Implication of the ryanodine receptor in TRPV4-induced calcium response in pulmonary arterial smooth muscle cells from normoxic and chronically hypoxic rats

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In vascular smooth muscle cells (VSMC), including pulmonary artery smooth muscle cells (PASMC), an increase in intracellular calcium concentration ([Ca2+]i) is a key step in the initiation of a variety of cellular functions such as contraction, proliferation, and migration (31, 40). In PASMC, agonists or hypoxia increase [Ca2+]i, which results from the release of intracellular stored calcium followed by an influx of extracellular calcium (8, 15, 51, 53).

Intracellular released calcium essentially originates from the sarcoplasmatic reticulum (SR) via the activation of two types of receptor/channels: the inositol 1,4,5-trisphosphate receptor/channel (IP3R) and the ryanodine receptor/channel (RyR) (1, 2, 33, 35). RyR are homotetrameric proteins with a total molecular mass greater than 2,000,000 Da and are controlled by several cytosolic or luminal proteins (30, 55). Three different subtypes of RyR, namely RyR1, RyR2, and RyR3, have been initially characterized in skeletal muscle, cardiac muscle, and brain, respectively (28, 46, 55). The expression of multiple RyR subtypes has been subsequently demonstrated in a variety of vascular smooth muscles (9, 38, 56), including pulmonary artery (7, 63, 65). In PASMC, RyR2 expression appears the most abundant (63), and the three isoforms of RyR are differentially localized in the cell. Indeed, RyR1 and RyR3 appear mainly concentrated in the cell cortex, whereas RyR2 seems to be present in more central regions of the cell (7, 63, 66). As a consequence, the implication of RyR in both the contractile response to acute hypoxia and agonists (e.g., norepinephrine) and in vasorelaxation has been addressed (17, 66).

Extracellular calcium ions enter the smooth muscle cell following the activation of voltage-operated calcium channels (L- and T-type calcium channels) or nonselective cation channels (NSCC) that are permeable to calcium (25). Part of NSCC belongs to the large family of transient receptor potential (TRP) proteins. Based on structural homology, the mammalian TRP superfamily has been subdivided into two groups of a total of seven subfamilies. Group 1 consists of the classical/canonical (TRPC), melastatin (TRPM), vanilloid-related (TRPV), ankyrin-related (TRPA), and no mechanoreceptor potential C (TRPN) subfamilies; group 2 includes the polycystin-related (TRPP) and mucolipin-related (TRPML) subfamilies (45, 58). All TRP proteins share the following common features: six transmembrane domains, a pore-forming loop between the fifth and sixth transmembrane segments, and the highly conserved TRP domains (45, 58). To date, 28 mammalian TRP homologs have been found in a wide variety of cells and tissues, and at least 10 TRPs have been identified as functional channels in VSMC (11, 16, 24, 32). Among them, TRPC, TRPM, and TRPV are widely expressed in VSMC and are implicated in a variety of physiological and pathophysi-
logical processes such as VSMC contraction, relaxation, growth, migration, and proliferation and control of blood pressure, arterial myogenic tone, and pulmonary hypertension (11, 16, 24, 32).

In PASMC, TRPC, TRPV, and TRPM are expressed and functional (59, 62), and, among the TRPV subfamily, type 4 vanilloid TRP subfamily (TRPV4) is the most abundantly expressed (62). TRPV4 is a Ca\(^{2+}\)-permeable NSCC, activated by a wide variety of stimuli, including physical, thermal, and chemical stimuli such as the synthetic agonist 4α-phorbol-12,13-didecanoate (4α-PDD), the arachidonic acid (AA) precursor, the endocannabinoid anandamide, and the AA metabolite epoxideicosatrienoic acid (EET) (19, 48). In rat pulmonary artery, it was previously shown that 4α-PDD induces a TRPV4-like current and increases [Ca\(^{2+}\)](i) of PASMC (15, 62). In other cell types, TRPV4 has been demonstrated to be the molecular basis of stretch-activated channels (39, 42), and, interestingly, the activity of stretch-activated channels in PASMC is increased under chronic hypoxia (14, 61).

