

Orai1 and TRPC1 Proteins Co-localize with Ca_v1.2 Channels to Form a Signal Complex in Vascular Smooth Muscle Cells*

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Voltage-dependent Ca_v1.2 L-type Ca²⁺ channels (LTCC) are the main route for calcium entry in vascular smooth muscle cells (VSMC). Several studies have also determined the relevant role of store-operated Ca²⁺ channels (SOCC) in vascular tone regulation. Nevertheless, the role of Orai1- and TRPC1-dependent SOCC in vascular tone regulation and their possible interaction with Ca_v1.2 are still unknown. The current study sought to characterize the co-activation of SOCC and LTCC upon stimulation by agonists, and to determine the possible crosstalk between Orai1, TRPC1, and Ca_v1.2. Aorta rings and isolated VSMC obtained from wild type or smooth muscle-selective conditional Ca_v1.2 knock-out (Ca_v1.2^{KO}) mice were used to study vascular contractility, intracellular Ca²⁺ mobilization, and distribution of ion channels. We found that serotonin (5-HT) or store depletion with thapsigargin (TG) enhanced intracellular free Ca²⁺ concentration ([Ca²⁺]_i) and stimulated aorta contraction. These responses were sensitive to LTCC and SOCC inhibitors. Also, 5-HT- and TG-induced responses were significantly attenuated in Ca_v1.2^{KO} mice. Furthermore, hyperpolarization induced with cromakalim or valinomyacin significantly reduced both 5-HT and TG responses, whereas these responses were enhanced with LTCC agonist Bay-K-8644. Interestingly, *in situ* proximity ligation assay revealed that Ca_v1.2 interacts with Orai1 and TRPC1 in untreated VSMC. These interactions enhanced significantly after stimulation of cells with 5-HT and TG. Therefore, these data indicate for the first time a functional interaction between Orai1, TRPC1, and Ca_v1.2 channels in VSMC, confirming that upon agonist stimulation, vessel contraction involves Ca²⁺ entry due to co-activation of Orai1- and TRPC1-dependent SOCC and LTCC.

Vasoactive agonists are known to promote vessel contraction by a rise in intracellular free Ca²⁺ concentration ([Ca²⁺]_i). This increase in [Ca²⁺]_i has been classically considered to occur first,

due to a rapid Ca²⁺ release from sarcoplasmic reticulum (SR)² stimulated by inositol 1,4,5-trisphosphate (InsP₃), and then to a transmembrane Ca²⁺ influx through L-type Ca²⁺ channels (LTCC), especially Ca_v1.2 channels, which are the main path for Ca²⁺ entry responsible for the excitation-contraction coupling process in excitable vascular smooth muscle cells (VSMC) (1). Other voltage-independent channels are also involved in transmembrane Ca²⁺ influx, such as store-operated Ca²⁺ channels (SOCC) responsible of extracellular Ca²⁺ entry, known as store-operated Ca²⁺ entry (SOCE) (2, 3). SOCC have been characterized both in freshly dispersed and in primary cultured VSMC from systemic and resistance vessels (4–6). It is well established that SOCE is mainly due to the activation of the Ca²⁺-sensing regulatory protein stromal interaction molecule 1 (STIM1) and Orai1, the pore-forming subunit of SOCC in a wide range of non-excitabile cells (7). Additionally, Orai1 was suggested to form a non-Ca²⁺ selective SOCC due to its association with TRPC1 in excitable cells (8, 9). Interestingly, evidence showed that TRPC1, Orai1, and Ca_v1.2 might interact with the proteins of other channels to form a signal complex in VSMC (10–12).

Taking into consideration that Ca²⁺ enters mainly through LTCC in VSMC, further understanding of how Orai1 and TRPC1 might influence the role of LTCC in Ca²⁺ signaling and contractility is needed to explain the physiological role of SOCC in vessel contraction, which still remains under debate (13). We hypothesized that Ca²⁺ release from the SR, induced by a vasoactive agonist such as serotonin (5-HT), could activate SOCE, leading to depolarization of the VSMC, which would stimulate LTCC. Therefore, the main aims of this study were first, to investigate whether SOCC activity might substitute LTCC function in VSMC, and second, to determine the endogenous distribution of Orai1, TRPC1, and Ca_v1.2 in VSMC.

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² The abbreviations used are: SR, sarcoplasmic reticulum; LTCC, L-type Ca²⁺ channel(s); SOCC, store-operated Ca²⁺ channel(s); SOCE, store-operated Ca²⁺ entry; VSMC, vascular smooth muscle cell(s); InsP₃, inositol trisphosphate; 2APB, 2-aminoethoxydiphenyl borate; 5-HT, serotonin; BayK, Bay-K-8644; GSK, GSK-7975A; ML-9, 1-(5-chloronaphthalen-1-yl) sulfonyl-1,4-diazepane; PLA, proximity ligation assay; SERCA, sarco-endoplasmic reticulum calcium ATPase; TG, thapsigargin; PS, physiological solution; SMA, smooth muscle actin; Nif, nifedipine; Caf, caffeine; Crom, cromakalim; WT, wild type; Gd³⁺, gadolinium.

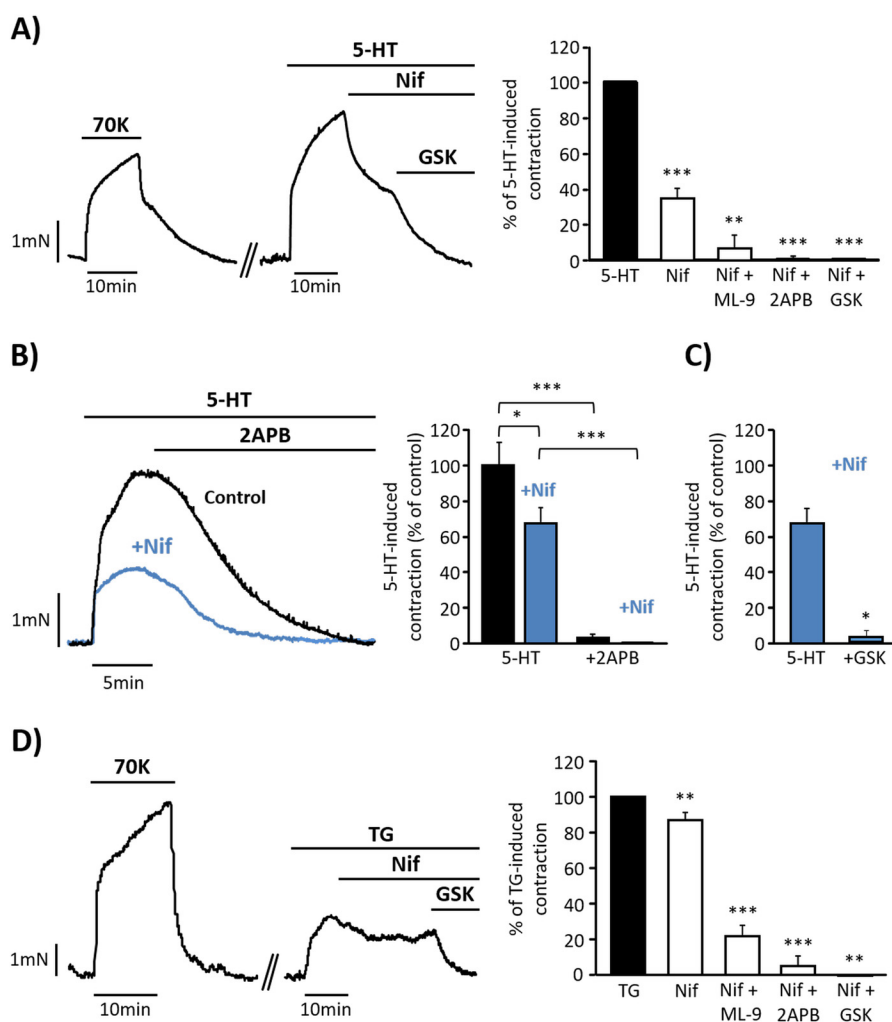


FIGURE 1. **Isometric contractions induced by serotonin and thapsigargin in mouse endothelium-denuded aorta.** *A*, representative recording showing high KCl (70 mM; 70K) and 5-HT (10 μM) evoked contractions in endothelium-denuded mouse aorta. The cumulative addition of nifedipine (Nif, 1 μM) and GSK-7975A (GSK, 10 μM) fully relaxes 5-HT-induced contraction. GSK-7975A was substituted by 2APB (50 μM) or ML-9 (25 μM) in other experiments to inhibit SOCC. The right panel shows a data summary of the effects of Nif (34.08% ± 4.25; *n* = 16), Nif + ML-9 (7.28% ± 7.28; *n* = 3), Nif + 2APB (0.87% ± 0.65; *n* = 8), and Nif + GSK (0.57% ± 0.57; *n* = 4) on 5-HT-induced contraction (control; *n* = 17). *B*, representative traces and data summary showing 5-HT- (10 μM) induced contraction in control rings (black bar and trace; 100% ± 13.01; *n* = 10) and rings pre-treated with 1 μM Nif for 10 min (blue trace and bars; 69.30% ± 8.45; *n* = 16). 50 μM 2APB was added as indicated in control (3.13% ± 2.40) and in rings pretreated with nifedipine (0.18% ± 0.13). Values were normalized to 70K responses. *C*, data summary showing 5-HT- (10 μM) induced contraction in rings pre-treated with 1 μM nifedipine (69.31% ± 8.45; *n* = 16) and after GSK-7975A (10 μM) administration in rings pretreated with Nif (3.20% ± 3.20; *n* = 3). *D*, representative recording and data summary of 10 μM TG- (*n* = 20) elicited aorta contraction. 1 μM Nif (86.05% ± 3.84; *n* = 22) was added followed by GSK (0% ± 0; *n* = 3), 50 μM 2APB (5.37% ± 5.37; *n* = 7), or 50 μM ML-9 (21.97% ± 6.20; *n* = 12). Values are the percentage of mean ± S.E. *, *p* < 0.05, **, *p* < 0.01 and ***, *p* < 0.001.

