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RESEARCH PAPER

K_V7 channels are involved in hypoxia-induced vasodilatation of porcine coronary arteries

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BACKGROUND AND PURPOSE

Hypoxia causes vasodilatation of coronary arteries, but the underlying mechanisms are poorly understood. We hypothesized that hypoxia reduces intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by opening of K channels and release of H_2S.

EXPERIMENTAL APPROACH

Porcine coronary arteries without endothelium were mounted for measurement of isometric tension and $[Ca^{2+}]_i$, and the expression of voltage-gated K channels K_V7 channels (encoded by KCNQ genes) and large-conductance calcium-activated K channels ($K_{Ca}1.1$) was examined. Voltage clamp assessed the role of K_V7 channels in hypoxia.

KEY RESULTS

Gradual reduction of oxygen concentration from 95 to 1% dilated the precontracted coronary arteries and this was associated with reduced $[Ca^{2+}]_i$ in $PGF_{2\alpha}$ (10 μM)-contracted arteries whereas no fall in $[Ca^{2+}]_i$ was observed in 30 mM K-contracted arteries. Blockers of ATP-sensitive voltage-gated potassium channels and $K_{Ca}1.1$ inhibited hypoxia-induced dilatation in $PGF_{2\alpha}$-contracted arteries; this inhibition was more marked in the presence of the K_V7 channel blockers, XE991 and linopirdine, while a $K_V7.1$ blocker, failed to change hypoxic vasodilatation. XE991 also inhibited H_2S- and adenosine-induced vasodilatation. PCR revealed the expression of $K_V7.1$, $K_V7.4$, $K_V7.5$ and $K_{Ca}1.1$ channels, and $K_{Ca}1.1$, $K_V7.4$ and $K_V7.5$ were also identified by immunoblotting. Voltage clamp studies showed the XE991-sensitive current was more marked in hypoxic conditions.

CONCLUSION

The $K_V7.4$ and $K_V7.5$ channels, which we identified in the coronary arteries, appear to have a major role in hypoxia-induced vasodilatation. The voltage clamp results further support the involvement of K_V7 channels in this vasodilatation. Activation of these K_V7 channels may be induced by H_2S and adenosine.

Abbreviations

4-AP, 4-aminopyridine; AOA, amino-oxyacetate; $K_{Ca}1.1$, large-conductance calcium-activated K channels; $[Ca^{2+}]_i$, intracellular calcium; IbTX, iberiotoxin; K_{125}PSS, PSS containing 125 mM K; K_{30}PSS, PSS containing 30 mM K; K_{ATP}, ATP-sensitive K channels; K_V, voltage-gated K channels; PPG, propargyl glycine; XE991, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride

Introduction

Coronary artery dilatation to hypoxia is a fast, protective response that increases flow to endangered myocardium. The hypoxic vasodilatation is of importance for vasomotor tone in the coronary microcirculation but is also present in conduit vessels (Wadsworth, 1994; Barron and Gu, 2000; Shimizu *et al.*, 2000). While the results from clinical trials of oxygen therapy are awaited (Stub *et al.*, 2012), the basal mechanisms linking oxygen concentration and coronary vascular tone need to be elucidated. In porcine coronary arteries, NO contributes to hypoxia-induced vasodilatation, but hypoxia still induces significant vasodilatation in arteries without endothelium (Hedegaard *et al.*, 2011).

Hypoxia is known to have a direct effect on the vascular smooth muscle, and hypoxia-induced vasodilatation may be dependent or independent of changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Shimizu *et al.*, 2000; Thorne *et al.*, 2004). Hypoxic vasodilatation independent of changes in vascular smooth muscle calcium has been ascribed to $[Ca^{2+}]_i$ desensitization (Somlyo and Somlyo, 2003; Wardle *et al.*, 2006), or may be mediated by force suppression due to thin filament phosphorylation of, for example heat shock protein 20 (Rembold *et al.*, 2000; Frobert *et al.*, 2005; Meeks *et al.*, 2005). Hypoxia-induced vasodilatation with a reduction in smooth muscle $[Ca^{2+}]_i$ can involve K channel opening (Lopez-Barneo *et al.*, 2004) and/or L-type Ca^{2+} channel closure (Franco-Obregon and Lopez-Barneo, 1996; Smani *et al.*, 2002). Several types of K channels including ATP-sensitive K channels (K_{ATP}) in the coronary circulation (Daut *et al.*, 1990; Dart and Standen, 1995; Liu and Flavahan, 1997; Lee *et al.*, 1998; Kamekura *et al.*, 1999), large-conductance calcium-activated K channels ($K_{Ca}1.1$, also known as BK_{Ca}) in coronary and cerebral arteries (Gebremedhin *et al.*, 1994; Nelson and Quayle, 1995; Lopez-Barneo *et al.*, 2004) and voltage-gated K_V channels of the $K_V1.5$ and $K_V2.1$ subtype in the coronary circulation (Shimizu *et al.*, 2000; Thorne *et al.*, 2002) have been suggested to be O_2 sensors and/or involved in hypoxic vasodilatation. Thus, the type of K channel involved in hypoxia-induced coronary vasodilatation is contentious. Furthermore, K_V7 channel openers were recently suggested to counteract changes induced by chronic hypoxia in the pulmonary circulation (Morecroft *et al.*, 2009), and, therefore, these channels may also be involved in hypoxic vasodilatation. The nomenclature used to denote the K channels conforms to Br J Pharmacol's *Concise Guide to PHARMACOLOGY* (Alexander *et al.*, 2013).

K_V7 blockers increase vascular tone in a number of rodent blood vessels including the portal vein, aorta and mesenteric, pulmonary and cerebral arteries (Yeung and Greenwood, 2005; Joshi *et al.*, 2006; Yeung *et al.*, 2007; Mackie *et al.*, 2008; Zhong *et al.*, 2010). Experiments have also revealed that activators of $K_V7.2$–7.5 channels, retigabine and flupirtine, cause relaxation (Yeung *et al.*, 2007; 2008b; Mackie *et al.*, 2008). Moreover, K_V7 channels sensitive to the blocker XE991 are involved in H_2S-induced relaxation in rat aorta (Schleifenbaum *et al.*, 2010). Also H_2S may act as an oxygen sensor and cause vasodilatation (Skovgaard *et al.*, 2011; Whiteman *et al.*, 2011). Therefore, based on these observations, we hypothesized that hypoxia reduces smooth muscle $[Ca^{2+}]_i$ by opening K channels and the release of H_2S.

To investigate this hypothesis, the following measurements were performed: (i) changes in $[Ca^{2+}]_i$ were measured to determine whether lowering $[Ca^{2+}]_i$ contributes to hypoxia-induced vasodilatation; (ii) the involvement of different K channels was investigated using selective K channel blockers; (iii) the presence of K_V7 channels was established by PCR and immunoblotting; (iv) XE991-sensitive currents were investigated by voltage patch clamp; (v) the role of H_2S was examined by inhibition of H_2S synthesis in hypoxic conditions.

Methods

Hearts from Landrace-Yorkshire hogs were obtained at a local slaughterhouse. All experiments conformed to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, 2010).

Chemicals and materials

SNP was purchased from VWR-Bie & Berntsen (Herlev, Denmark). 4-Aminopyridine (4-AP), glibenclamide, $PGF_{2\alpha}$, TEA, flupirtine, linopirdine, chromanol 293B, XE991 (10,10-*bis*(4-pyridinylmethyl)-9(10*H*)-anthracenone dihydrochloride), sodium hydrosulfide (NaHS), propargyl glycine (PPG) and amino-oxyacetate (AOA) were obtained from Sigma-Aldrich, St Louis, MO, USA. Iberiotoxin (IbTX) was purchased from Latoxan, Valence, France. The composition of PSS was (mM): NaCl 119 , KCl 4.7, $MgSO_4 \bullet 7H_2O$ 1.2 , $NaHCO_3$ 25, KH_2PO_4 1.2, EDTA 0.027, glucose 10, $CaCl_2$ 1.6, adjusted to pH 7.4. In PSS containing 125 mM or 30 mM K (K_{125}PSS and K_{30}PSS, respectively), NaCl was replaced by KCl on an equimolar basis to achieve the desired concentration.