It is thus evident that TRPV4 channels and RyR are two key actors of calcium signaling in VSMC. In cerebral arteries, a functional link between TRPV4 and RyR has been demonstrated, i.e., influx of calcium ions through TRPV4 can induce calcium release from SR via stimulation of RyR. Such a calcium-induced calcium release mechanism subsequently activates closely located large-conductance calcium-activated potassium (BKCa) channels and thus induces hyperpolarization and vasodilatation (17). However, with respect to vasoconstriction, the question of a possible direct link between TRPV4 and RyR2 has not been addressed so far. Because it is also known that calcium signaling in PASMC is altered in hypoxic conditions (3, 50, 53), we thus investigated the implication of RyR in the calcium and the contractile response induced by the TRPV4 agonist 4α-PDD in pulmonary artery from normoxic (Nx) and chronically hypoxic (CH) rats, a well-established animal model of pulmonary hypertension (4, 54).

MATERIALS AND METHODS

Animal model. The investigation was carried out in agreement with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH publication no. 85–23, revised 1996) and European Directives (86/609/CEE). Wistar male rats (200–300 g) were separated into two groups. The first group, control or Nx rats, was housed in ambient room air, whereas the second group, CH rats, was exposed to chronic hypoxia for 3 wk in a hypobaric chamber (50 kPa). Pulmonary hypertension was assessed by measuring the pulmonary artery pressure (PAP) and the ratio of right ventricle to left ventricle plus septum weight (Fulton’s index). To measure PAP, rats were anesthetized with 10 mg/kg xylazine and 50 mg/kg ketamine by intraperitoneal injection, and mean PAP was measured, in closed-chest rats, through a catheter inserted in the right jugular vein, then through the right atria and the right ventricle into the pulmonary artery and attached to a Baxter Uniflow gauge pressure transducer. Pressure was recorded with an 4.2-mm CaCl\(_2\), first, small pieces (1 × 1 mm) of IPAI and IPAA2 were then dissected free from surrounding connective tissues under binocular control.

PASMC isolation and culture. As previously described (14), primary cultured cells were obtained using a dispersal procedure with a low-calcium dissociation solution named DS (done with Hanks’ balanced salt solution supplemented with 100 μM CaCl\(_2\); first, small pieces (1 × 1 mm) of IPAI and IPAA2 and endothelium removed were washed for 15 min at room temperature. Next, tissue was transferred in DS containing 1 mg/ml papain and 0.3 mg/ml dithioerythritol (DTT) after enzymatic treatment, tissue was washed three times and suspended in DS before to be gently triturated (15–20 min at room temperature). Tissue dissociated was removed, and suspended cells were centrifuged at 250 g for 5 min at 4°C. Pellet was resuspended, and cells were immediately seeded in sterile glass cover slips with culture medium enriched with 10% fetal bovine serum. Cells were placed in a humidified incubator under 5% CO\(_2\) at 37°C and were used between 4 and 16 h after isolation.

Western blot. After removal of endothelium using 0.3% 3-[3-cholamidopropilidimethylammonio]1-propanesulfonate (37), all IPAI were dissected and extracted using RIPA buffer [1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 150 mM NaCl, and 20 mM sodium or potassium phosphate, pH 7.2] with 5 mM EDTA and anti-protease cocktail (P8340; Sigma) for 1 h on ice. After scraping, any insoluble material was removed by centrifugation at 15,000 g for 10 min at 4°C, and the amount of protein was assessed by the Lowry method. After heating during 6 min at 90°C, equal amounts of proteins were subjected to electrophoresis on a 8–14% acrylamide reducing gel. The proteins were transferred onto polyvinylidene fluoride membranes (Immobilon-P; Millipore) using a semidry electrophoretic system (Bio-Rad) for TRPV4 during 50 min. RyR proteins were transferred using liquid transfer with a first migration at 4°C overnight at 30 V and the next day a second migration at 100 V during 1.25 h at room temperature. Membranes were saturated in nonfat milk during 1 h and incubated overnight at 4°C with the following diluted primary antibodies: rabbit anti-TRPV4 (1:400; Abcam) or rabbit anti-RyR1 (1:200; Alomone), RyR2 (1:400; Alomone), and RyR3 (1:200; gift from Dr. V. Sorrentino). The membranes were then washed with a 1% PBS-Tween buffer and treated with the corresponding horseradish peroxidase-linked secondary antibodies (anti-mouse or anti-rabbit; Pierce) for 2 h at room temperature. After several washes in 1% PBS-Tween buffer, the membranes were processed for chemiluminescent detection using the Super Signal West Pico or Femto chemiluminescent detection using the Super Signal West Pico or Femto (Pierce) for 2 h at room temperature. After several washes in 1% PBS-Tween buffer, the membranes were processed for chemiluminescent detection using the Super Signal West Pico or Femto chemiluminescent substrate (Pierce), according to the manufacturer’s instructions. Immunoblots were then revealed by enhanced chemiluminescence acquired using Kodak Image Station 4000 MM. Band densities were quantified using GeneTool software (SynGene). Immunoblots were then stripped and revealed with mouse anti-β-actin.