Results

Agonist-induced Vasoconstriction Involves Ca²⁺ Entry through SOCC and LTCC in Endothelium-denuded Mouse Aorta—The role of SOCC and LTCC in contractile responses of aorta was studied using 5-HT as vasoactive agonist. Fig. 1A shows that 5-HT (10 μM) evoked a potent vasoconstriction in endothelium-denuded aorta, which was partially inhibited by nifedipine (1 μM), a specific inhibitor of LTCC in VSMC (14). The cumulative addition of GSK-7975A (10 μM), considered a specific inhibitor of Orai1 (15), further produced the complete relaxation of the vessel. Similar effects were also observed when other less specific inhibitors of SOCC, 2APB (50 μM) or ML-9 (25 μM) (3), were added after nifedipine, as summarized in Fig. 1A. Additionally, pre-treatment of aortic rings with 1 μM nifedipine attenuated but did not prevent 5-HT responses (Fig. 1, B and C); meanwhile the supplementary addition of 2APB (50

μM) or GSK-7975A (10 μM, Fig. 1C) produced the complete relaxation of the vessel. As shown in Fig. 1B, the addition of 2APB alone (50 μM) promoted the full relaxation of 5-HT-induced contraction, in contrast to the effect of nifedipine (Fig. 1A). Interestingly, the specific activation of SOCC with thapsigargin (TG, 10 μM), a SERCA inhibitor (16), evoked a nifedipine- (1 μM) sensitive vasoconstriction, although the inhibitory effect of nifedipine was smaller in comparison with its effect on 5-HT responses (Fig. 1D). Further relaxation was also produced by the addition of GSK-7975A (10 μM), 2APB (50 μM), or ML-9 (50 μM), indicating that TG-induced vasoconstriction involves LTCC and SOCC co-activation.

In experiments performed in isolated VSMC, administration of 5-HT (10 μM), applied in the continuous presence of extracellular Ca²⁺, evoked a transient followed by a sustained elevation of [Ca²⁺]_i (Fig. 2A). Both fast and sustained 5-HT-induced

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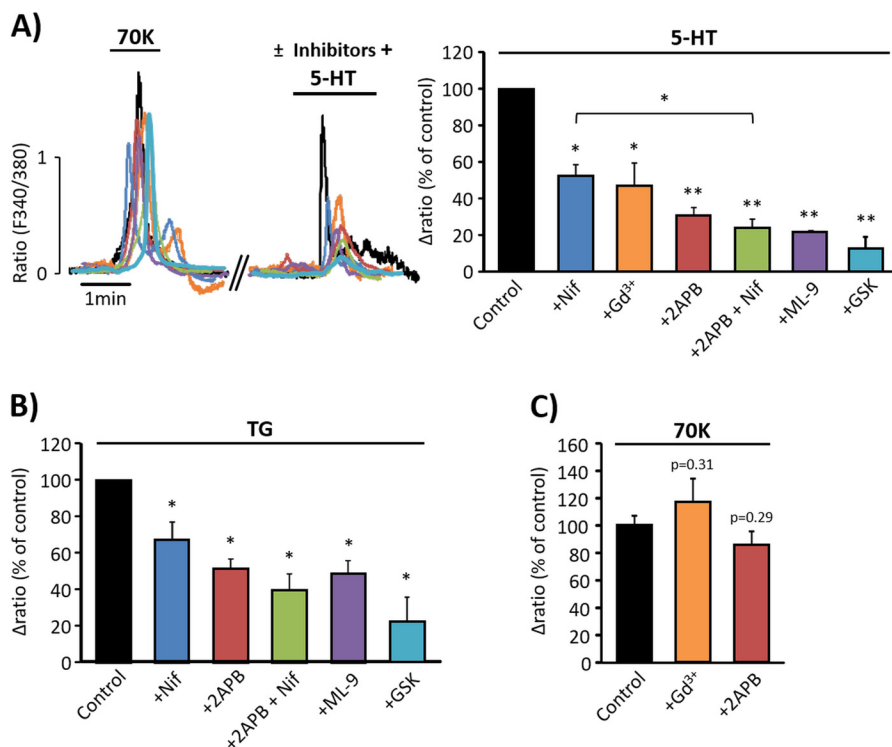


FIGURE 2. [Ca²⁺]_i changes elicited by serotonin and thapsigargin in vascular smooth muscle cell. *A, left panel*, representative traces of [Ca²⁺]_i responses in VSMC presented as fura-2 ratio (F_{340}/F_{380}). High KCl (70 mM; 70K) was applied, and then 5-HT (10 μ M) was added in the presence or absence of inhibitors. In the *right panel*, the bar graph summarizes 5-HT-evoked [Ca²⁺]_i increases in untreated cells (control, $n = 486$) and cells pre-treated with 100 nM Nif (52.30% \pm 6.20; $n = 65$), 5 μ M Gd³⁺ (47.15% \pm 12.40; $n = 48$), 25 μ M 2APB (30.69% \pm 4.40; $n = 111$), 25 μ M 2APB + 100 nM Nif (23.89% \pm 5.00; $n = 96$), 25 μ M ML-9 (21.67% \pm 0.50; $n = 102$), and 5 μ M GSK (12.67% \pm 6.53; $n = 160$). *B*, relative data summary of TG- (2 μ M) induced [Ca²⁺]_i changes in VSMC ($n = 379$) and in cells pre-treated with 100 nM Nif (67.51% \pm 9.66; $n = 141$), 25 μ M 2APB (51.49% \pm 5.45; $n = 148$), 25 μ M 2APB + 100 nM Nif (39.86% \pm 8.89; $n = 120$), 10 μ M ML-9 (48.70% \pm 7.36; $n = 110$), and 5 μ M GSK (22.10% \pm 13.71; $n = 152$). *C*, data summary of [Ca²⁺]_i increases evoked by 70K in VSMC (100% \pm 7.86; $n = 85$) or in the presence of 5 μ M Gd³⁺ (105.18% \pm 15.31; $n = 27$) and 50 μ M 2APB (76.74% \pm 9.15; $n = 88$). Values are the percentage of mean \pm S.E. normalized to the first response to 70K from at least 3 different experiments. *, $p < 0.05$, **, $p < 0.01$ versus control.

[Ca²⁺]_i increases were sensitive to nifedipine (100 nM) and to the SOCC inhibitors, GSK-7975A (5 μ M), Gd³⁺ (5 μ M), 2APB (25 μ M), and ML-9 (10 μ M). Pre-treatment of cells with 2APB (25 μ M) together with nifedipine (100 nM) caused significantly stronger suppression of 5-HT-evoked [Ca²⁺]_i responses, as compared with the effect of nifedipine alone. Similarly, the addition of TG (2 μ M) in the presence of extracellular Ca²⁺ induced an increase in [Ca²⁺]_i, which was sensitive to nifedipine (100 nM), GSK-7975A (5 μ M), 2APB (25 μ M), and ML-9 (10 μ M), as shown in Fig. 2B. The effect of 2APB (25 μ M) applied together with nifedipine (100 nM) caused additional reduction of TG-evoked [Ca²⁺]_i increase, like their effects in 5-HT response. In similar experiments, we tested a higher concentration of nifedipine (500 nM), and the effects were not significantly different from those obtained using 100 nM (data not shown). Fig. 2C shows data from control experiments in which Gd³⁺ (5 μ M) and 2APB (50 μ M) did not affect high KCl-induced [Ca²⁺]_i responses. Altogether, these data suggest that 5-HT- and TG-induced [Ca²⁺]_i increase and artery vasoconstriction involve Ca²⁺ entry through both LTCC and SOCC.

