 505 nm. To obtain high temporal profiles of Ca^{2+} sparks, line-scan images were acquired using a Leica TCS SP2 laser-scanning confocal microscope (Leica, Mannheim, Germany) with a Leica $\times 40$ oil immersion objective (NA 1.3), as reported previously [6, 8, 12]. The recording time was set at 5 s to obtain a number of Ca^{2+} sparks and minimize laser toxicity. Spatiotemporal characteristics of Ca^{2+} sparks were analyzed using the Leica Image Examiner and Interactive Data Language software (Research Systems, Boulder, CO, USA).

Measurements of whole-cell $[\text{Ca}^{2+}]_i$

Whole-cell $[\text{Ca}^{2+}]_i$ was measured using a dual-excitation wavelength fluorescence method with a TILL fluorescence imaging system (TILL Photonics, Gräfelfing, Germany) and a Nikon $\times 20$ oil immersion objective, as described previously [4]. PSMCs were loaded with 5 μM fura-2/AM in PSS for 30 min. The dye was excited alternatively at 340 and 380 nm wavelength (Xenon 75 W arc lamp), and the emission fluorescence was detected at 510 nm. Background fluorescence was determined by measuring an adjacent noncell region of the field. $[\text{Ca}^{2+}]_i$ was calculated by the ratio of fluorescence intensity at 340 and 380 nm excitation wavelength. In Ca^{2+} calibration experiments, 340 and 380 nm fluorescence at Ca^{2+} -free and saturating Ca^{2+} concentrations were determined using nominally Ca^{2+} -free PSS containing 10 mM ionomycin and 10 mM ethylene glycol bis(2-aminoethyl ether)- N,N,N,N -tetraacetic acid (EGTA) and PSS containing 10 mM ionomycin and 10 mM Ca^{2+} , respectively.

Patch clamp recordings

Voltage-dependent Ca^{2+} currents (I_{Ca}) were recorded using the nystatin-perforated patch clamp recordings with the EPC-9 patch clamp system (HEKA Electronics, Lambrecht, Germany), as described previously [4]. Nystatin was included in the pipette solution at a final concentration of 300 $\mu\text{g}/\text{ml}$. Patch pipettes had a resistance of 3–4 M Ω . Junction potentials between the pipette and bath solutions were compensated just before seal formation. Membrane capacitance and series resistance were compensated. An access resistance of less than 40 M Ω was accepted for current recordings. Holding potential was set at -40 mV. K^+ currents were blocked by using tetraethylammonium (TEA) bath solution and Cs^+ patch pipette solution (see below). Inward I_{Ca} was elicited by various depolarizing pulses ranging from -30 to 40 mV with 10 mV increment for 200 ms. The composition of the bath solution was (in mM): 130 TEA-Cl, 2 CaCl_2 , 1.2 MgCl_2 , 10 glucose, and 10 HEPES (pH 7.3 with CsOH). The patch pipette solution contained (in mM): 80 Cs-acetate, 50 CsCl, 5 MgCl_2 , 1 CaCl_2 , 3 EGTA, and 10 HEPES (pH 7.3 with CsOH).

In some experiments, the classic patch clamp technique was used to dialyze IP_3 into cells. Cells were voltage-clamped at -55 mV. The bath solution was normal Ca^{2+} (1.8 mM) PSS. The pipette solution contained (in mM): 140 CsCl, 2 MgCl_2 , 10 HEPES, 1 CaCl_2 , and 2 EGTA (pH 7.4). Fura-2 (pentapotassium salt, 50 μM) was included in the patch pipette solution to avoid a potential decrease in fluorescence.

Measurement of muscle tension

The third branches of the pulmonary arteries from adult $\text{RyR1}^{+/-}$ and $\text{RyR1}^{+/+}$ mice were sliced into rings of 1.5-mm length and placed in 2-ml tissue baths (Radnoti, Monrovia, CA, USA). One end of the vessel segments was fixed to a clip, and the other end was connected to a highly sensitive force transducer (Harvard Apparatus, Holliston, MA, USA). Bath

solution contained (in mM) 110 NaCl, 3.4 KCl, 2.4 CaCl₂, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 glucose (pH 7.4); was aerated with 20% O₂, 5% CO₂, and 75% N₂; and warmed at 37°C. Pulmonary artery rings were set at a resting tone of 200 mg. Muscle tension was recorded using a PowerLab/4SP recording system (AD Instruments, Colorado Springs, CO, USA).

Reagents

Fura-2/AM and Fluo-4/AM were purchased from Molecular Probes. Fura-2 (pentapotassium salt) and caffeine were obtained from Calbiochem (La Jolla, CA, USA). All other chemicals used in this study were purchased from Sigma (St Louis, MO, USA).

Data analysis

All values were expressed as the means±SEM of *n* cells or tissues investigated. Student's *t* test was used to determine the significance of differences, and a *P* value of <0.05 was considered to be statistically significant.

Results

The activity of spontaneous Ca²⁺ sparks is significantly reduced in RyR1^{-/-} mouse PSMCs

Considering that the functional role of RyR1 is unclear in vascular SMCs, we first investigated whether RyR-mediated Ca²⁺ sparks were altered in isolated PSMCs from embryonic RyR1^{-/-} mice. As shown in Fig. 1, we observed spontaneous Ca²⁺ sparks in both isolated RyR1^{+/+} and RyR1^{-/-} PSMCs in a normal Ca²⁺ (1.8 mM) PSS using a Leica TCS SP2 laser-scanning confocal microscope. Compared with RyR1^{+/+} cells, however, the frequency of Ca²⁺ sparks was significantly reduced in RyR1^{-/-} cells. The mean frequency was reduced from 0.071±0.009 sparks/s/μm in RyR1^{+/+} cells (*n*=31 from 14 mice) to 0.034±0.003 sparks/s/μm in RyR1^{-/-} cells (*n*=22 from 11 mice; *P*<0.05). On the other hand, the mean amplitude of Ca²⁺ sparks was similar in RyR1^{+/+} and RyR1^{-/-} cells (0.176±0.006 vs. 0.180±0.10 *F*/*F*₀). Moreover, Ca²⁺ spark rise time, duration, and width were unaltered in RyR1^{-/-} cells, either. The mean rise time, full duration at half-maximal amplitude (FDHM), and full width at half-maximal amplitude (FWHM) were 64.9±4.0 ms, 67.5±9.5 ms, and 1.96±0.14 μm in RyR1^{+/+} cells and 62.5±7.1 ms, 54.8±6.7 ms, and 1.98±0.26 μm in RyR1^{-/-} cells, respectively.

