

# Characterisation of K<sup>+</sup> Channels in Human Fetoplacental Vascular Smooth Muscle Cells

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## Abstract

Adequate blood flow through placental chorionic plate resistance arteries (CPAs) is necessary for oxygen and nutrient transfer to the fetus and a successful pregnancy. In non-placental vascular smooth muscle cells (SMCs), K<sup>+</sup> channels regulate contraction, vascular tone and blood flow. Previous studies showed that K<sup>+</sup> channel modulators alter CPA tone, but did not distinguish between effects on K<sup>+</sup> channels in endothelial cells and SMCs. In this study, we developed a preparation of freshly isolated CPASMCs of normal pregnancy and investigated K<sup>+</sup> channel expression and function. CPASMCs were isolated from normal human term placentas using enzymatic digestion. Purity and phenotype was confirmed with immunocytochemistry. Whole-cell patch clamp was used to assess K<sup>+</sup> channel currents, and mRNA and protein expression was determined in intact CPAs and isolated SMCs with RT-PCR and immunostaining. Isolated SMCs expressed  $\alpha$ -actin but not CD31, a marker of endothelial cells. CPASMCs and intact CPAs expressed h-caldesmon and non-muscle myosin heavy chain-2; phenotypic markers of contractile and synthetic SMCs respectively. Whole-cell currents were inhibited by 4-AP, TEA, charybdotoxin and iberiotoxin implicating functional K<sub>v</sub> and BK<sub>Ca</sub> channels. 1-EBIO enhanced whole cell currents which were abolished by TRAM-34 and reduced by apamin indicating activation of IK<sub>Ca</sub> and SK<sub>Ca</sub> respectively. BK<sub>Ca</sub>, IK<sub>Ca</sub> and SK<sub>Ca</sub>3 mRNA and/or protein were expressed in CPASMCs and intact CPAs. This study provides the first direct evidence for functional K<sub>v</sub>, BK<sub>Ca</sub>, IK<sub>Ca</sub> and SK<sub>Ca</sub> channels in CPASMCs. These cells display a mixed phenotype implicating a dual role for CPASMCs in controlling both fetoplacental vascular resistance and vasculogenesis.

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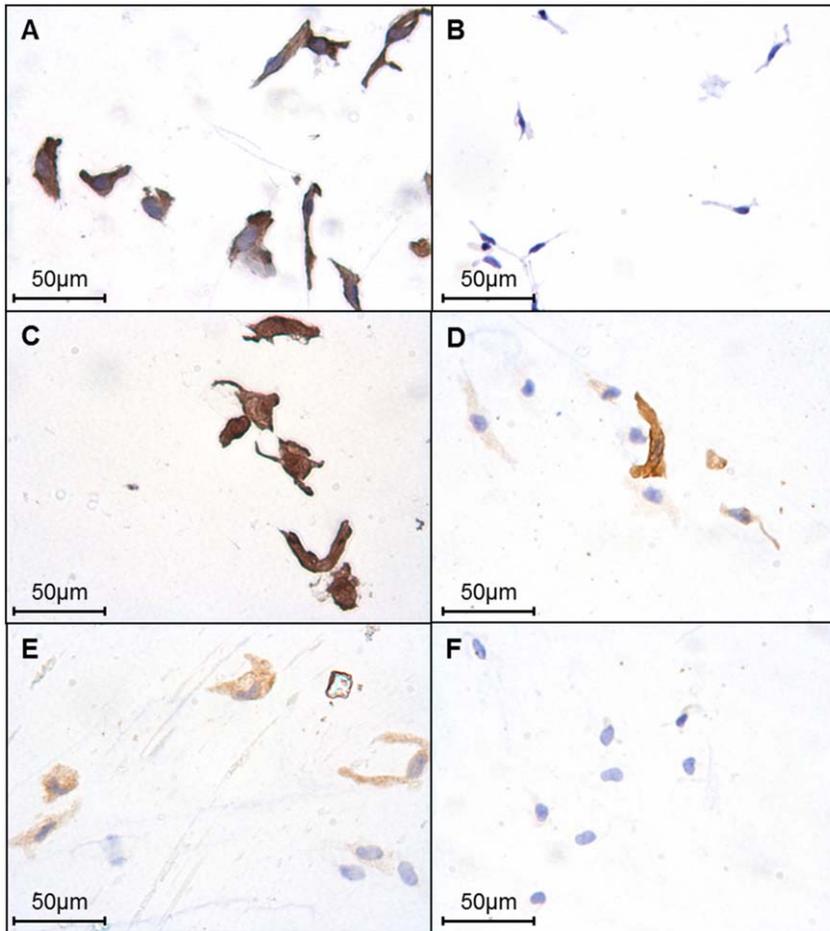
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## Introduction

Appropriate control of human placental blood flow is necessary for maximal exchange of oxygen and nutrients to the growing fetus and a successful pregnancy. Placental chorionic plate arteries (CPAs) branch from the umbilical arteries and are likely the primary determinants of fetoplacental vascular resistance as they have similar size characteristics (<500  $\mu$ m) to systemic resistance vessels [1]. Fetoplacental vascular resistance falls across gestation, indicated clinically by umbilical artery Doppler waveform analysis. Fetoplacental blood vessels lack innervation and respond poorly to potent vasoactive agents of the systemic circulation [1,2]. The primary mechanism to elicit vasodilation and maintain low vascular resistance throughout gestation is flow-induced nitric oxide (NO) release [3]. A high flow/low resistance circulation is essential to promote sufficient maternal-fetal exchange of oxygen and nutrients. Appropriate regulation of SMC function, and therefore fetoplacental vascular tone and blood flow, is necessary to facilitate maximal exchange of these substances and thereby support fetal growth. However, CPA smooth muscle cell (CPASMC) excitation-contraction coupling is poorly understood and studies are currently hindered by the lack of a suitable single cell model of isolated CPASMCs.

In non-placental vascular SMCs, potassium (K<sup>+</sup>) channels are important in controlling excitation-contraction coupling [4]. K<sup>+</sup> channels are important determinants of the resting membrane potential in vascular SMCs and are regulated by circulating vasoconstrictors and vasodilators. Membrane depolarisation, resulting from K<sup>+</sup> channel closure, provides the trigger for opening of voltage-gated Ca<sup>2+</sup> channels and the subsequent Ca<sup>2+</sup> influx to promote vasoconstriction [5]. Conversely, K<sup>+</sup> efflux due to K<sup>+</sup> channel openings causes membrane hyperpolarisation and therefore vasodilation. Non-placental VSMCs express members from all four K<sup>+</sup> channel families; K<sub>v</sub>, K<sub>IR</sub>, K<sub>2P</sub> and K<sub>Ca</sub> [4].

