TRPV4 channel contributes to serotonin-induced pulmonary vasoconstriction and the enhanced vascular reactivity in chronic hypoxic pulmonary hypertension

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TRPV4 channel contributes to serotonin-induced pulmonary vasoconstriction and the enhanced vascular reactivity in chronic hypoxic pulmonary hypertension. Am J Physiol Cell Physiol 305: C704–C715, 2013. First published June 5, 2013; doi:10.1152/ajpcell.00099.2013.—Transient receptor potential vanilloid 4 (TRPV4) is a mechanosensitive channel in pulmonary arterial smooth muscle cells (PASMCs). Its upregulation by chronic hypoxia is associated with enhanced myogenic tone, and genetic deletion of trpv4 suppresses the development of chronic hypoxic pulmonary hypertension (CHPH). Here we further examine the roles of TRPV4 in agonist-induced pulmonary vasoconstriction and in the enhanced vasoreactivity in CHPH. Initial evaluation of TRPV4-selective antagonists HC-067047 and RN-1734 in KCl-contracted pulmonary arteries (PAs) of trpv4−/− mice found that submicromolar HC-067047 was devoid of off-target effect on pulmonary vasoconstriction. Inhibition of TRPV4 with 0.5 μM HC-067047 significantly reduced the sensitivity of serotonin (5-HT)-induced contraction in wild-type (WT) PAs but had no effect on endothelin-1 or phenylephrine-activated response. Similar shift in the concentration–response curve of 5-HT was observed in trpv4−/− PAs, confirming specific TRPV4 contribution to 5-HT-induced vasoconstriction. 5-HT-induced Ca2+ response was attenuated by HC-067047 in WT PASMCs but not in trpv4−/− PASMCs, suggesting TRPV4 is a major Ca2+ pathway for 5-HT-induced Ca2+ mobilization. Nifedipine also attenuated 5-HT-induced Ca2+ response in WT PASMCs but did not cause further reduction in the presence of HC-067047, suggesting interdependence of TRPV4 and voltage-gated Ca2+ channels in the 5-HT response. Chronic exposure (3–4 wk) of WT mice to 10% O2 caused significant increase in 5-HT-induced maximal contraction, which was partially reversed by HC-067047. In concordance, the enhancement of 5-HT-induced contraction was significantly reduced in PAs of CH trpv4−/− mice and HC-067047 had no further effect on the 5-HT-induced response. These results suggest unequivocally that TRPV4 contributes to 5-HT-dependent pharmaco-mechanical coupling and plays a major role in the enhanced pulmonary vasoreactivity to 5-HT in CHPH.

TRPV4; serotonin; pulmonary arteries; chronic hypoxia; pulmonary hypertension

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TRPV4-dependent Ca\(^{2+}\) influx, elevation of resting [Ca\(^{2+}\)]\(_i\) in pulmonary arterial smooth muscle cells (PASMCs), and the appearance of an intravascular pressure-activated myogenic tone. Moreover, the development of chronic hypoxic pulmonary hypertension (CHPH) and vascular remodeling is delayed and suppressed in *trpv4*\(^{-/-}\) mice. These findings suggest that TRPV4 is an important contributing player in the pathogenesis of CPHH.

Besides these observations, our knowledge on the physiological functions of TRPV4 in PASMCs is very limited. For example, the contribution of TRPV4 in agonist-induced contraction and its role in the enhanced pulmonary vascular reactivity in pulmonary hypertension have not been examined. Previous studies show that serotonin (5-HT) activates an arachidonic acid-dependent nonselective cation current, which has pharmacological properties similar to TRPV4 in PASMCs (8, 22). In heterologous expression systems and endothelial cells, TRPV4 coassembles with TRPC1 forming heteromeric channels. In PAs, most agonists induce vasoconstriction through phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) to form inositol 1,4,5-trisphosphate (InsP\(_3\)), which releases Ca\(^{2+}\) from sarcoplasmic reticulum and activates SOCE, and diacylglycerol, which activates protein kinase C and ROCE. Many agonists also stimulate phospholipase A\(_2\) (PLA\(_2\)) to mediate release of arachidonic acid and its metabolites (26, 33, 34, 62). Hence, we hypothesize that TRPV4 of PASMCs may contribute to agonist-induced vasoconstriction and participate in the regulation of pulmonary vascular reactivity in pulmonary hypertension. In the present study, we aimed to test this hypothesis by using the currently available TRPV4-selective antagonists and *trpv4*\(^{-/-}\) mice to probe the contribution of TRPV4 in agonist-induced contraction in PAs of normal and CHPH mice.