Caffeine-induced global Ca²⁺ release is greatly inhibited in RyR1^{-/-} mouse PSMCs

To further test the functional activity of RyR1, we examined global Ca²⁺ release following the application of the prototypical RyR activator caffeine (30 mM) in RyR1^{+/+} and RyR1^{-/-} PSMCs. Ca²⁺ release was determined by assessing a change in Fluo-4 fluorescence intensity using a Leica laser-scanning confocal microscope with a line-scan mode. Consistent with the reduced spontaneous local Ca²⁺ release, caffeine-induced global Ca²⁺ release was also inhibited in RyR1^{-/-} cells under normal extracellular Ca²⁺ conditions. The mean amplitude of caffeine-induced increase in [Ca²⁺]_i was dramatically decreased from 2.1±0.4 (*F*/*F*₀) in control cells (*n*=11 from nine mice) to 0.9±0.1 (*F*/*F*₀) in RyR1^{-/-} cells (*n*=12 from seven mice; *P*<0.05; Fig. 2a).

As the functional activity of targeted molecules can be significantly inhibited in heterozygous gene deletion animals, we tested whether caffeine-induced global Ca²⁺ release was also reduced in isolated PSMCs from adult RyR1^{+/-} mice using a TILL fluorescence imaging system. Comparable to RyR1^{+/+} cells, caffeine-induced increase in [Ca²⁺]_i was greatly attenuated as well in RyR1^{+/-} cells in the presence of normal extracellular Ca²⁺ (Fig.

2b). Thus, caffeine-induced Ca^{2+} release is inhibited in both embryonic $\text{RyR1}^{-/-}$ and adult $\text{RyR1}^{+/-}$ PSMCs.

To verify that the reduced Ca^{2+} release in $\text{RyR1}^{-/-}$ and $\text{RyR1}^{+/-}$ PSMCs was due to a change in RyR1 protein expression, we examined and compared its expression in $\text{RyR1}^{-/-}$, $\text{RyR1}^{+/-}$, and their corresponding $\text{RyR1}^{+/+}$ cells using immunofluorescence staining. The results indicate that the expression of RyR1 was absent in $\text{RyR1}^{-/-}$ cells. In addition, RyR1 expression level was significantly lower in $\text{RyR1}^{+/-}$ than in $\text{RyR1}^{+/+}$ cells (Fig. 3).

Membrane depolarization-induced increase in $[\text{Ca}^{2+}]_i$ is markedly attenuated in $\text{RyR1}^{-/-}$ mouse PSMCs

RyR1 is known to be physically coupled to plasmalemmal VDCCs in skeletal muscle by which membrane depolarization activates VDCCs and subsequently RyR1, leading to SR Ca^{2+} release and cell contraction. This led us to wonder whether RyR1 contributed to membrane depolarization-induced increase in $[\text{Ca}^{2+}]_i$ in PSMCs. RyR1 gene deletion did not have a significant effect on the resting $[\text{Ca}^{2+}]_i$ in the presence of normal extracellular Ca^{2+} . The mean level was 95.5 ± 11.0 nM in $\text{RyR1}^{+/+}$ cells ($n=28$ cells from seven mice) and 84.6 ± 15.4 nM in $\text{RyR1}^{-/-}$ cells ($n=63$ cells from seven mice), respectively ($P>0.05$). However, membrane depolarization following the application of high K^+ (50 mM) resulted in a much smaller increase in $[\text{Ca}^{2+}]_i$ in $\text{RyR1}^{-/-}$ than $\text{RyR1}^{+/+}$ PSMCs. The mean increase in $[\text{Ca}^{2+}]_i$ was decreased from 260.3 ± 34.0 nM in $\text{RyR1}^{+/+}$ cells to 152.2 ± 13.4 nM in $\text{RyR1}^{-/-}$ cells ($P<0.05$). Similarly, high K^+ (100 mM)-evoked increase in $[\text{Ca}^{2+}]_i$ was also significantly attenuated in $\text{RyR1}^{-/-}$ cells in the presence of normal extracellular Ca^{2+} (Fig. 4a). In addition, 100 mM K^+ -induced increase in $[\text{Ca}^{2+}]_i$ is greater than 50 mM K^+ -evoked response in both $\text{RyR1}^{+/+}$ and $\text{RyR1}^{-/-}$ cells.

To further determine the potential physical coupling of RyR1 to VDCCs in PSMCs, we examined and compared membrane depolarization-induced increase in $[\text{Ca}^{2+}]_i$ in $\text{RyR1}^{+/+}$ and $\text{RyR1}^{-/-}$ PSMCs in Ca^{2+} -free bath solution (nominally Ca^{2+} -free plus 0.5 mM EGTA). Under these Ca^{2+} -free conditions, high K^+ (50 or 100 mM)-induced increase in $[\text{Ca}^{2+}]_i$ was also significantly decreased in $\text{RyR1}^{-/-}$ cells (Fig. 4b). These results provide additional evidence that RyR1 is importantly involved in membrane depolarization-induced Ca^{2+} release from the SR through its physical coupling to plasmalemmal VDCCs in PSMCs.

