

RESEARCH ARTICLE

The Effect of pH and Ion Channel Modulators on Human Placental Arteries

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Abstract

Chorionic plate arteries (CPA) are located at the maternofetal interface where they are able to respond to local metabolic changes. Unlike many other types of vasculature, the placenta lacks nervous control and requires autoregulation for controlling blood flow. The placental circulation, which is of low-resistance, may become hypoxic easily leading to fetal acidosis and fetal distress however the role of the ion channels in these circumstances is not well-understood. Active potassium channel conductances that are subject to local physicochemical modulation may serve as pathways through which such signals are transduced. The aim of this study was to investigate the modulation of CPA by pH and the channels implicated in these responses using wire myography. CPA were isolated from healthy placentae and pre-contracted with U46619 before testing the effects of extracellular pH using 1 M lactic acid over the pH range 7.4 - 6.4 in the presence of a variety of ion channel modulators. A change from pH 7.4 to 7.2 produced a $29 \pm 3\%$ ($n=9$) relaxation of CPA which increased to $61 \pm 4\%$ at the lowest pH of 6.4. In vessels isolated from placentae of women with pre-eclampsia ($n=6$), pH responses were attenuated. L-methionine increased the relaxation to $67 \pm 7\%$ ($n=6$; $p<0.001$) at pH 6.4. Similarly the TASK 1/3 blocker zinc chloride (1 mM) gave a maximum relaxation of $72 \pm 5\%$ ($n=8$; $p<0.01$) which compared with the relaxation produced by the TREK-1 opener riluzole ($75 \pm 5\%$; $n=6$). Several other modulators induced no significant changes in vascular responses. Our study confirmed expression of several ion channel subtypes in CPA with our results indicating that extracellular pH within the physiological range has an important role in controlling vasodilatation in the human term placenta.



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Introduction

Throughout pregnancy, it is essential for the placenta to manage acid-base balance within a narrow pH range in order to minimise adverse effects on fetal growth and development. This is accomplished by eliminating acids formed by normal fetal and placental metabolism via the maternal circulation and through buffering provided principally by hemoglobin and bicarbonate [1, 2]. In addition, during labor, uterine contractions can occlude blood flow hence placental perfusion by compression of the uterine artery [3]. This can lead to fetal hypoxia and acidosis thus monitoring of umbilical cord blood pH is a useful measure of fetal wellbeing, indicating the need for clinical intervention when pH poses a danger to fetal health [2].

The fetoplacental circulation consists of arteries and veins of the umbilical cord, chorionic plate and stem villi. Non-innervated, chorionic plate arteries (CPA) and veins branch directly from the umbilical cord over the fetal surface of the placenta, lying closest to the fetus. Fetoplacental arteries are also much less sensitive to vasoactive molecules that have potent effects in other vascular beds [4, 5] however the effects of molecules and factors produced locally that have the capacity to alter placental vascular function have not been widely studied yet may highlight mechanisms by which control of fetoplacental vascular tone is achieved.

The placental circulation shares many similarities with the pulmonary circulation including vasoconstriction in response to hypoxia (hypoxic pulmonary vasoconstriction, HPV) [6]. The placental counterpart to this phenomenon, hypoxic fetoplacental vasoconstriction (HFPV) is mediated by small (<500 μM diameter) fetoplacental arteries [7]. Hypoxia can in turn lead to the accumulation of H^+ ions and alter the pH of the cell's microenvironment [7]. In contrast, using *ex vivo* placental cotyledons instead of isolated blood vessels or cells, altering the pH with or without altering the pO_2 of the perfusate had little effect on HFPV [8], perfusion pressure or the pressor response [9]. In some pregnancies, inadequate or shallow invasion of the maternal spiral arteries at the uteroplacental interface leads to poor vascular perfusion of the placenta which is observed in pre-eclampsia and is seen in some forms of intra-uterine growth restriction [10]. Moreover acidosis is associated with intrauterine growth-restricted pregnancies indicating detrimental outcomes with chronic hypoxia.

A plethora of ion channels has been described in controlling the tone of vascular smooth muscle (VSM). Depolarisation of the myocyte membrane results in opening of L-type voltage-gated calcium (Ca^{2+}) channels (LTCC) that elevate cytosolic Ca^{2+} to drive vasoconstriction. Relaxation involves interplay of several channels with calcium-activated potassium (K^+) channels having key roles. Of these, the large -conductance calcium-activated K^+ channel, BK_{Ca} (MaxiK) enjoys widespread distribution in many vascular beds [11, 12] while channels of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance are primarily localised to the endothelium and are targets of endothelium-derived hyperpolarising factors [13]. Calcium-activated and voltage-gated K^+ (Kv) channels are expressed in the human placenta with a role for (Kv) channels proposed in the placental response

to HFPV where the Kv blocker 4-aminopyridine (4-AP) mimicked this effect [7]. While good evidence exists for functional expression for the aforementioned channels in CPA [14, 15], there are less data for two-pore potassium (K2P) channels in the placenta. These channels first described in yeast [16, 17], play a role in determining resting membrane potential and generate leak currents [18]. Intriguingly, they are subject to local regulation by physical and chemical factors in the vicinity that include pH, oxygen tension and lipids [18]. K2P channels are distinguished from other K⁺ channels by the presence of four transmembrane segments circumscribing two pore forming domains [18]. For example, TWIK and TWIK-related K⁺ (TREK) demonstrate high sensitivity to acidic pH inside the cell while the activity of the TWIK related acid-sensitive K⁺ (TASK) is inhibited by weak changes in extracellular pH [17, 19, 20]. TASK channels open at rest maintain the resting membrane potential and are inhibited by extracellular acidic pH which makes two of the main subtypes TASK-1 and TASK-3, key candidates for responding to pH stress within the placenta. Earlier work has identified the expression of TASK-1 and TREK-1 in placental cytotrophoblast with little evidence of their functional role. Ion channel expression and function in the fetoplacental vasculature is likely to exhibit a similar repertoire of channels found in most vascular beds that differ in their functional input to the control of vessel tone. The aim of this study was to identify the ion channels in resistance-sized CPA arteries, in particular the channel subtypes mediating the effects of low extracellular pH.

Materials and Methods

Ethical approval for the study was obtained from the Derbyshire Research Ethics Committee (04/Q2401/13) with patients providing fully informed, written consent prior to elective lower segment Caesarean section (ELLSCS) surgery. Whole placentae and myometrial biopsies were obtained from term (≥ 37 wks), non-laboring, singleton pregnancies following delivery at the Royal Derby Hospital and transported to the laboratory within twenty minutes. Reasons for ELLSCS included maternal request, previous ELLSCS or breech presentation. Patients with diabetes (pre-existing or gestational), high blood pressure, a history of cardiac or renal disease, or patients receiving pharmacological treatment other than iron supplementation and smokers were excluded from the study. Pre-eclamptic patients were recruited based on criteria of hypertension ($>140/90$ mm Hg) on two separate occasions, more than 4 hrs apart after 20th week of gestation in previously normotensive woman and proteinuria (0.3g/L over 24 hours [21]). [Table 1](#) shows the demographics of patients recruited.

For isolation of CPA, the amnion was gently peeled away by hand to reveal the network of arteries at the chorionic surface. An artery in isolation from a neighbouring vein was identified by following the point of cord insertion and tracking smaller branches of the selected artery. The fourth order branch of each CPA was used for the study and was dissected intact and free of adherent tissue.

Table 1. Patient demographics for normal and pre-eclamptic (pregnancies). Data are presented as mean (\pm SEM) or median [\pm IQR]. * $p < 0.05$.

