Ca\(^{2+}\) signaling in hypoxic pulmonary vasoconstriction: effects of myosin light chain and Rho kinase antagonists

Jian Wang, Letitia Weigand, Joshua Foxson, Larissa A. Shimoda, and J. T. Sylvester

Division of Pulmonary and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Submitted 9 April 2007; accepted in final form 12 June 2007

Ca\(^{2+}\) signaling in hypoxic pulmonary vasoconstriction: effects of myosin light chain and Rho kinase antagonists. Am J Physiol Lung Cell Mol Physiol 293: L674–L685, 2007. First published June 15, 2007; doi:10.1152/ajplung.00141.2007.—Antagonists of myosin light chain (MLC) kinase (MLCK) and Rho kinase (ROK) are thought to inhibit hypoxic pulmonary vasoconstriction (HPV) by decreasing the concentration of phosphorylated MLC at any intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in pulmonary arterial smooth muscle cells (PASMC); however, these antagonists can also decrease [Ca\(^{2+}\)]\(_j\). To determine whether MLCK and ROK antagonists alter Ca\(^{2+}\) homeostasis in HPV, we measured the effects of ML-9, ML-7, Y-27632, and HA-1077 on [Ca\(^{2+}\)]\(_i\), Ca\(^{2+}\) entry, and Ca\(^{2+}\) release in rat distal PASMC exposed to hypoxia or depolarizing concentrations of KCl. We performed parallel experiments in isolated rat lungs to confirm the inhibitory effects of these agents on pulmonary vasoconstriction. Our results demonstrate that MLCK and ROK antagonists caused concentration-dependent inhibition of hypoxia-induced increases in [Ca\(^{2+}\)]\(_i\) in PASMC and HPV in isolated lungs and suggest that this inhibition was due to blockade of Ca\(^{2+}\) release from the sarcoplasmic reticulum and Ca\(^{2+}\) entry through store- and voltage-operated Ca\(^{2+}\) channels in PASMC. Thus MLCK and ROK antagonists might block HPV by inhibiting Ca\(^{2+}\) signaling, as well as the actin–myosin interaction, in PASMC. If effects on Ca\(^{2+}\) signaling were due to decreased phosphorylated myosin light chain concentration, their diversity suggests that MLCK and ROK antagonists may have acted by inhibiting myosin motors and/or altering the cytoskeleton in a manner that prevented achievement of required spatial relationships among the cellular components of the response.

isolated rat lung; pulmonary vascular resistance; angiotensin II; vascular smooth muscle; calcium channels

HYPOXIC PULMONARY VASOCONSTRICTION (HPV) is triggered by an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in pulmonary arterial smooth muscle cells (PASMC) caused by Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) and Ca\(^{2+}\) entry through voltage-operated Ca\(^{2+}\) channels (VOCC) (57, 71). Recently, we and others demonstrated that Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels (SOCC) is required for HPV (42, 66, 77). SOCC are activated by depletion of SR Ca\(^{2+}\) stores (44, 45). The resulting Ca\(^{2+}\) influx, known as capacitative Ca\(^{2+}\) entry (CCE), is an important determinant of [Ca\(^{2+}\)]\(_i\) in a wide variety of vasoconstrictor responses (19, 35, 50, 54).

Numerous studies in smooth muscle indicate that, at high concentrations, Ca\(^{2+}\) forms complexes with calmodulin, which activate myosin light chain (MLC) kinase (MLCK), causing phosphorylation of MLC (29). Phosphorylated MLC (P-MLC) then facilitates stimulation of myosin ATPase activity by actin, leading to cross-bridge cycling and contraction. Evidence indicates that this sequence of events occurs during HPV. Hypoxia increased MLC phosphorylation in PASMC and pulmonary arteries in association with contraction (33, 38, 68, 69, 83). In pulmonary arteries, tension development during hypoxia correlated directly with P-MLC concentration ([P-MLC]) and was abolished by the MLCK antagonist ML-9 (83).

[P-MLC] can be increased not only by activation of MLCK, but also by inhibition of myosin phosphatase (MP), the enzyme that dephosphorylates P-MLC (28, 29). In vascular smooth muscle, the principal regulator of MP appears to be Rho kinase (ROK). ROK is activated by the GTP-complexed form of the monomeric GTPase, Rho, which is produced when G protein-linked receptors interact with their ligands (52, 56). ROK inhibits MP by phosphorylating its myosin-binding subunit and/or by activating an endogenous inhibitor known as CPI-17 (17). ROK may also phosphorylate MLC directly (31). By these means, ROK can increase [P-MLC] at a given rate of MLC phosphorylation via MLCK. Because MLCK is activated by Ca\(^{2+}\)-calmodulin complexes produced in proportion to [Ca\(^{2+}\)]\(_i\), activation of ROK is said to increase myofilament Ca\(^{2+}\) sensitivity, defined as an increase in contractile force at a given [Ca\(^{2+}\)]\(_i\) (17, 53).