We also found that high K^+ -induced increase in $[\text{Ca}^{2+}]_i$ was significantly lower in $\text{RyR1}^{+/+}$ cells in the absence of extracellular Ca^{2+} than that in the presence of normal extracellular Ca^{2+} . The mean increase in $[\text{Ca}^{2+}]_i$ caused by 50 mM K^+ was 260.3 ± 34.0 nM in normal Ca^{2+} bath medium (28 cells from seven mice; Fig. 4a) and 166.3 ± 16.6 nM in Ca^{2+} -free bath medium (45 cells from seven mice; Fig. 4b), a decrease of 36.1% ($P<0.05$). Similarly, high K^+ (100 mM)-induced response was reduced by 44.6% (from 508.2 ± 104.8 nM in normal Ca^{2+} PSS [25 cells from six mice] to 281.4 ± 32.3 nM in Ca^{2+} -free PSS [37 cells from seven mice], $P<0.05$). Besides, in $\text{RyR1}^{-/-}$ cells, 50 mM K^+ -induced increase in $[\text{Ca}^{2+}]_i$ was reduced by 23.3% (from 152.2 ± 13.4 nM in normal Ca^{2+} PSS [63 cells from seven mice] to 116.7 ± 12.2 nM in Ca^{2+} -free PSS [58 cells from six mice], $P=0.05$), whereas 100 mM K^+ -evoked response was similar in normal Ca^{2+} and Ca^{2+} -free PSS.

Membrane depolarization-induced increase in $[\text{Ca}^{2+}]_i$ are also diminished in $\text{RyR1}^{+/-}$ mouse PSMCs

Resembling embryonic $\text{RyR1}^{-/-}$ PSMCs, high K^+ -induced increase in $[\text{Ca}^{2+}]_i$ was greatly attenuated as well in adult $\text{RyR1}^{+/-}$ cells under normal extracellular Ca^{2+} conditions (Fig. 5a). The application of ryanodine (50 μM) for 8 min to specifically inhibit RyRs blocked

high K^+ (50 or 100 mM)-induced increase in $[Ca^{2+}]_i$ in RyR1^{+/+} cells. However, following treatment with ryanodine, high K^+ -evoked responses were not further reduced in RyR1^{+/-} cells (Fig. 5b). High K^+ (50 or 100 mM)-induced increase in $[Ca^{2+}]_i$ was also reduced in adult RyR1^{+/-} cells in the absence of extracellular Ca^{2+} (Fig. 5c).

Under normal extracellular Ca^{2+} conditions, high K^+ (50 mM) evoked a much smaller Ca^{2+} response in adult than in embryonic RyR1^{+/+} cells. The mean increase in $[Ca^{2+}]_i$ was 162.0 ± 27.8 nM in adult RyR1^{+/+} cells ($n=23$ from five mice; Fig. 5a) and 260.3 ± 34.0 nM in embryonic RyR1^{+/+} cells ($n=28$ from seven mice; Fig. 4a); it was decreased by 37.8% ($P < 0.05$). Likewise, high K^+ (100 mM)-evoked response was reduced by 32.2% in adult cells ($n=32$ from five mice) compared with embryonic cells ($n=25$ from six mice). Furthermore, in the absence of extracellular Ca^{2+} , high K^+ -induced response was smaller as well in adult than in embryonic RyR1^{+/+} cells. The mean increase in $[Ca^{2+}]_i$ by 50 mM K^+ was decreased from 166.3 ± 16.6 nM in embryonic cells ($n=45$ from seven mice; Fig. 4b) to 39.1 ± 3.1 nM in adult cells ($n=59$ from five mice; Fig. 5c; $P < 0.05$), whereas the mean increase in $[Ca^{2+}]_i$ by 100 mM K^+ was reduced from 281.4 ± 32.3 nM in embryonic cells ($n=37$ from seven mice) to 58.7 ± 7.5 nM in adult cells ($n=38$ from five mice; $P < 0.05$).

It is unlikely that inhibited Ca^{2+} release following membrane depolarization with high K^+ is due to a depression of VDCC expression in RyR1^{-/-} and RyR1^{+/-} cells. Even so, in order to exclude this possibility, we examined and compared I_{Ca} in RyR1^{+/+} and RyR1^{+/-} PSMCs. As shown in Fig. 6, we were able to record nifedipine-sensitive I_{Ca} in both RyR1^{+/+} and RyR1^{+/-} cells. There was no difference in the current-voltage curve of I_{Ca} between RyR1^{+/+} and RyR1^{+/-} cells, indicating that I_{Ca} is unaltered in RyR1^{+/-} PSMCs.

Muscle contraction following membrane depolarization with high K^+ is decreased in RyR1^{+/-} mouse pulmonary arteries

Consistent with the significant role of RyR1 in membrane depolarization-induced increase in $[Ca^{2+}]_i$, muscle contraction following membrane depolarization with high K^+ was diminished as well in isolated pulmonary arteries from adult RyR1^{+/-} mice (Fig. 7a). Under normal extracellular Ca^{2+} conditions, high K^+ (50 mM)-elicited muscle tension was decreased from 116.0 ± 8.4 mg in RyR1^{+/+} pulmonary arteries ($n=9$ from five mice) to 86.9 ± 9.8 mg in RyR1^{+/-} pulmonary arteries ($n=11$ from five mice; $P < 0.05$). Muscle contraction triggered by high K^+ (100 mM) was also significantly lessened in RyR1^{+/-} pulmonary arteries in the presence of normal extracellular Ca^{2+} .

Moreover, we found that high K^+ (100 mM) evoked a smaller muscle contraction in RyR1^{+/-} than in RyR1^{+/+} pulmonary arteries under Ca^{2+} -free extracellular conditions (Fig. 7b). The mean muscle contraction was 43.5 ± 8.4 mg in RyR1^{+/+} pulmonary arteries ($n=11$ from five mice) to 21.1 ± 2.1 mg in RyR1^{+/-} pulmonary arteries ($n=11$ from five mice; $P < 0.05$).

RyR1 is importantly involved in neurotransmitter-induced increase in $[Ca^{2+}]_i$ and contraction in mouse PSMCs

RyRs may amplify neurotransmitter-induced increase in $[Ca^{2+}]_i$ via a CICR process due to the local interaction of IP₃Rs with RyRs in PSMCs [4, 7–9]. In order to test the potential role of RyR1 in this local CICR process, we examined and compared neurotransmitter-triggered increase in $[Ca^{2+}]_i$ in RyR1^{+/+} and RyR1^{-/-} mouse PSMCs. As shown in Fig. 8a, the application of adenosine triphosphate (ATP) to stimulate purinergic receptors resulted in a smaller increase in $[Ca^{2+}]_i$ in RyR1^{-/-} cells than that in RyR1^{+/+} cells in the presence of normal extracellular Ca^{2+} . The mean increase in $[Ca^{2+}]_i$ induced by 3 μ M ATP was decreased from 545.4 ± 76.8 nM in RyR1^{+/+} cells ($n=47$ from eight mice) to 227.3 ± 59.9 nM

in RyR1^{-/-} cells ($n=25$ from seven mice; $P<0.05$). Likewise, ATP (30 μ M)-induced response was also greatly reduced in RyR1^{-/-} cells. Moreover, ATP (3 μ M)-triggered increase in $[Ca^{2+}]_i$ was reduced as well in RyR1^{-/-} PSMCs under Ca^{2+} -free extracellular conditions (Fig. 8b).