Isometric tension recordings

Immediately after the animals had been killed, the coronary circulation was perfused with physiological salt solution similar to PSS except that it contained glucose 5.5 mM, bubbled with 5% CO_2 in O_2 and was buffered with HEPES [for composition see (Pasgaard *et al.*, 2007)]. The hearts were bathed in this solution at 5°C until the start of the experiment. The left anterior descending coronary artery was carefully dissected and cut into 2 mm segments. In all preparations, the endothelium was removed by means of either a cotton bud or rubbing with a hair. For isometric tension recordings, artery segments were mounted in a four-chamber wire myograph (Tissue Bath System 700MO, Danish Myo Technology, Aarhus, Denmark) containing PSS bubbled with 95% O_2, 5% CO_2 at 37°C and the artery tension was normalized according to a standard procedure (Mulvany and Warshaw, 1979). Preparation viability was examined by exposing arteries to K_{125}PSS. Removal of the endothelial layer was evaluated by lack of response (<10% vasodilatation) to bradykinin (30 nM) in $PGF_{2\alpha}$ (10 μM)-contracted arteries.

Appropriate gas composition was obtained by mixing 95% O_2, 5% CO_2 with 95% N_2, 5% CO_2 (Digamix, H. Wösthoff GmbH, Bochum, Germany or Environics 4040, Tolland, CT, USA). The organ bath was bubbled through pimpstones allowing rapid equilibration of the oxygen tension. Oxygen

concentration was measured with an oxygen electrode (Unisense, Aarhus, Denmark). An oxygen concentration-response curve was made by maintaining the oxygen level until the arterial tone reached a steady state before changing to the next level.

Arteries were contracted with $PGF_{2\alpha}$ (10 μM) or K_{30}PSS. The experiments were run in parallel, blockers were administered as a pretreatment before the addition of $PGF_{2\alpha}$ and $PGF_{2\alpha}$ contracted artery segments served as controls. The following incubation periods and concentrations were used: 4-AP (0.5 mM), IbTX (100 nM), glibenclamide (3 μM), PPG (10 mM) and AOA (5 mM), TEA (1 and 10 mM), chromanol 293B (10 μM), XE991 (10 μM) and linopirdine (10 μM) all 30 min. Arteries were allowed to attain a stable tone before dilatation was induced by gradually reducing oxygen concentration.

Simultaneous measurements of $[Ca^{2+}]_i$ and tension

Arterial segments were mounted in a single chamber myograph and bubbled with 20% O_2, 5% CO_2 in N_2. A load mix was made from 1 mL DMSO, 200 μL cremophore and 4 mg pluronic acid. A total of 50 μg fura-2AM was dissolved in 20 μL of load mix and added to the chamber resulting in a final fura-2AM concentration of 6.5 $\mu g \cdot mL^{-1}$ as previously described (Rodriguez-Rodriguez *et al.*, 2008). Arteries were loaded for 3 h and subsequently washed thoroughly. The myograph was placed on an inverted microscope and force and fluorescence intensities were measured simultaneously at a sampling rate of 5 Hz using a computer equipped with dedicated software (Felix, Photon Technology International, South Brunswick, NJ, USA). Excitation wavelengths of 340 and 380 nm were used and emission signals measured through a 530 nm filter. At the end of the experiment, 10 μM ionomycin was added to PSS to achieve maximal saturation of Fura-2 with calcium. Then the fluorescence was quenched using nominally calcium-free PSS and 15 mM $MnCl_2$ to measure the background signal, which was subtracted from the recordings.

PCR

Porcine arterial samples were kept in RNA later buffer RLT (Qiagen, Copenhagen, Denmark) until homogenization in 350 μL RLT with β-mercaptoethanol and 5 μL carrier RNA and homogenized for 3 min in Tissuelyser from Qiagen. The samples were centrifuged for 2 min at 13 000 x *g*. The isolation of total RNA from the arteries was done with the RNeasy plus mini kit by using Qiacube (Qiagen) according to the manufacturer's instructions, after which cDNA was synthesized from total RNA using SuperScriptIII reverse transcriptase, SuperAse In and random decamer primers according to the manufacturer's instructions. As K_V7 channels had not previously been examined in the pig, Stratagene reference total mRNA from human ventricle (Stratagene, La Jolla, CA, USA) was included as a positive control for the expression of K_V7 channels. Two microlitres of cDNA was amplified in a thermal cycler (Peqlab, Eurofins MWG Operon, Ebersberg, Germany) in a reaction (25 μL) containing 0.2 mM deoxynucleoside 5′-triphosphate mix, 0.4 μM of each primer, and 0.03 U of TaKaRa Ex Taq Hot start version DNA polymerase. A 'hot-start' procedure was employed (1 min at 95°C) and thermal cycling conditions were 95°C, 12 s, 56–58°C, 1 min and 70°C, 45 s for 40 cycles with a final extension of 4 min at 72°C. PCR reaction products were resolved by agarose gel electrophoresis (2.5% w v^{-1}) and stained with ethidium bromide (0.5 $\mu g \cdot mL^{-1}$). Two or more sets of primers were used for each gene. The primers and expected product size are listed in Table 1. To determine the identity of the amplification products the agarose gel bands were excised and the DNA purified using a Qiaquick gel extraction kit (Quiagen). The purified PCR product was sequenced by Eurofins MWG Operon (Hedegaard and Mogensen, 2013a,b,c,d,e).

Immunoblotting

For immunoblotting of $K_V7.4$ and $K_V7.5$, arteries were frozen in cold acetone/dry ice (20 g/20 mL) at –78°C. For immunoblotting of $K_{Ca}1.1\alpha$ and $K_{Ca}1.1\beta$, the arteries were frozen at –80°C at the end of the experiment. In both cases, protein was extracted in lysis buffer using a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The samples were exposed to three cycles at 5000 rpm for 30 s each. After homogenization, they were left on ice for 20 min before being centrifuged for 15 min at 13,000 x *g* at 4°C. The supernatant was transferred to a new tube and frozen at –80°C. Total protein was quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Protein lysate was mixed with sample buffer and loaded with a pre-stain marker (Bio-Rad) onto the gel. The proteins were separated by SDS-PAGE through a 4–12% Criterion XT Bis-Tris gel (Bio-Rad) at 200 V in a criterion cell (Bio-Rad). Transfer to a membrane was achieved for 1 h at 100 V in a criterion blotter (Bio-Rad). For detection of $K_{Ca}1.1\alpha$ and $K_{Ca}1.1\beta$, the membrane was washed for 10 min in Tris buffered saline with Tween 20 (TBS-T) and blocked in 5% skimmed milk in TBS-T for 2 h before being incubated overnight at 4°C with primary antibody against $K_{Ca}1.1\alpha$ (1:1000) (Alomone, Jerusalem, Israel) and $K_{Ca}1.1\beta$ (1:200) (Abcam, Cambridge, UK). The membrane was washed in TBS-T before incubation with secondary antibody goat anti-rabbit IgG conjugated to HRP (Santa-Cruz Biotechnology, Santa Cruz, CA, USA) 1:4000 in TBS-T with 5% skimmed milk followed by washing. For detection of $K_V7.4$ and $K_V7.5$ channels, the membrane was washed for 10 min in TBS-T before it was blocked for 2 h in 0.3% I-block (Applied Biosystems, Foster City, CA, USA) and incubated overnight at 4°C with primary antibody ($K_V7.4$ 1:50 SC50417 and $K_V7.5$ 1:100 SC50416, Santa-Cruz Biotechnology), where specificity was previously tested in human embryonic kidney cells overexpressing the channels (Jepps *et al.*, 2011; Zhang *et al.*, 2011). The membrane was washed in PBS with Tween 20 before incubation with secondary antibody goat anti-rabbit IgG conjugated to HRP (Santa-Cruz Biotechnology) followed by washing. The membranes were developed by using the ECL-Plus kit (GE Healthcare, Copenhagen, Denmark). The blot was placed in an X-ray film cassette and developed in photographic developer.

