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Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca^{2+} and nonselective cation channels

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Weigand, Letitia, Joshua Foxson, Jian Wang, Larissa A. Shimoda, and J. T. Sylvester. Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca^{2+} and nonselective cation channels. *Am J Physiol Lung Cell Mol Physiol* 289: L5–L13, 2005. First published February 18, 2005; doi:10.1152/ajplung.00044.2005.—Previous studies indicated that acute hypoxia increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), Ca^{2+} influx, and capacitative Ca^{2+} entry (CCE) through store-operated Ca^{2+} channels (SOCC) in smooth muscle cells from distal pulmonary arteries (PASMC), which are thought to be a major locus of hypoxic pulmonary vasoconstriction (HPV). Moreover, these effects were blocked by Ca^{2+}-free conditions and antagonists of SOCC and nonselective cation channels (NSCC). To test the hypothesis that in vivo HPV requires CCE, we measured the effects of SOCC/NSCC antagonists (SKF-96365, $NiCl_2$, and $LaCl_3$) on pulmonary arterial pressor responses to 2% O_2 and high-KCl concentrations in isolated rat lungs. At concentrations that blocked CCE and $[Ca^{2+}]_i$ responses to hypoxia in PASMC, SKF-96365 and $NiCl_2$ prevented and reversed HPV but did not alter pressor responses to KCl. At 10 μM, $LaCl_3$ had similar effects, but higher concentrations (30 and 100 μM) caused vasoconstriction during normoxia and potentiated HPV, indicating actions other than SOCC blockade. Ca^{2+}-free perfusate and the voltage-operated Ca^{2+} channel (VOCC) antagonist nifedipine were potent inhibitors of pressor responses to both hypoxia and KCl. We conclude that HPV required influx of Ca^{2+} through both SOCC and VOCC. This dual requirement and virtual abolition of HPV by either SOCC or VOCC antagonists suggests that neither channel provided enough Ca^{2+} on its own to trigger PASMC contraction and/or that during hypoxia, SOCC-dependent depolarization caused secondary activation of VOCC.

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

IN ISOLATED PULMONARY ARTERIES (PA) and pulmonary arterial smooth muscle cells (PASMC), acute hypoxia caused depolarization (1, 18, 27, 38, 39, 69) and increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (2, 3, 7, 8, 38, 41, 45, 47, 58, 59, 70) and contraction (20, 24, 26, 28, 32). Moreover, removal of extracellular Ca^{2+} or antagonists of voltage-operated Ca^{2+} channels (VOCC) blocked hypoxic responses in PA (20, 24, 26) and PASMC (3, 7, 58) as well as hypoxic pulmonary vasoconstriction (HPV) in isolated lungs (30, 40, 53) and intact animals (33, 50, 52, 57). These results indicate that HPV requires Ca^{2+} influx through VOCC in PASMC.

Other findings suggest that Ca^{2+} release from sarcoplasmic reticulum (SR) and influx through pathways other than VOCC may also be important in HPV. For example, ryanodine, which blocks SR Ca^{2+} release, inhibited hypoxic responses in PASMC (59), PA (9, 19, 26), and isolated lungs (31). VOCC antagonists inhibited, but did not completely block, $[Ca^{2+}]_i$ responses to hypoxia in PASMC (7, 45) and HPV in isolated lungs (30). Depolarization with KCl plus inhibition of L-type VOCC with nifedipine or verapamil did not prevent contractile or $[Ca^{2+}]_i$ responses to hypoxia in isolated PA (43). One possible explanation for these findings is that hypoxia caused release of Ca^{2+} from SR in PASMC, leading to SR depletion, activation of store-operated Ca^{2+} channels (SOCC), and capacitative Ca^{2+} entry (CCE).

Recently, we reported that several so-called "canonical transient receptor potential (TRPC)" proteins were expressed in smooth muscle of distal PA (60), which are thought to be the major vascular locus of HPV (49). These proteins are homologs of TRP and TRP-like proteins that make up Ca^{2+} channels in *Drosophila* photoreceptors and are thought to compose mammalian SOCC, many forms of which may also be permeable to Na^+ and other cations and therefore function as nonselective cation channels (NSCC) (34, 37, 51). Consistent with TRPC expression, we and others demonstrated the presence of CCE in distal PA (51, 60). More recently, we found that acute hypoxia increased $[Ca^{2+}]_i$, Ca^{2+} influx, and CCE in distal PASMC and that these effects were completely blocked by removal of extracellular Ca^{2+} or SOCC/NSCC antagonists, but not nifedipine (61). These findings are consistent with recent observations from other laboratories (21, 22, 35) and suggest that HPV may require activation of SOCC in PASMC; however, it is well known that cell isolation and culture can alter cell phenotype. Moreover, changes in PASMC $[Ca^{2+}]_i$ do not necessarily translate to changes in pulmonary vascular resistance. In the present study, therefore, we assessed the contribution of SOCC and CCE to HPV in isolated lungs, where physiologically relevant pulmonary vasomotor responses can be measured directly.

METHODS

Isolated Lung Preparation

Our protocol was approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University. Male Wistar rats (200–400 g) were anesthetized 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 a rate of 30 min^{-1} (Harvard Rodent Ventilator model 883; Harvard Apparatus, Holliston, MA). A thoracotomy was performed, heparin (100 units) was injected into the right ventricular cavity, and the animal was exsanguinated from the femoral artery. The ventilating gas was changed to 16% O_2-5% CO_2. Cannulae were inserted into the main PA and left atrium, which drained into a heated reservoir. The lungs were perfused with

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a peristaltic pump (Ismatec Reglo Analog Pump; Cole Parmer, Vernon Hills, IL) at 40 $ml \cdot kg^{-1} \cdot min^{-1}$ with Krebs Ringer bicarbonate solution (KRBS) containing (in mM) 118 NaCl, 4.7 KCl, 0.57 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, and 10 glucose. Ficoll (4 g/dl) and sodium meclofenamate (3.1 μM) were added to provide oncotic pressure and prevent release of vasodilator prostaglandins. After the vasculature was flushed free of residual blood, the perfusate was recirculated. Lungs treated with $LaCl_3$ were perfused with bicarbonate- and phosphate-free HEPES-buffered salt solution (HBSS) to prevent precipitation of the antagonist (43, 51, 60) and ventilated without CO_2. HBSS contained (in mM) 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, and 10 glucose, as well as Ficoll (4 g/dl) and sodium meclofenamate (3.1 μM). To mitigate edema formation caused by high perfusion pressures, lungs exposed to KCl were perfused with a 70:30 mixture of KRBS or HBSS and the animal's own blood. Perfusate temperature was monitored with a thermistor in the left atrial effluent and maintained at 37°C with a heat exchanger. Pulmonary arterial, left atrial, and tracheal pressures were measured at end expiration 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. Because perfusate flow was also constant, increases in pulmonary arterial pressure (Ppa) were assumed to result from pulmonary vasoconstriction.