Compared with the normal Ca^{2+} extracellular solution, ATP (3 μ M)-induced increase in $[Ca^{2+}]_i$ in embryonic RyR1^{+/+} cells was smaller in Ca^{2+} -free extracellular solution. The mean increase in $[Ca^{2+}]_i$ was 545.4 ± 76.8 nM (47 cells from eight mice; Fig. 8a) and 344.0 ± 45.5 nM (60 cells from eight mice; Fig. 8b), respectively ($P<0.05$). However, in embryonic RyR1^{-/-} cells, ATP (3 μ M)-induced response was not different in normal Ca^{2+} extracellular solution (25 cells from seven mice) and in Ca^{2+} -free extracellular solution (36 cells from seven mice).

Akin to that in embryonic RyR1^{-/-} cells, ATP (30 μ M)-induced increase in $[Ca^{2+}]_i$ was significantly inhibited in adult RyR1^{+/-} cells, relative to adult RyR1^{+/+} cells (Fig. 9a). However, ATP (30 μ M)-caused response was not different between embryonic (Fig. 8a) and adult RyR1^{+/+} cells (Fig. 9a).

ATP can activate P2X ionotropic receptors causing direct extracellular Ca^{2+} influx through the channels and P2Y metabotropic receptors inducing SR Ca^{2+} release via IP₃Rs. Thus, we next investigated the effect of 2,3 -*O*-trinitro-phenyl-ATP (TNP-ATP), known to block P2X, but not P2Y receptors, on ATP-triggered Ca^{2+} responses in RyR1^{+/+} and RyR1^{+/-} cells. Following treatment with TNP-ATP (10 μ M) for 8 min, ATP-induced increase in $[Ca^{2+}]_i$ was much smaller in RyR1^{+/-} than that in RyR1^{+/+} cells. The mean increase in $[Ca^{2+}]_i$ was 528.2 ± 83.4 nM in RyR1^{+/+} cells ($n=37$ from five mice) and 307.2 ± 45.8 nM in RyR1^{+/-} cells ($n=35$ from five mice), respectively ($P<0.05$). These data further suggest that RyR1 contributes to neurotransmitter-induced Ca^{2+} release from the SR through IP₃Rs in PSMCs. In support of this view, we found that dialysis of IP₃ (20 μ M) to directly activate IP₃Rs caused a much smaller increase in $[Ca^{2+}]_i$ in RyR1^{+/-} than in RyR1^{+/+} cells (Fig. 9b).

Moreover, muscle contraction evoked by the adrenergic receptor neurotransmitter norepinephrine (300 μ M) was significantly reduced as well in RyR1^{+/-} mouse pulmonary arteries (Fig. 9c). The mean muscle tension elicited by norepinephrine was decreased from 90.8 ± 15.7 mg in RyR1^{+/+} pulmonary arteries ($n=12$ from six mice) to 34.0 ± 6.6 mg in RyR1^{+/-} pulmonary arteries ($n=14$ from seven mice; $P<0.05$).

RyR1 mediates hypoxia-induced increase in $[Ca^{2+}]_i$ and contraction in mouse PSMCs

Different from systemic vascular SMCs, PSMCs show an increase in $[Ca^{2+}]_i$ and contraction in response to hypoxia. Thus, we tested whether RyR1 contributed to the hypoxic responses. In these experiments, hypoxia was induced by perfusing bath solution aerated with 1% O₂, 5% CO₂ and 94% N₂, whereas normoxia was achieved by perfusing bath solution equilibrated with 20% O₂, 5% CO₂, and 74% N₂ [4, 6, 13]. As shown in Fig. 10a, hypoxic exposure for 5 min caused a smaller increase in $[Ca^{2+}]_i$ in RyR1^{-/-} mouse PSMCs than that in RyR1^{+/+} PSMCs. The mean increase in $[Ca^{2+}]_i$ was 87.2 ± 13.5 nM in RyR1^{+/+} PSMCs ($n=9$ from five mice) and 45.8 ± 9.5 nM in RyR1^{-/-} PSMCs ($n=23$ from nine mice), respectively ($P<0.05$).

Hypoxic increase in $[Ca^{2+}]_i$ was also significantly decreased in adult RyR1^{+/-} mouse PSMCs. The mean increase in $[Ca^{2+}]_i$ was reduced from 193.3 ± 35.4 nM in RyR1^{+/+} PSMCs ($n=49$ from five mice) to 94.5 ± 11.1 nM in RyR1^{+/-} PSMCs ($n=30$ from five mice; $P<0.05$; Fig. 10a).

To further test the contribution of RyR1 to hypoxic responses, we examined and compared hypoxia-induced vasoconstriction in adult RyR1^{+/+} and RyR1^{+/-} mouse pulmonary arteries. As shown in Fig. 10b, hypoxic vasoconstriction was much smaller in RyR1^{+/-} pulmonary arteries relative to RyR1^{+/+} pulmonary arteries. The mean hypoxic vasoconstriction was, respectively, 68.9±8.6 mg in RyR1^{+/+} pulmonary arteries (*n*=8 from four mice) and 36.4±6.9 mg in RyR1^{+/-} pulmonary arteries (*n*=8 from four mice; *P*<0.05).

