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Conformation of ryanodine receptor-2 gates store-operated calcium entry in rat pulmonary arterial myocytes

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Aims Store-operated Ca^{2+} entry (SOCE) contributes to a multitude of physiological and pathophysiological functions in pulmonary vasculatures. SOCE attributable to inositol 1,4,5-trisphosphate receptor ($InsP_3R$)-gated Ca^{2+} store has been studied extensively, but the role of ryanodine receptor (RyR)-gated store in SOCE remains unclear. The present study aims to delineate the relationship between RyR-gated Ca^{2+} stores and SOCE, and characterize the properties of RyR-gated Ca^{2+} entry in pulmonary artery smooth muscle cells (PASMCs).

Methods and results PASMCs were isolated from intralobar pulmonary arteries of male Wister rats. Application of the RyR1/2 agonist 4-chloro-*m*-cresol (4-CmC) activated robust Ca^{2+} entry in PASMCs. It was blocked by Gd^{3+} and the RyR2 modulator K201 but was unaffected by the RyR1/3 antagonist dantrolene and the $InsP_3R$ inhibitor xestospongin C, suggesting RyR2 is mainly involved in the process. siRNA knockdown of STIM1, TRPC1, and Orai1, or interruption of STIM1 translocation with ML-9 significantly attenuated the 4-CmC-induced SOCE, similar to SOCE induced by thapsigargin. However, depletion of RyR-gated store with caffeine failed to activate Ca^{2+} entry. Inclusion of ryanodine, which itself did not cause Ca^{2+} entry, uncovered caffeine-induced SOCE in a concentration-dependent manner, suggesting binding of ryanodine to RyR is permissive for the process. This Ca^{2+} entry had the same molecular and pharmacological properties of 4-CmC-induced SOCE, and it persisted once activated even after caffeine washout. Measurement of Ca^{2+} in sarcoplasmic reticulum (SR) showed that 4-CmC and caffeine application with or without ryanodine reduced SR Ca^{2+} to similar extent, suggesting store-depletion was not the cause of the discrepancy. Moreover, caffeine/ryanodine and 4-CmC failed to initiate SOCE in cells transfected with the ryanodine-binding deficient mutant RyR2-I4827T.

Conclusions RyR2-gated Ca^{2+} store contributes to SOCE in PASMCs; however, store-depletion alone is insufficient but requires a specific RyR conformation modifiable by ryanodine binding to activate Ca^{2+} entry.

Keywords Store-operated Ca^{2+} entry (SOCE) • Ryanodine receptor (RyR) • Stromal interaction molecule 1 (STIM1) • Canonical transient receptor potential 1 (TRPC1) • Pulmonary arterial smooth muscle cells

1. Introduction

Store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+} entry[1] has been implicated in diverse vascular functions, such as regulation of arterial tone, agonist-induced vasoconstriction, vascular smooth muscle cell (VSMC) proliferation, and vascular remodelling.[2] It is established that vasoactive agonists of G_q-protein-coupled receptors and receptor tyrosine kinases activate phospholipase C, generating inositol 1,4,5-trisphosphate ($InsP_3$) to activate $InsP_3$-receptors ($InsP_3Rs$) for Ca^{2+} release from sarcoplasmic reticulum (SR). The subsequent decrease in SR Ca^{2+} concentration ($[Ca^{2+}]_{SR}$) is detected by the Ca^{2+} sensor stromal interaction molecule (STIM), which oligomerizes and translocates to the SR-plasma membrane (PM) junctions where it couples and activates store-operated Ca^{2+} channels, including Orai and canonical transient receptor potential (TRPC) channels, to mediate Ca^{2+} influx. Despite SR is a continuous interconnected tubular network, SR Ca^{2+} stores in VSMCs are functionally segregated, attributable to the differences in spatial distributions and properties of $InsP_3Rs$, ryanodine

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receptors (RyRs) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCA).[3] Hence, SOCE is likely to be regulated differentially by functionally distinct SR Ca^{2+} stores.

In addition to $InsP_3R$, RyR-gated Ca^{2+} release plays many pivotal roles in Ca^{2+} signalling in VSMCs, including excitation–contraction/pharmaco-mechanical coupling, transcription regulation, and activation of enzymatic processes.[4] Depending on the physiological stimuli, Ca^{2+} release from RyR can cause global elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to activate actin–myosin interaction for vasoconstriction, and generate local Ca^{2+} signals in SR–PM junctions to stimulate Ca^{2+}-activated channels to modulate membrane potential and Ca^{2+} influx via voltage-dependent Ca^{2+} channels. However, the role of RyR-gated stores in SOCE in VSMCs has not been clearly defined. Previous studies have reported caffeine and ryanodine-sensitive SOCE in several cell types[5–8]; but the properties of RyR-mediated SOCE are quite different from the conventional SOCE. For example, robust RyR-sensitive SOCE is present only in embryonic and neonatal cardiac myocytes, but it is absent in adult myocytes,[9] despite $InsP_3$-R-gated SOCE and its machineries STIM1, Orai1, and TRPC channels are all fully operational.[10,11] In skeletal muscle, RyR-sensitive SOCE is dependent on RyR1 and the integrity of the junctional complex, but it is distinguishable from the thapsigargin-induced SOCE.[5,12] A SOCE independent RyR1-mediated 'excitation-coupled Ca^{2+} influx' has also been reported in skeletal muscle.[13] Furthermore, physical association between RyR1 and TRPC channels have been demonstrated in heterologous systems, suggesting conformational coupling can be involved in RyR1-mediated Ca^{2+} entry.[14,15]

In pulmonary arteries (PAs), SOCE is a major Ca^{2+} pathway responsible for vasomotor tone,[16] agonist-induced contraction,[17] hypoxic pulmonary vasoconstriction (HPV),[18] and pulmonary hypertension.[16,19] RyR-dependent Ca^{2+} release is also crucial for agonist-induced contraction, HPV, and spontaneous contraction of chronic hypoxic PAs.[20–23] However, the mechanistic connection between RyR and SOCE in PASMCs has not been established.[24,25] We have previously demonstrated that the three RyR subtypes are differentially expressed in peripheral and perinuclear SR of PASMCs, and are associated with local Ca^{2+} events of distinctive spatial and temporal characteristics.[26–28] RyR-dependent Ca^{2+} events can serve as the signal multiplier for amplifying the $InsP_3$- and NAADP-induced Ca^{2+} response,[20,29] and as the trigger for activation of Ca^{2+}-activated Cl^- channel.[30] However, whether RyR-gated Ca^{2+} store can independently activate SOCE in PASMCs is unclear.[24,25] In the present study, we characterized the physiological and molecular properties of RyR-dependent SOCE in PASMCs. We found that depletion of RyR-sensitive stores alone is insufficient but a specific change of RyR conformation is required for the activation of RyR-gated Ca^{2+} entry. These observations provide novel information on RyR-gated Ca^{2+} entry and suggest a unique mechanism of SOCE activation in pulmonary vasculature.

2. Methods

2.1 PASMC isolation

All animal procedures conformed to the NIH guidelines for the care and use of laboratory animals and approved by the Johns Hopkins Animal Care and Use Committee. Lungs were harvested from male Wistar rats (150–250 g) anaesthetized with sodium pentobarbital (130 mg/kg) intraperitoneally. Intralobar PAs were isolated and de-endothelialized. PASMCs were enzymatically dissociated and cultured transiently (16–24 h) before experiment as described previously.[20]

2.2 Cytosolic and SR Ca^{2+} measurements

Cytosolic and SR $[Ca^{2+}]$ were monitored using fluo-3 AM and fluo-5N AM, respectively.[26,29] Fluorescent dyes were excited at 488 nm and emission light at >515 nm was detected using a Nikon Diaphot microscope equipped with epifluorescence attachments and a microfluorometer. Ca^{2+} transients were recorded and calibrated as previously described.[29]

2.3 Transfection of PASMCs with siRNA

Freshly isolated PASMCs were cultured for 16–18 h and then transiently transfected with siRNA specific for STIM1 (5′-GGGAAGACCUCAAUUACCAUU-3′), TRPC1 (5′-GAAUUUAAGUCGUCUGAAAUU-3′), ORAI1 (5′-AGUUCUUACCGCUCAAGAGGCAGGC-3′), or a random non-silencing sequence with DharmaFECT transfection reagent (Dharmacon) according to manufacturer's instruction. PASMCs were further cultured with transfection mixture in serum-free OptiMEM medium for 48 h before experiment.

