Functional characterization of voltage-dependent Ca\(^{2+}\) channels in mouse pulmonary arterial smooth muscle cells: divergent effect of ROS

Eun A. Ko, Jun Wan, Aya Yamamura, Adriana M. Zimmnicka, Hisao Yamamura, Hae Young Yoo, Haiyang Tang, Kimberly A. Smith, Premanand C. Sundivakkam, Amy Zeifman, Ramon J. Ayon, Ayako Makino, and Jason X.-J. Yuan

Department of Medicine, Section of Pulmonary, Critical Care, Sleep and Allergy Medicine; Institute for Personalized Respiratory Medicine; Department of Pharmacology; and Center for Cardiovascular Research, University of Illinois at Chicago, Chicago, Illinois

Submitted 17 September 2012; accepted in final form 19 February 2013

Ko EA, Wan J, Yamamura A, Zimmnicka AM, Yamamura H, Yoo HY, Tang H, Smith KA, Sundivakkam PC, Zeifman A, Ayon RJ, Makino A, Yuan JX. Functional characterization of voltage-dependent Ca\(^{2+}\) channels in mouse pulmonary arterial smooth muscle cells: divergent effect of ROS. Am J Physiol Cell Physiol 304: C1042–C1052, 2013. First published February 20, 2013; doi:10.1152/ajpcell.00304.2012.—Electromechanical coupling via membrane depolarization-mediated activation of voltage-dependent Ca\(^{2+}\) channels (VDCC) in the plasma membrane of PA smooth muscle cells (PASMC) is an efficient mechanism in regulating pulmonary vascular tone, while mouse is an animal model often used to study pathogenic mechanisms of pulmonary vascular disease. The function of VDCC in mouse pulmonary artery (PA) smooth muscle cells (PASMC), however, has not been characterized, and their functional role in reactive oxygen species (ROS)-mediated regulation of vascular function remains unclear. In this study, we characterized the electrophysiological and pharmacological properties of VDCC in PASMC and the divergent effects of ROS produced by xanthine oxidase (XO) and hypoxanthine (HX) on VDCC in PA and mesenteric artery (MA). Our data show that removal of extracellular Ca\(^{2+}\) or application of nifedipine, a dihydropyridine VDCC blocker, both significantly inhibited 80 mM K\(^{+}\)-mediated PA contraction. In freshly dissociated PASMC, the maximum inward Ca\(^{2+}\) currents were \(-2.6 \pm 0.2\) pA/pF at \(+10\) mV (with a holding potential of \(-70\) mV). Window currents were between \(-40\) and \(+10\) mV with a peak at \(-15.4\) mV. Nifedipine inhibited currents with an IC\(_{50}\) of 0.023 \(\mu\)M, and 1 \(\mu\)M Bay K8644, a dihydropyridine VDCC agonist, increased the inward currents by 61%. XO/HX attenuated 60 mM K\(^{+}\)-mediated increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)]) due to Ca\(^{2+}\) influx through VDCC in PASMC. Exposure to XO/HX caused relaxation in PA preconstricted by 80 mM K\(^{+}\) but not in aorta and MA. In contrast, H\(_2\)O\(_2\) inhibited high K\(^{+}\)-mediated increase in [Ca\(^{2+}\)\(_{cyt}\)], and caused relaxation in both PA and MA. Indeed, RT-PCR and Western blot analysis revealed significantly lower expression of Cav1.3 in MA compared with PA. Thus, our study characterized the properties of VDCC and demonstrates that ROS differentially regulate vascular contraction by regulating VDCC in PA and systemic arteries.

vascular contraction; reactive oxygen species; pulmonary arterial smooth muscle

INTRACELLULAR FREE CALCIUM (Ca\(^{2+}\)) ions play a major role in the regulation of arterial smooth muscle tone under physiological and pathological conditions (36, 38). In vascular smooth muscle cells, membrane depolarization serves as a fundamental regulator of the open state probability of voltage-dependent Ca\(^{2+}\) channels (VDCC) in the plasma membrane. Alterations in membrane potential regulate cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)]) via Ca\(^{2+}\) entry through VDCC and consequently modulate vascular muscle cell excitability and vascular contraction and relaxation (37). Abnormal voltage-dependent Ca\(^{2+}\) influx has been implicated as a mechanism associated with the pathogenesis of idiopathic pulmonary arterial hypertension (IPAH) and hypoxia-induced pulmonary hypertension (5, 19). An experimental approach by Hirensallulr et al. (19) corroborates the findings that the activation of VDCC by high K\(^{+}\) solution or Bay K8644 elicited greater contractions in the pulmonary artery (PA) isolated from animals with pulmonary hypertension. Thus targeting VDCC channels in the plasma membrane of PA smooth muscle cells (PASMC) is an efficient approach for the development of novel therapies for pulmonary hypertension.