Discussion

Ca²⁺ sparks due to the opening of multiple RyRs have been detected in a variety of cell types including systemic and pulmonary vascular smooth muscle cells using a laser-scanning confocal microscope, and may play an important role in physiological regulation of intracellular Ca²⁺ homeostasis and contractile responses. It has been reported that Ca²⁺ sparks lead to vasodilation in cerebral (systemic) arteries by causing the opening of big-conductance Ca²⁺-activated K⁺ channels and subsequently hyperpolarizing STOCs [1], whereas these local Ca²⁺ release events may result in vasoconstriction in pulmonary arteries by activating Ca²⁺-activated Cl⁻ channels and then generating depolarizing spontaneous transient inward currents [8, 14]. However, the molecular nature of Ca²⁺ sparks remains unclear. In this study, we examined and compared spontaneous Ca²⁺ sparks in PSMCs from embryonic RyR1^{+/+} and RyR1^{-/-} mice. The results indicate that RyR1 gene deletion dramatically reduces the frequency of Ca²⁺ sparks, albeit it does not affect the amplitude, rise time, duration, and size of Ca²⁺ sparks (Fig. 1). As Ca²⁺ spark frequency and amplitude are both predominantly controlled by the number of functional RyRs, the distinct effects of RyR1 gene deletion on these two properties suggest that these Ca²⁺ release channels may generate Ca²⁺ sparks on their own. Alternately, RyR1 may form a local Ca²⁺ release unit with RyR2 and/or RyR3; as such, RyR1 is likely to be obligatory for the operation of the mixed functional units. It might also be envisaged that RyR1 gene deletion could upregulate RyR2 and/or RyR3 expression through an unknown compensatory process by which the amplitude of Ca²⁺ sparks might remain unchanged. However, this is an unlikely case because a very recent study has shown that there is no significant change in RyR2 and RyR3 expression in RyR1^{-/-} mouse bladder SMCs [15]. In addition, we have found that the resting level of [Ca²⁺]_i is equivalent in RyR1^{+/+} and RyR1^{-/-} PSMCs. Consistent with our finding that the activity of Ca²⁺ sparks is greatly suppressed in PSMCs, it has been reported that suppression of RyR1 expression using antisense oligonucleotides inhibits depolarization-induced Ca²⁺ sparks in cultured rat portal vein myocytes [3]. In addition, RyR1 gene deletion has been found to abolish depolarization-induced Ca²⁺ sparks in mouse bladder SMCs [15]. Taken together, these data indicate that RyR1 is important for the generation of Ca²⁺ sparks in pulmonary artery and other types of SMCs.

It is conceivably believed that RyR2 participates in the generation of Ca²⁺ sparks in vascular SMCs. Indeed, Coussin et al. have reported that antisense oligonucleotide-mediated inhibition of RyR2 expression blocks depolarization-evoked Ca²⁺ sparks in cultured portal vein SMCs [3]. The activity of Ca²⁺ sparks is significantly increased in FKBP12.6^{-/-} mouse bladder myocytes [16]. This result, together with the previous findings that FKBP12.6 physiologically binds to and inhibits RyR2 in systemic and pulmonary artery SMCs [13, 17], further suggests the contribution of RyR2 to the generation of Ca²⁺ sparks in vascular myocytes. Moreover, a recent study has found that the frequency and amplitude of Ca²⁺ sparks are reduced in bladder SMCs from RyR2^{+/-} mice [18]. As for the role of RyR3 in the formation of Ca²⁺ sparks in vascular SMCs, experimental data are inconsistent. RyR3 gene deletion increases the activity of Ca²⁺ sparks in mouse cerebral artery myocytes [2], while RyR3 gene suppression has no effect on depolarization-induced Ca²⁺ sparks in rat portal vein SMCs [3]. The activity of Ca²⁺ sparks is unaltered as well in RyR3^{-/-} mouse bladder

SMCs [16]. As such, it is worthwhile to perform additional studies to determine the role of RyR2 and RyR3 in the generation of Ca^{2+} sparks in PSMCs.

We and other investigators have previously described spontaneous Ca^{2+} sparks in adult rat and mouse PSMCs [6, 8, 14]. Compared with adult mouse PSMCs [6], Ca^{2+} sparks in embryonic mouse PSMCs (this study) exhibit a higher frequency (0.07 vs. 0.04 sparks/s/ μm), lower amplitude (0.17 vs. $0.31 \Delta F/F_0$), longer duration (FDHM; 68 vs. 39 ms), and larger size (FWHM; 2.0 vs. 0.8 μm). Overall, Ca^{2+} spark frequency, amplitude, FDHM, and FWHM in adult rat PSMCs are 0.02 sparks/s/ μm , $1.0 \Delta F/F_0$, 34 ms, and 1.8 μm , respectively [8, 14]. Relative to those in adult cells, Ca^{2+} sparks in embryonic PSMCs have a higher frequency, lower amplitude, longer duration, and similar size. Our previous studies have also shown that Ca^{2+} sparks comprise a higher frequency, higher amplitude, longer duration, and larger size in adult mouse pulmonary than in mesenteric artery SMCs [6]. Thus, Ca^{2+} sparks may display distinct spatiotemporal characteristics between PSMCs from different animal species and also between different types of SMCs.

Consistent with the inhibitory effect on RyR-mediated local Ca^{2+} release, we have found that RyR1 gene deletion also inhibits global Ca^{2+} release following the application of prototypical RyR agonist caffeine in PSMCs. Relative to RyR1^{+/+} cells, caffeine-induced increase in $[\text{Ca}^{2+}]_i$ is significantly reduced in RyR1^{-/-} cells. Similarly, caffeine-induced global Ca^{2+} release is significantly attenuated as well in RyR1^{+/-} cells (Fig. 2). In parallel to the reduced Ca^{2+} release, RyR1 expression level is absent in RyR1^{-/-} cells and largely reduced in RyR1^{+/-} cells (Fig. 3). As it is commonly thought that caffeine-induced Ca^{2+} release mirrors the SR Ca^{2+} load, one might deduce that the inhibition of caffeine-induced responses in RyR1^{-/-} and RyR1^{+/-} cells could be due to a reduction in the SR Ca^{2+} load. However, native RyR1 in skeletal myocytes normally shows a spontaneous Ca^{2+} release activity, generating Ca^{2+} sparks. Apparently, the reduction or lack of RyR1 expression may reduce spontaneous Ca^{2+} release, which consequently augments, rather than depresses the SR Ca^{2+} load. Furthermore, as stated above, RyR1 may form the structural and functional Ca^{2+} release units with RyR2 and/or RyR3. As such, these structurally or functionally mixed Ca^{2+} release units can be significantly suppressed in RyR1^{-/-} and RyR1^{+/-} cells. Taken together, the inhibition of caffeine-induced increase in $[\text{Ca}^{2+}]_i$ in RyR1^{-/-} and RyR1^{+/-} cells may result from a reduction in the functional activity of RyR1-formed Ca^{2+} release units, rather than in the SR Ca^{2+} load. Compatible with these results, previous studies have revealed that caffeine-induced global Ca^{2+} response is greatly attenuated by RyR1 gene suppression with antisense oligonucleotides in rat portal vein SMCs [3] and by RyR1 gene deletion in mouse bladder myocytes as well [15]. These results provide additional evidence showing that RyR1 is a functional component of RyRs/ Ca^{2+} release channels in pulmonary and systemic vascular SMCs.

