Mobilization of sarcoplasmic reticulum stores by hypoxia leads to consequent activation of capacitative Ca^{2+} entry in isolated canine pulmonary arterial smooth muscle cells

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Capacitative Ca^{2+} entry (CCE) has been speculated to contribute to Ca^{2+} influx during hypoxic pulmonary vasoconstriction (HPV). The aim of the present study was to directly test if acute hypoxia causes intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) rises through CCE in canine pulmonary artery smooth muscle cells (PASMCs). In PASMCs loaded with fura-2, hypoxia produced a transient rise in $[Ca^{2+}]_i$ in Ca^{2+}-free solution, indicating Ca^{2+} release from the intracellular Ca^{2+} stores. Subsequent addition of 2 mM Ca^{2+} in hypoxia elicited a sustained rise in $[Ca^{2+}]_i$, which was partially inhibited by 10 μM nisoldipine. The dihydropyridine-insensitive rise in $[Ca^{2+}]_i$ was due to increased Ca^{2+} influx, because it was abolished in Ca^{2+}-free solution and hypoxia was shown to significantly enhance the rate of Mn^{2+} quench of fura-2 fluorescence. The dihyropyridine-insensitive rise in $[Ca^{2+}]_i$ and the increased rate of Mn^{2+} quench of fura-2 fluorescence were inhibited by 50 μM SKF 96365 and 500 μM Ni^{2+}, but not by 100 μM La^{3+} or 100 μM Gd^{3+}, exhibiting pharmacological properties characteristic of CCE. In addition, predepletion of the intracellular Ca^{2+} stores inhibited the rise in $[Ca^{2+}]_i$ induced by hypoxia. These results provide the first direct evidence that acute hypoxia, by causing Ca^{2+} release from the intracellular stores, activates CCE in isolated canine PASMCs, which may contribute to HPV.

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Hypoxic pulmonary vasoconstriction (HPV) diverts blood flow from poorly ventilated areas of the lung to well-ventilated regions. This process is unique to the pulmonary circulation during alveolar hypoxia to optimize arterial oxygenation by matching the local ventilation with perfusion (von Euler and Liljestrand, 1946). However, the cellular mechanisms responsible for the acute vasoconstrictor response of small distal pulmonary arteries to hypoxia remain unclear (Sylvester, 2001; Weir *et al.* 2002). In isolated pulmonary arterial smooth muscle cells (PASMCs), hypoxia has been shown to inhibit voltage-dependent K^+ currents, possibly leading to membrane depolarization and activation of Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Post *et al.* 1992; Yuan *et al.* 1993; Cornfield *et al.* 1994; Osipenko *et al.* 2000). Recent attention has also focused on the role of hypoxic-induced release of Ca^{2+} from intracellular sarcoplasmic reticulum (SR) Ca^{2+} stores as an early essential event in the acute hypoxic response (Salvaterra and Goldman, 1993; Jabr *et al.* 1997; Dipp *et al.* 2001; Morio and McMurtry, 2002).

In the case of hypoxic-induced release of Ca^{2+} from intracellular Ca^{2+} stores, Dipp *et al.* (2001) suggest that released Ca^{2+} may be the trigger that activates constriction, and that Ca^{2+} influx across the plasmalemma is not essential for HPV. On the other hand, depletion of intracellular stores, but not the released Ca^{2+} *per se*, has been suggested to mediate HPV through activation of a separate voltage-independent extracellular Ca^{2+} entry pathway (Jabr *et al.* 1997; Robertson *et al.* 2000), so-called capacitative Ca^{2+} entry (CCE) (Putney and McKay, 1999). The reason for these inconsistencies is unclear, but it could be explained by the variations in animal species, degree of vessel wall pretone and experimental protocols used (see Weissmann *et al.* 2001 for review).

Several recent studies have confirmed the existence of CCE in PASMCs, and confirmed the expression of several homologues of transient receptor potential proteins, putative candidates for store-operated channels, in PASMCs (Ng and Gurney, 2001; Walker *et al.* 2001; Wilson *et al.* 2002; Snetkov *et al.* 2003; Wang *et al.* 2003). In cultured rabbit PASMCs, Kang *et al.* (2003) demonstrated the presence of CCE and hypoxia enhanced the depletion-activated rise in $[Ca^{2+}]_i$. They also found that hypoxia caused Ca^{2+} release from the SR Ca^{2+} stores and a nifedipine-insensitive rise in $[Ca^{2+}]_i$ (Kang *et al.*

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2002). These observations led to the suggestion that hypoxia may activate CCE by releasing Ca^{2+} from the SR. However, no direct evidence was provided on the nature of the pathway involved. Thus, the possibility that acute hypoxia causes activation of CCE remains to be demonstrated. More recently, chronic hypoxic exposure of cultured rat PASMCs was found to up-regulate store-operated channels, which may contribute to the enhanced vascular tone in HPV (Lin *et al.* 2004). This study suggested that HPV may activate CCE, but the question of whether acute hypoxia may activate CCE, and whether this is a direct action of hypoxia on CCE channels or is mediated indirectly by depletion of intracellular stores, has yet to be directly tested. We have recently characterized the properties of CCE activated by intracellular Ca^{2+} store depletion in freshly isolated canine PASMCs by measuring changes in cytosolic Ca^{2+} concentration, and by direct measurement of activation of a store-operated Ca^{2+} permeability pathway by monitoring the rate of Mn^{2+} quench of fura-2 fluorescence (Wilson *et al.* 2002). The purpose of the present study was to utilize these same experimental approaches to directly test the hypothesis that acute hypoxia, by causing Ca^{2+} release from the SR Ca^{2+} stores, indirectly leads to activation of CCE in freshly isolated canine PASMCs.

Methods

Cell isolation

PASMCs were isolated from canine pulmonary artery as previously described (Wilson *et al.* 2002). Cross-breed dogs of either sex were killed with pentobarbital sodium (45 mg kg^{-1} I.V.) and ketamine (15 mg kg^{-1} I.V.), as conforming with the requirements of the University of Nevada at Reno Institutional Care and Use Committee. The heart and lungs were removed *en bloc*, and the third and fourth branches of the pulmonary artery were dissected at 5°C to decrease cellular metabolic activity. The arteries were flushed with a low-Ca^{2+} physiological salt solution (PSS) composed of the following (mM): NaCl 125, KCl 5.36, Na_2HPO_4 0.34, K_2HPO_4 0.44, $MgCl_2$ 1.2, Hepes 11, glucose 10 and $CaCl_2$ 0.05 (pH 7.4 adjusted with Tris). Arteries were cleaned of connective tissue, cut into small pieces and placed in a tube containing fresh low-Ca^{2+} PSS. Tissue was immediately digested or cold stored at 5°C for up to 24 h. To disperse cells, pulmonary arterial tissue was incubated with the low-Ca^{2+} PSS containing (mg ml^{-1}): collagenase type XI 0.5, elastase type IV 0.04 and bovine serum albumin 0.5 (fat-free) for 16–18 h at 5°C. The tissue was then transferred to an enzyme-free, low-Ca^{2+} PSS, and triturated with a fire-polished Pasteur pipette. The resulting dispersed PASMCs were cold-stored in the refrigerator at 5°C until use, where they remained viable for 8–10 h.