**MATERIALS AND METHODS**

**Chronic hypoxia exposure.** Adult male *trpv4*\(^{-/-}\) mice and age-matched wild-type (WT) mice (C57BL/6J; 8 wk old) were placed in a hypoxic chamber (10% O\(_2\)) for 3–4 wk to develop hypoxic pulmonary hypertension as described and characterized previously (38). The chamber was constantly flushed with hypoxic air to maintain normal CO\(_2\) concentration. Cages were cleaned and replenished with food and water once a week. Normoxic mice were housed in room air as controls.

**Measurement of hemodynamic parameters and right heart hypertrophy.** Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Pulmonary hypertension was evaluated by measuring right ventricular systolic pressure (RVSP), mean pulmonary arterial pressure (mean PAP), pulmonary vascular resistance (PVR), and right ventricular hypertrophy. The mice were ventilated by a volume-controlled ventilator (0.018 ml/g body wt and 130 cycles/min, Inspira ASV; Harvard Apparatus, Holliston, MA). RVSP and PAP were monitored using a Mikro-Tip pressure catheter (SPR-1000; Millar Instruments, Houston, TX) via direct puncture of the end of each experiment. Pulmonary vascular resistance (PVR) was measured by the normalization module of Labchart7 with tension was calculated by the resistance (PVR), and right ventricular hypertrophy. The mice were isolated and the endothelium was removed. Endothelium-denuded PAs were allowed to recover for 30 min in cold (4°C) HBSS, followed by 20 min in reduced-Ca\(^{2+}\) (20 \(\mu\)M) HBSS at room temperature. The tissue was digested at 37°C for 15 min in 20 \(\mu\)M Ca\(^{2+}\)-HBSS containing collagenase (type I; 1,750 U/ml), papain (9.5 U/ml), BSA (2 mg/ml), and dithiothreitol (1 mM) and then washed with Ca\(^{2+}\)-free HBSS to stop digestion. PASMCs were dispersed gently by trituration with a small-bore pipette in Ca\(^{2+}\)-free HBSS at room temperature. Cells were then placed on 25-mm glass coverslips. PASMCs were cultured transiently (12 h) in Ham’s F-12 medium (with l-glutamine) supplemented with 0.5% FCS (Mediatech, Herndon, VA), 100 U/ml streptomycin, and 0.1 mg/ml penicillin. Culture medium was then changed to SmGM (Lonza, Walkersville, MA) supplemented with growth factor and 10% FCS. PASMCs were allowed to grow for 3–4 more days and were then serum starved for 12 h before experiments.

**Measurement of intracellular [Ca\(^{2+}\)]\(_i\).** [Ca\(^{2+}\)]\(_i\) was monitored using the membrane-permeable Ca\(^{2+}\)-sensitive fluorescent dye fluo 3-AM. PASMCs were loaded with 5–10 \(\mu\)M fluo 3-AM (dissolved in DMSO with 20% pluronic acid) for 45 min at room temperature (~23°C) in normal Tyrode solution containing the following (in mM): 137 NaCl, 5.4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Cells were then washed and rested for 15–30 min to allow for complete deesterification of cytosolic dye. Fluoro-3 was excited at 488 nm, and emission light at >515 nm was detected using a Nikon Diaphot microscope equipped with a microfluorometer. Protocols were executed and data were collected online with a Digidata analog-to-digital interface and a pClamp software package (Axon Instruments, Foster City, CA). [Ca\(^{2+}\)]\(_i\) was calibrated using a pseudoratio method (4) with the following equation: [Ca\(^{2+}\)]\(_i\) = ([K] * R)/([([K]/[Ca\(^{2+}\)]\(_{\text{free}}\) + 1] – R), where R is F/F_0, the
dissociation constant (K) of fluo 3 is 1.1 μM, and resting Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{\text{rest}}\)] is assumed to be 150 nM.

**Data analysis.** All data are represented as means ± SE; n indicates number of animals or experiments as specified in the text. Concentration-response curves were fitted with the three-parameter logistic model, \(R = E_{\text{max}}/[1 + ([A]/EC_{50})^b]\), where \(R\) is the normalized developed tension, \(E_{\text{max}}\) is the maximal response, \([A]\) is the concentration of the agonist, and \(EC_{50}\) is the effective concentration for 50% response, and \(b\) is the slope factor. Statistical significance (\(P < 0.05\)) of the changes was compared with paired or unpaired Student’s t-tests, the nonparametric Mann-Whitney U-tests, or by one- or two-way ANOVA with Bonferroni post hoc test, wherever applicable.

**Chemicals and drugs.** RN-1734 was purchased from Calbiochem (Billerica, MA) and HC-067047, 5-HT, collagenase, protease, papain, serum albumin, dithiothreitol, antibiotics, and other chemicals were purchased from Sigma Chemical (St. Louis, MO). Ham’s F-12 medium was purchased from Mediatech (Herndon, VA), and SmGM and growth factors were purchased from Lonza. Fluo 3-AM and pluronic acid were purchased from Molecular Probes (Eugene, OR). Stock solutions of HC-067047 and RN-1734 were prepared in DMSO and diluted 1:1,000 in 2 mM Ca\(^{2+}\) solutions of HC-067047 and RN-1734 were prepared in DMSO and diluted 1:1,000 in 2 mM Ca\(^{2+}\)-Tyrode solution before use.