Parameter	NORMAL	PE	p
	N=40	N=6	ns: not significant
Maternal age (yrs)	31.25 \pm 0.9	29 \pm 2.3	0.3 ns
Primipara n (%)	11 (27.5%)	4 (66%)	-
Prepregnancy Body mass index (kg/m ²)	25 [22–32]	26.5 [23–30]	0.9 ns
Max. systolic blood pressure outside labour (mmHg)	125 \pm 3.2	150 \pm 6.1	0.01 *
Max. diastolic blood pressure outside labour (mmHg)	63 \pm 1.5	96 \pm 4	0.003 *
Proteinuria g/L	-	3.97 \pm 0.7	-
Gestational age at delivery (days)	271 \pm 1.2	236 \pm 8.0	0.0009 *
Birth weight (g)	3242 \pm 73	2128 \pm 320	0.0003 *
Birth weight centile	48 [15–73]	30 [18–50]	0.4 ns
Placenta/Birth weight ratio	0.2 \pm 0.02	0.3 \pm 0.09	0.2 ns
Cord blood pH	Venous (N)	7.28 \pm 0.02 (30)	7.27 \pm 0.03 (6)
	Arterial (N)	7.34 \pm 0.01 (30)	7.32 \pm 0.01 (6)

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Three separate samples from each placenta were taken in this way. Following fine dissection to remove any connective tissue, CPA were transferred to physiological salt solution (PSS; composition in mmol/l: 119 NaCl, 4.7 KCl, 25 NaHCO₃, 2.5 MgSO₄, 1.6 CaCl₂, 1.2 KH₂PO₄, 5.5 glucose, 0.034 EDTA pH 7.4) at room temperature, 21–26°C) and used immediately for wire myography.

Wire myography

Once all connective tissue had been removed, CPA were transferred to cold (4–6°C PSS) and used immediately for wire myography. Each vessel was mounted onto the free end of a 50 μ m tungsten wire before securing with a second fixing screw. A second wire was passed through the lumen and fixed to the opposite jaw so that the two wires were in parallel and any remaining connective tissue removed. The remaining vessel segments were mounted in the same way and left to equilibrate and heat to 37°C before normalisation. The effective pressure was determined for each vessel segment and normalized to an effective pressure of 5.1 kPa and set at a circumference of 0.9. The resting pressure of 5.1 kPa was applied to closely match the transmural pressure recorded across the intervillous space of the placenta ([22, 23]. CPA were precontracted with the thromboxane mimetic U46619 (1 nM–1 μ M in PSS), and responses to a number of ion channel and vasoactive modulators investigated. The tissue baths were continuously perfused with 5% O₂, 5% CO₂, 90% N₂ (BOC, UK) and maintained at 37°C. The effects of extracellular acidification were assessed by applying decreasing doses of 1M lactic acid in 0.2 units (pH 7.6–6.4) to the bath, in the presence or absence of various ion channel modulators and vasoactive agents. The pH of the bath solutions was routinely checked. Each compound was added directly to the chamber twenty minutes prior to stimulation with U46619. The baseline tension of each vessel was assessed pre- and 5 mins post-addition of each pharmacological

agent. One vessel in each experiments to which no drugs were added was used as a time control or as a vehicle control where the solvent alone was added to differentiate specific drug-induced effects. Concentrations of agents used were based on experiments performed in our lab along with published literature.

Placental VSM primary culture

VSM were cultured using explants of CPA based on the methodology of Leik *et al.*, [24]. One mm² square pieces of artery were excised and each piece placed in the well of a 12-well plate with the endothelium facing downwards on to the plastic surface. Dulbecco's Modified Eagle Medium (DMEM; containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin) were added to each well and plates maintained in a humidified incubator in a 5% CO₂/air environment. Apart from regular media changes, culture plates were left for a period of 3–4 weeks at which point outgrowths could be subcultured by simply removing the explant and trypsinising (0.025% in calcium - and magnesium -free Hanks Balanced Salt Solution, HBSS) the sample followed by washing in serum-containing DMEM to inactivate trypsin. The cell pellet was washed again and centrifuged at 200 g before resuspending cells for transfer into a T-25 tissue culture flask. Myometrial biopsies from non-laboring patients were enzymatically dispersed immediately after collection [25] to produce single, smooth muscle cells and cultured to be used as a positive control for immunofluorescence studies.

Confocal immunofluorescence

Placental and myometrial smooth muscle cells were cultured on glass coverslips in DMEM (with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin) until 70% confluent. Culture media was aspirated then cells fixed with 4% w/v paraformaldehyde (pH 7.4) for 5 mins at 4°C. Fixative was then gently removed and 3% bovine serum albumin (BSA)/1% glycine in PBS added to cells for 15 mins followed by blocking with a mixture of 10% goat and 10% chick serum in PBS. After 1 hour, the blocking solution was aspirated and cells incubated with 200 µl of primary antibody against the channels TASK-3 (1:50), TREK-1 (1:100) or TWIK-2 (1:50), and CaV1.2 (1:50) and left overnight at 4°C with gentle shaking. A negative control was also used where primary antibody was replaced with either control IgG or antibody was incubated with excess control peptide according to the manufacturer's instructions before adding to the cells. Following removal of the primary antibody, the coverslips were coated with either anti-mouse FITC or anti-rabbit TRITC conjugated secondary antibodies (1:5000) in 10% goat serum in PBS for 1.5h in the dark with gentle shaking then mounted in PBS:glycerol. Mounted slides were placed face down on the slide stage and viewed using a Leica TCS SP2 confocal microscope with Acousto-Optical Beam Splitter (AOBS) scan head and a 63x glycerol immersion objective lens. Images were captured using three laser lines in single scans to eliminate any overlap

between the channels. DAPI IF was viewed using an Ultraviolet laser (372 nm excitation and 385nm emission). FITC was viewed using 488 nm excitation with 505–530 emission (Argon laser) and TRITC was viewed with 546 nm excitation and 560 nm emission (Helium//Neon laser). Each individual sample was run at least twice for immunofluorescence studies.

Drugs and solutions

All reagents were obtained from Sigma Aldrich (UK). Drugs used were curcumin, L-methionine and ruthenium red as TREK-1 inhibitors; bupivacaine, methanadamide, copper chloride, lidocaine and zinc chloride to inhibit either TASK-1 or TASK-3; quinidine and barium chloride as inhibitors of TWIK channels, iberiotoxin to block BK_{Ca} channels; apamin as an SK_{Ca} channel blocker and nifedipine for L-type Ca²⁺ channels. Concentrations used were based on published data or preliminary experiments carried out in our lab. Drugs were prepared daily from fresh stock solutions or from frozen stocks. Curcumin and ruthenium red were prepared in ethanol while nifedipine, quinidine and riluzole were added to DMSO then diluted in PSS. All other compounds used were soluble in PSS with pH adjusted to 7.4 prior to addition to the bath. For wire myography, the total addition of each compound to the bath was maintained at <1% of the total chamber volume. Time controls or vehicle controls (diluent only) were also included in each experiment. Ion channel antibodies were from Caltag Medsystems, UK.

Data and statistical analysis

Concentration response curves were analysed by fitting the data to a 3-parameter logistic equation and compared by global curve fitting (GraphPad Software Inc Version 5, San Diego, CA, USA). The top and bottom plateaus were constrained and shared; the logEC₅₀ values were compared with the control group. Normalcy was assessed using the Shapiro-Wilks normality test. Differences amongst responses were assessed using a one-way ANOVA with Bonferroni post hoc comparison across each concentration range used. Data are presented as mean \pm SEM of n vessels used. A p value of <0.05 was considered to be statistically significant.