Voltage clamp

To assess membrane conductance, smooth muscle cells from the left anterior descending coronary artery were isolated by incubation with an enzyme solution containing collagenase (1.5 $mg \cdot mL^{-1}$ type I, Worthington Biochem Corp., Lakewood,

Table 1

Primers used for PCR

Gene	Sense	Antisense	Bp
KCNQ1	(+) 5′-ttcctccagatcctgcggatgc-3′	(–)5′-gttgggctcttccttacagaact-3′	716
	(+) 5′-tcatcttctcctcctacttcgtg-3′	**(–) 5′-tgttggttctcatgaaatggg-3′**	633
KCNQ2	**(+) 5′-tctacatcctggaaatcgtgact-3′**	**(–) 5′-tcagcagctccagctggttcag-3′**	777
	(+) 5′-aacgtctttgccacatctgcgct-3′	(–) 5′-cacgatctttcaaactgaccttc-3′	676
	(+) 5′-tcggtgtctccttcttcgcgct-3′	(–) 5′-tgtcatccacaatgtcctctcc-3′	579
	(+) 5′-ttctacgccaccaacctctcg-3′	(–) 5′-acagcatgtccaggtggccg-3′	621
KCNQ3	(+) 5′-ccttccacttagacatgacgttc-3′	(–) 5′-acagccataagcttcaaactcag-3′	865
	(+) 5′-ctgagtttgaagcttatggctgt-3′	(–) 5′-acgactgattcatagaacctcca-3′	932
	(+) 5′-gtattatgctaccaaccccaaca-3′	(–) 5′-atatggttcattcctgggagatt-3′	661
	(+) 5′-ggaaagctatttaccctctgaa-3′	**(–) 5′-ggaatacctgttgtcctccttct-3′**	711
KCNQ4	(+) 5′-ggccacctggtactactatgaca-3′	(–) 5′-tcttcacagcaggcataacatcg-3′	506
	(+) 5′-agaagagctaccagagcgaact-3′	**(–) 5′-cgtcccatcatactgatttcatc-3′**	314
KNCQ5	(+) 5′-tctgcaaaaactcagggtaacat-3′	(–) 5′-tcctccactgatacatcacactg-3′	944
	(+) 5′gccagaaacactttgagaaaaga-3′	**(–) 5′-acatcatatgggcgtaatgtttc-3′**	619
Kcnma1	**(+) 5′-ggatgcgctcatcatcccggt-3′**	**(–) 5′-ggcgcccgagtgtggtttttg-3′**	902
	(+) 5′-tcctgtcagccaatcagaataat-3′	(–) 5′-ctcttcctgcacgtacttctgtt-3′	909

The primers in bold are the ones depicted in Figure 4.

NJ, USA) in nominally Ca^{2+}-free solution (in mM): 120 NaCl, 6 KCl, 1.2 $MgCl_2$, 10 glucose, 10 HEPES at pH 7.3 adjusted with NaOH. A few 2–3 mm segments of coronary arteries were placed in a microtube containing an enzyme solution and incubated at 37°C for 10–15 min. Single cells were released by trituration with a polyethylene pipette into the nominally Ca^{2+}-free solution and, then, added into Petri dish containing the bath solution. Cells attached to the bottom of Petri dish were used in the whole-cell voltage clamp experiments 20 min after isolation during next 2.5 h.

The bath solution contained (in mM): 120 NaCl, 4.2 KCl, 1.2 KH_2PO_4, 0.5 $MgSO_4$, 3 $NaHCO_3$, 1.8 $CaCl_2$, 10 HEPES and 10 glucose at pH 7.4 adjusted with NaOH. During experiments, the oxygen tension in the bath solution was modified by gassing the solution with either 20% O_2 and 5% CO_2 in N_2 or 5% CO_2 in N_2 (this gives a concentration of 1% O_2) for a minimum of 20 min and continued during the whole experiment. The patch pipette solution contained (in mM) 110 potassium gluconate, 30 KCl, 5 HEPES, 0.5 $MgCl_2$, 5 Na_2ATP, 1 GTP and 0.1 EGTA at pH 7.2 adjusted with KOH (Zhong *et al.*, 2010).

Patch pipettes were prepared from borosilicate glass (PG15OT-7.5; Harvard Apparatus, Kent, UK) and fire polished to achieve tip resistances in the range of 4–8 MΩ. Membrane current recordings were made with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) in whole-cell configuration at room temperature (22–24°C). Data acquisition and analysis were done with software packages Clampex 9.0 for Windows (Axon Instruments) and Microcal Origin v.8.1 for Windows (Microcal Software, Northampton, MA, USA). Only cells with essentially no leak current (seal resistance ≥1.5 GΩ) and a low access resistance (5–10 MΩ) were used. Series resistance and capacitive current were routinely compensated for electronically. The stability of these parameters was tested regularly during the course of the experiment, that is before and after each intervention.

Current-voltage (*I-V*) relations were determined using 500 ms step pulses to between –95 and +55 mV in increments of 10 mV from a holding potential of –75 mV. Averaged current amplitude at the last 50 ms of voltage-step pulses was used to construct *I-V* relations. *I-V* relations were determined for control conditions and after treatment with 10 μM XE991. The XE991-sensitive current was calculated by subtraction of the *I-V* relation after XE991 treatment from the *I-V* relation under control conditions.

Data analysis and statistics

Data are presented as means ± SEM. Paired *t*-tests were used to analyse whether incubation with different blockers altered the baseline tone, contraction and coronary artery tone remaining at 1% O_2. Dilatation responses were calculated as percentage reduction of the contraction induced by either K_{30}PSS or $PGF_{2\alpha}$ (10 μM). The response curves to decreasing oxygen concentrations were compared by means of two-way ANOVA followed by a Bonferroni *post hoc* test or by a *t*-test. When the effect of a combination treatment with two different K channel blockers was compared to the effect of single channel-block treatment, the two control groups were pooled and concentration-response curves were compared by means of two-way ANOVA for concentration-dependence and effect of treatment.

In experiments where tension recordings and $[Ca^{2+}]_i$ measurements were performed simultaneously, responses were compared by means of an unpaired *t*-test. The background signal at each of the excitation wavelengths was subtracted from the fluorescence intensities and the ratio F_{340}/F_{380}

was calculated. The change in ratio that occurred with hypoxia was expressed as a percentage of the ratio increase induced by the contracting agent (K_{30}PSS or $PGF_{2\alpha}$). $P < 0.05$ was considered significant.

Results

Role of $[Ca^{2+}]_i$ in hypoxia-induced dilatation in porcine coronary arteries

Figure 1A is a typical trace illustrating hypoxia-induced dilatation in a $PGF_{2\alpha}$-contracted coronary artery. In Figure 1B, a typical trace for hypoxia-induced dilatation in a 30 mM K-depolarized artery is depicted. In $PGF_{2\alpha}$-contracted arteries and in K_{30}PSS-contracted arteries, a gradual reduction in oxygen concentration caused oxygen-dependent dilatation ($P < 0.0001$, $n = 6$, Figure 1C) and the coronary artery tone remaining at 1% O_2 was reduced from 50 ± 8% in K_{30}PSS to 11 ± 4% in $PGF_{2\alpha}$-contracted arteries. The contractile tension evoked by K_{30}PSS did not differ from that induced by $PGF_{2\alpha}$ (23 ± 4 Nm^{-1} versus 23 ± 5 Nm^{-1} respectively, $P = 0.9$, $n = 6$).

Simultaneous tension recordings and $[Ca^{2+}]_i$ measurements were performed to investigate to what extent hypoxia-induced dilatation was dependent on lowering of $[Ca^{2+}]_i$ or rather caused by Ca^{2+}-desensitization and/or force suppression. $PGF_{2\alpha}$ (10 µM) and K_{30}PSS caused similar increases in $[Ca^{2+}]_i$ (Figure 2A and 2B). In $PGF_{2\alpha}$-contracted arteries, an abrupt reduction from 20 to 1% O_2 lowered $[Ca^{2+}]_i$ and induced vasodilatation (Figure 2A and 2C). However, in K_{30}PSS-contracted arteries, hypoxia induced a smaller degree of vasodilatation than in $PGF_{2\alpha}$-contracted arteries, but without decreasing the level of $[Ca^{2+}]_i$ (Figure 2B and 2C) suggesting that depolarization with K prevents the reduction in $[Ca^{2+}]_i$ caused by hypoxia.

Effect of K channel blockers on hypoxia-induced dilatation

As the hypoxia-induced dilatation was smaller in K-contracted arteries than in those contracted with $PGF_{2\alpha}$, we investigated the effect of blocking various K channels. Incubation with the voltage-dependent K channel blocker 4-AP (0.5 mM) and the K channel blocker, TEA (10^{-2} M) increased baseline artery tone, but not the response to $PGF_{2\alpha}$ as compared to vehicle-treated preparations. Incubation with blockers of $K_{Ca}1.1$ channels, TEA (10^{-3} M) and IbTX or a blocker of ATP-sensitive K channels, glibenclamide did not alter baseline tone or response to $PGF_{2\alpha}$ as compared to parallel control preparations (Table 2).