**RESULTS**

**Evaluation of TRPV4 antagonists for the study of pulmonary vascular reactivity.** To evaluate the contribution of TRPV4 in agonist-induced vasoactivity, we first examined the effect of the TRPV4 antagonist HC-067047 on PE-induced isometric contraction of endothelium-denuded (EC−) PAs of WT mice. Application of 1 nM to 10 μM PE activated concentration-dependent contraction of PAs with a maximal response (\(E_{\text{max}}\)) of 96.7 ± 2.3% and a pD\(_2\) (defined as \(-\log EC_{50}\)) of 7.7 ± 0.1 (n = 6; Fig. 1A). Preincubation of PA rings with 5 μM HC-067047, a concentration used previously on PASMCs (6, 47), for 15 min caused significant reduction in the maximal response (\(E_{\text{max}} = 63.0 ± 7.6, n = 10, P < 0.01\)) and the pD\(_2\) (7.2 ± 0.08, P < 0.01) of PE (Fig. 1, A–C). To validate that the vasorelaxant effect of HC-067047 was specifically due to TRPV4 inhibition, the experiments were repeated in PAs of trpv4\(^{-/-}\) mice. However, the concentration-response curve of PE generated in PAs of trpv4\(^{-/-}\) mice was identical to that of WT (\(E_{\text{max}} = 96.5 ± 2.3, \text{pD}_2 = 7.8 ± 0.06, n = 8\); Fig. 1, A–C). Preincubation of trpv4\(^{-/-}\) PAs with 5 μM HC-067047 caused significant suppression of PE-induced contraction (\(E_{\text{max}} = 57.9 ± 8.9\) and \(\text{pD}_2 = 7.3 ± 0.06, n = 4\)) similar to that observed in WT PAs (Fig. 1, D–F). Moreover, pretreatment of trpv4\(^{-/-}\) PAs with another TRPV4 antagonist, RN-1734 (30 μM), also significantly inhibited the PE-induced contraction (\(E_{\text{max}} = 74.9 ± 5.3\) and \(\text{pD}_2 = 7.2 ± 0.1, n = 4\)). These results indicate that both HC-047067 and RN-1734 at these commonly used concentrations could suppress PE-induced PA contraction through mechanisms other than TRPV4 inhibition.
To determine the optimal concentration of HC-047067 and RN-1734 with minimal off-target effects, we tested the vasorelaxant effect of the two antagonists at various concentrations on K⁺ (60 mM)-induced contraction in PAs of trpv4−/− mice. HC-047067 at 1 and 5 μM caused concentration-dependent relaxation of both endothelium-intact (EC+) and EC− PAs, while 0.5 μM HC-047067 had no significant effect on K⁺-induced PA contraction (Fig. 2, A and B). RN-1734 at concentrations of 5, 10, and 30 μM caused significant relaxation of K⁺-precontracted PAs of trpv4−/− mice (Fig. 2, C and D). Since the IC₅₀ of HC-047067 and RN-1734 for TRPV4 inhibition is 17 nM and 5.9 nM, respectively (17, 63), the significant TRPV4-independent effects of RN-1734 at 5 μM precluded its use for the experiments on pulmonary vascular reactivity. In contrast, HC-067047 at 0.5 μM has minimal off-target effects on voltage-gated Ca²⁺ channels (VDCC) and the downstream mechanisms for PA contraction; hence this concentration of HC-067047 was used in all other experiments through the study.

Contributions of TRPV4 in pulmonary vasoreactivity to 5-HT. To evaluate the contribution of the TRPV4 channel in agonist-induced pulmonary vascular reactivity, we examined the effects of HC-067047 and trpv4 gene knockout on the contractile effects of PE, endothelin-1 (ET-1), and 5-HT in isolated PAs with and without endothelium. Concentration-response curves of PE, ET-1, and 5-HT generated in EC+ PAs of WT mice generally had significantly lower E₅₀ and pD₂ values compared with those in EC− PAs, consistent with a vasodilatory influence from endothelium (Figs. 3 and 4). Inhibition of TRPV4 with 0.5 μM HC-067047, however, had no noticeable effect on the E₅₀ and pD₂ of PE or ET-1-induced contraction in both EC+ and EC− PAs (Fig. 3). In addition, concentration-response relations of PE and ET-1 obtained from PAs of trpv4−/− mice were similar to those of WT control. These results suggest that TRPV4 is not involved in PE and ET-1-mediated pulmonary vasoconstriction. In contrast, HC-067047 caused a significant reduction in pD₂ of 5-HT (control: 7.8 ± 0.06, n = 5; HC-067047: 7.5 ± 0.09, n = 5, P < 0.05), without affecting the maximal response in EC− PAs of WT mice (Fig. 4, A–C). The contribution of TRPV4 to the sensitivity of 5-HT-induced contraction was confirmed in endothelium-denuded PAs of trpv4−/− mice, where the concentration-response curve was shifted significantly to the right and overlapped with the relation generated in WT PAs in the presence of HC-067047. There was no significant difference in the 5-HT-induced contraction in EC+ WT PAs after HC-067047 treatment or in trpv4−/− PAs, suggesting that endothelial TRPV4 inhibition counteracts the influence due to inhibition of TRPV4 of PASMCs. These results suggest that TRPV4 in PASMCs participates in the 5-HT-activated pharmacomechanical coupling for PA contraction.