In rat distal pulmonary arteries, the slowly developing phase 2 of hypoxic contraction was associated with an elevated, but constant, [Ca\(^{2+}\)]\(_i\) (46). Hypoxia increased ROK activity in PASMC, and this increase was blocked by exoenzyme C3 or toxin B, antagonists of Rho (68). Hypoxia increased phosphorylation of MLC and the myosin-binding subunit of MP and decreased MP activity in PASMC (69), Y-27632 and HA-1077, antagonists of ROK, blocked hypoxia-induced MP inactivation and MLC phosphorylation in PASMC, phase 2 hypoxic contraction in distal pulmonary arteries, and HPV in isolated lungs and intact animals (16, 39, 47, 68, 69). Taken together, these results suggest that HPV requires an increase in Ca\(^{2+}\) sensitivity, as well as [Ca\(^{2+}\)]\(_i\), and that the increase in Ca\(^{2+}\) sensitivity is mediated by Rho-activated ROK.

In several cell types, inhibitors of ROK and MLCK have altered increases in [Ca\(^{2+}\)]\(_i\) induced by a variety of agonists. For example, Y-27632 blocked [Ca\(^{2+}\)]\(_i\) responses to norepinephrine in rat mesenteric artery and rat aorta (18). Similarly, HA-1077 blocked increases in [Ca\(^{2+}\)]\(_i\) induced by norepinephrine in rat aorta (18, 59) and by bradykinin or thapsigargin in porcine aortic endothelial cells (72). ML-9 or a related MLCK antagonist, ML-7, blocked [Ca\(^{2+}\)]\(_i\) responses to KCl in coronary artery (80); methacholine, thapsigargin, or KCl in guinea pig trachealis (23); bradykinin, thapsigargin, or shear stress in...
endothelial cells (43, 58, 72, 73); and UTP in bovine adrenocortical fasciculata cells (30). In endothelial and adrenocortical fasciculata cells, isolated trabeculae, and human blood monocytes, ML-9 blocked CCE through SOCC (23, 30, 43, 61). In portal vein myocytes, ML-7 and ML-9 blocked norepinephrine-induced increases in currents through nonspecific cation channels (2). These results suggest that, in addition to inhibition of the actin-myosin interaction, antagonists of MLCK and ROK can reduce \( [Ca^{2+}] \), through effects on \( Ca^{2+} \) channel activation.

It is not known whether MLCK and ROK antagonists alter \( Ca^{2+} \) signaling during HPV, which requires \( Ca^{2+} \) release from the SR and \( Ca^{2+} \) entry through VOCC and SOCC; therefore, we measured the effects of ML-9, ML-7, Y-27632, and HA-1077 on \( [Ca^{2+}]_i \), \( Ca^{2+} \) entry, and \( Ca^{2+} \) release in rat distal PASMC exposed to hypoxia or depolarizing concentrations of KCl. Parallel experiments were performed in isolated rat lungs to determine the effects of these agents on pulmonary vasocostriction.

**METHODS**

**PASMC**

As previously described (65, 66), distal (>4th generation) intrapulmonary arteries were dissected from lungs of pentobarbital sodium (65 mg/kg ip)-anesthetized male Wistar rats (300–500 g body wt). The luminal surfaces were rubbed with a cotton swab for removal of endothelial cells from thevascular segments. Smooth muscle cells were obtained by enzymatic digestion and cultured for 3–6 days on glass coverslips in Smooth Muscle Growth Medium 2 (Clonetics, Walkersville, MD) containing 5% serum in a humidified atmosphere of 5% CO\(_2\)–95% air at 37°C. Serum concentration in the medium was decreased to 0.3% 24 h before an experiment to stop cell growth. Cellular purity was >95%, as assessed by morphological appearance under phase-contrast microscopy and immunofluorescence staining for \( \alpha \)-actin (65, 66).

For measurement of \( [Ca^{2+}]_i \), and \( Ca^{2+} \) entry, cells were incubated with 7.5 \( \mu M \) fura 2-AM (Molecular Probes, Eugene, OR) for 60 min at 37°C under an atmosphere of 5% CO\(_2\)–95% air. Coverslips were then mounted in a closed polycarbonate chamber clamped to a heated aluminum platform (model PH-2, Warner Instrument, Hamden, CT) on the stage of a inverted microscope (TSE 100 Ellipse, Nikon, Melville, NY) and perfused at 0.5 ml/min with Krebs-Ringer bicarbonate solution (KRBS), which contained (in mM) 118 NaCl, 4.7 KCl, 0.57 MgSO\(_4\), 1.18 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 10 glucose. Perfusate was equilibrated in heated reservoirs with 5% CO\(_2\) and either 16% O\(_2\) (normoxia) or 4% O\(_2\) (hypoxia) and led to the chamber through stainless steel tubing. For depolarization of the cells, perfusate KCl was increased to 60 mM and NaCl was decreased to 82.7 mM. Chamber temperature was maintained at 37°C with an in-line heat exchanger and dual-channel heater controller (models SF-28 and TC-344B, Warner Instrument).