A small number of studies have indirectly assessed the role of K<sup>+</sup> channels in CPASMC excitation-contraction coupling in normal pregnancy. Using a range of K<sup>+</sup> channel modulators in the *in vitro* perfused placenta and isolated CPAs, K<sub>v</sub>, K<sub>ATP</sub>, K<sub>2P</sub> and K<sub>Ca</sub> channels have been implicated in regulating basal and agonist-induced tone [6,7,8,9,10,11,12]. K<sup>+</sup> channels have also been implicated in the maintenance of the resting membrane potential of CPASMCs around -38 mV and its sensitivity to high external K<sup>+</sup> [13]. Membrane depolarisation and hyperpolarisation elicited by serotonin (5-HT) and acetylcholine (ACh) respectively, was modulated by charbydotoxin and glibenclamide suggesting the presence of K<sub>Ca</sub> and K<sub>ATP</sub> channel conductances in CPASMCs



**Figure 1. Phenotype characterisation of isolated CPASMCs.** Representative examples of immunocytochemistry (A)  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), (B) CD31, (C) myosin heavy chain-2 (MHC-2), (D) h-caldesmon, (E) non-muscle myosin heavy chain-B (NMMHC-B), and (F) negative control; non-immune IgG. Positive immunostaining (DAB; brown) and nuclei (haematoxylin; blue).  
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[14]. Previous expression studies demonstrate mRNA and protein for  $BK_{Ca}$  and some  $K_v$  isoforms in whole placental homogenate or intact CPAs [8,10,11,15]. However, functional studies of CPA constriction or relaxation with channel modulators have not distinguished between effects on  $K^+$  channels expressed in endothelial cells and smooth muscle cells. The ion channel physiology of SMCs from resistance CPAs has yet to be investigated directly.

In this study, we developed a preparation of freshly isolated SMCs from CPAs of normal pregnancy and characterised  $K^+$  channel currents, mRNA and protein expression using whole-cell electrophysiology, immunocytochemistry and RT-PCR.

## Methods

### Ethical Approval

This work was performed with ethical approval from the North West (Haydock Park) Research Ethics Committee (Ref: 08/H1010/55) and informed written consent obtained for all collected tissue. Term placentas (37–42 weeks gestation; N = 40) were collected within 30 min of delivery (vaginal delivery or elective caesarean section) from women with uncomplicated pregnancies (no evidence of hypertension, FGR or other medical disorders).

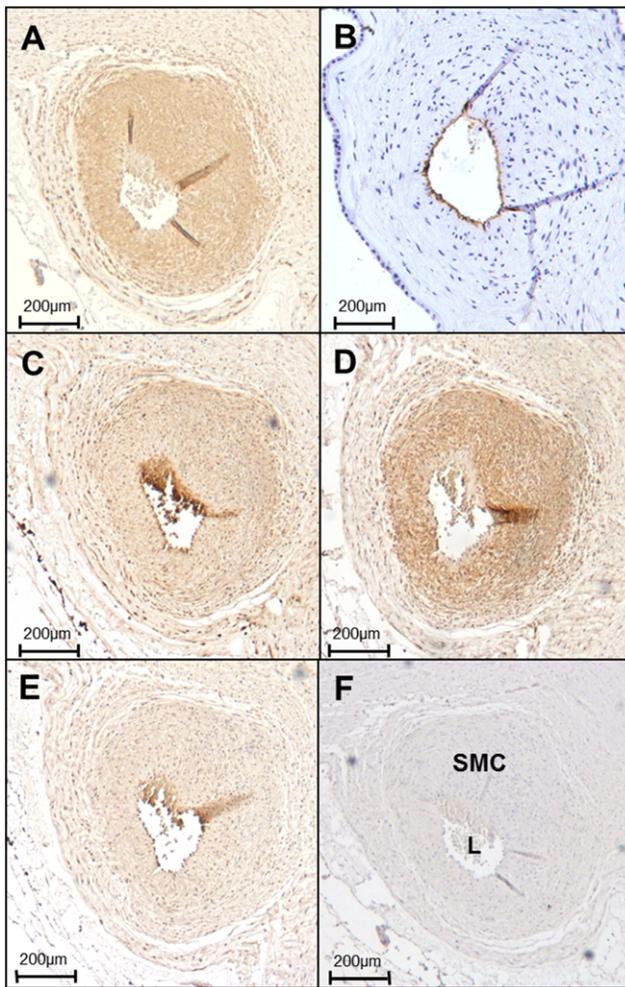
The investigation conforms to the principles outlined in the Declaration of Helsinki.

### Isolation of chorionic plate arterial smooth muscle cells

Small (150–500  $\mu$ m internal diameter) CPAs were dissected from placental biopsies using fine dissecting forceps. CPAs were cut into 5 mm lengths and maintained in  $Ca^{2+}$  free-dissociation media (DM containing in mM: 120 NaCl, 25  $NaHCO_3$ , 4.2 KCl, 0.6  $KH_2PO_4$ , 1.2  $MgCl_2$ , 11 Glucose; pH 7.4 for 5 min). CPAs were transferred into a tube containing 1 ml DM containing papain, and DTT (both 1.0 mg/ml) for 20 min at 37°C. The tissue was washed three times in ice-cold  $Ca^{2+}$  free-DM and transferred to 0.01 mM  $Ca^{2+}$ -DM containing; collagenase type 1A and collagenase type F (both 1.0 mg/ml) and incubated at 37°C for 10 min. The vessels were washed three times in ice-cold 0.01 mM  $Ca^{2+}$ -DM and triturated with a fire-polished glass Pasteur pipette.

### Characterisation of chorionic plate arterial smooth muscle cells

Immunocytochemistry was performed on the cell isolates following methanol fixation to determine; (1) the purity of the isolation technique using SMC markers  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; 15  $\mu$ g/ml; A2547 Sigma-Aldrich), myosin-heavy chain-2



**Figure 2. Phenotype characterisation of CPASMCs.** Representative examples of immunohistochemistry (A)  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), (B) CD31 (C) myosin-heavy chain-2 (MHC-2) (D) h-caldesmon, (E) non-muscle myosin heavy chain-B (NMMHC-B), and (F) negative control; non-immune IgG. Positive immunostaining (DAB; brown) and nuclei (haematoxylin; blue). SMC; smooth muscle cell, L; lumen. doi:10.1371/journal.pone.0057451.g002

(MHC-2; 1:250; ab683 Abcam), and the endothelial cell marker CD31 (5  $\mu$ g/ml; M0823 DAKO), (2) SMC phenotype using the contractile marker h-caldesmon (7.7  $\mu$ g/ml; C4562 Sigma-Aldrich) and synthetic marker non-muscle myosin heavy chain-B (NMMHC-B; 1:1000; ab684 Abcam). Endogenous peroxidase activity was quenched with 3%  $H_2O_2$  for 10 min. Non-specific binding was prevented by incubation for 30 min with non-immune block (10% goat serum, 2% human serum, and 0.1% Tween 20 in TBS). Primary antibodies were optimised and diluted in non-immune block and incubated overnight at 4°C. Negative controls were performed following substitution of the primary antibody with the corresponding concentration of non-immunised IgG. Antibody binding was detected by application of secondary antibody (biotinylated goat anti-mouse IgG; E0433; DAKO) for 30 min and avidin peroxidase (100  $\mu$ g/ml in TBS) for a further 30 min. Staining was developed using diaminobenzidine (0.75% solution in TBS) and counterstained with Harris's haematoxylin, dehydrated and then mounted.

SMC marker protein expression was confirmed in intact CPAs. CPA sections were formalin fixed for 24 h, dewaxed, rehydrated,

and microwaved for antigen retrieval in 0.01 M sodium citrate (pH 6.0). The subsequent incubation steps were repeated as described above.

