

# A Role for Receptor-Operated $\text{Ca}^{2+}$ Entry in Human Pulmonary Artery Smooth Muscle Cells in Response to Hypoxia

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## Summary

Hypoxic pulmonary vasoconstriction (HPV) is an important homeostatic mechanism in which increases of  $[\text{Ca}^{2+}]_i$  are primary events. In this study, primary cultured, human pulmonary artery smooth muscle cells (hPASMC) were used to examine the role of TRPC channels in mediating  $[\text{Ca}^{2+}]_i$  elevations during hypoxia. Hypoxia ( $\text{PO}_2$  about 20 mm Hg) evoked a transient  $[\text{Ca}^{2+}]_i$  elevation that was reduced by removal of extracellular calcium. Nifedipine and verapamil, blockers of voltage-gated calcium channels (VGCCs), attenuated the hypoxia-induced  $[\text{Ca}^{2+}]_i$  elevation by about 30 %, suggesting the presence of alternate  $\text{Ca}^{2+}$  entry pathways. Expression of TRPC1 and TRPC6 in hPASMC were found by RT-PCR and confirmed by Western blot analysis. Antagonists for TRPC, 2APB and SKF96365, significantly reduced hypoxia-induced  $[\text{Ca}^{2+}]_i$  elevation by almost 60 %. Both TRPC6 and TRPC1 were knocked down by siRNA, the loss of TRPC6 decreased hypoxic response down to 21 % of control, whereas the knockdown of TRPC1 reduced the hypoxia response to 85 %, suggesting that TRPC6 might play a central role in mediating hypoxia response in hPASMC. However, blockade of PLC pathway caused only small inhibition of the hypoxia response. In contrast, AICAR, the agonist of AMP-activated kinase (AMPK), induced a gradual  $[\text{Ca}^{2+}]_i$  elevation, whereas compound C, an antagonist of AMPK, almost abolished the hypoxia response. However, co-immunoprecipitation revealed that AMPK $\alpha$  was not colocalized with TRPC6. Our data supports a role for TRPC6 in mediation of the  $[\text{Ca}^{2+}]_i$  elevation in response to hypoxia in hPASMC and suggests that this response may be linked to cellular energy status *via* an activation of AMPK.

## Key words

Hypoxic pulmonary vasoconstriction (HPV) • hPASMC • Hypoxia • Transient receptor potential channel (TRPC) • AMP-activated kinase (AMPK)

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## Introduction

Hypoxic pulmonary vasoconstriction (HPV) is the rapid, reversible increase in pulmonary vascular resistance that occurs when the alveolar oxygen tension falls below a threshold level (Fishman 1976, Ward and Aaronson 1999). This shift in blood flow from poorly ventilated to better ventilated areas improves the matching of ventilation and perfusion which minimize arterial hypoxemia (Grover *et al.* 1983). Although the mechanism responsible for HPV has still not been fully elucidated, an increase of  $[\text{Ca}^{2+}]_i$  is a central event in the contraction of pulmonary artery smooth muscle cells (PASMC). The general concept of HPV is that inhibition of  $\text{O}_2$ -sensitive  $\text{K}^+$  channels depolarizes the PASMC membrane potential ( $E_M$ ), and induces  $\text{Ca}^{2+}$  influx through L-type voltage-gated  $\text{Ca}^{2+}$  channels (VGCC) which is sufficiently causing vasoconstriction (Sweeney and Yuan 2000, Archer and Michelakis 2002, Gurney 2002, Waypa and Schumacker 2002, Lopez-Barneo *et al.* 2004, Michelakis *et al.* 2004).

PASMC display a stable, negative membrane potential, which is normally maintained between  $-50$  and  $-60$  mV without spontaneous generation of action potentials (McCulloch *et al.* 1999, Sweeney and Yuan, 2000). However, hypoxia depolarizes PASMC by an

average of only 10-20 mV (Osipenko *et al.* 1997) and by itself this may not be sufficient to activate  $\text{Ca}^{2+}$  influx through VGCC in PASMCM, as the activation threshold for VGCC is about -30 mV in these cells (Clapp and Gurney 1991). In addition, HPV can be achieved without affecting membrane potential as 8-bromo-cADPR blocks HPV induced by alveolar hypoxia in rat lung *in situ* and this inhibition is thus independent of initiation of voltage-gated  $\text{Ca}^{2+}$  influx (Dipp and Evans 2001, Dipp *et al.* 2001). All evidences thus suggested that VGCCs are not the sole contributor to the elevation of  $[\text{Ca}^{2+}]_i$  during hypoxia and there is a significant contribution of non-voltage-gated cation channels.

Other possible candidates for  $\text{Ca}^{2+}$  entry include the transient receptor potential (TRP) channels, which are non-selective cation channels with a  $\text{Na}^+ : \text{Ca}^{2+}$  selectivity (Henderson *et al.* 2000, Clapham 2003). Upon activation, cation entry *via* TRP would cause membrane depolarization that could be of sufficient magnitude to induce additional  $\text{Ca}^{2+}$  entry *via* VGCC. Previous studies have shown that the hypoxia induced  $\text{Ca}^{2+}$  entry in rat distal PASMCM were mediated by SOC (Wang *et al.* 2005, Weigand *et al.* 2005). In addition to TRPC1 and TRPC4, TRPC6 has also been identified in rat PASMCM, but relatively few studies have addressed the role of ROC (receptor-operated calcium entry) in mediating HPV (Beech 2005, Weissmann *et al.* 2006), as these channels, if activated, could also contribute to membrane depolarization during hypoxia (Rosker *et al.* 2004).

In this study, we aimed to determine the effect of hypoxia upon calcium entry pathways, especially TRPC channel, and its relevant activation and regulation pathways in hPASMCM. Our data suggests a key role for  $\text{Ca}^{2+}$  entry *via* TRPC6 in the mediation of HPV that may be linked to the activation of AMPK.

## Methods and Materials

### Cell culture

Second passaged primary cultured human pulmonary artery smooth muscle cells (hPASMCM: total 4 batches, the population doubling time 46 h, 42 h, 65 h and 38 h, respectively) (Promocell catalogue no. c-12521 shown positive to smooth muscle specific  $\alpha$ -actin) were cultured in smooth muscle growing medium (Promocell Ltd) under 5 %  $\text{CO}_2$  at 37 °C. Cells were used within 5 passages. When cells in the culture flasks reached 70 % confluence, they were seeded at medium density onto coverslips. Experiments were performed until cells

reached 70 % confluence in a coverslip.