After removal of extracellular dye by 10 min of normoxic perfusion, \( [Ca^{2+}]_i \) was determined at 6- to 60-s intervals from the ratio of fura 2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (\( F_{340}/F_{380} \)) measured in 10–30 cells using a xenon arc lamp, interference filters, an electronic shutter, a \( \times 20 \) fluorescence objective, and a cooled charge-coupled device imaging camera. Data were collected online with InCyte software (Intracellular Imaging, Cincinnati, OH). \( [Ca^{2+}]_i \) was estimated from \( F_{340}/F_{380} \) measured in vitro in calibration solutions with 0–1,350 nM Ca\(^{2+} \) (Molecular Probes). After a 5-min control period, cells were exposed to 4% O\(_2\) for 20 min or 60 mM KCl for 15 min followed by a 10-min normoxic recovery period, during which perfusate KCl concentration ([KCl]) was normal. During exposure to hypoxia or high K\(^+\) concentration ([K\(^+\)]), antagonists of MLCK (ML-9 and ML-7) or ROX (Y-27632 and HA-1077) were added to the perfusate in amounts sufficient to achieve the desired concentrations: 10, 30, or 100 \( \mu M \) ML-9 (\( n = 4 \)–5/group), 1, 10, or 100 \( \mu M \) ML-7 (\( n = 4 \)–5/group), 1, 3, or 10 \( \mu M \) Y-27632 (\( n = 4 \)–5/group), and 10, 30, or 100 \( \mu M \) HA-1077 (\( n = 4 \)–5/group). In each experiment, cells were exposed to hypoxia or high \([K^+]_i\) and treated with a single concentration of a single antagonist. Untreated cells from the same isolate served as controls (\( n = 4–9 \)).

To assess CCE, we perfused PASMC with normoxic (16% O\(_2\)) or hypoxic (4% O\(_2\)) Ca\(^{2+}\)-free KRBS containing 0.5 mM EGTA to chelate residual Ca\(^{2+}\), 5 \( \mu M \) nifedipine to prevent Ca\(^{2+}\) entry through L-type VOCC, and 10 \( \mu M \) cyclopiazonic acid (CPA) to deplete SR Ca\(^{2+}\) stores. In our first series of experiments, we estimated CCE after 10 min by measuring the increase in \( [Ca^{2+}]_i \), caused by restoration of perfusate \( [Ca^{2+}]_i \) to 2.5 mM in cells exposed to 10, 30, or 100 \( \mu M \) ML-9 (\( n = 4 \)–5/group), 1, 3, or 10 \( \mu M \) Y-27632 (\( n = 4 \)–5/group), or 10, 30, or 100 \( \mu M \) HA-1077 (\( n = 3–4 \) group). Antagonists were given simultaneously with CPA. Untreated cells from the same isolate served as controls (\( n = 4–10 \)). In our second series of experiments, we estimated CCE after 10 min by measuring the rate at which 200 \( \mu M \) MnCl\(_2\) quenched fura 2 fluorescence excited at 360 nm over the ensuing 10 min in untreated cells (\( n = 3 \) during normoxia and \( n = 4 \) during hypoxia) and cells exposed to hypoxia + ML-9 (100 \( \mu M \), \( n = 4 \)) or hypoxia + Y-27632 (10 \( \mu M \), \( n = 4 \)).

To assess \( Ca^{2+} \) release from SR, we perfused PASMC with normoxic Ca\(^{2+}\)-free KRBS containing 0.5 mM EGTA to chelate residual Ca\(^{2+}\). After 10 min, the cells were perfused with hypoxic 4% O\(_2\) Ca\(^{2+}\)-free KRBS for 5 min. We estimated \( Ca^{2+} \) release as the maximum increase in \( [Ca^{2+}]_i \), caused by hypoxia in PASMC exposed to 100 \( \mu M \) ML-9 (\( n = 4 \)) or 10 \( \mu M \) Y-27632 (\( n = 4 \)), which were added to the perfusate 8 min before hypoxia. Untreated cells served as controls (\( n = 4 \)).

To assess voltage-operated \( Ca^{2+} \) entry, we perfused PASMC with normoxic Ca\(^{2+}\)-free KRBS containing 0.5 mM EGTA to chelate residual Ca\(^{2+}\), 50 \( \mu M \) SKF-96365 to prevent CCE (65), and 60 \( \mu M \) KCl to cause depolarization. After 10 min, we estimated voltage-operated \( Ca^{2+} \) entry from the rate at which 200 \( \mu M \) MnCl\(_2\) quenched fura 2 fluorescence in PASMC exposed to 100 \( \mu M \) ML-9 (\( n = 3 \)) or 5 \( \mu M \) nifedipine (\( n = 2 \)). Untreated cells served as controls (\( n = 3 \)).