SOCE Does Not Compensate LTCC Function in Endothelium-denuded Aorta of Ca_v1.2 Knock-out Mice—Next, we examined whether SOCC can substitute LTCC function in vascular tone regulation. We used aorta from a conditional Ca_v1.2 knock-out (Ca_v1.2^{KO}) mouse model to assess 5-HT and TG effects. Fig. 3A indicates that Ca_v1.2 protein expression was

efficiently decreased in Ca_v1.2^{KO} mice as compared with wild type (WT). Consistently, aorta contraction induced by depolarizing stimulus, high KCl (70 mM), was significantly attenuated in Ca_v1.2^{KO} mice as compared with WT (Fig. 3, B and F). In the same way, 5-HT (10 μ M, Fig. 3, C and F) and TG (10 μ M, Fig. 3, D and F) induced significantly smaller contractions in Ca_v1.2^{KO} aorta as compared with WT. 5-HT- and TG-evoked vasoconstrictions in Ca_v1.2^{KO} aorta were still somewhat sensitive to nifedipine (1 μ M), probably due to the presence of the remaining functional Ca_v1.2 channels (Fig. 3, C–H). Interestingly, the evoked contractions were largely inhibited by 2APB (50 μ M) or ML-9 (25 μ M), as shown in Fig. 3, C and D and summarized in Fig. 3, G and H. Moreover, in freshly isolated Ca_v1.2^{KO} VSMC, the addition of high KCl, 5-HT (10 μ M), or TG (2 μ M) evoked significantly reduced [Ca²⁺]_i responses, as compared with WT (Fig. 4, A–C). Because the effect of vasoconstrictor agonists depends on the initial InsP₃-induced Ca²⁺ release from intracellular stores, we checked the integrity of the SR in Ca_v1.2^{KO} mice using caffeine, to release Ca²⁺ from ryanodine-sensitive stores (17). We observed that vessel contractions (Fig. 3, E and F) and [Ca²⁺]_i increases (Fig. 4, A and C) induced by caffeine (10 mM) stimulation were not affected in Ca_v1.2^{KO} mice, confirming that 5-HT- and TG-reduced responses are not due to differences in SR Ca²⁺ load between WT and Ca_v1.2^{KO} mice.

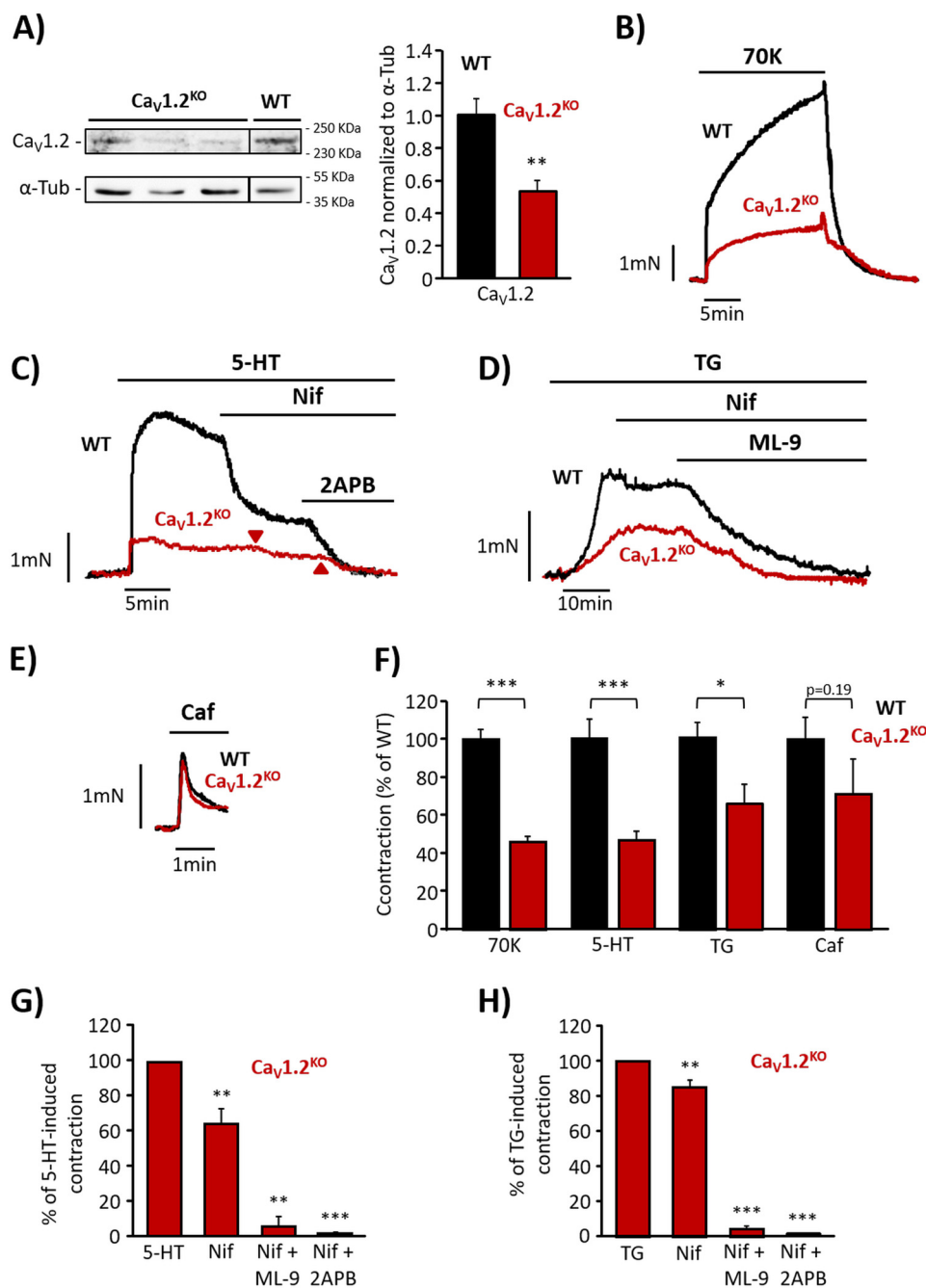


FIGURE 3. Effect of serotonin in endothelium-denuded aorta isolated from Ca_v1.2 knock-out mice. *A*, Western blots and data summary of Ca_v1.2 protein expression in wild type (WT, 1.00 ± 0.10; *n* = 4) and Ca_v1.2 knock-out mice (Ca_v1.2^{KO}, 0.59 ± 0.06; *n* = 4). *α-Tub*, *α*-tubulin. *B*, representative traces of aortic ring contractions induced by high KCl (70 mM; 70K) in aorta from WT and Ca_v1.2^{KO} mice. *C*, representative recordings showing 5-HT- (10 μM) evoked contraction and the effects of the addition of nifedipine (Nif, 1 μM) and 2APB (50 μM) in aortic rings from WT and Ca_v1.2^{KO} mice. The arrows indicate the time when nifedipine and 2APB were applied in Ca_v1.2^{KO} aortic ring. *D*, representative traces of TG- (10 μM) evoked contraction and the effects of the cumulative addition of nifedipine (1 μM) and ML-9 (25 μM) in aortic rings from WT and Ca_v1.2^{KO} mice. *E*, representative contraction of aortic rings from WT and Ca_v1.2^{KO} mice induced by caffeine (Caf, 10 mM) and the effects of the addition of nifedipine (1 μM) and ML-9 (25 μM) in aortic rings from WT and Ca_v1.2^{KO} mice. *F*, data summary of aortic ring contractions induced by 70K, 5-HT, TG, and Caf from WT (black bars; 70K, 100% ± 5.58; 5-HT, 100% ± 11.23; TG, 100% ± 10.95; Caf, 100% ± 12.10; *n* = 11–42) and Ca_v1.2^{KO} mice (red bars; 70K, 51.24% ± 3.17; 5-HT, 46.02% ± 6.42; TG, 66.23% ± 10.22; Caf, 71% ± 18.68; *n* = 10–31). *G*, data summary of 5-HT-induced contractions from Ca_v1.2^{KO} aorta (*n* = 11) and the effects of 1 μM Nif (64.16% ± 8.70; *n* = 10), 1 μM Nif + 50 μM 2APB (1.34% ± 0.63; *n* = 6), or 1 μM Nif + 25 μM ML-9 (5.61% ± 5.61; *n* = 3). *H*, data summary showing the effects of 1 μM Nif (85.14% ± 4.15; *n* = 12), 1 μM Nif + 50 μM 2APB (0.83% ± 0.83; *n* = 5), and 1 μM Nif + 50 μM ML-9 (4.42% ± 1.93; *n* = 7) on TG-induced aorta contractions from Ca_v1.2^{KO} mice (*n* = 13). Values are the percentage of mean ± S.E. *, *p* < 0.05, **, *p* < 0.01, and ***, *p* < 0.001.