PC12 cells (gift from Prof. A. Logan at University of Birmingham and Prof. J. St John at Warwick University) were cultured in DMEM medium containing 7.5 % FCS and 7.5 % horse serum at 5 %  $\text{CO}_2$  in a 37 °C incubator (Del Toro *et al.* 2003). The cells were passaged every five days. Sterilized coverslips were coated with poly-L-lysine (Sigma) according to the manufacturer's protocol. The cells were then loaded onto the poly-L-lysine coated coverslip at a high density.

### RNAi

Stealth siRNA was obtained from Invitrogen. hPASMCM cells were passaged onto coverslips in 500  $\mu\text{l}$  Opti-MEM (Invitrogen) one day before transfection and reached about 40-50 % confluence at the time of transfection. 20 pmol siRNA, against TRPC6 (Invitrogen catalogue no. HSS110994) or TRPC1 (Invitrogen catalogue no. HSS110980) with a 1:125 final dilution of lipofectamine 2000 (Invitrogen catalogue no. 11668) was used according to the manufacturer's instructions. Transfection rate was calculated by eGFP transfection and it was over 60 %. The knockdown effects were examined at 48 h and the results were compared with duplex control, i.e. control without knockdown and control with another relative TRPC knockdown. The knockdown experiments were triplicates and knockdown effects on HPV response were averaged from three different batches of hPASMCM.

### RT-PCR and Western blots

RT-PCR experiments followed standard protocols. Primers against human TRPC channels were used as previously described. The program used: 94 °C 2 min followed by 30 cycles of 94 °C for 0.5 min, 54 °C for 1 min, 72 °C for 1 min and finished with a single 10 min cycle at 72 °C for extension.

Cultured hPASMCM cells were homogenized with CellLytic M lysis/extraction reagent (Sigma c2978) containing Protease Inhibitor cocktail (Sigma P8340). Protein concentrations were determined by the Bradford Assay method. Equal amounts of total protein were loaded into the SDS-PAGE gel wells.  $\beta$ -actin was used as the inner marker to calibrate the protein quantity. Samples were prepared with 5x loading buffer and separated on 10 % SDS-PAGE gels and then transferred to PVDF membranes, which were rinsed with Tris-buffered saline Tween-20 (TBS-T) and then blocked in TBS-T containing 5 % milk for one hour (at room

temperature). The membrane was incubated with the first antibody, TRPC1 antibody (1:500, Santa Cruz Biotechnology CA, sc 15055) or TRPC6 antibody (1:500, Santa Cruz Biotechnology CA, sc19196) overnight at 4 °C, followed by washes in TBS-T and incubation with the secondary antibody (1:10000, HRP, BioRad) for one hour at room temperature. Membranes were washed with TBS-T and blots were detected by Pico chemiluminescent substrate (Pierce 34080). A negative control was performed without incubation of either primary antibody or secondary antibody.

Co-immunoprecipitation was performed using PriFound™ Mammalian Co-Immunoprecipitation Kit (Pierce 23605) according to the manufacturer's instruction. In brief, the antibody coupling gel was loaded into a Handee Spin Cup column. Thereafter, the baiting antibody (TRPC6) about 50 µg diluted in coupling buffer was added to the gel. Samples were added into column and incubated overnight with gentle end-over-end mixing. Two control samples were prepared: 1) control gel without TRPC6 antibody. 2) gel with a non-related antibody (cPLA, Santa Cruz Biotechnology CA, sc1724), which is expressed in hPASM (Ichinose *et al.* 2002). The column was then washed with Co-IP buffer and all elution were collected separately followed by SDS-PAGE. The optimal number of washes was determined when there is no protein in the final wash fraction. After adding elution buffer to the gel, the final samples were collected separately by centrifuge. All the samples including controls were separated by SDS-PAGE and detected by Western blot using anti-AMPK antibody. The elution was also checked by Western blot using anti-TRPC6 antibody.

#### Calcium imaging

The growth medium was removed and cells were rinsed once in Earl's Balanced Salt Solution (EBSS). 50 µg Fura-2 AM (F1221, Molecular Probe, Ltd.) was dissolved in 20 µl 20 % pluronic acid (0.01 g in 50 µl DMSO) as the stock concentration. Prior to the experiment, a mixture of 1 µl stock dye in 200 µl EBSS was loaded onto the cells and incubated for at least 30 min. Prior to placing the coverslips into the recording chamber, cells were rinsed in normal Tyrode's medium to remove residual dye. Data acquisition and analysis were performed by Wasabi programming (Hamamatsu, Ltd.). A CCD camera (C9102, Hamamatsu, Ltd.) was used to capture the fluorescent image. Images were collected in a format of 1344 x 1024 pixels. Fluorescent changes in

Fura-2 were measured with double wavelength excitation at 340 and 380 nm, and emission at 510 nm. Absolute  $Ca^{2+}$  was calibrated by using Fura-2 calcium imaging calibration kit (Invitrogen F6774). Changes in  $Ca^{2+}$  concentration in the region of interest were calculated according to a ratio of 340/380. Time lapse recording initially captured the images at 1 s intervals. In order to minimize cell photobleaching in the long experimental protocol, 2 or 3 s intervals were applied in the experiments. Most data presented in the figures were acquired at 3 s intervals.

*Post-hoc* analysis was carried out using Wasabi programming. Individual cells or groups of cells were outlined and analyzed. Data from one coverslip were averaged and presented in the figures. The same experiments were repeated on different coverslips, the total number of cells and batches were presented using *n* and *N*, respectively. Data are presented as means ± S.E.M. Statistical differences were compared using a Student's paired *t* test, taking  $P < 0.05$  as significant value. The traces in most figures represented the average results from one coverslip over 15 cells.

#### Solution and chemicals

Modified Tyrode's solution for  $Ca^{2+}$  imaging recording contained (mM): NaCl 117, KCl 4.46,  $MgCl_2$  1.5,  $CaCl_2$  1.8,  $NaHCO_3$  23, D-glucose 11, HEPES 10, pH 7.2. The hypoxia medium was made of modified Tyrode's buffer bubbled with  $N_2$ . The  $O_2$  level in the recording chamber was measured by using an OCM-2 amplifier (Cameron Instrument Company) and a mini Clark-style  $O_2$  electrode (Diamond General Development Corp). EGTA solution for  $Ca^{2+}$  imaging recording contained (mM): NaCl 117, KCl 4.46,  $NaHCO_3$  23, D-glucose 11, HEPES 10, and EGTA 0.2. High  $K^+$  bath solution contained (mM): NaCl 66.3, KCl 45.3,  $MgCl_2$  1,  $CaCl_2$  1.8,  $NaHCO_3$  23, D-glucose 11, and HEPES 10. All solutions were made on the day of experiments.