The K channel blocker, TEA significantly inhibited dilatation induced by lowering O_2 (10^{-3} M $P < 0.05$, $n = 7$ and 10^{-2} M $P < 0.001$, $n = 7$, Table 2). Furthermore, we observed an effect of TEA on adenosine-induced dilatation (10^{-3} M $P < 0.001$, $n = 7$ and 10^{-2} M $P < 0.001$, $n = 7$, data not shown). Blocking of K_V channels with 4-AP, large-conductance $K_{Ca}1.1$ channels with IbTX or K_{ATP} channels with glibenclamide reduced hypoxia-induced dilatation (Table 2). Blocking K_{ATP} channels with glibenclamide caused a pronounced inhibition of dilatation induced by cromakalim ($n = 7$), an opener of K_{ATP} channels, while these dilatations were not affected by incubation with 4-AP ($n = 6$).

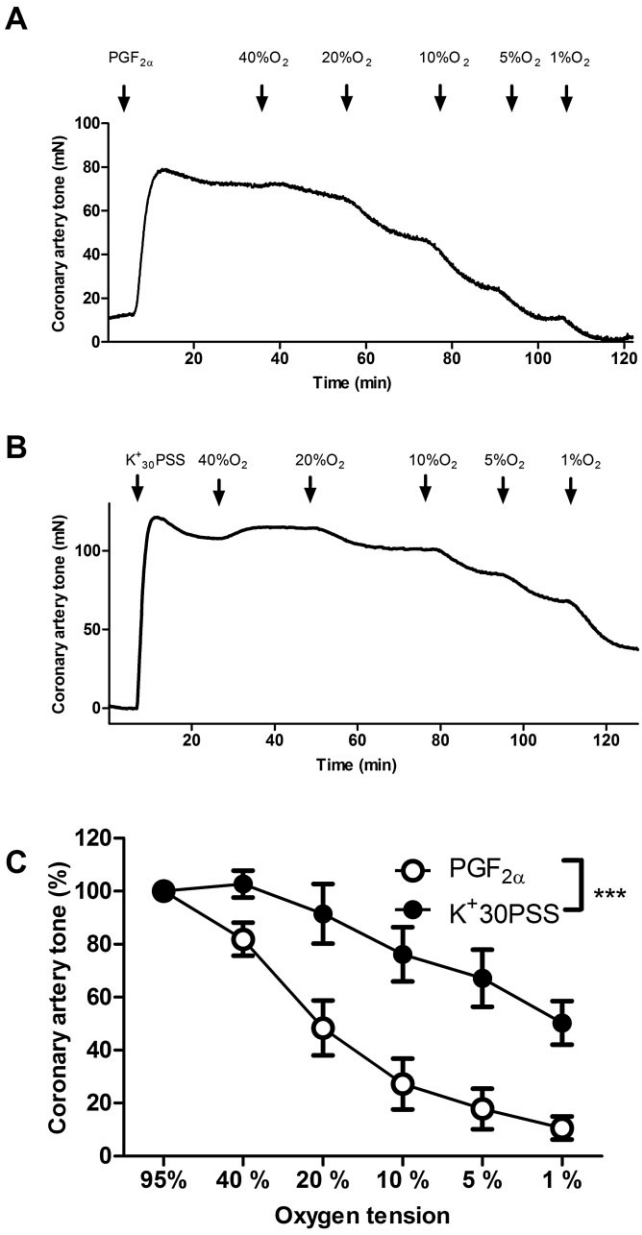

Figure 1

Hypoxia-evoked vasodilatation in porcine coronary artery. Original tracing illustrating the dilatation induced by decreasing O_2 concentration in the organ bath (A) $PGF_{2\alpha}$ (10 µM)- and (B) K_{30}PSS-contracted porcine coronary artery without endothelium. (C) Concentration-response curves for O_2 lowering in coronary arterial segments without endothelium contracted with K_{30}PSS or $PGF_{2\alpha}$ (10 µM) ($n = 6$, *$P < 0.05$ by two-way ANOVA).

To investigate whether the effects of K channel blockers on hypoxia-induced dilatation were additive, we used a combination of different K channel blockers. The combination of 4-AP and IbTX (blockers of K_V and $K_{Ca}1.1$ channels, respectively) significantly diminished hypoxia-induced dilatation (Table 2) as did a combination of 4-AP and glibenclamide (blockers of K_V and K_{ATP} channels respectively) (Table 2). However, combinations of either 4-AP and IbTX or 4-AP and

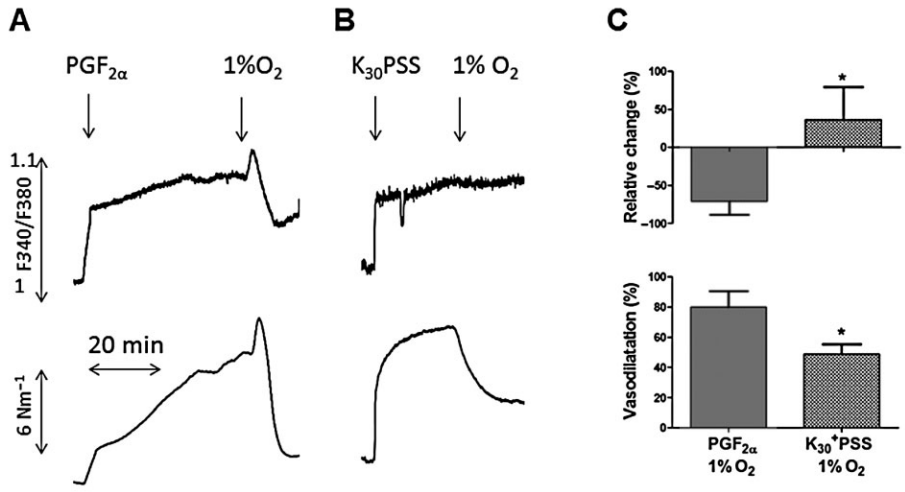

Figure 2

Effects of hypoxia on $[Ca^{2+}]_i$ and tension in pig coronary artery. Effect of hypoxia on (A) $PGF_{2\alpha}$ (10 μM)- and (B) 30 mM K ($K_{30}PSS$)-contracted arteries without endothelium. (C): Average data showing the change in $[Ca^{2+}]_i$ and tension in $PGF_{2\alpha}$- and $K_{30}PSS$-contracted preparations in response to reducing organ bath O_2 concentration from 20 to 1% (n = 6, *P < 0.05 by Student's t-test).

Table 2

Effect of K channel blockers on hypoxia-induced dilatation

	n	Baseline tone (Nm^{-1})	$PGF_{2\alpha}$ contraction (Nm^{-1})	Coronary artery tone (%) remaining at 1% O_2
Control	7	1.2 ± 2.2	34.6 ± 10.5	22.6 ± 9.8
TEA 10^{-3} M	7	2.6 ± 2.2	35.1 ± 7.5	30.2 ± 17.4
TEA 10^{-2} M	7	18.3 ± 12.3*	37.0 ± 10.1	37.0 ± 22.8
Control	12	0.1 ± 0.1	18.2 ± 1.0	14.1 ± 2.7
4-AP	12	2.4 ± 0.8*	19.8 ± 1.5	30.4 ± 2.7*
Control	6	–0.1 ± 0.1	24.3 ± 3.4	14.5 ± 1.9
IbTx	6	1.9 ± 1.1	22.0 ± 2.5	27.6 ± 1.9*
Control	7	0.1 ± 0.1	18.6 ± 1.8	16.5 ± 4.4
Glib	7	–0.1 ± 0.3	19.8 ± 2.8	20.2 ± 3.4*
Control	6	0.9 ± 0.6	21.2 ± 1.8	18.7 ± 3.0
4-AP+IbTx	6	8.6 ± 3.2*	29.3 ± 3.5	34.5 ± 5.7*
Control	6	1.0 ± 0.7	16.6 ± 1.1	14.0 ± 2.4
4-AP+Glib	6	3.8 ± 1.3*	18.3 ± 1.6	33.6 ± 8.7*
Control	12	–0.4 ± 0.7	25.5 ± 1.8	17.5 ± 5.2
XE991	11	4.8 ± 2.7*	18.7 ± 2.6	49.2 ± 2.9*
Linopirdine	7	3.0 ± 3.1*	19.84 ± 2.0	42.7 ± 2.3*
Control	7	2.0 ± 1.4	26.1 ± 2.5	11.9 ± 4.3
XE991+4-AP+IbTx	7	22.3 ± 3.0*	17.5 ± 4.0	62.1 ± 2.0*
Control	5	–0.5 ± 0.3	23.7 ± 1.7	26.9 ± 1.6
Chromanol 293B	5	–0.8 ± 1.1	20.2 ± 4.6	30.0 ± 4.3

Effect on baseline tone, $PGF_{2\alpha}$ contraction and coronary artery tone (%) remaining at 1 % O_2 in the absence and presence of K channels blocker. Results are means +/– SEM. n = number of animals examined. *P<0.05 by Student's t-test.