The intensity of the signals was evaluated by densitometry and semiquantified as the intensity of band corresponding to the protein of interest divided by the intensity of the band corresponding to actin for each experiment.

Electrophysiological recordings. Channel activity was recorded using the whole cell patch-clamp technique (29). The electrodes were pulled on a PC-10 (Narishige) puller in two stages from borosilicate glass capillaries (1.5 mm OD, 1.66 mm ID; Harvard Apparatus). The pipettes had a mean resistance of 3–4 MΩ when measured in standard recording conditions. Cells were viewed under phase contrast with a Nikon Diaphot inverted microscope. A RK 400 patch amplifier (Biologic) was used for current recordings. Stimulus control, data acquisition, and processing were carried out on a personal computer fitted with a Digidata interface (Axon Instruments), using pclamp 10.2 software. Current records were filtered with a Bessel filter at 1 kHz and digitized at 4 kHz for storage and analysis. Data were analyzed using Clampfit 10.2 software. Extracellular solution contained (in
mM) 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 8 HEPES, and 11 glucose at pH 7.4. Intracellular solution contained (in mM) 140 sodium glutamate, 2 MgCl₂, and 10 HEPES, pH 7.3.

Microspectrofluorimetric assay of cytosolic calcium. The Ca²⁺-sensitive fluorescent probe indo 1 was used to record changes in [Ca²⁺]ᵢ. The cells, plated on glass cover slips, were incubated with 5 μM indo 1 penta-acetoxyethyl ester in Krebs-HEPES solution (composition in mM: 118.4 NaCl, 4.7 KCl, 2 CaCl₂, 1.2 MgSO₄, 4 NaHCO₃, 1.2 KH₂PO₄, 6 d-glucose, and 10 HEPES, pH 7.4) at room temperature for 40 min, then washed, and again maintained at room temperature in the same saline solution before the fluorescence measurements. For single cell measurements, the dual emission microscope constructed from a Nikon Diaphot inverted microscope fitted with epifluorescence (×40 oil immersion fluorescence objective; numerical aperture, 1.3). For excitation of indo 1, a collimated light beam from a 100-watt mercury arc lamp (Nikon) was filtered at 355 nm and reflected from a dichroic mirror (380 nm). The emitted fluorescence signal was passed through a pinhole diaphragm slightly larger than the selected cell and directed onto another dichroic mirror (455 nm). Transmitted light was filtered at 480 nm, reflected from 405 nm, and the intensities were recorded by separate photometers (P100; Nikon). Under these experimental conditions, the fluorescence ratio (F₄₀₅/F₄₈₀) was calculated and recorded on-line as a voltage signal.

Isometric force measurement. IPA2 (internal diameter <200 μm) were dissected free of connective tissue. Segments of 1.6 to 2 mm length were mounted in a Mulvany myograph (Multi Myograph system, model 610M, J. P. Trading), bathed in 5 ml Krebs solution maintained at 37°C, and gassed with a mixture of 95% O₂-5% CO₂ (pH 7.4). As determined in preliminary experiments (14), the optimal resting tension for IPA removed from rats under either normoxic or hypoxic conditions corresponds to an equivalent transmural pressure of 30 mmHg (equivalent to 3.99 mN/mm²) (36) or 50 mmHg (equivalent to 6.65 mN/mm²), respectively. Cumulative addition of 4α-PDD (1–20 μM) was performed in the absence or in the presence of ryanodine (100 μM, added 30 min before), an inhibitor of the three RyR isoforms, or dantrolene (10 μM, added 15 min before), an inhibitor of RyR1 and RyR3 only.

Chemicals. General salts were from VWR. All other chemicals were purchased from Sigma, except HC-067047 and paxilline were from Tocris Bioscience and DMEM-HEPES, FCS, and penicillin-streptomycin were from Gibco.

