A role for receptor operated Ca^{2+} entry in human pulmonary artery smooth muscle cells in response to hypoxia

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Short title: Role of ROC in HPV

Abstract

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 *ca.* 20mmHg) 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 around 30%, suggesting the presence of alternate Ca^{2+} entry pathways . Expression of TRPC1 and 6 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 knock down of TRPC1reduced the hypoxia response to 85%, suggesting that TRPC6 might play a central role in mediating hypoxia response in hPASMC. However, blockage 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 and compound C, an antagonist of AMPK, almost abolished the hypoxia response. However, co-immunoprecipitation revealed that AMPKα was not co-localised 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 word: hypoxic pulmonary vasoconstriction (HPV), hPASMC, hypoxia, transient receptor potential channel (TRPC), AMP-activated kinase (AMPK)

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., 1983a; Grover et al., 1983b). 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 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 sufficiently causing vasoconstriction (Archer and Michelakis, 2002; Gurney, 2002; Lopez-Barneo et al., 2004; Michelakis et al., 2004; Sweeney and Yuan, 2000; Waypa and Schumacker, 2002).

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 depolarises pulmonary artery smooth muscle cells by an 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 around -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 significant contribution via 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 (Clapham, 2003; Henderson et al., 2000). Upon activation, cation entry via TRP would cause membrane depolarisation 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 4, 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 depolarisation 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 activation of AMPK.

Methods and Materials

Cell culture 2nd 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) from Promocell (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 manufacture instructions. Transfection rate was calculated by eGFP transfection and it was over 60%. The knockdown effects were examined at 48 hours and the results were compared with control duplex, control without knockdown and control with another relative TRPC knockdown. The knockdown experiments were triplicates and knockdown effects on HPV response were averaged from 3 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 2min 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 1 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 1 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^{TM} Mammalian Co-Immunoprcipitation Kit (Pierce 23605) according to the manufacture 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 (0.01g 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 minutes. 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., and statistical differences were compared using a Student's paired *t* test, taking $P < 0.05$ as significant *. The trace was represented in most figures were the average results from 1 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 bubbling 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). The chamber tube passed an electrical valve (Automation 8) and reached the recording chamber. The media flowed into recording chamber (RG 26, Warner) if the valve was open; otherwise they continually circulated to reduce procedure with 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 around 20 mmHg.

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. 68.8%± 4.4% (n=72, N=3, P<0.05, fig1a) 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 30s, 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-induce high K^+ induced $[Ca^{2+}]_i$ elevation in PC12 cells (fig1b). The inlets in fig1b showed the inhibition by verapamil of high K-induced $[Ca^{2+}]_i$ elevation. 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,

fig1d) 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 the results of RT-PCR from primary cultured hPASMC. However, mRNA of α1G, and α1H subunits, encoding T-type VGCCs was not detected（fig1e）.

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

The expression of Na^{+}-Ca^{2+} exchanger (NCX), TRPC1 and TRPC6 but not TRPC3, 4, 5 and 7 were detected by RT-PCR in hPASMC (data not shown) and the presences of TRPC1 and 6 proteins were further confirmed by western blot (fig 1f). 2APB (50μM) and SKF 96365 (20μM) were applied as antagonists of TRPC channels and they respectively, 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）.

The non-specific effects on VGCCs of 2APB and SKF96365 were investigated in PC12 cells (inlets of fig2a and fig2b) and no significant change in VGCCs activity was observed (both n>100, P<0.05). The amount of hypoxic response inhibited by these TRPC antagonists was significant in comparing with 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}=1.1-2.2μM) (Iwamoto and Shigekawa, 1998), enhanced the hypoxia response (n=84, N=3, P<0.05, fig2c) in hPASMC.

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 respectively by use of corresponding siRNAs. After the introducing of siRNA, the protein levels of TRPC 6/TRPC1was detected at 24h and 48h by western blot, which both reduced gradually. At 48 hours, 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 TRPC1,4 and 5 which are activated by store depletion; receptor operated channels (ROC) consisting of TRPC3,6 and 7, 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 (100μM), was only present in control cells but almost absent in cells treated with siRNA against TRPC6 over 48 hours. The hypoxic responses were significantly reduced by ca. 79 ± 5.5% (n=270, N=3, $P<0.05$, fig 3b) in cells lacking of functional TRPC6. In the same TRPC6 knock down cells, the calcium entry activated by store depletion via TRPC1 was virtually unaffected (fig3c). This result demonstrated the specificity in knockdown of TRPC6 expression.

