Expression and Function of Potassium Channels in Human Placental Chorionic Plate Arteries

‘A thesis submitted to the University of Manchester for the degree of PhD in the Faculty of Medical and Human Sciences’

2012

Melissa Faith Brereton

School of Medicine
CONTENTS

LIST OF FIGURES ................................................................. 6
LIST OF TABLES ............................................................... 10
LIST OF APPENDICES .......................................................... 11
ABSTRACT ........................................................................... 12
DECLARATION ....................................................................... 13
COPYRIGHT STATEMENT ..................................................... 14
ABBREVIATIONS .................................................................. 15
PUBLICATIONS AND PRESENTATIONS .................................... 17
ACKNOWLEDGEMENTS ......................................................... 19
DEDICATION ......................................................................... 20

1. INTRODUCTION .................................................................. 21

1.1. PLACENTA ....................................................................... 21
  1.1.1. Structure and function of the placenta ....................... 21
  1.1.2. Fetoplacental vasculature ........................................ 24

1.2. CONTROL OF VASCULAR TONE ....................................... 26
  1.2.1. Neuronal Factors ...................................................... 26
  1.2.2. Humoral Factors ...................................................... 27
    1.2.2.1. Vasoconstrictors .............................................. 27
    1.2.2.2. Vasodilators .................................................. 29
  1.2.3. Metabolic Factors .................................................... 30
  1.2.4. Ion Channels .......................................................... 32

1.3. CONTROL OF FETOPLACENTAL VASCULAR TONE ............ 37
  1.3.1. Experimental methods of assessment ......................... 38
    1.3.1.1. Placental perfusion ........................................... 38
    1.3.1.2. Intact vessels ................................................. 38
    1.3.1.3. Isolated endothelial and smooth muscle cells ....... 39
  1.3.2. Neuronal Factors .................................................... 39
  1.3.3. Humoral Factors ..................................................... 40
    1.3.3.1. Vasoconstrictors .............................................. 40
    1.3.3.2. Vasodilators .................................................. 41
  1.3.4. Metabolic Factors .................................................... 42
  1.3.5. Ion Channels .......................................................... 48
  1.3.6. Summary .............................................................. 49

1.4. K⁺ CHANNELS: STRUCTURE AND FUNCTION .................... 50
  1.4.1. Structure .............................................................. 50
    1.4.1.1. Voltage-Gated K⁺ Channels ................................ 51
    1.4.1.2. Ca²⁺-Activated K⁺ Channels .............................. 52
    1.4.1.3. Inwardly-Rectifying K⁺ Channels ....................... 53
    1.4.1.4. Twin-Pore K⁺ Channels .................................... 53
  1.4.2. Function: Control of Vascular Tone ......................... 57
    1.4.2.1. Voltage-Gated K⁺ Channels .............................. 57
    1.4.2.2. Ca²⁺-Activated K⁺ Channels .............................. 59
    1.4.2.3. Inwardly-Rectifying K⁺ Channels ....................... 61
    1.4.2.4. Twin-Pore K⁺ Channels .................................... 62
1.5. K⁺ CHANNEL EXPRESSION AND FUNCTION IN THE FETOPLACENTAL VASCULATURE ................................................................. 65
1.5.1. Expression .................................................................................. 65
  1.5.1.1. Voltage-Gated K⁺ Channels ...................................................... 66
  1.5.1.2. Ca²⁺-Activated K⁺ Channels .................................................. 66
  1.5.1.3. Inwardly-Rectifying K⁺ Channels ............................................ 66
  1.5.1.4. Twin-Pore K⁺ Channels ......................................................... 67
1.5.2. Function: Control of Fetoplacental Vascular Tone ......................... 67
  1.5.2.1. Voltage-Gated K⁺ Channels .................................................. 67
  1.5.2.2. Ca²⁺-Activated K⁺ Channels .................................................. 69
  1.5.2.3. Inwardly-Rectifying K⁺ Channels ............................................ 70
  1.5.2.4. Twin-Pore K⁺ Channels ......................................................... 71
1.5.3. Summary .................................................................................... 71

1.6. AIMS AND HYPOTHESES ............................................................... 73

2. ROLE OF OXYGEN-SENSITIVE Kᵥ CHANNEL BLOCKERS IN REGULATING CHORIONIC PLATE ARTERIAL TONE 74

2.1. INTRODUCTION ............................................................................. 74

2.2. MATERIALS AND METHODS .......................................................... 78
  2.2.1. Vessel Preparation .................................................................... 78
  2.2.2. Wire Myography ....................................................................... 78
  2.2.3. Chemicals and Statistical Analyses ............................................. 80

