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A Role for Receptor-Operated 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ elevations during hypoxia. Hypoxia (PO_2 about 20 mm Hg) evoked a transient $[Ca^{2+}]_i$ elevation that was reduced by removal of extracellular calcium. Nifedipine and verapamil, blockers of voltage-gated calcium channels (VGCCs), attenuated the hypoxia-induced $[Ca^{2+}]_i$ elevation by about 30 %, suggesting the presence of alternate 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ elevation, whereas compound C, an antagonist of AMPK, almost abolished the hypoxia response. However, co-immunoprecipitation revealed that AMPKα was not colocalized with TRPC6. Our data supports a role for TRPC6 in mediation of the $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ is a central event in the contraction of pulmonary artery smooth muscle cells (PASMC). The general concept of HPV is that inhibition of O_2-sensitive K^+ channels depolarizes the PASMC membrane potential (E_M), and induces Ca^{2+} influx through L-type voltage-gated 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

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average of only 10-20 mV (Osipenko *et al.* 1997) and by itself this may not be sufficient to activate Ca^{2+} influx through VGCC in PASMC, 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 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 $[Ca^{2+}]_i$ during hypoxia and there is a significant contribution of non-voltage-gated cation channels.

Other possible candidates for Ca^{2+} entry include the transient receptor potential (TRP) channels, which are non-selective cation channels with a Na^+: 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 Ca^{2+} entry *via* VGCC. Previous studies have shown that the hypoxia induced Ca^{2+} entry in rat distal PASMC 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 PASMC, 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 hPASMC. Our data suggests a key role for 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 (hPASMC: 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 α-actin) were cultured in smooth muscle growing medium (Promocell Ltd) under 5 % 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 % 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. hPASMC cells were passaged onto coverslips in 500 µl 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 hPASMC.

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 hPASMC cells were homogenized with CelLytic 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. β-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 hPASMC (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-riboside), 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_2 level in the medium. The O_2 level of the hypoxia medium in the recording chamber was about 20 mm Hg.

Results

Source of Ca^{2+} elevation induced by hypoxia

Four minutes of exposure to hypoxia induced a significant and reversible Ca^{2+} 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^{2+} (+EGTA). In a control, when hPAMSC were exposed to high K^+ solution (40 mM) for 30 s, a transient Ca^{2+} elevation was evoked, as predicted (Fig. 1a). This suggests that hypoxia-induced $[Ca^{2+}]_i$ elevations were primarily, although not solely, due to Ca^{2+} 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^+ solution (40 mM) induced a significant $[Ca^{2+}]_i$ elevation in PC12 cells, due to the opening of L-type VGCCs (Green *et al.* 2002). Verapamil dose-dependently inhibited high K^+-induced $[Ca^{2+}]_i$ elevation in PC12 cells (Fig. 1b). The inlet in Figure 1b shows the inhibition of high K-induced $[Ca^{2+}]_i$ elevation