### Whole-cell $K^+$ current recordings

Spindle-shaped, relaxed SMCs obtained within 4 h of isolation were selected for electrophysiology experiments. Aliquots of the cell suspension (50  $\mu$ l) were left to settle and attach for 20–30 min before addition of extracellular solution (2 ml). Recordings were made using the whole-cell patch-clamp technique [16]. Haematocrit glass patch pipettes (3–6  $M\Omega$  resistance) were pulled using a vertical pipette puller (PC-10, Narishige). Voltage protocols were applied using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) with pCLAMP 10.2 software (Axon Instruments). Cells were voltage clamped at  $-60$  mV and step depolarised from  $-70$  mV to  $+80$  mV for 500 ms in 10 mV increments and repolarised to  $-40$  mV. Membrane capacitance was calculated using manual whole-cell capacitance controls on the Axopatch amplifier. All recordings were performed at room temperature (22–25°C).

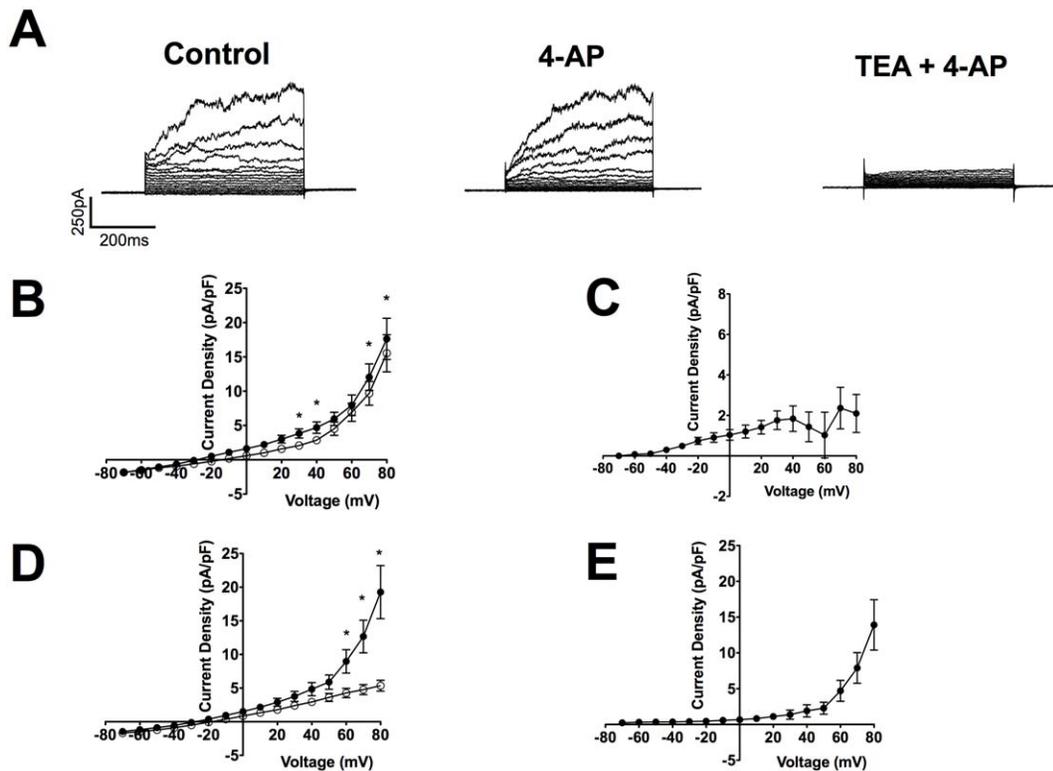
Cells were bathed with extracellular solution containing (mM): 140 NaCl, 5 KCl, 1  $CaCl_2$ , 1  $MgCl_2$ , 5 HEPES, 10 Mannitol and 5 Glucose (pH 7.3 with NaOH). Patch pipettes were filled with (mM): 120 K-aspartate, 20 KCl, 1  $MgCl_2$ , 0.5 EGTA, 35 Mannitol and 5 HEPES (pH 7.2 with KOH).  $K^+$  channel function was assessed by extracellular application of 4-aminopyridine (4-AP;  $K_v$  inhibitor; 5 mM), tetraethylammonium (TEA;  $K_{Ca}$  inhibitor; 5 mM), charybdotoxin (ChTx;  $BK_{Ca}$  and  $IK_{Ca}$  inhibitor 100 nM), iberiotoxin (IbTx;  $BK_{Ca}$  inhibitor; 100 nM), TRAM-34 ( $IK_{Ca}$  inhibitor; 10  $\mu$ M), apamin ( $SK_{Ca}$  inhibitor; 100 nM) and 1-EBIO ( $IK_{Ca}$  opener; 100  $\mu$ M). Drug concentrations were chosen according to previous electrophysiology experiments performed in non-placental vascular SMCs [17,18,19,20,21,22,23]. All  $K^+$  channel modulators were diluted in extracellular solution. Once whole-cell currents had stabilised in control solutions,  $K^+$  channel modulators were microinjected into the bath solution and currents allowed to approach a new steady state prior to recording. Poor adhesion of the cell isolates to the recording chamber necessitated the use of a static perfusion system and prevented washout of drugs.

### $K^+$ channel protein expression in chorionic plate arterial SMCs

Expression of  $BK_{Ca}$  (10  $\mu$ g/ml; APC-107 Alomone Labs) and  $IK_{Ca}$  (8  $\mu$ g/ml; APC-064 Alomone Labs) was assessed in CPASMCs by immunocytochemistry and confirmed in CPA sections.

### $K^+$ channel mRNA expression in chorionic plate arteries

Total RNA was extracted from CPAs (approximately 3 cm lengths; diameter  $<500$   $\mu$ m) using the RNeasy<sup>®</sup> Fibrous Tissue Mini Kit (QIAGEN<sup>®</sup>, Crawley, UK) according to the manufacturer's instructions, including a DNase incubation step. RNA purity was assessed using spectrophotometric analysis and quantified using a Quant-iT<sup>™</sup> Ribogreen<sup>®</sup> RNA assay kit with reference to rRNA standards (Molecular Probes, Invitrogen). RNA extracted from CPAs isolated from 10 placentas was stored at  $-80^\circ C$ . cDNA was generated in duplicate from 25 ng RNA using a Stratagene Affinity Script Multi-temperature cDNA synthesis kit (Agilent, Stockport, UK). Real-time PCR was performed for a housekeeping gene and cDNA pooled if acceptable duplicates using a Stratagene MX3000P system (Agilent) using Brilliant II SYBR<sup>®</sup> Green Master Mix (Stratagene). Primers used have either



**Figure 3. Characterisation of functional voltage-gated ( $K_v$ ) and  $Ca^{2+}$ -activated  $K^+$  channels ( $K_{Ca}$ ) in CPASMCs.** Representative example of the inhibition of outward currents in CPASMCs at negative membrane potentials by the  $K_v$  blocker 4-aminopyridine (4-AP; 5 mM; **A**) and at depolarised potentials following co-application of the  $K_{Ca}$  inhibitor tetraethylammonium (TEA; 5 mM; **A**). Mean current-voltage relationships measured at the end of the 500 ms voltage step ranging from  $-70$  mV to  $+80$  mV were obtained in the absence ( $\bullet$ ) and presence ( $\circ$ ) of 4-AP (**B**;  $n = 11$ ,  $N = 8$ ) and TEA alone (**D**;  $n = 11$ ,  $N = 6$ ). \* indicates currents (mean  $\pm$  SEM)  $P < 0.05$ ; Two-way ANOVA followed by Bonferroni Post Hoc Test. 4-AP (**C**) and TEA (**E**) sensitive currents were linear and outwardly rectifying respectively. doi:10.1371/journal.pone.0057451.g003