Effects of Membrane Potential Manipulation on 5-HT and TG Responses—In light of the previous data demonstrating that 5-HT and TG co-activate SOCC and LTCC, we examined whether changes in membrane potential are relevant for the responses to agonists. Therefore, we tested the effect of cro-

makalim, an agonist of ATP-sensitive K⁺ channel (K_{ATP}) widely used to promote significant hyperpolarization (18). Fig. 5, *A* and *B*, show that aorta pre-treatment with cromakalim (20 μM) significantly reduced 5-HT- (10 μM) and TG- (10 μM) induced vasoconstriction, whereas pre-treatment of vessels

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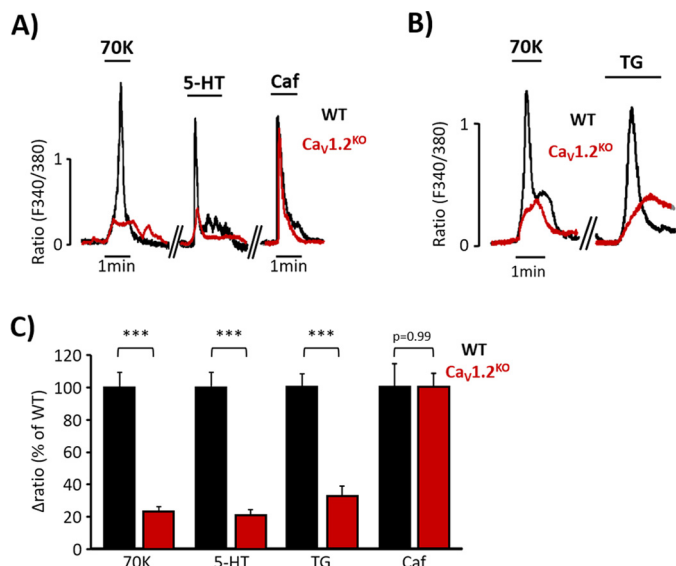


FIGURE 4. [Ca²⁺]_i increases elicited by serotonin or thapsigargin are diminished in Ca_v1.2^{KO} mice. *A*, representative [Ca²⁺]_i changes in aortic VSMC from wild type (WT, black traces) and Ca_v1.2 knock-out mice (Ca_v1.2^{KO}, red traces) mice evoked by high KCl (70 mM; 70K), 5-HT (10 μM), and Caf (10 mM). *B*, representative [Ca²⁺]_i changes of aortic VSMC from WT (black traces) and Ca_v1.2^{KO} (red traces) mice evoked by 70K and TG (10 μM). *C*, data summary of [Ca²⁺]_i changes induced by 70K, 5-HT, TG, and Caf in VSMC from WT (black bars; 70K, 100% ± 8.77; 5-HT, 100% ± 13.18; TG, 100% ± 8.57; Caf, 100% ± 14.23; *n* = 379–1157) and Ca_v1.2^{KO} mice (red bars; 70K, 22.92% ± 2.82; 5-HT, 20.76% ± 3.94; TG, 26.83% ± 6.18; Caf, 99.88% ± 8.60; *n* = 190–640). Values are the percentage of mean ± S.E. ***, *p* < 0.001 versus control.

with glibenclamide (3 μM), an inhibitor of K_{ATP} channel (14) that blocks the hyperpolarizing action of cromakalim, efficiently antagonized its effects on 5-HT-induced contraction (Fig. 5A). Moreover, the increase in [Ca²⁺]_i induced by both 5-HT (Fig. 5C) and TG (Fig. 5D) in isolated VSMC was also inhibited by cromakalim (5 μM). Nevertheless, the responses to high KCl or caffeine were not significantly affected by the application of cromakalim neither in aortic rings (Fig. 5E) nor in isolated VSMC (Fig. 5F). To confirm these findings, we explored the effect of valinomycin, a potassium-selective ionophore that promotes hyperpolarization, bringing the membrane potential to values close to the Nernst potential for potassium (19). As shown in Fig. 6, *A* and *B*, pre-treatment of aortic rings with valinomycin (500 nM) significantly reduced the effects of 5-HT and TG on vasoconstriction. In addition, incubation of VSMC with valinomycin (100 nM) significantly inhibited 5-HT- (Fig. 6C) and TG- (Fig. 6D) evoked [Ca²⁺]_i increase, whereas the high KCl and caffeine responses were not affected.

To corroborate the physiological relevance of the co-activation of LTCC and SOCC during agonist stimulation, we explored whether LTCC agonist Bay-K-8644 (BayK) (20) could enhance aorta responses elicited by 5-HT and TG. As shown in Fig. 7, *A* and *B*, aortic rings pre-treated with BayK (100 nM) exhibited significantly higher contractions when stimulated with 5-HT and TG as compared with untreated aortic rings. The addition of 2APB (50 μM) efficiently relaxed 5HT- and TG-induced vasoconstriction in BayK-treated arterial rings. Similar increased responses were also observed when high KCl was applied in BayK-treated aorta (Fig. 7C).

Altogether, these data suggest that vasoconstrictions initiated by 5-HT or SOCE activation with TG are attenuated in

hyperpolarized arteries, whereas these responses are potentiated when the LTCC activation threshold is shifted toward hyperpolarized potentials.

Endogenous Distribution of Orai1, TRPC1, and Ca_v1.2 in VSMC—Orai1 and TRPC1 are suggested to interact to form non-selective SOCC in VSMC (8). Several lines of evidence suggest that the Ca_v1.2 isoform might form a different signal complex with other channels to handle [Ca²⁺]_i in VSMC (10–12). Here, we examined the endogenous subcellular localization of Ca_v1.2, Orai1, and TRPC1 and their possible interaction by the *in situ* proximity ligation assay (PLA). Fig. 8, *A* and *C*, show a large number of PLA red puncta in VSMC when incubated with primary antibodies against Ca_v1.2 and Orai1. Meanwhile, no PLA signal was detected in VSMC conjugated only with anti-Orai1 antibody, but without anti-Ca_v1.2 antibody (Fig. 8, *B* and *C*). Similarly, Fig. 9A shows that Ca_v1.2 interacts with TRPC1, as indicated by a large number of red PLA puncta in VSMC. Interestingly, VSMC stimulation with 5-HT (10 μM) and TG (2 μM), but not with high KCl, significantly increased puncta signals, indicating a significant rise in the interaction of Ca_v1.2 with Orai1 (Fig. 8, *A* and *C*) and TRPC1 (Fig. 9, *A* and *C*) after agonist stimulation. These data suggest that Orai1 and TRPC1 interact with Ca_v1.2 in basal conditions and upon agonist stimulation, which will certainly favor their functional communication upon agonist stimulation to promote intracellular Ca²⁺ signaling in VSMC.

Discussion

Although it is widely accepted that LTCC and SOCC contribute to the physiopathology of VSMC, their direct functional relationship had remained virtually unexplored. The present study provides new data confirming the role of SOCC in vascular tone regulation, unveiling for the first time a functional crosstalk between Ca_v1.2, Orai1, and TRPC1 channels that might serve for fine-tuning of vascular smooth muscle Ca²⁺ signaling, as summarized in the scheme shown in Fig. 10. Routinely, to activate SOCE, pharmacological or physiological agonists were added in the absence of Ca²⁺, and then extracellular Ca²⁺ was restored in the well known “Ca²⁺-free/Ca²⁺-readmission” or “Ca²⁺ add-back” protocols (see for example Ref. 5). Nevertheless, there is little information about physiological agonists that can activate SOCE in the continuous presence of extracellular Ca²⁺, without store depletion, as discussed elsewhere (13). In this study, we demonstrated that 5-HT applied in the presence of extracellular Ca²⁺ evoked a fast increase of [Ca²⁺]_i, followed by a sustained phase, in isolated VSMC. 5-HT also activated a sustained vasoconstriction in aortic rings. These responses were sensitive to inhibitors of SOCC, supporting the involvement of SOCE in vessel contraction. In fact, we showed that GSK-7975A, which is considered a specific inhibitor of Orai1 (15), as well as other blockers, efficiently inhibited 5-HT-induced responses. Gd³⁺, 2APB, and ML-9 are still widely used as SOCE inhibitors in different cell types (3), despite their lack of specificity. In this study, the involvement of SOCC in vasoconstriction is also supported by the specific activation with TG, which induced aorta vasoconstriction, indicating that SOCE contributes to vascular tone regulation, in agreement with previous studies (21, 22). Interestingly, we also