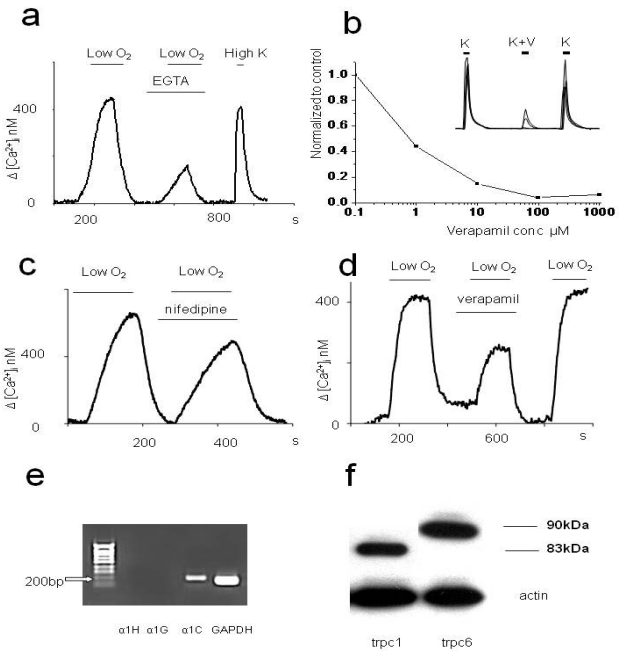

Fig. 1. Contribution of VGCCs to hypoxia-induced $[Ca^{2+}]_i$ elevation in hPASMC. **a.** Four minutes of exposure to hypoxia (pO_2 20 mm Hg) caused a significant and reversible $[Ca^{2+}]_i$ elevation in hPASMC. Hypoxia-induced $[Ca^{2+}]_i$ elevation was reduced to 31.2±4.4 % after removal of extracellular Ca^{2+} (+EGTA). **b.** Verapamil inhibited high K^+ induced $[Ca^{2+}]_i$ elevation in a dose-dependent manner in O_2 sensitive cells (PC12). Inlets (PC12 cells) showed the inhibition effects of verapamil at four different dose on high K^+-induced $[Ca^{2+}]_i$ elevation (1 μM, 10 μM, 100 μM and 1000 μM). Nifedipine (20 μM) (**c**) and verapamil (40 μM) (**d**) inhibited hypoxia-induce $[Ca^{2+}]_i$ elevation in hPASMC 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 hPASMC but not α1H and α1G. (**f**) The presence of TRPC1 and 6 proteins in hPASMC 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 hPASMC, respectively. Nifedipine (20 μM) and verapamil (40 μM) inhibited the hypoxia-induced $[Ca^{2+}]_i$ 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 hPASMC, but a considerable (>60 %) part of response was retained, implicating the activity of other Ca^{2+} 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 hPASMC, whereas mRNA of α1G, and α1H subunits, encoding T-type VGCCs, was not detected (Fig. 1e).

Role of other Ca^{2+} entry pathways in hypoxia

The expression of Na^+-Ca^{2+} exchanger (NCX), TRPC1 and TRPC6 but not TRPC3, TRPC4, TRPC5 and

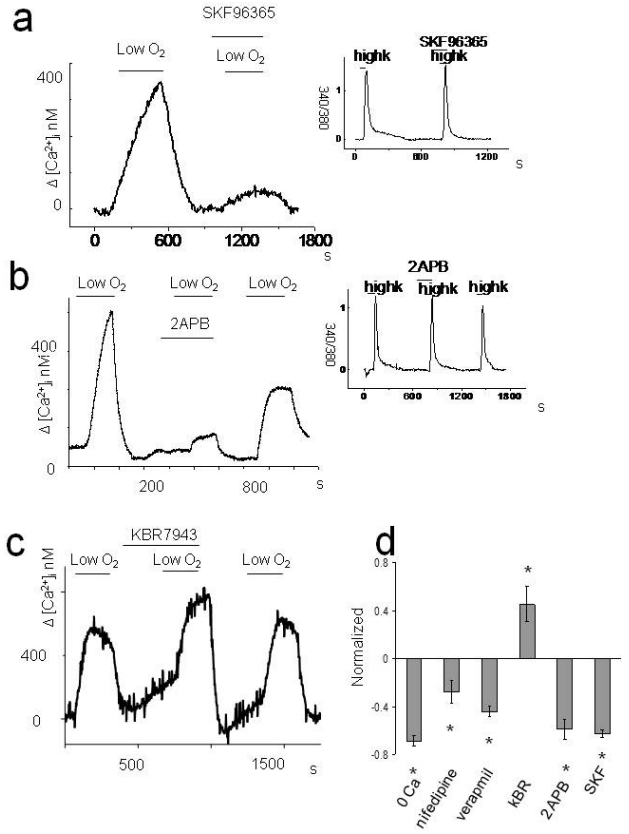

Fig. 2. Contributions of non-VGCC Ca^{2+} entry pathways to hypoxia-induced $[Ca^{2+}]_i$ elevation in hPASMC. **a. b.** SKF96365, 2APB, antagonists of TRPC receptors, inhibited the hypoxia-induced $[Ca^{2+}]_i$ elevation in hPASMC. The effects on VGCCs of these TRPC antagonists observed in PC12 cells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