2.4 DNA transfection and preparation of HEK293 cell lysates

RyR2 wild-type and RyR-I4827T mutant plasmids were supplied by Dr S.R. Wayne Chen (University of Calgary). HEK293 cells were transfected with RyR2 plasmids using Ca^{2+} phosphate precipitation, and cell lysates were prepared from transfected cells as described previously.[31]

2.5 Immunoblotting analysis

Solubilized protein samples were resolved on a 5–8% (w/v) gradient SDS–PAGE gel and electrotransferred onto a PVDF membrane at 45 V for 20–22 h at 4°C in Towbin buffer with 10% methanol and 0.01% (w/v) SDS. Standard western blot protocol was then followed. Specific primary antibodies against RyR2 (Thermo Scientific, 1:1000), STIM1 (BD Biosciences, 1:1000), TRPC1 (Alomone, 1:1000), Orai1 (Thermo Scientific, 1:1000), or actin (Santa Cruz, 1:5000) were used. The band intensities were normalized with actin.

2.6 Statistical analysis

Data are expressed as means ± SEM. *N* denotes the number of experiments, each represents the response recorded from a group of cells under the field of the fluorescence microscope. Each set of experiments were performed in cells isolated from three or more animals. Statistical significance ($P < 0.05$) was assessed by unpaired Student's *t*-test, one-way ANOVA with Holm–Sidak method for multiple comparison, non-parametric Mann–Whitney Rank Sum Test, or Kruskal–Wallis one-way ANOVA on Ranks with Dunn's test for multiple comparisons wherever applicable.

3. Results

3.1 The RyR agonist, 4-chloro-*m*-cresol (4-CmC), induced Ca^{2+} entry in PASMCs

To study RyR-gated Ca^{2+} entry, the RyR agonist 4-CmC was applied to PASMCs under Ca^{2+} free condition to activate RyR (*Figure 1A*). Ca^{2+} entry was elicited by reintroduction of extracellular Ca^{2+} (2 mmol/L) after 1000 s in the presence of 1 μmol/L nifedipine, the peak and maximum rate of change of Ca^{2+} transient at 1300–1500 s was quantified for comparison. 4-CmC (1 mmol/L) activated a fast robust Ca^{2+} release that last for 100–200 s under Ca^{2+}-free condition and a sustained Ca^{2+} influx ($\Delta[Ca^{2+}]_i$: 246 ± 68 nmol/L, $n = 9$) (*Figure 1C*) upon reintroduction of extracellular Ca^{2+}. The 4-CmC-induced Ca^{2+} influx was inhibited by a low concentration of Gd^{3+} (1 μmol/L; $\Delta[Ca^{2+}]_i$: 65 ± 23 nmol/L, $n = 10$; $P < 0.05$). SOCE was elicited by thapsigargin using the same protocol for comparison (*Figure 1B*). Depletion of SR Ca^{2+} stores of PASMCs with 10 μmol/L thapsigargin activated similar

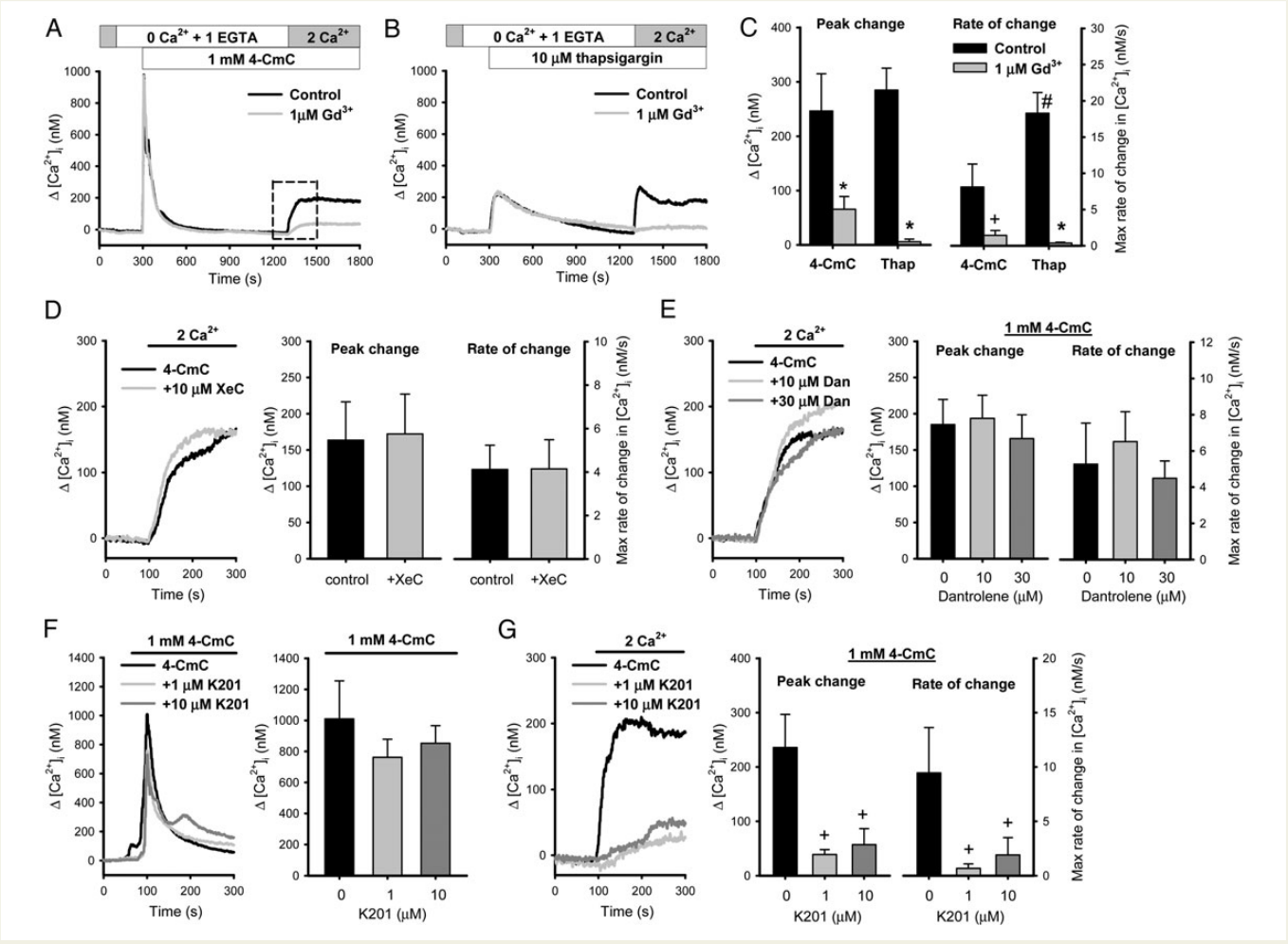

Figure 1 The RyR agonist 4-chloro-*m*-cresol (4-CmC) induced Ca^{2+} entry in PASMCs. Averaged tracings showing (*A*) 4-CmC (1 mmol/L) and (*B*) thapsigargin (10 μmol/L)-induced Ca^{2+} entry in the absence or presence of Gd^{3+} (1 μmol/L). (*C*) The Ca^{2+} entry component (1300–1500 s) quantified as averaged peak and maximum rate of increase of Ca^{2+} transients [$n = 5–10$ experiments from six animals, 'asterisk' indicates $P < 0.05$ vs. control (unpaired *t*-test); 'plus' indicates $P < 0.05$ vs. control, and 'hash' indicates $P < 0.05$ vs. 4-CmC (Mann–Whitney Rank Sum Test)]. (*D*) 4-CmC-induced Ca^{2+} entry in PASMCs with or without pre-incubation with xestospongin C (XeC)(10 μmol/L) ($n = 16$ experiments from eight animals). (*E*) Effect of dantrolene (Dan; 10, 30 μmol/L) on 4-CmC-induced Ca^{2+} influx ($n = 8–11$ experiments from six animals). (*F* and *G*) The effect of K201 (1, 10 μmol/L) on the Ca^{2+} release and Ca^{2+} entry induced by 4-CmC ($n = 8–12$ experiments from five animals, 'plus' indicates $P < 0.05$ using Kruskal–Wallis one-way ANOVA on Ranks).

peak Ca^{2+} entry but with significantly faster rate of increase compared with 4-CmC, and Ca^{2+} entry was abolished by Gd^{3+} (*Figure 1C*).