Contributions of TRPV4 to 5-HT-induced Ca²⁺ response in PASMCs. To further examine whether 5-HT mobilizes intracellular Ca²⁺ via TRPV4 channels, the specificity of HC-067047 on Ca²⁺ response activated by 5-HT was evaluated in PASMCs of trpv4−/− mice. 5-HT (1 μM) elicited Ca²⁺ response with a transient peak followed by a sustained Ca²⁺ response (Fig. 5, A and B). The response was unaltered in PASMCs after preincubation with 0.5 μM HC-067047 for 20 min, confirming that HC-067047 at 0.5 μM does not have a

Fig. 2. Vasorelaxant effect of TRPV4 antagonists on K⁺ precontracted PAs of trpv4−/− mice. A: representative traces showing the effect of 0.5, 1, and 5 μM HC-067047 on EC+ and EC− trpv4−/− PAs precontracted by 60 mM K⁺. B: bar graph summarizing percent inhibition of K⁺-induced contraction by HC-067047 (n = 5–7). C: traces showing the effect of 5, 10, and 30 μM RN-1734 on EC+ and EC− trpv4−/− PAs precontracted by 60 mM K⁺ (n = 6–9). D: bar graph showing percent inhibition of K⁺ induced contraction by RN-1734. Arrows indicate application of HC-067047 and RN-1734 at various concentrations.
side effect on Ca\(^{2+}\) influx or release. In contrast, HC-067047 caused significant inhibition of both the peak (control: 657.8 ± 96.4 nM, n = 14; HC-067047: 220.9 ± 39.7 nM, n = 17, P < 0.001) and the sustained Ca\(^{2+}\) response (control: 171.8 ± 35.1 nM, n = 14; HC-067047: 43.6 ± 15.2 nM, n = 17, P < 0.001) activated by 5-HT in PASMCs of WT mice (Fig. 5, C and D). The reduction in the peak response was comparable to, while the inhibition of the sustained response was stronger than, that caused by the voltage-dependent Ca\(^{2+}\) channel blocker nifedipine (1 μM), which is known to inhibit 5-HT-induced contraction of PAs (20, 58). Treatment of PASMCs with both HC-067047 and nifedipine reduced the Ca\(^{2+}\) response to the same extent as HC-067047 alone. These results suggest that the Ca\(^{2+}\) response activated by 5-HT is mediated mainly by Ca\(^{2+}\) influx via TRPV4 and voltage-gated Ca\(^{2+}\) channels, which may operate in an interdependent manner. For example, cation entry via TRPV4 may cause membrane depolarization to activate voltage-gated Ca\(^{2+}\) channels. The major contribution of Ca\(^{2+}\) influx was confirmed by almost complete inhibition of Ca\(^{2+}\) response after removal of extracellular Ca\(^{2+}\) 100 s before 5-HT application. In addition, inhibition of InsP\(_3\) receptors with xestospongin C (3 μM) caused only a small reduction in the peak response without altering the sustained Ca\(^{2+}\) increase (Fig. 5, E and F). These results are in line with the myograph
data suggesting that 5-HT activates Ca\(^{2+}\) influx via TRPV4 channels to participate in the 5-HT-mediated pulmonary vasoreactivity. To verify the successful deletion of the TRPV4 gene, long-term PAP were elevated in WT mice (Fig. 6). After 3–4 wk of hypoxia exposure, systolic, diastolic, and mean PAP were all elevated in WT mice (Fig. 6, A–D). Therefore, we further evaluated the contribution of TRPV4 in 5-HT-induced contraction in CH WT and trpv4\(^{-/-}\) mice. To compare the contribution of TRPV4 in the enhanced vascular reactivity in PASMCs of CH mice, 5-HT-induced contraction was normalized with the developed tension activated by 60 mM KCl. K\(^{+}\)-induced PA contractions were similar among PASMCs of normoxic and CH WT and trpv4\(^{-/-}\) mice, except for a small reduction (~20–30%) in the developed tension in the endothelium-denuded PASMCs, presumably due to minimal mechanical injury of PASMCs during endothelium denudation. 5-HT-induced maximal contraction was enhanced in both endothelium intact (normoxia: 101.8 ± 1.09% n = 6; CH: 136.6 ± 6.4%, n = 7, P < 0.001) and denuded PASMCs (normoxia: 114.3 ± 2.0%, n = 5; CH: 168.6 ± 8.4%, n = 13, P < 0.01) of CH WT mice (Fig. 7, A and C). The enhancement of 5-HT response was apparently greater in EC\(^+\) PASMCs. HC-067047 caused a small but significant suppression of the concentration-response relation in EC\(^+\) PASMCs (n = 6, P < 0.05), and the inhibition was more pronounced in EC\(^-\) PASMCs (n = 6, P < 0.05). 5-HT-induced contraction was also significantly potentiated in both EC\(^+\) PASMCs (normoxia: 104.6 ± 1.2%, n = 8; CH: 121.9 ± 2.6%, n = 16, P < 0.01) and EC\(^-\) PASMCs (normoxia: 113.3 ± 1.8%, n = 8; CH: 147.8 ± 3.8%, n = 16, P < 0.001) of CH trpv4\(^{-/-}\) mice. The enhancement of 5-HT maximal response in CH trpv4\(^{-/-}\) PASMCs, however, was significantly less compared with those of WT, when it is evaluated as % increase in maximal response (EC\(^+\): WT = 34.8 ± 6.4%, trpv4\(^{-/-}\) = 17.4 ± 2.7% P < 0.05; EC\(^-\): WT = 54.3 ± 3.8%, trpv4\(^{-/-}\) = 34.4 ± 3.8, P < 0.05)(Fig. 7E). HC-067047 had no further effect on the maximal response or pD\(_2\) of 5-HT-induced contraction in PASMCs of CH trpv4\(^{-/-}\) mice further substantiate our previous finding that the TRPV4 gene contributes critically to the development of CPHP (72).