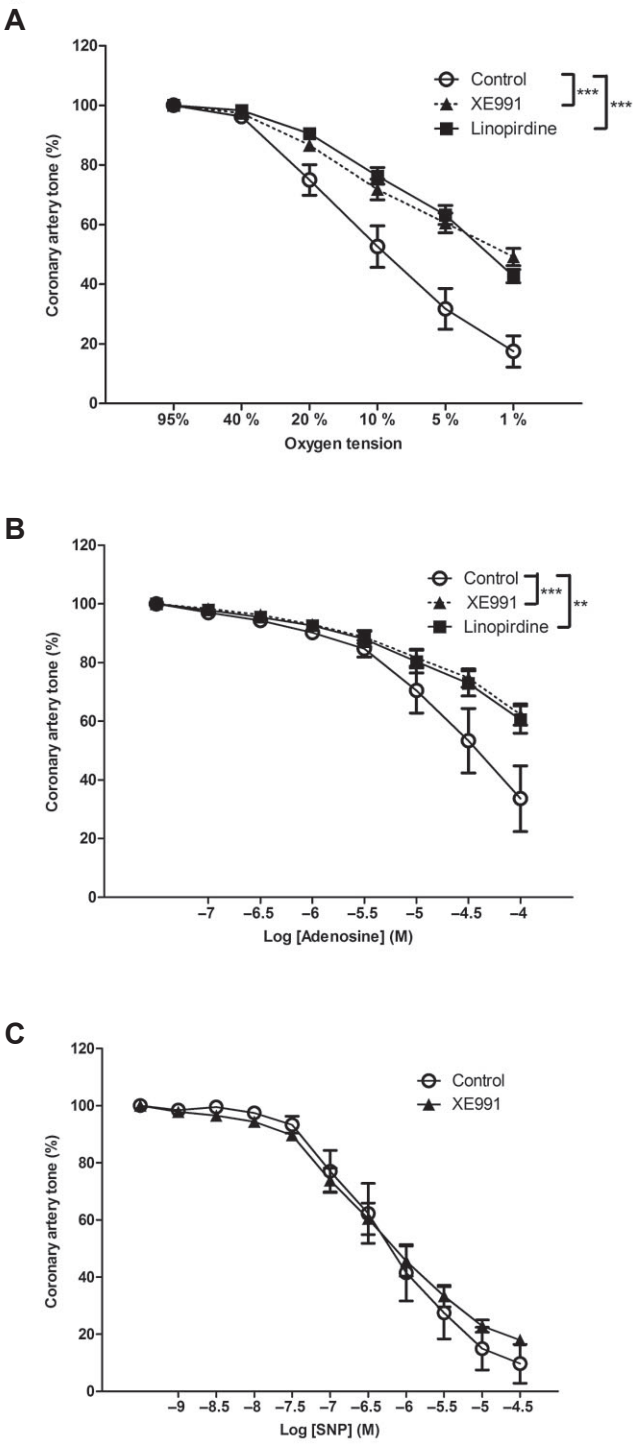

Figure 3

Effect of K_V7 channel inhibition on hypoxia-induced dilatation in pig coronary artery. (A) Concentration-response curves for O_2 lowering in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the $K_V7.1$–7.5 inhibitors XE991 (10 μM) and linopirdine (10 μM) (n = 7–12, ***P < 0.001 by two-way ANOVA). (B) Concentration-response curves for adenosine in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the $K_V7.1$–7.5 inhibitor XE991 (10 μM) and linopirdine (10 μM) (n = 5, **P < 0.01, ***P < 0.001 by two-way ANOVA). (C) Concentration-response curves for SNP in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the $K_V7.1$–7.5 inhibitors XE991 (10 μM) (n = 4, P = 0.65 by two-way ANOVA).

glibenclamide did not add to the blocking effect of either blocking agent alone (Table 2).

Role of K_V7 channels

Blocking the KCNQ encoded K_V7 channels by either XE991 (10 μM) or linopirdine (10 μM) reduced hypoxia-induced dilatation (P < 0.0001, n = 11 and P < 0.0001, n = 7 Figure 3A). Blocking $K_V7.1$ with chromanol 293B (10 μM) failed to inhibit hypoxia-induced dilatation (Supporting Information Figure S1 and Table 2). Incubation with either XE991 (10 μM) or linopirdine (10 μM) increased baseline artery tone as compared to controls, whereas incubation with chromanol 293B did not alter baseline tone (Table 2), neither of them changed the response to $PGF_{2\alpha}$. A combination of XE991, 4-AP and IbTx showed an additional effect as compared to XE991 alone (see Table 2 and Supporting Information Figure S4).

Adenosine-induced dilatations were also inhibited by XE991 (10 μM) and linopirdine (10 μM) (P < 0.001 n = 5 and P < 0.01 n = 5 Figure 3B) whereas chromanol 293B (10 μM) did not affect adenosine-induced dilatations (P = 0.12, n = 5 results not shown). Blockade of K_V7 channels with XE991 caused a pronounced inhibition of dilatations induced by flupirtine (P < 0.001, n = 7, results not shown), an opener of $K_V7.2$–7.5 channels, while these dilatations were not affected by incubation with chromanol 293B (P = 0.85, n = 7, results not shown). To test the selectivity of XE991, we used SNP to induce vasodilatation and found this response was not affected by the presence of XE991 (P = 0.65, n = 4, Figure 3C).

PCR and immunoblotting

We examined the presence of $K_V7.1$–$K_V7.5$ and $K_{Ca}1.1$ (BK_{Ca}) mRNA by reverse transcriptase (RT)-PCR and sequence analysis. We found expression of $K_V7.1$, $K_V7.4$, $K_V7.5$ and $K_{Ca}1.1$ (BK_{Ca}) in porcine coronary arteries (Figure 4A, D, E and F) but we could not observe the presence of $K_V7.2$ and $K_V7.3$ (Figure 4B and 4C) despite using four different sets of primers directed against each of these channels. At least one of the primer sets for $K_V7.2$ and $K_V7.3$ resulted in a positive result in tissue from the human heart, which was used as a positive control.

The presence of $K_{Ca}1.1$ (BK_{Ca}) channels was demonstrated by immunoblotting, this revealed the expression of both $K_{Ca}1.1$ (BK_{Ca}) channel subunits in the porcine coronary arteries (Figure 5A and B). We also detected the expression of $K_V7.4$ and $K_V7.5$ channel proteins in tissue from porcine coronary arteries (Figure 5C and D).

Whole-cell voltage clamp studies

Further evidence for the importance of K_V7 channels in hypoxia-induced vasodilatation was obtained from voltage-clamp experiments which showed that the XE991-sensitive current was more marked when bubbling with 5% CO_2 in N_2

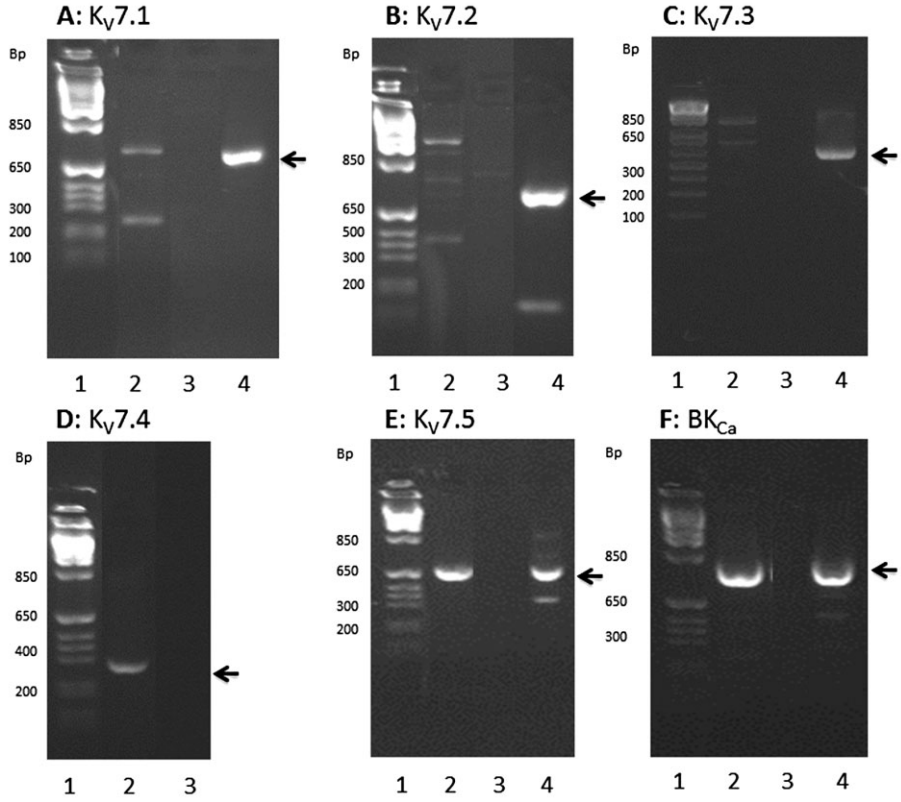

Figure 4

Detection of K_V7 and $K_{Ca}1.1$ (BK_{Ca}) channels by RT-PCR. In all panels, lane 1 is a marker, lane 2 is RT-positive samples, lane 3 is RT-negative samples and lane 4 is a control with human heart. The bands analysed and confirmed as the right product are marked with an arrow. Primers can be seen in Table 1, (n = 6). (A) $K_V7.1$ (B) $K_V7.2$ (C) $K_V7.3$ (D) $K_V7.4$ (E) $K_V7.5$ and (F) $K_{Ca}1.1$ (BK_{Ca}).