There is substantial evidence showing cross-talks between $InsP_3R$ and RyR via Ca^{2+}-induced Ca^{2+} release,[20] and depletion of $InsP_3R$-gated store activates SOCE.[1] The possible involvement of $InsP_3R$ in 4-CmC-induced Ca^{2+} entry was examined. Pre-incubation of PASMCs with the $InsP_3R$ inhibitor xestospongin C (10 μmol/L) for 1 h, which effectively inhibits InP_3R-mediated Ca^{2+} mobilization in PASMCs,[29] had no significant effect on the 4-CmC-induced Ca^{2+} influx (*Figure 1D*), suggesting that it is independent of the $InsP_3R$-gated SOCE pathway.

3.2 RyR2 mediates RyR-gated Ca^{2+} entry in PASMCs

RyR1, RyR2, and RyR3 are expressed in PASMCs.[26] Specific RyR agonists and inhibitors were used to identify the RyR subtype involved. Maurocalcine (400 nmol/L), which specifically activates RyR1,[32] failed to induce Ca^{2+} release and Ca^{2+} entry in PASMCs (data not shown, $n = 9$). Dantrolene, which inhibits Ca^{2+} release through RyR1 and RyR3 but not RyR2,[33] had no significant effect on 4-CmC-induced Ca^{2+} entry (*Figure 1E*). In contrast, the anti-arrhythmic drug K201, which increases the affinity of FKBP12.6 (calstabin2) for stabilizing the closed-state of RyR2 at rest,[34,35] had no significant effect on 4-CmC-induced Ca^{2+} release but caused significant inhibition of 4-CmC-induced Ca^{2+} entry (*Figure 1F* and *G*). The peak Ca^{2+} responses were 39 ± 9 and 57 ± 30 nmol/L ($n = 8–11$; $P < 0.05$) at 1 and 10 μmol/L K201, respectively, compared with the control of 236 ± 61 nmol/L ($n = 12$). The rates of increase in $[Ca^{2+}]_i$ were also significantly slower in the presence of K201. These data indicate that RyR2 is the major RyR subtype responsible for the RyR-gated Ca^{2+} entry in PASMCs.

3.3 RyR-gated Ca^{2+} entry is mediated through STIM1, TRPC1, and Orai1

The molecular partners in the RyR-gated Ca^{2+} entry were examined by using siRNA. Transfection of PASMCs with siRNA against STIM1

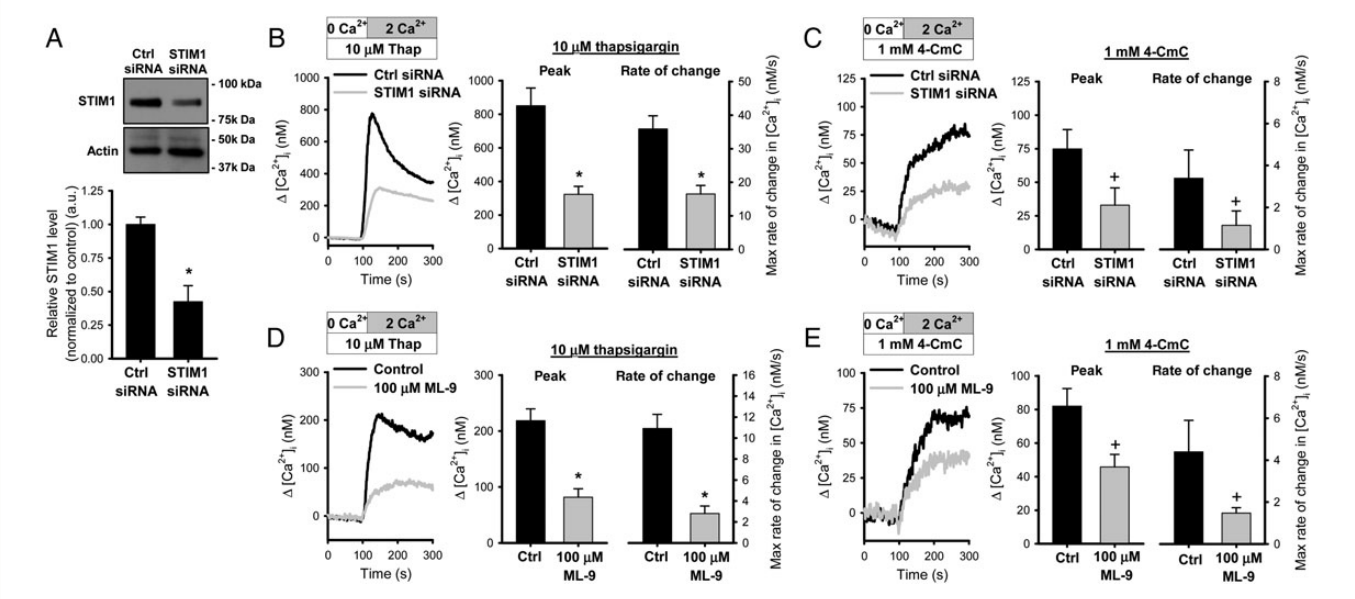

Figure 2 RyR-gated Ca^{2+} entry is mediated by STIM1. (*A*) Western blot and normalized amount of STIM1 protein in PASMCs transfected with STIM1 siRNA or control scrambled siRNA ($n = 5$ experiments from five animals). (*B*) Ca^{2+} influx induced by thapsigargin or (*C*) 4-CmC in PASMCs transfected with siRNA against STIM1 or control siRNA ($n = 7–8$ experiments from five animals). (*D*) Effect of ML-9 (100 μmol/L) on thapsigargin-induced Ca^{2+} entry ($n = 6–7$ experiments from three animals) or (*E*) 4-CmC-induced Ca^{2+} entry ($n = 7–8$ experiments from three animals). 'Asterisk' indicates $P < 0.05$ using unpaired *t*-test and 'plus' indicates $P < 0.05$ using Mann–Whitney Rank Sum Test.

resulted in 58% reduction in STIM1 protein compared with a non-silencing scrambled siRNA ($n = 5$, $P < 0.05$) (*Figure 2A*). The thapsigargin-induced SOCE was significantly attenuated in STIM1 siRNA-transfected cells (*Figure 2B*). 4-CmC-induced Ca^{2+} entry was also reduced in STIM1 siRNA-transfected PASMCs ($n = 8$; $P < 0.05$) (*Figure 2C*). Moreover, interruption of the STIM1 translocation using ML-9 (100 μmol/L)[36] significantly inhibited thapsigargin (*Figure 2D*) and 4-CmC-induced Ca^{2+} entry (*Figure 2E*). Previous studies showed that TRPC1 and Orai1 are store-operated cation channels in PASMCs.[16,37] Their contributions to RyR-gated Ca^{2+} entry were examined. TRPC1 and Orai1 protein expression were reduced by 62% and 53%, respectively, in the siRNA-transfected PASMCs (*Figure 3A* and *D*). Knockdown of TRPC1 or Orai1 caused significant reduction of thapsigargin-induced SOCE (*Figure 3B* and *E*). Moreover, 4-CmC-induced Ca^{2+} entry was significantly attenuated in TRPC1 siRNA-transfected cells (control: 94 ± 20 nmol/L, $n = 10$; TRPC1 siRNA: 40 ± 10 nmol/L, $n = 8$; $P < 0.05$) (*Figure 3C*) and Orai1 siRNA-transfected cells (control: 131 ± 20 nmol/L, $n = 7$; Orai1 siRNA: 61 ± 22 nmol/L, $n = 7$; $P < 0.05$) (*Figure 3F*). siRNA of STIM1, TRPC1, and Orai1 and the blockers had no or little effect on the resting $[Ca^{2+}]_i$ (Supplementary material online, *Figure S1*). These results clearly showed that RyR-gated Ca^{2+} influx activated by 4-CmC share similar properties with thapsigargin-induced SOCE and is mediated through STIM1, TRPC1, and Orai1 in PASMCs.