Results

The effects of voltage-gated, calcium-sensitive and K₂P channel modulators were investigated by direct application to resistance-sized CPA (vessel diameter $418 \pm 12 \mu\text{m}$; n=96) precontracted with U46619. No significant effect ($p > 0.05$) of 10 μM glibenclamide, a K_{ATP} channel inhibitor (n=9) on the U46619 concentration-response curve was observed. Pre-incubation with 10 μM apamin or 1 mM 4-AP enhanced basal tone and produced a leftward shift of the concentration-response curve ([Fig. 1A](#)). This shift was accompanied by altered log

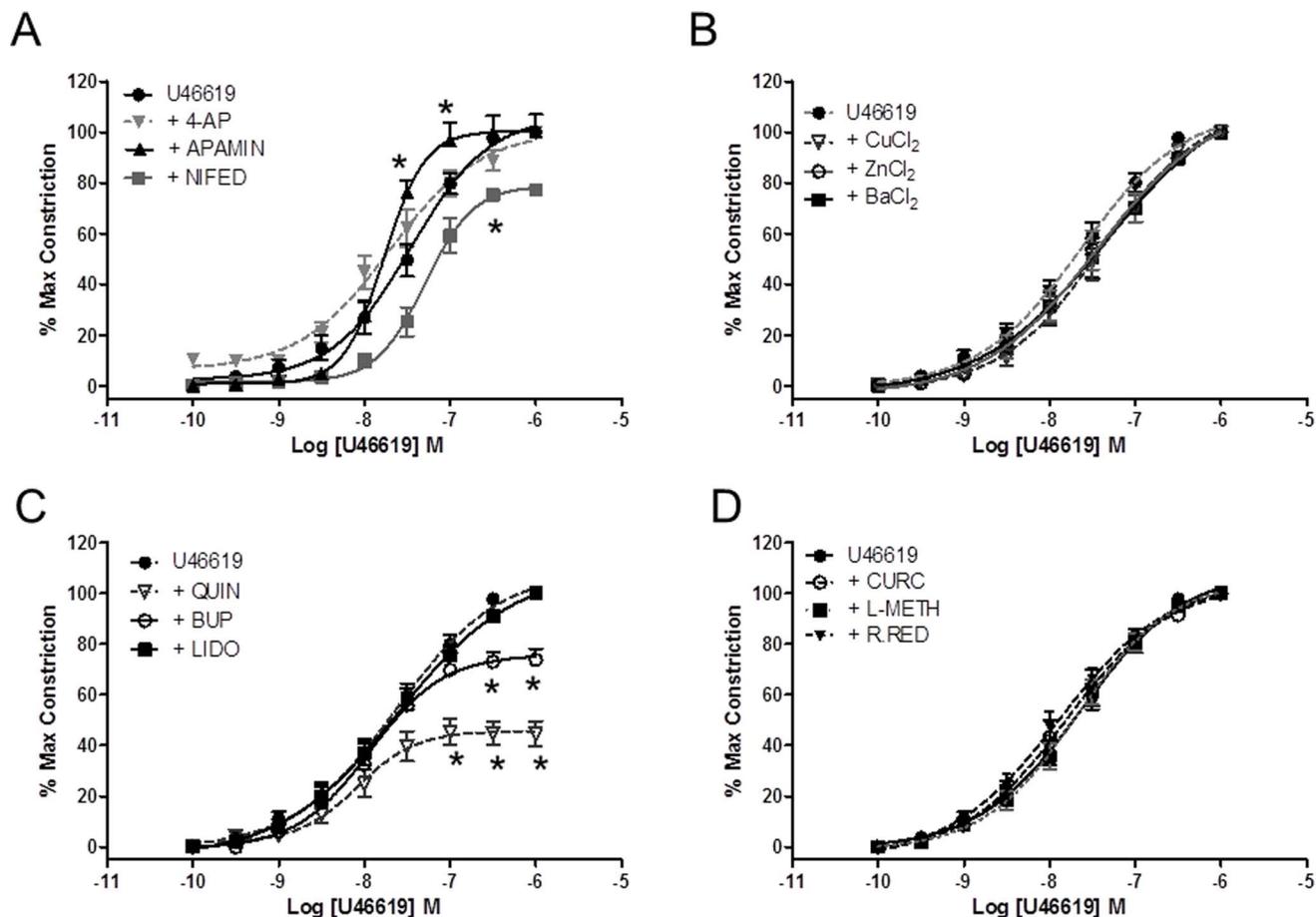


Fig. 1. Modulation of the U4661-induced vasoconstriction by ion channel modulators. Wire myography of CPA was carried out in physiological salt solution with vessels mounted under tension. The effects of channel modulators were evaluated by preincubating CPA with test drug and comparing responses of CPA precontracted with U46619. (A) The effect of voltage-gated channel blockers. Nifedipine caused a rightward shift in the dose-response curve. The Kv inhibitor 4-AP increased basal tension while apamin significantly altered the response to U46619 at higher concentrations. (B) The inorganic compounds BaCl₂, CuCl₂ and ZnCl₂ had no effect on the U46619 curve (C) Bupivacaine and quinidine both reduced maximal vasoconstriction in CPA. No effect of non-specific TASK or TREK inhibitors was evident. For all graphs in this and subsequent figures, data are presented as mean ± SEM with p<0.05 (*) taken as a significant finding.

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EC₅₀ values of -7.8 ± 0.1 for both apamin (n=9) and 4-AP (n=9) compared with -7.5 ± 0.1 for U46619 alone (n=12). The CaV1.2 blocker nifedipine displaced the control curve to the right with an EC₅₀ of -7.3 ± 0.1 (n=9) when vessels were challenged with U46619 concentrations above 10⁻⁸M. Nifedipine (10 μM) also reduced the maximal response to 79.01% (n=9). The inorganic compounds CuCl₂, ZnCl₂ and BaCl₂ (n=9 for all), each at a bath concentration of 1 mM, had no effect on CPA responses to U46619-induced vasoconstriction (Fig. 1B). The lack of effect of the local anesthetic lidocaine (100 μM), was in contrast to a decrease in maximal contraction by $26.1 \pm 4.2\%$ and $55.4 \pm 3.7\%$ respectively elicited by pre-incubation with either 200 μM bupivacaine (n=9) or 200 μM quinidine (n=9; Fig. 1C). Neither L-methionine (n=9), ruthenium red (n=9)

nor curcumin (n=9) significantly altered the contractile performance of U46619-induced vasoconstriction ($p > 0.05$; [Fig. 1D](#)).

On examining vasodilatory responses of U46619-precontracted vessels, the BK_{Ca} channel opener NS1619 (10^{-10} - 10^{-5} M) resulted in a significant, concentration-dependent relaxation of $91.5 \pm 8.3\%$ (n=4), an effect that was maximally inhibited to $74.2 \pm 8.4\%$ with TEA (n=4; $p > 0.05$) and even further ($46.9 \pm 11.9\%$; n=4) following pretreatment with the BK_{Ca} channel antagonist iberiotoxin. These responses differed significantly from controls ($p < 0.05$; [Fig. 2A](#)). The widely-used TREK-1/TRAAK channel opener, riluzole, also caused effective relaxation ($83.0 \pm 3.8\%$) of precontracted arteries (n=18), which was inhibited by 5 mM TEA ($56.8 \pm 7.1\%$; n=6) but unaffected by 100 μ M methanandamide (n=6), 100 μ M lidocaine (n=6; [Fig. 2B](#)), 10 μ M ruthenium red (n=6), L-methionine (n=6), 10 μ M curcumin (n=13), 200 μ M bupivacaine (n=6), 200 nM IbTX (n=4), 1 mM 4-AP (n=6) and 10 μ M apamin (n=6; data not shown).