Experimental Protocols

Prevention of pressor responses to hypoxia and angiotensin II. After a 20-min stabilization period of perfusion and ventilation with 16% O_2, lungs were alternately exposed four times to angiotensin II (ANG II; 0.05 μg bolus into the main PA) and hypoxia (ventilation with 2% O_2 for 5 min) at 5- to 10-min intervals. Perfusate P_{O_2} measured with an oxygen microelectrode (Microelectrodes, Londonderry, NH) in the left atrial effluent of eight lungs averaged 112 (SD 4.6) mmHg during steady-state ventilation with 16% O_2 and 15.1 (SD 3.7) mmHg after 5 min of ventilation with 2% O_2. The CCE antagonists SKF-96365 (5, 16, or 50 μM; $n = 3$, 4, and 5, respectively), $NiCl_2$ (100, 200, or 500 μM; $n = 3$, 4, and 5, respectively), and $LaCl_3$ (1, 10, 30, or 100 μM; $n = 6$, 6, 4, and 3, respectively) were added to perfusate after the second hypoxic exposure. Additional lungs were exposed similarly to the L-type VOCC antagonist nifedipine (0.05, 0.5, or 5 μM; $n = 4$, 3, and 3, respectively) or Ca^{2+}-free KRBS perfusate containing 1 mM EGTA ($n = 4$). Untreated lungs perfused with KRBS ($n = 10$) or HBSS ($n = 6$) served as controls.

Reversal of HPV. After the 20-min normoxic stabilization period, lungs were exposed to ANG II (0.05 μg bolus injected into main PA) and hypoxia (ventilation with 2% O_2 for 5 min). After 10 min of normoxic recovery, lungs were then ventilated with 2% O_2 for 45 min. To assess the ability of CCE antagonists to reverse established HPV, SKF-96365 (50 μM; $n = 4$), $NiCl_2$ (500 μM; $n = 4$), or $LaCl_3$ (10 μM; $n = 4$) was added to the perfusate during the last 15 min of the 45-min hypoxic exposure. Additional lungs were exposed similarly to nifedipine (5 μM; $n = 4$) or perfusion with Ca^{2+}-free KRBS containing 1 mM EGTA ($n = 4$). Untreated lungs perfused with KRBS ($n = 5$) or HBSS ($n = 5$) and subjected to the same protocol served as controls. Experiments were not accepted for analysis if Ppa after 30 min of hypoxia was not at least 3 mmHg higher than Ppa at the onset of exposure. On this basis, one lung was excluded from each of the KRBS control, SKF-96365, and Ca^{2+}-free perfusate groups, four from the HBSS control group, and two from the $LaCl_3$ group.

Prevention of pressor responses to KCl. After the 20-min stabilization period, 4 M KCl was added to the reservoir in amounts adequate to increase perfusate [KCl] initially by 5 and subsequently by 10 mM increments. After each increment, sufficient time was allowed for Ppa to stabilize. KCl was administered in this manner until Ppa achieved a maximum or the lungs developed edema, as indicated by sudden loss of reservoir volume and increases in Ppa and end-inspiratory tracheal pressure. To examine the effects of CCE antagonists on the pressor response to KCl, SKF-96365 (50 μM; $n = 6$), $NiCl_2$ (500 μM; $n = 6$), or $LaCl_3$ (10 μM, $n = 4$) was administered 10 min before KCl. Additional experiments were performed in lungs exposed to nifedipine (5 μM; $n = 6$) or Ca^{2+}-free perfusate ($n = 3$) in a similar manner. Untreated lungs perfused with KRBS ($n = 11$) or HBSS ($n = 4$) served as controls.

Drugs and Materials

Stock solutions of SKF-96365 (10 mM) and KCl (4 M) were made up in water and stored at 4°C for ≤7 days before use. Stock solutions of $NiCl_2$ (10 mM) and $LaCl_3$ (1 and 10 mM) were made up in water on the day of the experiment. Nifedipine was dissolved in dimethyl sulfoxide to make a 30 mM stock solution and stored at −4°C until use. All agents were obtained from Sigma Chemical (St. Louis, MO).

Statistical Analysis

Data are expressed as means (SD). Statistical analysis was performed using Student's *t*-test or analysis of variance. If a significant interaction F-ratio was obtained with the latter, pairwise comparison of individual means was performed by calculating the least significant difference. Comparison of group means with control was performed using Dunnett's test. Differences were considered significant when $P < 0.05$.

RESULTS

Prevention of Pressor Responses to Hypoxia and ANG II

Figure 1 shows representative recordings of four exposures to ANG II and hypoxia in control lungs (Fig. 1*A*) and lungs perfused with Ca^{2+}-free KRBS after *exposure 2* (Fig. 1*B*). Average Ppa at baseline and peak response to ANG II and hypoxia for each of the four exposures in these groups are shown in Fig. 1, *C* and *D*. Both ANG II and hypoxia caused rapid reversible increases in Ppa at constant flow, left atrial pressure, and end-expiratory tracheal pressure, indicating vasoconstriction. In control lungs, Ppa at baseline and peak response to ANG II and hypoxia increased slightly over time. Perfusion with Ca^{2+}-free KRBS abolished the increase in baseline Ppa as well as the pressor responses to hypoxia and ANG II.