Measurement of intracellular Ca^{2+}

The cytosolic Ca^{2+} concentration was estimated in PASMCs loaded with fura-2 acetoxymethyl ester (fura-2 AM) (Molecular Probes, Eugene, OR, USA) using a dual excitation digital Ca^{2+} imaging system (IonOptix, Inc., Milton, MA, USA) equipped with an intensified CCD camera as previously described (Wilson *et al.* 2002). PASMCs were loaded with 10 μM Fura-2 AM for 30 min in the dark at room temperature, and placed on the coverslip in a 0.2 ml perfusion chamber mounted on an epifluorescence microscope (Nikon). To remove extracellular fura-2 AM, cells were washed several times with 2 mM Ca^2-PSS composed of the following (mM): NaCl 113, KCl 5, MgCl 1, $CaCl_2$ 2, NaH_2PO_4 0.5, $NaHCO_3$ 24 and glucose 10 (gassed continuously with 21% O_2/5% CO_2/74% N_2, pH 7.4). Cells were illuminated with xenon arc lamp at 340 ± 15 and 380 ± 12 nm (Omega Optical, Brattleboro, VT, USA), and from regions that encompassed single cells, emitted light was collected with a CCD camera at 510 nm (Nikon). All experiments were performed at 35–37°C, and images were acquired at 1 Hz, and stored on the compact disk for later analysis. Background fluorescence was collected automatically and subtracted from the acquired fluorescence video images during each experiment. The ratio of fluorescence (R) excited at the two excitation wavelengths was used to estimate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), as described by Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d(S_{f_2}/S_{b_2})[(R - R_{min})/(R_{max} - R)]$$

The values for S_{f_2} (fluorescence measured at 380 nm in Ca^{2+}-free solution), S_{b_2} (fluorescence measured at 380 nm in Ca^{2+}-saturating conditions), R_{min} (minimum ratio) and R_{max} (maximum ratio) were determined from *in situ* calibrations of fura-2 for each cell. The dissociation constant for Ca^{2+} binding (K_d) was assumed to be 224 nM (Grynkiewicz *et al.* 1985). To determine R_{min}, cells were dialysed at the end of each experiment with 4 μM ionomycin in Ca^{2+}-free PSS containing 10 mM EGTA. R_{max} was determined from cells dialysed with 4 μM ionomycin in PSS containing 10 mM $CaCl_2$.

In control experiments, PASMCs were superfused continuously with normoxic PSS in an open air recording chamber. Normoxic PSS was prepared by continuous gassing with certified gas mixture containing 21% O_2, 5% CO_2 and 74% N_2 (Air Liquide, Santa Fe Springs, CA, USA). In experiments where the effect of hypoxia was investigated, hypoxia was induced by switching normoxic PSS to hypoxic PSS, which continuously superfused the cells in the recording chamber. Hypoxic PSS was prepared by continuous gassing with uncertified gas mixture containing 95% N_2 and 5% CO_2 (Sierra Welding, Sparks, NV, USA). The uncertified gas mixture contained minimal amount of

oxygen, which equilibrated with PSS to avoid exposure of cells to anoxic condition. All solutions were placed in a water bath at 37°C and saturated with either normoxic or hypoxic gas mixtures for at least 30 min before the start of perfusion, and maintained at pH 7.4. The P_{O_2} was measured in preliminary experiments with an oxygen-sensitive electrode (MI-730; Microelectrodes, Inc., Bedford, NH, USA) to be 145 ± 1 mmHg during normoxic PSS perfusion, and falling to 15 ± 1 mmHg within 79 ± 2 s of hypoxic exposure. The P_{O_2} of hypoxic solutions was measured at the end of each experiment, and was found to be 15–18 mmHg, which ensured that the P_{O_2} did not approach anoxia during the recording of each experiment. The effect of hypoxia was examined in cells incubated in Ca^{2+}-free PSS for 10 min, followed by the re-exposure of cells with 2 mM Ca^{2+} PSS for another 10–15 min. Ca^{2+}-free PSS was identical to 2 mM Ca^{2+} PSS, but with $CaCl_2$ omitted and 1 mM EGTA added. An elevation in $[Ca^{2+}]_i$ above basal levels during 2 mM Ca^{2+} re-addition was used as a marker of hypoxia-induced extracellular Ca^{2+} entry. In experiments where the Ca^{2+} influx pathway was studied, the rate of Mn^{2+}-induced quenching of fura-2 fluorescence was recorded during excitation at 360 nm in nominally Ca^{2+}-free PSS containing 10 μM nisoldipine (Wilson *et al.* 2002). Nominally Ca^{2+}-free solutions were similar to 0 Ca^{2+} PSS, but with EGTA omitted. In experiments where CCE was investigated, a store-depletion protocol previously described by Wilson *et al.* (2002) was used. The SR Ca^{2+} stores were maximally depleted by exposure of cells to a cocktail containing 10 μM cyclopiazonic acid (CPA) and 10 μM ryanodine, followed by a 30 s exposure to 10 μM 5-HT and 10 mM caffeine in Ca^{2+}-free solutions. All experiments were performed on viable isolated PASMCs. Cell viability was assessed by visual examination to ensure that cells remained relaxed under resting unstimulated conditions, and Mn^{2+} quench experiments were only performed on cells in which the rate of Mn^{2+} quench of the fura-2 signal in the presence of ionomycin was at least fourfold greater than the basal rate, as previously described (Wilson *et al.* 2002).

Drug solutions and data analysis

CPA, nisoldipine and ionomycin were dissolved in dimethylsulphoxide (DMSO). Other drugs were dissolved in deionized water. Data are expressed as means ± S.E.M. of n cells isolated from at least three dogs. Statistical comparisons employed Student's paired t tests or one-way analysis of variance (ANOVA) with Tukey's pairwise comparison. A value of $P < 0.05$ was considered significant.