Animal models have long since been used to study the disease development and progression, since the investigations in human specimens are mainly restricted to end-stage pulmonary hypertension. Several animal models in different species have been used, among which rats and mice were commonly used for most studies. Although the rat has been a preferred animal model for mechanistic studies of pulmonary hypertension for more than 50 yr, recently there is an understandable desire among the investigators to use mice, as they offer a variety of tools often lacking for other species. Approaches ranging from generating a transgenic or knock-out models have strongly influenced the way we think of the pathogenesis of pulmonary hypertension and helped shift our understanding on the mechanisms such as cell-cell interactions, metabolic changes, cell phenotypic alterations, and immune dysregulation (16, 22, 27, 45). In recent years, a significant number of knockout and transgenic mouse models has been used to investigate pathogenic mechanisms associated with pulmonary hypertension (1, 8, 34). However, using mouse models in investigating the electrophysiological properties of VDCC has been limited due to technical difficulties in isolating the PA and single smooth muscle cells from mouse arteries. With this in mind, we sought to examine the electrophysiological and pharmacological characteristics of VDCC in freshly dispersed mouse PASMC.

In parallel, the ability of reactive oxygen species (ROS) to modify vascular tone has been a topic of great interest for the past several decades (3, 13). ROS-mediated vasconstriction, smooth muscle cell proliferation, and vascular remodeling are likely to play a critical role in many forms of pulmonary hypertension. Several studies proposed that hypoxia paradoxically initiates an increase in ROS signaling in the PA. It was also shown that the resulting shift in the redox status to a more oxidized state triggers the release of intracellular Ca\(^{2+}\) stores
and recruitment of Ca\textsuperscript{2+} channels in the plasma membrane and thereby mediates contraction associated with the pathogenesis of systemic or pulmonary hypertension (2, 4, 10, 23, 24, 50). It is therefore of particular interest to determine the response of Ca\textsuperscript{2+} current and the vascular response to ROS in mouse PA (and PASM C) compared with systemic arteries. To facilitate our study of ROS effects, we used xanthine oxidase (XO) and hypoxanthine (HX) as well as H\textsubscript{2}O\textsubscript{2} (35). Herein, we examined the effects of ROS on VDCC and [Ca\textsuperscript{2+}]\textsubscript{i} in mouse PASM C. Data presented here also show evidence of the divergent effect of ROS on arterial contraction in mouse PA compared with systemic arteries such as the aorta and mesenteric artery (MA).

METHODS AND MATERIALS

Cell isolation. Freshly dissociated mouse PASM C were used in this study to functionally characterize VDCC. Male C57BL/6 mice (7–8 wk) were killed by cervical dislocation in accordance with the approved protocol by the University of Illinois at Chicago Institutional Animal Care and Use Committee. Lungs were removed from the chest cavity and washed in a normal Tyrode solution composed of the following (mM): 143 NaCl, 5.4 KCl, 0.33 NaH\textsubscript{2}PO\textsubscript{4}, 0.5 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 5 HEPES, and 16.6 glucose (adjusted with NaOH to pH 7.4). The second- or third-order branches of intrapulmonary arteries (<400-μm external diameter) were isolated by dissecting the surrounding connective tissues from lung in a normal Tyrode solution.

The isolated arteries were then cut open longitudinally, and smooth muscle cells were isolated from the PA segment by treatment of enzyme with collagenase type XI (1 mg/ml), protease type XXIV (0.45 mg/ml), bovine serum albumin (2 mg/ml), and trypsin inhibitor (2 mg/ml) at 4°C for 30 min, followed by 37°C for 8 min. The digested arteries were washed with Ca\textsuperscript{2+} free solution for several times and then placed in the storage solution containing the following (mM): 70 KOH, 50 l-glutamate, 55 KCl, 20 taurine, 20 KH\textsubscript{2}PO\textsubscript{4}, 3 MgCl\textsubscript{2}, 20 glucose, 10 HEPES, and 0.5 EGTA (pH 7.4). Cells were dispersed by trituration with a fire-polished glass pipette to make a suspension of asingle PASM C. All experiments were carried at room temperature (22–24°C). Cells were used within 6 h and stored at 4°C until use.

Whole cell patch clamp. Ca\textsuperscript{2+} currents were recorded with whole cell patch-clamp technique using an Axopatch-1D amplifier and a DigiData 1322 interface ( Molecular Devices, Sunnyvale, CA). Borosilicate patch pipettes (3–4 MΩ) were used to make a suspension of single PASM C. The cell was then placed in a recording chamber containing the following (mM): 70 KCl, 75 l-glutamate, 20 taurine, 20 KH\textsubscript{2}PO\textsubscript{4}, 3 MgCl\textsubscript{2}, 10 glucose, 10 HEPES, and 0.5 EGTA (pH 7.4). Cells were superfused with a normal Tyrode solution, and the experiments were performed at room temperature (22–24°C).

Measurement of [Ca\textsuperscript{2+}]\textsubscript{i}. Cells on coverslips were incubated at room temperature for 30 min in normal Tyrode solution containing the membrane-permeable Ca\textsuperscript{2+}-sensitive fluorescent indicator fura-2 ace-toxymethyl ester (fura-2 AM; 4 μM) to load cells with dye. Cells on coverslips were then placed in a perfusion chamber on the stage of an inverted Nikon Eclipse/TE 200 microscope equipped with the TE-FM epifluorescence. The fura-2 AM-loaded cells were then washed with normal Tyrode solution for 20 min to remove excess extracellular dye before the experiments started. The fluorescence for fura-2 was recorded as 510 nm wavelength light emission with excitation wave-lengths of 340 and 380 nm by use of the digital fluorescent imaging system from Intracellular Imaging. [Ca\textsuperscript{2+}]\textsubscript{i} was measured as the ratio of fluorescence intensities (F\textsubscript{340}/F\textsubscript{380}) in PASM C loaded with fura-2 AM.