been previously described by our group in chorionic plate arteries; BK<sub>Ca</sub> (F)5'-AAGCAACGGAATGGAGGCAT-3' (R)5'-CCAGTGAACATCCAGTAGAGT-3', or obtained from PrimerBank and optimised for use in this system; IK<sub>Ca</sub> (F)5'-GCTGCTGCGTCTCTACCTG-3' (R)5'-AAGCGGACTTGAT TGAGAGCG-3'; SK<sub>Ca3</sub> (F)5'-GGCGGATAGCCATGACC-TAC-3' (R)5'-CGTGCCGTCCAGAAGAAGCTT-3'. BLAST searches were performed to ensure primers had no homology with other known gene products. Cycling parameters were: 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 30 s (denature), X°C for 1 min (X denotes the annealing temperature specific for each primer set; BK<sub>Ca</sub>; X = 60, IK<sub>Ca</sub> and SK<sub>Ca3</sub>; X = 61) and 72°C for 1 min (extension). Fluorescence measurements were calculated after each anneal and extension step. A dissociation curve was performed for all PCRs to ensure specificity of the reaction. All PCRs were conducted in duplicate with negative controls on the same plate. cDNA generated from human reference RNA was used as a positive control. PCR products were resolved using 2% agarose gel electrophoresis.

### Chemicals and statistical analysis

General chemicals and pharmacological agents were purchased from Sigma-Aldrich, Poole, Dorset, UK including; collagenase type F, collagenase type 1A, DTT (DL-Dithiothreitol), papain, goat serum, avidin peroxidase, Tween-20, 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB), Harris's haematoxylin, 4-AP TEA, 1-EBIO and TRAM-34. Swine serum was obtained from Vector Laboratories Inc., Burlingame, CA, USA. Charybdotoxin,

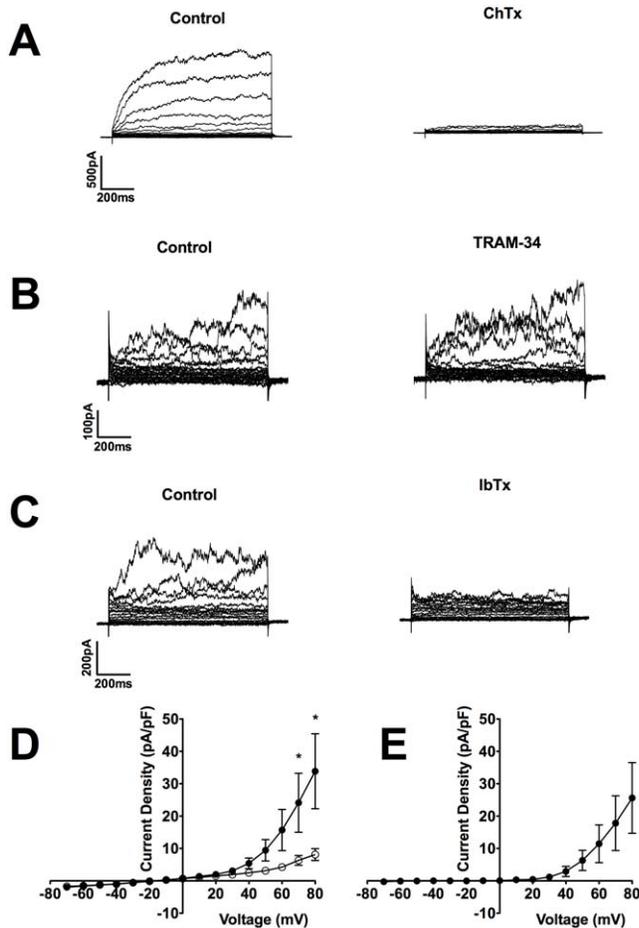
iberiotoxin and apamin were purchased from Alomone Labs, Jerusalem, Israel. Whole-cell currents were normalised to cell capacitance and results represented as mean  $\pm$  S.E.M ( $n =$  no. cells;  $N =$  no. placentas; statistical evaluation was performed using Wilcoxon-matched pairs signed rank test and Two-way ANOVA with  $n$  as the number of cells obtained from a minimum of  $N = 3$  placentas). Current-voltage relationships were constructed from currents measured at 490 ms and significance considered at the  $P < 0.05$  level.

## Results

### Characterisation of chorionic plate arterial SMCs

Cell isolates had a long, oval shaped morphology. They displayed positive immunostaining for the SMC markers  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA;  $N = 4$ ; Figure 1A) and myosin heavy chain-2 (MHC-2;  $N = 3$ ; Figure 1C), and an absence of staining for the endothelial cell marker CD31 ( $N = 3$ ; Figure 1B), confirming that they were SMCs. Consistent with previous studies in CPAs *in situ* [24], CPASMCs expressed the contractile and synthetic SMC phenotypic markers h-caldesmon ( $N = 3$ ; Figure 1D) and non-muscle myosin heavy chain-B (NMMHC-B;  $N = 3$ ; Figure 1E) respectively. Staining for these proteins was variable as cells displayed both positive and negative staining for h-caldesmon and NMMHC-B in the same field of vision (Figure 1 D, E).

In keeping with isolated SMCs, SMCs of CPA sections expressed proteins that are associated with both contractile,  $\alpha$ -SMA ( $N = 4$ ; Figure 2A), MHC-2 ( $N = 4$ ; Figure 2C), h-caldesmon



**Figure 4. Characterisation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel isoforms in CPASMCs.** Representative example of the inhibition of outward currents by the  $\text{BK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$  blocker charybdotoxin (ChTx;  $n=4$ ;  $N=2$ ; 100 nM; **A**), but not the specific  $\text{IK}_{\text{Ca}}$  inhibitor TRAM-34 ( $n=3$ ;  $N=2$ ; 10  $\mu\text{M}$ ; **B**). The specific  $\text{BK}_{\text{Ca}}$  blocker iberitoxin (IbTx; 100 nM; **C**) inhibited outward currents at depolarised potentials. Mean current-voltage relationships measured at the end of the 500 ms voltage step ranging from  $-70$  mV to  $+80$  mV were obtained in the absence ( $\bullet$ ) and presence ( $\circ$ ) of IbTx (**D**);  $*P<0.05$ ; Two-way ANOVA followed by Bonferroni Post Hoc Test; mean  $\pm$  SEM;  $n=8$ ,  $N=4$ ). IbTx-sensitive currents (**E**) were outwardly rectifying. doi:10.1371/journal.pone.0057451.g004

( $N=4$ ; Figure 2D) and synthetic NMMHC-B ( $N=4$ ; Figure 2E) SMCs. The endothelium was clearly visible with CD31 staining ( $N=4$ ; Figure 2B). No staining was evident in either CPASMCs ( $N=4$ ; Figure 1F) or CPA sections ( $N=4$ ; Figure 2F) following substitution of primary antibody with non-immune IgG at an equivalent concentration.

#### Characterisation of passive membrane properties and whole-cell current profiles

Membrane capacitance of CPASMCs ranged from 11.0 pF to 41.8 pF ( $23.2\pm 7.6$  pF; mean  $\pm$  SEM;  $n=66$ ;  $N=24$ ). Step depolarisation from a holding potential of  $-60$  mV to a series of test potentials between  $-70$  mV and  $+80$  mV elicited outward currents with a threshold potential of activation between  $-40$  mV and  $-10$  mV. In the majority of cells (56/66; 85%), currents displayed time-dependent activation, were outwardly rectifying and current magnitude fluctuated at depolarised potentials (e.g. Figure 3A) characteristic of transient openings of

$\text{BK}_{\text{Ca}}$  channels described in SMCs of several vascular beds [17,18,19,20,21,25]. These currents were superimposed upon a smaller, time-independent current. In 15% of cells; the smaller current was only recorded under basal conditions. The number of voltage steps displaying fluctuating currents, their magnitude and time course was variable between cells and did not correlate with any apparent visual differences in cell morphology under a light microscope.