The following chemicals were used: verapamil, 2APB, nifedipine, SKF96365, U73122 and D609 (all Sigma), KBR7943 (Torcis) AICA (5-aminoimidazole-4-carboxamide-1-β-ribose), compound C (Calbiochem). Most chemicals, which dissolved in ethanol or DMSO were made up as 1000 times stock. All chemical solutions were made as required on the day of experiments. The solvents, ethanol and DMSO at the same vehicle concentration, were tested alone in controls and had no effect.

### Perfusion system for exchange of the gas bubbled media

The media were bubbled in para-film sealed bottles. Each bottle was connected to two TYGON tubes; a 1.6 mm ID tube carried the medium out of the bottle, and a 0.8 mm ID tube returned the medium to the bottle. The bubbled medium circulated in the system constructed of the medium bottle and two tubes, and was driven by a peristaltic pump (Gilson minipuls 3 with four pumping heads). A tri-connector (Y shape) was placed very close to the electric valve and the recording chamber. This Y connector connected the delivery and return tubes, and another tube to the recording chamber (chamber tube). There is an electrical valve in control of fluid flow through chamber tube (Automation 8). The media flowed into recording chamber (RG 26, Warner) if the valve was open; otherwise they continually circulated to reduce dead space. The flow rate was 5 ml/min. After the coverslip was loaded into the recording chamber, a glass cover-slide (20 x 60 mm) was placed on top of the recording chamber to reduce surface gas exchange. An oxygen electrode was placed into the recording chamber to detect the O<sub>2</sub> level in the medium. The O<sub>2</sub> level of the hypoxia medium in the recording chamber was about 20 mm Hg.

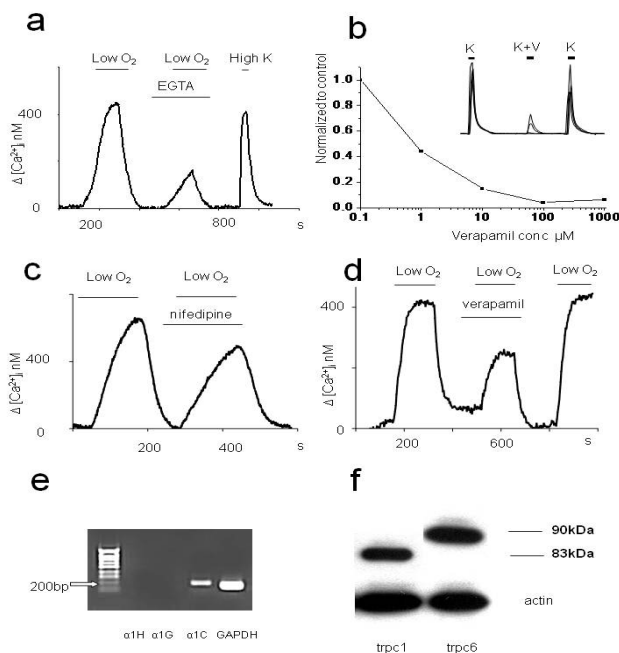
## Results

### Source of Ca<sup>2+</sup> elevation induced by hypoxia

Four minutes of exposure to hypoxia induced a significant and reversible Ca<sup>2+</sup> elevation (Fig. 1a) in these cells. A part (68.8±4.4 %) (n=72, N=3, P<0.05, Fig. 1a) of this elevation was reduced by removal of extracellular Ca<sup>2+</sup> (+EGTA). In a control, when hPAMSC were exposed to high K<sup>+</sup> solution (40 mM) for 30 s, a transient Ca<sup>2+</sup> elevation was evoked, as predicted (Fig. 1a). This suggests that hypoxia-induced [Ca<sup>2+</sup>]<sub>i</sub> elevations were primarily, although not solely, due to Ca<sup>2+</sup> entry *via* the plasma membrane.

### Role of VGCCs in hypoxia

As positive controls, effects of common antagonists of VGCCs were examined in PC12 cells, because of predominant expression of VGCCs in PC12 cells. High concentration K<sup>+</sup> solution (40 mM) induced a significant [Ca<sup>2+</sup>]<sub>i</sub> elevation in PC12 cells, due to the opening of L-type VGCCs (Green *et al.* 2002). Verapamil dose-dependently inhibited high K<sup>+</sup>-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation in PC12 cells (Fig. 1b). The inlet in Figure 1b shows the inhibition of high K-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation

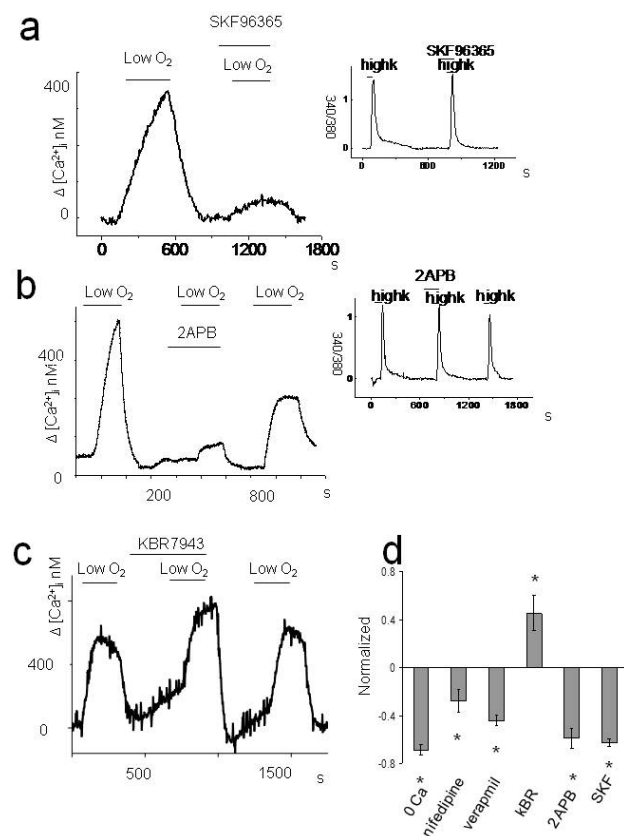


**Fig. 1.** Contribution of VGCCs to hypoxia-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation in hPAMSC. **a.** Four minutes of exposure to hypoxia (pO<sub>2</sub> 20 mm Hg) caused a significant and reversible [Ca<sup>2+</sup>]<sub>i</sub> elevation in hPAMSC. Hypoxia-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation was reduced to 31.2±4.4 % after removal of extracellular Ca<sup>2+</sup> (+EGTA). **b.** Verapamil inhibited high K<sup>+</sup> induced [Ca<sup>2+</sup>]<sub>i</sub> elevation in a dose-dependent manner in O<sub>2</sub> sensitive cells (PC12). Insets (PC12 cells) showed the inhibition effects of verapamil at four different dose on high K<sup>+</sup>-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation (1 μM, 10 μM, 100 μM and 1000 μM). Nifedipine (20 μM) (**c**) and verapamil (40 μM) (**d**) inhibited hypoxia-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation in hPAMSC by 27.6±9.3 % and 33.9±4.3 % respectively. **(e)** RT-PCR results show that only the α1C subunit of VGCCs was found in hPAMSC but not α1H and α1G. **(f)** The presence of TRPC1 and 6 proteins in hPAMSC was shown by Western blot.

by verapamil. The inhibitory effects of verapamil were fully reversed when verapamil was washed off. 40 μM of verapamil and 20 μM of nifedipine were thereafter chosen for further experiments in hPAMSC, respectively. Nifedipine (20 μM) and verapamil (40 μM) inhibited the hypoxia-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation by 27.6±9.3 % (n=145, N=4, P<0.05, Fig. 1c) and 33.9±4.3 % (n=137, N=4, P<0.05, Fig. 1d) respectively in hPAMSC, but a considerable (>60 %) part of response was retained, implicating the activity of other Ca<sup>2+</sup> channels during acute hypoxic response. mRNA of the α1C subunit of VGCCs, which specifically encodes L-type VGCCs, was detected by RT-PCR from primary cultured hPAMSC, whereas mRNA of α1G, and α1H subunits, encoding T-type VGCCs, was not detected (Fig. 1e).

### Role of other Ca<sup>2+</sup> entry pathways in hypoxia

The expression of Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (NCX), TRPC1 and TRPC6 but not TRPC3, TRPC4, TRPC5 and



**Fig. 2.** Contributions of non-VGCC  $\text{Ca}^{2+}$  entry pathways to hypoxia-induced  $[\text{Ca}^{2+}]_i$  elevation in hPASC. **a.** **b.** SKF96365, 2APB, antagonists of TRPC receptors, inhibited the hypoxia-induced  $[\text{Ca}^{2+}]_i$  elevation in hPASC. The effects on VGCCs of these TRPC antagonists observed in PC12 cells are shown on upright corner of the figure. **c.** Example averaged trace showed the effect of KBR7943 on hypoxia response in hPASC. **d.** Bar figure shows effects of antagonists of  $\text{Ca}^{2+}$  channels on hypoxic response in hPASC. All the data were normalized to the control. 0 represented the control level. KBR7943 enhanced hypoxia-induced  $\text{Ca}^{2+}$  elevation to  $45.2 \pm 14.7\%$ . 2APB and SKF96365 reduced hypoxia-induced  $[\text{Ca}^{2+}]_i$  elevation by  $58.5 \pm 8.2\%$  and  $62.5 \pm 3.3\%$ , respectively.

TRPC7 were detected by RT-PCR in hPASC (data not shown) and the presence of TRPC1 and TRPC6 proteins were further confirmed by Western blot (Fig. 1f). 2APB (50  $\mu\text{M}$ ) and SKF 96365 (20  $\mu\text{M}$ ) were applied as antagonists of TRPC channels and both chemicals significantly reduced the hypoxia-induced  $\text{Ca}^{2+}$  response by  $58.5 \pm 8.2\%$  ( $n=180$ ,  $N=4$ ,  $P<0.05$ , Fig. 2b) and  $62.6 \pm 3.3\%$  ( $n=175$ ,  $N=4$ ,  $P<0.05$ , Fig. 2a), respectively. The non-specific effects on VGCCs of 2APB and SKF96365 were investigated in PC12 cells (inlets of Figs 2a and 2b), but no significant change in VGCCs activity was observed (both  $n>100$ ,  $P<0.05$ ). The extent of hypoxic response inhibited by these TRPC antagonists was significant than that by VGCC antagonist, suggesting that TRPC channels primarily mediate  $\text{Ca}^{2+}$  entry in

hypoxic response of hPASC. In addition, KBR7943 (5  $\mu\text{M}$ ), an antagonist of NCX, which specifically blocks reverse model of NCX at low concentration ( $\text{IC}_{50}=1.1\text{--}2.2\ \mu\text{M}$ ) (Iwamoto and Shigekawa 1998), enhanced the hypoxia response in hPASC ( $n=84$ ,  $N=3$ ,  $P<0.05$ , Fig. 2c).

#### Role of TRPC6 and TRPC1 in mediating hypoxia-induced $\text{Ca}^{2+}$ entry

The role of TRPC channels in mediating the hypoxic response was further validated by gene knockdown experiments. TRPC6 and TRPC1 in hPASC were knocked down by using corresponding siRNAs. After the application of siRNA, the protein levels of TRPC6/TRPC1 detected at 24 h and 48 h by Western blot were both gradually reduced. At 48 h, the amount of protein was almost undetectable (Fig. 3a). Both a housekeeping protein ( $\beta$ -actin) and the other TRPC family member protein (either TRPC1 or TRPC6) were used as controls. The expression of control protein was detectable and exhibited no changes.

TRPC family can be divided further into two functional groups: store-operated channels (SOC) consisting of TRPC1, TRPC4 and TRPC5 which are activated by store depletion; receptor operated channels (ROC) consisting of TRPC3, TRPC6 and TRPC7, which are activated by ligands (Beech 2005). TRPC6 channels could be activated by diacylglycerol (DAG), resulting in  $\text{Ca}^{2+}$  entry via TRPC6. 1-oleoyl-2-acetyl-sn-glycerol (OAG) is commonly used as a DAG analogue (Thebault *et al.* 2005). To confirm a functional knockdown of TRPC6,  $\text{Ca}^{2+}$  entry induced by OAG (100  $\mu\text{M}$ ), was only present in control cells but almost absent in cells treated with siRNA against TRPC6 over 48 h. The hypoxic responses were significantly reduced by about  $79 \pm 5.5\%$  ( $n=270$ ,  $N=3$ ,  $P<0.05$ , Fig. 3b) in cells lacking of functional TRPC6. In the same TRPC6 knockdown cells, the calcium entry activated by store depletion via TRPC1 was virtually unaffected (Fig. 3c). This result demonstrated the specificity in knockdown of TRPC6 expression.