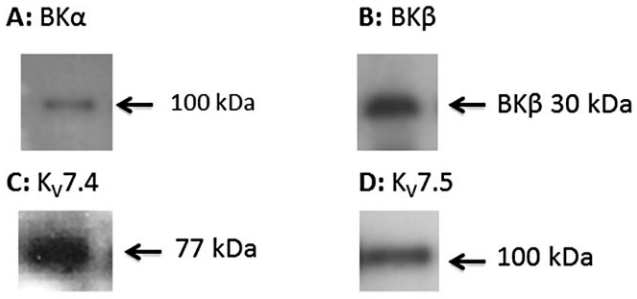

Figure 5

Detection of $K_{Ca}1.1$ (BK_{Ca}) and K_V7 channels by immunoblotting. Immunoblot with samples from a normoxic artery (n = 3) showing the presence of (A) $K_{Ca}1.1\alpha$ (BKα) located at 100 kDa (B) $K_{Ca}1.1\beta$ (BKβ) located at 30 kDa (C) $K_V7.4$ located at 77 kDa and (D) $K_V7.5$ located at 100 kDa.

(which gives approximately 1% O_2 in the bath) than when bubbling with 21% O_2 and 5% CO_2 in N_2 (Figure 6C). In Figure 6D, we show the current remaining after subtracting currents obtained after XE991-treatment from basal currents. The significantly higher XE991-sensitive current under hypoxic conditions suggests that K_V7 channels are activated by hypoxia.

Effect of H_2S

Hydrogen sulphide (H_2S) has been shown to be involved in vasodilatation responses mediated through the K_V7 channels (Schleifenbaum *et al.*, 2010). We, therefore, tested the effect of XE991 on NaHS-induced vasodilatation and found that XE991(10 μM) inhibited vasodilatation induced by NaHS ($P < 0.0001$, n = 6 Figure 7A). The overall relaxation curves induced by lowering oxygen were not significantly changed by incubation with a combination of PPG (10 mM) and AOA (5 mM) which respectively inhibits the H_2S producing enzymes cystathionine γ-lyase and cystathionine β-synthase, when analysed with a two-way ANOVA. However, a t-test analysis of the vasodilatation induced by 1% O_2 revealed that hypoxia-induced vasodilatation at 1% O_2 was significantly inhibited (Figure 7B). Combining PPG, AOA and XE991 gave an additional effect compared to XE991 alone indicating that even though the effect of NaHS can be suppressed by inhibiting K_V7 channels, part of the NaHS relaxation comes from pathways not affected by K_V7 channel blockers.

Discussion

The main findings of the present study were that the hypoxia-induced reduction in $[Ca^{2+}]_i$ in $PGF_{2\alpha}$-contracted

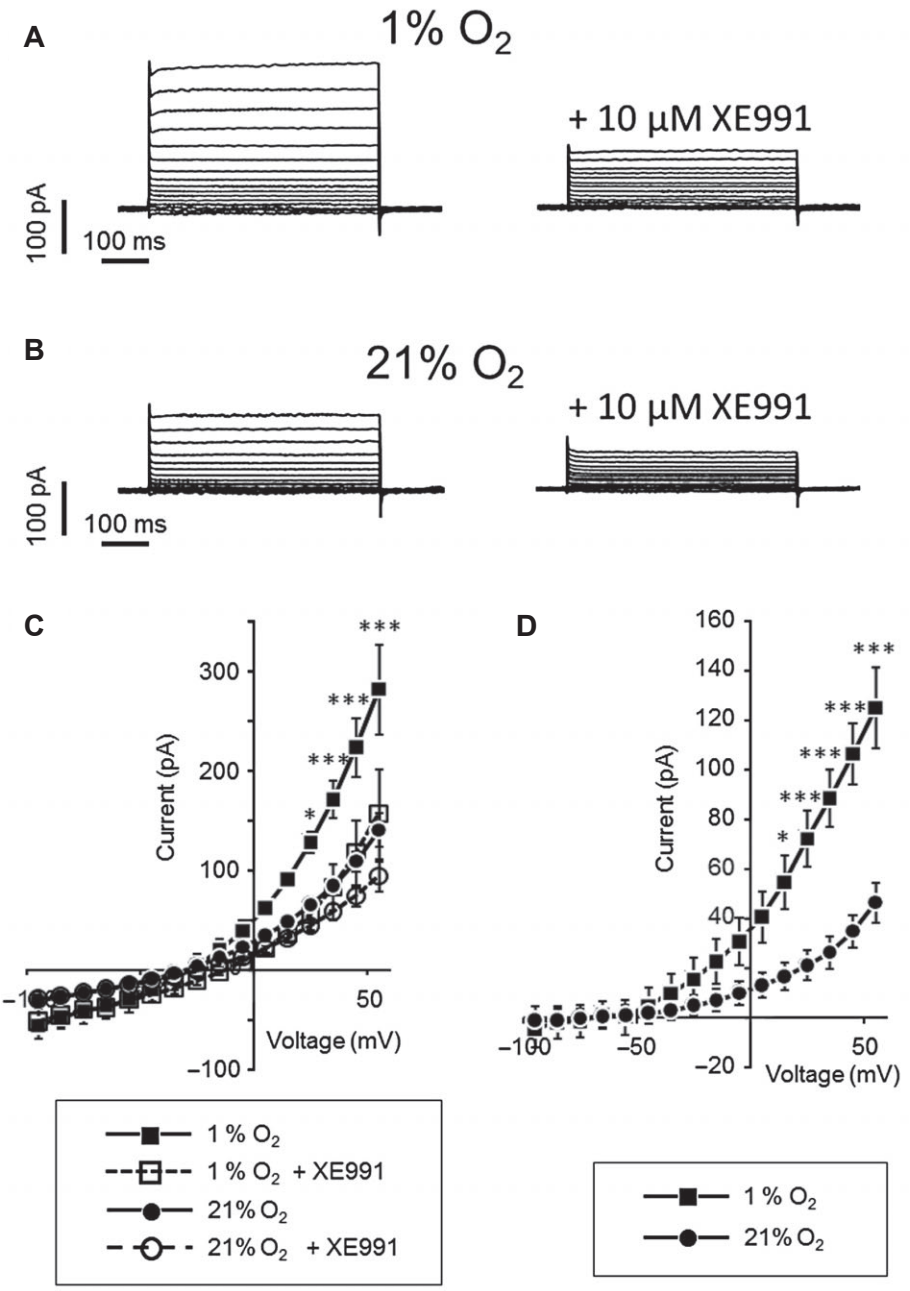

Figure 6

The current activated by hypoxia in the membrane of freshly isolated smooth muscle cells from pig coronary arteries is sensitive to XE991. (A): Representative traces from the whole-cell voltage-step experiments where the current was recorded by stepping membrane voltage between –95 and +55 mV. The traces were obtained in the bath solution that was virtually free from O_2 (~1% O_2) by bubbling with 5% CO_2 in N_2. Representative traces before and after treatment with XE991 are shown. (B) Representative traces from the whole-cell voltage-step experiments similar to those in (A) but obtained in the bath solution with 21% O_2. (C) Averaged current-voltage traces for whole-cell current at ~1% O_2 and at 21% O_2 under control conditions and after addition of XE991. Two-way ANOVA indicates significant effect of different oxygen tensions on membrane current ($F(1,12) = 8.9$, $P > 0.011$). Bonferroni post-test indicates * and *** $P < 0.05$ and <0.001, $n = 7$. (D): Averaged current-voltage traces for XE991 currents obtained by subtraction of the current after XE991 treatment from the control current at ~1% O_2 and at 21% O_2. Two-way ANOVA indicates different effect of XE991 at ~1% O_2 and at 21% O_2 ($F(1,12) = 8.3$, $P > 0.014$). Bonferroni post-test indicates * and *** $P < 0.05$ and <0.001, $n = 7$.