2.3. RESULTS .......................................................................................... 82
  2.3.1. Effect of acute exposure to different oxygen tensions on contractile responses ........................................................................ 82
  2.3.2. Effect of chronic exposure to different oxygenations on contractile responses ........................................................................ 84
  2.3.3. Effect of hyperoxia on the ability of Kᵥ channel blockers to regulate chorionic plate arterial tone .................................................... 87
  2.3.4. Effect of normoxia on the ability of Kᵥ channel blockers to regulate chorionic plate arterial tone .................................................... 97
  2.3.5. Effect of hypoxia on the ability of Kᵥ channel blockers to regulate chorionic plate arterial tone .................................................... 107
  2.3.6. Summary ................................................................................... 117

2.4. Discussion ....................................................................................... 119
  2.4.1. Effect of acute exposure to different oxygenations on contractile responses ........................................................................ 119
  2.4.2. Effect of chronic exposure to different oxygenations on contractile responses ........................................................................ 120
  2.4.3. Effect of acute exposure to different oxygenations on the ability of Kᵥ channels to modulate chorionic plate arterial constriction ............... 121
  2.4.4. Effect of chronic exposure to different oxygenations on the ability of Kᵥ channel blockers to modulate chorionic plate arterial constriction ........... 122
  2.4.5. Effect of acute exposure to different oxygenations on the ability of the oxygen-sensitive Kᵥ channel Kᵥ₁.₅ blocker to modulate chorionic plate arterial constriction .............. 123
2.4.6.  Effect of chronic exposure to different oxygenations on the ability of the oxygen-sensitive \( K_v \) channel \( K_v_{1.5} \) blocker to modulate chorionic plate arterial constriction .................................................. 124

2.4.7.  Conclusion ........................................................................................................... 125

3.  K\(^+\) CHANNEL EXPRESSION AND FUNCTION IN CHORIONIC PLATE ARTERIES .................................................. 126

3.1.  PROTEIN EXPRESSION OF K\(^+\) CHANNELS IN CHORIONIC PLATE ARTERIAL SMOOTH MUSCLE CELLS ................................................................. 126

3.1.1.  Introduction ........................................................................................................... 126

3.1.2.  Materials and Methods ..................................................................................... 129

3.1.2.1.  Cultured cell isolation protocol ..................................................................... 129

3.1.2.2.  Acute cell isolation protocol ........................................................................ 129

3.1.2.3.  Immunocytochemistry ................................................................................ 137

3.1.2.4.  Chemicals ....................................................................................................... 137

3.1.3.  Results ................................................................................................................ 139

3.1.3.1.  Characterisation of cultured cell phenotype .................................................. 139

3.1.3.2.  Characterisation of acutely isolated cell phenotype .................................... 145

3.1.3.3.  K\(^+\) channel protein expression .................................................................. 149

3.1.4.  Discussion ......................................................................................................... 152

3.1.4.1.  Characterisation of SMC culture model ...................................................... 152

3.1.4.2.  Acutely isolated cell model characterisation ................................................ 154

3.1.4.3.  K\(^+\) channel expression in chorionic plate arterial SMCs ............................ 157

3.2.  mRNA EXPRESSION OF K\(^+\) CHANNELS IN CHORIONIC PLATE ARTERIES .................................................................................. 159

3.2.1.  Introduction ........................................................................................................ 159

3.2.2.  Materials and Methods ................................................................................... 159

3.2.2.1.  Vessel Preparation ...................................................................................... 159

3.2.2.2.  RNA extraction ........................................................................................... 160

3.2.2.3.  Spectrophotometry ....................................................................................... 160

3.2.2.4.  Ribogreen Assay .......................................................................................... 161

3.2.2.5.  Reverse transcription .................................................................................... 161

3.2.2.6.  Quantitative Real Time Polymerase Chain Reaction (qPCR) ..................... 162

3.2.3.  Results ............................................................................................................... 167

3.2.3.1.  K\(^+\) channel mRNA expression in chorionic plate arteries ............................ 167

3.2.3.2.  Effect of 48h culture on K\(^+\) channel mRNA expression ............................. 170

3.2.4.  Discussion ......................................................................................................... 173