Tension measurement. The PA ring was cut and two tungsten hooks were carefully passed through the lumen of ring in an organ bath. One hook was connected on the bottom of a perfusion chamber, and the other was attached to an isometric force transducer (Harvard Apparatus). The resting passive tension was maintained at 300 mg, and the rings were allowed to stabilize at resting tension for 1 h. Isometric tension was continuously recorded, and data were acquired using DATAQ software (DATAQ Instruments). The PA rings were then applied with 40 mM K\textsuperscript{+} for three times to obtain a stable contractile response before challenge drugs. Isolated PA rings were perfused with modified Krebs solution (MKS; at 37°C) consisting of the following (in mM): 138 NaCl, 1.8 CaCl\textsubscript{2}, 4.7 KCl, 1.2 MgSO\textsubscript{4}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 5 HEPES, and 10 glucose (pH 7.4). The absolute amount of force relative to the basal tension was measured, which was expressed as the net increase in tension (mg). The endothelium was removed for all experiments except in experiments assessing endothelium function.

Quantitative real-time RT-PCR. Total RNA was prepared from the isolated PA and MA from C57BL mice using TRIzol reagent (Invitrogen) and quantified with NanoDrop 2000c (Thermo Scientific). The equal amount of RNA from each sample was reversely transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The quantitative real-time PCR analysis was performed for the following gene-specific primers: CaV 1.1 (forward, 5′-aatt gcc acca tta ttg ctc gtt-3′; reverse, 5′-ttg gtc cca gag cta ctg cat gct ggt-3′), CaV 1.2 (forward, 5′-ttg gtc cca gag cta ctg cat gct ggt-3′; reverse, 5′-ttg gtc cca gag cta ctg cat gct ggt-3′), CaV 1.3 (forward, 5′-ttg gtc cca gag cta ctg cat gct ggt-3′; reverse, 5′-ttg gtc cca gag cta ctg cat gct ggt-3′), and CaV 1.4 (forward, 5′-ttc tta tgg ctc gcc gga tgg cca tta ttc-3′; reverse, 5′-ttg gtc cca gag cta ctg cat gct ggt-3′). Quantitative RT-PCR was performed in a Real-Time Cycler (Bio-Rad CFX384, C1000 Thermal Cycler; Roche) using 1 μg of RNA, which was added to a final volume of 10 μl, which contained 5 μl of Taq Universal SYBR Green Supermix (Bio-Rad, Qiagen, Hilden, Germany) and 500 nmol/l of each primer. The reaction was initiated at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 30 s, and extension at 72°C for 15 s. After amplification, a melting curve analysis from 50 to 95°C with a heating rate of 0.2°C/s with continuous fluorescence acquisition was performed to assure correct PCR amplification. The relative quantification method was used whereby the change in expression of the target genes relative to the housekeeping gene (GAPDH) was calculated.

Western blotting. Protein samples were prepared from isolated PA and MA of C57BL/6 mice in 1× RIPA lysis buffer (Milipore), supplemented with 2% n-dodecyl-β-D-maltoside (Thermo Scientific) and protease inhibitor cocktail (Roche). Tissues were homogenized on ice with a glass Dounce homogenizer, followed by brief pulses of sonication until completely uniform. The crude lysate was briefly centrifuged, and protein concentrations were determined using a BCA Protein Assay Kit (Pierce). Equal amounts (30 μg) of total protein from each sample were loaded onto 4–20% SDS gradient gels (Mini-PROTEAN TGX; Bio-Rad) and separated by gel electrophoresis. Proteins were transferred onto PVDF membranes and stained with Ponceau S. Blots were probed with polyclonal rabbit anti-Cav1.2 and anti-Cav1.3 (1:200; Alomone) and monoclonal β-actin (1:1,000; Santa Cruz Biotechnology). Bands were visualized using ImageJ software.

Chemicals. Nifedipine, HX, XO, Bay K8644, phenylephrine (PE), and acetycholine (ACH) were obtained from Sigma (St. Louis, MO), and fura-2 AM was obtained from Molecular Probes. A stock solution (10 mM) of nifedipine and fura-2 AM was dissolved in DMSO and kept away from light to avoid photodegradation. The stock solution of 250 mM HX was prepared by dissolving in distilled water and adding a few drops of NaOH to adjust to pH 7.4: aliquots of the stock solution
were diluted 1:1,000 into the bath solution to a final concentration of 250 μM.

Data analysis and statistics. Data analysis and curve fitting were presented with pCLAMP 8.1 and SigmaPlot 2000 software. Activation and inactivation curves, including half activation and inactivation potentials, were determined after normalization to maximal current and fit to Boltzmann equations. Time constants were derived from single exponential curves. The results are expressed as means ± SE. Statistical comparison was performed with a Student’s t-test, with values of P < 0.05 being considered significant.