#### $\text{K}^+$ channel currents in chorionic plate arterial SMCs

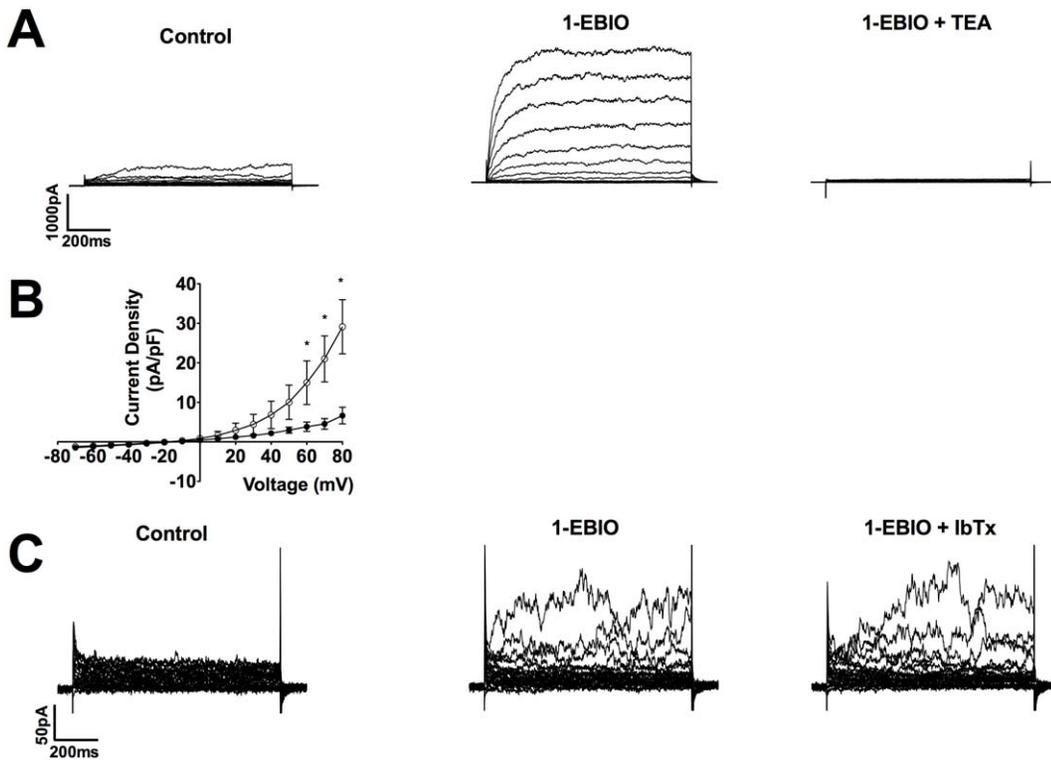
To identify  $\text{K}^+$  channels that may contribute to whole-cell currents in CPASMCs, a pharmacological approach was employed.

The broad-spectrum  $\text{K}_v$  channel blocker 4-AP (5 mM), had a small but significant inhibitory effect on the small time-independent current at potentials between 0 mV and  $+40$  mV (at  $+40$  mV: control  $4.7\pm 0.8$  pA/pF; 4-AP  $2.9\pm 0.4$  pA/pF;  $P<0.05$ ; Wilcoxon matched-pairs signed rank test;  $n=11$ ,  $N=8$ ; Figure 3A). Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence ( $\bullet$ ) and presence ( $\circ$ ) of 5 mM 4-AP with significant inhibition of currents evident at  $+30$  mV and  $+40$  mV, and potentials depolarised to  $+70$  mV ( $P<0.05$ ; Two-way ANOVA followed by Bonferroni Post Hoc Test; Figure 3B). 4-AP sensitive current reversed close to the  $\text{K}^+$  equilibrium potential implicating the presence of  $\text{K}_v$  channel current (Figure 3C). The majority of the outward currents were not affected by 4-AP.

Application of the broad-spectrum  $\text{K}_{\text{Ca}}$  blocker TEA (5 mM) abolished the majority of outward currents in CPASMCs that were insensitive to 4-AP (Figure 3A). TEA preferentially inhibited at potentials positive to  $+50$  mV, with a  $66\pm 5\%$  decrease observed at  $+80$  mV ( $n=11$ ;  $N=6$ ). Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence ( $\bullet$ ) and presence ( $\circ$ ) of TEA alone with significant inhibition of currents evident at potentials depolarised to  $+60$  mV ( $P<0.05$ ; Two-way-ANOVA followed by Bonferroni Post Hoc Test Figure 3D). TEA-sensitive currents were outwardly rectifying and reversed close to the  $\text{K}^+$  equilibrium potential indicating  $\text{K}_{\text{Ca}}$  channel currents (Figure 3E).

Identifying the specific  $\text{K}_{\text{Ca}}$  isoform responsible for CPASMC currents utilised application of pharmacological blockers. Inhibition of  $\text{BK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$  isoforms with ChTx abolished outward currents ( $n=4$ ;  $N=2$ ; Figure 4A). Selective blockade of  $\text{IK}_{\text{Ca}}$  channels with TRAM-34 was without effect on outward currents ( $n=3$ ,  $N=2$ ; Figure 4B). Conversely, inhibition of  $\text{BK}_{\text{Ca}}$  channels with IbTx abolished outward currents at  $+80$  mV by  $61\pm 12\%$  ( $n=10$ ,  $N=4$ ; Figure 4C), in common with TEA (Figure 3) and ChTx (Figure 4A). Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence ( $\bullet$ ) and presence ( $\circ$ ) of IbTx with significant inhibition evident at potentials depolarised to  $+70$  mV ( $P<0.05$ ; Two-way-ANOVA followed by Bonferroni Post Hoc Test; (Figure 4D). IbTx-sensitive currents were outwardly rectifying and reversed close to the  $\text{K}^+$  equilibrium potential indicating that they were mediated by  $\text{BK}_{\text{Ca}}$  channels (Figure 4E).