**Isolated Lungs**

Male Wistar rats (200–400 g body wt) were given heparin (1,000 U ip) and anesthetized 20–30 min later with pentobarbital sodium (65 mg/kg ip). A tracheostomy was performed, and the animal was ventilated with room air at a tidal volume of 10 ml/kg and rate of 30 min\(^{-1}\) (rodent ventilator 883, Harvard Apparatus, Holliston, MA). After exsanguination from the femoral artery, the ventilating gas was changed to 16% O\(_2\)-5% CO\(_2\). A thoracotomy was performed, and cannulas were inserted into the main pulmonary artery and left atrium, which drained into a heated reservoir. The lungs were perfused with a peristaltic pump (Ismatec Regulo Analog Pump, Cole Parmer, Vernon Hills, IL) at 40 ml/h \(-1\)-min\(^{-1}\) with KRBS containing (in mM) 118 NaCl, 4.7 KCl, 0.57 MgSO\(_4\), 1.18 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 10 glucose. Ficoll (4 g/dl) and sodium methylfenamate (3.1 \( \mu M \)) were added to provide onotic pressure and prevent release of vasodilator prostaglandins. After the vasculature was flushed free of residual blood, the perfusate was recirculated. In KCl experiments, lungs were perfused with a 70:30 mixture of KRBS and the animal’s own blood to mitigate edema formation caused by high perfusion pressures. Temperature in the left atrial effluent was maintained at 37°C with a heat exchanger. Pulmonary arterial pressure (Ppa), left atrial pressure, and tracheal pressure were measured relative to the bottom of the lung with pressure transducers (model P10EZ, Spectramed, Oxnard, CA) and recorded with a computer-linked recording system (Powerlab, ADInstruments, Colorado Springs, CO). End-expiratory tracheal and
left atrial pressures were maintained at 3–4 and 0mmHg, respectively. Since perfusate flow was also constant, increases in Ppa were assumed to reflect pulmonary vasoconstriction.

After a 20-min stabilization period, lungs were subjected to one of two protocols: 1) four alternating exposures to ANG II (0.05-μg bolus into the main pulmonary artery) and hypoxia (ventilation with 2% O₂ for 5 min) at 5- to 10-min intervals or 2) progressive 5–10 mM increases in perfusate [KCl] at 5- to 10-min intervals until Ppa achieved a maximum or the lungs developed edema. In protocol 1, antagonists of MLCK (30 or 100 μM ML-9 or ML-7) or ROK (3, 10, 30, or 100 μM Y-27632 or HA-1077) were added to the perfusate after the second hypoxic exposure (n = 4–6 at each antagonist concentration). In protocol 2, the antagonists (10, 30 or 100 μM ML-9, ML-7, Y-27632, or HA-1077; n = 4–5 at each antagonist concentration) were given 10–15 min before administration of KCl. In both protocols, a lung was exposed to a single concentration of a single antagonist. Untreated lungs served as controls (n = 9–10).

**Drugs and Materials**

Stock solutions of Y-27632, HA-1077, SKF-96935, and KCl were made up in water and stored at 4°C. Stock solutions of ML-9, ML-7, and ANG II were made up in water on the day of the experiment. Stock solutions of nifedipine and CPA were made up in DMSO and stored at −20°C. ML-9, ML-7, Y-27632, and nifedipine were obtained from CalBiochem (La Jolla, CA). All other agents were obtained from Sigma Chemical (St. Louis, MO).

**Data Analysis**

To estimate the concentrations at which MLCK or ROK antagonists inhibited responses by 50% (IC₅₀), the mean Δ[Ca²⁺]ᵢ or maximum ΔPpa elicited by hypoxia or KCl in antagonist-treated preparations was expressed as a percentage of the corresponding mean values in untreated preparations. To obtain IC₅₀, we used an iterative least-squares method to fit the relationship between percent response and log inhibitor concentration.
and antagonist concentration to the Hill equation (66, 77). On two occasions in PASMC, mean Δ[C\(_{a}^{2+}\)] at the high antagonist concentration was either negative (Fig. 1, A and B; ML-7) or plateaued at >0 nM (Fig. 2, A and B: Y-27632, hypoxia). In these cases, average Δ[C\(_{a}^{2+}\)], at that concentration was subtracted from all observations before data were expressed as percentage of control.

In PASMC isolates from a specific animal, we measured [C\(_{a}^{2+}\)], in each cell and then entered the average of these values into statistical analysis as an n of 1. Thus, in PASMC and isolated lung experiments, n refers to the number of animals. Statistical analyses were performed using Student’s t-test or ANOVA. If a significant interaction F-ratio was obtained with the latter, pair-wise comparison of individual means was performed by calculating the least significant difference. Comparisons of group means with control were performed using Dunnett’s test. Differences were considered significant when P < 0.05. Values are means ± SD unless otherwise stated.

RESULTS

Responses to Hypoxia

PASMC. Hypoxia caused rapid increases in PASMC [C\(_{a}^{2+}\)], that were sustained for the duration of exposure (Fig. 1A). ML-9 and ML-7, antagonists of MLCK, caused concentration-dependent inhibition of this response (Fig. 1, A and B), with estimated IC\(_{50}\) of 6.3 and 10.8 µM, respectively (Fig. 1C). Y-27632 and HA-1077, antagonists of ROK, also inhibited [C\(_{a}^{2+}\)], responses to hypoxia in a concentration-dependent manner (Fig. 1, A and B). Estimated IC\(_{50}\) was 2.5 µM for Y-27632 and 20.2 µM for HA-1077 (Fig. 1C).