3.3.  FUNCTION OF K\(^+\) CHANNELS IN CHORIONIC PLATE ARTERIAL SMOOTH MUSCLE CELLS ................................................................. 176

3.3.1.  Introduction ........................................................................................................ 176

3.3.2.  Materials and Methods ................................................................................... 177

3.3.2.1.  SMC Isolation Procedure ............................................................................ 177

3.3.2.2.  Whole-cell Electrophysiology ..................................................................... 177

3.3.2.3.  Solutions ...................................................................................................... 178

3.3.2.4.  Chemicals and statistical analysis ............................................................... 179

3.3.3.  Results ............................................................................................................... 180

3.3.3.1.  Characterisation of passive membrane properties and whole-cell current profiles ........................................................................................................... 180

3.3.3.2.  Voltage-gated K\(^+\) Channel Pharmacology (\( K_v \)) .................................... 183

3.3.3.3.  \( \text{Ca}^{2+} \)-activated K\(^+\) Channel Pharmacology (\( K_{\text{Ca}} \)) ...................... 186
3.3.4. Discussion ................................................................. 196
  3.3.4.1. Characterisation of whole-cell currents ................................... 196
  3.3.4.2. Voltage-gated $K^+$ channels ($K_v$) ....................................... 198
  3.3.4.3. Ca$^{2+}$-activated $K^+$ channels ($K_{Ca}$) ......................... 201

4. GENERAL DISCUSSION .................................................................. 207

4.1. Summary of Findings ................................................................. 207

4.2. Physiological Relevance and Interpretation ................................. 209
  4.2.1. Fetoplacental vasculature Vs. Pulmonary vasculature: Regulation by oxygen
         ............................................................................................ 209
  4.2.2. Smooth muscle cell phenotype: Implications on fetoplacental vascular physiology
         ............................................................................................. 212
  4.2.3. Implications for pregnancy pathologies: Fetal Growth Restriction ........ 215

4.3. Future Work ............................................................................. 218
  4.3.1. Chorionic plate arterial SMCs: Phenotype ................................ 218
  4.3.2. Chorionic plate arterial SMCs: $K^+$ channel expression and function ...... 218
  4.3.3. Fetal Growth Restriction ....................................................... 219

4.4. Conclusion .............................................................................. 220

5. REFERENCES .............................................................................. 221

6. APPENDIX .................................................................................. 244

FINAL WORD COUNT 67,344
LIST OF FIGURES

1. INTRODUCTION

Figure 1-1: Term human placenta .................................................................23
Figure 1-2: Diagrammatic representation of the vascular supply of the human placenta. ........................................................................................................25
Figure 1-3: Smooth muscle cell ion channel involvement in vasoconstriction ........35
Figure 1-4: Smooth muscle cell ion channel involvement in vasodilation .............36
Figure 1-5: Oxygen gradients within the placental cotyledon ................................47
Figure 1-6: Structural Classification of K⁺ Channels ........................................55

2. ROLE OF OXYGEN-SENSITIVE Kᵥ CHANNEL BLOCKERS IN REGULATING CHORIONIC PLATE ARTERIAL TONE

Figure 2-1: Wire Myography Protocol ..............................................................80
Figure 2-2: Effect of acute exposure to different oxygen tensions on chorionic plate artery constriction. ..................................................................................83
Figure 2-3: Effect of chronic (24 h) exposure to different oxygen tensions on chorionic plate artery constriction .................................................................85
Figure 2-4: Effect of chronic (48 h) exposure to different oxygen tensions on chorionic plate artery constriction .................................................................86
Figure 2-5: Effect of chronic exposure to hyperoxia on chorionic plate artery constriction ..............................................................................................................89
Figure 2-6: Depolarisation-induced constriction of chorionic plate arteries under hyperoxia .........................................................................................................90
Figure 2-7: Effect of vehicle incubation on chorionic plate artery constriction under hyperoxia .....................................................................................................91
Figure 2-8: Effect of vehicle incubation on chorionic plate artery sensitivity to U-46619 and maximal constriction under hyperoxia ........................................92
Figure 2-9: Effect of 4-AP on agonist-induced constriction in chorionic plate arteries under hyperoxia .................................................................93
Figure 2-10: Effect of 4-AP on basal tone in chorionic plate arteries under hyperoxia. 94
Figure 2-11: Effect of DPO-1 on agonist-induced constriction in chorionic plate arteries under hyperoxia .................................................................95
Figure 2-12: Effect of DPO-1 on basal tone in chorionic plate arteries under hyperoxia. ................................................................. 96