Data and statistical analysis. For fluorescence experiments, the delta fluorescence ratio (Δ_ratio) was determined as the difference between the basal fluorescence ratio recorded before the addition of the drug and the fluorescence recorded at the peak response. In some experiments, the kinetics of the [Ca²⁺]ᵢ response was analyzed by calculating t½, the time to return to 50% of the peak amplitude. Results are expressed as means ± SE; n indicates the number of preparations, tissues, tested cells, or arterial rings with a number of rats used for each condition from five to eight individuals indicated by N. Statistical analyses were performed using a nonparametric test for

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Fig. 1. Effect of type 4 vanilloid transient receptor potential subfamily (TRPV4) activation on the intracellular calcium concentration ([Ca²⁺]ᵢ) in pulmonary artery smooth muscle cells (PASMC) from normoxic rats. A: typical traces of [Ca²⁺]ᵢ under application of 4α-phorbol-12,13-didecanoate (4α-PDD, 5 μM), a specific activator of TRPV4, in the absence (control) or in the presence of caffeine (5 mM) or xestopongin C (10 μM). 4α-PDD was applied during the time indicated by the horizontal bar, whereas caffeine and xestopongin C were applied, respectively, 5 and 20 min before and during application of 4α-PDD. Ordinate: the calcium response was estimated by the fluorescence ratio (F₄₀₅/F₄₈₀) of indo 1. B: histograms are frequency distributions (means ± SE) for the effect of 4α-PDD on the [Ca²⁺]ᵢ in the absence (control, N = 5, n = 29) or in the presence of 1 (N = 5, n = 20) and 5 (N = 6, n = 24) μM HC-067047 (HC). C: histograms are frequency distributions (means ± SE) for the effect of 4α-PDD on the [Ca²⁺]ᵢ in the absence (control) or in the presence of caffeine (5 mM, N = 5, n = 22), thapsigargin (TG) plus cyclopiazonic acid (CPA) (N = 6, n = 24), or xestopongin C (10 μM, N = 5, n = 22). Ordinate in B and C: calcium response estimated by the delta fluorescence ratio (Δ_ratio) that was determined as the difference between the basal fluorescence ratio recorded before the addition of the drug and the fluorescence recorded at the peak response. *P < 0.05, **P < 0.01 compared with the control response in Kruskal and Wallis tests.
unpaired samples (Mann-Witney and Kruskal-Wallis tests) based on \( N \) numbers. Values of \( P < 0.05 \) were considered significant.

**RESULTS**

**Chronic hypoxia induced pulmonary hypertension.** Chronic hypoxia (3 wk at 50 kPa) increased both the mean PAP (15.6 \( \pm \) 1.6 vs. 25 \( \pm \) 1.2 mmHg, \( N = 6 \), in control and CH rats, respectively) \( (P < 0.05) \) and the Fulton’s index (0.26 \( \pm \) 0.04 vs. 0.5 \( \pm \) 0.02, \( N = 6 \), in control and CH rats, respectively) \( (P < 0.05) \).

**Role of intra- and extracellular calcium in TRPV4-induced \([Ca^{2+}]_i\) response in PASMC from normoxic rats.** Application of 4\( \alpha \)-PDD (5 \( \mu \)M), a non-protein kinase C-activating phorbol derivative and specific TRPV4 agonist (60), induced a \([Ca^{2+}]_i\] increase in PASMC from control (normoxic) rats \( (\Delta_{\text{ratio}}: 0.22 \pm 0.01, N = 5 \) rats, \( n = 29 \) cells) \( (\text{Fig. 1}) \). This 4\( \alpha \)-PDD-induced \([Ca^{2+}]_i\] response was inhibited by the selective TRPV4 blocker HC-067047 (20) in a concentration-dependent manner \( (\Delta_{\text{ratio}}: 0.14 \pm 0.02, N = 5 \) rats, \( n = 20 \) cells, \( P < 0.05 \) and \( \Delta_{\text{ratio}}: 0.06 \pm 0.01, N = 6 \) rats, \( n = 24 \) cells, \( P < 0.01 \), in the presence of 1 and 5 \( \mu \)M HC-067047, respectively) \( (\text{Fig. 1B}) \). The 4\( \alpha \)-PDD-induced \([Ca^{2+}]_i\] response was fully inhibited in the absence of external \( Ca^{2+} \) \( (N = 5, n = 35 \) cells) or in the presence of external \( Ni^{2+} \) \( (300 \mu \text{M}, N = 5, n = 35 \) cells), a potent inhibitor of NSCC (data not shown). In a subsequent series of experiments, we tested the effect of caffeine and of thapsigargin (TG) and cyclopiazonic acid (CPA), well known compounds to empty internal \( Ca^{2+} \) store in muscle cells, including PASMC.