RESULTS

Functional role of VDCC in pulmonary vasoconstriction. The PA rings isolated from intrapulmonary arteries of mice were used in this study. For the measurements of vascular tension, two tungsten hooks were carefully passed through the lumen of PA rings (Fig. 1A). To evaluate the contribution of VDCC-mediated Ca\(^{2+}\) influx to mediating vasoconstriction, PA rings were exposed to high K\(^+\)-solutions ranging from 10 to 120 mM. Raising extracellular K\(^+\) concentration ([K\(^+\)]\(_{\text{ext}}\)) from 4.7 to 120 mM shifts the K\(^+\) equilibrium potential and resulting membrane depolarization. This subsequently opens the VDCC, which in turn enhances Ca\(^{2+}\) influx, increasing [Ca\(^{2+}\)]\(_{\text{cyt}}\) and causing vasoconstriction. With this available knowledge, we exposed PA rings, for which the basal tension was set at 300 mg, to increasing [K\(^+\)]\(_{\text{i}}\) from 4.7 to 20, 40, 60, 80, and 120 mM, respectively. We observed a significant increase in the amplitude of active tension (Fig. 1B, top). The dose-response curve showed an almost linear relationship at [K\(^+\)]\(_{\text{i}}\), between 15 and 40 mM and arrived at a plateau phase when [K\(^+\)]\(_{\text{i}}\) reached 60–80 mM (EC\(_{50}\) = 26.5 mM; Fig. 1B, bottom). These data suggest that high K\(^+\)-mediated VDCC activation plays a significant role in pulmonary vasoconstriction.

Removal of extracellular Ca\(^{2+}\) almost abolished the 80 mM K\(^+\) (80K)-mediated PA contraction (Fig. 1C, left). In addition, extracellular application of the VDCC blocker nifedipine (300 nM) also significantly and reversibly inhibited 80K-induced active tension (Fig. 1C, right). The 80K-induced active tension was 0.2981 ± 0.0278 g in the PA rings superfused with 1.8 mM Ca\(^{2+}\)-containing MKS, 0.0073 ± 0.0137 g (a 99% inhibition).

![Fig. 1](http://ajpcell.physiology.org/)
endothelium-derived hyperpolarizing factors. These findings suggest that the endothelium was intact in the PA (Fig. 1D) we used to test the functional role of VDCC.

**Passive membrane properties of mouse PASMC.** Freshly dissociated PASMC from mouse PA isolated from second- to third-order intrapulmonary arterial branches (100- to 400-μm diameters) were used to measure the electrophysiological properties of VDCC. As previously reported (28), the freshly dissociated mouse PASMC from intrapulmonary arteries were oblong, unlike the long spindle-shaped cells isolated from other species (Fig. 2A). To measure the basic biophysical properties of VDCC, the maximum inward current was evoked by a +10-mV depolarizing pulse from a holding potential of −70 mV (Fig. 2B, left). The membrane capacitance (Cm), in mouse PASMC (Fig. 2B, right) was 21.6 ± 0.6 pF (n = 45). Peak currents were determined at +10 mV and normalized to cell capacitance (current density) were −2.6 ± 0.2 pA/pF (n = 35; Fig. 2C), and the averaged resting membrane potential was −31.5 ± 0.8 mV (n = 32; Fig. 2D). The activation time constant of maximum currents, τact, was measured by fitting a monoexponential curve to the rising phase of each type of current. The currents were maximally activated at 4.2 ± 0.3 ms (n = 37) after depolarization, with peak activation occurring at +10 mV (Fig. 2E, left). The current then decayed exponentially with a mean time constant of 195.5 ± 18.6 ms (n = 37; Fig. 2E, right).

**Biophysical properties of VDCC.** Figure 3A shows representative traces of currents recorded to examine the currents of Ca2+ (Ica) and Ba2+ (Iba) through VDCC channels. When cells were superfused with 2 mM Ca2+-containing solution, depolarization from a holding potential of −70 to +10 mV induced a relative rapidly inactivating inward current (Fig. 3A). Substitution of 2 mM Ca2+ with 2 mM Ba2+ slightly increased the amplitude, which was followed by a slower inactivation phase of the inward currents. Increasing extracellular Ba2+ concentration from 2 to 10 mM significantly increased the amplitude of the currents (Fig. 3A). When the external solution with 2 mM Ba2+ was substituted with 10 mM Ba2+, the amplitude of the peak currents increased by almost threefold (from −17.3 ± 2.5 to −57.3 ± 5.5 pA; Fig. 3B). Inactivation time constants (τinact) were calculated via a monoexponential fit on the decaying phase of the inactivation curves for 2 mM Ba2+ and 2 mM Ca2+; τinact decreased from 116.5 ± 8.9 ms (2 mM Ba2+) to 57.2 ± 6.0 ms (2 mM Ca2+; Fig. 3C). This difference in the current-voltage (I-V) relationship profile between Ba2+ and Ca2+ solutions is consistent with the contention that the amplitude of Ba2+ currents is much higher than that of Ca2+ currents through VDCC, while the inactivation time constant of Ba2+ currents is greater than that of Ca2+ currents.