In the functional experiments of TRPC1 knock down 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) with 0 bath Ca^{2+} (with EGTA) solution(fig 3d). The hypoxic responses were reduced by ca 15 ± 9.2 %(n=135, N=3, P<0.05, fig 3e) in cells lacking functional TRPC1 in comparing with the control over 48 hours. 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 the 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, only inhibited the hypoxia-induced Ca^{2+} response by 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 and therefore PLC activation does not appear to be a major regulator of TRPC6 during hypoxia. In the interest of finding other pathway, which may regulate TRPC6, AICA-riboside (1mM), 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, fig4c). The reduction rate of the hypoxia-induced $[Ca^{2+}]_i$ elevation caused by compound C was so high, and the calcium entry blocked by it must partially mediated by 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 the determination of a functional response to hypoxia in hPASMC and suggests that consequent membrane depolarisation 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 (Harhun et al., 2006; Thebault et al., 2005). The dosages used were comparable to those used in other studies (Chinopoulos et al., 2004; Harhun et al., 2006; Thebault et al., 2005) 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 knock down TRPC in hPASMC. Since knockdown techniques could cause unspecific and compensational effects of related subfamily members (Seth, M 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 knock down cells. These findings with those inhibitions by 2APB and SKF 96365 together provide strong evidence implicating TRPC6, particularly, in mediating HPV.

Elevation of $[Ca^{2+}]_i$ mediated by hypoxia initiates smooth muscle contraction (Ward et al., 2004). The source of the Ca^{2+} rise induced by hypoxia remains contentious. Our data shows 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 elevation in Ca^{2+} 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 deriving 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 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 depolarisation 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 (Michelakis et al., 2004; Ward and Aaronson, 1999).

In support of previous studies (Robertson et al., 2000; Wadsworth, 1994; Ward and Aaronson, 1999), we have shown that block 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 depolarisation and Ca^{2+} elevation during hypoxia. TRPC1, 4 & 6 protein expression has been found previously in rat PASMC (Wang et al., 2006) and we have found expression of TRPC1 & 6 but not 4 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 6 will lead to large amounts of Na^+ influx with smaller amounts of Ca^{2+} (Minke, 2006; Minke and Cook, 2002; Rosker et al., 2004). The elevation of $[Na^+]_i$ would depolarize the membrane and the simultaneous closure of K^+ channels by hypoxia would potentiate the depolarisation by preventing a counter hyperpolarisation as well as by contributing to the degree of depolarisation (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 depolarisation. Consistent to previous studies (Weissmann et al., 2006; Yu et al., 2004), our finding that pharmacological block of TRP channels or selective knockdown of TRPC6 almost abolished Ca^{2+} entry, supports that TRPC6 plays a central role in the hypoxia response of hPASMC. In addition, our results also suggested that operation of TRPC6 might not 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 signalling the present 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 increases in ROS during hypoxia exists (Archer et al., 2004; Michelakis et al., 2004; Moudgil et al., 2005; Ward et al., 2004; Waypa and Schumacker, 2005). Some of the controversy may reside with technical considerations but presently 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 only had a small effect on the hypoxia response and this suggests that an alternative signalling pathway other than PLC could lead to 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+} mobilisation from intracellular SR stores. Our data confirms 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 depolarisation for the activation of a greater Ca^{2+} conductance via VGCCs.

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Figure legend

Fig. 1. Contribution of VGCCs to hypoxia-induced $[Ca^{2+}]_i$ elevation in hPASMC.

a. Four minutes of exposure to hypoxia (pO_2 20 mmHg) 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 dosage dependent manner in O2 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 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.

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 were obtained from PC12 cells and shown on upright corner of the figure. c. Example averaged trace showed the effect of KBR7943 on hypoxia response in hPASMC. d. Bar figure showed 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.

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 24h and 48h by western blot analysis. At 48 hours, 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 hours 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 hours and to 21.3 ± 8.9% over 48 hours, whereas in cells with trpc1 knockdown hypoxia-induced $[Ca^{2+}]_i$ elevation insignificantly reduced over 24 hours and reduced to 84.5 ± 9.2 % over 48 hours, in comparison with the control.

Fig. 4. Hypoxia-induced $[Ca^{2+}]_i$ elevation in hPASMC 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 AMPK α. 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.