Since H_2O_2 may also relax arteries, we tested the effects of catalase on the H_2O_2 -mediated relaxation of U46619-precontracted CPAs. Vasorelaxation to H_2O_2 was considerably attenuated by catalase at 10^{-7} M and 10^{-5} M (n=5; [Fig. 2C](#)). TEA with a log IC_{50} of -3.8 ± 0.1 demonstrated an inhibition of the H_2O_2 response at the lower doses of 10^{-8} – 10^{-7} M ($p < 0.05$) and also at 10^{-5} M ($p < 0.05$; n=4). In contrast, SNP caused $49.9 \pm 7.9\%$ relaxation of the U46619 response which was greater than that induced either by ACh ($24.2 \pm 4.2\%$) or bradykinin ($13.2 \pm 2.1\%$) as shown in [Fig. 2D](#). Direct application of H_2O_2 produced variable, transient and desensitising responses when added directly to CPA but these were not investigated further. The non-specific TREK-1 blocker curcumin [26], also known for its antioxidant properties, produced no significant shift in the response of CPA to H_2O_2 (n=5) while iberiotoxin had little effect on the vasodilatation evoked by application of H_2O_2 .

The effect of acidification

Incremental decreases in extracellular pH (7.4–6.4) by addition of lactic acid resulted in concentration-dependent relaxation of U46619-precontracted CPA ([Fig. 3](#), top trace). The vascular responses were transient nature, characterised by an immediate downward deflection followed by a return to baseline that we have termed the recovery period, as seen in [Fig. 3](#) (top trace). Maximal relaxation ($54.2 \pm 3.5\%$) of the U46619-precontraction was observed at a pH of 6.4 (n=16). In order to investigate the contribution of channel families underlying the relaxation induced by pH, a variety of channel blockers were tested. Pre-incubation of CPA with 1mM $ZnCl_2$ produced a greater degree of relaxation ($75.1 \pm 4.7\%$; n=6) than pH alone and prolonged the recovery period as pH was lowered ([Fig. 3B](#)), achieving significance at $pH < 7.0$ ([Fig. 5A](#)). Curcumin inhibited the relaxant effect induced by pH ([Fig. 4A](#); [Fig. 5B](#)) while pre-incubation with L-methionine, an inhibitor of stretch-activated channels thought to be TREK-1, accelerated the downward deflection of the relaxant response and

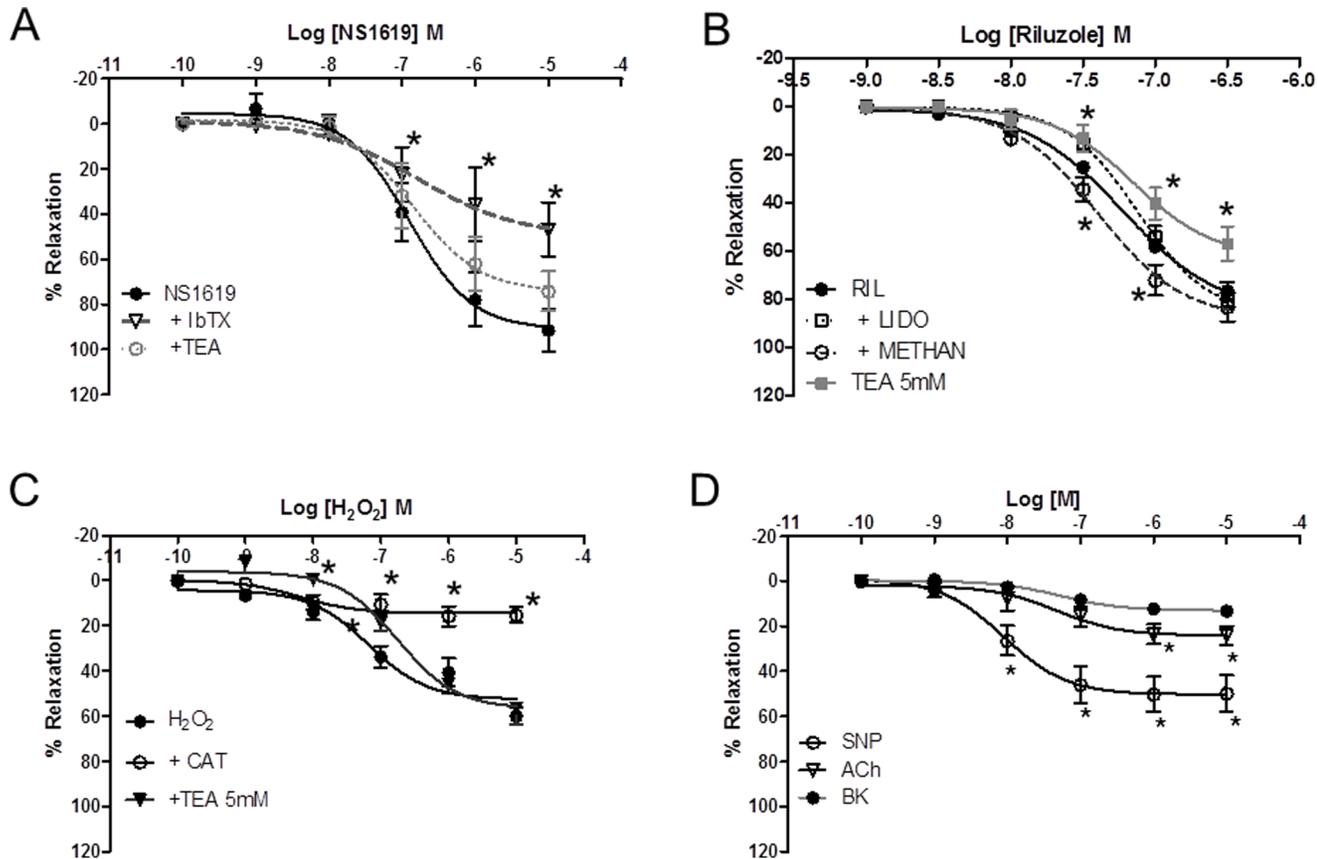


Fig. 2. CPA relaxant effects induced by vasomodulators. (A) The BK_{Ca} channel opener NS1619 produced relaxation of precontracted CPA. The response was significantly inhibited by iberiotoxin but not by TEA. (b) Riluzole also caused relaxation that was unaffected by lidocaine, and methanandamide but significantly inhibited by TEA (C) Catalase inhibited the H₂O₂ vasorelaxation while TEA had no effect on the maximal relaxation but caused a rightward shift of the curve (D) SNP, bradykinin and ACh induced vasorelaxation to differing extents.

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increased the magnitude of the response as pH was reduced (Fig. 4B; Fig. 5B). CuCl₂ also affected the recovery phase inhibiting the contractile effect and enhancing relaxation to $72.8 \pm 4.9\%$ (n=6; Fig. 5A). BaCl₂ had no significant effect (p>0.05) on pH responses (Fig. 5A). Neither iberiotoxin nor 4-AP had any significant effect on the pH response while nifedipine significantly increased the relaxation to pH to $74.0 \pm 5.0\%$ (p<0.05; Fig. 5C). Ruthenium red (Fig. 5B), amiloride and omeprazole had no effect on the response of CPA to pH although a slight effect of ouabain was significant (p<0.05) at lower pH values compared with responses to pH alone (Fig. 5D).