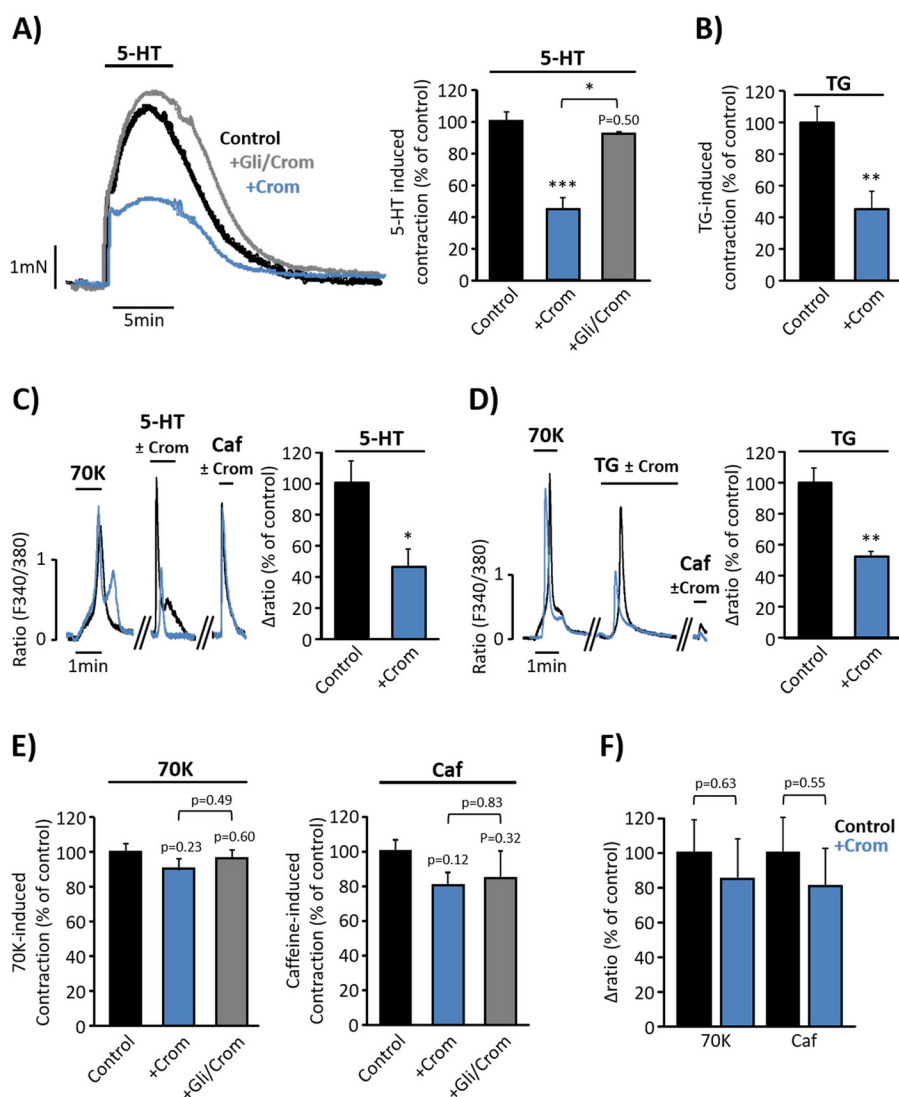


FIGURE 5. Cromakalim attenuates serotonin and thapsigargin responses. *A*, representative traces and data summary of 5-HT- (10 μM) evoked contraction in aortic rings pre-incubated for 15 min with vehicle (control, black trace and bar; 100% ± 5.73; n = 6), with 20 μM cromakalim (+Crom; blue trace and bar; 44.59% ± 7.16; n = 3) or with 3 μM glibenclamide together with 20 μM cromakalim (+Gli/Crom, gray trace and bar; 92.47% ± 0.72; n = 3). *B*, data summary of TG- (10 μM) induced contraction in aortic rings pre-incubated for 15 min with vehicle (control, 100% ± 9.70; n = 4) or 20 μM Crom (45.15% ± 10.86; n = 4). *C*, representative traces of [Ca²⁺]_i changes elicited by high KCl (70 mM, 70K), 10 mM 5-HT, and 10 mM Caf in VSMC pre-incubated for 3 min with vehicle (control, black trace and bar), or with 5 μM Crom (blue trace and bar). Data summaries of 5-HT responses in the presence of vehicle (control, 100% ± 14.44; n = 79) and in the presence of Crom (46.39% ± 10.94; n = 87) are shown. *D*, representative traces of [Ca²⁺]_i changes elicited by 70K, 2 μM TG, and 10 mM Caf in VSMC pre-incubated for 3 min with vehicle (control, black trace and bar), or with 5 μM Crom (blue trace and bar). Data summaries of [Ca²⁺]_i changes elicited by 2 μM TG applied in control (100% ± 9.30, n = 151) or in the presence of cromakalim (52.36% ± 2.95, n = 94) are shown. *E*, bar graphs summarize 70K- and caffeine-induced vasoconstriction in control (70K = 100% ± 4.33 and Caf = 100% ± 6.80; n = 6), in rings pretreated for 15 min with 20 μM Crom (70K = 90.09% ± 6.39 and Caf = 80.44% ± 7.68; n = 3), and in rings treated for 15 min with 3 μM glibenclamide + 20 μM cromakalim (+Gli/Crom; 70K = 96.13% ± 5.00; Caf = 84.42% ± 16.32; n = 3). *F*, data summary of [Ca²⁺]_i changes produced by 70K and Caf (10 mM) in VSMC pre-treated for 3 min with vehicle (control, 70K = 100% ± 18.67 and Caf = 100% ± 20.13; n = 95), or with 5 μM Crom (70K = 84.97% ± 22.55 and Caf = 80.91% ± 21.81; n = 74). Values are the percentage of mean ± S.E. normalized to 70K responses. *, p < 0.05, **, p < 0.01, and ***, p < 0.001.

demonstrated that SOCC sustained Ca²⁺ entry and vasoconstriction in Ca_v1.2^{KO} mice, although not enough to completely compensate for the absence of functional LTCC. Our results using the Ca_v1.2^{KO} mice confirmed the requirement of functional LTCC for vessel contraction even when vasoconstriction was specifically activated through SOCE using TG. These data agree with previous studies using Ca_v1.2^{KO} mice, which demonstrated that Ca_v1.2 is essential to control blood pressure and vasoconstrictor responses (17, 23).

Furthermore, increasing lines of evidence suggested that SOCE activation could serve not only as an important path for

Ca²⁺ entry, but also as a depolarizing trigger for a secondary activation of LTCC in VSMC (24). Knowing that sarcolemmal K⁺ channels are key regulators of resting potential in VSMC and vascular tone (25), we demonstrated that 5-HT and TG responses were sensitive to membrane potential changes, as they were attenuated by cromakalim or valinomyin, suggesting a smaller contribution of LTCC under these conditions. Valinomyin is expected to maintain the driving force for Ca²⁺ entry upon SOCE activation, as it impairs membrane depolarization. This might result in a slight increase in Ca²⁺ entry via SOCE, which, in our hands did not compensate for the effect of

Orai1, TRPC1, and Ca_v1.2 Interaction

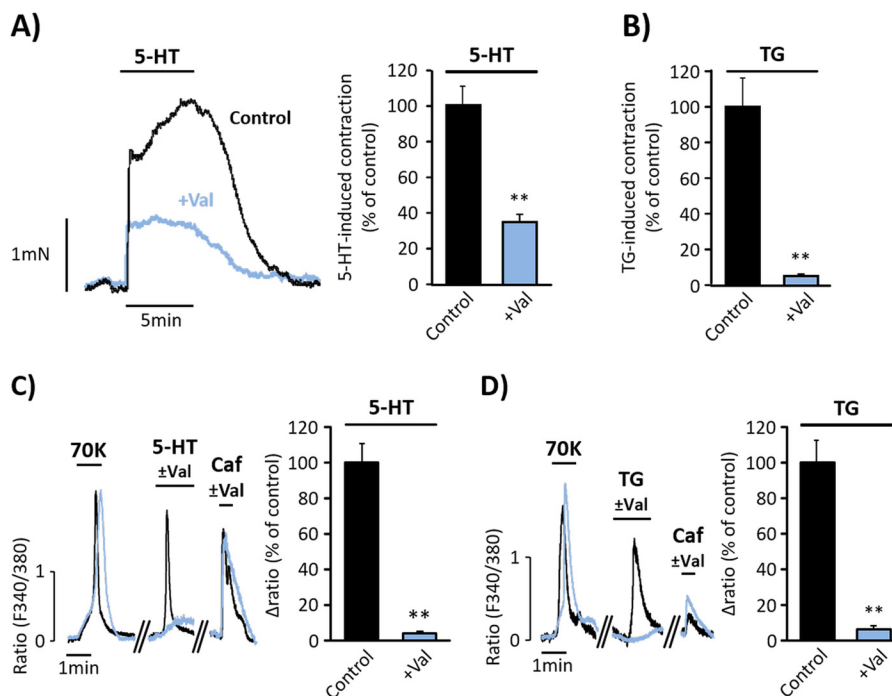


FIGURE 6. Valinomycin attenuates serotonin- and thapsigargin-induced vasoconstriction and $[Ca^{2+}]_i$ increase. *A* and *B*, representative traces and data summary of 5-HT- ($10 \mu M$) and TG- ($10 \mu M$) evoked contractions in control aortic rings (in black; 5-HT = $100\% \pm 10.62$, $n = 9$; and TG = $100\% \pm 16.07$; $n = 5$), and in rings pre-incubated for 15 min with 500 nM valinomycin (+Val, in blue; 5-HT = $35.10\% \pm 4.67$, $n = 4$ and TG = $5.57\% \pm 0.53$, $n = 3$). *C* and *D*, representative traces and data summary of $[Ca^{2+}]_i$ changes elicited by high KCl (70K, 70 mM) 10 μM 5-HT, and 2 μM TG and in control VSMC (5-HT = $100\% \pm 11.24$, $n = 93$ and TG = $100\% \pm 12.64$, $n = 90$) and in cells pre-incubated for 3 min with 100 nM valinomycin (5-HT = $3.68\% \pm 0.63$, $n = 89$ and TG = $6.38\% \pm 1.95$, $n = 114$). 10 mM Caf was applied at the end of each experiment as indicated. Values are the percentage of mean \pm S.E. normalized to 70K responses. **, $p < 0.01$.