It is noted that the thapsigargin-induced SOCE was enhanced and the 4-CmC-induced SOCE was reduced in the control siRNA-transfected PASMCs compared with the control PASMCs (*Figure 1*). Examining the pooled data found that the resting $[Ca^{2+}]_i$ was significantly elevated, the thapsigargin-induced Ca^{2+} release was significantly enhanced, while the 4-CmC-induced Ca^{2+} release was reduced in the control siRNA-transfected cells (see Supplementary material online, *Figure S2*). These results suggest that the siRNA transfection procedure have different effects on the thapsigargin- and 4-CmC-sensitive Ca^{2+} stores. There was a report suggested that 4-CmC at 1 mM have a minor inhibitory effect (10–15%) on SERCA activity.[38] The differential changes in the thapsigargin and 4-CmC-induced SOCE in the control siRNA-transfected cells indicates that 4-CmC activates SOCE through a mechanism independent of SERCA inhibition.

3.4 Caffeine induces Ca^{2+} entry only in the presence of ryanodine

In contrast to 4-CmC, application of 5 mmol/L caffeine activated large Ca^{2+} release, but failed to induce SOCE (*Figure 4A*). The inability of caffeine to induce SOCE was not due to insufficient activation of RyR-gated Ca^{2+} store because the amplitudes of Ca^{2+} release induced by caffeine and 4-CmC were comparable (*Figure 4B*). It was also not due to an inhibitory effect of caffeine on SOCE, because caffeine did not alter the thapsigargin-induced SOCE in PASMCs (*Figure 4C*). These results suggest that activation of RyR-gated Ca^{2+} store alone is insufficient to cause RyR-gated Ca^{2+} entry.

Caffeine-induced Ca^{2+} response was further examined in the presence of ryanodine because previous studies had successfully used caffeine and ryanodine simultaneously to activate SOCE in canine renal arterial smooth muscle cells.[25] Ryanodine (50 μmol/L) alone did not induce Ca^{2+} influx, but application of caffeine and ryanodine together caused robust Ca^{2+} entry (*Figure 4D*). Addition of ryanodine 300 s after caffeine application also activated significant Ca^{2+} influx (see Supplementary material online, *Figure S3*) suggesting ryanodine exerts its effect after RyR activation. Inclusion of ryanodine at various concentrations had no significant effect on caffeine-induced Ca^{2+} release (*Figure 4E*), but uncovered caffeine-induced Ca^{2+} influx in a concentration-dependent manner (*Figure 4F*). These data suggest that ryanodine is capable of conferring Ca^{2+} influx to

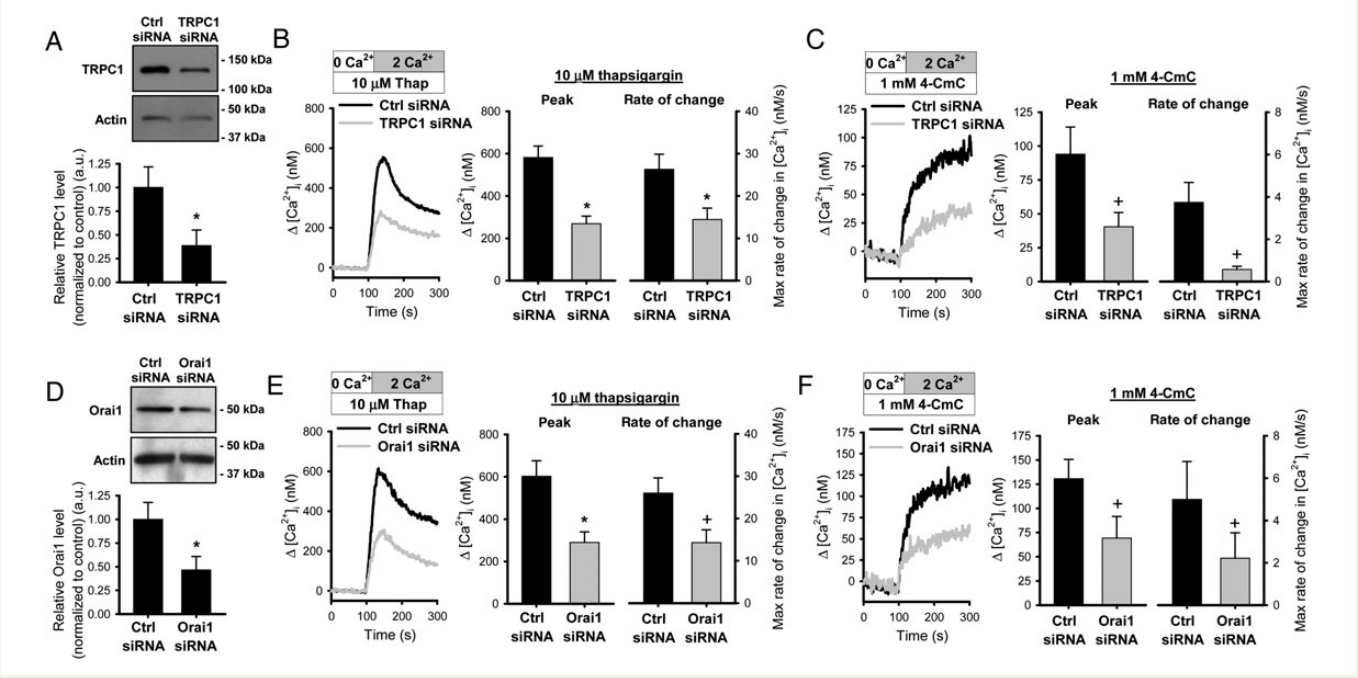

Figure 3 RyR-gated Ca^{2+} entry is mediated by TRPC1 and Orai1. (*A*) Western blot and normalized TRPC1 protein level in PASMCs transfected with TRPC1 siRNA or control siRNA ($n = 7$ experiments from seven animals). (*B*) Ca^{2+} influx induced by thapsigargin or (*C*) by 4-CmC in PASMCs transfected with siRNA against TRPC1 or control siRNA ($n = 8–10$ experiments from three animals). (*D*) Western blot and normalized amount of Orai1 protein in Orai1 siRNA or scrambled control-transfected PASMCs ($n = 7$ experiments from seven animals). (*E*) Ca^{2+} influx induced by thapsigargin or (*F*) by 4-CmC in PASMCs transfected with Orai1-specific or control scrambled siRNA ($n = 7–13$ experiments from five animals). 'Asterisk' indicates $P < 0.05$ using unpaired *t*-test and 'plus' indicates $P < 0.05$ using Mann–Whitney Rank Sum Test.