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**A**

**Normoxic rat**

- **4\( \alpha \)-PDD**
- **Control**
- **Ryanodine (100 \( \mu \)M)**

**B**

**Chronically hypoxic rat**

- **4\( \alpha \)-PDD**
- **Control**
- **Ryanodine (100 \( \mu \)M)**

**C**

**Delta Ratio (405/480)**

- **Nx**
- **CH**

Fig. 2. Implication of ryanodine receptor (RyR) subtype in the TRPV4-induced calcium response in PASMC from normoxic and chronically hypoxic rats. A: experiments were performed on PASMC from normoxic rats. Typical traces of 4\( \alpha \)-PDD (5 \( \mu \)M)-induced calcium response in the absence (control) and in the presence of ryanodine (100 \( \mu \)M). **B**: experiments were performed on PASMC from chronically hypoxic rats. Typical traces of 4\( \alpha \)-PDD (5 \( \mu \)M)-induced calcium response in the absence (control) and in the presence of ryanodine (100 \( \mu \)M). **C**: histograms are frequency distributions (means \( \pm \) SE) for the caffeine (5 \( \mu \)M)-induced calcium response in PASMC from normoxic (Nx, open bar) and chronically hypoxic (CH, filled bar) rats \( (N = 7, n = 31 \) and \( N = 5, n = 29 \) respectively). AB: 4\( \alpha \)-PDD was applied during the time indicated by the horizontal bar, whereas ryanodine was applied 30 min before and during application of 4\( \alpha \)-PDD. Ordinate: the calcium response was estimated by the fluorescence ratio \( (F_{405}/F_{480}) \) of indo 1. Ordinate in ABb and C: the calcium response estimated by \( \Delta_{\text{ratio}} \), that was determined as the difference between the basal fluorescence ratio recorded before the addition of the drug and the fluorescence recorded at the peak response. \(*P < 0.05 \) and \(**P < 0.01 \) compared with the control response. Kruskal and Wallis tests were used for A and B, whereas the Mann-Whitney test was applied for C.
(23, 27, 33). When PASMC were preincubated with caffeine (5 mM) for 5 min, we observed that the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response was significantly reduced (Δratio: 0.15 ± 0.01, N = 5, n = 22, P < 0.01) compared with the absence of caffeine (Fig. 1A). A similar decrease (i.e., 65%) of the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response was also observed when PASMC were preincubated for 15 min with TG (1 μM) and CPA (10 μM) (Fig. 1C). Altogether, these experiments strongly suggest that both calcium influx and calcium release from \([\text{Ca}^{2+}]_i\) stores are implicated in the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response.

Identification of the intracellular calcium-release channel implicated in the TRPV4-induced \([\text{Ca}^{2+}]_i\) response. In PASMC, release of \([\text{Ca}^{2+}]_i\) from the internal store is operative by at least two types of receptor/channels proteins, RyR and IP₃R. When PASMC from Nx rats were preincubated for 20 min with xestospongin C (10 μM), an inhibitor of IP₃R (35), the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response was not modified (Δratio: 0.23 ± 0.02, N = 5, n = 32, P > 0.05) (Fig. 1A). In another set of experiments, we then focused our attention on the implication of RyR subtypes in the TRPV4-activated \([\text{Ca}^{2+}]_i\) signaling using RyR inhibitors. When PASMC from control rats were preincubated for 30 min with ryanodine (100 μM), a nonselective inhibitor of all RyR subtypes (RyR1, RyR2, and RyR3), the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response was significantly reduced (Δratio: 0.27 ± 0.01, N = 8, n = 29 and 0.16 ± 0.02, N = 5, n = 29, P < 0.05, in the absence and in the presence of ryanodine) (Fig. 2Aa,b). However, when PASMC from control rats were preincubated for 15 min with dantrolene (10 μM), an inhibitor of RyR1 and RyR3 subtypes only (57), the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response was not significantly reduced (Δratio: 0.26 ± 0.02, N = 6, n = 28, P > 0.05, compared with the absence of dantrolene) (Fig. 2Ab). When experiments were performed on PASMC from CH rats, we observed that 1) the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response was significantly higher (Δratio: 0.34 ± 0.03, N = 6, n = 33, P < 0.05) than the one in control rats (Fig. 2B); 2) the slope of the recovery phase of the \([\text{Ca}^{2+}]_i\) response was slower (t_{1/2} = 134.2 ± 11.2 s, N = 5, n = 15 and 270.6 ± 19.2 s, N = 5, n = 16, P < 0.05, in PASMC from Nx and CH rats, respectively); 3) ryanodine and dantrolene induced similar effects (Fig. 2Bb), i.e., ryanodine decreased the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response by 40.7 and 64.7% (P < 0.05) in PASMC from Nx and CH rats (0.16 ± 0.02, N = 5, n = 29 and 0.12 ± 0.01, N = 5, n = 29), respectively, whereas dantrolene did not change this response in CH rats (Δratio: 0.33 ± 0.03, N = 5, n = 20); and 4) the caffeine-induced \([\text{Ca}^{2+}]_i\) response was increased (Δratio: 0.15 ± 0.03, N = 7, n = 31 and 0.22 ± 0.02, N = 5, n = 29, P < 0.05, in PASMC from Nx and CH rats, respectively) (Fig. 2C). These experiments suggest that RyR2 is the RyR subtype implicated in the TRPV4-activated \([\text{Ca}^{2+}]_i\) signaling pathway.