Figure 2-13: Effect of chronic exposure to normoxia on chorionic plate artery constriction. ................................................................. 99

Figure 2-14: Depolarisation-induced constriction of chorionic plate arteries under normoxia. ................................................................. 100

Figure 2-15: Effect of vehicle incubation on chorionic plate artery constriction under normoxia. ................................................................. 101

Figure 2-16: Effect of vehicle incubation on chorionic plate artery sensitivity to U-46619 and maximal constriction under normoxia. ................................................................. 102

Figure 2-17: Effect of 4-AP on agonist-induced constriction in chorionic plate arteries under normoxia. ................................................................. 103

Figure 2-18: Effect of 4-AP on basal tone in chorionic plate arteries under normoxia. ................................................................. 104

Figure 2-19: Effect of DPO-1 on agonist-induced constriction in chorionic plate arteries under normoxia. ................................................................. 105

Figure 2-20: Effect of DPO-1 on basal tone in chorionic plate arteries under normoxia. ................................................................. 106

Figure 2-21: Effect of chronic exposure to hypoxia on chorionic plate artery constriction. ................................................................. 109

Figure 2-22: Depolarisation-induced constriction of chorionic plate arteries under hypoxia. ................................................................. 110

Figure 2-23: Effect of vehicle incubation on chorionic plate artery constriction under hypoxia. ................................................................. 111

Figure 2-24: Effect of vehicle incubation on chorionic plate artery sensitivity to U-46619 and maximal constriction under hypoxia. ................................................................. 112

Figure 2-25: Effect of 4-AP on agonist-induced constriction in chorionic plate arteries under hypoxia. ................................................................. 113

Figure 2-26: Effect of 4-AP on basal tone in chorionic plate arteries under hypoxia. ................................................................. 114

Figure 2-27: Effect of DPO-1 on agonist-induced constriction in chorionic plate arteries under hypoxia. ................................................................. 115

Figure 2-28: Effect of DPO-1 on basal tone in chorionic plate arteries under hypoxia. ................................................................. 116
3. K⁺ CHANNEL EXPRESSION AND FUNCTION IN CHORIONIC PLATE ARTERIES

3.1 Protein expression of K⁺ channels in chorionic plate arterial smooth muscle cells

Figure 3-1: Phase contrast images of arterial smooth muscle outgrowths close to the vessel explant with time in culture. ................................................................. 140

Figure 3-2: Phase contrast images of arterial smooth muscle cells in the centre and edge of the outgrowth colonies with time in culture. ................................. 142

Figure 3-3: Immunofluorescence staining of arterial cells utilising an antibody to α-smooth muscle actin. ................................................................. 144

Figure 3-4: Phase contrast images of freshly isolated arterial smooth muscle cells. 145

Figure 3-5: Phenotype characterisation of acutely isolated chorionic plate arterial SMCs............................................................................................... 147

Figure 3-6: Phenotype characterisation of chorionic plate arterial sections. ......... 148

Figure 3-7: K⁺ channel expression in acutely isolated SMCs............................. 150

Figure 3-8: K⁺ channel expression in chorionic plate arterial sections. .............. 151

3.2 mRNA expression of K⁺ channels in chorionic plate arteries

Figure 3-9: qPCR performed on candidate K⁺ channel........................................ 166

Figure 3-10: K⁺ channel transcripts in chorionic plate arteries.......................... 168

Figure 3-11: Relative abundance of K⁺ channels in freshly isolated chorionic plate arteries. ........................................................................................................... 169

Figure 3-12: Relative change in K⁺ channel mRNA expression in freshly isolated and cultured chorionic plate arteries. ...................................................... 171

Figure 3-13: Relative abundance of K⁺ channels in chorionic plate arteries cultured for 48h at 6 % O₂. ................................................................. 172

3.3 Function of K⁺ channels in chorionic plate arterial smooth muscle cells

Figure 3-14: Properties of chorionic plate arterial SMCs. ............................... 181

Figure 3-15: Characterisation of whole-cell current recordings in chorionic plate arterial SMCs............................................................. 182
Figure 3-16: Effect of inhibition of voltage-gated K⁺ channels with 4-aminopyridine (4-AP) on whole-cell currents in chorionic plate arterial SMCs. ........................................ 184