CPAs from patients with pre-eclampsia obtained at ELLSCS were also included in some of our functional studies. Fig. 6A shows that using the same 4th branch of the CPA from PE placental samples, vessel diameter was not found to be significantly different to CPAs for normotensive patients (p>0.05). Neither was the dose-response curve to U46619 of CPA significantly different between normal and PE vessels (p>0.05; Fig. 6B). Vascular responses, in terms of maximal

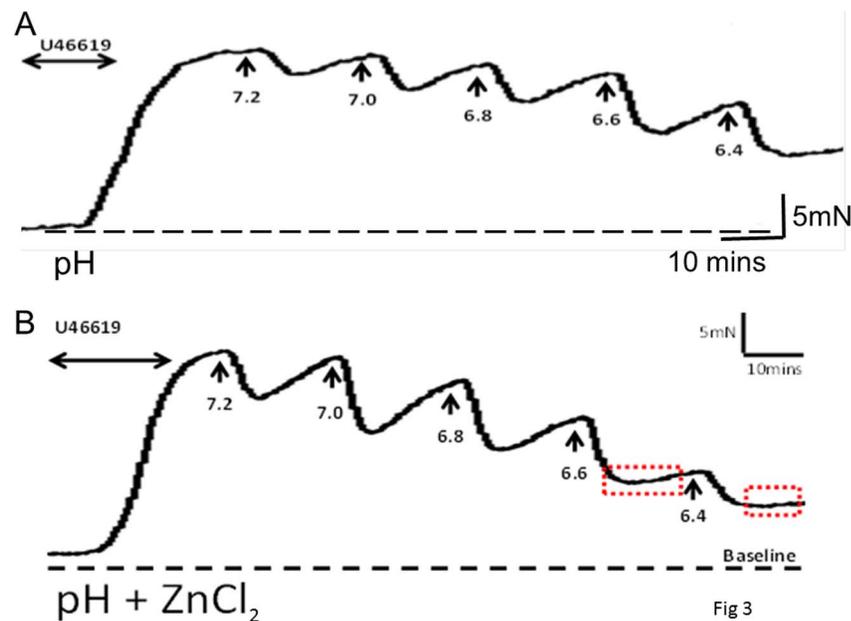


Fig. 3. The effects of pH on CPA of normal vessels. Incremental decreases in pH were introduced by direct addition of lactic acid to precontracted vessels. (A) Transient relaxations were immediately induced followed by recovery and a return of tone. (B) Preincubation with ZnCl₂ enhanced the relaxant effects of pH as well as inhibiting the recovery phase shown by the boxed regions.

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relaxation and log EC₅₀ values to the relaxants sodium nitroprusside log EC₅₀s (-5.5 ± 0.1 and -5.7 ± 0.1 for normal and PE respectively; Fig. 6C) and riluzole (log EC₅₀s -6.6 ± 0.1 and -6.7 ± 0.1 ; for normal and PE respectively; Fig. 6D) were also unaltered between the two groups ($p > 0.05$ in both cases). Interestingly PE vessels demonstrated an attenuated vasorelaxant response on acidification compared with normal vessels (Fig. 7A and Fig. 7B).

Protein expression and localisation of TREK-1 (n=12; Fig. 8A) and TASK-3 (Fig. 8B) was confirmed by confocal immunofluorescence compared with control samples where primary antibody was replaced with the relevant control IgG. TREK-1 immunofluorescence was characteristically linear across the cell membrane (Fig. 8A). Representative images show TASK-3 (Fig. 8B) expression was abundant around perinuclear regions but was also membrane-associated (Fig. 8B). CaV1.2 (corresponding to LTCC) immunofluorescence was clearly observed along VSM of CPA (n=10; Fig. 8C) and intense TWIK-2 expression was also noted (n=5; Fig. 8D).

Discussion

Excess protons are potentially harmful to cells as they can cross the cell membrane with ease and interfere with key cellular functions and pathways. This has important consequences in the placenta which needs to maintain continuous

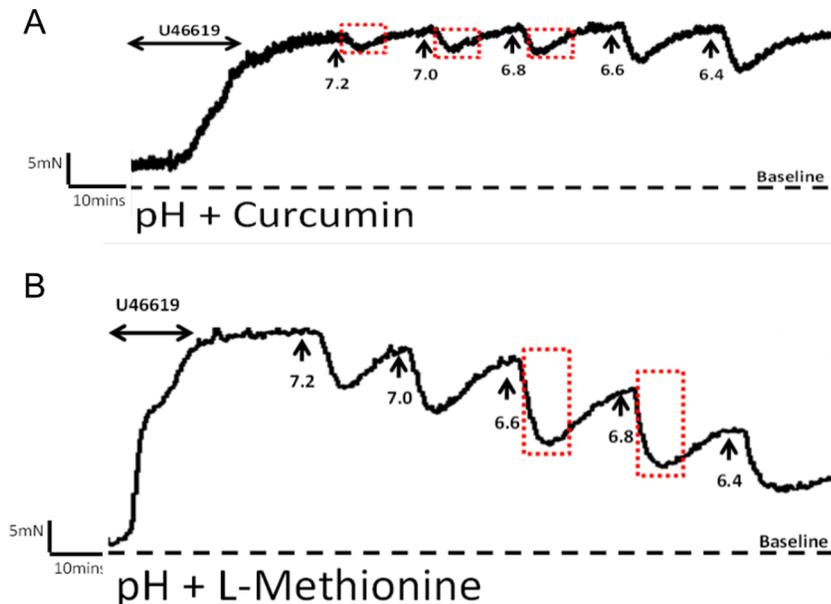


Fig. 4. The effects of TREK-1 modulators. (A) 10 μ M Curcumin inhibited both the maximal relaxant effect by pH and accelerated the recovery phase. (B) pH effects were potentiated by 1 mM L-methionine shown by the boxed area.

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blood flow through which effective fetomaternal transfer for elimination of waste metabolites into the maternal circulation is achieved. Acidic pH may also lead to chronic metabolic inhibition as ATP levels fall adding to the pH insult [27]. Levels of metabolic acids such as lactic acid have been shown to increase when tissue perfusion is low as may occur during certain pregnancy disorders or during uterine contractions accompanying normal labour [28,29]. Umbilical cord arterial pH values in normal term infants lie in the range 7.2–7.3 while cord arterial pH values below a threshold of 7.0 are associated with perinatal mortality and morbidity as well as cerebral palsy [30]. We used lactic acid for the experiments described herein to demonstrate that lowering extracellular pH presents a significant H^+ challenge resulting in an immediate and initial loss of arterial tone in placental resistance vessels. This response is transient and reversible as the vessel recovers its contractile function probably as a consequence of buffering agents within the saline. These effects of pH afforded an opportunity to investigate the specific ion channels expressed in CPA as well as the main subtypes underlying the pH responses.