Further analysis of the contribution of  $\text{K}_{\text{Ca}}$  channels to whole-cell currents was performed with extracellular application of 1-EBIO ( $\text{IK}_{\text{Ca}}$  and  $\text{SK}_{\text{Ca}}$  channel activator). 1-EBIO increased whole-cell currents in all cells tested (control:  $6.6\pm 2.1$  pA/pF; 1-EBIO:  $29.1\pm 6.9$  pA/pF at  $+80$  mV;  $P<0.05$ ;  $n=22$ ,  $N=10$ ; Wilcoxon matched-pairs signed rank test; Figure 5A). Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence ( $\bullet$ ) and presence ( $\circ$ ) of 1-EBIO with significant activation evident at potentials depolarised to  $+60$  mV ( $P<0.05$ ; Two-way-ANOVA followed by Bonferroni Post Hoc Test;



**Figure 5. Characterisation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel openers in CPASMCs.** The  $\text{IK}_{\text{Ca}}$  and  $\text{SK}_{\text{Ca}}$  channel activator 1-EBIO (100  $\mu\text{M}$ ) increased outwards currents in CPASMCs (A). Mean current-voltage relationships obtained at the end of the 500 ms voltage step in the absence (●) and presence (○) of 1-EBIO (B; \* $P < 0.05$ ; Two-way ANOVA followed by Bonferroni Post Hoc Test; mean  $\pm$  SEM;  $n = 22$ ;  $N = 10$ ). The 1-EBIO sensitive current was inhibited by the  $\text{K}_{\text{Ca}}$  inhibitor TEA (A; 5 mM;  $n = 2$ ;  $N = 2$ ), but not the specific  $\text{BK}_{\text{Ca}}$  inhibitor iberiotoxin (IbTx; 100 nM; C). doi:10.1371/journal.pone.0057451.g005

Figure 5B). Tail-currents were observed in 2 out of 22 recordings following 1-EBIO application (see Figure 5A) characteristic of  $\text{K}_{\text{v}}$  channels. However, the low incidence of these currents prevented further analysis. Abolition of the 1-EBIO response with TEA confirmed this increase in whole-cell current was mediated by a  $\text{K}_{\text{Ca}}$  channel (Figure 5A). Experiments to determine the  $\text{K}_{\text{Ca}}$  isoform responsible for 1-EBIO-sensitive current excluded a role for  $\text{BK}_{\text{Ca}}$  as selective blockade of these channels with IbTx in the continued presence of 1-EBIO, had no effect on the magnitude of outward currents (1-EBIO:  $15.2 \pm 11.2$  pA/pF; 1-EBIO + IbTx:  $14.2 \pm 9$  pA/pF at +80 mV;  $P > 0.05$ ; Wilcoxon matched-pairs signed rank test;  $n = 3$ ,  $N = 3$ ; Figure 5C).

Selective inhibition of  $\text{IK}_{\text{Ca}}$  isoforms with TRAM-34 ( $n = 7$ ,  $N = 4$ ) reduced 1-EBIO sensitive currents (1-EBIO:  $34.5 \pm 10.0$  pA/pF; 1-EBIO + TRAM-34:  $7.1 \pm 2.7$  pA/pF at +80 mV;  $P < 0.05$ ; Wilcoxon matched-pairs signed rank test;  $n = 7$ ,  $N = 4$ ; Figure 6A). Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence (●) and presence of 1-EBIO (○) and presence of 1-EBIO with TRAM-34 (x). Addition of TRAM-34 following 1-EBIO application restored the current-voltage relationship to the basal level (Figure 6B). In addition, inhibition of  $\text{SK}_{\text{Ca}}$  isoforms with apamin reduced 1-EBIO sensitive currents (+80 mV from  $17.5 \pm 5.0$  pA/pF to  $10.7 \pm 2.4$  pA/pF;  $P < 0.05$ ; Wilcoxon matched-pairs signed rank test;  $n = 7$ ;  $N = 3$  Figure 6C). The remaining currents were blocked by TRAM-34. Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence (●) and presence of 1-EBIO (○) and presence of 1-EBIO with apamin (x) (Figure 6D).

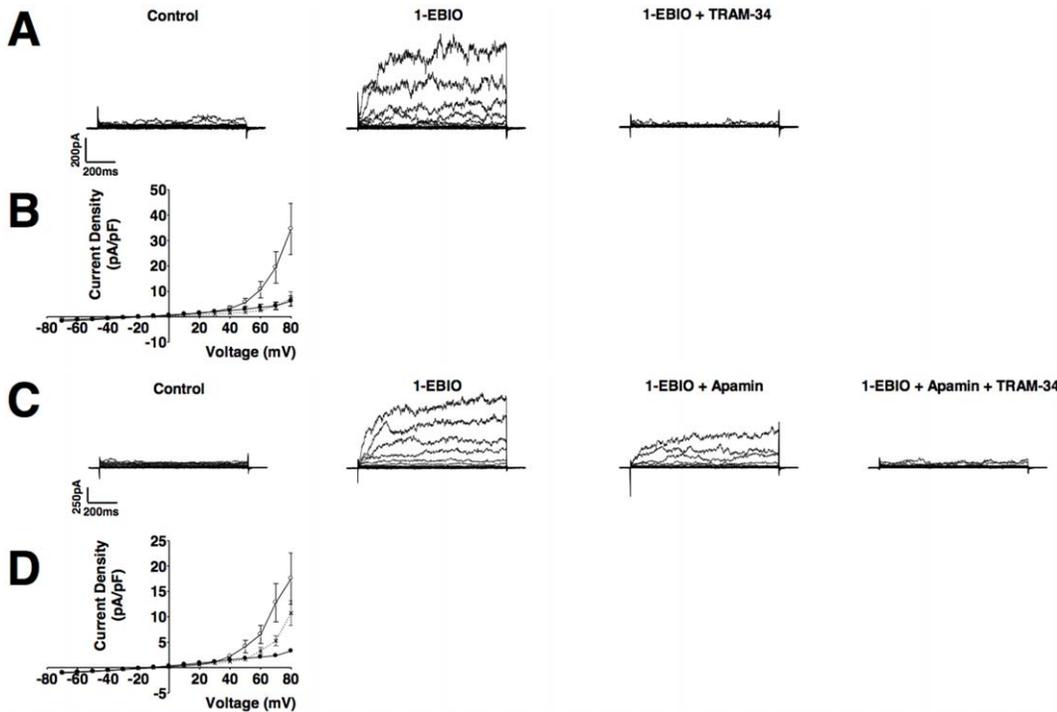
### $\text{K}_{\text{Ca}}$ channel expression in chorionic plate arteries

Protein expression of the pore-forming  $\alpha$ -subunits of  $\text{BK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$  was evident in isolated CPASMCs ( $N = 3$ ; Figure 7A, B) and intact CPA sections ( $N = 3$ ; Figure 7D, E). Non-specific staining in either isolated SMCs ( $N = 4$ ; Figure 7C) or CPA sections ( $N = 4$ ; Figure 7F) was not observed following substitution of primary antibody with non-immune IgG at an equivalent concentration. mRNA for  $\text{BK}_{\text{Ca}}$  (Figure 7G,  $32 \pm 1$ ; CT values median  $\pm$  IQR),  $\text{IK}_{\text{Ca}}$  channels (Figure 7H,  $35 \pm 2$ ; CT values median  $\pm$  IQR) and  $\text{SK}_{\text{Ca}3}$  (Figure 7I,  $33 \pm 2$ ; CT values median  $\pm$  IQR) was also expressed in intact CPA sections.

### Discussion

Currently, no studies have systematically investigated  $\text{K}^+$  channel activity in SMCs of resistance arteries in the human placenta that regulate fetoplacental blood flow. In this study, a method was developed to isolate SMCs from placental chorionic plate arteries with size characteristics of resistance vessels for whole cell recording to characterise  $\text{K}^+$  currents. We showed that the cells express several  $\text{K}^+$  currents that, on the basis of their pharmacology, could be attributed to  $\text{K}_{\text{v}}$ ,  $\text{BK}_{\text{Ca}}$ ,  $\text{IK}_{\text{Ca}}$ , and  $\text{SK}_{\text{Ca}}$  channels. The contribution of these channels was supported by their mRNA and/or protein expression in intact arteries and in SMCs after isolation.