Results

To determine whether hypoxia increases $[Ca^{2+}]_i$, canine PASMCs superfused with normoxic 2 mM Ca^{2+} PSS were first exposed to normoxic Ca^{2+}-free PSS, followed by perfusing the bath with hypoxic Ca^{2+}-free PSS (Fig. 1*A*). When applied in Ca^{2+}-free solution, hypoxia caused a transient increase in $[Ca^{2+}]_i$ indicative Ca^{2+}

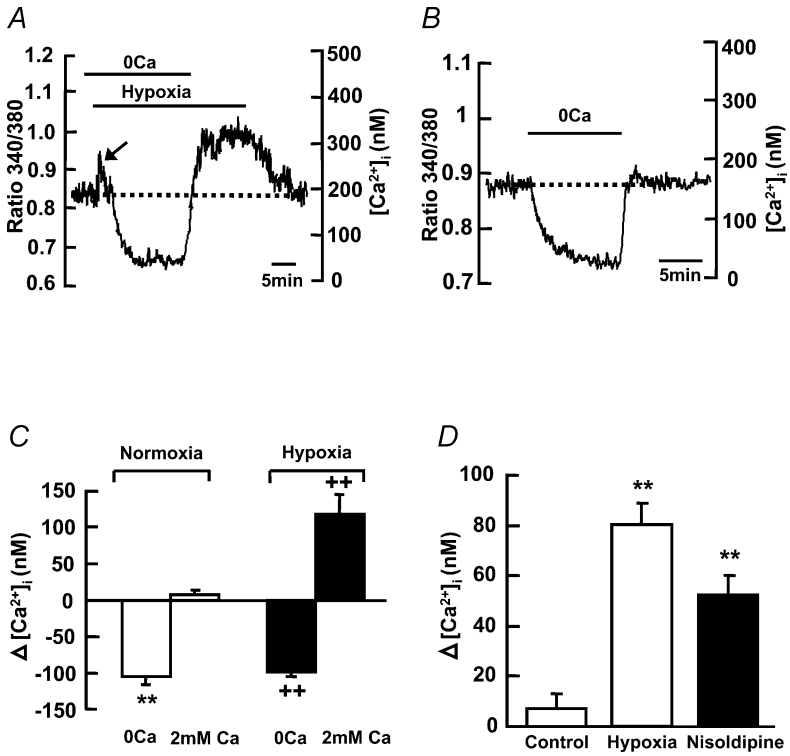

Figure 1. Hypoxia increases $[Ca^{2+}]_i$ in freshly isolated pulmonary artery smooth muscle cells
A, when applied in Ca^{2+}-free solution, hypoxia transiently elevated fura-2 fluorescence ratio, indicating Ca^{2+} release from the intracellular stores (arrow). Readmission of 2 mM Ca^{2+} in hypoxic solution caused an increase in fluorescence ratio, which slowly returned to basal levels (dotted line) upon reoxygenation. *B*, in control experiments, pulmonary artery smooth muscle cells (PASMCs) were exposed to normoxic solutions throughout recording. Removal of external Ca^{2+} caused a decrease in fluorescence ratio below basal level. Subsequent addition of 2 mM Ca^{2+} restored the fluorescence ratio to the basal levels. *C*, mean changes in $[Ca^{2+}]_i$ compared to the resting $[Ca^{2+}]_i$ in normoxia ($n = 66$) and hypoxia ($n = 148$) experiments as indicated. $^{**}P < 0.01$ and $^{++}P < 0.01$, compared to the resting $[Ca^{2+}]_i$ (ANOVA). *D*, bar graph showing the mean changes in $[Ca^{2+}]_i$ after the addition of 2 mM Ca^{2+} in normoxia (control, $n = 66$) and in the continued presence of hypoxia ($n = 119$), with and without 10 μM nisoldipine. $^{**}P < 0.01$ (ANOVA).

release from the intracellular stores. The transient rise in $[Ca^{2+}]_i$ decayed slowly to a mean level (41 ± 7 nM, $R = 0.68 \pm 0.01$, $n = 148$, $P < 0.01$) below baseline (139 ± 10 nM, $R = 0.82 \pm 0.01$). Subsequent addition of 2 mM Ca^{2+} in hypoxia elicited a significant rise in $[Ca^{2+}]_i$ to 256 ± 32 nM ($R = 0.90 \pm 0.01$), 117 ± 27 nM ($\Delta R = 0.08 \pm 0.01$) above basal levels (Fig. 1*C*, $n = 148$, $P < 0.01$). The response to hypoxia was sustained and reversed slowly upon reoxygenation. In control experiments, cells were exposed to normoxic solutions throughout the protocol (Fig. 1*B*). Removal of extracellular Ca^{2+} caused a mean 104 ± 12 nM ($\Delta R = 0.14 \pm 0.01$) decrease in $[Ca^{2+}]_i$, from a basal value of 153 ± 22 nM ($R = 0.83 \pm 0.02$) to 49 ± 13 nM ($R = 0.69 \pm 0.02$, $n = 66$, $P < 0.01$). Subsequent addition of 2 mM Ca^{2+} caused an increase in $[Ca^{2+}]_i$ to 160 ± 20 nM ($R = 0.85 \pm 0.02$, $n = 66$), only 7 ± 6 nM (Fig. 1*B*; $\Delta R = 0.02 \pm 0.01$, $n = 66$) higher than the basal values (not significant). Figure 1*D* shows that the increase in $[Ca^{2+}]_i$ induced by hypoxia was significantly reduced by approximately 35% ($n = 119$, $P < 0.01$) by 10 μM nisoldipine, a dihydropyridine Ca^{2+} antagonist. Therefore, the hypoxic induced rise in $[Ca^{2+}]_i$ involves both dihydropyridine-sensitive and -insensitive Ca^{2+} entry pathways. This contrasts with the Ca^{2+} store depletion-induced rise in $[Ca^{2+}]_i$ in these cells, which was previously shown to only involve a dihydropyridine-insensitive Ca^{2+} entry pathway (Wilson *et al.* 2002).

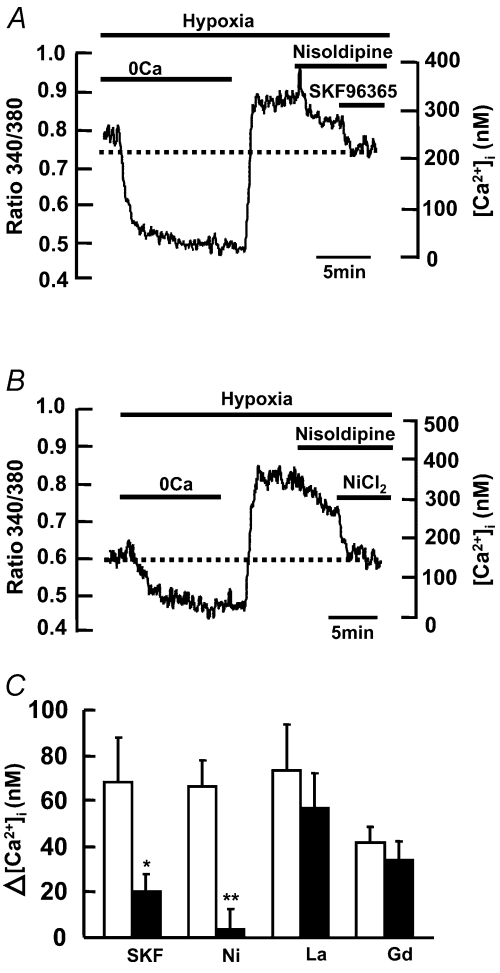

Figure 2. Sensitivity of nisoldipine-insensitive rise in $[Ca^{2+}]_i$ to blockers of store-operated channels
In the continued presence of 10 μM nisoldipine, the hypoxia-induced rise in fura-2 fluorescence ratio was blocked by 50 μM SKF 96365 (*A*) and 500 μM Ni^{2+} (*B*). *C*, bar graph showing the mean changes in $[Ca^{2+}]_i$ after addition of 2 mM Ca^{2+} in hypoxic solution containing 10 μM nisoldipine, with and without SKF 96365 ($n = 39$), Ni^{2+}($n = 28$), La^{3+} ($n = 11$) and Gd^{3+} ($n = 20$). $^*P < 0.05$ and $^{**}P < 0.01$, compared with the absence of blocker (Student's paired *t* test).