In most vascular preparations, lowering pH results in vasodilatation and is consistent with the responses we observed in of CPA. Our findings corroborate observations by others on the role of LTCC in the placenta with the dihydropyridine blocker, nifedipine, inhibiting the U46619 constriction [6] in accordance with its role in HFPV ([7] [31]). Pre-treatment with nifedipine enhanced the response to acidification and may be linked to the reduced contractile effect of U46619 produced in the presence of nifedipine. CPA were also

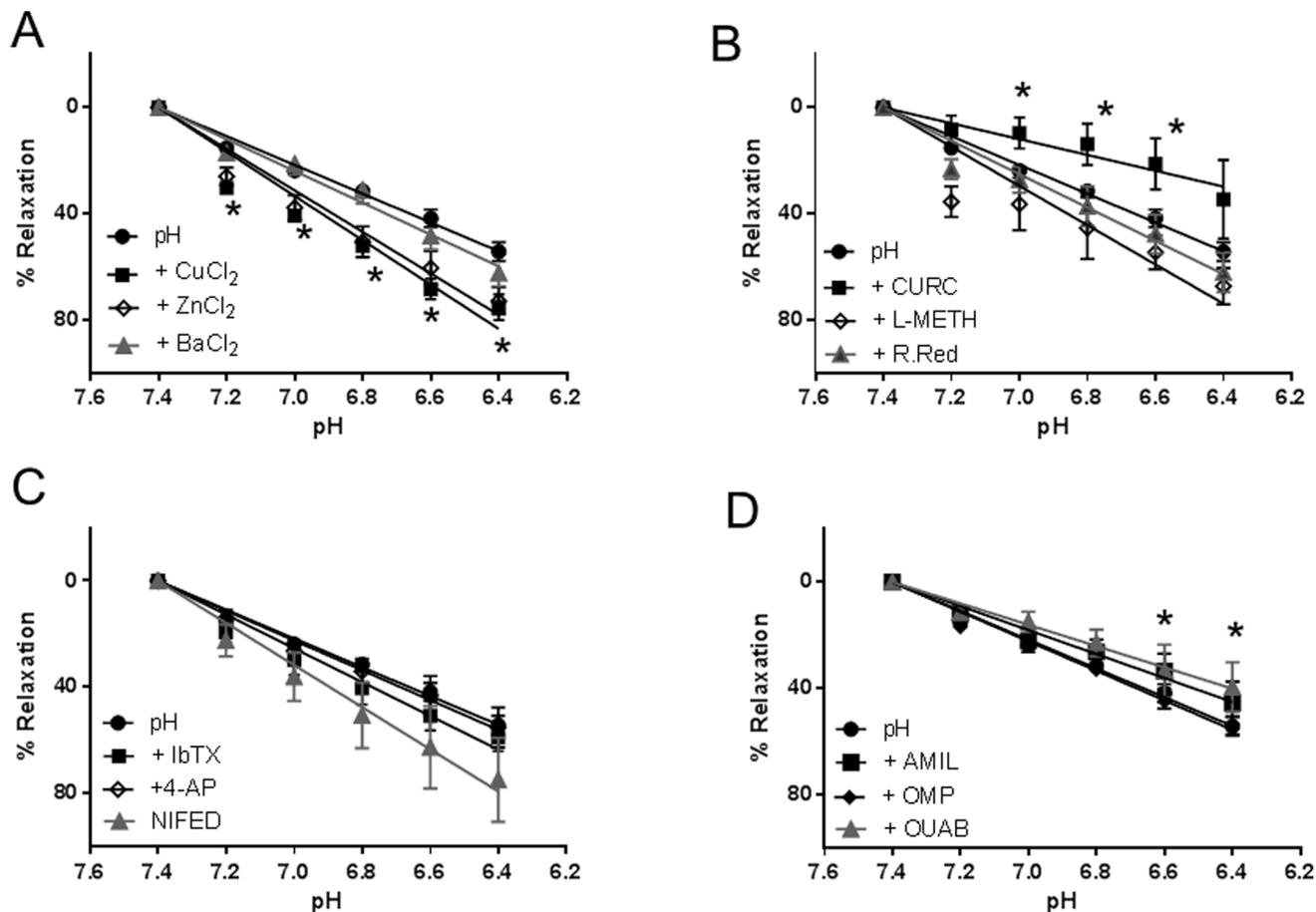


Fig. 5. The effect of channel modulators on the pH response of U46619-precontracted vessels. (A) 1 mM BaCl₂ had no effect on relaxation while both 1 mM CuCl₂ and 1 mM ZnCl₂ significantly increased the relaxation evoked. Curcumin inhibited relaxation while 10 μM ruthenium red had no effect (C) L-methionine induced relaxation at (C) 10 μM Nifedipine inhibited the response while 1 mM 4-AP and 200 nM IbTX had no effect, (D) Neither 10 μM amiloride, 10 μM omeprazole nor 50 μM ouabain had any effect on the pH response while ouabain was seen to relax CPA

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able to produce a transient response upon stimulation with lactic acid in the presence of this LTCC channel blocker indicating other channels may underlie this response.

Vascular effects implicating the BK_{Ca} channel in CPA function are interesting. Although we failed to demonstrate a role for this channel either on basal tone or pH responses, the BK_{Ca} channel opener NS1619 evoked a potent iberiotoxin-sensitive relaxation in CPA compared to that seen with SNP suggesting it may form part of an important feedback loop that counteracts depolarisation. A lack of effect of low pH on BK_{Ca} while consistent with data from other vascular beds [32, 33] arteries contrasts with findings in cerebral smooth muscle such as parenchymal brain arteries where acidosis is linked with vasodilation through BK_{Ca} channels [34, 35] therefore this channel does not appear to play [6] a direct role in combatting acidosis or ischemia. Our immunofluorescence observations confirmed the localisation of both BK_{Ca} and LTCC proteins to the membrane of

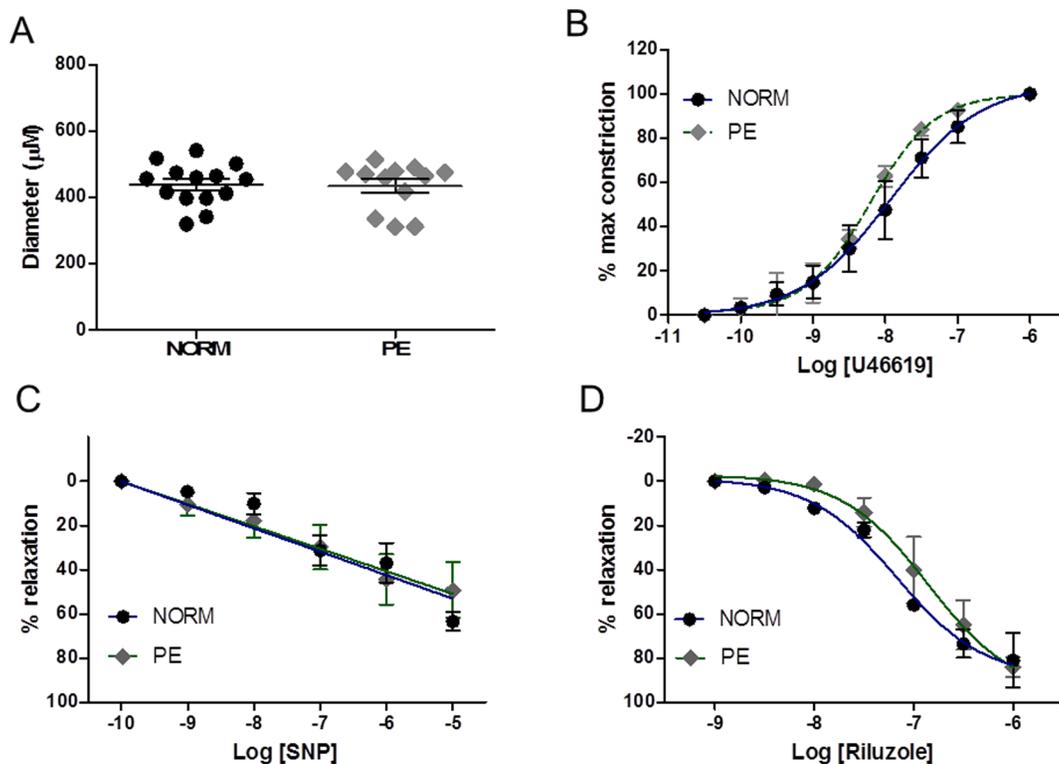


Fig. 6. Comparison of *ex vivo* responses of CPA vessels from pre-eclamptic patients with those of normal pregnancy. (A) CPA isolated from pre-eclamptic (PE) and normal (NORM) pregnancies used for myography had similar diameters ($p > 0.05$). (B) Concentration-response curves of CPA of PE and NORM patients exhibited similar responses to U46619. Vasorelaxation of vessels to SNP (C) and riluzole (D) was unchanged between normal and PE pregnancies ($p > 0.05$).