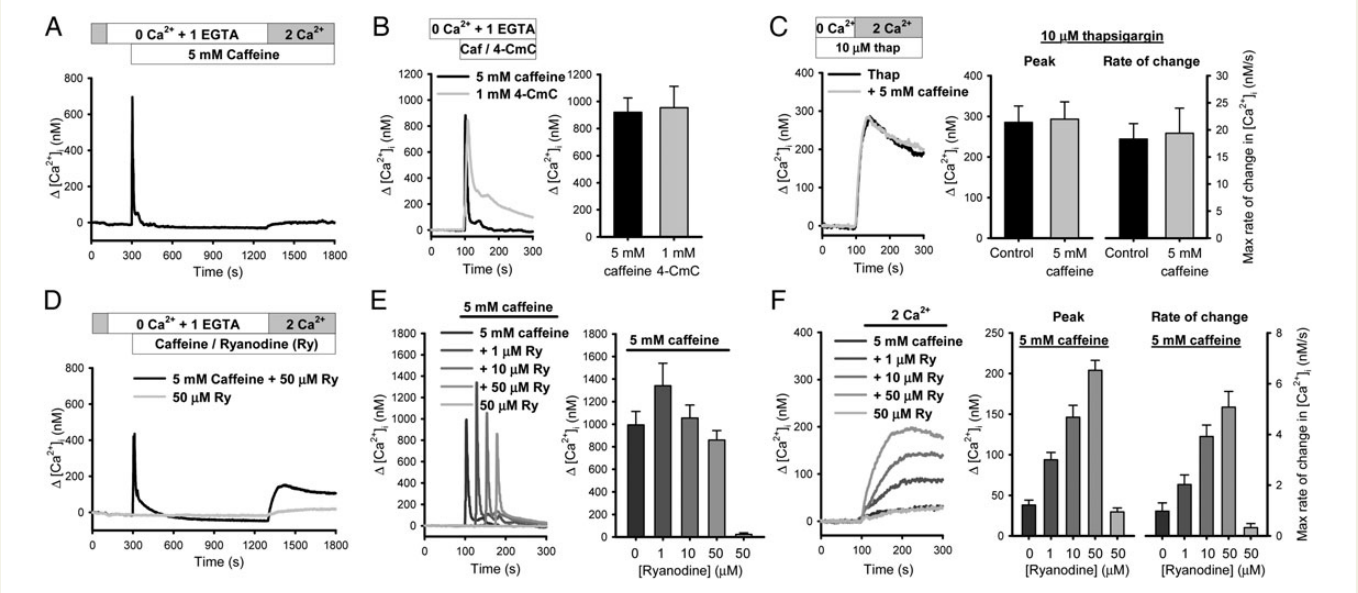

Figure 4 Caffeine induces RyR-gated Ca^{2+} entry only in the presence of ryanodine. (*A*) Caffeine (5 mmol/L) failed to induce Ca^{2+} entry in PASMCs. (*B*) Averaged Ca^{2+} traces and peak $[Ca^{2+}]_i$ values of caffeine and 4-CmC-induced Ca^{2+} release ($n = 10–23$ experiments from 20 animals). (*C*) Caffeine had no inhibitory effect on thapsigargin-induced SOCE ($n = 9$ experiments from three animals). (*D*) Effect of ryanodine (50 μmol/L) and ryanodine+caffeine (5 mmol/L) on Ca^{2+} influx in PASMCs. (*E*) Mean traces and averaged peak values showing the change in $[Ca^{2+}]_i$ during initial Ca^{2+} release activated by 5 mmol/L caffeine (Caf) in the absence or presence of different concentrations of ryanodine (Ry) or by 50 μmol/L ryanodine alone. The traces were shifted by 50 s for better display. (*F*) Mean traces, peak change, and maximum rate of change in $[Ca^{2+}]_i$ evoked by 5 mmol/L caffeine (Caf) in the absence or presence of different concentrations of ryanodine (Ry) or by ryanodine alone ($n = 8–15$ experiments from nine animals).

caffeine-induced RyR activation and the effect is not related to promoting caffeine-induced Ca^{2+} release for further SR Ca^{2+} depletion.

The caffeine-induced Ca^{2+} influx in the presence of 50 μmol/L ryanodine was abolished by Gd^{3+} (*Figure 5A*). Pre-incubation of PASMCs with xestospongin C did not alter the caffeine/ryanodine-induced

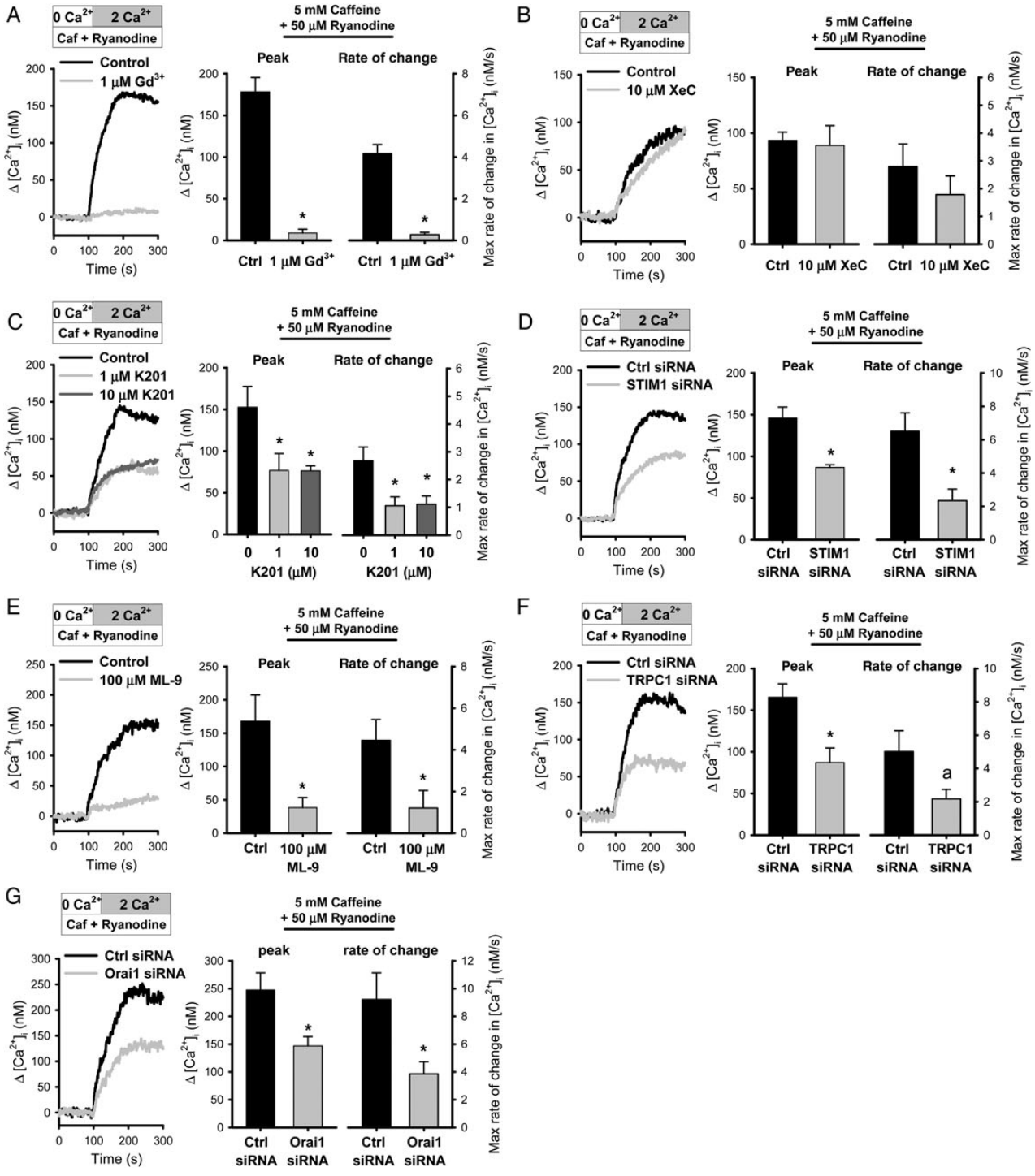

Figure 5 Caffeine and ryanodine activates Ca^{2+} entry with characteristics of RyR-gated Ca^{2+} entry. (*A*) Effect of 1 μmol/L Gd^{3+} on Ca^{2+} influx induced by 5 mmol/L caffeine and 50 μmol/L ryanodine ($n = 8$ experiments from two animals, $^*P \leq 0.001$, unpaired *t*-test). (*B*) Ca^{2+} influx induced by caffeine and ryanodine with or without pre-incubation with 10 μmol/L xestospongin C (XeC) ($n = 6–7$ experiments from three animals). (*C*) Inhibition of caffeine/ryanodine-induced Ca^{2+} entry by K201 (1, 10 μmol/L) ($n = 8–9$, $^*P < 0.05$, one-way ANOVA). (*D*) Ca^{2+} influx induced by caffeine/ryanodine in PASMCs transfected with siRNA against STIM1 ($n = 8–9$ experiments from four animals, $^*P < 0.05$, unpaired *t*-test). (*E*) Effect of interruption of STIM1 translocation by ML-9 (100 μmol/L) on caffeine/ryanodine-induced Ca^{2+} entry ($n = 6$ experiments from two animals, $^*P < 0.05$, unpaired *t*-test). (*F*) Ca^{2+} influx induced by caffeine/ryanodine in PASMCs transfected with siRNA against TRPC1, (*G*) Orai1 or control scrambled siRNA ($n = 6–9$ experiments from three animals, 'asterisk' indicates $P < 0.05$ and 'a' indicates $P = 0.6$ unpaired *t*-test).

response (*Figure 5B*). K201, which had no effect on the Ca^{2+} release, significantly attenuated Ca^{2+} influx activated by caffeine and ryanodine ($n = 8–9$, $P < 0.05$)(*Figure 5C*). Moreover, transfection of PASMCs with siRNA against STIM1 and interruption of STIM1 translocation with ML-9 significantly attenuated the Ca^{2+} entry (*Figure 5D* and *E*). Caffeine/ryanodine-induced Ca^{2+} entry was also significantly inhibited in TRPC1 siRNA and Orai1 siRNA-transfected cells (*Figure 5F* and *G*). These results demonstrated that the molecular and pharmacological properties of caffeine/ryanodine-induced Ca^{2+} entry are similar to the RyR-gated Ca^{2+} entry activated by 4-CmC.