TRPC7 were detected by RT-PCR in hPASMC (data not shown) and the presence of TRPC1 and TRPC6 proteins were further confirmed by Western blot (Fig. 1f). 2APB (50 μM) and SKF 96365 (20 μM) were applied as antagonists of TRPC channels and both chemicals significantly reduced the hypoxia-induced Ca^{2+} response by 58.5±8.2 % (n=180, N=4, P<0.05, Fig. 2b) and 62.6±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 Ca^{2+} entry in hypoxic response of hPASMC. In addition, KBR7943 (5 μM), an antagonist of NCX, which specifically blocks reverse model of NCX at low concentration (IC_{50}=1.1-2.2 μM) (Iwamoto and Shigekawa 1998), enhanced the hypoxia response in hPASMC (n=84, N=3, P<0.05, Fig. 2c).

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

The role of TRPC channels in mediating the hypoxic response was further validated by gene knockdown experiments. TRPC6 and TRPC1 in hPASMC 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 (β-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 Ca^{2+} entry *via* TRPC6. 1-oleolyl-2-acetyl-sn-glycerol (OAG) is commonly used as a DAG analogue (Thebault *et al.* 2005). To confirm a functional knockdown of TRPC6, Ca^{2+} entry induced by OAG (100 μM), 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±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 β-actin were not affected by SiRNA against TRPC1, hPASMC possessed the similar response to OAG but smaller $[Ca^{2+}]_i$ elevation in response to store depletion by tharpsigargin (1 μM) in the absence of bath Ca^{2+} (with EGTA) solution (Fig. 3d). The hypoxic responses were reduced by about 15±9.2 % (n=135, N=3, P<0.05,