**Contribution of TRPV4 in altered pulmonary vasoreactivity in CH mice.** To compare the contribution of TRPV4 in the enhanced vascular reactivity in PASMCs of CH mice, 5-HT-induced contraction was normalized with the developed tension activated by 60 mM KCl. K\(^{+}\)-induced PA contractions were similar among PASMCs of normoxic and CH WT and trpv4\(^{-/-}\) mice, except for a small reduction (~20–30%) in the developed tension in the endothelium-denuded PASMCs, presumably due to minimal mechanical injury of PASMCs during endothelium denudation. 5-HT-induced maximal contraction was enhanced in both endothelium intact (normoxia: 101.8 ± 1.09%, n = 6; CH: 136.6 ± 6.4%, n = 7, P < 0.001) and denuded PASMCs (normoxia: 114.3 ± 2.0%, n = 5; CH: 168.6 ± 8.4%, n = 13, P < 0.01) of CH WT mice (Fig. 7, A and C). The enhancement of 5-HT response was apparently greater in EC\(^+\) PASMCs. HC-067047 caused a small but significant suppression of the concentration-response relation in EC\(^+\) PASMCs (n = 6, P < 0.05), and the inhibition was more pronounced in EC\(^-\) PASMCs (n = 6, P < 0.05). 5-HT-induced contraction was also significantly potentiated in both EC\(^+\) PASMCs (normoxia: 104.6 ± 1.2%, n = 8; CH: 121.9 ± 2.6%, n = 16, P < 0.01) and EC\(^-\) PASMCs (normoxia: 113.3 ± 1.8%, n = 8; CH: 147.8 ± 3.8%, n = 16, P < 0.001) of CH trpv4\(^{-/-}\) mice. The enhancement of 5-HT maximal response in CH trpv4\(^{-/-}\) PASMCs, however, was significantly less compared with those of WT, when it is evaluated as % increase in maximal response (EC\(^+\): WT = 34.8 ± 6.4%, trpv4\(^{-/-}\) = 17.4 ± 2.7% P < 0.05; EC\(^-\): WT = 54.3 ± 3.8%, trpv4\(^{-/-}\) = 34.4 ± 3.8, P < 0.05)(Fig. 7E). HC-067047 had no further effect on the maximal response or pD\(_2\) of 5-HT-induced contraction in PASMCs of CH trpv4\(^{-/-}\) mice further substantiate our previous finding that the TRPV4 gene contributes critically to the development of CPHP (72).