3.5 Caffeine-induced store-depletion was insufficient for activation of RyR-gated Ca^{2+} entry

To examine agonist-induced SR Ca^{2+} depletion, the intra-SR Ca^{2+} content was measured using the low affinity Ca^{2+} dye Fluo-5N-AM.[39] 4-CmC caused a rapid sustained reduction in Fluo-5N signal indicating Ca^{2+} depletion in the RyR-gated SR Ca^{2+} stores (*Figure 6A*). Application of caffeine with/without ryanodine under Ca^{2+}-free condition caused a transient increase in Fluo-5N signal presumably due to an increase in local Ca^{2+} microdomains detected by the residual cytosolic Fluo-5N (*Figure 6B*). SR store depletion was revealed by the subsequent fall in Fluo-5N signal (*Figure 6C*). SR Ca^{2+} content was reduced to the same level as 4-CmC by caffeine in the absence or presence of ryanodine, when quantified at 1000 s for comparison ($\Delta F/F_0$ of 4-CmC: 0.48 ± 0.06, $n = 5$; caffeine: 0.42 ± 0.04, $n = 7$; caffeine + ryanodine: 0.43 ± 0.05, $n = 5$; $P = 0.67$).

In another set of experiment, we tested if RyR locked by ryanodine in a subconductance state[40] can activate Ca^{2+} influx. A short 10 s-pulse of caffeine was applied to PASMCs pre-treated with (10 min) and in the continuous presence of 50 μmol/L ryanodine and 2 mmol/L Ca^{2+}. The caffeine-pulse induced a Ca^{2+} transient with a peak followed by a sustained plateau phase ($\Delta[Ca^{2+}]_i$: 69.9 ± 26.9 nmol/L, $n = 6$) (*Figure 6D* and *E*), which persisted long after caffeine was washed out. The plateau phase was abolished by the removal of external Ca^{2+} and was recovered after reintroduction of Ca^{2+}, showing that it was exclusively caused by Ca^{2+} entry. The same caffeine-pulse in the absence of ryanodine activated comparable peak Ca^{2+} release,

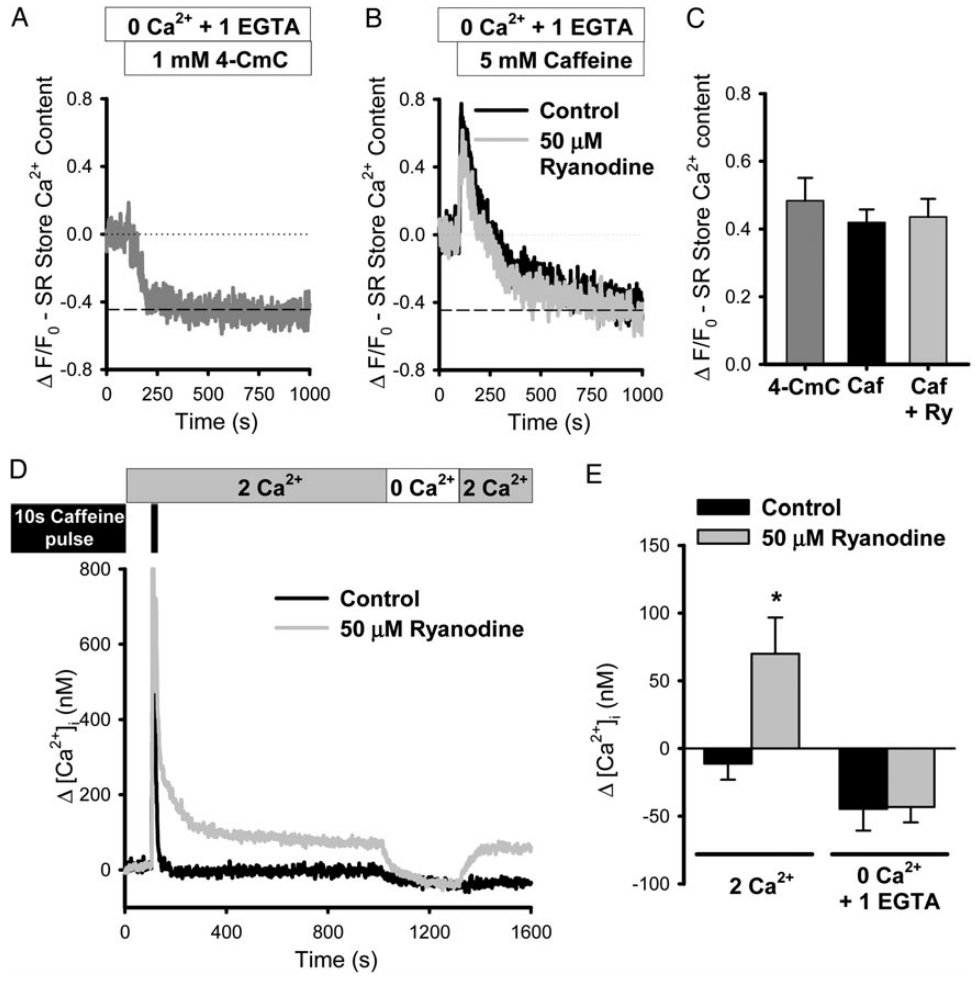

Figure 6 Store release is insufficient for activation of RyR-gated Ca^{2+} entry. (*A*) Averaged luminal $[Ca^{2+}]_{SR}$ traces showing store depletion by 4-CmC, or (*B*) by caffeine in the absence or presence of ryanodine. (*C*) Mean reduction in $[Ca^{2+}]_{SR}$ signal caused by 4-CmC and caffeine in the presence or absence of ryanodine measured at 900–1000 s after application ($n = 5–7$ experiments from four animals). (*D*) Measurement of cytosolic $[Ca^{2+}]_i$ after application of a 10 s-pulse of caffeine to PASMCs superfused with Ca^{2+}-containing solution with/without ryanodine. (*E*) Mean values of change in $[Ca^{2+}]_i$ during Ca^{2+} entry (700–800 s) and after removal of external Ca^{2+} (1200–1300 s) ($n = 6$ experiments from four animals, $^*P < 0.05$, unpaired *t*-test).

but failed to activate a sustained Ca^{2+} response. Collectively, these data suggest that depletion of RyR-gated store with caffeine alone is insufficient to induce RyR-gated Ca^{2+} entry, but requires a specific change of RyR conformation that can be achieved through ryanodine binding.