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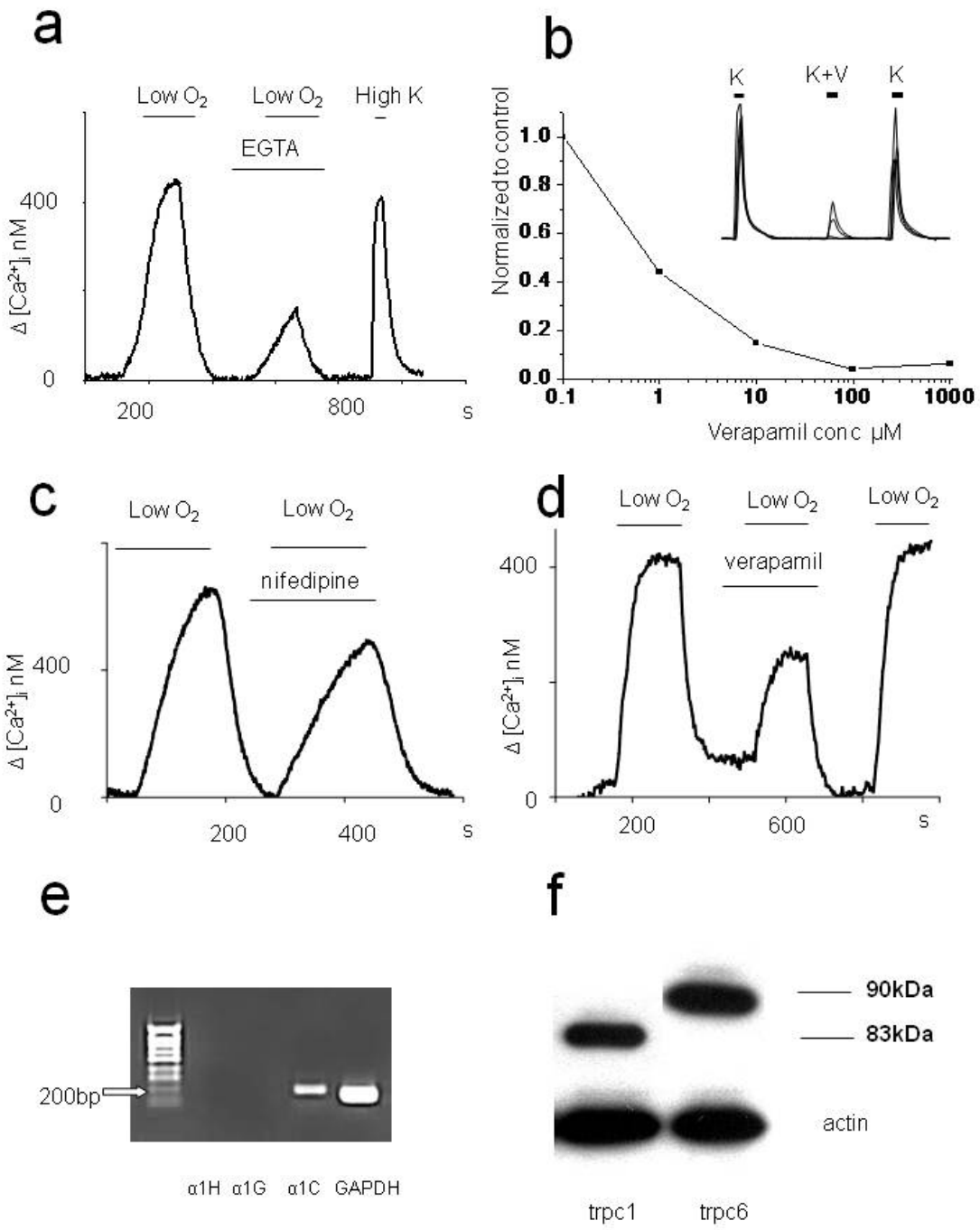
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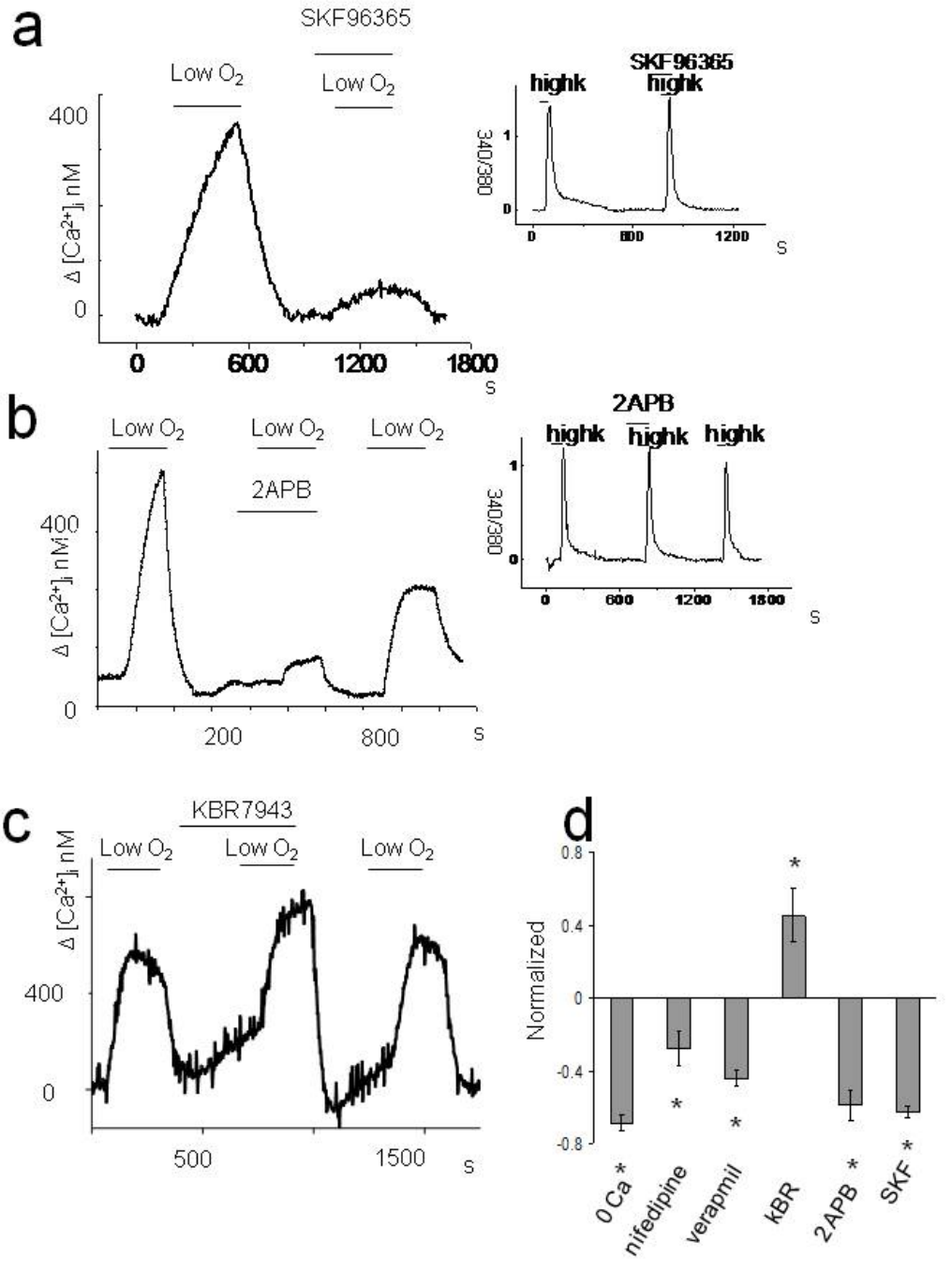
Low O2
High K
EGTA
400
0
Δ [Ca2+]i nM
200
800
s
b
K
K+V
K
Normalized to control
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
1000
Verapamil conc μM
c
Low O2
Low O2
nifedipine
400
0
Δ [Ca2+]i nM
200
400
s
d
Low O2
Low O2
Low O2
verapamil
400
0
Δ [Ca2+]i nM
200
600
s
e
200bp
α1H α1G α1C GAPDH
f
90kDa
83kDa
actin
trpc1
trpc6