In the functional experiments with TRPC1 knockdown, where proteins of TRPC6 and  $\beta$ -actin were not affected by siRNA against TRPC1, hPASC possessed the similar response to OAG but smaller  $[\text{Ca}^{2+}]_i$  elevation in response to store depletion by thapsigargin (1  $\mu\text{M}$ ) in the absence of bath  $\text{Ca}^{2+}$  (with EGTA) solution (Fig. 3d). The hypoxic responses were reduced by about  $15 \pm 9.2\%$  ( $n=135$ ,  $N=3$ ,  $P<0.05$ ,

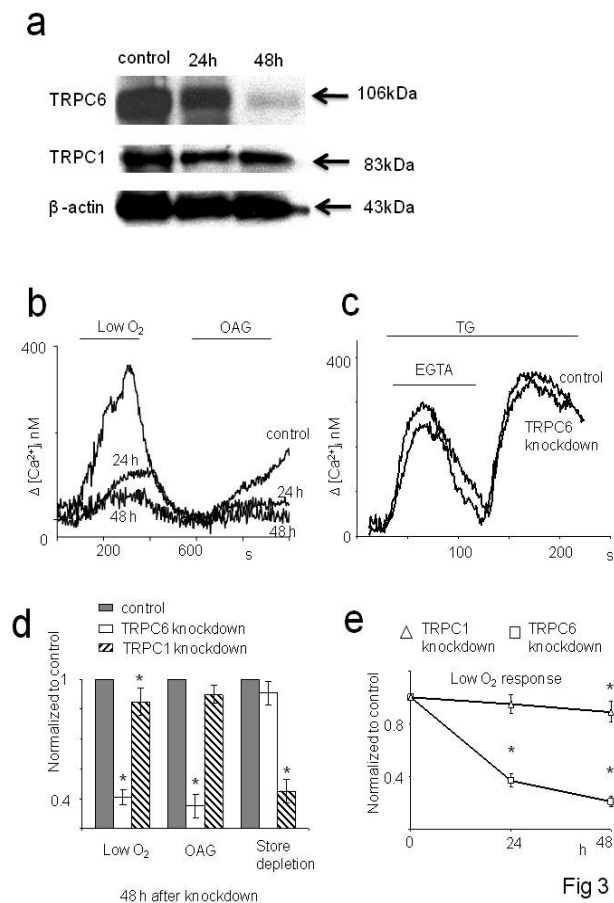
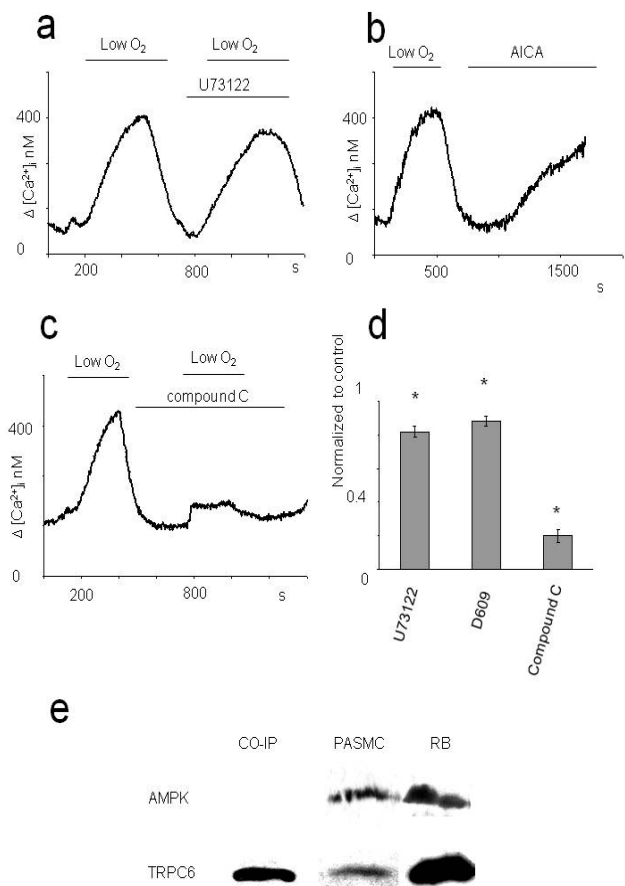


Fig. 3e) in cells lacking functional TRPC1 as compared to the controls over 48 h. These results suggest a key role for TRPC6, and thus ROC, in mediating the  $Ca^{2+}$  entry during hypoxia in hPASMC.

#### The hypoxia-induced $Ca^{2+}$ elevation was via AMPK but independent of PLC

Since DAG is produced *via* PLC (phospholipase C) pathway following the activation of G-protein, the PLC pathway could be potentially an essential link



between G-protein activation and TRPC6 activation. However, U73122 (10  $\mu$ M) (Fig. 4a), an antagonist of PLC-PI and D609 (10  $\mu$ M), an antagonist of PLC-PC, inhibited the hypoxia-induced  $Ca^{2+}$  response by only  $18.3 \pm 3.4\%$  ( $n=120$ ,  $N=4$ ,  $P<0.05$ , Fig. 4d) and  $11.8 \pm 3\%$  ( $n=102$ ,  $N=3$ ,  $P<0.05$ ), respectively. Therefore PLC activation does not appear to be a major regulator of TRPC6 during hypoxia.

To find other pathway, which may regulate TRPC6, AICA-riboside (1 mM), an AMP mimetic agent that activates AMPK, induced a slow and gradual  $Ca^{2+}$  elevation in hPASMC (Fig. 4b) and Compound C (40  $\mu$ M), an antagonist of AMPK, almost completely

abolished the hypoxia-induced  $\text{Ca}^{2+}$  elevation ( $n=75$ ,  $N=3$ ,  $P<0.05$ , Fig. 4c). The inhibition effect of compound C on hypoxia-induced  $[\text{Ca}^{2+}]_i$  elevation indicated that calcium entry must be partially mediated through TRPC6, suggesting a possible linkage (functional or physical) between TRPC6 and AMPK. However, co-immunoprecipitation results failed to reveal any physical connection between TRPC6 and AMPK (Fig. 4e).