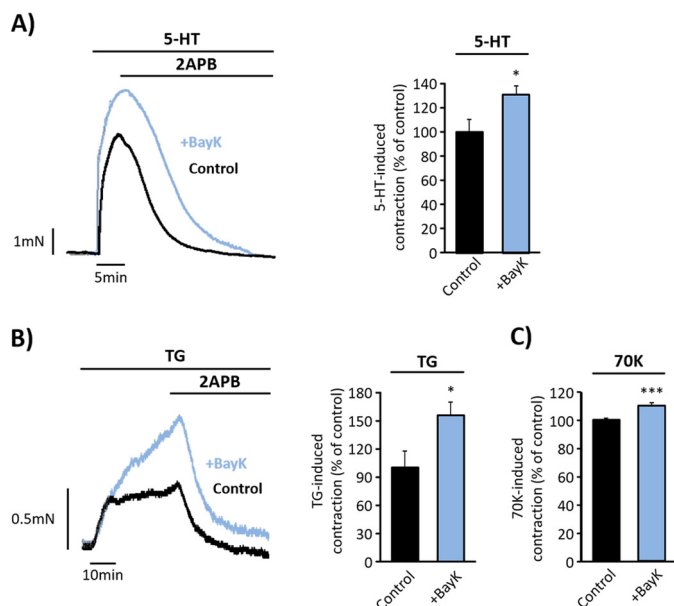


FIGURE 7. LTCC agonist, BayK, increases endothelium-denuded aorta responses. *A* and *B*, representative traces and data summary of 5-HT- ($10 \mu M$) and TG- ($10 \mu M$) evoked contractions in control aortic rings (in black; 5-HT = $100\% \pm 10.53$, $n = 3$; and TG = $100\% \pm 17.57$, $n = 6$) and in rings pre-incubated for 15 min with 100 nM BayK (in blue; 5-HT = $131.30\% \pm 7.16$, $n = 4$ and TG = $155\% \pm 13.65$, $n = 5$). 2APB (50 μM) was added as indicated. *C*, bar graph shows a data summary of high KCl (70K, 70 mM) responses in control rings ($100\% \pm 1.55$, $n = 11$) and in rings pre-incubated with BayK for 15 min ($110\% \pm 1.83$, $n = 12$). Values are the percentage of mean \pm S.E. normalized to 70K responses. *, $p < 0.05$, ***, $p < 0.001$.

the secondary activation of LTCC. To our knowledge, few studies have suggested that hyperpolarization, due to K_{Ca} channel activation, sustained Ca^{2+} entry through SOCE (for example, in

chondrocytes) (26). On the other hand, BayK, which shifts LTCC activation to hyperpolarized potentials, enhanced agonist responses. Thus, our results indicated that agonist responses can be attenuated or potentiated significantly depending on the open probability of LTCC. Similarly, a recent study demonstrated that depletion of SR stimulated SOCE, producing depolarization and LTCC activation in rat myometrium (27). Recently, we have determined that the transient expression of Ca_v1.2 channel subunits in HEK cells resulted in a significant increase in Ca^{2+} entry induced by TG, attributed to secondary activation of Ca_v1.2 channels induced by cation influx via SOCC (28). We have also demonstrated that upon store depletion, STIM1 inhibits Ca^{2+} entry through LTCC (28), in agreement with studies by other groups (29). Our data suggest that store depletion might promote two independent mechanisms involving the interaction of different components of SOCE with Ca_v1.2 to fine-tune its activity: Ca^{2+} influx via SOCE promotes secondary activation of LTCC, and STIM1 modulates Ca_v1.2 function. Certainly, further investigations are needed to shed more light on this intriguing reciprocal regulation of LTCC by store depletion. In fact, the dual regulation of LTCC by $[Ca^{2+}]_i$ increase has been extensively studied in excitable cells, as reviewed recently (30). $[Ca^{2+}]_i$ enhancement is known to promote the well characterized Ca^{2+} -dependent inactivation process likely to prevent Ca^{2+} overload (31), whereas $[Ca^{2+}]_i$ increase can also stimulate Ca^{2+} -dependent facilitation of Ca_v1.2 to potentiate Ca^{2+} influx, for example, during the excitation-contraction coupling in VSMC (32).

Another important issue that remains under debate is the identity of SOCC in excitable VSMC. Several groups have

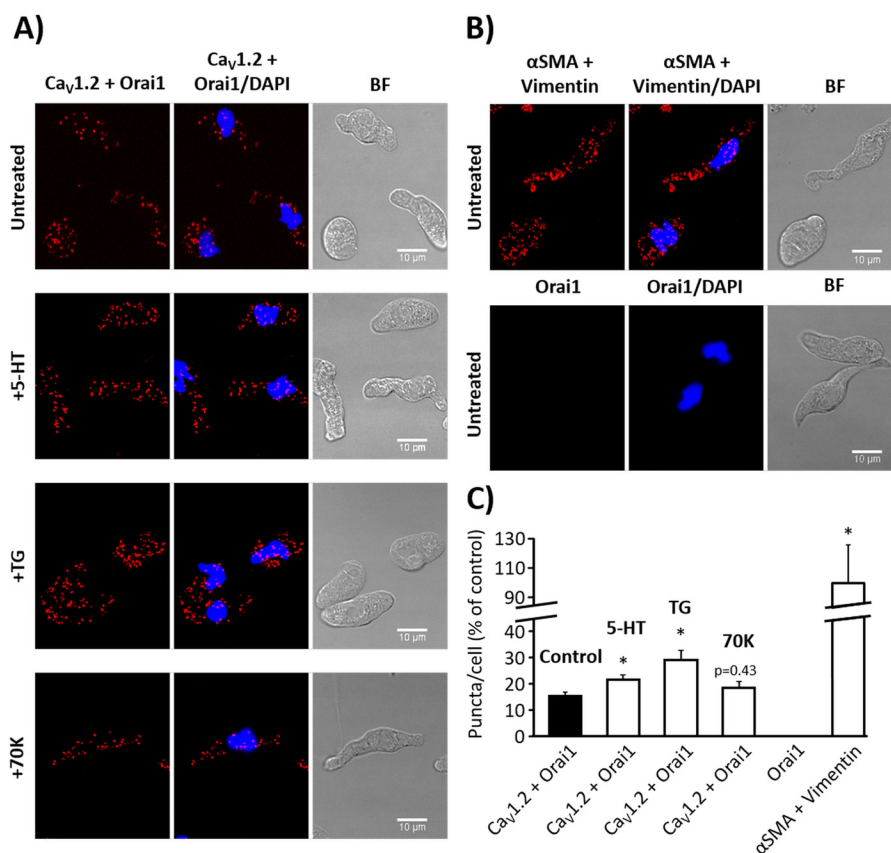


FIGURE 8. Orai1 and Ca_v1.2 co-localization in aortic myocytes. *A*, representative images of aortic VSMC using primary antibodies against Ca_v1.2 and Orai1 conjugated with the appropriate PLA probes, from untreated control cells or from cells stimulated with 5-HT (10 μM), TG (2 μM), or high KCl (70K, 70 mM). Red puncta indicate that proteins are in close proximity (<40 nm). The left panels show VSMC images when antibodies were conjugated with PLA probes, the middle panels show merges images from cells also stained with DAPI (blue), and the right panels show bright field (BF) pictures. *B*, images from VSMC using primary antibodies against α-SMA and vimentin used as positive control (upper panel) and from VSMC conjugated with only anti-Orai1 primary antibody as negative control (lower panel). *C*, the bar graph summarizes the mean number of PLA signals in untreated control VSMC (15.42% ± 1.50; *n* = 6), and in VSMC stimulated with 5-HT (21.60% ± 1.65; *n* = 10), TG (28.90% ± 3.81; *n* = 8), or 70K (18.33% ± 2.45; *n* = 8). No detectable PLA puncta were observed in control experiments using cells conjugated only with anti-Orai1 antibody (Orai1, *n* = 8). The interaction between anti-α-SMA and anti-vimentin antibodies was used as positive control (100% ± 25.88; *n* = 5). Similar experiments were repeated 3 times. Values are the percentage of mean ± S.E., normalized to positive control. Significance values indicate difference as compared with control, *, *p* < 0.05.

shown interactions between different proteins to form the SOCE signaling complex as discussed elsewhere (33, 34). Here, using *in situ* PLA assay, we showed for the first time that endogenous Orai1, TRPC1, and Ca_v1.2 are distributed in close vicinity. Indeed, hybridization of the PLA probes that occurs when proteins are <40 nm apart (35) confirmed a strong co-localization between these channels. Remarkably, we observed a significant increase of puncta signals in cells incubated with agonists that involve SOCE activation (5-HT through the InsP₃ signaling pathway and TG via SERCA inhibition), but not with depolarizing stimulus with high KCl. These data suggest that agonist-induced Ca²⁺ influx is likely due to a functional interaction/communication between TRPC1- and Orai1-dependent SOCC and Ca_v1.2 channels in VSMC. Recently, independent studies showed that Orai1 associates with other channels to form the arachidonate-regulated Ca²⁺ (ARC) channels (36), associates with TRPC1 to form non-selective SOCC (8, 37), or even associates with small conductance Ca²⁺-activated potassium channel 3 (SK3) (11). Therefore, we provided several lines of evidence demonstrating that the SOCC components, Orai1 and TRPC1, form a macromolecular complex with Ca_v1.2 LTCC to regulate [Ca²⁺]_i signaling and vascular tone.