All groups of lungs were treated similarly during the first two exposures to hypoxia and ANG II. Table 1 shows mean baseline Ppa and peak change in Ppa (ΔPpa) caused by ANG II or hypoxia during *exposure 2* for each group. Values measured in lungs subsequently treated with SKF-96365, $NiCl_2$, nifedipine, or Ca^{2+}-free perfusate did not differ from values measured in KRBS-perfused control lungs. Baseline values of Ppa measured during *exposure 2* in HBSS-perfused control lungs and HBSS-perfused lungs subsequently treated with $LaCl_3$ were slightly higher than values measured in KRBS-perfused control lungs; however, baseline responses to angiotensin and hypoxia were not different.

Because changes in baseline vasomotor tone might alter pressor responses, we first asked whether any treatment changed baseline Ppa. Figure 2*A* shows the change in baseline Ppa between *exposure 2* (before treatment) and *exposure 4* (end of treatment) in lungs perfused with KRBS. In control lungs, baseline Ppa increased 1.1 (SD 0.82) mmHg during this time. This value did not differ from that in lungs treated with

SKF-96365 (50 μM), $NiCl_2$ (500 μM), or nifedipine (5 μM). Lungs treated with lower concentrations of these agents behaved similarly. In contrast, baseline Ppa fell 0.72 (SD 0.13) mmHg in lungs exposed to Ca^{2+}-free KRBS. In HBSS-perfused control lungs, baseline Ppa increased 1.3 (SD 0.57) mmHg from *exposure 2* to *exposure 4* (Fig. 2*C*). This value did not differ from that measured in KRBS-perfused control lungs or HBSS-perfused lungs treated with 1 or 10 μM $LaCl_3$; however, it was significantly less than the increases caused by 30 and 100 μM $LaCl_3$, which averaged 3.2 (SD 0.78) and 7.2 (SD 1.0) mmHg, respectively (Fig. 2, *B* and *C*).

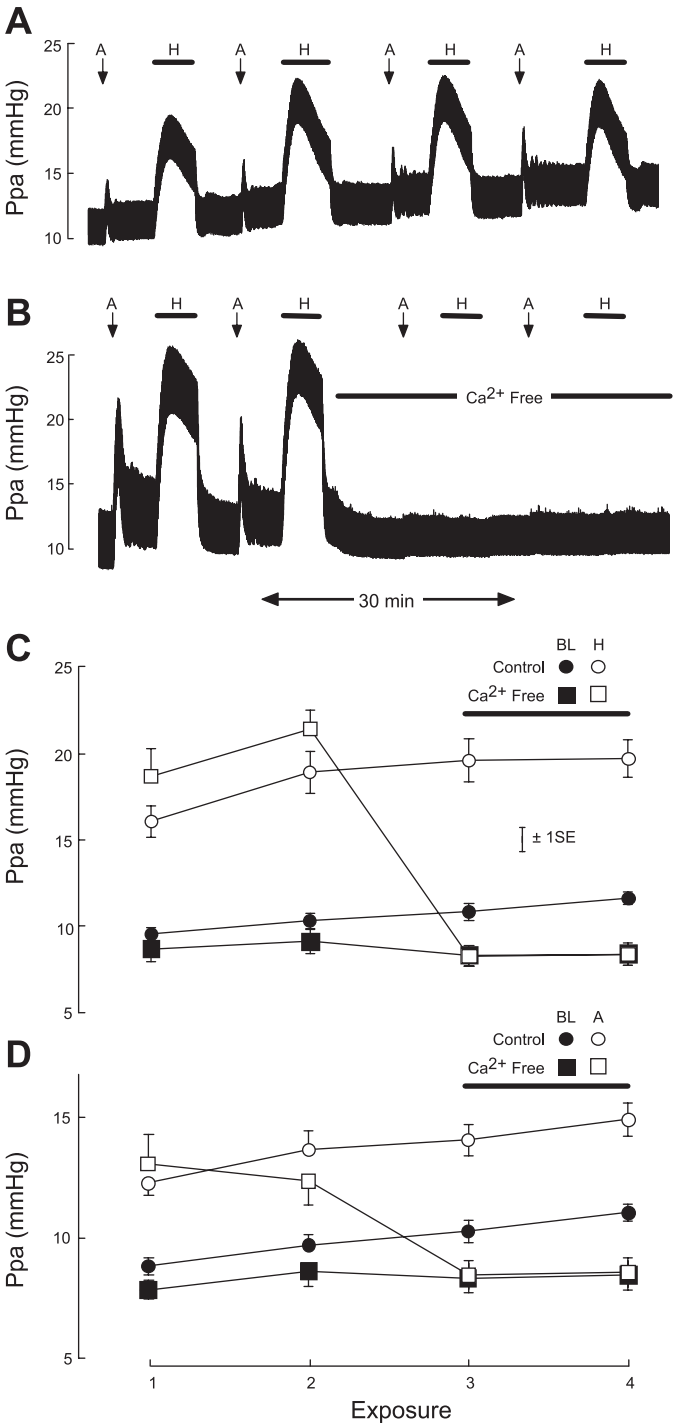

Fig. 1. Pressor responses to angiotensin II (ANG II; A, 0.05-μg bolus into the main pulmonary artery) and hypoxia (H, ventilation with 2% O_2) in isolated rat lungs perfused with a constant flow of Krebs Ringer bicarbonate solution (KRBS) containing Ficoll (4 g/dl) and sodium meclofenamate (3.1 μM). *A* and *B*: typical recordings of experiments in control lungs and lungs perfused with Ca^{2+}-free KRBS after the 2nd hypoxic exposure, respectively. *C* and *D*: mean values for pulmonary arterial pressure (Ppa) at baseline (BL) and peak response to ANG II and hypoxia for each of the 4 exposures are shown, respectively.

Table 1. *Baseline values of normoxic Ppa and ΔPpa to ANG II (0.05 μg into main pulmonary artery) or hypoxia (ventilation with 2% O_2) measured just before treatment (exposure 2) in all groups of lungs*

Group	*n*	Baseline Ppa (mmHg)	Peak ΔPpa (mmHg)	
			ANG II	Hypoxia
Control (KRBS)	11	9.6 (1.2)	4.1 (1.9)	8.7 (3.3)
SKF-96365	12	10.2 (1.1)	4.4 (2.7)	10.4 (3.3)
$NiCl_2$	12	10.2 (1.7)	3.5 (1.8)	11.0 (5.0)
Nifedipine	10	9.6 (1.1)	2.4 (1.6)	8.4 (5.2)
Ca^{2+} free	4	8.6 (1.2)	3.8 (1.1)	12.3 (6.7)
Control (HBSS)	6	12.0 (1.6)*	4.8 (2.8)	10.8 (6.8)
$LaCl_3$	19	12.0 (2.3)*	3.4 (1.6)	8.6 (5.0)

Values are means (SD). *$P < 0.05$ compared with control (Krebs Ringer bicarbonate solution, KRBS). HBSS, HEPES-buffered salt solution; ANG II, angiotensin II; Ppa, pulmonary arterial pressure; ΔPpa, peak pressor responses.