## Discussion

These data demonstrate a central role for TRPC6-mediated cation entry in hypoxia-mediated hPASMCM response and suggests that consequent membrane depolarization could induce further  $\text{Ca}^{2+}$  entry via VGCCs activation. Furthermore, our data suggests that AMPK might provide the link between cellular energy status and TRPC activation.

Our conclusions are partly based upon the use of relatively selective antagonists of TRP channels. 2APB was initially designed as an  $\text{IP}_3$  antagonist but is widely used to block TRP (Iwasaki *et al.* 2001, Thebault *et al.* 2005, Xu *et al.* 2005), whilst SKF96365 is commonly used as a blocker of non-selective cation channels (Thebault *et al.* 2005, Harhun *et al.* 2006). The dosages used were comparable to those used in other studies (Chinopoulos *et al.* 2004, Thebault *et al.* 2005, Harhun *et al.* 2006) and we found consistent results with both drugs, suggesting that TRP channels are most likely activated during hypoxia. To overcome the non-selectivity of these drugs, we performed additional experiments using a targeted siRNA approach to knockdown TRPC in hPASMCM. Since knockdown techniques could cause unspecific and compensational effects of related subfamily members (Seth *et al.* 2004), examination of the expression levels of TRPC1 was performed when TRPC6 was knocked down and examination of TRPC6 was performed in TRPC1 knockdown cells. These findings together with those inhibitions by 2APB and SKF 96365 provide strong evidence implicating TRPC6, particularly, in mediating HPV.

Elevation of  $[\text{Ca}^{2+}]_i$  induced by hypoxia initiates smooth muscle contraction (Ward *et al.* 2004). The source of the  $\text{Ca}^{2+}$  rise induced by hypoxia remains contentious. Our data show that  $\text{Ca}^{2+}$  entry is a major component but a considerable intracellular  $\text{Ca}^{2+}$  release must also occur as we were unable to prevent the  $[\text{Ca}^{2+}]_i$  elevation even in  $\text{Ca}^{2+}$ -free solutions. This dual source of  $\text{Ca}^{2+}$  has often been remarked upon (Ward and Aaronson

1999) but the relative amounts derived from each source are not yet known with certainty and may vary with species and/or age. In our hPASMCM, the majority of the  $\text{Ca}^{2+}$  rise induced by hypoxia was due to  $\text{Ca}^{2+}$  entry, but  $\text{Ca}^{2+}$  release also contributed over 30 % of the response.  $\text{Ca}^{2+}$  entry is believed to occur primarily as a consequence of VGCCs activation, subsequent to membrane depolarization following hypoxia-induced closure of  $\text{K}^+$  channels. Whilst the molecular identity of the specific  $\text{K}^+$  channel inhibited is argued over, a definitive role for  $\text{K}^+$  channels as the primary mediators of HPV is still not yet established (Ward and Aaronson 1999, Michelakis *et al.* 2004).

In support of previous studies (Wadsworth 1994, Ward and Aaronson 1999, Robertson *et al.* 2000), we have shown that the blockade of VGCCs with either verapamil or nifedipine, does not prevent  $\text{Ca}^{2+}$  elevation, suggesting that VGCCs do not offer a unique entry pathway for  $\text{Ca}^{2+}$ . Another possible candidate to handle the  $\text{Ca}^{2+}$  entry across the membrane is the NCX. The functional presence of NCX in rat pulmonary artery (Wang *et al.* 2000) is demonstrated by the finding that removal of extracellular  $\text{Na}^+$ , potentiated the peak of  $[\text{Ca}^{2+}]_i$  under normoxia. We have now shown the expression of NCX in hPASMCM. The mechanism for hypoxia-induced elevation of  $\text{Ca}^{2+}$  via NCX remains controversial. Mild hypoxia can inhibit NCX, partly resulting in the elevation of  $[\text{Ca}^{2+}]_i$  (Wang *et al.* 2000), but HPV was not prevented by inhibition of NCX (Becker *et al.* 2006). Thus, although present, NCX may not be a source of  $\text{Ca}^{2+}$  in HPV and our results appear to confirm this concept.

TRPC are non-selective cation channels mediating  $\text{Ca}^{2+}$  entry in response to a wide range of stimuli (Hardie 2003) and offer another route for cation entry that might contribute to the membrane depolarization and  $\text{Ca}^{2+}$  elevation during hypoxia. TRPC1, TRPC4 and TRPC6 protein expression has been found previously in rat PASMCM (Wang *et al.* 2006) and we have found expression of TRPC1 and TRPC6 but not TRPC4 in hPASMCM. This species difference may reflect a difference in function, which has yet to be determined, but nevertheless demonstrates a potential for TRPC in mediating HPV. Activation of TRPC1 and TRPC6 will lead to large  $\text{Na}^+$  influx with smaller amounts of  $\text{Ca}^{2+}$  (Minke and Cook 2002, Rosker *et al.* 2004, Minke 2006). The elevation of  $[\text{Na}^+]_i$  would depolarize the membrane and the simultaneous closure of  $\text{K}^+$  channels by hypoxia would potentiate the depolarization by preventing a

counter hyperpolarization as well as by contributing to the degree of depolarization (Rosker *et al.* 2004). This fall in membrane potential could reach the threshold level of L-type VGCCs, resulting in a significant  $\text{Ca}^{2+}$  entry. Thus, activation of TRPC and inactivation of  $\text{K}^+$  channels, during hypoxia, could be seen as complementary mechanisms for initiating and maintaining a sufficient membrane depolarization. Consistent to previous studies (Yu *et al.* 2004, Weissmann *et al.* 2006), our finding that pharmacological blockade of TRP channels or selective knockdown of TRPC6 almost abolished  $\text{Ca}^{2+}$  entry, suggests that TRPC6 plays a central role in the hypoxia response of hPASC. In addition, our results also suggested that operation of TRPC6 might not be regulated through PLC pathway.

The question remains as to the identity of the  $\text{O}_2$  sensor in PASC and its link with  $\text{Ca}^{2+}$  elevation during hypoxia. During hypoxia, either the loss of a signal present during normoxia or the appearance of a hypoxia-dependent factor must occur for signaling the presence of a reduced  $\text{O}_2$ . Reactive oxygen species (ROS), derived from mitochondrial or non-mitochondrial sources, have been suggested as a mediator of HPV, but their role is controversial and evidence for and against increased ROS during hypoxia exists (Archer *et al.* 2004, Michelakis *et al.* 2004, Ward *et al.* 2004, Moudgil *et al.* 2005, Waypa and Schumacker 2005). Some of the controversy may reside with technical considerations but actually the role of ROS is not definitive.