arteries can be attributed to opening of one or more K channel types. The $K_V7.4$ and $K_V7.5$ channels, which we identified in the coronary arteries, appear to be particularly important in the hypoxia-induced vasodilatation. The hypoxic vasodilatation was inhibited by two different blockers of K_V7 channels and the voltage clamp results further indicate that K_V7 channels are involved in this response to hypoxia. Our findings suggest that hypoxia may

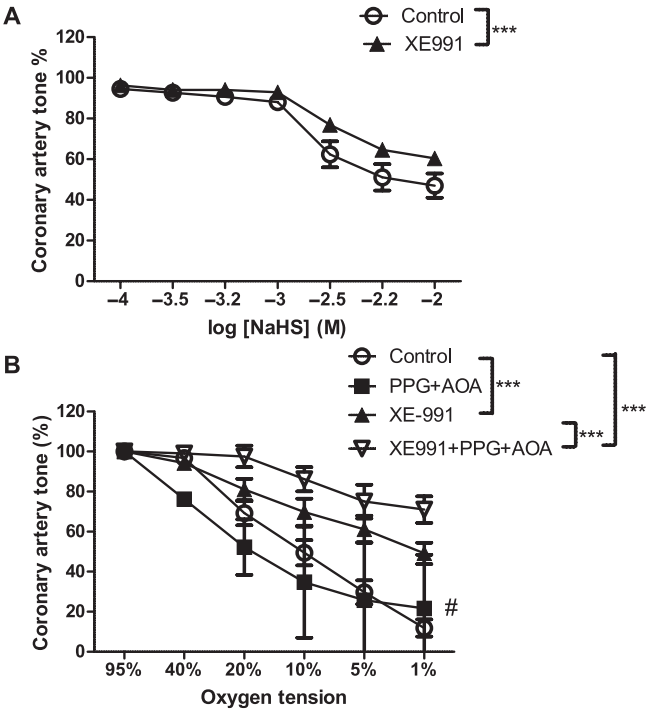

Figure 7

Effect of K_V7 channel inhibition on NaHS-induced dilatation in pig coronary artery. (A): Concentration-response curves for NaHS in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the $K_V7.1–7.5$ inhibitor XE991 (10 μM) (n = 6, ***P < 0.0001 by two-way ANOVA) (B): Concentration-response curves for O_2 lowering in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the $K_V7.1–7.5$ inhibitor XE991 (10 μM) and the inhibitors of the H_2S producing enzymes PPG (10 mM) and AOA (5 mM) (n = 6–16, ***P < 0.001 by two-way ANOVA, #P < 0.05 by Student t-test).

act via K channel opening, which is, in part mediated by H_2S.

Role of intracellular calcium and K channels in hypoxia-induced dilatation

Previous studies have shown that $[Ca^{2+}]_i$ is reduced during hypoxia (Shimizu *et al.*, 2000), but it has also been suggested that reduced $[Ca^{2+}]_i$ cannot fully account for hypoxia-induced dilatation (Aalkjaer and Lombard, 1995; Shimizu *et al.*, 2000; Thorne *et al.*, 2001; Gu *et al.*, 2005). We found that in $PGF_{2\alpha}$-contracted arteries, hypoxia lowered $[Ca^{2+}]_i$ and induced dilatation. In K_{30}PSS-contracted arteries, hypoxia did not alter $[Ca^{2+}]_i$ but caused dilatation. In our experiments, the level of contraction induced by K_{30}PSS or $PGF_{2\alpha}$ did not differ. Since hypoxia-induced dilatation was only partially inhibited by K_{30}PSS contraction, part of this dilatation must be caused by mechanisms independent of changes in $[Ca^{2+}]_i$, such as force suppression or Ca^{2+} desensitization. The decrease in $[Ca^{2+}]_i$ observed in $PGF_{2\alpha}$-contracted arteries suggested the involvement of K channels, since opening of these will lead to hyperpolarization, which decreases $[Ca^{2+}]_i$. Blocking calcium influx may also contribute to the hypoxia-induced relaxation (Franco-Obregon and Lopez-Barneo, 1996; Smani *et al.*, 2002), but the effect of the K channel blockers indicate that K channels are involved in the hypoxic relaxation.

Several K channels including K_{ATP} (Daut *et al.*, 1990; Dart and Standen, 1995; Liu and Flavahan, 1997; Lee *et al.*, 1998; Kamekura *et al.*, 1999), $K_{Ca}1.1$ (BK_{Ca};Gebremedhin *et al.*, 1994; Nelson and Quayle, 1995; Lopez-Barneo *et al.*, 2004) and certain K_V channel subtypes $K_V1.5$ and $K_V2.1$ (Shimizu *et al.*, 2000; Thorne *et al.*, 2002) have been suggested to be involved in hypoxia-induced vasodilatation. In the present study, glibenclamide significantly inhibited the relaxations induced by the K_{ATP} opener, cromakalim, but in the same preparations only slightly inhibited the relaxations evoked by lowering oxygen. These findings agree with the observations in rat and human coronary small arteries, which also suggested that the contribution of K_{ATP} channels to hypoxic vasodilatation is negligible (Lynch *et al.*, 2006). Although 4-AP can also inhibit K_{ATP} currents at concentrations above 0.2 mM (Beech and Bolton, 1989; Nelson and Quayle, 1995), in our experiments the concentration of 4-AP used (0.5 mM) has been shown to be relatively selective for K_V channels (Nelson and Quayle, 1995). In the present study, 4-AP did not affect the cromakalim-induced relaxation, and, therefore, we believe that the reduction in hypoxic vasodilatation observed in the present study can be ascribed to inhibition of 4-AP-sensitive K_V channels.

In coronary arteries with endothelium, NO release is increased and contributes to hypoxic vasodilatation (Lynch *et al.*, 2006; Hedegaard *et al.*, 2011) and, recently, we found that NO induces vasodilatation by activation of TEA-sensitive channels (Hedegaard *et al.*, 2011). In the present study of porcine coronary arteries without endothelium, TEA also caused a small reduction in the hypoxia-induced vasodilatation at concentrations (10^{-3} M) selective for $K_{Ca}1.1$ (BK_{Ca}) channels (Lang and Ritchie, 1990). The observed expression of the α and β subunits of $K_{Ca}1.1$ (BK_{Ca}) in these arteries, and the finding that IbTX, a selective blocker of $K_{Ca}1.1$ channels, reduced the hypoxic vasodilatation, further indicate that smooth muscle $K_{Ca}1.1$ channels contribute to the hypoxic vasodilatation. The combined blockade of 4-AP-sensitive K_V channels and $K_{Ca}1.1$ channels did not cause further inhibition of hypoxic vasodilatation. Thus, $K_{Ca}1.1$ channels may mediate the endothelium-dependent component, involving NO, of the hypoxic vasodilatation (Hedegaard *et al.*, 2011). However, in porcine coronary arteries, in the absence of endothelium, our results show that K_{ATP}, 4-AP-sensitive K_V and $K_{Ca}1.1$ channels contribute little to hypoxic vasodilatation, not enough to explain the pronounced inhibition of hypoxic vasodilatation observed in the preparations contracted by increasing the extracellular K concentration.

Role of K_V7 channels in hypoxic vasodilatation

K_V7 channels are involved in the regulation of vascular tone in a number of rodent blood vessels (Yeung and Greenwood, 2005; Joshi *et al.*, 2006; Yeung *et al.*, 2007; Mackie *et al.*, 2008; Zhong *et al.*, 2010) and it has also been suggested that they counteract changes induced by chronic hypoxia in the pulmonary circulation (Morecroft *et al.*, 2009). Therefore, these channels may also be involved in hypoxic vasodilatation. Indeed, in the present study we found that two different blockers of $K_V7.1–7.5$ channels XE991 and linopirdine inhib-

ited the dilatation induced by hypoxia, adenosine and flupirtine, an opener of $K_V7.2$–7.5 channels, whereas the $K_V7.1$ inhibitor, chromanol 293B did not affect the dilatation induced by these agents. The effect of XE991 appears to be specific, since the SNP-induced relaxation was not changed in the presence of XE991.