Fig 1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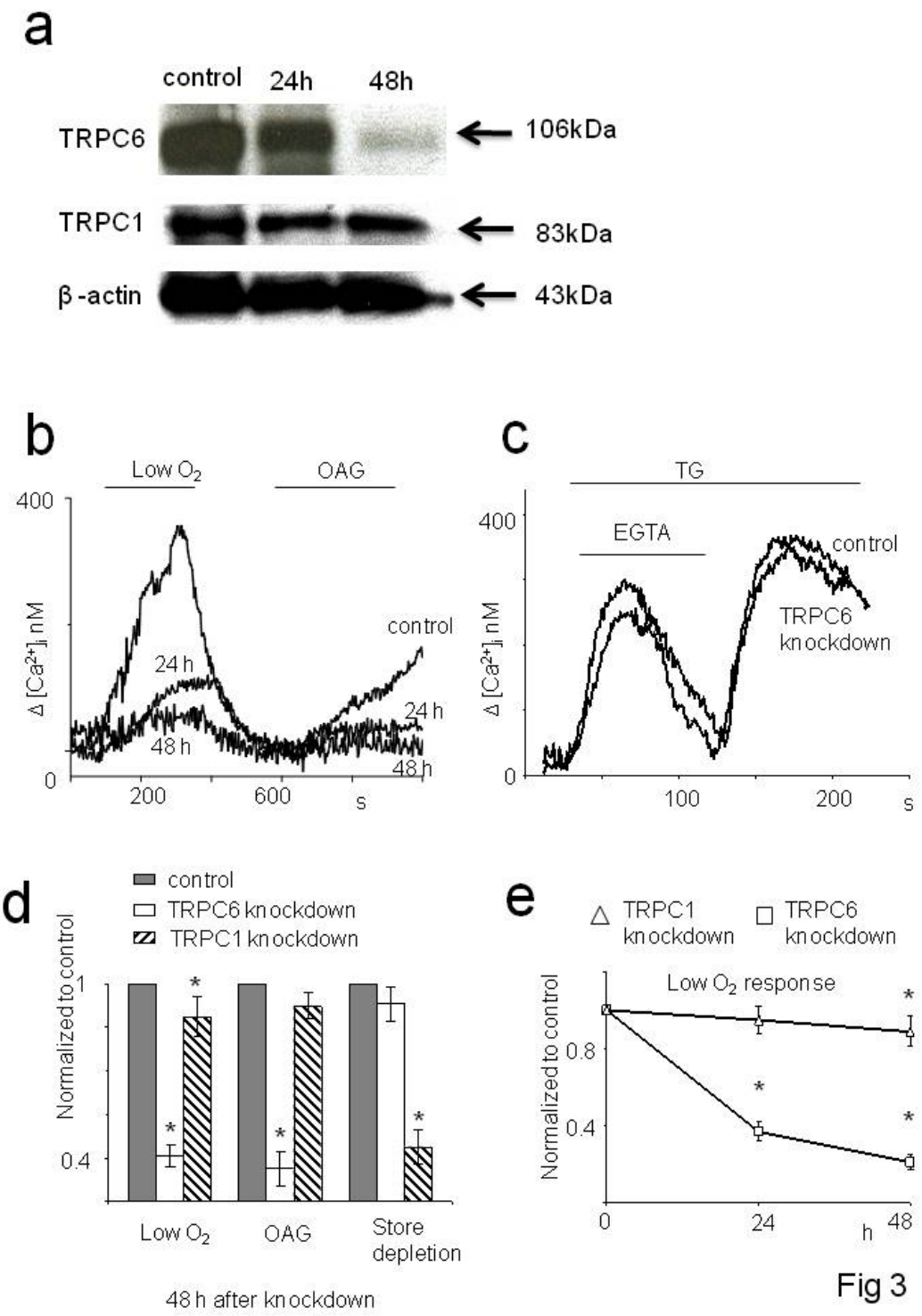
SKF96365
Low O2
Low O2
400
Δ [Ca2+]i nM
0
0
600
1200
1800
s
highk
SKF96365
highk
340/380
1
0
0
800
1200
s
b
Low O2
Low O2
Low O2
2APB
400
Δ [Ca2+]i nM
0
200
800
s
2APB
highk
highk
highk
340/380
1
0
0
600
1200
1800
s
c
KBR7943
Low O2
Low O2
Low O2
Δ [Ca2+]i nM
400
0
500
1500
s
d
Normalized
0.8
0.4
0
-0.4
-0.8
*
*
*
*
*
*
0 Ca
nifedipine
verapmil
kBR
2APB
SKF