We also determined the steady-state activation and inactivation kinetics of VDCC. Steady-state activation data were derived from the I-V relationship, measured by depolarization from a holding potential of −70 mV to a series of test potentials ranging from −60 to +50 mV in 10-mV increments. A representative record of superimposed currents is shown in Fig. 3D. The normalized activation curve indicates that the channels were activated at −30 mV, and the channel activation maximized at approximately +10 mV. To examine the inactivation of VDCC in more detail, a standard two-pulse protocol was applied. Figure 3F shows a typical experiment in which a cell was held at conditioning potentials ranging from −90 to +20 mV for 2 s before depolarizing to the test potential of +10 mV for 200 ms to elicit the peak inward currents through VDCC. Figure 3F, filled circles, shows steady-state activation and inactivation curves plotted from the data shown in Fig. 3D. The normalized inactivation curve implies that the channels were inactivated at potentials from −60 mV and the maximal inactivation occurred at +10 mV. The window currents of VDCC fell within a voltage range of −40 to +10 mV (half activation and inactivation of Ica in mouse PASMC was −11.2 ± 2.0 and −17.3 ± 1.3 mV, respectively), with a peak window current of −15.4 mV.
VOLTAGE-DEPENDENT Ca\(^{2+}\) CHANNELS IN MOUSE PASMC

Pharmacological properties of VDCC. Next, we used nifedipine (a known dihydropyridine blocker of VDCC) to characterize the channels function in PASMC. Cells treated with nifedipine (10 or 30 nM) showed a rapid attenuation of peak inward currents (Fig. 4A). Furthermore, a dose-dependent inhibition of inward current at the concentrations ranging from 0.001 to 3 \(\mu\)M of nifedipine was observed (Fig. 4B, left). There was progressive inhibition of the peak, with increasing concentrations of nifedipine. Blockade was not complete (on average it was 91.3 \pm 4.3\% with nifedipine, 3 \(\mu\)M) but showed a tendency towards saturation at the concentration of 3 \(\mu\)M. The concentration-inhibition curve illustrating the effects of nifedipine on VDCC was constructed, and IC\(_{50}\) value for nifedipine was \(\approx 0.023 \mu\)M (Fig. 4B, right). We next observed the effect of Ba\(^{2+}\) removal on the amplitude of the peak inward currents (Fig. 4C). Following removal of external Ba\(^{2+}\), the amplitude of the VDCC was significantly reduced from \(-56.9 \pm 5.3\) to \(-13.7 \pm 2.6\) pA (Fig. 4C). The currents in Ba\(^{2+}\)-free conditions could imply that the remaining currents were not carried by Ba\(^{2+}\) (potentially carried by other monovalent cations). Several studies have shown that in the absence of extracellular Ca\(^{2+}\), Ca\(^{2+}\) channels are permeable to monovalent cations and that Mg\(^{2+}\) blocks this current.

Extracellular application of 1 \(\mu\)M Bay K8644, a VDCC activator, significantly and reversibly increased the amplitude of inward currents in freshly dissociated mouse PASMC (Fig. 4, D and E). The peak amplitude increased from 40.5 \pm 9.1 to \(-102.8 \pm 16.1\) pA in the presence of Bay K8644 at +10-mV depolarizing pulse (Fig. 4D, right). In addition, the augmenting effect of Bay K8644 was fairly rapid, taking <3 min for maximal changes, and the I-V for the peak of Bay K8644-mediated currents was shifted 10 mV in the hyperpolarizing direction (Fig. 4E).

Divergent effect of ROS on VDCC, [Ca\(^{2+}\)\]\(_{cyt}\), and vascular contraction. VDCC on the plasma membrane are affected by exogenous and endogenous redox compounds (21). A study in isolated guinea pig ventricular myocytes demonstrated that oxidation of thiol groups in L-type VDCC channels inhibited Ca\(^{2+}\) entry (30). To examine the effects of ROS on PASMC, we exposed the cells to XO (0.2 mU/ml) and HX (250 \(\mu\)M). The inward currents through VDCC, elicited by a depolarizing pulse of +10 mV, were inhibited to 49.2 \pm 5.9\% by XO/HX in cells superfused with vehicle (Fig. 5, A, left, and B). In addition, the inward currents through VDCC in the presence of 1 \(\mu\)M Bay K8644 were inhibited to 48.0 \pm 4.2\% with exposure to XO/HX (Fig. 5, A, middle, and B). However, the challenge of HX alone had no effect on the inward currents through VDCC (Fig. 5A, middle). Furthermore, the inhibitory effect of XO/HX on VDCC was prevented following treatment with apocynin, used as an inhibitor of NADPH oxidase (Fig. 5, A, right, and B). These results suggested that ROS produced by HO/HX (in the presence of NADPH oxidase) significantly inhibited VDCC in freshly dissociated mouse PASMC. Indeed, treatment of PASMC with XO/HX, although it slightly increased the basal [Ca\(^{2+}\)\]\(_{cyt}\), significantly inhibited 60 mM K\(^{+}\)-induced increase in [Ca\(^{2+}\)\]\(_{cyt}\) (Fig. 5, C and D).