To determine if the dihydropyridine-insensitive component activated by hypoxia involved CCE, its sensitivity to known blockers of store-operated channels Ni^{2+}, La^{3+}, Gd^{3+} and SKF 96365 (Parekh and Penner, 1997) were tested. In these experiments, 10 μM nisoldipine was used to block the dihydropyridine-sensitive component. SKF 96365 (50 μM) reduced the increase in nisoldipine-insensitive rise in $[Ca^{2+}]_i$ by about 71%, from 69 ± 19 nM ($\Delta R = 0.06 \pm 0.01$) to 20 ± 7 nM ($\Delta R = 0.02 \pm 0.01$) (Fig. 2*A* and *C*; $n = 39$, $P < 0.05$), and 500 μM Ni^{2+} reduced it by about 94%, from 67 ± 11 nM ($\Delta R = 0.08 \pm 0.01$) to 4 ± 8 nM ($\Delta R = 0.005 \pm 0.007$) (Fig. 2*B* and *C*; $n = 28$, $P < 0.01$). La^{3+} (100 μM) (Fig. 2*C*; $n = 11$) and Gd^{3+} (100 μM) (Fig. 2*C*; $n = 20$) did not significantly reduce the increase in $[Ca^{2+}]_i$.

To determine if hypoxia increases $[Ca^{2+}]_i$ by recruiting a new Ca^{2+} influx pathway similar to CCE, the effect of hypoxia on Mn^{2+} quench of fura-2 fluorescence was tested under comparable conditions. In control experiments, the effect of store-depletion on Mn^{2+} quench of fura-2 was examined as described in our previous study (Wilson *et al.* 2002). We previously found in canine PASMCs that voltage-gated Ca^{2+} channels did not contribute to store-depletion activated Ca^{2+} entry, since 10 μM nisoldipine had no effect on the rise in $[Ca^{2+}]_i$ caused by store-depletion (Wilson *et al.* 2002). Thus, nisoldipine was omitted in the study of store-depletion on Mn^{2+} quench of fura-2 fluorescence. Figure 3*A* shows the fluorescence intensity recorded at an excitation wavelength of 360 nm in a single PASMC. Removal of extracellular Ca^{2+} did not cause any decline in fluorescence intensity. The addition of 30 μM $MnCl_2$ caused the fluorescence to decline at a rate of 0.036 arbitrary units (a.u.) s^{-1}. Subsequent depletion of the SR Ca^{2+} stores resulted in a 131% increase in the rate of decline of fluorescence to 0.083 a.u. s^{-1}, corresponding to enhanced Mn^{2+} quench indicative of store-depletion activated Ca^{2+} entry (Wilson *et al.* 2002).

The pharmacology of the store-depletion-activated Ca^{2+} entry was further studied by testing the effects of known blockers of store-operated channels on Mn^{2+}

quench of fura-2. Figure 3*B* and *C* shows that Ni^{2+} and SKF 96365 inhibited store-depletion-activated Mn^{2+} quench of fura-2. In the presence of 500 μM Ni^{2+} (Fig. 3*B*), the rate of Mn^{2+} quench was not significantly altered by store depletion (from 0.025 to 0.028 a.u. s^{-1}). Similarly, in the presence of 50 μM SKF 96365 (Fig. 3*C*), the rate of Mn^{2+} quench was not significantly altered by store depletion (from 0.026 to 0.029 a.u. s^{-1}). On the other hand, Fig. 3*D* and *E* shows that La^{3+} and Gd^{3+} did not inhibit the store-depletion-activated Mn^{2+} quench of fura-2. In the presence of 100 μM La^{3+} (Fig. 3*D*), the rate of Mn^{2+} quench was significantly increased by store depletion (from 0.04 to 0.086 a.u. s^{-1}). Similarly, in the presence of 100 μM Gd^{3+} (Fig. 3*E*), the rate of Mn^{2+} quench was also significantly increased by store depletion (from 0.03 to 0.067 a.u. s^{-1}). Figure 3*F* summarizes these results, showing that in the absence of blockers, depletion of SR Ca^{2+} stores caused a mean increase in Mn^{2+} quench of fura-2 of 140 ± 6% ($n = 142$). This increase in Mn^{2+} quench rate caused by store-depletion was significantly reduced by Ni^{2+} to only 15 ± 6% ($n = 26$, $P < 0.01$) and by SKF 96365 to 9 ± 6% ($n = 22$, $P < 0.01$), but was not affected by La^{3+} (131 ± 14%, $n = 16$) or Gd^{3+} (153 ± 12%, $n = 26$).

We next investigated the effect of hypoxia on Mn^{2+} quench of fura-2 fluorescence in PASMCs using a similar experimental approach. Figure 4*A* shows in a single PASMC that removal of extracellular Ca^{2+} alone did not cause any decline in fluorescence. Addition of $MnCl_2$ (in the presence of 10 μM nisoldipine to inhibit dihydropyridine-sensitive Ca^{2+} entry) reduced fluorescence intensity at a rate of 0.033 a.u. s^{-1}. Subsequent exposure to hypoxia resulted in a 97% increase in the rate of decline of fluorescence intensity to 0.065 a.u. s^{-1}, indicating hypoxic activation of a nisoldipine-insensitive Ca^{2+} entry pathway. To determine if CCE contributes to hypoxia-activated Ca^{2+} entry, the pharmacology of hypoxia-activated Ca^{2+} entry was examined by using blockers of store-operated channels. Figure 4*B* and *C* shows that