Figure 3-17: Effect of inhibition of the voltage-gated K⁺ channel isoform Kv1.5 with DPO-1 on whole-cell currents in chorionic plate arterial SMCs. ................................. 185

Figure 3-18: Effect of inhibition of Ca²⁺-activated K⁺ channels with tetraethylammonium (TEA) on whole-cell currents in chorionic plate arterial SMCs. ......................... 188

Figure 3-19: Effect of inhibition of the Ca²⁺-activated K⁺ channel isoforms BKCa and IKCa with charybdotoxin (ChTx) on whole-cell currents in chorionic plate arterial SMCs. ...189

Figure 3-20: Effect of inhibition of the Ca²⁺-activated K⁺ channel isoform IKCa with TRAM-34 on whole-cell currents in chorionic plate arterial SMCs. ............................. 189

Figure 3-21: Effect of inhibition of the Ca²⁺-activated K⁺ channel isoform BKCa with iberiotoxin (IbTx) on whole-cell currents in chorionic plate arterial SMCs. ................. 190

Figure 3-22: Effect of activation of the Ca²⁺-activated K⁺ channel isoforms IKCa and SKCa with 1-EBIO on whole-cell currents in chorionic plate arterial SMCs. .................. 191

Figure 3-23: Effect of inhibition of Ca²⁺-activated K⁺ channels with tetraethylammonium (TEA) on 1-EBIO-sensitive currents in chorionic plate arterial SMCs. ...................... 192

Figure 3-24: Effect of inhibition of the Ca²⁺-activated K⁺ channel isoform BKCa with iberiotoxin (IbTx) on 1-EBIO-sensitive currents in chorionic plate arterial SMCs. ...... 193

Figure 3-25: Effect of inhibition of the Ca²⁺-activated K⁺ channel isoform IKCa with TRAM-34 on 1-EBIO-sensitive currents in chorionic plate arterial SMCs. ................. 194

Figure 3-26: Effect of inhibition of the Ca²⁺-activated K⁺ channel isoform SKCa with apamin on 1-EBIO-sensitive currents in chorionic plate arterial SMCs. ...................... 195
LIST OF TABLES

1. INTRODUCTION

Table 1-1: K+ channel Groups. ................................................................. 56
Table 1-2: Functional roles for K\textsubscript{2P} channels. ................................. 64

2. ROLE OF OXYGEN-SENSITIVE K\textsubscript{v} CHANNEL BLOCKERS IN REGULATING CHORIONIC PLATE ARTERIAL TONE

Table 2-1: Summary- Effect of different oxygenations on agonist- and depolarisation-induced constriction. ................................................................. 118
Table 2-2: Summary- Effect of different oxygenations on the ability of 4-AP to modulate agonist-induced constriction and basal tone. ......................................................... 118
Table 2-3: Summary- Effect of different oxygenations on the ability of DPO-1 to modulate agonist-induced constriction and basal tone. ......................................................... 118

3. K+ CHANNEL EXPRESSION AND FUNCTION IN CHORIONIC PLATE ARTERIES

3.1 Protein expression of K+ channels in chorionic plate arterial smooth muscle cells

Table 3-1: Summary of isolation adaptations based on methods described in human placental arteries. ................................................................. 134
Table 3-2: Summary of isolation adaptations based on methods performed under Ca\textsuperscript{2+} free conditions. ................................................................. 135
Table 3-3: Summary of isolation adaptations based on methods performed under low Ca\textsuperscript{2+} conditions. ................................................................. 136
Table 3-4: Primary and secondary antibodies used for immunostaining studies........ 138

3.2 mRNA expression of K+ channels in chorionic plate arteries

Table 3-5: Primers and experimental conditions used for qPCR. ................................. 165
LIST OF APPENDICES

Appendix 1: Non-specific staining for SMC phenotypic markers.................................244

Appendix 2: Mean current-voltage relationship for cultured smooth muscle cells.....246
ABSTRACT

Appropriate control of fetoplacental vascular tone by chorionic plate resistance arteries is necessary for adequate oxygen and nutrient transfer to the growing fetus and a successful pregnancy. Arterial smooth muscle cells (SMCs) express potassium (K⁺) channels which regulate tone in response to humoral vasoactive agents and oxygen. Previous studies of human placental chorionic plate arteries show alterations in vascular reactivity by oxygen and K⁺ channel modulators. However, it remains to be determined whether K⁺ channels are localised to the SMCs of chorionic plate arteries where they directly control excitation-contraction coupling. The overall hypothesis for this thesis is that K⁺ channels are expressed in chorionic plate arterial SMCs and regulate fetoplacental vascular tone in response to altered oxygenation.