Previous studies have shown that mRNA for $K_V7.1$, $K_V7.4$ and $K_V7.5$ is readily detectable in mice portal vein, thoracic aorta, carotid and femoral artery smooth muscle (Yeung *et al.*, 2007; 2008b). $K_V7.1$ and $K_V7.5$ transcripts have also been detected in adult rat aorta (Brueggemann *et al.*, 2007) and $K_V7.1$, $K_V7.4$ and $K_V7.5$ in rat mesenteric artery smooth muscle cells (Mackie *et al.*, 2008). In human arteries, $K_V7.4$ was shown to be expressed in all the arteries examined with variable contributions from $K_V7.1$, $K_V7.3$ and $K_V7.5$ (Ng *et al.*, 2011). We were able to detect mRNA transcripts from $K_V7.1$, $K_V7.4$ and $K_V7.5$, but not $K_V7.2$ and $K_V7.3$, in the coronary artery, even though we tested four different sets of primers and obtained a positive result in control tissue from human heart. Our results agree with previous results showing that $K_V7.1$, $K_V7.4$ and $K_V7.5$ are the dominantly expressed genes in the vasculature, and there is accumulating evidence indicating that $K_V7.4$ and $K_V7.5$ are responsible for the activity in the vasculature (Jepps *et al.*, 2011). Immunoblotting showed that $K_V7.4$ and $K_V7.5$ channel proteins are also expressed in porcine coronary arteries. Taken together with the observations that XE991 inhibits $K_V7.1$–7.4 with IC_{50} values ~1–5 $\mu mol \cdot L^{-1}$ (Yeung *et al.*, 2007; 2008a) and $K_V7.5$ with an IC_{50}~60 $\mu mol \cdot L^{-1}$ (Jensen *et al.*, 2005; Yeung *et al.*, 2008a), and since we used 10 μM XE991, our results suggest that the effect we observed is likely to be mediated by $K_V7.4$ channels.

The combination of IbTX, 4-AP and XE991 had a greater inhibitory effect on the hypoxic vasodilatation compared to XE991 alone and the effect of this combination was similar to that observed in preparations contracted with 30 mM potassium (e.g. Figure 2 versus Supporting Information Figure S4). These results suggest that XE991 has an effect on the K_V7 channels, which can be separated from that induced by blocking other potassium channels.

In previous studies, resting membrane potential in pinned coronary arteries from pigs has been measured to be approximately –50 mV (Edwards *et al.*, 2000) and $PGF_{2\alpha}$ (2–6 μM) was found to depolarize the membrane to –40 to –35 mV (Thollon *et al.*, 2002). Resting membrane potential is more depolarized in pressurized preparations, while agonist-induced depolarization is less (Schubert *et al.*, 1996). This confirms that the XE991-sensitive currents activated above –40 mV are within the range of physiologically relevant membrane potentials.

Interestingly, in the voltage-clamp study, the membrane conductance was higher under hypoxic conditions. This hypoxia-induced current was sensitive to XE991 at a concentration that has previously been shown to inhibit K_V7 currents (Yeung and Greenwood, 2005). Although recent studies have suggested that XE991 can also attenuate $K_V1.2/K_V1.5$ and $K_V2.1/K_V9.3$ channels, an overexpression study confirmed that its major effect is on K_V7 channels (Zhong *et al.*, 2010). We thus propose that members of the K_V7 channel family (i.e. $K_V7.2$-$K_V7.5$ channels) are activated by hypoxia in coronary artery smooth muscle cells and are involved in the vasodilator response.

Role of K_V7 channels in H_2S and adenosine induced vasodilatation

A fall in oxygen availability during hypoxia decreases mitochondrial H_2S oxidation resulting in an increase in biologically active H_2S, and therefore, H_2S has been suggested to mediate hypoxic vasodilatation (Skovgaard *et al.*, 2011). Moreover, H_2S has been shown to cause relaxation by activation of K_{ATP} channels and K_V7 channels (Zhang *et al.*, 2007; Schleifenbaum *et al.*, 2010). Incubation of the porcine coronary arteries with inhibitors of enzymes synthesizing H_2S attenuated the vasodilatation induced by lowering oxygen tension to 1% and the combination of these enzyme inhibitors with XE991 inhibited the hypoxic vasodilatation. In addition, the inhibitory effect of XE991 on the H_2S-induced relaxation suggests that part of the K_V7 activation in hypoxia may be attributed to H_2S.

Another metabolite released from the myocardium in ischaemic conditions is adenosine (Decking *et al.*, 1997). Adenosine increases cAMP and a study in renal arteries demonstrated that K_V7 channels are regulated by cAMP-dependent processes (Chadha *et al.*, 2012). This finding is in line with our observation that adenosine-induced relaxation was inhibited in the presence of XE991. Although further studies are required to reveal the source of the mediators, these results suggest that H_2S and adenosine may lead to the activation of K_V7 channels during hypoxia in porcine coronary arteries.

Conclusion

Our findings suggest that smooth muscle $K_V7.4$ and 7.5 channels are the main components involved in the hypoxic vasodilatation in porcine coronary arteries with other K channels contributing to a much lesser extent. The results from the voltage clamp experiments further supported a role for K_V7 channels during hypoxia. We also showed that H_2S and adenosine are likely to induce the activation of K_V7 channels in hypoxia. Based on these preclinical findings, it would be interesting to investigate the therapeutic potential of modulators of K_V7 channels already in use. Flupirtine is approved as a treatment for pain in Europe, and retigabine has shown promising results in phase III clinical trials as an antiepileptic agent (Mackie and Byron, 2008), these openers could potentially form the basis for testing in patients with cardiac ischaemia.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Effect of K channel inhibition with TEA on hypoxia- and adenosine-induced dilatation in pig coronary artery. (A) Concentration-response curves for O_2 lowering in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the K channel inhibitor TEA (1 mM and 10 mM) ($n = 7$, $^*P = 0.046$, $^{***}P < 0.001$ by two-way ANOVA). (B) Concentration-response curves for adenosine in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the K channel inhibitor TEA (1 mM and 10 mM) ($n = 7$, $^{***}P < 0.001$ by two-way ANOVA).
Figure S2 Effect of K channel inhibition on hypoxia-induced dilatation in pig coronary artery. Concentration-response curves for O_2 lowering in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of (A): 4-AP 0.5 mM ($n = 12$, $^*P < 0.001$ by two-way ANOVA), (B): IbTX 100 nM ($n = 6$, $^*P < 0.001$ by two-way ANOVA) or (C): glibenclamide 3 μM ($n = 7$, $^*P < 0.05$ by two-way ANOVA).

Figure S3 Effect of K channel inhibition on hypoxia-induced dilatation in pig coronary artery. Concentration-response curves for O_2 lowering in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of (A): 4-AP 0.5 mM and IbTX 100 nM ($n = 6$, $^*P < 0.01$ by two-way ANOVA), (B): glibenclamide 3 μM and 4-AP 0.5 mM ($n = 6$, $^*P < 0.001$ by two-way ANOVA)

Figure S4 Effect of K channel inhibition on hypoxia-induced dilatation in pig coronary artery. Concentration-response curves for O_2 lowering in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of XE991 10 μM, 4-AP 0.5 mM and IbTX 100 nM ($n = 7$–8, $^{***}P < 0.001$ by two-way ANOVA).

Figure S5 Effect of $K_V7.1$ channel inhibition on hypoxia- and adenosine-induced dilatation in pig coronary artery. (A) Concentration-response curves for O_2 lowering in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the $K_V7.1$ channel inhibitor chromanol 293B (10 μM) ($n = 5$, $P = 0.22$ by two-way ANOVA). (B) Concentration-response curves for adenosine in coronary arterial segments without endothelium contracted with $PGF_{2\alpha}$ (10 μM) in the absence and presence of the $K_V7.1$ channel inhibitor chromanol 293B (10 μM) ($n = 5$, $P = 0.45$ by two-way ANOVA).

Figure S6 Detection of $K_{Ca}1.1$ and K_V7 channels by immunoblotting. Immunoblot with samples from a normoxic artery in lane 1 and from a hypoxic artery in lane 2 and showing the presence of (A) $K_{Ca}1.1\alpha$ located at 100 kDa (B) $K_{Ca}1.1\beta$ located at 30 kDa (C) $K_V7.4$ located at 77 kDa and (D) $K_V7.5$ located at 100 kDa. (E) Incubated with secondary antibody goat anti-rabbit IgG conjugated to HRP.