Experimental Procedures

Ethical Approval—All experiments were conducted in accordance with the Spanish legislation on protection of animals (Royal Decree 53/2013), conformed to the Directive 2010/63/EU of the European Parliament, and were approved by the local Ethics Committee of Animal Care of the University Hospital Virgen del Rocío (HUVR) of Seville. Mice (strain C57BL/6) were sacrificed by intraperitoneal administration of a lethal dose of sodium thiopental (200 mg/kg).

Ca_v1.2^{KO} Mouse Model—We used WT and Ca_v1.2^{KO} mice generated at the Institut für Pharmakologie und Toxikologie, München, Germany (17, 23). Ca_v1.2^{KO} mice express a tamoxifen-inducible Cre recombinase under control of the SM22 promoter (SM-Cre ERT2(ki)). To induce smooth muscle-specific Cre recombination, adult mice were treated with freshly prepared tamoxifen solution dissolved in corn oil at 10 mg/ml (Sigma) by intraperitoneal injection once a day for 5 days at a dosage of 1 mg/day. Ca_v1.2^{KO} mice were analyzed between 16 and 18 days after the first injection of tamoxifen, as these animals die between 18 and 21 days (23). The background mouse strain was C57BL/6.

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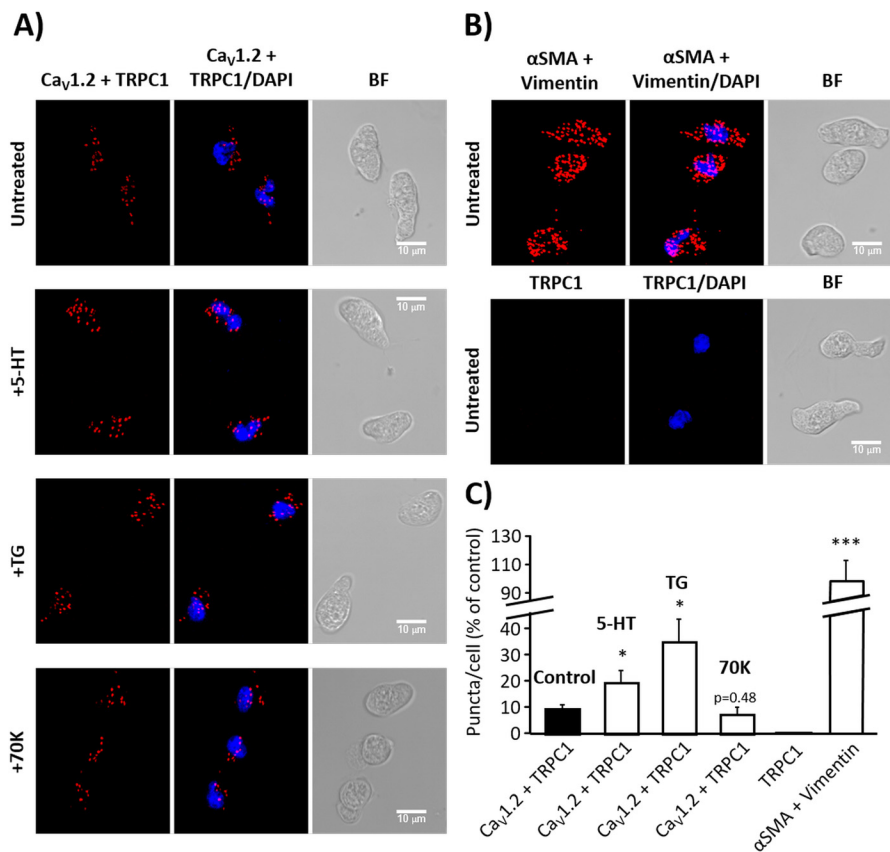


FIGURE 9. TRPC1 and Ca_v1.2 co-localization in aortic myocytes. *A*, representative images of aortic VSMC using primary antibodies against Ca_v1.2 and TRPC1 conjugated with the appropriate PLA probes, from untreated control cells or from cells stimulated with 5-HT (10 μM), TG (2 μM), or high KCl (70K, 70 mM). Red puncta indicate that proteins are in close proximity (<40 nm). The left panels show VSMC images when antibodies were conjugated with PLA probes, the middle panels show merge images from cells also stained with DAPI (blue), and the right panels show bright field (BF) pictures. *B*, images from VSMC using primary antibodies against α-SMA and vimentin used as positive control (upper panel) and from VSMC conjugated with only anti-TRPC1 primary antibody as negative control (lower panel). *C*, the bar graph summarizes the mean number of PLA signals in control VSMC (9.85% ± 1.53; n = 9), in 5-HT- (19.98% ± 4.72; n = 8), TG- (35.53% ± 8.78; n = 9), or 70K- (7.72% ± 2.76; n = 4) treated VSMC. No detectable PLA puncta were observed in control experiments using cells conjugated only with anti-TRPC1 antibody (TRPC1, n = 10). The interaction between anti-α-SMA and anti-vimentin antibodies was used as positive control (100% ± 14.63; n = 7). Similar experiments were repeated 3 times. Values are the percentage of mean ± S.E., normalized to positive control. Significance values indicate difference as compared with control, *, p < 0.05, ***, p < 0.001.

Measurement of Contractility in Arterial Rings—Thoracic aorta was quickly removed and placed in ice-cold Krebs solution (in mM: 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 24.8 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 10 glucose). Then, aorta was cleaned from connective tissue, cut in rings (~2 mm), and mounted on a small-vessel myograph (J.P. Trading, Aarhus, Denmark) to measure isometric tension connected to a digital recorder (Myodataq-2.01, Myodata-2.02 Multi-Myograph System) as described previously (5). Aorta rings were placed on a chamber filled with Krebs solution at 37 °C bubbled with 95% O₂ and 5% CO₂. Before the experiments, segments were subjected to a basal tension of 2.5 micronewtons and stabilized for at least 1 h. The endothelium was mechanically removed by rubbing the luminal surface of the ring with a small plastic tube, and the integrity of the endothelium was tested at the beginning of each experiment by the addition of acetylcholine (up to 10 μM) as described previously (38). The data summary presented in bar graphs shows normalized responses of the increment and the difference between the maximum contraction and resting tone of the vasoconstriction.

Preparation of Aortic Smooth Muscle Cells—The segment of thoracic aorta was quickly removed and placed in cold physio-

logical solution (PS) (in mM: 137 NaCl, 5.4 KCl, 0.2 CaCl₂, 4.17 NaHCO₃, 2 MgCl₂, 0.44 KH₂PO₄, 0.42 NaH₂PO₄, 10 HEPES, 11.11 glucose, 0.05 EGTA). Aorta was dissected, cleaned, cut into pieces, and incubated with 1–2 mg/ml elastase (4 units/mg) and 4 mg/ml collagenase type I (125 units/mg) (Sigma) in PS for 1 h at 4 °C and then for 10–15 min at 37 °C. Cells were mechanically dispersed using fire-polished glass pipettes and plated on coverslips. VSMC were easily distinguished by their size and typical elongated shape, and samples from dispersed cells were stained with mouse anti-α-SMA antibody (Sigma) or phalloidin (Sigma), a marker for F-actin, to verify preparation of VSMC and to rule out any major presence of fibroblasts or endothelial cells.