Next, in the tension measurements, we set out to investigate the differences in vascular contraction among mouse PA, aorta, and MA in response to XO/HX. The XO/HX challenged to PA was the same in the same concentration as that used in the electrophysiological study. To activate VDCC, 80K was applied to the

---

**Fig. 3.** Ca\(^{2+}\) permeability and steady-state voltage dependence of inactivation and activation of VDCC in freshly dissociated mouse PASMC. A: representative recording of inward currents through VDCC elicited by a depolarizing pulse of +10 mV from a holding potential of −70 mV (left). Maximum currents were superimposed in the presence of 10 mM Ba\(^{2+}\), 2 mM Ba\(^{2+}\), and 2 mM Ca\(^{2+}\). Right: mean current-voltage (I-V) curves for 10 mM Ba\(^{2+}\), 2 mM Ba\(^{2+}\), and 2 mM Ca\(^{2+}\). Peak currents were determined at each voltage, normalized to cell capacitance. Voltage-clamp steps were applied from a holding potential of −70 mV to test potentials ranging from −60 to +50 mV with increments of 10 mV (300-ms steps). B: bar graph shows the peak currents in the presence of 10 mM Ba\(^{2+}\), 2 mM Ba\(^{2+}\), and 2 mM Ca\(^{2+}\). C: inactivation time constant, \(\tau_{max}\), was measured by fitting a monoeponential curve to the decaying phase in the presence of 2 mM Ba\(^{2+}\) and 2 mM Ca\(^{2+}\), respectively. D: representative family of superimposed currents, elicited by depolarization from a holding potential of −70 mV to test potentials (300 ms) ranging from −60 to +50 mV (in 10-mV increments). E: representative family of currents, elicited by a double-pulse protocol, to construct steady-state inactivation curve. The cell was held at −70 mV and stepped to a series of conditioning pulses (2 s) ranging from −90 to +20 mV before depolarization to +10 mV for 200 ms. Inset: magnified inactivation currents. F: averaged data showing the activation curve (closed circle, \(n = 11\) cells) and the inactivation curve (open circle, \(n = 7\) cells). Smooth curves through inactivation and activation data points are the best fit generated by the computer. Peak current was determined at each voltage, normalized to cell capacitance. Data are expressed as means ± SE. Cells were isolated from 5–9 mice. *** \(p < 0.001\), statistical difference from the control value.
Fig. 4. Pharmacological properties of VDCC in freshly dissociated mouse PASMC. A: Superimposed representative currents were elicited by a depolarizing pulse of +10 mV from a membrane holding potential of −70 mV before (control) and during application of 10 and 30 nM nifedipine. Right: mean I-V curves for control in the presence of 10 and 30 nM nifedipine, respectively. Voltage-clamp steps were applied from a holding potential of −70 mV to test potentials of −60 to +50 mV with increments of 10-mV, 300-ms steps. Peak current was determined at each voltage. B: Superimposed currents obtained before and during subsequent application of various concentrations of nifedipine (from 0.003 to 3 μM). Right: concentration-inhibition curves for nifedipine from 0.0001 to 3 μM. C: Representative recordings of inward currents through VDCC elicited by a depolarizing pulse to +10 mV from a holding potential of −70 mV in PASMC superfused with 10 mM Ba²⁺ (10Ba) or Ba²⁺-free (0Ba) solutions. Right: bar graph shows current amplitudes in the absence (0Ba) or presence of 10 mM Ba²⁺ (10Ba). D: Representative recordings of inward currents through VDCC elicited by a depolarizing pulse of 10 mV from a holding potential of −70 mV before (Cont), during (BayK), and after (Wash) exposure to 1 μM Bay K8644. Bar graph shows current amplitudes in PASMC before, during and after application of Bay K8644. Data are expressed as means ± SE. Cells were isolated from 7–10 mice. **p < 0.01 and ***p < 0.001, statistical difference from the control value. E: mean I-V curves in PASMC before (control) and during (BayK) application of BayK 8644.

PA to elicit a contraction as demonstrated in Fig. 6A. An additional transient contraction was observed following addition of XO/HX when 80K-mediated active tension reached plateau; however, it caused gradual relaxation to 73.2 ± 4.8% of 80K-induced vasoconstriction (Fig. 6, A, left, and B). These data are consistent with the observation that XO/HX inhibits VDCC and decreases Ca²⁺ influx through VDCC in PASMC. In addition, we determined the effect of XO/HX on the vascular contraction in both aorta and MA. In contrast to the findings observed in PA, aorta and MA exposed to XO/HX did not show relaxation and returned to the baseline upon a washout with MKS solution (Fig. 6, A, middle and right, and B).