In skeletal muscle, RyR1 is known to be physically coupled to plasmalemmal VDCCs. As such, membrane depolarization can activate VDCCs and subsequently RyRs without the involvement of extracellular Ca^{2+} influx, leading to a massive Ca^{2+} release from the SR to trigger cell contraction. In the present study, we have discovered that under normal extracellular Ca^{2+} conditions, membrane depolarization with high K^+ at 50 or 100 mM induces a much smaller increase in $[\text{Ca}^{2+}]_i$ in embryonic RyR1^{-/-} relative to RyR1^{+/+} PSMCs. A similar reduction in high K^+ -induced increase in $[\text{Ca}^{2+}]_i$ is observed in embryonic RyR1^{-/-} cells in Ca^{2+} -free extracellular medium (Fig. 4). Comparable to that in RyR1^{-/-} cells, high K^+ (50 or 100 mM)-evoked increase in $[\text{Ca}^{2+}]_i$ is dramatically decreased as well in adult RyR1^{+/-} PSMCs in the presence and absence of extracellular Ca^{2+} . In support of the role of RyR1 in membrane depolarization-triggered Ca^{2+} release from the SR, high K^+ (50 or 100 mM)-induced increase in $[\text{Ca}^{2+}]_i$ is not further reduced in RyR1^{+/-} cells following treatment with ryanodine to inhibit RyRs (Fig. 5). One might assume that the

depressed Ca^{2+} release following membrane depolarization with high K^+ in $\text{RyR1}^{-/-}$ and $\text{RyR1}^{+/-}$ cells could be due to a reduction in the expression or function of VDCCs. However, this is not the case, as our patch clamp studies demonstrate that there is no difference in VDCC current density between $\text{RyR1}^{+/+}$ and $\text{RyR1}^{+/-}$ cells (Fig. 6). We have also observed that high K^+ (50 mM)-induced Ca^{2+} response in $\text{RyR1}^{-/-}$ cells is smaller by 23.3% in Ca^{2+} -free than in normal Ca^{2+} extracellular solution, while by 36.1% in $\text{RyR1}^{+/+}$ cells; high K^+ (100 mM)-evoked response in $\text{RyR1}^{-/-}$ cells is similar in the presence to that in the absence of extracellular Ca^{2+} , even as it is reduced by 44.6% in $\text{RyR1}^{+/+}$ cells. Apparently, Ca^{2+} release triggered by membrane depolarization with high K^+ is greatly depressed in $\text{RyR1}^{-/-}$ cells. Moreover, muscle contraction following membrane depolarization with high K^+ is largely reduced in $\text{RyR1}^{+/-}$ pulmonary arteries under normal Ca^{2+} and Ca^{2+} -free extracellular conditions (Fig. 7). Taken together, RyR1 can be physically coupled to plasmalemmal VDCCs to cause Ca^{2+} release from the SR in response to membrane depolarization without the involvement of extracellular Ca^{2+} influx, leading to contraction in PSMCs.

Collier et al. have reported that VDCCs and RyRs in rabbit bladder SMCs are “loosely coupled,” by which activation of VDCCs following membrane depolarization produces nonobligate Ca^{2+} release from the SR, and sufficient Ca^{2+} influx through VDCCs is required to activate CICR [19]. This CICR via the “loose coupling mechanism” in bladder myocytes has been suggested to be mediated by RyR2 , as it is significantly promoted by FKBP12.6 gene deletion and not affected by RyR3 gene deletion [16]. Supportedly, depolarization-induced Ca^{2+} sparks and waves are reduced in adult $\text{RyR2}^{+/-}$ mouse bladder SMCs [18]. Our current findings that RyR1 mediates Ca^{2+} release from the SR and associated contraction following membrane depolarization through its direct coupling to VDCCs provide an additional mechanism for excitation-contraction coupling in SMCs. In agreement with this concept, membrane depolarization-induced Ca^{2+} sparks are abolished in embryonic $\text{RyR1}^{-/-}$ mouse bladder myocytes [15]. Moreover, a previous study using antisense oligonucleotides indicate that RyR1 is involved in CICR in cultured portal vein myocytes [3].