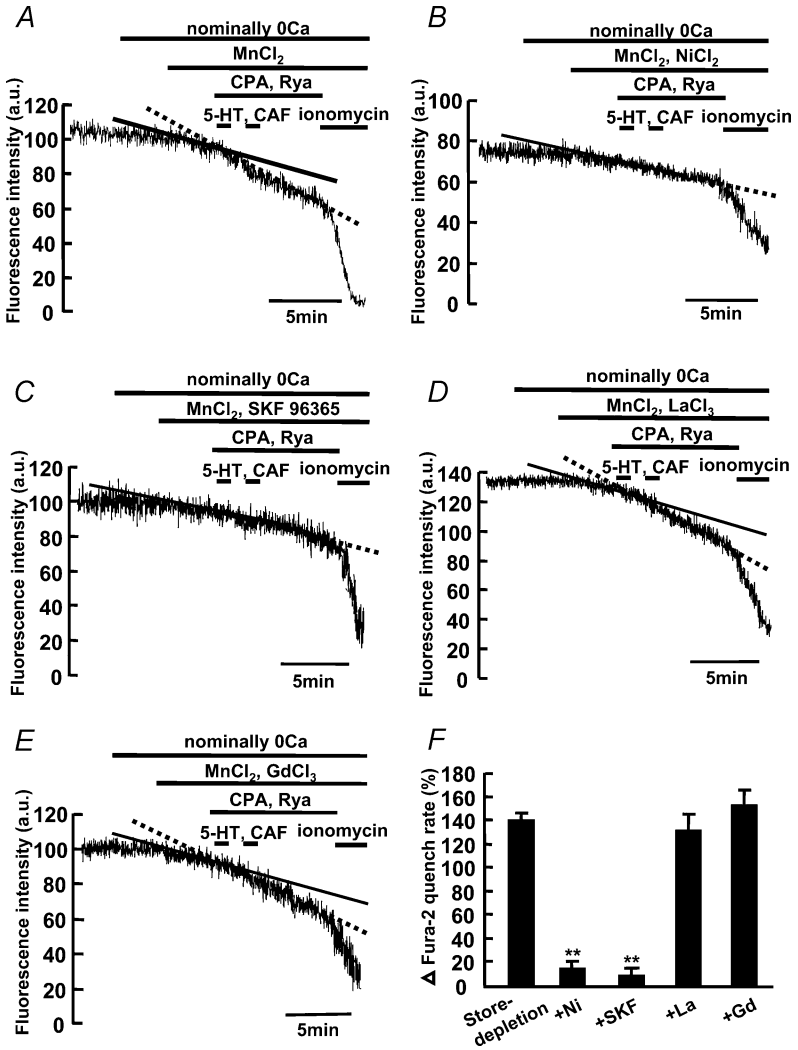

Figure 3. Store-depletion increases the rate of Mn^{2+} quench of fura-2 fluorescence in PASMCs
A, changes in flourescence intensity (arbitrary units, a.u.) were continuously recorded in nominally Ca^{2+}-free solution, followed by addition of 30 μM $MnCl_2$; store-depletion was induced by sustained exposure to cyclopiazonic acid (CPA; 10 μM) and ryanodine (10 μM) and transient exposures to 5-HT (10 μM) and caffeine (10 mM) as indicated. *B–E*, effects of blockers of store-operated channels on store-depletion-activated Mn^{2+} quench of fura-2 fluorescence. The increase in Mn^{2+} quench of fura-2 activated by store-depletion was inhibited by 500 μM Ni^{2+} (*B*) and 50 μM SKF 96365 (*C*), but not blocked by 100 μM La^{3+} (*D*) or 100 μM Gd^{3+} (*E*). *F*, bar graph showing percentage change in fura-2 quench after store-depletion in the absence ($n = 142$) and presence of Ni^{2+} ($n = 26$), SKF 96365 ($n = 22$), La^{3+} ($n = 16$) and Gd^{3+} ($n = 26$). $^{**}P < 0.01$ (ANOVA).

Ni^{2+} and SKF 96365 inhibited hypoxic activated Mn^{2+} quench of fura-2. In the presence of 500 μM Ni^{2+} (Fig. 4*B*), prior to hypoxic challenge, the rate of quench was 0.020 a.u. s^{-1}, and following exposure of the cell to hypoxia, the rate increased by only 15% to 0.023 a.u. s^{-1}. Similarly, in the presence of 50 μM SKF 96365 (Fig. 4*C*), hypoxia increased the rate of quench by only 12%, from 0.025 to 0.028 a.u. s^{-1}. In contrast, Fig. 4*D* and *E* shows that La^{3+} and Gd^{3+} did not inhibit the hypoxia-activated Mn^{2+} quench of fura-2. In the presence of 100 μM La^{3+} (Fig. 4*D*), prior to hypoxic challenge, the rate of quench was 0.026 a.u. s^{-1}, and following exposure of cell to hypoxia, the rate increased by 96% to 0.051 a.u. s^{-1}. Similarly, in the presence of 100 μM Gd^{3+} (Fig. 4*E*), hypoxia increased the rate of quench by 117%, from 0.030 to 0.065 a.u. s^{-1}. Figure 4*F* summarizes these results. In the absence of blockers, hypoxia caused a mean increase in the rate of Mn^{2+} quench of fura-2 of $102 \pm 5\%$ ($n = 157$). The increase in Mn^{2+} quench rate activated by hypoxia was significantly reduced by Ni^{2+} to only $5 \pm 11\%$ ($n = 16$, $P < 0.01$), and by SKF 96365 to $19 \pm 4\%$ ($n = 34$, $P < 0.01$), but not affected by La^{3+} ($95 \pm 11\%$, $n = 25$) or Gd^{2+} ($101 \pm 11\%$, $n = 33$).

If CCE contributes to the hypoxic-induced rise in $[Ca^{2+}]_i$, then depletion of the SR Ca^{2+} stores may be a prerequisite for hypoxia to activate nisoldipine-insensitive Ca^{2+} entry. Thus predepletion of the stores might prevent the hypoxic induced rise in $[Ca^{2+}]_i$. In accordance with this, pre-exposure of cells to hypoxia might also prevent the store-depletion activated rise in $[Ca^{2+}]_i$. To test these possibilities, SR Ca^{2+} stores were maximally depleted by exposure of cells to a cocktail including CPA (10 μM) and ryanodine (10 μM), followed by a 30 s exposures to 5-HT (10 μM) and caffeine (10 mM) in Ca^{2+}-free solutions (Wilson *et al.* 2002). Figure 5*A* shows that following the store depletion, 2 mM Ca^{2+} re-addition caused a 102 ± 9 nM (Fig. 5*C*; $\Delta R = 0.041 \pm 0.005$, $n = 41$, $P < 0.01$) increase in $[Ca^{2+}]_i$ above the basal values. Subsequent exposure of cells to hypoxia caused a small, but not significant increase in the depletion-activated rise