The effects of SKF-96365, $NiCl_2$, $LaCl_3$, and nifedipine on pressor responses to hypoxia and ANG II, expressed as percentages of responses just before treatment (*exposure 2*), are shown in Fig. 3. With the exception of $LaCl_3$, all agents caused concentration-dependent inhibition of vasoconstriction induced by hypoxia and ANG II. Inhibition by SKF-96365 and nifedipine was greatest during *exposure 3*, whereas inhibition by $NiCl_2$ was greatest during *exposure 4*. When all concentrations were included in the analysis of variance, $LaCl_3$ had no effect at 1, 10, and 30 μM and potentiated vasoconstriction at 100 μM. When only those concentrations that did not alter baseline Ppa were included (1 and 10 μM; Fig. 2, *B* and *C*), $LaCl_3$ inhibited HPV by 31% at 10 μM but did not affect the response to ANG II (Fig. 4).

The relationships between antagonist concentration and pressor response, expressed as a percentage of that measured in control lungs, are shown in Fig. 5. As estimated from the Hill equation, IC_{50} values for SKF-96365 and $NiCl_2$ against hypoxia (19 and 173 μM, respectively; Fig. 5*A*) were similar to those against ANG II (24 and 173 μM, respectively; Fig. 5*B*). Nifedipine was a potent inhibitor of both responses but was more effective against hypoxia (IC_{50} = 0.034 vs. 0.96 μM). IC_{50} for $LaCl_3$ could not be determined because of the variable effects of this agent (Figs. 3 and 4).

Reversal of HPV

In KRBS-perfused control lungs exposed to hypoxia for 45 min (Fig. 6, *A* and *B*), Ppa increased initially to a peak [ΔPpa = 8.2 (SD 2.0) mmHg at 4 min] before stabilizing at a lower value for the remainder of the exposure [ΔPpa = 5.6 (SD 1.7) mmHg at 30 min and 5.0 (SD 1.3) mmHg at 45 min]. The minimum ΔPpa between 30 and 45 min, expressed as a percentage of ΔPpa at 30 min, averaged 91.3% (SD 8.2) in KRBS-perfused control lungs (Fig. 6*C*). SKF-96365 (50 μM), $NiCl_2$ (500 μM), nifedipine (5 μM), or perfusion with Ca^{2+}-free KRBS abolished this sustained HPV (Fig. 6, *B* and *C*). In

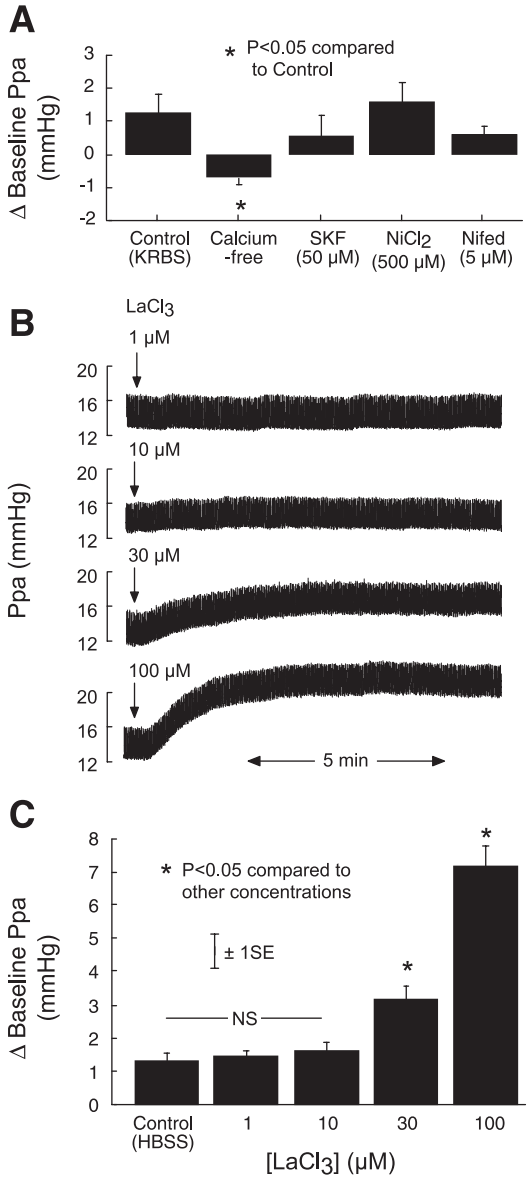

Fig. 2. *A*: average change in normoxic baseline Ppa (Δ baseline Ppa) between the 2nd and 4th exposures to ANG II and hypoxia for each group of isolated rat lungs perfused with KRBS. *B*: typical recordings of Ppa after administration of 1, 10, 30, or 100 μM $LaCl_3$ after the 2nd hypoxic exposure in normoxic rat lungs perfused with HEPES-buffered salt solution (HBSS). *C*: average change in normoxic baseline Ppa between the 2nd and 4th hypoxic exposures in untreated control HBSS-perfused rat lungs and lungs treated with $LaCl_3$.

HBSS-perfused lungs, 10 μM $LaCl_3$, which inhibited initial HPV by 31% (Fig. 4), did not alter sustained HPV; i.e., minimum ΔPpa between 30 and 45 min, expressed as a percentage of ΔPpa at 30 min, averaged 60.1% (SD 33.8) in control lungs and 79.1% (SD 25.3) in lungs treated with 10 μM $LaCl_3$ ($P = 0.382$).