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CPA supporting their functional contributions to the control of placental arterial tone [6, 7, 15]. However, as our experiments relied on a month-long culture period of explants before sufficient single VSM cells were obtained for immunofluorescence, expression may not reflect channel expression in native cells, largely due to channel expression being modified by *in vitro* conditions including oxygen tension.

The effects of apamin on CPA are consistent with a role for SK_{Ca} channels reported to be present in myocytes of CPA [14]. However the concentration used in our study was greater than required for specific blockade of SK_{Ca} and therefore our observation is of limited value. The fact that pH did not alter the effect of the U46619-induced vasoconstriction observed in the presence of apamin suggests no role for these channels in acidosis. The modified CPA response in the presence of 4-AP lends further support for the role of Kv channels in regulation of placental tone [7] but unaffected by pH.

We provide evidence for the first time for K2P channel protein expression in CPA of TREK-1, TWIK-2 and, TASK-1. TREK-1 currents were initially reported in rat brain slices where this channel has the highest expression levels [36]. Subsequent studies have shown that TREK-1 is also widely expressed in many

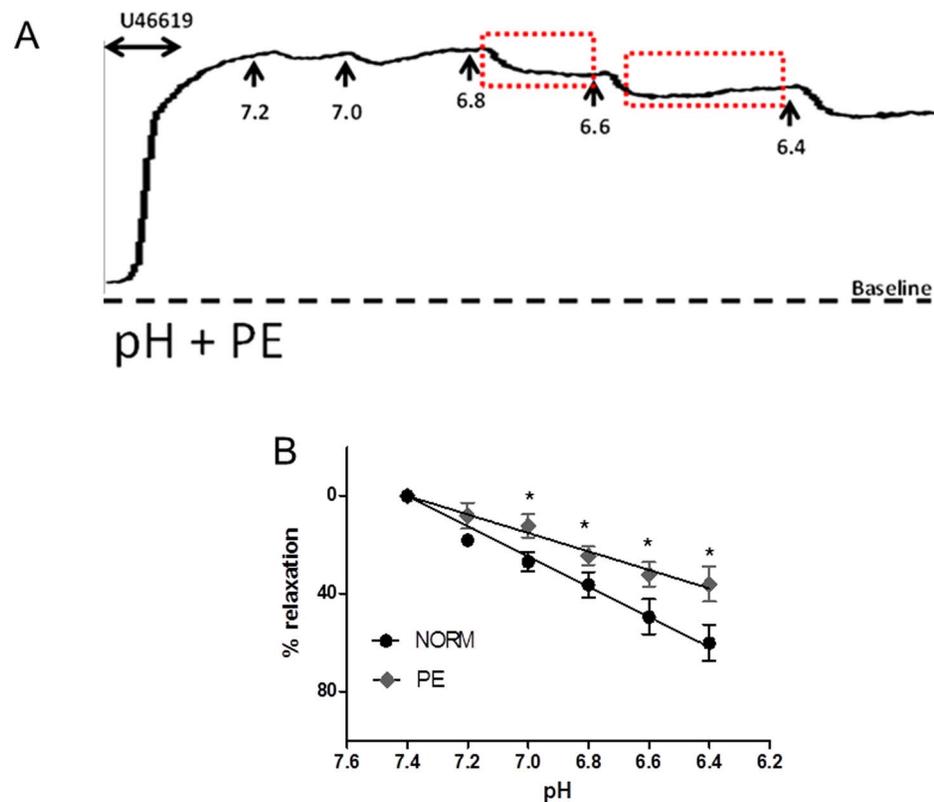


Fig. 7. Effects of pH on vessels from women with pre-eclampsia. (A) Representative trace of CPA from a pre-eclamptic patient to stepwise addition of pH. Lower pH reduced the ability of the vessel to return to baseline (red box). (B) Concentration-response curve to pH showed a significant inhibition in the relaxant response of PE samples across the lower pH range.

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tissue types [37,38]. Its capacity to control cellular function through its sensitivity to diverse stimuli including temperature, intracellular pH, lipids and stretch [39–41] identifies it as a target for the convergence of multiple signals and therefore is of potential therapeutic interest. TREK-1 is also present in cerebral arteries where it is a target for the action of polyunsaturated fatty acids and underpins the neuroprotection attributed to this group of lipids [42,43]. The importance of the TREK-1 response to stretch was shown in a recent using bladder smooth muscle cells [44]. The bladder wall stretches during the process of filling with stretch-sensitive ion channels implicated in maintaining quiescence to prevent unregulated contraction of the bladder wall. In bladder myocytes, L-methionine, but not its inactive enantiomer D-methionine, blocked the response by bladder myocytes to stretch with TREK-1 the most likely stretch-dependent channel mediating mechanotransduction [44]. In our study, L-methionine potentiated instead of blocking vasorelaxation indicating other routes for vasoactive effects of this amino acid. One of these may be through the metabolism of L-methionine to L-cysteine which replenishes sulfhydryl pools necessary for biotransformation of glyceryl trinitrate [45].

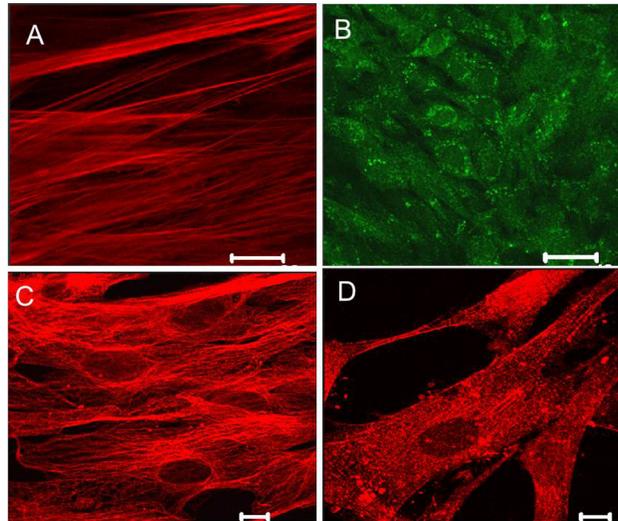


Fig. 8. Immunolocalisation of placental ion channels. Cultured CPA were fixed then incubated with primary antibody to the channel of interest then visualised. Immunofluorescence for (A) TREK-1; scale bar 10 μ m, (B) TASK-3; scale bar 40 μ m (C) CaV1.2 channels; scale bar 10 μ m, was apparent. Patterns of expression varied with TREK-1 characterised by linear expression while TASK-3 was perinuclear. (D) Representative TWIK-2 expression in CPA; scale bar 10 μ m.