Our previous studies indicated that the [C\(_{a}^{2+}\)], response to hypoxia in PASMC required CCE through SOCC (65, 66). To determine whether inhibition of this response by antagonists of MLCK and ROK was due to blockade of CCE, we determined the effects of ML-9, Y-27632, and HA-1077 on [C\(_{a}^{2+}\)], responses to restoration of extracellular Ca\(_{a}^{2+}\) in PASMC perfused with Ca\(_{a}^{2+}\)-free KRBS containing CPA to deplete Ca\(_{a}^{2+}\) stores in the SR and nifedipine to prevent Ca\(_{a}^{2+}\) entry through VOCC (Fig. 2). Administration of CPA in the absence of extracellular Ca\(_{a}^{2+}\) caused a transient increase in [C\(_{a}^{2+}\)], which was presumably due to Ca\(_{a}^{2+}\) release from the SR followed by Ca\(_{a}^{2+}\) uptake into intracellular storage sites not dependent on SR Ca\(_{a}^{2+}\)-ATPase, such as mitochondria, and/or Ca\(_{a}^{2+}\) efflux via plasma membrane Ca\(_{a}^{2+}\)-ATPase or Na\(^+\)/Ca\(_{a}^{2+}\) exchange (Fig. 2A). The maximum increase in [C\(_{a}^{2+}\)], induced by CPA in the absence of extracellular Ca\(_{a}^{2+}\) averaged 126 ± 66 nM in normoxic control PASMC and was not altered by hypoxia or antagonists of MLCK and ROK. In contrast (Fig. 2), the maximum increase in [C\(_{a}^{2+}\)], caused by subsequent restoration of extracellular Ca\(_{a}^{2+}\) was markedly enhanced by hypoxia, as we observed previously (66). Moreover, this enhancement was abolished by ML-9, Y-27632, or HA-1077 (IC\(_{50}\) = 6.4, 1.2, and 23.9 µM, respectively; Fig. 2C). These IC\(_{50}\) values were virtually identical to those estimated for [C\(_{a}^{2+}\)], responses to hypoxia (6.3, 2.5, and 20.2 µM, respectively; Fig. 1C). To confirm that these effects were due to inhibition of Ca\(_{a}^{2+}\) influx through SOCC, rather than enhance-

Fig. 2. Effects of antagonists of MLCK (ML-9) and ROK (Y-27632 and HA-1077) on [C\(_{a}^{2+}\)], responses to restoration of extracellular Ca\(_{a}^{2+}\) concentration ([Ca\(_{a}^{2+}\)]) to 2.5 mM during normoxia (N) and hypoxia (H) in rat distal PASMC perfused with Ca\(_{a}^{2+}\)-free Krebs-Ringer bicarbonate solution (KRBS) containing 0.5 mM EGTA, 5 µM nifedipine (NIF), and 10 µM cyclopiazonic acid (CPA). A: time course of [C\(_{a}^{2+}\)], at different antagonist concentrations. B: maximum increase in [C\(_{a}^{2+}\)], after restoration of extracellular [C\(_{a}^{2+}\)] in untreated control PASMC and cells treated with MLCK and ROK antagonists. Concentrations of ROK antagonists that caused maximum inhibition during hypoxia (H) had no effect during normoxia (N). C: concentration-response relations determined by least-squares iterative fit of Hill equation to mean data in B, expressed as percentages of values measured in control cells.
ment of Ca\(^{2+}\) efflux or uptake, we measured the effects of ML-9 and Y-27632 on quenching of fura 2 fluorescence by Mn\(^{2+}\) in PASMC perfused with Ca\(^{2+}\)-free KRBS containing CPA and nifedipine (Fig. 3). Hypoxia increased the rate at which Mn\(^{2+}\) quenched fura 2 fluorescence more than twofold. This increase was eliminated by ML-9 (100 \(\mu M\)) or Y-27632 (10 \(\mu M\)).

The MLCK antagonist ML-9 also inhibited CCE during normoxia (Fig. 2). The IC\(_{50}\) for this effect (5 \(\mu M\)) was equivalent to that observed for ML-9 vs. hypoxia-induced increases in CCE (6.4 \(\mu M\); Fig. 2C) or [Ca\(^{2+}\)]\(_{i}\) (6.3 \(\mu M\); Fig. 1C). In contrast, neither ROK antagonist (Y-27632 nor HA-1077) altered CCE during normoxia (Fig. 2).

In untreated control PASMC perfused with Ca\(^{2+}\)-free KRBS, hypoxia caused a transient increase in [Ca\(^{2+}\)]\(_{i}\) (128 ± 19 nM), indicating intracellular Ca\(^{2+}\) release (Fig. 4). This increase was virtually abolished in cells treated with 100 \(\mu M\) ML-9 or 10 \(\mu M\) Y-27632 (17 ± 14 and 18 ± 11 nM, respectively, \(P < 0.0001\)). Baseline [Ca\(^{2+}\)]\(_{i}\) under normoxic Ca\(^{2+}\)-free conditions averaged 106 ± 52 nM, did not differ among groups, and was not altered by ML-9 or Y-27632.