Wire myography was used to assess whether chorionic plate arterial tone was modulated (1) by acute (3h) and chronic (24h, 48h culture) exposure to placental hyperoxia, normoxia or hypoxia, or (2) following blockade of voltage-gated K⁺ channels (Kᵥ) and the oxygen-sensitive Kᵥ channel, Kᵥ1.5. Acute and chronic exposure to different oxygenations did not affect constriction to the thromboxane mimetic U-46619. Acutely, inhibition of Kᵥ channels with 4-AP (1 mM) enhanced basal tone at all oxygenations and U-46619 constriction under hypoxia. Inhibition of Kᵥ1.5 channels with DPO-1 (3 μM) had no effect on basal tone or U-46619 constriction.

An in vitro model was developed of freshly isolated chorionic plate arterial SMCs to investigate protein expression and function of K⁺ channels including Kᵥ1.5. Using immunocytochemistry, the cell isolates were confirmed as SM due to expression of α-smooth muscle actin and markers of SMC contractile and synthetic phenotype, in common with native arteries. 4-AP (5 mM) and DPO-1 (3 μM) inhibited a small but significant component of the whole-cell current at negative membrane potentials, and the SMCs expressed Kᵥ1.5 protein. The small contribution to whole-cell currents from 4-AP and DPO-1 sensitive channels supports the myography data suggesting that these channels do not have a marked effect on chorionic plate arterial tone. The majority of whole-cell current was inhibited by TEA (5 mM), charybdotoxin (100 nM) and iberiotoxin (100 nM) and therefore mediated by the Ca²⁺-activated K⁺ channel, BKCa. IKCa and SKCa channels are functional in chorionic plate arterial SMCs as activation with 1-EBIO (100 μM) markedly enhanced whole-cell currents; an effect that was abolished by the IKCa inhibitor TRAM-34 and reduced by the SKCa inhibitor apamin (100 nM). BKCa and IKCa protein was expressed in SMCs after isolation and in the native artery.

These studies demonstrate expression and function of the oxygen-sensitive K⁺ channel, Kᵥ1.5, in chorionic plate arterial SMCs. However, Kᵥ channels do not appear to have a prominent role in controlling vascular tone in the whole vessel. Furthermore, these data highlight a more important role for KCa channels in chorionic plate arteries where all three isoforms are expressed in the SMCs and responsible for the majority of whole-cell K⁺ current. The localisation of diverse K⁺ channels to chorionic plate arterial SMCs may be related to their phenotype, which displays both contractile and synthetic characteristics, and implicates a dual role for these SMCs to control both fetoplacental vascular resistance and vasculogenesis throughout pregnancy.
DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institution of learning.
COPYRIGHT STATEMENT

I. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester the right to use such Copyright, including for administrative purposes.

II. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

III. The ownership of certain Copyright, patents, designs, trade marks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

IV. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfor.aspx?DocID=487), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on Presentation of Theses.
ABBREVIATIONS