**Chronic hypoxia-induced pulmonary hypertension in WT and trpv4\(^{-/-}\) mice.** It is well documented that pulmonary vasoreactivity to many vasoactive agonists, including 5-HT, is enhanced in CPHP (39, 45, 46, 58). Therefore, we further evaluated the contribution of TRPV4 in 5-HT-induced contraction in CH WT and trpv4\(^{-/-}\) mice. To verify the successful generation of a CPHP model, hemodynamic parameters of normoxic and CH mice were measured using an open-chest approach and right heart hypertrophy was determined. Consistent with our previous observation (72), deletion of the TRPV4 gene had little effect on RVSP and RV/(LV + S) ratio in normoxic mice, but significantly diminished the CH-induced increase in RVSP and right heart hypertrophy (Fig. 6, C–E). After 3–4 wk of hypoxia exposure, systolic, diastolic, and mean PAP were all elevated in WT mice (Fig. 6, F–H). Mean PAP was increased from 11.5 ± 0.18 mmHg (n = 6) to 19.8 ± 0.62 mmHg (n = 10, P < 0.001). In contrast, trpv4\(^{-/-}\) mice were relatively resistant to CH, showing ablated systolic, diastolic, and mean PAP. Mean PAP of CH trpv4\(^{-/-}\) mice was 16.8 ± 0.45 mmHg (n = 10) compared with 12.5 ± 0.27 mmHg (n = 5, P < 0.001) in normoxic animals. Likewise, CH caused a robust elevation of PVR in CH WT mice, which was markedly suppressed in trpv4\(^{-/-}\) mice (CH WT: 3.25 ± 0.22 vs. CH trpv4\(^{-/-}\): 2.47 ± 0.23 mmHg, n = 8, P < 0.05)(Fig. 6f). Cardiac output was similar in WT and trpv4\(^{-/-}\) mice with and without CH exposure (Fig. 6j). These hemodynamic data further substantiate our previous finding that the TRPV4 gene contributes critically to the development of CPHP (72).
Fig. 5. The contribution of TRPV4 in 5-HT-induced Ca^{2+} response in pulmonary arterial smooth muscle cells (PASMCs). A: mean traces of Ca^{2+} response activated by 5-HT in PASMCs of trpv4^{−/−} mice in the absence (n = 11) and presence (n = 8) of 0.5 μM HC-067047. B: mean peak and sustained Ca^{2+} response measured at 50 s after 5-HT application in experiments shown in A. C: mean traces of Ca^{2+} response activated by 5-HT in PASMCs of WT mice in the absence (n = 14) or presence of 0.5 μM HC-067047 (n = 18), 1 μM nifedipine (n = 8), or HC-067047 and nifedipine (n = 9). D: mean peak and sustained Ca^{2+} response derived from experiments in C. E: mean traces of Ca^{2+} response activated by 5-HT in PASMCs of WT mice in solution containing 2 mM Ca^{2+} (control, n = 14), Ca^{2+} free (+1 mM EGTA, n = 8), or in the presence of 3 μM xestospongin C (n = 9). F: mean peak and sustained Ca^{2+} response derived from experiments in E. Xestospongin C. *P < 0.05, **P < 0.01, ***P < 0.001 vs. controls; +P < 0.05 vs. +Nif.

(Fig. 7F). These results clearly suggest that TRPV4 in PASMCs contributes to the enhanced 5-HT-induced maximal contraction in CH PAs.

DISCUSSION

The present study exploited both TRPV4-specific antagonists and trpv4 knockout mice to probe the contributions of TRPV4 in pulmonary vasoreactivity under normal conditions and in CHPH. The major findings are as follows: 1) inhibition of TRPV4 with a low concentration of HC-067047 or deletion of trpv4 gene caused a reduction in the sensitivity of 5-HT-induced PA contraction but had no effect on ET-1 or PE-activated pulmonary vasoconstriction; 2) Ca^{2+} response elicited by 5-HT was significantly attenuated by HC-067047 in WT PASMCs but was not altered by the antagonist in trpv4^{−/−} PASMCs; 3) 5-HT-induced maximal contraction was augmented in PAs of CH WT mice and the enhanced response was partially reversed by TRPV4 inhibition; and 4) CH-induced pulmonary hypertension was significantly attenuated in trpv4^{−/−} mice, and the enhancement in 5-HT induced contraction was reduced in trpv4^{−/−} PAs. These results suggest that TRPV4 is associated specifically with 5-HT-dependent pharmaco-mechanical coupling in murine PAs and plays a significant role in the altered vasoreactivity in CHPH. In addition, our evaluation of TRPV4 antagonists in PAs of trpv4^{−/−} mice raises concern for the use of high concentrations of TRPV4 antagonists, especially RN-1734, in the studies of vascular functions due to their nonspecific off-target effects.