Isolated lungs. Figure 5 shows representative recordings of four alternating exposures to ANG II and hypoxia in untreated control lungs and lungs treated with an antagonist of MLCK (ML-9) or ROK (HA-1077) after the second exposure. Before treatment, ANG II and hypoxia caused rapid reversible increases in Ppa at constant flow, left atrial pressure, and end-expiratory tracheal pressure, indicating vasoconstriction. Peak ΔPpa from baseline caused by hypoxia at the second exposure averaged 10.6 ± 1.3 mmHg in control lungs and did not differ among groups. B: mean maximum increases in [Ca\(^{2+}\)]\(_{i}\), determined by measuring maximum value during hypoxia for each cell in an experiment, averaging these values across cells, and entering this average value as an \(n\) of 1 in the calculation of group means.

Fig. 3. Effects of antagonists of MLCK (100 \(\mu M\) ML-9) and ROK (10 \(\mu M\) Y-27632) on capacitative Ca\(^{2+}\) entry, measured as quenching of fura 2 fluorescence at 360 nm (F\(_{360}\)) by 200 \(\mu M\) MnCl\(_2\) in PASMC perfused with Ca\(^{2+}\)-free KRBS containing CPA and nifedipine (Fig. 3). Hypoxia increased the rate at which Mn\(^{2+}\) quenched fura 2 fluorescence more than twofold. This increase was eliminated by ML-9 (100 \(\mu M\)) or Y-27632 (10 \(\mu M\)).
Responses to Depolarization

**PASMC.** On exposure to 60 mM KCl, $[\text{Ca}^{2+}]_i$ exhibited an initial rapid overshoot followed by a sustained elevation (Fig. 7A). The peak increase caused by depolarization was usually larger than that caused by hypoxia (Fig. 1). ML-9 and ML-7, antagonists of MLCK, caused concentration-dependent inhibition of this response ($IC_{50} = 19.1$ and 4.6 μM, respectively; Fig. 7). Similar to their lack of effect on CCE during normoxia (Fig. 2), the ROK antagonists Y-27632 (10 μM) and HA-1077 (100 μM) did not alter depolarization-induced increases in $[\text{Ca}^{2+}]_i$ during normoxia (Fig. 7).

To confirm that the inhibitory effect of ML-9 on the $[\text{Ca}^{2+}]_i$ response to depolarization was due to inhibition of $\text{Ca}^{2+}$ entry through VOCC, we measured Mn$^{2+}$ quenching of fura 2 fluorescence in PASMC perfused with $\text{Ca}^{2+}$-free KRBS containing 60 mM KCl to activate VOCC and 50 μM SKF-96365 to block CCE through SOCC (65, 66). Under these conditions, 100 μM ML-9 and 5 μM nifedipine markedly reduced the rate at which Mn$^{2+}$ quenched fura 2 fluorescence (Fig. 8).

**Isolated lungs.** Figure 9 shows representative recordings of pressor responses to increased perfusate $[\text{KCl}]$ in untreated control lungs and lungs treated with an antagonist of MLCK (ML-9) or ROK (HA-1077). In control lungs, increases in perfusate $[\text{KCl}]$ caused progressive increases in Ppa from $9.7 \pm 0.62 \text{ mmHg}$ at 5 mM to $33.8 \pm 2.8 \text{ mmHg}$ at 40 mM, the highest concentration achieved in all lungs of this group. Antagonists of MLCK and ROK inhibited pressor responses to KCl (Fig. 10A). Estimated IC$_{50}$ values for ML-9, ML-7, Y-27632, and HA-1077 were 57.3, 79.0, 6.0, and 11.6 μM, respectively (Fig. 10B). In the case of ML-9 and ML-7, these IC$_{50}$ values were 0.5–1.2 log units greater than IC$_{50}$ values measured vs. $[\text{Ca}^{2+}]_i$ responses to depolarization in PASMC (19.1 and 4.6 μM, respectively; Fig. 7B). This was not true for the ROK antagonists Y-27632 and HA-1077, which inhibited pressor responses to depolarization in isolated lungs (Fig. 10B) but had no effect on $[\text{Ca}^{2+}]_i$ responses to depolarization in PASMC (Fig. 7B).

**DISCUSSION**

In our prior studies (66, 77), we found that HPV in isolated lungs required $\text{Ca}^{2+}$ entry through SOCC and VOCC. In the present study, we confirm that HPV was blocked by antagonists of MLCK or ROK and test the possibility that this inhibition was caused by effects on $\text{Ca}^{2+}$ signaling in PASMC.

In rat distal PASMC, hypoxia induced a rapid reversible increase in $[\text{Ca}^{2+}]_i$ (Fig. 1) and enhanced CCE (Figs. 2 and 3),
as previously observed in this preparation (66). Both effects were blocked by antagonists of MLCK or ROK. Moreover, antagonist IC₅₀ values vs. [Ca²⁺]ᵢ responses to hypoxia were virtually identical to IC₅₀ values vs. hypoxic enhancement of CCE (Figs. 1C and 2C). Since CCE through SOCC was essential for the [Ca²⁺]ᵢ response to hypoxia in PASMC (66), these results suggest that MLCK and ROK antagonists blocked [Ca²⁺]ᵢ responses to hypoxia by blocking CCE. This could occur if the antagonists 1) blocked Ca²⁺ release from the SR, thereby preventing or limiting depletion of SR Ca²⁺ stores, and/or 2) inhibited transduction pathways signaling activation of SOCC in response to SR Ca²⁺ store depletion.