To ensure the specificity of dantrolene on RyR, we checked the effect of dantrolene on the caffeine-induced \([\text{Ca}^{2+}]_i\) response. Preincubation of PASMC from Nx rats with dantrolene (10 μM) for 15 min strongly reduced the percentage of responding cells to caffeine (5 mM) (80%, N = 5, n = 64 and 25%, N = 5, n = 57, in the absence and in the presence of dantrolene, respectively) (data not shown).

TRPV4 and RyR expression in pulmonary arteries from Nx and CH rats. Western blot analysis revealed the expression of TRPV4 (band at ~98 kDa) in IPA from Nx and CH rats (Fig. 3Aa). Control positive experiments also showed the presence of TRPV4 in aorta from Nx rats (Fig. 3Aa). TRPV4 expression was increased by 154% in CH rats compared with Nx rats (OD: 0.54 ± 0.04, N = 5 and 0.21 ± 0.05, N = 6, respectively, P < 0.05) (Fig. 3Ab). RyR2 (double bands at ~250 kDa) were expressed in IPA from both Nx and CH rats (Fig. 3Ba), with a higher expression (68%) in CH rats (0.22 ± 0.05, N = 6 and 0.37 ± 0.04, N = 6, respectively, P < 0.05) (Fig. 3Bb). These data support the difference of amplitude of the 4α-PDD-induced \([\text{Ca}^{2+}]_i\) response noted Fig. 2 between Nx and CH rats. We also observed the expression of RyR1 and RyR3 isoforms, but
the variations were not significant between Nx and CH rats (N = 5, data not shown).

Effects of 4α-PDD on outwardly rectifying currents in PASMC from Nx and CH rats. To test the TRPV4 channel activity, ionic current induced by 4α-PDD was recorded on freshly dissociated PASMC. In PASMC from Nx rats, 4α-PDD (5 μM) increased the current elicited by a ramp depolarization from −100 to +80 mV (Fig. 4Aa). This current was an outwardly rectifying current, with a reversal potential of −3.45 ± 2.2 mV, N = 6, n = 12 and a slope conductance (between −80 and 0 mV) of 452 ± 63 pS, N = 6, n = 12 (Fig. 4Aa). The 4α-PDD-induced current was fully inhibited by 5 μM HC-067047 (Fig. 4Ab) and by ruthenium red (10 μM), another inhibitor of TRPV4 channels (N = 5, n = 10, data not shown). 4α-PDD induced a current with similar properties in PASMC from CH rats (reversal potential of −5.3 ± 2.4 mV and slope conductance of 745 ± 150 pS, N = 5, n = 10) (Fig. 4B).

However, the current density was statistically larger (+73.5%) in PASMC from CH rats compared with the Nx rat (−5.26 ± 0.22 pA/pF, N = 5, n = 10, and −3.03 ± 0.2 pA/pF, N = 6, n = 12, respectively at −80 mV) (P < 0.05) (Fig. 4C).