Prevention of Pressor Responses to KCl

In KRBS-perfused control lungs, increases in perfusate [KCl] caused progressive increases in Ppa from 10.7 (SD 1.3) mmHg at 5 mM to 34.7 (SD 9.9) mmHg at 40 mM, the highest concentration achieved by all lungs in this group (Fig. 7, *A* and *B*). The same maximum [KCl] was achieved by all lungs treated with SKF-96365 (50 μM) or $NiCl_2$ (500 μM). Neither agent altered the Ppa-[KCl] relationship (Fig. 6*B*). $LaCl_3$ (10 μM), which caused some prevention of HPV (Fig. 4), did not prevent vasoconstriction to KCl (Fig. 7*C*). In contrast, nifedipine (5 μM) or Ca^{2+}-free perfusate almost abolished vasoconstrictor responses to KCl and allowed all lungs to achieve maximum KCl concentrations of 80 and 70 mM, respectively (Fig. 7, *A* and *B*).

DISCUSSION

HPV in isolated lungs depended critically on extracellular [Ca^{2+}] (Fig. 1), confirming the requirement for Ca^{2+} influx in this preparation (12), as in PA (20, 26) and PASMC (8, 45, 58,

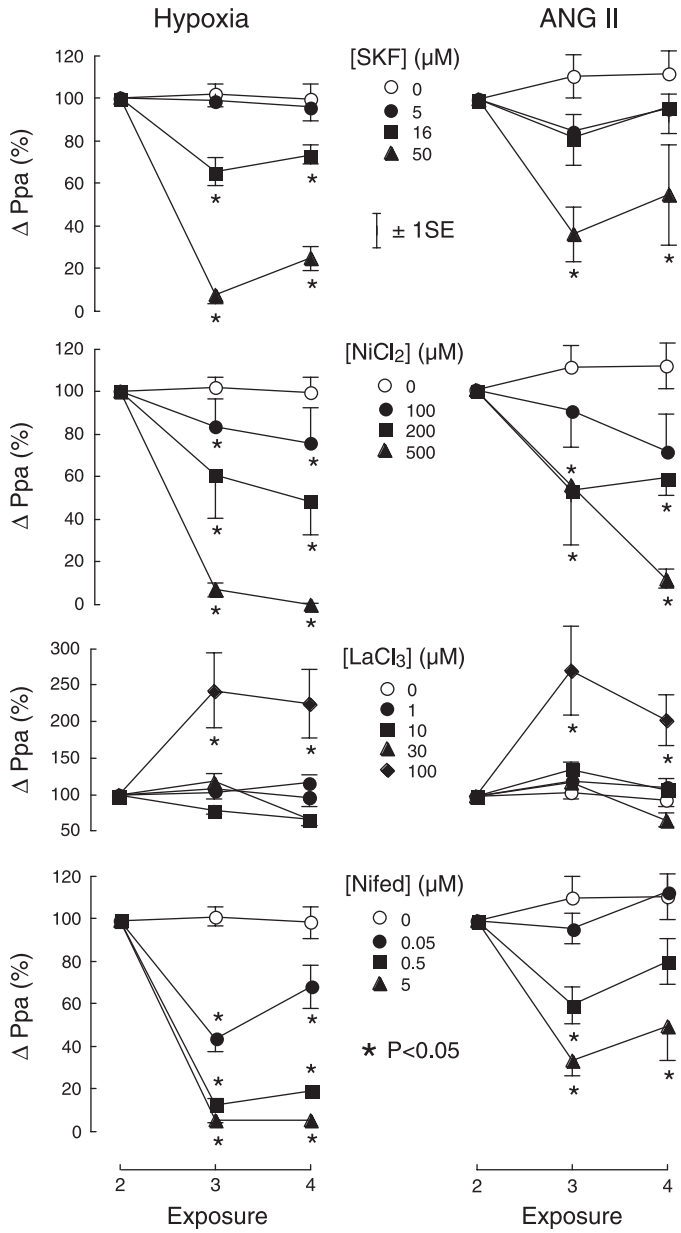

Fig. 3. Peak pulmonary arterial pressor responses (ΔPpa) to hypoxia and ANG II, expressed as a percentage of ΔPpa during *exposure 2*, after which treatment with different concentrations of SKF-96365 (SKF), $NiCl_2$, $LaCl_3$, or nifedipine (Nifed) began.

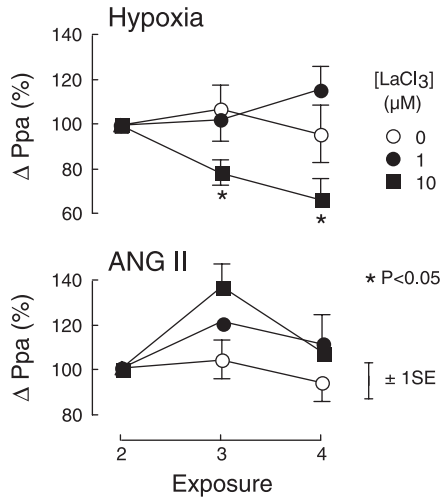

Fig. 4. ΔPpa to hypoxia (*top*) and ANG II (*bottom*), expressed as a percentage of ΔPpa during *exposure 2*, after which treatment with 1 or 10 μM $LaCl_3$ began.

59, 61). In addition, SOCC/NSCC antagonists prevented and reversed HPV (Figs. 1 and 3–6). The antagonist concentrations required for these effects also blocked CCE and hypoxia-induced increases in $[Ca^{2+}]_i$ and CCE in rat distal PASMC (60, 61) but did not block $[Ca^{2+}]_i$ responses to KCl in PASMC (60) or pressor responses to KCl in isolated lungs (Fig. 7), indicating inhibition of SOCC but not VOCC. These results suggest that HPV required CCE in PASMC.

The validity of this suggestion depends on the specificity of the antagonists. Inhibition of HPV by SKF-96365 (50 μM) and $NiCl_2$ (500 μM) did not result from nonspecific alterations of baseline vasomotor tone or toxic effects on contractile machinery because baseline Ppa (Fig. 2) and pressor responses to KCl (Fig. 7) in lungs exposed to these antagonists were not different from those in control lungs. Their lack of effect on KCl-induced vasoconstriction also indicates that SKF-96365 and $NiCl_2$ did not inhibit VOCC, confirming previous results in PASMC (60). Although structurally dissimilar, SKF-96365 and $NiCl_2$ clearly shared the intended action in rat distal PASMC, where IC_{50} against CCE were estimated to equal 6.3 and 191 μM, respectively (60). These values were within ranges of potency previously reported for inhibition of CCE in pulmonary arterial and other smooth muscles (10, 14, 15, 19, 29, 34, 43, 56, 64, 68). Furthermore, the concentrations of SKF-96365 and $NiCl_2$ that blocked CCE in PASMC were similar to those that blocked HPV in isolated lungs (IC_{50} = 19 and 173 μM, respectively; Fig. 5) and $[Ca^{2+}]_i$ responses to hypoxia in PASMC (IC_{50} = 3.2 and 36.7 μM, respectively) (61), suggesting a common mode of action.