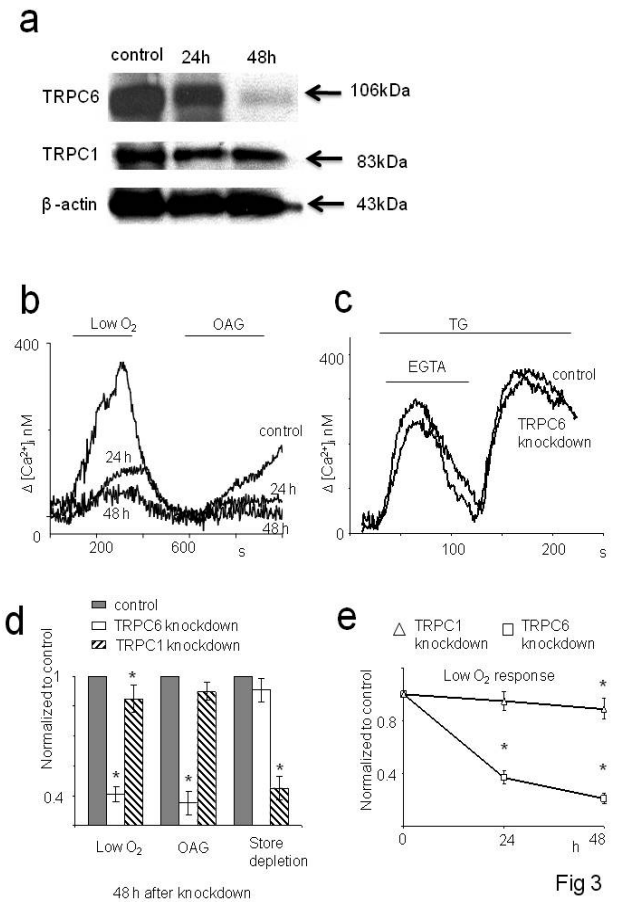

Fig. 3. Effect of siRNA knockdown of TRPC in hPASMC on hypoxia-induced $[Ca^{2+}]_i$ elevation. **a.** After the introducing of siRNA, the protein level of TRPC 6 was detected at 24 h and 48 h by Western blot analysis. At 48 h, the amount of protein was almost undetectable. Both housekeeping protein (β-actin) and TRPC1 were used as controls. The expression of control protein was detectable and exhibited no changes, suggesting the specificity of knockdown effect on TRPC6. **b.** Following TRPC6 knocking down, hypoxia-induced Ca^{2+} response was also significantly decreased over 48 h while cells exhibited very small response to OAG. **c.** Cells with TRPC6 knockdown maintained response to store depletion as the control. **d.** Summary of hypoxic responses of cells with TRPC knockdown, OAG and store depletion. **e.** In hPASMC cells with TRPC6 knockdown, hypoxia-induced $[Ca^{2+}]_i$ elevation decreased to 37.1±9.5 % over 24 h and to 21.3±8.9 % over 48 h, whereas in cells with TRPC1 knockdown hypoxia-induced $[Ca^{2+}]_i$ elevation insignificantly reduced over 24 h and reduced to 84.5±9.2 % over 48 h, in comparison with the control.

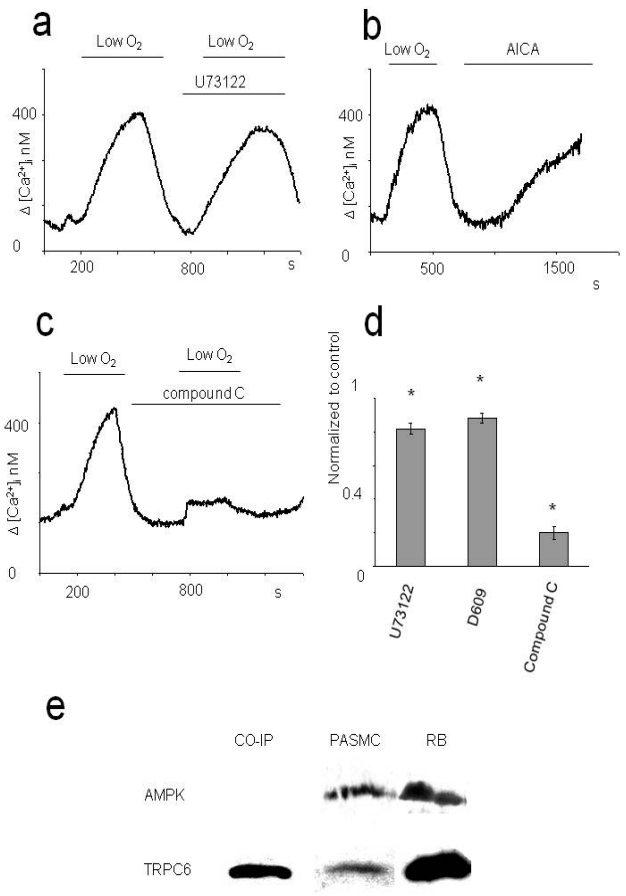

Fig. 4. Hypoxia-induced $[Ca^{2+}]_i$ elevation in hPASMC is independent of PLC. **a.** U73122 (10 μM), an antagonist of PLC, slightly decreased the Ca^{2+} elevation induced by hypoxia. **b.** AICA (100 μM), an agonist of AMPK, induced a slow $[Ca^{2+}]_i$ elevation. **c.** Compound C (40 μM), an antagonist of AMPK, significantly inhibited Ca^{2+} elevation induced by hypoxia. **d.** U73122 and D609, which inhibit PK-PLC, had a small effect on Ca^{2+} elevation induced by hypoxia in hPASMC. However, compound C significantly inhibited hypoxia-induced $[Ca^{2+}]_i$ elevation. **e.** The products of immunoprecipitation by TRPC6 from hPASMC were examined by the antibody against AMPKα. The clear blots in hPASMC and rat brain indicated the presence of AMPK protein in these cells. The blank in TRPC6 precipitation suggested there was no physical linkage between TRPC6 and AMPK.