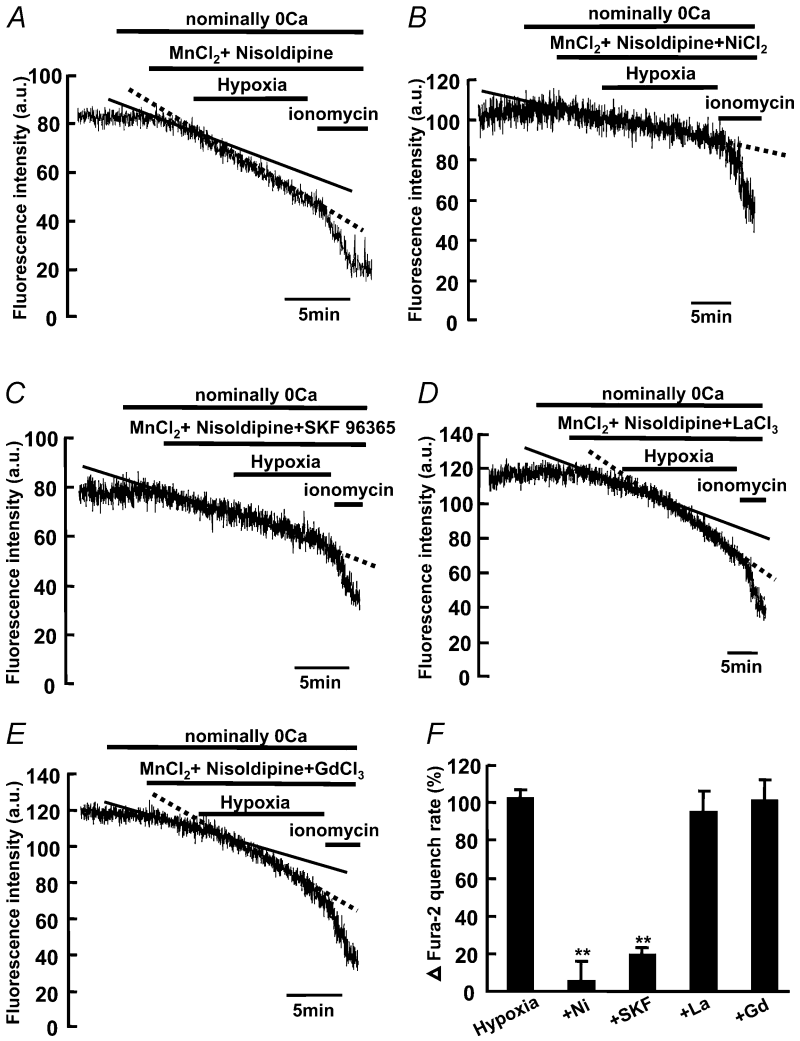

Figure 4. Hypoxia increases the rate of Mn^{2+} quench of fura-2 fluorescence in PASMCs
A, changes in flourescence intensity were continuously recorded in nominally Ca^{2+}-free solution, followed by addition of 30 μM $MnCl_2$ and 10 μM nisoldipine, in normoxic and hypoxic solutions as indicated. *B–E*, effects of blockers of store-operated channel on hypoxia-activated Mn^{2+} quench of fura-2. The increase in Mn^{2+}-quench of fura-2 induced by hypoxia was inhibited by 500 μM Ni^{2+} (*B*) and 50 μM SKF 96365 (*C*), but not inhibited by 100 μM La^{3+} (*D*) or 100 μM Gd^{3+} (*E*). *F*, bar graph showing percentage change in fura-2 quench after exposure of cells to hypoxic solution, in the absence ($n = 157$) and presence of Ni^{2+} ($n = 16$), SKF 96365 ($n = 34$), La^{3+} ($n = 25$) and Gd^{3+} ($n = 33$). $^{**}P < 0.01$ (ANOVA).

in $[Ca^{2+}]_i$ (Fig. 5*C*, $n = 41$). Similarly, pre-exposure of cells with hypoxia prevented the store-depletion-activated rise in $[Ca^{2+}]_i$. Figure 5*B* illustrates that hypoxia caused a 175 ± 14 nM increase in $[Ca^{2+}]_i$ in the absence of nisoldipine (Fig. 5*C*; $\Delta R = 0.059 \pm 0.004$, $n = 48$, $P < 0.01$). Subsequent depletion of the intracellular stores did not enhance the sustained rise in $[Ca^{2+}]_i$ induced by hypoxia (Fig. 5*C*; $n = 48$). Thus, predepletion of the stores prevented hypoxic induced rise in $[Ca^{2+}]_i$, and hypoxia did not affect the rise in $[Ca^{2+}]_i$ induced by store-depletion (Fig. 5*A*). On the other hand, pre-exposure of cells with hypoxia prevented the store-depletion activated rise in $[Ca^{2+}]_i$, and store-depletion did not affect the rise in $[Ca^{2+}]_i$ induced by hypoxia (Fig. 5*B*). It is noteworthy that the increase in $[Ca^{2+}]_i$ induced by hypoxia was significantly higher than that induced by store-depletion (Fig. 5*C*; $P < 0.01$). This may be due to an additional component of Ca^{2+} influx through voltage-gated Ca^{2+} channels in the absence of nisoldipine.

Discussion

This is the first direct evidence that hypoxia causes a rise in $[Ca^{2+}]_i$ through activation of CCE in PASMCs. We previously found in canine PASMCs that simultaneous depletion of the functionally separate inositol 1,4,5-trisphosphate (IP_3)-sensitive and ryanodine-sensitive stores activates CCE, which causes a rise in $[Ca^{2+}]_i$ of ~50–100 nM (Wilson *et al.* 2002). This rise in $[Ca^{2+}]_i$ was insensitive to nisoldipine, and inhibited by 500 μM Ni^{2+} but not by 100 μM Gd^{3+}. Experiments in intact vessels revealed a potentiated contractile response to hypoxia in the presence of CPA or thapsigargin (Jabr *et al.* 1997). This response was partially sensitive to nisoldipine, but abolished in the absence of extracellular Ca^{2+}, suggesting a recruitment of a novel Ca^{2+} entry pathway. This pathway was similar to CCE described in rat PASMCs (Ng and Gurney, 2001), since it was also blocked by 50 μM SKF 96365. In the present study,