Although these observations are consistent with the possibility that SKF-96365 and $NiCl_2$ blocked HPV by inhibiting CCE, other actions have been described in other tissues. For example, SKF-96365 was found to prevent Ca^{2+} influx through receptor-operated Ca^{2+} channels, promote Ca^{2+} influx through nonspecific cation channels, and inhibit SR Ca^{2+} pumps (25). $NiCl_2$ is thought to be a nonspecific blocker of Ca^{2+} entry (25) and has been shown to inhibit sarcolemmal Na^+-Ca^{2+} exchange (5). It seems unlikely that enhanced Ca^{2+} entry, reduced SR Ca^{2+} uptake, or blockade of Na^+-Ca^{2+} exchange could explain the inhibitory effects of SKF-96365 and $NiCl_2$ on HPV; however, it remains possible that these agents blocked Ca^{2+} influx through channels other than SOCC and VOCC, such as the voltage- and store-independent channels hypothesized by Robertson et al. (43).

The effects of $LaCl_3$ are more difficult to interpret. In some previous studies (34, 35), chelation or precipitation of La^{3+} may have limited bioavailability. To avoid this problem, we used an HBSS perfusate that contained neither bicarbonate nor phosphate (43, 51, 60). In our studies, $LaCl_3$ increased $[Ca^{2+}]_i$ in PASMC perfused with HBSS under baseline normoxic conditions (61) and caused concentration-dependent vasoconstriction in normoxic HBSS-perfused lungs (Fig. 2, *B* and *C*). These findings suggest that $LaCl_3$ had actions other than inhibition of SOCC, such as inhibition of Na^+-Ca^{2+} exchange and Ca^{2+} pumps at the plasma membrane (5, 48). They also suggest that effects of $LaCl_3$ on HPV could be the nonspecific result of increased baseline tone. At 10 μM, however, $LaCl_3$ altered neither baseline tone (Fig. 2*C*) nor the pressor response to KCl (Fig. 7*C*) but inhibited HPV by 31% (Fig. 4). Because IC_{50} for $LaCl_3$ against CCE in distal PASMC was 40.4 μM (60), these results are consistent with the hypothesis that HPV requires CCE. At 100 μM, however, $LaCl_3$ increased baseline tone (Fig. 2, *B* and *C*) and potentiated HPV (Fig. 3). Because

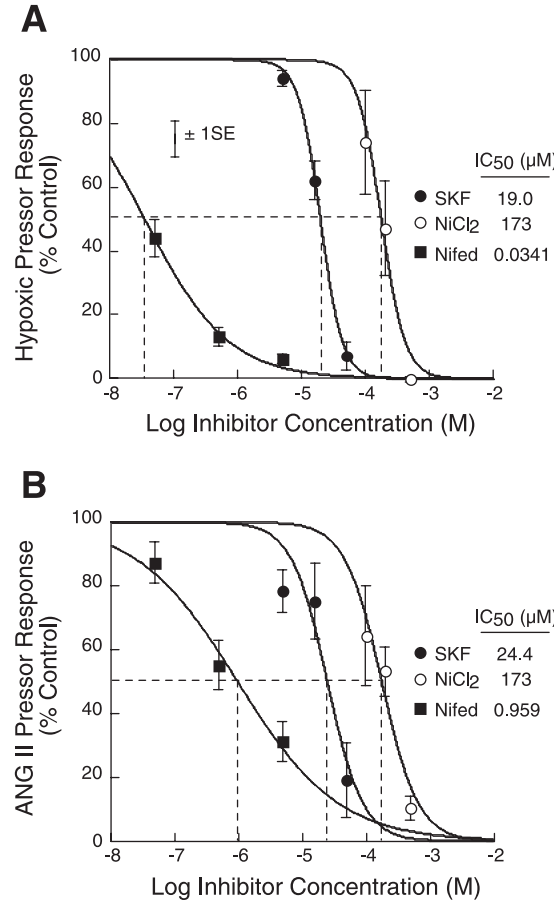

Fig. 5. Concentration-effect relationships for SKF-96365, $NiCl_2$, and nifedipine against pressor responses to hypoxia (*A*) and ANG II (*B*). Pressor responses are expressed as percentages of mean values observed in untreated control lungs. The curves represent least-squares fits of the Hill equation, from which antagonist concentrations causing IC_{50} were estimated.