The isolation protocol produced a high yield of relaxed CPASMCs, which displayed positive immunostaining for SMC markers and negative staining for an endothelial cell marker. Phenotypic characterisation of CPASMCs confirmed our previous electron microscopy studies of the native artery [24]; isolated



**Figure 6. Functional intermediate-conductance ( $IK_{Ca}$ ) and small-conductance ( $SK_{Ca}$ )  $Ca^{2+}$ -activated  $K^+$  channels in CPASMCs.** The specific  $IK_{Ca}$  inhibitor TRAM-34 (10  $\mu$ M) abolished 1-EBIO sensitive currents (A). Mean current-voltage relationships obtained at the end of the 500 ms voltage step under control conditions (●), and following application of 1-EBIO (○) and TRAM-34 in the continued presence of 1-EBIO (x) (mean  $\pm$  SEM; n = 7; N = 4; B). The specific  $SK_{Ca}$  inhibitor apamin (100 nM) partially inhibited 1-EBIO sensitive currents and the remaining current was abolished by the subsequent addition of TRAM-34 (C). Mean current-voltage relationships obtained at the end of the 500 ms voltage step under control conditions (●), and following application of 1-EBIO (○) and apamin in the continued presence of 1-EBIO (x) (mean  $\pm$  SEM; n = 7; N = 3; D). doi:10.1371/journal.pone.0057451.g006

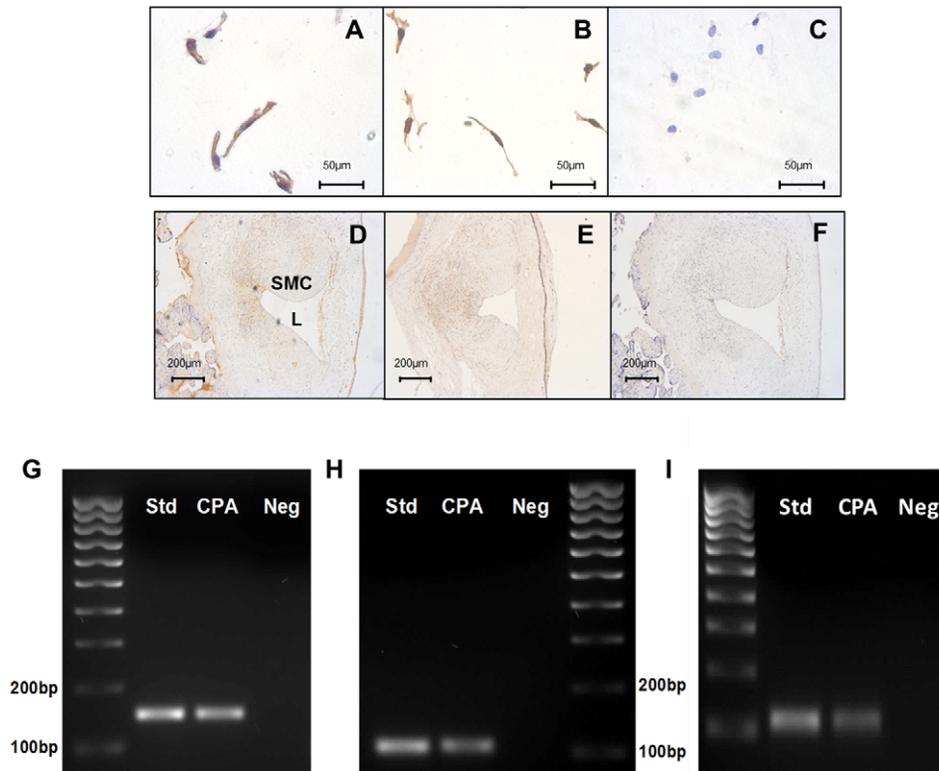
CPASMCs and intact CPAs displayed a mixed phenotype, expressing markers of both contractile (h-caldesmon) and synthetic (NMMHC-B) SMC phenotypes. The h-caldesmon and NMMHC-B staining intensity was variable between the isolates suggesting a range of intermediate phenotypes between purely contractile and synthetic. The presence of synthetic SMCs in CPAs, which are important in controlling vasculogenesis, is consistent with the fetal origin of the tissue [26]. A high incidence of synthetic relative to contractile SMCs will impact upon the response of CPAs to modulators of vascular tone and may explain why these vessels are relatively unresponsive to potent vasoactive agents of the systemic circulation [1].

The major  $K^+$  currents expressed in CPASMCs under basal conditions were investigated. Whole-cell currents comprised a linear, time-independent component and an outwardly rectifying component characterised by the presence of fluctuating currents at depolarised potentials. Current magnitude was variable between cells and was not related to cell size or obvious differences in cell morphology; only relaxed SMCs with a defined membrane were used for electrophysiology experiments. Variability in the current profiles could relate to their phenotype (i.e. synthetic/contractile). However, linking current profiles to SMC phenotype necessitates staining cells that have been recorded which was beyond the scope of this initial investigation to screen  $K^+$  currents in CPASMCs for the first time.

A pharmacological approach was employed to identify the  $K^+$  channels responsible for outward currents in CPASMCs. Voltage-gated  $K^+$  channels ( $K_v$ ) were initially targeted given the previous expression and functional data demonstrating a role for these channels in modulating basal and agonist-induced tone in the

perfused placenta and isolated CPAs using the  $K_v$  channel blocker 4-AP [8,10,11]. In CPASMCs, 4-AP inhibited currents at relatively negative membrane potentials. This suggests  $K_v$  channels may be important at physiological membrane potentials and is consistent with 4-AP enhancing basal tone in the intact vessel [8]. The precise  $K_v$  channel isoforms responsible for 4-AP sensitive currents in CPASMCs remains unknown but previous studies have demonstrated  $K_v1.5$  [27],  $K_v7.4$  [28] and  $K_v9.3$  [29] protein expression in the SMCs of intact CPA sections. Microelectrode impalement studies have demonstrated that the membrane potential of CPASMCs in the intact vessel is approximately  $-38$  mV and is sensitive to high external  $K^+$  [13]. Membrane depolarisation and hyperpolarisation elicited by serotonin (5-HT) and acetylcholine (ACh) respectively, was modulated by ChTx and glibenclamide suggesting the presence of  $K_{Ca}$  and  $K_{ATP}$  conductances in CPASMCs [14]. However, it remains to be determined in isolated CPASMCs, whether  $K_v$  channels *per se* and specifically which isoforms contribute to maintaining the resting membrane potential and are therefore directly responsible for 4-AP induced alterations in basal tone.