To test the first possibility, we determined whether ML-9 or Y-27632 altered the effects of hypoxia on [Ca²⁺]ᵢ in PASMC perfused with Ca²⁺-free KRBS (Fig. 4). Under these conditions, any increase in [Ca²⁺]ᵢ must be due to release of Ca²⁺ from intracellular stores. Consistent with previous evidence in PASMC and pulmonary arteries indicating that hypoxia stimulates release of Ca²⁺ from the SR (14, 24, 49, 64, 67, 84, 85), we found that hypoxia caused a transient increase in [Ca²⁺]ᵢ in these cells. This increase was abolished by ML-9 or Y-27632 (Fig. 4), suggesting that ML-9 and Y-27632 inhibited [Ca²⁺]ᵢ
responses to hypoxia (Fig. 1) by blocking hypoxia-induced release of Ca\(^{2+}\) from the SR, thereby preventing SOCC activation. Neither ML-9 nor Y-27632 alone altered \([\text{Ca}^{2+}]_i\) in PASMC exposed to Ca\(^{2+}\)-free conditions; therefore, it is unlikely that these antagonists inhibited hypoxia-induced Ca\(^{2+}\) release by causing prior depletion of SR Ca\(^{2+}\) stores (43). Rather, they must have interfered with the poorly understood mechanisms by which hypoxia activates Ca\(^{2+}\) release in PASMC.

With respect to the second possibility, we found that hypoxia did not alter Ca\(^{2+}\) release induced by CPA but, nevertheless, enhanced CPA-induced CCE (see RESULTS, Figs. 2 and 3) (66), suggesting that hypoxia facilitated transduction pathways linking store depletion to SOCC activation. Antagonists of MLCK and ROK blocked hypoxic enhancement of CPA-induced CCE, suggesting interference with this facilitation (Figs. 2 and 3). Thus MLCK and ROK antagonists appeared to block \([\text{Ca}^{2+}]_i\) responses to hypoxia by inhibiting hypoxic release of Ca\(^{2+}\) from the SR and hypoxic facilitation of the transduction pathways leading to SOCC activation; however, if SR Ca\(^{2+}\) stores acted upon by hypoxia differed from those acted upon by CPA, as suggested by some investigators (20, 24, 25, 62), hypoxic facilitation of transduction pathways leading to SOCC activation would not necessarily be implicated by our results. More work is needed to clarify the mechanisms by which hypoxia activates CCE.

Hypoxia activated MLCK and ROK in PASMC and pulmonary arteries, leading to MLC phosphorylation and contraction that was inhibited by ML-9 or Y-27632 (33, 38, 69, 83). Inhibition of HPV in isolated lungs by Y-27632 has also been reported (16, 47). Our results confirm these findings and, in addition, demonstrate inhibition of HPV in isolated lungs by ML-9, ML-7, and HA-1077 (Figs. 5 and 6). Although MLCK and ROK antagonists may have blocked HPV by inhibiting actin-myosin cross-bridge cycling and contraction, as commonly assumed, our results in PASMC (Fig. 1) suggest that they also may have acted by inhibiting Ca\(^{2+}\) signaling.

This deduction assumes that the behavior of our in vitro PASMC was similar to that of PASMC in vivo. In support of this assumption, acute hypoxia caused a rapid reversible increase in \([\text{Ca}^{2+}]_i\) in our PASMC (but not in similarly treated aortic smooth muscle cells), which was half-maximal at 39 mmHg PO\(_2\) and inhibited by exposure to Ca\(^{2+}\)-free medium or antagonists of voltage- and store-operated channels (66). Although these response characteristics were shared by HPV in isolated rat lungs (77), more direct evidence is needed to confirm that Ca\(^{2+}\) signaling in our PASMC reflected that of in vivo cells.

Because HPV in isolated lungs and \([\text{Ca}^{2+}]_i\) responses to hypoxia in PASMC depended on Ca\(^{2+}\) influx through SOCC and VOCC (42, 66, 77), we determined the effects of MLCK and ROK antagonists on responses to activation of VOCC with high extracellular [K\(^+\)]. In PASMC, ML-9 and ML-7 blocked...
As expected, MLCK antagonists blocked pulmonary vasoconstrictor responses to high perfusate \([K^+]\) in isolated lungs (Figs. 9 and 10). Because of our results in PASMC (Figs. 8 and 9), this effect could be due to inhibition of the actin-myosin interaction and/or inhibition of voltage-operated \(Ca^{2+}\) entry. In contrast to their lack of effect on \([Ca^{2+}]_i\), responses to high \([K^+]\) in PASMC, ROK antagonists were potent inhibitors of pulmonary pressor responses to high \([K^+]\) in isolated lungs, as previously reported (16). ROK antagonists have had similar inhibitory effects on contractile responses to high \([K^+]\) in most (18, 27, 32, 37, 59), but not all (22, 47, 63), studies of airway and vascular tissue. These effects may have occurred because ROK had been activated by paracrine mediators released from depolarized neural, endothelial, or other cells present in isolated lungs, vessels, and airways, but not our PASMC preparation. Consistent with this possibility, we found that Y-27632 blocked responses to high \([K^+]\) in intact, but not endothelium-denuded, rat distal pulmonary arteries (78).