TRPV4 is a multimodal channel sensitive to diverse stimuli including osmotic cell swelling, mechanical stress, heat, endogenous ligands, and chemical stimulants, serving a wide spectrum of physiological functions in different cell types (16). But its role in agonist-induced vasoreactivity has only been sparingly studied. There is ample evidence in other cell sys-
tems suggesting that TRPV4 can be regulated by receptor-mediated signaling pathways. Studies on osmo-mechanosensitivity of TRPV4 show that its activity is dependent on phospholipase A (PLA) activation and subsequent generation of arachidonic acid metabolites epoxyeicosatrienoic acids (5,6-EET, 8,9-EET) by cytochrome P450 epoxygenase (64, 65, 66) and/or is related to Src family tyrosine kinase-mediated tyrosine phosphorylation (69, 71). Protein kinase C (PKC) and protein kinase A (PKA)-dependent phosphorylation can sensitize osmotic activation of TRPV4 in HEK293 cells, and the sensitization is mimicked by bradykinin, which activates PKC via a G protein-coupled mechanism (18). Multiple PKC-dependent phosphorylation sites involved in TRPV4 sensitization have been identified (18, 51). A recent study suggests that acetylcholine stimulates TRPV4-dependent Ca release in endothelial cells through activation of PKC (1). Besides PKC, InsP3 can sensitize TRPV4 to the stimulation of 5,6-EET in ciliated epithelial cells, presumably through binding of the InsP3 receptor to a TRPV4 COOH-terminal domain (19, 21). Moreover, TRPV4 forms heteromeric channels with TRPC1 in endothelial cells and contributes to store-operated Ca entry (42–44), which is a major Ca influx pathway activated by InsP3-induced Ca release. Based on these findings in other cell systems, it seems justified to postulate that agonist receptors coupled to PLA, phospholipase C (PLC), and/or tyrosine kinase are capable of stimulating TRPV4 channels in vascular smooth muscle cells.

Our experiments using three different vasoactive agonists, 5-HT, PE, and ET-1, clearly show that TRPV4 contributes specifically to the 5-HT-induced pulmonary vasoactivity but it is not involved in PE or ET-1-mediated contractile response. In nonhypoxic mice, the contribution of TRPV4 to 5-HT-induced pulmonary vasoreactivity is evident by the rightward shift in the concentration-response relation after TRPV4 inhibition by HC-067047 and by a similar shift observed in PASMs of trpv4 mice. It is further supported by the marked inhibition of 5-HT-induced Ca release by HC-067047 in WT PASMCs, and the absence of the inhibitory effect of the antagonist in trpv4 PASMCs. The specific involvement of TRPV4 in 5-HT-mediated response suggests unique coupling of the TRPV4 channel to the 5-HT-dependent signaling pathways. Previous studies showed that the 5-HT receptors 5-HT2A, 5-HT1B, and 5-HT2B are expressed in PAs of rats and mice (28, 35, 46). 5-HT-induced pulmonary vasoconstriction is mediated mainly by 5-HT2A and to a lesser extent by 5-HT1B in normoxic PAs (28, 46, 54). Since 5-HT2A stimulates PLC through Gq and activates PLAr through a PLC-independent signaling cascade (33, 34, 53), its activation may act as a dual...
stimulus for TRPV4 activation through the synergistic actions of PLC-dependent activation of PKC and InsP3 release, and PLA2-dependent production of the arachidonic acid metabolites EETs. Our results, therefore, provide the evidence for substantiating the claim that 5-HT indeed stimulates TRPV4 channels (8, 22), which represents an important component of Ca2+ mobilization that contributes to 5-HT-induced pulmonary vasoconstriction.

PE and ET-1 bind to \(\alpha_1\)-adrenergic receptors and ET\(_A\) receptors, respectively, both of which activate PLC through \(G_\alpha\). ET-1 and PE have been reported to stimulate arachidonic acid release in vascular and nonvascular smooth muscles (7, 26, 62, 70), but the participation of PLA2 activation in ET-1 or PE-mediated pulmonary reactivity is unclear (57). It is now recognized that G protein-coupled receptors are spatially organized with members of their signaling pathways and ion channels as signalomes or channelsomes in specialized membrane domains for efficacious signal transduction (41, 49). The selective activation of TRPV4 by 5-HT but not by ET-1 and PE despite their similar signaling mechanisms suggests that TRPV4 is perhaps associated discriminatorily with 5-HT-specific signalomes in PASMCs.