$Ca^{2+}$ -activated  $K^+$  channels were next targeted given previous studies demonstrating a role for these channels in modulating fetoplacental vascular tone [8,10,11]. The marked inhibition by TEA ( $K_{Ca}$  inhibitor), charybdotoxin ( $BK_{Ca}$  and  $IK_{Ca}$  inhibitor), iberiotoxin ( $BK_{Ca}$  inhibitor) but not TRAM-34 ( $IK_{Ca}$  inhibitor) suggests that  $BK_{Ca}$  channels are responsible for the majority of outward currents in CPASMCs under the recording condition used in the present study. Expression of  $BK_{Ca}$  mRNA and protein was confirmed in CPAs and protein localised to the SMCs. In common with non-placental vascular SMCs [17,18,19,20,21], the



**Figure 7. mRNA and protein expression of K<sub>Ca</sub> channels in CPAs.** Isolated CPASMCs expressed BK<sub>Ca</sub> (A) and IK<sub>Ca</sub> (B) protein. BK<sub>Ca</sub> (D) and IK<sub>Ca</sub> (E) protein was localised to the SMCs in intact CPA sections. Negative control (C) and (F); substitution of primary antibody with non-immune IgG. RT-PCR detection of BK<sub>Ca</sub> (G) IK<sub>Ca</sub> (H) and SK<sub>Ca</sub>3 (I) transcripts in human reference cDNA (Std), chorionic plate artery (CPA). Negative (Neg) is omission of cDNA. Amplicon lengths; BK<sub>Ca</sub> 147, IK<sub>Ca</sub> 102 and SK<sub>Ca</sub>3 106. SMC; smooth muscle cell, L; lumen. doi:10.1371/journal.pone.0057451.g007

large single-channel conductance of BK<sub>Ca</sub> likely accounts for the characteristic noise and fluctuating currents observed at depolarised potentials in CPASMCs. BK<sub>Ca</sub> activity in the present study likely reflects membrane depolarisation induced Ca<sup>2+</sup> entry given the low intracellular Ca<sup>2+</sup> recording concentrations employed (0.5 mM [EGTA]<sub>i</sub>; predicted [Ca<sup>2+</sup>]<sub>i</sub> ~10 nM). Compared to pulmonary resistance arterial SMCs which similarly carry deoxygenated blood but express primarily K<sub>v</sub> channels, the predominance of K<sub>Ca</sub> over K<sub>v</sub> channels in CPASMCs is interesting. Extrapolation of this *in vitro* finding to the physiological situation in the intact vessel or whole placenta where there is a complex ionic environment should be made with caution. However, together with previous observations demonstrating functional BK<sub>Ca</sub> channels in the whole-perfused placenta, intact artery and large diameter CPASMCs using iberiotoxin and charybdotoxin [8,10,11], this study implicates a role for BK<sub>Ca</sub> channels in CPASMC excitation-contraction coupling.

Further analysis of the role of K<sub>Ca</sub> channels in CPASMCs demonstrated IK<sub>Ca</sub> currents. Application of the SK<sub>Ca</sub> and IK<sub>Ca</sub> activator 1-EBIO significantly increased outward currents. 1-EBIO sensitive currents were reduced by TEA (K<sub>Ca</sub> inhibitor), TRAM-34 (IK<sub>Ca</sub> inhibitor), but unaffected by the BK<sub>Ca</sub> inhibitor iberiotoxin indicating functional IK<sub>Ca</sub> channels. In non-placental blood vessels, immunohistochemical and electrophysiological studies do not implicate IK<sub>Ca</sub> channel expression or function in SMCs, rather, they are localised to the endothelium [23,30,31,32,33]. Electrophysiology experiments in SMCs isolated from the rat hepatic artery [32] and mouse portal vein [23] failed to observe an effect of 1-EBIO on whole-cell K<sup>+</sup> currents. This is

in marked contrast to CPASMCs where 1-EBIO consistently increased currents at +80 mV by over 400%. The effect of 1-EBIO is strongly Ca<sup>2+</sup> dependent [34] and stimulation of IK<sub>Ca</sub> usually requires high intracellular Ca<sup>2+</sup> concentrations or addition of the Ca<sup>2+</sup> ionophore ionomycin. Why 1-EBIO is causing significant current activation in CPASMCs given the low recording intracellular Ca<sup>2+</sup> concentration in this study is unclear. Similarly, the apparent time-dependent activation of the 1-EBIO-sensitive current is inconsistent with the voltage-independent nature of IK<sub>Ca</sub> channels. However, 1-EBIO-sensitive currents will be modulated by other underlying ion conductances native to these cells. In addition, kinetic analyses were not performed to quantify differences in current profiles following 1-EBIO application given the inherent variability in outward currents recorded. The data demonstrating abolition of 1-EBIO-activated currents with TRAM-34, together with those demonstrating IK<sub>Ca</sub> mRNA and protein expression, provides strong evidence for the presence of IK<sub>Ca</sub> channels in CPAs and their localisation to SMCs.

1-EBIO can also activate SK<sub>Ca</sub> channels. It was therefore investigated whether the SK<sub>Ca</sub> inhibitor apamin had any effect on 1-EBIO sensitive currents. Application of apamin at a concentration that inhibits both SK<sub>Ca</sub> isoforms [35] reduced 1-EBIO sensitive currents but to a lesser extent than TRAM-34. In common with IK<sub>Ca</sub> channels, SK<sub>Ca</sub> are thought to predominantly localise to the endothelium where they participate in the EDHF response [30,36]. Therefore, functional SK<sub>Ca</sub> channels in CPASMCs were unexpected and may be mediated by the SK<sub>Ca</sub>3 isoform given mRNA expression for the pore forming  $\alpha$ -subunit in the intact artery. Protein expression of SK<sub>Ca</sub>3 remains to be

determined in the intact CPA and CPASMCs. The functional significance of SK<sub>Ca</sub>3 channels in CPASMCs is not known but they may be important in dampening excitation and promoting vasodilation following a rise in intracellular Ca<sup>2+</sup> in common with BK<sub>Ca</sub> channels.

It is evident that CPASMCs are a heterogeneous population of cells; they express diverse K<sup>+</sup> channel currents with varying magnitude and activation profiles, and display a mixed phenotype with both contractile and synthetic characteristics. In other vascular SMCs, a close relationship exists between phenotype and K<sup>+</sup> channel expression [37,38,39]. SMCs with a contractile phenotype predominantly express BK<sub>Ca</sub> and K<sub>v</sub> channels which are important in mediating excitation-contraction coupling [18,22,25,40,41,42]. Conversely, synthetic SMC express IK<sub>Ca</sub> channels that control proliferation and migration of SMCs during vasculogenesis by promoting Ca<sup>2+</sup> entry and activation of Ca<sup>2+</sup>-dependent growth factors [38,43,44,45]. The mixed phenotype of CPASMCs with both contractile and synthetic characteristics may account for the functional expression of BK<sub>Ca</sub>, K<sub>v</sub> and IK<sub>Ca</sub> channels. BK<sub>Ca</sub> and K<sub>v</sub> channel activity in CPASMCs are important in controlling excitation-contraction coupling under basal and agonist-stimulated conditions as pharmacological modulation of these channels alters CPA tone in the intact vessel and perfused placenta [8,10,11]. The physiological significance of IK<sub>Ca</sub> channels in CPASMCs is unexplored. Similarly, whether the existence of this channel in CPASMCs is linked to the synthetic

phenotype remains to be confirmed. In addition to a putative role in regulating fetoplacental vascular tone, IK<sub>Ca</sub> channels may play an important role in controlling CPASMC proliferation and placental vasculogenesis throughout gestation. Further studies are required to directly correlate whole-cell K<sup>+</sup> currents and the contribution from specific channels with the expression of SMC phenotypic markers.

In conclusion, this study provides the first direct evidence for K<sub>v</sub>, BK<sub>Ca</sub>, IK<sub>Ca</sub> and SK<sub>Ca</sub> channel currents in CPASMCs. These cells display a mixed phenotype implicating a dual role for CPASMCs in controlling both fetoplacental vascular resistance and vasculogenesis throughout pregnancy.

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## Author Contributions

Conceived and designed the experiments: MFB MW SLG RLJ. Performed the experiments: MFB. Analyzed the data: MFB. Contributed reagents/materials/analysis tools: MFB SLG MW RLJ. Wrote the paper: MFB.

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