We also examined the effect of H₂O₂ on VDCC, [Ca²⁺]ᵣ, and vascular contraction. Addition of 0.3 μM H₂O₂ to the bath solution inhibited inward currents through VDCC to 34.6% of control (Fig. 7A). In PASMC loaded with fura-2, H₂O₂ (0.3 μM) elicited a transient increase in [Ca²⁺]ᵣ which declined to the basal level within 3–5 min. In the presence of H₂O₂, the 60 mM K⁺-mediated increase in [Ca²⁺]ᵣ was, however, significantly inhibited compared with [Ca²⁺]ᵣ in the absence of H₂O₂ (Fig. 7B). As for tension measurement experiments, H₂O₂ (0.3 μM) caused relaxation in 80K-induced contractions for PA preparations (Fig. 7C). However, exogenous H₂O₂ exerted a differential effect on the 80K-induced contractions in...
indicate that Cav1.3, an L-type VDCC, is approximately sevenfold in mouse PA (Fig. 8A). These data suggest increased mRNA levels of Cav1.3 in PA of mice, as measured by Real-time RT-PCR experiments, were performed to compare the mRNA expression level of various VDCC channels in PA and MA. As shown in Fig. 9, both PA and MA express multiple VDCC including Cav1.1, Cav1.2, Cav1.3, and Cav1.4. The expression of Cav1.1, Cav1.2, and Cav1.4 channels was similar between PA and MA in mice, whereas the expression level of Cav1.3 was significantly higher in PA than in MA (Fig. 8A).

Western blot analysis was performed to examine whether increased mRNA levels of Cav1.3 in PA of mice, as measured by RT-PCR, correspond to enhanced Cav1.3 protein expression. Consistent with the RT-PCR experiments, the protein expression of Cav1.2 was not significantly different in PA and MA preparations, whereas Cav1.3 expression increased approximately sevenfold in mouse PA (Fig. 8B). These data indicate that Cav1.3, an L-type VDCC α-subunit, is differentially expressed in PA and MA and the diverging effects of ROS on high K⁺-mediated contraction in PA and MA may be related to this variation in expression of Cav1.3.

**DISCUSSION**

The expression and appropriate functioning of VDCC channels in PASMC play an indispensable role in regulating pulmonary vascular tone and contributes significantly to the pathological states. Previously, it has been reported that dysfunction and upregulation of Ca²⁺ channels in animal and human PASMC are linked to the development of sustained pulmonary vasoconstriction and vascular remodeling in pulmonary hypertension (6, 19). While mice are commonly used as models for...
half activation and inactivation of the currents were +5 and −17 mV (55), and −4 and −28 mV (33), respectively. These data suggest that the open probability of VDCC from freshly dissociated mouse PASMC is enhanced at more negative potentials, which could indicate that differential expression of VDCC subtypes alters the gating kinetics of Ca\textsuperscript{2+} channels in mouse PASMC compared with other forms of arterial smooth muscle cells.

Nifedipine relaxes high K\textsuperscript{+}-induced contraction of mouse PA, supporting the role of VDCC in pulmonary vascular contraction. In the present study, electrophysiological data clearly demonstrate that nifedipine caused the inhibition of VDCC in mouse PASMC (IC\textsubscript{50} = 23 nM) more effectively than that observed in single rabbit ear artery cells (17), where nifedipine produced a 50% reduction in current. In rat MA, nifedipine caused half inhibition of VDCC and vascular contraction at a concentration of 100 nM. In addition, nifedipine caused the inhibition of Ca\textsuperscript{2+} current with a half-maximal effective dose, 159 ± 54 nM in human urethra smooth muscle cells (20).

The opposite effects of ROS on vascular contraction in different types of vasculature have been reported. For example, superoxide and H\textsubscript{2}O\textsubscript{2} caused vasoconstriction and attenuated endothelium-dependent dilation (2, 25), whereas in vessels preconstricted with agonists H\textsubscript{2}O\textsubscript{2} caused a relaxation response in rat and rabbit aorta (23, 58), porcine and canine coronary arteries (4, 46), cat and canine cerebral arteries (12, 56), and rabbit MA (14). Even in the same artery, either constriction or relaxation can be induced depending on the concentration of ROS. For example, in the MA, a biphasic response is caused: a transient contraction followed by a persistent relaxation at high concentrations of H\textsubscript{2}O\textsubscript{2}, whereas the MA only caused contraction at low concentrations of H\textsubscript{2}O\textsubscript{2} (15). In our study, we observed the reciprocal effect of ROS on vascular contraction in the PA and systemic (aortic and MA) arteries. Both aorta and MA slightly contracted further from the elicited contraction induced by 80K followed by the gradual return to the contraction elicited by 80K with exposure of exogenous ROS generated by XO/HX. However, in PA, exposure to XO/HX caused a relaxation of 80K-induced vasoconstriction after slightly further contraction. These responses appear to be mediated via the impaired Ca\textsuperscript{2+} influx due to VDCC inhibition. Increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} by high K\textsuperscript{+} in PASMC were also inhibited by exposure to XO/HX. When XO/HX was removed, the effects of relaxation were sustained or accelerated in mouse PA, and also the inhibitory effect on VDCC increased. However, the arterial contraction evoked by 80K returned to basal tension when the arteries were washed with MKS solution in the aorta and MA. These findings suggest a fundamental difference in the way that basic signaling pathways interact with VDCC, which ultimately have a greater influence on the vascular tone of PA. There are several reports that suggest a direct correlation between ROS and VDCC activation. Amberg et al. (2) reported that XO/HX elicit the activation of VDCC in rat cerebral arterial smooth muscle cell. H\textsubscript{2}O\textsubscript{2} can activate Ca\textsuperscript{2+} channels in guinea pig ventricular myocytes when using a perforated patch configuration (52), whereas, in mouse cere-
bral cortical neurons, the hydroxyl ion suppressed Ca\(^{2+}\) influx through VDCC (49). These differential responses may relate to the concentration and type of ROS, vessel heterogeneity, artery size, species, and experimental conditions.