**IC\(_{50}\)** values of MLCK and ROK antagonists vs. \(Ca^{2+}\) signaling during hypoxia in PASMC (Fig. 1C) were slightly greater than \(IC_{50}\) values vs. kinase activities of the purified enzymes measured in vitro (3, 12, 48, 55) and less than \(IC_{50}\) values vs. HPV in isolated lungs (Fig. 6B); however, they were comparable to concentrations found by other laboratories to inhibit smooth muscle contraction or MLC phosphorylation induced by high extracellular \([K^+]\) or \([Ca^{2+}]_i\) in the case of ML-9 and ML-7 (23, 37, 48, 78, 80) or by G protein-linked receptor agonists or GTP\(\gamma\)S in the case of Y-27632 and HA-1077 (10, 18, 22, 26, 37, 40, 55, 59, 63, 78). Since MLCK and ROK antagonists act by competing with ATP for its binding sites on the enzymes, these differences could be due to different concentrations of ATP and/or enzymes among preparations (3, 40, 48, 55). Moreover, in isolated lungs and smooth muscle preparations, MLCK and ROK activity may have been increased by endogenous or exogenous agonists, thereby increasing antagonist concentrations required for inhibition (26, 47, 55, 70), an effect that would not be present in purified enzyme preparations (3, 12, 48, 55). For example, the presence of an endogenous agonist might explain why HPV was slightly, but significantly, increased by 30 \(\mu\)M ML-9 or ML-7 during the fourth exposure of our isolated lungs to hypoxia (Fig. 6A). Since MLCK antagonists blocked \(Ca^{2+}\) influx and nitric oxide production in endothelial cells (74), they may have decreased endothelium-dependent vasodilation, a known modulator of HPV (21). An exogenous agonist might explain why \(IC_{50}\) for Y-27632 vs. HPV was 31.2 \(\mu\)M in our isolated lungs, which were exposed to ANG II and hypoxia, but only 0.06 \(\mu\)M in the lungs studied by Robertson et al. (47), which were exposed to hypoxia alone. Alternatively, this difference in \(IC_{50}\) could be related to the phase of HPV, since phase 2 HPV in pulmonary arteries was more sensitive to Y-27632 than phase 1 (47) and since the duration of hypoxia in our lungs was only 10–15 min compared with 40 min in the study of Robertson et al. Additional factors that could decrease antagonist potency in isolated lungs include protein binding, diffusion barriers, catabolism, and a large volume of distribution. Such considerations emphasize that comparability of \(IC_{50}\) values does not provide much support for the possibility that the inhibitory effects of MLCK and ROK antagonists on \(Ca^{2+}\) signaling in HPV were indeed due to inhibition of MLCK or ROK, rather
than to nonspecific actions, such as inhibition of other kinases. Nevertheless, this possibility should be considered.

If MLCK and ROK antagonists acted by decreasing [P-MLC], we must explain how this decrease led to such diverse effects on Ca\(^{2+}\) signaling; i.e., MLCK and ROK antagonists blocked hypoxia-induced increases in [Ca\(^{2+}\)]\(_i\) (Fig. 1) by blocking Ca\(^{2+}\) release from the SR (Fig. 4), Ca\(^{2+}\) entry through SOCC (Figs. 2 and 3), and Ca\(^{2+}\) entry through VOCC (Figs. 7 and 8). One possible explanation is that antagonist-induced decreases in [P-MLC] inhibited myosin motors and/or altered the cytoskeleton in a manner that prevented necessary maintenance or rearrangement of spatial relationships among the cellular components of the hypoxic response. For example, hypoxic activation of SR Ca\(^{2+}\) release by cADP-ribose (13, 79) and/or short-lived reactive oxygen species released from mitochondria (75, 76) might require proximity of mitochondria to SR. SOCC activation might depend on translocation of channel proteins to the plasma membrane (5, 51) and/or assembly of channels and regulatory proteins into signaling scaffolds (4, 7, 8, 81). Maintenance of VOCC activation might require local sequestration of inactivating Ca\(^{2+}\) and mitochondrial reactive oxygen species. Such possibilities would be consistent with accumulating evidence that the cytoskeleton plays an important role in the regulation of integrin-linked transduction pathways by increased actin-myosin interaction could be essential components of the actin-myosin interaction.

In summary, our results indicate that the MLCK antagonists ML-9 and ML-7 and the ROK antagonists Y-27632 and HA-1077 blocked HPV in isolated lungs and increases in [Ca\(^{2+}\)]\(_i\) induced by hypoxia in distal PASM. The latter effect appeared to be due to inhibition of Ca\(^{2+}\) release from the SR, Ca\(^{2+}\) entry through SOCC, and Ca\(^{2+}\) entry through VOCC. If the behavior of our PASM resembled that of PASM in vivo, these results suggest that MLCK and ROK antagonists blocked HPV by inhibiting Ca\(^{2+}\) signaling, as well as the actin-myosin interaction.

**REFERENCES**