Fig 2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control
24h
48h
TRPC6
106kDa
TRPC1
83kDa
β-actin
43kDa
b
Low O2
OAG
400
Δ [Ca2+]i nM
0
control
24 h
48 h
24 h
48 h
200
600
s
c
TG
EGTA
400
control
TRPC6
knockdown
Δ [Ca2+]i nM
0
100
200
s
d
control
TRPC6 knockdown
TRPC1 knockdown
Normalized to control
1
0.4
Low O2
OAG
Store
depletion
48 h after knockdown
e
TRPC1
knockdown
TRPC6
knockdown
Low O2 response
Normalized to control
0.8
0.4
0
24
h
48

Fig 3

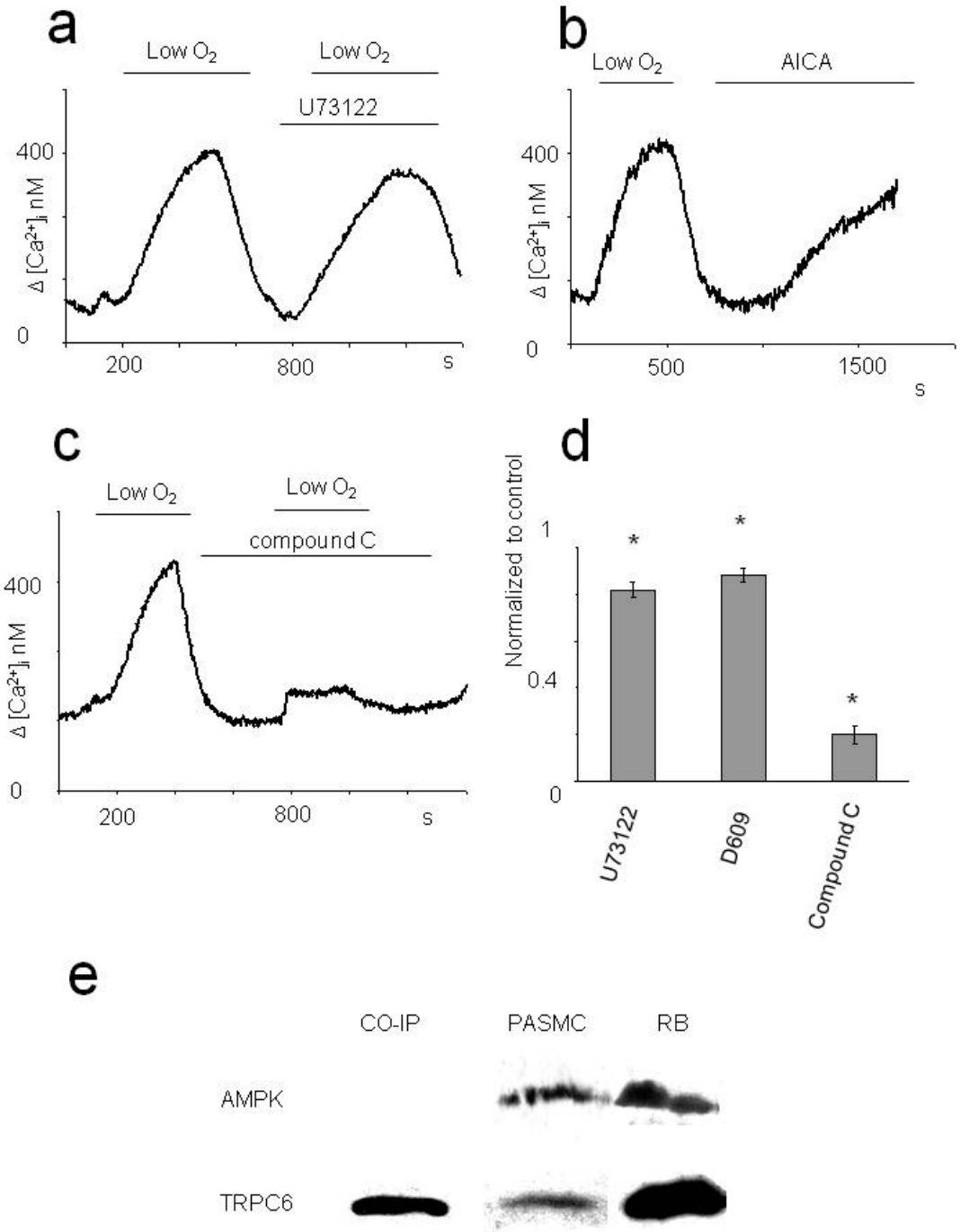
a
Low O2
Low O2
U73122
400
Δ [Ca2+]i nM
0
200
800
s
b
Low O2
AICA
400
Δ [Ca2+]i nM
0
500
1500
s
c
Low O2
Low O2
compound C
400
Δ [Ca2+]i nM
0
200
800
s
d
Normalized to control
1
0.4
0
*
*
*
U73122
D609
Compound C
e
CO-IP
PASMC
RB
AMPK
TRPC6

Fig 4