TRPC6 is activated by products of PLC, such as DAG. In our experiments, the pharmacological inhibition of PLC had only a small effect on the hypoxia response and this suggests that an alternative signaling pathway other than PLC could lead to the activation of TRPC6. The cellular AMP:ATP ratio is a sensitive measure of the cell energy status (Hardie *et al.* 2006) and elevations in

this ratio occur in a variety of stress situations including starvation, oxidative stress and hypoxia. These stressors activate AMPK which acts to attenuate or prevent falls in ATP concentration through a variety of downstream kinases that inhibit cellular anabolic processes whilst activating catabolic processes. Recently, a role for AMPK in HPV was demonstrated, whereby HPV and/or rat PASC  $\text{Ca}^{2+}$  elevations in hypoxia (Wyatt *et al.* 2006) could be attenuated with the AMPK antagonist, Compound C or mimicked with AICA-ribose with the action occurring consequent to cADP ribose dependent  $\text{Ca}^{2+}$  mobilization from intracellular SR stores. Our data confirm these actions of AMPK in hPASC, but our data would suggest that, in human cells, this action does not appear to be primarily *via*  $\text{Ca}^{2+}$  release and AMPK in these cells might signal to TRPC6, but the co-immunoprecipitation data revealed that if this were to occur, it would not be a direct effect *via* a physical link between AMPK and TRPC6.

In conclusion, we have demonstrated a functional role for TRPC6 in mediating the  $\text{Ca}^{2+}$  influx required for HPV in hPASC. We suggest that the activation of TRPC6 occurs subsequent to an elevation in cellular AMPK during hypoxia. The activation of TRPC6 would increase  $[\text{Ca}^{2+}]_i$  directly and may also, in concert with the hypoxia-mediated inactivation of  $\text{O}_2$ -sensitive  $\text{K}^+$  channels, act to ensure sufficient membrane depolarization for the activation of a greater  $\text{Ca}^{2+}$  conductance *via* VGCCs.

### Conflict of Interest

There is no conflict of interest.

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### References

- ARCHER S, MICHELAKIS E: The mechanism(s) of hypoxic pulmonary vasoconstriction: potassium channels, redox  $\text{O}_2$  sensors, and controversies. *News Physiol Sci* **17**: 131-137, 2002.
- ARCHER SL, WU XC, THEBAUD B, MOUDGIL R, HASHIMOTO K, MICHELAKIS ED:  $\text{O}_2$  sensing in the human ductus arteriosus: redox-sensitive  $\text{K}^+$  channels are regulated by mitochondria-derived hydrogen peroxide. *Biol Chem* **385**: 205-216, 2004.
- BECKER S, KNOCK GA, SNETKOV V, WARD JP, AARONSON PI: Role of capacitative  $\text{Ca}^{2+}$  entry but not  $\text{Na}^+/\text{Ca}^{2+}$  exchange in hypoxic pulmonary vasoconstriction in rat intrapulmonary arteries. *Novartis Found Symp* **272**: 259-268, 2006.



- BEECH DJ: Emerging functions of 10 types of TRP cationic channel in vascular smooth muscle. *Clin Exp Pharmacol Physiol* **32**: 597-603, 2005.
- CHINOPOULOS C, GERENCSEER AA, DOCZI J, FISKUM G, ADAM-VIZI V: Inhibition of glutamate-induced delayed calcium deregulation by 2-APB and  $\text{La}^{3+}$  in cultured cortical neurones. *J Neurochem* **91**: 471-483, 2004.
- CLAPHAM DE: TRP channels as cellular sensors. *Nature* **426**: 517-524, 2003.
- CLAPP LH, GURNEY AM: Modulation of calcium movements by nitroprusside in isolated vascular smooth muscle cells. *Pflugers Arch* **418**: 462-470, 1991.
- DEL TORO R, LEVITSKY KL, LOPEZ-BARNEO J, CHIARA MD: Induction of T-type calcium channel gene expression by chronic hypoxia. *J Biol Chem* **278**: 22316-22324, 2003.
- DIPP M, EVANS AM: Cyclic ADP-ribose is the primary trigger for hypoxic pulmonary vasoconstriction in the rat lung in situ. *Circ Res* **89**: 77-83, 2001.
- DIPP M, NYE PC, EVANS AM: Hypoxic release of calcium from the sarcoplasmic reticulum of pulmonary artery smooth muscle. *Am J Physiol* **281**: L318-L325, 2001.
- FISHMAN AP: Hypoxia on the pulmonary circulation. How and where it acts. *Circ Res* **38**: 221-231, 1976.
- GREEN KN, BOYLE JP, PEERS C: Hypoxia potentiates exocytosis and  $\text{Ca}^{2+}$  channels in PC12 cells via increased amyloid beta peptide formation and reactive oxygen species generation. *J Physiol* **541**: 1013-1023, 2002.
- GROVER R, WAGNER W, MCMURTRY I, REEVES J: Pulmonary circulation. In: *Handbook of physiology*. J SHEPHERD, F ABBOUD (eds), Williams and Wilkins, Baltimore, 1983, pp 103-137.
- GURNEY AM: Multiple sites of oxygen sensing and their contributions to hypoxic pulmonary vasoconstriction. *Respir Physiol Neurobiol* **132**: 43-53, 2002.
- HARDIE DG, HAWLEY SA, SCOTT JW: AMP-activated protein kinase – development of the energy sensor concept. *J Physiol Lond* **574**: 7-15, 2006.
- HARDIE RC: Regulation of TRP channels via lipid second messengers. *Annu Rev Physiol* **65**: 735-759, 2003.
- HARHUN M, GORDIENKO D, KRYSHAL D, PUCOVSKY V, BOLTON T: Role of intracellular stores in the regulation of rhythmical  $[\text{Ca}^{2+}]_i$  changes in interstitial cells of Cajal from rabbit portal vein. *Cell Calcium* **40**: 287-298, 2006.
- HENDERSON SR, REUSS H, HARDIE RC: Single photon responses in Drosophila photoreceptors and their regulation by  $\text{Ca}^{2+}$ . *J Physiol Lond* **524**: 179-194, 2000.
- ICHINOSE F, ULLRICH R, SAPIRSTEIN A, JONES RC, BONVENTRE JV, SEREHAN CN, BLOCH KD, ZAPOL WM: Cytosolic phospholipase  $\text{A}_2$  in hypoxic pulmonary vasoconstriction. *J Clin Invest* **109**: 1493-1500, 2002.
- IWAMOTO T, SHIGEKAWA M: Differential inhibition of  $\text{Na}^+/\text{Ca}^{2+}$  exchanger isoforms by divalent cations and isothiourea derivative. *Am J Physiol* **275**: C423-C430, 1998.
- IWASAKI H, MORI Y, HARA Y, UCHIDA K, ZHOU H, MIKOSHIBA K: 2-Aminoethoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-trisphosphate receptors. *Receptors Channels* **7**: 429-439, 2001.
- LOPEZ-BARNEO J, DEL TORO R, LEVITSKY KL, CHIARA MD, ORTEGA-SAENZ P: Regulation of oxygen sensing by ion channels. *J Appl Physiol* **96**: 1187-1195, 2004.
- MCCULLOCH KM, OSIPENKO ON, GURNEY AM: Oxygen-sensing potassium currents in pulmonary artery. *Gen Pharmacol* **32**: 403-411, 1999.
- MICHELAKIS ED, THEBAUD B, WEIR EK, ARCHER SL: Hypoxic pulmonary vasoconstriction: redox regulation of  $\text{O}_2$ -sensitive  $\text{K}^+$  channels by a mitochondrial  $\text{O}_2$ -sensor in resistance artery smooth muscle cells. *J Mol Cell Cardiol* **37**: 1119-1136, 2004.
- MINKE B: TRP channels and  $\text{Ca}^{2+}$  signaling. *Cell Calcium* **40**: 261-275, 2006.
- MINKE B, COOK B: TRP channel proteins and signal transduction. *Physiol Rev* **82**: 429-472, 2002.
- MOUDGIL R, MICHELAKIS ED, ARCHER SL: Hypoxic pulmonary vasoconstriction. *J Appl Physiol* **98**: 390-403, 2005.
- OSIPENKO ON, EVANS AM, GURNEY AM: Regulation of the resting potential of rabbit pulmonary artery myocytes by a low threshold,  $\text{O}_2$ -sensing potassium current. *Br J Pharmacol* **120**: 1461-1470, 1997.