Previous studies from our laboratory and others have shown that Ca^{2+} release from the SR through IP_3Rs in response to neurotransmitters or other agonists can activate RyRs via a local CICR process to induce further SR Ca^{2+} release, amplifying neurotransmitter- or agonist-evoked Ca^{2+} release and attendant contraction in pulmonary vascular SMCs [4, 7, 9, 10, 20]. Similar pharmacological evidence for RyR -mediated amplification of neurotransmitter- or agonist-induced Ca^{2+} response has also been obtained in systemic vascular myocytes and other type of SMCs as well [21–23]. We have previously found that neurotransmitter-induced increase in $[\text{Ca}^{2+}]_i$ and contraction are both similar in $\text{RyR3}^{+/+}$ and $\text{RyR3}^{-/-}$ mouse PSMCs [4], indicating that this Ca^{2+} release channel is not involved in the local CICR process due to the interaction of IP_3Rs and RyRs. In the present study, we have unveiled that the application of ATP to stimulate purinergic receptors elicits a smaller increase in $[\text{Ca}^{2+}]_i$ in $\text{RyR1}^{-/-}$ than in $\text{RyR1}^{+/+}$ PSMCs in the presence and absence of extracellular Ca^{2+} (Fig. 8). Similarly, ATP-induced increase in $[\text{Ca}^{2+}]_i$ is also attenuated in $\text{RyR1}^{+/-}$ cells. It is known that P2X ionotropic and P2Y metabotropic receptors are both expressed in PSMCs; ATP can stimulate P2X receptors leading to direct extracellular Ca^{2+} influx through the channels, whereas P2Y receptors inducing SR Ca^{2+} release via IP_3Rs [24–26]. Our current study reveals that treatment with TNP-ATP to specifically block P2X , but not P2Y receptors, significantly attenuates ATP-induced increase in $[\text{Ca}^{2+}]_i$ in $\text{RyR1}^{+/+}$ cells; moreover, ATP-evoked responses is further depressed in $\text{RyR1}^{+/-}$ cells following treatment with TNP-ATP. These findings suggest that RyR1 contributes to neurotransmitter-induced Ca^{2+} release from the SR through IP_3Rs in PSMCs. In support of this view, we have found that dialysis of IP_3 to directly activate IP_3Rs induces a much smaller Ca^{2+}

release in RyR1^{+/-} than in RyR1^{+/+} cells. Moreover, neurotransmitter-induced muscle contraction is significantly inhibited in pulmonary arteries from RyR1^{+/-} mice (Fig. 9). Collectively, these novel findings greatly extend previous pharmacological studies, indicating that RyR1 is a unique and important contributor to neurotransmitter-induced Ca²⁺ and contractile responses in pulmonary and also possibly systemic vascular myocytes.

Relative to embryonic RyR1^{+/+} PSMCs, membrane depolarization with high K⁺ induces a much smaller increase in [Ca²⁺]_i in adult RyR1^{+/+} PSMCs under normal extracellular Ca²⁺ conditions. Similarly, high K⁺-evoked Ca²⁺ response is reduced in adult RyR1^{+/+} PSMCs in the absence of extracellular Ca²⁺. In contrast, stimulation of purinergic receptors with ATP results in a similar increase in [Ca²⁺]_i in embryonic and adult RyR1^{+/+} cells. These data, along with the fact that both high K⁺- and ATP-elicited Ca²⁺ responses are blocked in RyR1^{-/-} cells, suggest that the cellular mechanism(s) underlying membrane depolarization-induced Ca²⁺ release may undergo a change with development.

Using pharmacological inhibitors, we and other investigators have demonstrated that the inhibition of RyRs blocks hypoxic increase in [Ca²⁺]_i and attendant contraction in PSMCs [4, 6, 8, 9, 13, 20, 27–30]. Our previous study using RyR3^{-/-} mice further demonstrates that RyR3 plays an important role in hypoxia-induced Ca²⁺ release and contraction in PSMCs [4]. Moreover, we have found that RyR antagonists can further inhibit the hypoxic responses in RyR3^{-/-} PSMCs and have accordingly proposed that RyR1 and/or RyR2 may also participate in the hypoxic responses [4]. Supportedly, in this study, we have revealed that hypoxia induces a much smaller increase in [Ca²⁺]_i in PSMCs from RyR1^{-/-} than that from RyR1^{+/+} mice. Consistently, hypoxic increase in [Ca²⁺]_i is inhibited as well in adult RyR1^{+/-} PSMCs. In the current study, we have also shown that hypoxic muscle contraction in isolated pulmonary arteries is significantly attenuated in RyR1^{+/-} mice relative to RyR1^{+/+} mice (Fig. 10). Together, these data indicate that RyR1, similar to RyR3, is important for hypoxia-mediated increase in [Ca²⁺]_i and contraction in PSMCs.

In conclusion, our findings provide evidence for the important functions of RyR1 as an essential molecular component of RyRs contributing to spontaneous local Ca²⁺ release, as well as membrane depolarization-, neurotransmitter-, and hypoxia-induced global Ca²⁺ release and attendant contraction in vascular (pulmonary artery) SMCs.

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Fig. 1.

Spontaneous local Ca^{2+} release is inhibited in RyR1^{-/-} mouse PSMCs. **a** Original line-scan confocal images show Ca^{2+} sparks in an RyR1^{+/+} and RyR1^{-/-} PSMC in normal Ca^{2+} (1.8 mM) extracellular solution. The images were taken using a Leica TCS SP2 laser-scanning confocal microscope. **b** Bar graph summarizes the spatiotemporal characteristics Ca^{2+} sparks in RyR1^{+/+} and RyR1^{-/-} PSMCs. Numbers in parentheses in the bar graph showing the frequency of Ca^{2+} sparks indicate the number of cells and mice tested; numbers in parentheses in the rest of bar graphs indicate the number of Ca^{2+} sparks. * $P < 0.05$ compared with RyR1^{+/+} cells

Fig. 2.

Caffeine-induced global Ca^{2+} release is reduced in RyR1^{-/-} and RyR1^{+/-} mouse PASMCs. **a** Original recordings show caffeine (*Caf*)-induced increase in fluo-4 fluorescence ($[Ca^{2+}]_i$), determined using a Leica TCS SP2 laser-scanning confocal microscope, in an embryonic RyR1^{+/+} and RyR1^{-/-} cell in the presence of normal extracellular Ca^{2+} . Bar graph summarizes caffeine-induced increase in $[Ca^{2+}]_i$ in RyR1^{+/+} and RyR1^{-/-} cells. Numbers in parentheses indicate the number of cells and mice tested, respectively. * $P < 0.05$ compared with RyR1^{+/+} cells. **b** Caffeine-induced increase in $[Ca^{2+}]_i$, assessed by using a TILL fluorescence imaging system, in adult RyR1^{+/+} and RyR1^{+/-} PASMCs under normal extracellular Ca^{2+} conditions

Fig. 3.