We also characterized the effect of H\(_2\)O\(_2\) on VDCC, [Ca\(^{2+}\)]\(_{cyt}\) and vascular contraction to identify whether exogenous H\(_2\)O\(_2\) elicits a response similar to that of XO/HX. Similar to the patch-clamp data from PASMCs treated with XO/HX, VDCC were inhibited by exposure to H\(_2\)O\(_2\). This effect was unlikely an artifact of I\(_{Ca}\) due to only as cells were intracellularly perfused with 5 mM ATP. It has been previously shown that ATP sustains the current amplitude of VDCC in guinea pig ventricular (7, 26) and MA cells (41), bovine chromaffin cells (11), and rat MA (57). In addition, our patch pipette solutions were made with 10 mM EGTA, which is reported to prolong the survival of I\(_{Ca}\) in ventricular myocytes (7).

Increases in [Ca\(^{2+}\)]\(_{cyt}\) induced by high K\(^+\) were impaired in the presence of H\(_2\)O\(_2\) after shown increases of basal [Ca\(^{2+}\)]\(_{cyt}\). Exogenous application of H\(_2\)O\(_2\) to PA preparations precontracted with 80K led to slightly enhanced constriction followed by relaxation. Consistent with the results of XO/HX treatment, impaired increases of [Ca\(^{2+}\)]\(_{cyt}\) through VDCC caused inhibition of 80K-induced contraction in PA. Similar to our results, others investigators have reported that H\(_2\)O\(_2\) caused relaxation in other types of arteries (4, 9, 54). However, it should be noted that other studies have divergently reported exogenous H\(_2\)O\(_2\) can significantly enhance [Ca\(^{2+}\)]\(_{cyt}\) and induce vasoconstriction in PA (32, 42, 44, 48). It is possible that the observed vasorelaxation of mouse PA in our studies is a result of H\(_2\)O\(_2\) inhibition on VDCC and/or ryanodine receptors (RyR). Functional evidence of RyR1 in Ca\(^{2+}\) release and contraction in mouse PA has been previously reported (31). In this study, local and global Ca\(^{2+}\) increases and high K\(^+\)-induced contractions are significantly reduced in RyR1 homozygous and heterozygous mice (31). As VDCC are known to be physically coupled to RyR1 in skeletal muscle (53), where membrane depolarization of VDCC subsequently opens RyR1 leading to SR Ca\(^{2+}\) release, it is possible that H\(_2\)O\(_2\) attenuates extracellular Ca\(^{2+}\) flux by inhibiting VDCC, which prevents the opening of RyR1 ultimately leading to vasorelaxation. Further studies are required to elucidate the effects of H\(_2\)O\(_2\) on vasomotor tone and Ca\(^{2+}\) mobilization. Additionally, we aim to determine which ion channels contribute to further contraction, followed by relaxation induced by H\(_2\)O\(_2\), which shows a different response compared with the treatment with XO/HX in MA.

As for ROS generation, XO/HX and H\(_2\)O\(_2\) are different sources of ROS; this resulted in different responses of vascular contraction in MA. However, in PA, the effect of XO/HX and H\(_2\)O\(_2\) showed consistent vascular response with relaxation, but MA exhibited vascular relaxation only with exposure to H\(_2\)O\(_2\). These results imply the existence of different susceptibilities, depending on the type of ROS generation, especially in MA.

In the present study, we also demonstrated that MA expresses lower levels of Ca\(_{1,3}\) channels compared with PA. Another report suggested that the lack of Ca\(_{1,3}\) Ca\(^{2+}\) channels in null mutant mice resulted in a depolarizing shift in the voltage-dependent activation in atrial myocytes (59). Therefore, it is possible that the differential expression of the Cav1.3 channel in PA and MA causes the changes in whole cell VDCC function and offer different reactivity to ROS. However, the physiological role of Cav1.3 in vascular contraction in PA must be determined, and the existence of a direct interaction with ROS remains unclear.

In summary, our study provides an extensive functional characterization of VDCC channels in mouse PA and PASMC. The electrophysiological and pharmacological properties demonstrated in the present study provide an important basis for using mice as a disease model to study the pathogenic roles of VDCC in pulmonary vascular disease. In addition, our investigation also demonstrates the novel finding that ROS differentially regulates vascular contraction between PA and systemic arteries. The mechanisms underlying these different responses may relate to the inhibition of different subtype of VDCC by ROS in PASMC and systemic arterial smooth muscle cells.

ACKNOWLEDGMENTS

Present address of A. Yamamura: Kinjo Gakuin University School of Pharmacy, Nagoya, Japan.
Present address of H. Yamamura: Nagoya City University Graduate School of Pharmaceutical Sciences, Nagoya, Japan.

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-115014, HL-066012, and HL-098053 and National Science Foundation of China Grants 81228001, 81270117, and 30810139004.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


AJP-Cell Physiol • doi:10.1152/ajpcell.00304.2012 • www.ajpcell.org