Cytosolic Ca²⁺ Measurement—VSMC plated on coverslips were incubated in PS with 2–5 μM Fura-2AM for 30 min at room temperature, and then cells were washed. For the experiments, a coverslip was placed on the stage of Nikon Eclipse TS-100 inverted microscope equipped with a 20× Fluor objective (0.75 NA), as described previously (5). Fluorescence images from a large number of loaded single cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Basic Im2, Image Solutions (UK) Ltd., Preston, UK) equipped

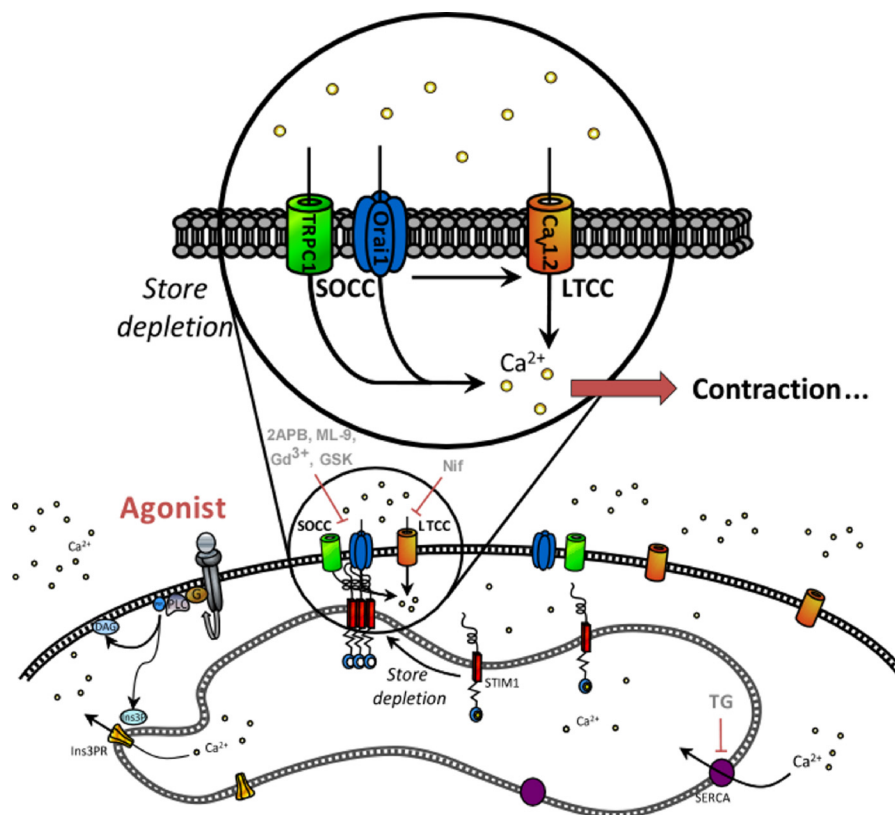


FIGURE 10. **Schematic model summarizing Orai1, TRPC1, and $Ca_v1.2$ co-activation upon 5-HT stimulation.** We propose that 5-HT binding to its receptors promotes $InsP_3$ -induced Ca^{2+} release, depletion of Ca^{2+} stores, activation of Ca^{2+} entry through Orai1, and TRPC1-dependent SOCC. The consequent SOCE will trigger secondary activation of LTCC, allowing a significant increase in intracellular free Ca^{2+} concentration and further aorta contraction.

with a light-sensitive CCD camera (Cooke PixelFly, Applied Scientific Instrumentation, Eugene, OR). Changes in $[Ca^{2+}]_i$ are represented as the ratio of Fura-2 fluorescence induced at an emission wavelength of 510 nm due to excitation at 340 and 380 nm (ratio = F_{340}/F_{380}). Ca^{2+} influx was calculated as the difference between the peak ratio before and after the addition of different drugs (Δ ratio). Data summaries are normalized to control values obtained in each cell preparation as indicated in the figure legends. Auto-fluorescence was determined at the end of each experiment by the addition of ionomycin and $MnCl_2$. Experiments were performed using a continuous perfusion system in physiological salt solution (in mM, pH = 7.4: 140 NaCl, 2.5 $CaCl_2$, 2.7 KCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose). High KCl solution was also used (in mM, pH = 7.4: 70 NaCl, 2.5 $CaCl_2$, 70 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose).

Protein Extraction and Western Blotting—Dissected arteries from mice were flash-frozen in an ice-cold mixture of 10% TCA and 10 mM DTT in acetone. Arteries were later washed in ice-cold acetone containing 10 mM DTT and lyophilized overnight. Prior to protein extraction, the lyophilized vessels were weighed in an ultra-precision scale to normalize the Western blotting load. 1 μ l of sample buffer (60 mM Tris HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 100 mM DTT) was added for each 2 μ g of artery for protein extraction. Samples were heated at 95 $^{\circ}C$ for 10 min and rotated overnight at 4 $^{\circ}C$ prior to electrophoresis. Similar amounts of protein samples extracted from WT and $Ca_v1.2^{KO}$ mice were subjected to SDS-PAGE (10%) and electro-transferred onto nitrocellulose

membranes. After blocking with 5% nonfat dry milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 2 h at room temperature, Western blots were probed overnight at 4 $^{\circ}C$ or for 1.5 h at room temperature with specific primary antibodies in blocking solution. After washing, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories) in TTBS. Detection was performed with the enhanced chemiluminescence reagent ECL Plus (Amersham Biosciences) and the ImageQuant LAS 4000 Mini Gold system. Primary antibodies used were: rabbit anti- $Ca_v1.2$ (1:200, Alomone Labs) and mouse anti- α -tubulin (1:5000, Sigma) as housekeeping loading control. For quantification, tiff images were analyzed with ImageJ software.

In Situ Proximity Ligation Assay—Spatial co-localization of $Ca_v1.2$ and Orai1 were analyzed with PLA technology in freshly isolated aortic myocytes using the Duolink *in situ* PLA detection kit Red (Sigma), following the manufacturer's instructions. VSMC were seeded in a six-channel μ -Slide from IBIDI and fixed with 100% cold methanol for 5 min. VSMC were blocked for 30 min with 3% heat-inactivated goat serum and 1% BSA in PBS and incubated with primary antibodies (rabbit anti- $Ca_v1.2$, 1:50 (Alomone Labs); mouse anti-Orai1, 1:100 (Novus Biologicals); or mouse anti-TRPC1 1:50 in blocking solution (Santa Cruz Biotechnology)) for 2 h at room temperature. Cells were labeled with Duolink PLA anti-rabbit PLUS and anti-mouse MINUS probes for 1 h at 37 $^{\circ}C$. The secondary antibody-

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ies of PLA PLUS and MINUS probes were attached to synthetic oligonucleotides that hybridize when they are in close proximity (*i.e.* <40 nm separation). The hybridized oligonucleotides were then ligated for 30 min at 37 °C prior to rolling circle amplification for 100 min at 37 °C. Fluorescently labeled oligonucleotides hybridized to the rolling circle amplification product. The red fluorescent fluorophore-tagged oligonucleotides were visualized using a confocal microscope (Leica TCS SP2). Maximum intensity projections of all z-sections (0.5 μm) were obtained by ImageJ software, and puncta of maximum intensity projections were analyzed by Duolink ImageTool software (Sigma). The interaction between anti-α-SMA and anti-vimentin antibodies was used as positive control (Fig. 8B). As a negative control, we conducted experiments using only one primary antibody (mouse anti-Orai1 or anti-TRPC1 antibodies), which did not show any detectable PLA signal (Fig. 8C).

Confocal Acquisition—Direct confocal acquisition of fluorescence was performed using a Leica TCS SP2 microscope (Leica) equipped with a blue diode at 405 nm, argon-krypton at 458–514 nm, helium-neon at 543 nm, and helium-neon at 633 nm. Images were acquired using a HCX PI Apo CS 63×/1.3 immersion objective in z-stack intervals of 0.5 μm. Confocal acquisition of fluorescence labels was performed as follows: DAPI (excited at 405 nm and recorded on 400–450 nm) and Alexa Fluor 594 (excited at 594 nm and recorded at 593–667 nm). All figures were processed and mounted by ImageJ software (ConfocalUniovi 1.5 ImageJ), and image deconvolution was conducted using an ImageJ plugin for spectral image deblurring (Parallel Spectral Deconvolution) based on a generalized Tikhonov regularization method (39).

Drugs—Drugs were purchased from Sigma, Invitrogen, and Aobious. The concentration of some inhibitors tested in this study varied when they were used in vessels or cells, but all were within the range of their optimum effects. Higher concentrations were used in rings to ensure inhibitor permeability in thick aorta.

Statistical Analysis—Data analysis was carried out using SigmaPlot software, version 11.0. A sample size calculation was performed prior to the start of this study. We expected a decrease of ~50–100% in vasoreactivity using Ca_v1.2^{KO} mice. We decided to include at least 3–5 subjects for each experiment, taking into consideration an α of 5% and power of 90% or an α of 1% and power of 80% and keeping in mind the failed experiments. Statistical analyses were performed by Student's *t* test for two-group comparison or one-way analysis of variance followed by Tukey multiple comparison post hoc tests comparing different groups. Group data are presented as the percentage of mean ± S.E. and *p* values < 0.05, <0.01, and <0.001 were considered significant as indicated in the figures with *, **, and ***, respectively.

Author Contributions—J. A. M., E. C. S., P. G. R., and T. S. conceived and designed experiments. J. A. M., E. C. S., P. G. R., F. M. Q., J. A. R., A. C., and T. S. collected, analyzed, and interpreted data. F. M. Q., J. A. R., A. C., A. O., and T. S. drafted the article or revised it critically for important intellectual content. All authors approved the final version for publication.

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