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Other non-specific modulators of stretch-dependent K^+ channels had varying effects on the pH response of CPA. Curcumin, the active ingredient of turmeric has been shown to inhibit bovine TREK-1 channels, coupling this response to the depolarisation required for cortisol secretion from adrenocortical cells [46]. This observed inhibition induced by low pH is arguably consistent with blockade of a K^+ conductance in CPA. Curcumin's effects include its antioxidant properties as well as inhibition of the transcription factor nuclear factor κ -B which given the immediacy of the response to low pH is unlikely to be the mechanism underlying the effects of this phytochemical on transcription. Interestingly, the neuroprotectant riluzole which inhibits voltage-gated sodium channels and SK_{Ca} channels [47] has dual effects on TREK-1 channels where it serves to activate and inhibit TREK-1 currents [48]. Its action on CPA was to produce relaxation of precontracted arteries that could occur via SK_{Ca} channel or TREK-1 modulation. In contrast to our findings where the riluzole effect was not altered by pH, the response of mouse taste-bud cells in to citric acid, was potentiated by riluzole via TREK-1 blockade by acid-induced cell depolarisation [20]. The mechanosensitivity of TREK-1 may be closely linked with α -actin expression. Lauritzen [49] showed that TREK-1 activity could be altered following disruption of the α actin network in embryonic striatal neurons with implications for synaptogenesis. The pattern of TREK-1 staining noted in cardiac ventricular myocytes is thought to detect longitudinal stretch [50]. Given this evidence, the colocalisation of TREK-1 and α -actin that we observed suggests a role for this channel in mechanotransduction in response to vessel stretch or vasoconstriction.

Our observation that both CuCl_2 and ZnCl_2 enhanced the relaxant effect of CPA at low pH does not point to the effect of acidification being mediated by K2P channels since blocking them would be expected to result in reduced relaxation. In fact, the increase in vessel tone seen during the recovery period immediately after acidification was blunted by both CuCl_2 and to a greater extent with ZnCl_2 . The effects of these inorganic ions are difficult to tease apart due to their varied effects at several ion channels. Electrophysiological recordings of channels that exhibit both pH and zinc sensitivity have shown that application of 100–200 μM of ZnCl_2 can reduce TASK currents by 50% [51]. At these concentrations, we saw no change in the baseline response of CPA to ZnCl_2 and opted to use the higher dose of 1 mM in order to unmask any effect on isolated arteries. The response to ZnCl_2 can also be considered by the effects the trace element has on Ca^{2+} channel currents. ZnCl_2 inhibits the entry of Ca^{2+} into cells and this may explain why we observed a blunting of the contraction that followed the initial relaxation. Ischemia is an important modulator of Zn^{2+} levels and an increase in free unbound Zn^{2+} (where it is normally bound to protein) can alter neuronal excitability [52]. Zn^{2+} can inhibit Ca^{2+} and Na^+ channel currents (along with TASK-1) to alter neurotransmitter release and in this context, the failure of the vessels to recover from relaxation in the presence of ZnCl_2 suggests a potential blocking effect on Ca^{2+} channels involved in depolarisation.

Copper ions can bind with high affinity to cysteine, histidine, or glutamate residues which can lead to a dissociation of disulfide bridges which in K2P channels hold the pore-forming domains in place. Gruss [51] examined the role of both ions in modulating HEK-293 cells stably transfected with human TREK-1 and TASK-3. It was shown that copper activated TREK-1 while an inhibition was seen with TASK-3 [51]. With respect to pH, TASK channels open at the resting membrane potential and are a target for anaesthetics and extracellular pH. TASK-1 mRNA has been shown to be high within the placenta ([17, 53]. External acidification will inhibit both TASK 1 and TASK-3 which would have an opposite effect to the relaxation we observed in CPA but studies have shown that the exact pH value has contrasting effects on each ion channel subtype. TASK-1 is inhibited at pH 7.1–7.4 while TASK-3 is inactive at pH <6.7 [17]. The two ion channels in CPA also appeared to colocalise and given their range of pH sensitivities suggests that TASK-3 and TASK-1 may be differentially activated. Methanandamide has also been shown to inhibit both TASK-1 and TASK-3 channels but in our hands a meagre effect of methanandamide on the riluzole-mediated vasorelaxant effect, unaffected by pH, was observed. Since methanandamide is an agonist at endocannabinoid receptors CB1 and CB2 [26, 54], the relevance of our observation is limited.

The broad-acting K2P channel antagonist quinidine has as its most likely targets the K2P channels TWIK-2, TREK-1, TREK-2 and TASK-2. The tissue distribution of human TWIK-2 is high in the placenta along with the aorta, oesophagus, stomach and spleen [19]. Expression for the ion channel was detected in CPA and mRNA for the K2P members TWIK 1/2 was present in mouse taste-buds with TWIK 1/2 levels evidently the highest [20]. The quinidine block of CPA

in the absence of pH with no change on acidification indicates that TWIK-2 channels are not implicated in responses to pH but supports a role for the channel in placental vascular function.

Other likely candidates implicated in the pH response of CPA are the acid-sensing family of ion channels (ASIC). H^+ ions are removed from the cell via ATP dependent Na^+ transporters [55]. A small effect of ouabain (Na^+/K^+ ATPase inhibitor), and absence of an effect of the NHE inhibitor omeprazole along and the epithelial Na^+ channel blocker amiloride suggest no role for ASICs in CPA tone when the extracellular face was acidified. However, an attenuation of the acidic pH response when NaCl was replaced with choline chloride suggests Na^+ are potentially involved in the recovery from acidic pH insults and could involve non-selective cations channels including those of the transient receptor potential family (TRPs) that are pH-sensitive [56].

The functional responses from a small population of CPA from PE placenta were examined as part of the study since myometrial arteries from pre-eclamptic women display altered endothelium-mediated vasorelaxation [57]. The main difference between vessels from PE and normal pregnancies was seen on exposure to acidic pH with PE samples exhibiting a reduced loss in tone across the same dose range. This diminished ability to relax in response to an acidic pH stimulus could potentially be harmful as the excess H^+ cannot be efficiently buffered in placental blood. This would carry the risk of spreading the low pH insult to other areas of the placenta and more importantly expose the fetus to the same stress. It was significant that the pH response was blunted in PE samples and suggests the vessels may be less sensitive to low pH or may reflect the altered vasoconstriction seen in PE placentae. In fact, it has been shown that severe PE may predispose the fetus to acute hypoxia and fetal acidosis resulting in fetal distress [58]. Reactive oxygen species generated as a result of hypoxia (which often precedes acidosis) but also raised in pre-eclampsia [59, 60] also affect vascular function as shown by H_2O_2 -mediated vasorelaxation of precontracted CPA. This conflicts with the observations of no effect of H_2O_2 on CPAs precontracted with U46619 [61]. Our limited data set using CPAs of pre-eclamptic women provides an insight into the altered vascular function of adverse pregnancy samples but this study needs to be expanded to better understand the importance of these differences.

This study reports for the first time the expression of key members of the K2P channel family, TWIK-2, TASK-3 and TREK-1 in intact CPA and within cultured smooth muscle cells isolated from CPA. We also report that low pH causes vasorelaxation of CPA. In an *in vivo* context, extracellular acidification would improve perfusion possibly to minimise local acidic insults. Our work and that of others working with K2P channels is hampered by the lack of specific K2P channel antagonists. As a result, we may have been observing the effects of blocking more than one ion channel compensated by activation of another to maintain resting membrane potential. Responses to acidic pH will activate a number of proton-sensing ion channels however the data provided imply that no single ion channel is the target of extracellular pH. Importantly, functional responses may also reflect the surgery beforehand. Allen showed that anaesthetics used during ELLSCS can

alter the function of placental vessels [62]. It is well known that ion channels especially some K₂P channels are modulated directly by anaesthetics [37, 43, 63] and therefore samples should also be studied following vaginal delivery where alternative forms of pain relief are administered. Evidence is provided to suggest that compensatory mechanisms may exist in the placental vasculature to facilitate the response to acidic pH stress. Deciphering the potential role of K₂P channels in CPA is complex and further work will need to make use of molecular methods such as RNAi to knockdown particular channels due to a lack of selective pharmacological agents active at subtypes of this channel family.

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Author Contributions

Conceived and designed the experiments: RNK FBP TYA. Performed the experiments: TYA. Analyzed the data: RNK TYA. Wrote the paper: RNK TYA.

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