3.6 The I4827T mutation on RyR2 abolished RyR-gated Ca^{2+} entry

To further test the concept that ryanodine binding modifies RyR to allow activation of Ca^{2+} influx, wild-type RyR2 (WT) and RyR2-I4827T mutant was transfected into HEK293 cells. RyR2-I4827T is a ryanodine-binding deficient mutant, which was shown to have no detectable [^{3}H] ryanodine binding.[41] WT and the mutant RyR2-transfected cells had similar RyR2 expression level, which was 2–3 times higher than that of untransfected control HEK293 cells ($n = 8–10$, $P < 0.05$) (*Figure 7A*). Application of caffeine together with ryanodine or 4-CmC caused similar Ca^{2+} release in HEK293 cells transfected with RyR2-WT and RyR2-I4827T, but did not cause significant Ca^{2+} release in the untransfected control (*Figure 7B*). This indicated functional RyRs were expressed and the SR Ca^{2+} stores were comparable in the WT and mutant RyR overexpressed cells. In contrast, caffeine/ryanodine activated Ca^{2+} entry with an averaged peak of 97 ± 18 nmol/L in RyR2-WT transfected cells ($n = 7$), but the Ca^{2+} entry was minimal in RyR2-I4827T mutant cells (27 ± 4 nmol/L, $n = 6$; $P < 0.05$) (*Figure 7C*). Furthermore, 4-CmC activated a small SOCE in the RyR2-WT transfected cells, and the RyR-gated SOCE was significantly attenuated in the RyR2-I4827T-transfected cells ($\Delta[Ca^{2+}]_i$ of WT: 21 ± 3 nmol/L, $n = 11$; RyR2-I4827T: 3 ± 2 nmol/L, $n = 8$; $P \leq 0.001$; *Figure 7D*). These data indicate that the I4827 residue of RyR is important for the gating of Ca^{2+} influx by the RyR-activating agents.

It is noted that 4-CmC activated a much smaller SOCE compared with caffeine and ryanodine in the RyR2-WT transfected HEK293 cells, despite Ca^{2+} release activated by both methods were similar. The reason for the discrepancy is unclear. A recent study suggested that 4-CmC may partially inhibit Orai-mediated Ca^{2+} entry,[42] which could be an important component of SOCE in the HEK293 cells.

4. Discussion

The present study characterized the functional and molecular properties of RyR-gated Ca^{2+} entry in PASMCs. The major findings are (i) the RyR agonist 4-CmC is capable of activating RyR-gated Ca^{2+} influx; (ii) the RyR-gated Ca^{2+} influx is mediated mainly through RyR2; (iii) the physiological and pharmacological properties of RyR-gated Ca^{2+} influx is indistinguishable from SOCE activated by thapsigargin; (iv) depletion of RyR-sensitive Ca^{2+} store with caffeine alone is insufficient to activate Ca^{2+} influx; (v) addition of ryanodine together with caffeine restored RyR-gated Ca^{2+} influx without altering SR Ca^{2+} depletion; and (vi) mutation of the I4827 residual of RyR2 abolished RyR-gated Ca^{2+} entry. These results revealed the unique property of RyR-gated Ca^{2+} entry

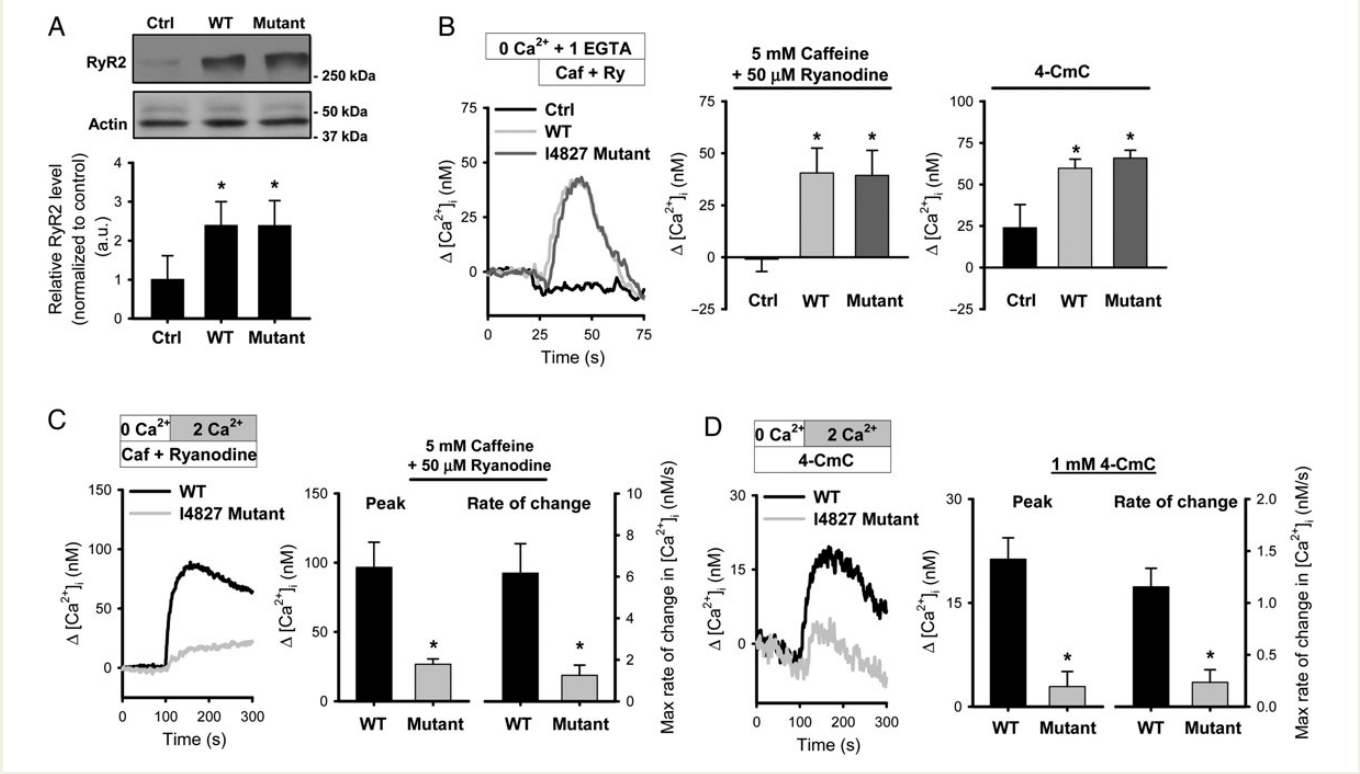

Figure 7 Effect of RyR2-I4827T mutant on RyR-gated SOCE. (*A*) Western blot and normalized amount of RyR2 protein in control and HEK293 cells transfected with RyR2-wild-type (WT) or RyR2-I4827T mutant ($n = 8–10$, $^*P < 0.05$, one-way ANOVA). (*B*) Caffeine in the presence of ryanodine-activated Ca^{2+} release in RyR2-WT and RyR2-I4827T mutant-transfected HEK293 cells, but not in untransfected control. (Middle and right panels) Averaged peak change in $[Ca^{2+}]_i$ activated by caffeine and 4-CmC, respectively, in control, WT, or mutant ($n = 6–7$, $^*P < 0.05$, one-way ANOVA). (*C*) Averaged Ca^{2+} traces showing Ca^{2+} entry activated by caffeine and ryanodine, and (*D*) by 4-CmC in HEK293 cells expressing RyR2-WT or RyR2-I4827T mutant ($n = 6–11$, $^*P < 0.05$, unpaired *t*-test).

in rat PASMCs that its activation requires both depletion of RyR-gated Ca^{2+} stores and a specific change of RyR conformation.

This study provided the direct evidence that activation of RyR with 4-CmC is capable of stimulating Ca^{2+} influx in PASMCs. This is in concordance with the reports that depletion of RyR-gated Ca^{2+} stores contributes to SOCE in skeletal muscle, lymphocytes, and airway smooth muscle.[5–8] This is also consistent with the observations that the endogenous RyR agonist cyclic-ADP-ribose (cADPR) activated Ca^{2+} influx in human myometrial cells and in RyR1 and TRPC3 co-expressing HEK293 cells; and the cADPR inhibitor 8-Br-cADPR attenuated Ca^{2+} influx induced by endothelin-1 in myometrial cells.[7,15] However, it is in contrast to the early study in canine PASMCs that depletion of both $InsP_3R$- and RyR-sensitive Ca^{2+} stores are required to elicit SOCE.[30,31] Since vasoactive agonist was not used in our experiments and inhibition of $InsP_3R$ with xestospongin C did not affect RyR-mediated Ca^{2+} entry, our results clearly show that stimulation of RyR can independently activate SOCE in rat PASMCs.