RyR1 protein expression is absent in RyR1^{-/-} mouse PSMCs and reduced in RyR1^{+/-} mouse PSMCs. Original images show an embryonic RyR1^{+/+} and RyR1^{-/-}, as well as adult RyR1^{+/+} and RyR1^{+/-} cell stained with specific anti-RyR1 antibody, followed by Alexa 488-conjugated antibody. Images were taken using a Zeiss LSM510 confocal microscope. Bar graph shows the average intensity of RyR1 immunofluorescence staining in RyR1^{+/+} and RyR1^{+/-} cells. The fluorescence intensity was corrected by subtracting the background signal. **P*<0.05 compared with RyR1^{+/+} cells

Fig. 4. Membrane depolarization-induced increase in $[Ca^{2+}]_i$ is blocked in $RyR1^{-/-}$ mouse PSMCs. **a** Recording traces illustrate an increase in $[Ca^{2+}]_i$ induced by membrane depolarization with high K^+ in an $RyR1^{+/+}$ and $RyR1^{-/-}$ PSMC in the presence of normal extracellular Ca^{2+} . Graph summarizes high K^+ -induced increase in $[Ca^{2+}]_i$ in $RyR1^{+/+}$ and $RyR1^{-/-}$ PSMCs under normal extracellular Ca^{2+} conditions. * $P < 0.05$ compared with $RyR1^{+/+}$ cells; † $P < 0.05$ compared with 50 mM K^+ . **b** Summary of high K^+ -induced increase in $[Ca^{2+}]_i$ in $RyR1^{+/+}$ and $RyR1^{-/-}$ PSMCs in Ca^{2+} -free extracellular medium (nominally Ca^{2+} -free and 0.5 mM EGTA)

Fig. 5. Membrane depolarization-induced increase in $[Ca^{2+}]_i$ is attenuated as well in $RyR1^{+/±}$ mouse PSMCs. **a** Representative recordings exhibit high K^+ -induced increase in $[Ca^{2+}]_i$ in an $RyR1^{+/+}$ and $RyR1^{+/±}$ PSMC in normal Ca^{2+} extracellular solution. Graph shows summary of high K^+ -evoked increase in $[Ca^{2+}]_i$ in $RyR1^{+/+}$ and $RyR1^{+/±}$ PSMCs. * $P < 0.05$ compared with $RyR1^{+/+}$ cells; † $P < 0.05$ compared with 50 mM K^+ . **b** High K^+ -induced increase in $[Ca^{2+}]_i$ in $RyR1^{+/+}$ cells and $RyR1^{+/±}$ as well as $RyR1^{+/±}$ PSMCs pretreated with ryanodine (50 μ M) for 8 min in the presence of normal extracellular Ca^{2+} . **c** Summary of high K^+ -induced increase in $[Ca^{2+}]_i$ in $RyR1^{+/+}$ and $RyR1^{+/±}$ PSMCs in the absence of extracellular Ca^{2+} .

Fig. 6. Voltage-dependent Ca^{2+} currents are unaltered in $RyR1^{+/-}$ mouse PSMCs. Representative current traces show I_{Ca} recorded in an $RyR1^{+/+}$ and $RyR1^{+/-}$ cell before (control, *Ctl*) and after treatment with nifedipine (*Nif*, 1 μM) for 5 min. Graph presents the current-voltage curve of I_{Ca} in $RyR1^{+/+}$ and $RyR1^{+/-}$ PSMCs in normal Ca^{2+} extra-cellular solution

Fig. 7. Membrane depolarization-caused muscle contraction is reduced in $RyR1^{+/-}$ mouse pulmonary arteries. **a** Original recordings show muscle contraction caused by membrane depolarization with high K^+ in an $RyR1^{+/+}$ and $RyR1^{+/-}$ pulmonary artery in the presence of normal Ca^{2+} bath solution. Bar graph summarizes high K^+ -elicited muscle contraction in $RyR1^{+/+}$ and $RyR1^{+/-}$ pulmonary arteries. * $P < 0.05$ compared with $RyR1^{+/+}$ pulmonary arteries. **b** Muscle contraction induced by high K^+ in $RyR1^{+/+}$ and $RyR1^{+/-}$ pulmonary arteries in the absence of extracellular Ca^{2+} .

Fig. 8.

Neurotransmitter-induced Ca^{2+} release is inhibited in RyR1^{-/-} mouse PSMCs. **a** Recordings display an increase in $[Ca^{2+}]_i$ induced by ATP in an RyR1^{+/+} and RyR1^{-/-} PSMC. Graph shows summary of ATP-induced increase in RyR1^{+/+} and RyR1^{-/-} PSMCs. * $P < 0.05$ compared with RyR1^{+/+} cells. **b** ATP-induced increase in $[Ca^{2+}]_i$ in an RyR1^{+/+} and RyR1^{-/-} PSMC in the absence of extracellular Ca^{2+}

Fig. 9. Neurotransmitter-induced Ca^{2+} release and contraction are depressed in RyR1^{+/-} mouse PSMCs. **a** ATP-induced increase in $[Ca^{2+}]_i$ in RyR1^{+/+} and RyR1^{+/-} PSMCs in normal Ca^{2+} PSS. * $P < 0.05$ compared with RyR1^{+/+} cells. **b** IP₃-induced increase in $[Ca^{2+}]_i$ in RyR1^{+/+} and RyR1^{+/-} PSMCs. Dialysis of IP₃ was achieved through the patch pipette using the classic patch clamp technique. Cells were clamped at -55 mV. **c** Muscle contraction elicited by the adrenergic receptor agonist norepinephrine (NE) in RyR1^{+/+} and RyR1^{+/-} pulmonary arteries. * $P < 0.05$ compared with RyR1^{+/+} pulmonary arteries

Fig. 10.

Hypoxic increase in $[Ca^{2+}]_i$ and contraction are diminished in RyR1^{-/-} and RyR1^{+/-} PSMCs. **a** Original recordings of hypoxic increase in $[Ca^{2+}]_i$ in an RyR1^{+/+} and RyR1^{-/-} cell. Bar graph summarizes the effect of hypoxia on $[Ca^{2+}]_i$ in RyR1^{+/+}, RyR1^{-/-}, and RyR1^{+/-} cells. * $P < 0.05$ compared with RyR1^{+/+} cells. **b** Hypoxic vasoconstriction in RyR1^{+/+} and RyR1^{+/-} pulmonary arteries. * $P < 0.05$ compared with RyR1^{+/+} pulmonary arteries