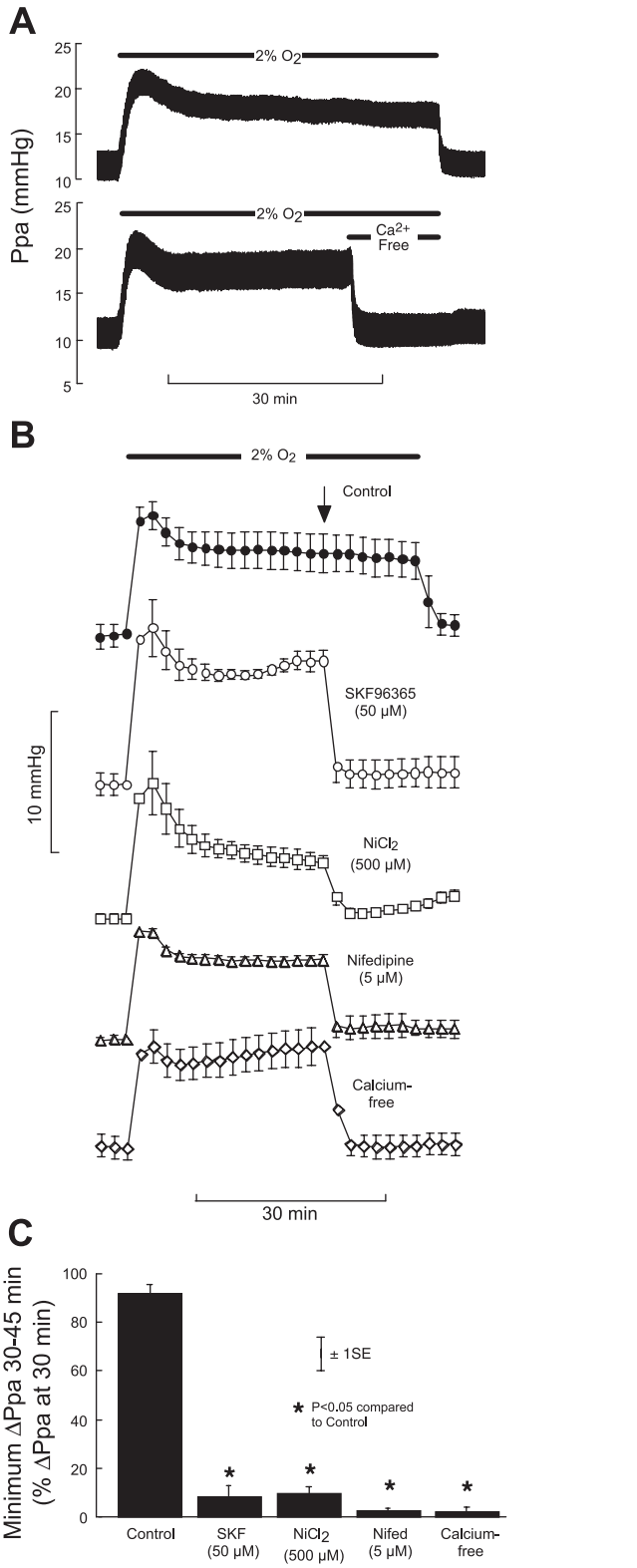

Fig. 6. *A*: typical recordings of Ppa during ventilation of isolated rat lungs with 2% O_2 for 45 min. In *A*, untreated control lungs are shown (*top*), and lungs perfused with Ca^{2+}-free KRBS during the last 15 min of hypoxia are shown (*bottom*). *B*: time course of mean Ppa during 45 min of hypoxia in untreated control lungs and lungs exposed to antagonists of store-operated Ca^{2+} channels (SKF-96365, $NiCl_2$), voltage-operated Ca^{2+} channels (nifedipine), or Ca^{2+}-free perfusate during the last 15 min of hypoxia (arrow). *C*: minimum change in Ppa from normoxic baseline values (ΔPpa) for each group during the last 15 min of hypoxia (30–45 min) expressed as a percentage of ΔPpa just before treatment (30 min).

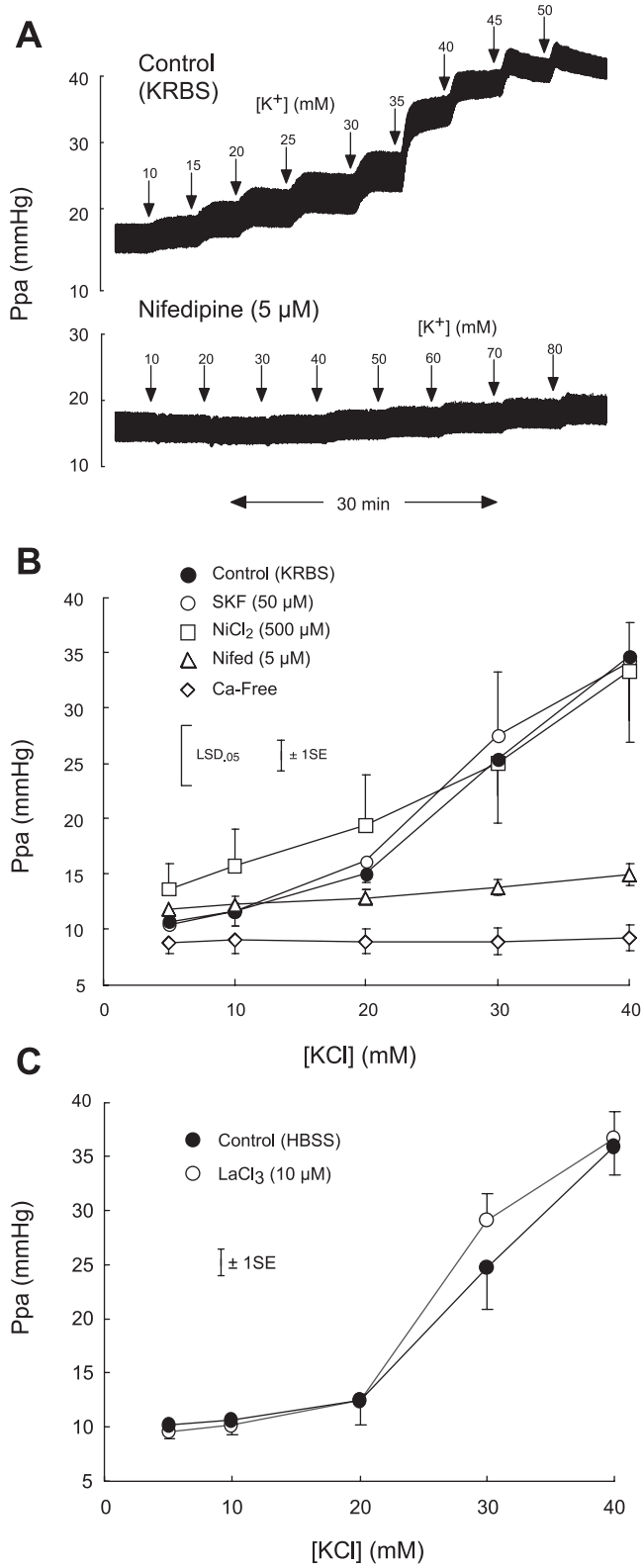

Fig. 7. *A*: typical recordings of Ppa in untreated (*top*) and nifedipine-treated (*bottom*) rat lungs exposed to increasing perfusate concentrations of KCl. Mean data for all groups of lungs perfused with KRBS are shown in *B*. Only Ca^{2+}-free perfusate and nifedipine inhibited KCl-induced vasoconstriction. Mean data for untreated and $LaCl_3$-treated rat lungs perfused with HBSS are shown in *C*. $LaCl_3$ (10 μM) did not alter the KCl response. $LSD_{0.05}$, least significant difference for $P = 0.05$.