Effect of TRPV4 and RyR2 on pulmonary arterial tone. Finally, we tested the effect of 4α-PDD on the pulmonary artery tone. In Nx rats, 4α-PDD (5–20 μM) induced concentration-dependent elevation of resting tone (Fig. 5). As for [Ca2+]i and current responses, the amplitude of the 4α-PDD-induced contraction was significantly reduced in the additional presence of HC-067047 (5 μM) (Fig. 6A). In the presence of 10 μM of dantrolene, the 4α-PDD-induced contractile response was unchanged, whereas, in the presence of 100 μM ryanodine, it was significantly decreased (53.05% reduction in the presence of 20 μM 4α-PDD, N = 5, n = 10, P < 0.05) (Fig. 5A). In CH rats, 1) the 4α-PDD-induced contractile responses were significantly increased compared with Nx rats.

![Image](http://ajplung.physiology.org/)

**Fig. 4.** Effects of 4α-PDD on ionic currents in PASMC from Nx and CH rats. A: whole cell patch-clamp recording on PASMC from normoxic rats. a, Representative current trace obtained before (control = ctr) or after 4α-PDD (5 μM) application. b, Representative current trace obtained in the presence of 4α-PDD (5 μM) alone and in the additional presence of the compound HC-067047 (HC, 5 μM). Inset shows the frequency distributions (means ± SE) for the 4α-PDD-induced current (measured at −80 mV) in the absence (open bar, N = 5, n = 8) and in the presence (filled bar, N = 5, n = 10) of HC-067047. Currents were elicited by 500-ms voltage ramps from −100 to +80 mV (see the inset). The holding potential was 0 mV. I, current; V, voltage. B: whole cell patch-clamp recording on PASMC from chronically hypoxic rats. Representative current trace obtained before (ctr) or after 4α-PDD (5 μM) application. C: frequency distributions for 4α-PDD-induced current densities at −80 mV in PASMC from Nx (open bar, N = 6, n = 12) or CH (filled bar, N = 5, n = 10) rats. Data are mean values ± SE. *P < 0.05 between PASMC from Nx and CH rats. Mann-Whitney tests were applied.
DISCUSSION

The present study reveals that, in PASMC, 1) TRPV4 and RyR2 ion channels are present and functional, 2) activation of TRPV4 with the specific agonist 4α-PDD induces a [Ca^{2+}]_i increase that is reduced by RyR inhibitors but not by the IP_{3}R inhibitor xestospongin C, and 3) chronic hypoxia increases TRPV4 and RyR2 expression and 4α-PDD-induced [Ca^{2+}]_i, currents, and contractile responses. These data support the existence of an interplay between TRPV4 and RyR2 in the control of [Ca^{2+}] of PASMC and thus of pulmonary vascular tone. This original signaling pathway could be reinforced under chronically hypoxic conditions and may thus be a critical mechanism in the pathophysiological process such as pulmonary hypertension.

In rat pulmonary artery, we show that 4α-PDD-induced [Ca^{2+}]_i, currents, and contractile responses were all reduced in the presence of the compound HC-067047, known as a selective TRPV4 channel blocker (20), reinforcing the idea that those responses are mediated by the activation of TRPV4 channels. TRPV4-induced [Ca^{2+}]_i increase is amplified by a subsequent Ca^{2+} release from internal store via activation of RyR channels. Our results provide further insights on the isoform identity of the RyR channel and show that isoform 2 (RyR2) is the main channel implicated in the evoked pathway. This study thus provides the first molecular and physiological evidence that TRPV4 and RyR2 channels together operate to increase cytosolic Ca^{2+} in pulmonary myocytes. Although some discrepancies between ryanodine and dantrolene effects have been reported (35, 44, 47), whereas dantrolene inhibits specifically RyR1 and RyR3 but not RyR2 (22, 34, 57, 64). The combination of these RyR antagonists with other drugs (caffeine, xestospongin C, 4α-PDD) allowed us to identify the
obtained in the absence (control) and in the presence of IbTx or paxilline in the presence of HC-067047 in Mann-Whitney test. Via activation of BKCa (17), in the pulmonary artery the same TRPV4-RyR pathway induces membrane hyperpolarization opposite to what has been shown in cerebral artery where the channel inhibitors iberiotoxin (IbTx, 100 nM) and paxilline (10 μM) on the amplitude of the contraction induced by either 5 or 20 μM 4α-PDD (N = 5, n = 8–10 for each condition). Data are means ± SE. *P < 0.05 between the contraction obtained in the absence (control) and in the presence of HC-067047 in Mann-Whitney test. B: effect of the BKCa channels inhibitors iberiotoxin (IbTx, 100 nM) and paxilline (10 μM) on the amplitude of the contraction induced by either 5 or 20 μM 4α-PDD (N = 5, n = 8–10 for each condition). Data are means ± SE. *P < 0.05 between the contraction obtained in the absence (control) and in the presence of IbTx or paxilline in Kruskal and Wallis tests.