- ROBERTSON TP, HAGUE D, AARONSON PI, WARD JP: Voltage-independent calcium entry in hypoxic pulmonary vasoconstriction of intrapulmonary arteries of the rat. *J Physiol Lond* **525**: 669-680, 2000.
- ROSKER C, GRAZIANI A, LUKAS M, EDER P, ZHU MX, ROMANIN C, GROSCHNER K:  $\text{Ca}^{2+}$  signaling by TRPC3 involves  $\text{Na}^+$  entry and local coupling to the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. *J Biol Chem* **279**: 13696-13704, 2004.
- SWEENEY M, YUAN JX: Hypoxic pulmonary vasoconstriction: role of voltage-gated potassium channels. *Respir Res* **1**: 40-48, 2000.
- THEBAULT S, ZHOLOS A, ENFISSI A, SLOMIANNY C, DEWAILLY E, ROUDBARAKI M, PARYS J, PREVARSKAYA N: Receptor-operated  $\text{Ca}^{2+}$  entry mediated by TRPC3/TRPC6 proteins in rat prostate smooth muscle (PS1) cell line. *J Cell Physiol* **204**: 320-328, 2005.
- WADSWORTH RM: Vasoconstrictor and vasodilator effects of hypoxia. *Trends Pharmacol Sci* **15**: 47-53, 1994.
- WANG J, SHIMODA LA, WEIGAND L, WANG W, SUN D, SYLVESTER JT: Acute hypoxia increases intracellular  $[\text{Ca}^{2+}]$  in pulmonary arterial smooth muscle by enhancing capacitative  $\text{Ca}^{2+}$  entry. *Am J Physiol* **288**: L1059-L1069, 2005.
- WANG J, WEIGAND L, LU W, SYLVERSTER JT, SEMENZA GL, SHIMODA LA: Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular  $\text{Ca}^{2+}$  in pulmonary arterial smooth muscle cells. *Circ Res* **98**: 1528-1537, 2006.
- WANG YX, DHULIPALA PK, KOTLIKOFF MI: Hypoxia inhibits the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger in pulmonary artery smooth muscle cells. *FASEB J* **14**: 1731-1740, 2000.
- WARD JP, AARONSON PI: Mechanisms of hypoxic pulmonary vasoconstriction: can anyone be right? *Respir Physiol* **115**: 261-271, 1999.
- WARD JP, SNETKOV VA, AARONSON PI: Calcium, mitochondria and oxygen sensing in the pulmonary circulation. *Cell Calcium* **36**: 209-220, 2004.
- WAYPA GB, SCHUMACKER PT:  $\text{O}_2$  sensing in hypoxic pulmonary vasoconstriction: the mitochondrial door re-opens. *Respir Physiol Neurobiol* **132**: 81-91, 2002.
- WAYPA GB, SCHUMACKER PT: Hypoxic pulmonary vasoconstriction: redox events in oxygen sensing. *J Appl Physiol* **98**: 404-414, 2005.
- WEIGAND L, FOXSON J, WANG J, SHIMODA LA, SYLVESTER JT: Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated  $\text{Ca}^{2+}$  and nonselective cation channels. *Am J Physiol* **289**: L5-L13, 2005.
- WEISSMANN N, DIETRICH A, FUCHS B, KALWA H, AY M, DUMITRASCU R, OLSCHESKI A, STORCH U, MEDEROS Y, SCHNITZLER M, GHOFrani HA, SCHERMULY RT, PINKENBURG O, SEEGER W, GRIMMINGER F, GUDERMANN T: Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. *Proc Natl Acad Sci USA* **103**: 19093-19098, 2006.
- WYATT CN, MUSTARD KJ, PEARSON SA, DALLAS ML, ATKINSON L, KUMAR P, PEERS C, HARDIE DG, EVANS AM: AMP-activated protein kinase mediates carotid body excitation by hypoxia. *J Biol Chem* **282**: 8092-8098, 2007.
- XU SZ, ZENG F, BOULAY G, GRIMM C, HARTENECK C, BEECH DJ: Block of TRPC5 channels by 2-aminoethoxydiphenyl borate: a differential, extracellular and voltage-dependent effect. *Br J Pharmacol* **145**: 405-414, 2005.
- YU Y, FANTOZZI I, REMILLARD CV, LANDSBERG JW, KUNICHIKA N, PLATOSHYN O, TIGNO DD, THISTLETHWAITE PA, RUBIN LJ, YUAN JX: Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci USA* **101**: 13861-13866, 2004.
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