It is well established that 5-HT plays a major role in the development of pulmonary hypertension, which is associated with increase in plasma 5-HT levels, upregulation of 5-HT\(_1B\) and 5-HT\(_3B\) receptors (28, 35, 55) and 5-HT transporter (12–14), as well as enhanced 5-HT-induced pulmonary vasoconstriction and PASMC proliferation (12, 15, 28, 45, 46). Consistent with observations in the CH rat model (45, 46, 58), we found a potentiation in the 5-HT-induced maximal contraction in PAs of CH WT-mice. The partial reversal of the enhanced 5-HT-induced contraction by HC-067047 in WT PAs and the significant reduction of the maximal response in trpv4\(^{-/-}\) PAs further suggest that TRPV4 contributes to the enhanced vasoactivity developed in CPH. It is interesting to note that the contribution of TRPV4 is apparent in the maximal response in CH PAs, instead of the sensitivity to 5-HT as observed in normoxic PAs. This could be related to alterations in 5-HT receptor subtypes in CH PAs, where 5-HT\(_1B\) and 5-HT\(_2B\) receptors expression are upregulated and the contribution of 5-HT\(_1B\) to pulmonary vasoconstriction is augmented (28, 35, 45, 55). The participation of TRPV4 in the maximal response elicited by 5-HT in CH PAs could be related to upregulation of TRPV4 channels similar to the CH rat model (72). It could also be related to the enhanced PLA2/arachidonic acid/cytochrome-P450 epoxygenase/EETs signaling pathway. Previous studies showed that exposure of mice to hypoxia upregulates cytochrome-P450 epoxygenases and downregulates the soluble epoxide hydrolase, resulting in the increase in EET production and accumulation in the lungs (29, 52). Stimulation of PLA2 by 5-HT, hence, may generate a stronger stimulus for TRPV4 activation to potentiate vasoconstriction in CH PAs. The enhanced EET-TRPV4 mechanism can be important for CPH development, as inhibition of cytochrome-P450 epoxygenases attenuates whereas deletion of the soluble epoxide hydrolase gene exaggerates CPH (29, 52).

It has to be emphasized, however, that TRPV4 is just one of the many pathways participating in 5-HT-induced pulmonary vasoconstriction, and other effectors such as NADPH oxidases, Ca\(^{2+}\)-activated Cl\(^-\) channels, and Rho-kinase are known to be
involved in the enhanced pulmonary vasoreactivity in CHPH (3, 20, 25, 39, 58). Nevertheless, TRPV4 may participate cooperatively with the other mechanisms to reinforce the alteration in pulmonary vascular reactivity.

We have previously shown that CH upregulates TRPV4 expression in rat PAs, and its upregulation is associated with enhanced basal vasomotor and myogenic tone. The increase in RVSP and right heart hypertrophy is delayed and suppressed, and vascular remodeling is attenuated in CH trpv4−/− mice (72). Here, by using an open-chest approach, we further confirm that the elevation of mean PAP and pulmonary vascular resistance is greatly suppressed in CH trpv4−/− mice without significant alteration of cardiac output. Hence, the participation of TRPV4 in CHPH is solely restricted to pulmonary vasculature and is independent of cardiac function. Recent evidence from others also shows that CH enhances TRPV4-induced Ca2+ release from ryanodine-sensitive Ca2+ stores in rat PASMCs (6) and activation of TRPV4 promotes PASMC migration (47). Taking all these pieces of information together, we postulate that TRPV4 in PASMCs contributes to the development of CHPH through multifaceted influences on Ca2+ influx and release, promoting basal and myogenic tone, cell migration, and vascular reactivity to collectively muster an elevation of pulmonary vascular resistance and vascular remodeling. Recent research in other areas has pinpointed the involvement of TRPV4 in many different diseases (50) and has suggested TRPV4 as an important therapeutic target (61). In view of its roles in multiple pathological changes in CHPH, TRPV4 can be considered as a potential target for the treatment of pulmonary hypertension.

Our experiments for evaluating TRPV4 antagonists in PAs of trpv4−/− mice raise concern for the use of high concentrations of TRPV4 antagonists. HC-067047 and RN-1747 have dramatic off-target effects at the concentrations of these antagonists need to be interpreted with caution. This precludes its use in experiments on vascular remodeling. Recent research in other areas has pinpointed the elevation of pulmonary vascular resistance and vascular reactivity in chronic hypoxic pulmonary hypertension. Taking all these pieces of information together, we postulate that TRPV4 in PASMCs contributes to the development of CHPH through multifaceted influences on Ca2+ influx and release, promoting basal and myogenic tone, cell migration, and vascular reactivity to collectively muster an elevation of pulmonary vascular resistance and vascular remodeling. Recent research in other areas has pinpointed the involvement of TRPV4 in many different diseases (50) and has suggested TRPV4 as an important therapeutic target (61). In view of its roles in multiple pathological changes in CHPH, TRPV4 can be considered as a potential target for the treatment of pulmonary hypertension.

In conclusion, we have used both pharmacological and genetic tools to evaluate the role of TRPV4 in pulmonary vascular reactivity. We found unequivocally that TRPV4 contributes to 5-HT-mediated pulmonary vasoconstriction and is responsible in part for the enhanced 5-HT-induced response in CHPH. Together with its roles in enhanced myogenic tone, PASMC migration, and pulmonary vascular remodeling, TRPV4 presents itself as an important player in the development of CHPH.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Y.X., Z.F., J.H., C.H., and O.P. performed experiments; Y.X. and Z.F. analyzed data; Y.X. and J.S.K.S. interpreted results of experiments; Y.X. prepared figures; Y.X. drafted manuscript; Y.X., S.C., W.L., and J.S.K.S. edited and revised manuscript; J.S.K.S. conception and design of research; J.S.K.S. approved final version of manuscript.

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