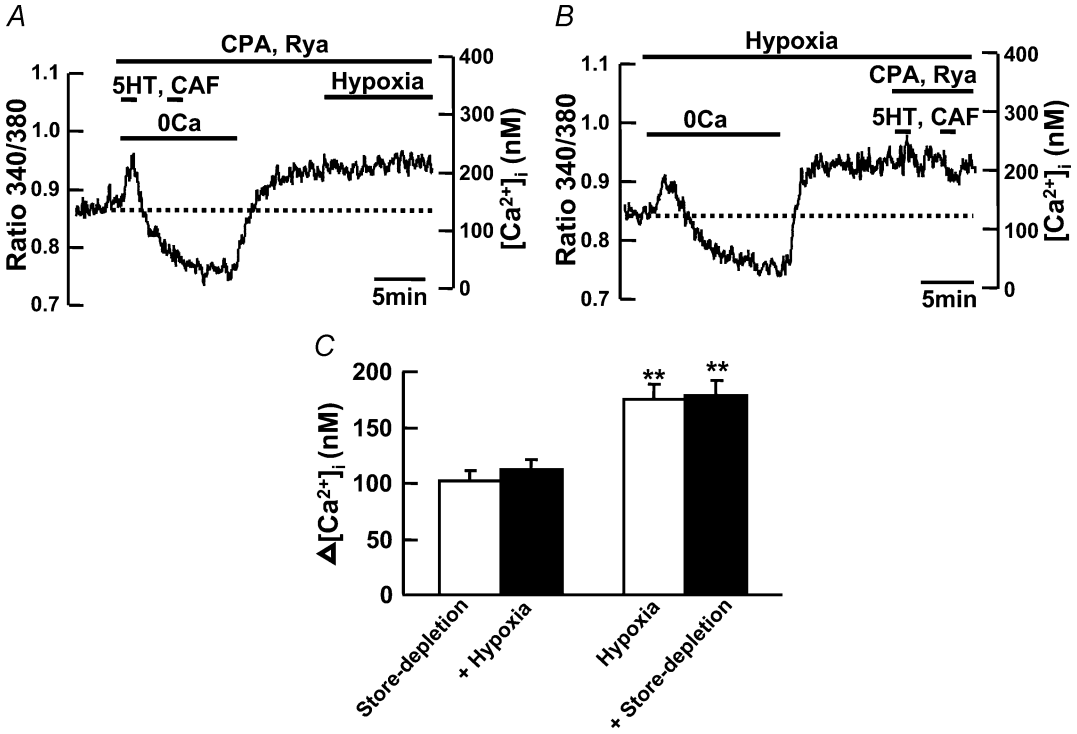

Figure 5. Predepletion of Ca^{2+} stores inhibits hypoxic-induced rise in $[Ca^{2+}]_i$ in PASMCs
A, in normoxic solutions, depletion of intracellular Ca^{2+} stores (as in Fig. 3) transiently increases the fura-2 fluorescence ratio in Ca^{2+}-free solution. Readmission of 2 mM Ca^{2+} caused a rise in fluorescence ratio above the basal level (dotted line). Subsequent exposure of cells to hypoxic solution did not further increase the ratio. *B*, a transient increase in the fluorescence ratio was observed in cells exposed to hypoxic Ca^{2+}-free solution. Readmission of 2 mM Ca^{2+} in hypoxia caused a rise in fluorescence ratio above the basal level. Subsequent depletion of the sarcoplasmic reticulum (SR) Ca^{2+} stores did not further increase the ratio. *C*, bar graph showing mean changes in $[Ca^{2+}]_i$ after addition of 2 mM Ca^{2+} in cells subjected to store-depletion (left, open bar, $n = 41$) or hypoxia (right, open bar, $n = 48$) alone, and cells exposed to hypoxia with the stores predepleted (left, filled bar, $n = 41$) or subjected to store depletion with pre-exposure of cells to hypoxia (right, filled bar, $n = 48$). $^{**}P < 0.01$ (ANOVA).

hypoxia caused intracellular Ca^{2+} release as indicated by a transient rise in $[Ca^{2+}]_i$ detected by fura-2 in Ca^{2+}-free solution. Subsequent addition of 2 mM Ca^{2+} elicited a nisoldipine-sensitive and nisoldipine-insensitive rise in $[Ca^{2+}]_i$. Interestingly, the pharmacological properties of the nisoldipine-insensitive rise in $[Ca^{2+}]_i$ (~50 nM) are similar to CCE. More importantly, hypoxia accelerated Mn^{2+} quench of fura-2 fluorescence at a rate similar to that found for store-depletion activated Mn^{2+} entry. Furthermore, the increase in fura-2 quench rates induced by store-depletion and hypoxia were both inhibited by SKF 96365 and Ni^{2+}, but not affected by Gd^{3+} and La^{3+}, confirming that hypoxia stimulates Ca^{2+} entry through a pathway similar to CCE. These data provide the first direct evidence in freshly isolated PASMCs that acute hypoxia, by releasing Ca^{2+} from the intracellular stores, activates a Ca^{2+} entry pathway resembling CCE.

In the present study, a rapid fall (~100 nM) in cytosolic Ca^{2+} concentration was observed in response to removal of extracellular Ca^{2+}. This is consistent with our previous published data in canine PASMCs (Wilson *et al.* 2002). In most of the cells studied in Ca^{2+}-free solution, $[Ca^{2+}]_i$ was well maintained at concentration between 20 and 50 nM, and not approaching zero. This observation is not uncommon but in fact consistent with many studies employing similar protocol, where removal of extracellular Ca^{2+} resulted in a rapid fall in cytosolic Ca^{2+} (e.g. Doi *et al.* 2000; Morales *et al.* 2004). None of these papers explain why removing extracellular Ca^{2+} causes a rapid fall in $[Ca^{2+}]_i$. However, the nonlinearity of the relationship between fluorescence and Ca^{2+} binding would predict this phenomenon. It is unlikely that the rapid fall in $[Ca^{2+}]_i$ seen in the present study is related to cell viability because re-addition of Ca^{2+} resulted in a rapid rise in $[Ca^{2+}]_i$ to the basal level in the control experiment (Fig. 1*B*), or above the basal level during hypoxic challenge (Figs 1*A*, and 2*A* and *B*), suggestive of good responsiveness of cells to external Ca^{2+}.

The possibility that hypoxia causes membrane depolarization leading to Ca^{2+} influx through the activation of L-type Ca^{2+} channels has long been considered a component of HPV in many pulmonary preparations (Madden *et al.* 1984; Harder *et al.* 1985; Archer *et al.* 1985; Woodmansey *et al.* 1995; Cornfield *et al.* 1994; Jabr *et al.* 1997; Robertson *et al.* 2000). However, the mechanism(s) underlying these events remains unclear. Our previous electrophysiological study in canine PASMCs showing that hypoxia inhibited K^+ currents (Post *et al.* 1992), together with the present study that hypoxia causes intracellular Ca^{2+} release and activates a nisoldipine-sensitive rise in $[Ca^{2+}]_i$, is consistent with the possibility that hypoxic release of Ca^{2+} from intracellular stores may inhibit Kv channels, leading to membrane depolarization and subsequent activation of L-type Ca^{2+} channels (Post *et al.* 1995). It is also possible that the Ca^{2+} release from the intracellular stores may activate Ca^{2+}-dependent Cl^- channels, leading to membrane depolarization and hence activation of L-type Ca^{2+} channels (Clapp *et al.* 1996; Ng and Gurney, 2001), or that hypoxia may directly activate L-type Ca^{2+} channels (Franco-Obregón and López-Barneo, 1996).