RyR-gated Ca^{2+} influx in PASMCs is mediated by RyR2. It is deduced from the facts that the RyR1 and RyR2 agonist 4-CmC[43,44] activated robust Ca^{2+} entry, but the RyR1 agonist maurocalcine[32] failed to stimulate Ca^{2+} entry; and the RyR1 and RyR3 antagonist dantrolene[33] had no effect, while K201 which stabilizes RyR2[34] abolished the 4-CmC induced Ca^{2+} influx. Our previous studies showed that all three RyRs are expressed in PASMCs with RyR2 being the predominant subtype.[26] RyR2 is expressed in the peripheral SR close to the sarcolemmal membrane, RyR3 is localized in perinuclear regions, and RyR1 is expressed in both peripheral and perinuclear SR.[26] The close proximity of RyR2-gated SR with plasma membrane is therefore essential for the SR–PM junctional interactions for RyR-gated Ca^{2+} entry. Recent studies from other groups showed RyR1 localization in the subsarcolemal regions of PASMCs,[27,28] and may perform other physiological functions including interactions with stretch-activated cation channels in PM.[28] It is noteworthy that the highly abundant RyR2s in the dyadic junctions of adult cardiac myocytes are not involved in SOCE despite $InsP_3R$-gated SOCE is intact.[9–11] Hence, the molecular compositions and functional properties of the SR–PM junctions of PASMCs and cardiac myocytes are clearly different despite their SRs are both gated by RyR2.

The RyR2-gated Ca^{2+} influx observed in the present study has similar pharmacological and molecular properties of conventional SOCE in PASMCs activated by depletion of SR using thapsigargin or cyclopiazonic acid. It was inhibited by lanthanides including Gd^{3+} and La^{3+}, blocked by ML-9,[36] and attenuated by siRNA knockdown of STIM1, TRPC1, and Orai1. The requirement of the SR Ca^{2+} sensor STIM1 in RyR-gated Ca^{2+} entry clearly indicates that it is indeed a component of SOCE in PASMCs.[37,45,46] The participation of TRPC1 in SOCE is consistent with previous studies from several laboratories using TRPC1 specific siRNA,[16] blocking antibody,[45] and TRPC1 overexpression.[17] Involvement of Orai1 in SOCE in PASMCs has also been established.[37,46] However, RyR-gated Ca^{2+} entry in PASMCs can be distinguished from conventional SOCE, because depletion of RyR-gated Ca^{2+} stores alone is insufficient to activate Ca^{2+} influx. Continuous activation of RyRs with caffeine failed to stimulate Ca^{2+} influx despite $[Ca^{2+}]_{SR}$ was reduced to the same level caused by 4-CmC. Furthermore, ryanodine, which itself does not activate SOCE, restores the ability of caffeine to induce Ca^{2+} influx without promoting Ca^{2+} release or causing further reduction of $[Ca^{2+}]_{SR}$. These results suggest that other factors in addition to SR depletion are required for the activation of RyR-gated Ca^{2+} entry.

The inability of caffeine to activate SOCE could not be explained by the interference of the STIM1, TRPC1, and/or Orai channels, because caffeine does not affect thapsigargin-induced SOCE in rat PASMCs. Rather, the differential effects of caffeine and 4-CmC on activating Ca^{2+} entry may underscore the importance of ligand-dependent conformational change of RyRs in the process. It is known that 4-CmC and caffeine bind to different sites of RyR.[43,47] Substitution of specific residuals in the C-terminus of RyR that renders 4-CmC ineffective has no effect on caffeine-induced Ca^{2+} release. Caffeine and 4-CmC also induce different conformational changes of RyR.[48,49] FRET-analysis of the interaction between the amino-terminal and the central region of RyR2 which is crucial for the channel gating showed that activation of RyR with caffeine enhanced, whereas 4-CmC and other physiological activators of RyR, including ATP and Ca^{2+}, decreased the domain–domain interactions.[48] Another study showed that caffeine caused conformational change in the clamp region of RyR2, but 4-CmC and Ca^{2+} did not.[49] Hence, 4-CmC and other physiological activators may confer a RyR conformation that facilitates RyR-gated Ca^{2+} entry.

The notion of specific RyR conformational change is required for gating Ca^{2+} influx is supported by the restoration of caffeine-induced Ca^{2+} influx by ryanodine without causing further reduction of $[Ca^{2+}]_{SR}$. Ryanodine binds to opened RyRs and locks the channels in a stable subconductance state which presents a large energy barrier for RyR closing.[40] Interestingly, it has been shown that ryanodine alone does not affect the N-terminal and the central domain interaction of RyRs, but the binding of ryanodine to RyR2 activated by caffeine reverts the domain–domain interaction in the direction similar to that induced by 4-CmC or by ATP and Ca^{2+}.[48] The conformational change of RyR induced by RyR and caffeine persisted after caffeine washout.[48] This is coherent with our finding that ryanodine by itself does not activate RyR-gated SOCE in PASMCs, but ryanodine in the presence of caffeine activates Ca^{2+} influx similar to those elicited by 4-CmC. The persistent Ca^{2+} influx observed long after caffeine washout is also consistent with the stable binding of ryanodine maintains the RyR conformation required for Ca^{2+} influx. Furthermore, the abolition of caffeine/ryanodine-activated Ca^{2+} influx in the RyR2-I4827T overexpressed cells which exhibited normal caffeine/ryanodine-induced Ca^{2+} release provides supportive evidence that ryanodine binding facilitates RyR-gated Ca^{2+} entry independent of RyR activation and SR Ca^{2+} release.

The requirement of both SR Ca^{2+} depletion and conformational change of RyR for the induction of Ca^{2+} entry in PASMCs suggests physical association of RyR to the SOCE machinery. It has been shown that the foot-structure (N-terminus cytoplasmic domains) of heterologously expressed RyR1 is required for caffeine-induced Ca^{2+} entry in CHO cells.[14] Co-immunoprecipitation of overexpressed RyR1 and TRPC3; and colocalization of the RyR2 and STIM1 had been reported in HEK293 cells.[15,50] Our study also shows that substitution of RyR2-I4827 disrupts RyR-gated Ca^{2+} entry. These observations suggest conformational coupling involving RyR may be an essential step of RyR-gated SOCE. Conformational coupling was proposed previously as one of the models of $InsP_3R$-gated SOCE,[51] but the attention was shifted after the discovery of STIM1 in SOCE. It is proposed more recently that the Homer proteins may act as the physical link between $InsP_3R$ and TRPC channels keeping the channels in the closed state; and dissociation of the TRPCs–Homer–$InsP_3R$ complex allows STIM1 to access and gate-open the TRPC channels for Ca^{2+} entry.[52,53] Since Homer proteins also bind and regulate RyRs,[54,55] similar mechanism may participate in RyR-gated SOCE. Moreover, other RyR-associated proteins in the SR–PM junctional complex may be also

involved. The intricate activation model of RyR-gated Ca^{2+} entry warrants further investigations.

RyR-gated SOCE may play specific roles in pulmonary vascular functions. Previous studies have established that SOCE is an important mechanism for the activation of HPV,[18] which can be effectively blocked by inhibiting RyR-gated Ca^{2+} stores.[21–23] RyR-gated SOCE can contribute to vasoactive agonist-induced Ca^{2+} response due to RyR activation by the endogenous RyR ligand cADPR and Ca^{2+}-induced Ca^{2+} release.[20,29] Furthermore, SOCE is known to play an essential role in enhanced pulmonary vascular tone, PASMC proliferation and migration, and vascular remodelling during the development of pulmonary hypertension.[16,19] However, it is unclear whether the RyR-gated and $InsP_3R$-gated SOCE operate independently or interdependently in these specific physiological processes. In conclusion, the present study identified and characterized a unique mode of Ca^{2+} entry gated by ryanodine-sensitive Ca^{2+} stores in PASMCs. Since SOCE and RyR-dependent Ca^{2+} pathways are known to play crucial roles in diverse vascular functions, new information on the mechanistic interactions between these two important processes may provide novel insights for the physiological and pathophysiological regulation of pulmonary and perhaps other vasculatures.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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