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 μM) (Fig. 4a), an antagonist of PLC-PI and D609 (10 μM), an antagonist of PLC-PC, inhibited the hypoxia-induced Ca^{2+} response by only 18.3±3.4 % (n=120, N=4, $P<0.05$, Fig. 4d) and 11.8±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 μM), an antagonist of AMPK, almost completely

abolished the hypoxia-induced Ca^{2+} elevation (n=75, N=3, P<0.05, Fig. 4c). The inhibition effect of compound C on hypoxia-induced $[Ca^{2+}]_i$ 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 hPASMC response and suggests that consequent membrane depolarization could induce further 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 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 hPASMC. 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 $[Ca^{2+}]_i$ induced by hypoxia initiates smooth muscle contraction (Ward *et al.* 2004). The source of the Ca^{2+} rise induced by hypoxia remains contentious. Our data show that Ca^{2+} entry is a major component but a considerable intracellular Ca^{2+} release must also occur as we were unable to prevent the $[Ca^{2+}]_i$ elevation even in Ca^{2+}-free solutions. This dual source of 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 hPASMC, the majority of the Ca^{2+} rise induced by hypoxia was due to Ca^{2+} entry, but Ca^{2+} release also contributed over 30 % of the response. Ca^{2+} entry is believed to occur primarily as a consequence of VGCCs activation, subsequent to membrane depolarization following hypoxia-induced closure of K^+ channels. Whilst the molecular identity of the specific K^+ channel inhibited is argued over, a definitive role for 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 Ca^{2+} elevation, suggesting that VGCCs do not offer a unique entry pathway for Ca^{2+}. Another possible candidate to handle the 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 Na^+, potentiated the peak of $[Ca^{2+}]_i$ under normoxia. We have now shown the expression of NCX in hPASMC. The mechanism for hypoxia-induced elevation of Ca^{2+} *via* NCX remains controversial. Mild hypoxia can inhibit NCX, partly resulting in the elevation of $[Ca^{2+}]_i$ (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 Ca^{2+} in HPV and our results appear to confirm this concept.

TRPC are non-selective cation channels mediating 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 Ca^{2+} elevation during hypoxia. TRPC1, TPRC4 and TPRC6 protein expression has been found previously in rat PASMC (Wang *et al.* 2006) and we have found expression of TRPC1 and TPRC6 but not TPRC4 in hPASMC. 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 TPRC6 will lead to large Na^+ influx with smaller amounts of Ca^{2+} (Minke and Cook 2002, Rosker *et al.* 2004, Minke 2006). The elevation of $[Na^+]_i$ would depolarize the membrane and the simultaneous closure of 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 Ca^{2+} entry. Thus, activation of TRPC and inactivation of 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 Ca^{2+} entry, suggests that TRPC6 plays a central role in the hypoxia response of hPASMC. 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 O_2 sensor in PASMC and its link with 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 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 PASMC 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 Ca^{2+} mobilization from intracellular SR stores. Our data confirm these actions of AMPK in hPASMC, but our data would suggest that, in human cells, this action does not appear to be primarily *via* 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 Ca^{2+} influx required for HPV in hPASMC. We suggest that the activation of TRPC6 occurs subsequent to an elevation in cellular AMPK during hypoxia. The activation of TRPC6 would increase $[Ca^{2+}]_i$ directly and may also, in concert with the hypoxia-mediated inactivation of O_2-sensitive K^+ channels, act to ensure sufficient membrane depolarization for the activation of a greater Ca^{2+} conductance *via* VGCCs.

Conflict of Interest

There is no conflict of interest.

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