In various vascular smooth muscle preparations, it has been reported that L-type Ca^{2+} channel blockers may inhibit Ca^{2+} release from intracellular Ca^{2+} stores at concentrations well above those use to block membrane L-type Ca^{2+} channels (Saida and van Breemen, 1983; Kanaide *et al.* 1988; Kalsner, 1997). However, the inhibitory effects on Ca^{2+} release vary among different blockers and vascular preparations. In rabbit mesenteric artery, diltiazem inhibited noradrenaline-induced contractions in Ca^{2+}-free medium at concentrations between 1 and 100 μM, but nisoldipine only partially inhibited the contractions at the same concentrations (Saida and van Breemen, 1983). In cultured rat aorta smooth muscle cells, verapamil and diltiazem partially inhibited noradrenaline-induced Ca^{2+} release at concentrations up to 100 μM (Kanaide *et al.*, 1988). In contrast, verapamil (10 μM) failed to inihibit Ca^{2+} release or contraction induced by noradrenaline in Ca^{2+}-free medium in rat aortas (Hagiwara *et al.* 1993). In the present study, 10 μM nisoldipine was used to inhibit voltage-gated Ca^{2+} entry activated by hypoxia to study CCE. Although we did not directly test if nisoldipine also inhibited Ca^{2+} release, in our experiments nisoldipine may have little or no effect on hypoxia-induced Ca^{2+} release because it was applied after re-addition of external Ca^{2+}, where the stores would have been depleted by hypoxia in Ca^{2+}-free solution (Fig. 2). Furthermore, in cells pretreated with nisoldipine, hypoxia increased the rate of Mn^{2+} quench of fura-2 fluorescence (Fig. 4). If nisoldipine prevented hypoxia from causing the SR Ca^{2+} release, the increase in the Mn^{2+} quench of fura-2 induced by hypoxia may not be observed because activation of CCE required depletion of the intracellular Ca^{2+} stores.

In intact arteries, agonist preconstriction or pretone is usually a prerequisite to demonstrate hypoxic pulmonary constriction (e.g. Rodman *et al.* 1989; Ogata *et al.* 1992; Demiryurek *et al.* 1993; Hoshino *et al.* 1994; Vandier *et al.* 1997; Robertson *et al.* 2000; Liu *et al.* 2001). On the other hand, hypoxia was found to induce pulmonary artery contraction and a rise in $[Ca^{2+}]_i$ in the absence of pretone (Dipp *et al.* 2001; Kang *et al.* 2002). The nature of this priming event is unresolved. It may involve increased myofilament Ca^{2+} sensitivity, partial depolarization of resting membrane potential or Ca^{2+} release (see Ward & Aaronson, 1999; Sylvester, 2001 for reviews). How the pretone helps to potentiate the effect of hypoxia is unclear, but the fact that it is needed in some preparations implies that there may be synergy between the pathways activated by agonists and hypoxia (Gurney,

2002). In canine pulmonary artery, hypoxia elicited a significant amount of contraction in vessels preconstricted with phenylephrine (Jabr *et al.* 1997). Surprisingly, pretreatment with agonist is not necessary for hypoxia to cause an increase in $[Ca^{2+}]_i$ in isolated PASMCs. This could be explained by the experimental protocol used in the present study in which cells were exposed to hypoxia in Ca^{2+}-free solution followed by readmission of external Ca^{2+}. When external Ca^{2+} was removed, a slow release of Ca^{2+} from the SR may occur (Morales *et al.* 2004), but this was not sufficient to activate CCE in canine PASMCs because no significant rise in $[Ca^{2+}]_i$ above basal level was observed after readmission of 2 mM Ca^{2+} in the control condition. In addition, removal of external Ca^{2+} may cause a partial depolarization due to increased Na^+ entry (Gonzalez-Martinez, 2003). Such depolarization, however, was not sufficient to activate L-type Ca^{2+} channels in canine PASMCs because we have found that nisoldipine had no effect on the Ca^{2+} entry upon re-addition of Ca^{2+} in unstimulated cells (Wilson *et al.* 2002). Nevertheless, the small amounts of Ca^{2+} release from the stores and the partial depolarization of the resting membrane potential may serve as a priming condition necessary for hypoxia to cause a significant rise in $[Ca^{2+}]_i$. Therefore, perhaps it is not the pretone *per se* that is important, but the activation of particular cellular pathways that acts in synergy with the mechanisms activated in smooth muscle by hypoxia (Gurney, 2002).

We previously demonstrated that ryanodine pretreatment with brief caffeine exposures attenuated the hypoxia-induced contraction in canine pulmonary arteries, suggesting that Ca^{2+} release from the ryanodine-sensitive stores plays an important early role in HPV (Jabr *et al.* 1997). While these results have been confirmed by other laboratories (Robertson *et al.* 2000; Dipp *et al.* 2001), it is unlikely that hypoxia depletes only the ryanodine-sensitive stores in the present study because hypoxia was found to activate CCE, and the activation of CCE required simultaneous depletion of IP_3-sensitive and ryanodine-sensitive stores in canine PASMCs (Wilson *et al.* 2002). We have previously found that depletion of IP_3-sensitive stores alone by CPA and angiotensin II failed to activate CCE in canine PASMCs (Wilson *et al.* 2002), but an enhanced hypoxia contraction was observed in pulmonary arteries pretreated with CPA or thapsigargin (Jabr *et al.* 1997). Therefore, it is possible that apart from causing Ca^{2+} release from the ryanodine-sensitive stores, hypoxia may also inhibit Ca^{2+}-ATPase pump of the SR, thereby depleting the IP_3-sensitive stores by preventing Ca^{2+} uptake (Evans and Dipp, 2002). Although we have no evidence to support this, our findings in canine PASMCs that IP_3-sensitive and ryanodine-sensitive stores are two separate entities in the SR (Janiak *et al.* 2001), and that simultaneous depletion of these stores attenuates the hypoxia-induced rise in $[Ca^{2+}]_i$, suggests that Ca^{2+} release from both stores may be an early event in the cascade activated by hypoxia, which leads to CCE activation.

In conclusion, hypoxia activates CCE involving a store depletion-induced mechanism, which contributes to the development of HPV. CCE has been shown to associate with cell proliferation, being greater in proliferating bronchial smooth muscle cells (Sweeney *et al.* 2002) and human PASMCs (Golovina, 1999; Golovina *et al.* 2001) compared to those that are growth-arrested. Furthermore, hypoxic-induced increases in AP-1 binding activity enhances CCE in human pulmonary artery endothelial cells, which could be implicated in stimulating PASMC proliferation, leading to the pulmonary vascular remodelling in patients with hypoxia-mediated pulmonary hypertension (Fantozzi *et al.* 2003). Although the mechanism(s) linking hypoxia to CCE activation remain to be elucidated, our finding that hypoxia activates CCE in PASMCs suggests that the molecular signal that links store-depletion to the activation of CCE may play an important role in hypoxia, which may be a useful target for the development of new vasodilators to treat pulmonary hypertension.

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