In summary, the study addresses unresolved issues concerning the role of TRPV4 in Ca2+ signaling of vascular cells. Moreover, in PASMC, our data point out the importance of the endothelial TRPV4 channel (18), the effect of which being opposite to that of the stimulation of myocyte TRPV4, could explain the modest amplitude of the 4α-PDD-induced contraction observed in the present study.

In accordance with a previous report from Earley’s group in cerebral myocytes (17), we have also observed that RyR are closely located in a submembranar region (57) and thus are related to a relaxation response, whereas, in pulmonary artery, only the RyR1 subtype is located in the same region (7). In PASMC, RyR3 is mainly located in a perinuclear region, and it is involved in the endothelin1-NAADP signaling pathway (7, 34), whereas RyR2 is present in a more central region of the cell (7) close to the contractile apparatus. This could explain that calcium release through the RyR2 subtype induces a contractile response as observed in the present study. However, we cannot totally rule out the implication of BKCa in the interplay between TRPV4 and RyR2 in IPA, since, for a high 4α-PDD concentration (20 μM), TRPV4-mediated contraction was increased in the presence of IbTx and paxilline (Fig. 6B). Contraction of vascular smooth muscle in response to agonist has indeed been previously reported despite the stimulation of BKCa (41). The possible involvement of BKCa together with the stimulation of an endothelial TRPV4 channel (18), the effect of which being opposite to that of the stimulation of myocyte TRPV4, could explain the modest amplitude of the 4α-PDD-induced contraction observed in the present study.

In the present work, we also addressed the potential implication of such a signaling pathway under pathophysiological conditions, e.g., pulmonary hypertension. Pulmonary hypertension often occurs as a consequence of respiratory diseases such as chronic obstructive pulmonary disease, inducing hypoxic alveolar conditions (6). Pulmonary hypertension is characterized by both a pulmonary vascular hyperreactivity and remodeling of the arterial wall, two phenomena that are calcium dependent (40, 43). In the present study, we used the chronically hypoxic rat, a well-established animal model of hypoxia-induced pulmonary hypertension (4, 54) in which we have previously shown that resting [Ca2+]i is increased in PASMC (3, 4). Compared with pulmonary artery from Nx rats, we show in pulmonary artery from CH rats an increase in 1) the expression of both TRPV4 and RyR2 and 2) the 4α-PDD-induced [Ca2+]i response, current, and tension. Because overexpression of other channels under CH has been demonstrated in PASMC (21, 26), our data cannot totally rule out the possibility that the increased [Ca2+]i response could be due to an increased calcium content of SR or modest expression variations of other RyR isoforms that will modify the calcium homeostasis or RyR2 activity (10). Indeed, it is known that hypoxia could activate other partners of the cellular Ca2+ homeostasis like IP3R or sarco(endo)plasmic reticulum calcium-ATPase (3) in parallel of acting directly on RyRs (13). However, taking into account that 4α-PDD, ryanodine, or dantrolene have specific sites on receptors, we propose that TRPV4 and RyR2 participate in the potentiation of vasoconstriction through activation of Ca2+ signaling under hypoxia. We finally suggest that the TRPV4-RyR2 signaling pathway could be an important factor responsible for the elevated pulmonary tone observed in pulmonary hypertension. Modulation of this pathway could thus represent a new strategy to treat pulmonary hypertension.

In summary, the study addresses unresolved issues concerning the role of TRPV4 in Ca2+ signaling of vascular cells. Moreover, in PASMC, our data point out the importance of the link between TRPV4-RyR2 in such calcium signaling. Because the expression of both TRPV4 and RyR is increased under hypoxic conditions known to lead to pulmonary hypertension, pharmacological or genomical modulation of this signaling pathway could be relevant for the development of innovative therapeutics for this disease.
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DISCLOSURES

No conflicts of interest are declared by the authors.

AUTHOR CONTRIBUTIONS


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