100 μM $LaCl_3$ increased basal $[Ca^{2+}]_i$, abolished CCE, and blocked $[Ca^{2+}]_i$ responses to hypoxia in PASMC (60, 61), potentiation of HPV in isolated lungs may have been due to an increase in myofilament Ca^{2+} sensitivity occurring in the face of increased basal $[Ca^{2+}]_i$. Several findings support this possibility. In PA exposed to sustained hypoxia, progressive contraction occurred at slightly elevated but constant $[Ca^{2+}]_i$ (41), and this response was enhanced by raising basal tone with vasoactive agonists (36). HPV in both isolated lungs and PA was blocked by antagonists of Rho kinase (11, 42, 62), which is thought to increase Ca^{2+} sensitivity by inhibiting myosin light chain phosphatase, thereby increasing phosphorylated myosin light chain concentration and vasomotor tone at constant levels of $[Ca^{2+}]_i$ and myosin light chain kinase activity (54, 62, 63). Further studies will be required to clarify the effects of high $[LaCl_3]$ on HPV.

As with hypoxia, the mechanisms by which ANG II changes vasomotor tone are complex and may vary among vessels (44). In PASMC, ANG II caused oscillations of $[Ca^{2+}]_i$ that appeared to result from cycling of inositol trisphosphate-dependent SR Ca^{2+} release (16). These $[Ca^{2+}]_i$ oscillations were thought to evoke oscillatory activation of Ca^{2+}-dependent Cl^- (Cl_{Ca}) channels, leading to depolarization, Ca^{2+} influx through VOCC, and contraction (17); however, nifedipine only partially inhibited ANG II-induced contractions in PA, suggesting contributions by SR Ca^{2+} release and/or CCE (17). Consistent with these possibilities, we found that SKF-96365 and $NiCl_2$ inhibited pressor responses to ANG II in a concentration-dependent manner, similar to their effects on HPV (Figs. 3 and 5). Indeed, IC_{50} for SKF-96365 and $NiCl_2$ against ANG II (24 and 173 μM, respectively) were almost identical to IC_{50} against hypoxia (19 and 173, respectively). Nifedipine was less potent against ANG II than HPV (IC_{50} = 0.96 and 0.034 μM, respectively), perhaps reflecting a greater contribution of SR Ca^{2+} release to the ANG II response due to the short duration of stimulation with ANG II, which was delivered as a bolus into the main PA.

In isolated PA, the response to hypoxia of more than a 30-min duration was usually biphasic, consisting of early transient contraction followed by late sustained contraction (4, 9, 23, 24). Similar temporal characteristics have been described for HPV in isolated lungs (65–67). Because the mechanisms responsible for the early and late phases of HPV might be different (55), we determined whether Ca^{2+}-free perfusate, nifedipine, and SOCC/NSCC antagonists could inhibit sustained HPV. Consistent with previous results (20, 24), Ca^{2+}-free perfusate or nifedipine reversed HPV after 30 min of hypoxia (Fig. 6). SKF-96365 (50 μM) and $NiCl_2$ (500 μM) had similar effects. These findings suggest that influx of extracellular Ca^{2+} through SOCC and VOCC was required not only to initiate but also to sustain HPV. In contrast, Dipp et al. (9) reported that late hypoxic contraction in rat PA was blocked by ryanodine plus caffeine, but not by Ca^{2+}-free conditions, suggesting that HPV was sustained by Ca^{2+} release from SR rather than Ca^{2+} influx. We cannot explain this discrepancy.

Our conclusion that HPV requires both CCE and voltage-gated Ca^{2+} influx is based on our observations that 50 μM SKF-96365 and 500 μM $NiCl_2$ (which blocked CCE but not responses to KCl) or 5 μM nifedipine (which blocked responses to KCl but not CCE) were all quite effective at preventing and reversing HPV (Figs. 1, 3, 5, and 6). In complex preparations like isolated lungs, one possible explanation for this dual requirement is that cells other than PASMC were involved in HPV. For example, endothelin-1, which is derived from endothelial cells and thought to be required for full expression of HPV in vivo (26, 46, 47), can activate SOCC (71); however, this possibility would not explain why $[Ca^{2+}]_i$ responses to hypoxia were SOCC dependent in isolated PASMC (35, 61). Another possibility is that SOCC and VOCC provided parallel pathways for influx of Ca^{2+} into PASMC during hypoxia, but neither pathway on its own was capable of increasing $[Ca^{2+}]_i$ by amounts sufficient to trigger contraction. This might occur if CCE and voltage-gated Ca^{2+} influx occurred in noncontractile and contractile intracellular compartments, respectively, which had limited access to one another (13, 51, 56). Alternatively, primary activation of SOCC by hypoxia could cause secondary activation of VOCC. For example, hypoxia-induced release of Ca^{2+} from SR (9, 19, 26, 31, 35, 59) could deplete SR Ca^{2+} stores, leading to SOCC activation and CCE. Increases in $[Ca^{2+}]_i$ due to CCE and/or SR Ca^{2+} release could then inhibit voltage-dependent K^+ channels and/or activate Cl_{Ca} channels (6, 38), leading to PASMC depolarization and Ca^{2+} influx through VOCC. The opposite sequence has also been suggested (7, 31); namely, that voltage-gated Ca^{2+} influx might cause secondary release of Ca^{2+} from SR. Previous findings that $[Ca^{2+}]_i$ responses to hypoxia were abolished by SOCC/NSCC antagonists but were inhibited only 50% by VOCC antagonists would seem to argue against this possibility (35, 61). Because SOCC/NSCC are thought to have reversal potentials near 0 mV (34, 37, 51), it is also possible that hypoxic activation of these channels depolarized PASMC directly, again leading to secondary VOCC activation and voltage-dependent Ca^{2+} influx. Moreover, if activated SOCC/NSCC were sufficiently permeable to Na^+, depolarization and increased $[Na^+]_i$ could promote Ca^{2+} influx via reverse-mode Na^+-Ca^{2+} exchange (5). More investigation is needed to evaluate these possibilities.

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