1-EBIO  1-Ethyl-2-benzimidazolinone
4-AP    4-aminopyridine
5-HT    5-hydroxytryptamine (serotonin)
α-SMA   α-smooth muscle actin
ACE     Angiotensin converting enzyme
ACh     Acetylcholine
AdR     Adrenoreceptor
AVP     Arginine vasopressin
ATP     Adenosine triphosphate
ATII    Angiotensin II
AT1R    Angiotensin I receptor
BSA     Bovine serum albumin
Ca^{2+} Calcium
CaCl₂   Calcium chloride
cAMP    Cyclic adenosine monophosphate
cGMP    Cyclic guanosine monophosphate
ChTx    Charybdotoxin
COX     Cyclooxygenase
CRH     Corticotrophin releasing hormone
DAB     3,3′-Diaminobenzidine tetrahydrochloride hydrate
DAG     Diacylglycerol
DAPI    4′,6-diamidino-2-phenylindole
DMEM    Dulbecco Modified Eagle Medium
DPO-1   Diphenyl phosphine oxide-1
dH₂O    Distilled water
DTT     Dithiothreitol
DM      Dissociation media
EDHF    Endothelial dependent hyperpolarisation factor
eNOS    Endothelial nitric oxide synthase
ET-1    Endothelin-1
FGR     Fetal growth restriction
GPCR    G protein coupled receptor
h       Hours
HBSS    Hank’s buffered salt solution
HFPV    Hypoxic fetoplacental vasoconstriction
HPV     Hypoxic pulmonary vasoconstriction
HUVEC   Human umbilical vein endothelial cells
IbTx    Iberiotoxin
IP₃     Inositol triphosphate
K⁺      Potassium
K₂P     Twin-pore potassium channels
K⁺ATP   ATP-sensitive potassium channels
K⁺Ca    Calcium-activated potassium channels
Kᵢ      Inwardly-rectifying potassium channels
Kᵣ      Voltage-gated potassium channels
KPSS    Potassium chloride solution
KOH     Potassium hydroxide
KCl     Potassium chloride
KH₂PO₄  Potassium dihydrogen phosphate
MgCl₂   Magnesium chloride
NaCl    Sodium chloride
NaOH    Sodium hydroxide
NaH₂PO₄ Sodium dihydrogenphosphate
NaHCO₃  Sodium bicarbonate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>Prostaglandin F₂α</td>
</tr>
<tr>
<td>PGH₂</td>
<td>Prostaglandin H₂</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PSG</td>
<td>Penicillin, streptomycin, glutamine</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological salt solution</td>
</tr>
<tr>
<td>ROC</td>
<td>Receptor-operated calcium channel</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic/endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SM</td>
<td>Smooth muscle</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SOC</td>
<td>Store-operated calcium channel</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered solution</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TRAM-34</td>
<td>1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vasodilatory endothelial growth factor</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channel</td>
</tr>
</tbody>
</table>
PUBLICATIONS AND PRESENTATIONS

Publications and presentations arising from the work described in this thesis:

Manuscripts in preparation


Abstracts

Brereton MF, Wareing M & Greenwood SL (2011). Characterisation of potassium (K⁺) currents in chorionic plate arterial smooth muscle cells *(International Federation of Placental Associations, Norway, abstract accepted)*.


Oral presentations

“O₂-Sensitive K⁺ Channels: Role for Kᵥ1.5 in Human Placental Arteries?” (Cardiac and Respiratory Themed Meeting, The Physiological Society, University of Manchester, UK, September 2010).

“Role of O₂-Sensitive K⁺ Channels in modulating chorionic plate arterial tone”. (Medical and Human Sciences Research Symposium, University of Manchester, UK, June 2010).

**Invited Speaker**

“Control of blood flow in the human placenta: Role of K⁺ channels” (Wake Forest University, North Carolina, USA, April 2010).

**Awards**

The Physiological Society Poster Competition Prize, Cardiac and Respiratory Themed Meeting, The University of Birmingham, September 2010.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr Mark Wareing and Dr Susan Greenwood. Mark provided technical assistance and statistical / data analysis advice during the myography experiments. Sue has provided continued help and support with many aspects of my PhD training which has proved an excellent foundation for an academic career in science. Her advice during the developmental stages of the smooth muscle cell isolation protocol and setting up the new patch clamp rig in the department was particularly valuable and much appreciated. Her door always remained open during the long frustrating months of troublesome cell preparations where she would always make time for me and provide intellectual discussions which have stimulated and shaped my scientific interests for the future.

I wish to thank Dr Peter Brown, University of Manchester, for his invaluable advice and use of his patch clamp rig and pipette puller during the early stages of my PhD. I am particularly grateful to Dr Iain Greenwood and all the members of his lab at St George’s, University of London, who allowed me to visit and gain their expertise in smooth muscle cell isolation. This opportunity provided me with the necessary confidence in my electrophysiology skills to overcome the problems with isolating chorionic plate arterial smooth muscle cells in Manchester. Without Iain’s support and kindness, obtaining the electrophysiology data in this thesis would not have been possible.

My extended gratitude goes to all the women who have donated their placentas for research and the clinicians and midwives in Maternal and Fetal Health Research Group and Central Delivery Unit at St Mary’s Hospital for taking the time to consent these patients. Without the use of this tissue, and funding from the Medical Research Council and Tommy’s the Baby Charity, the work in this thesis would not have been possible.

I am particularly grateful to Dr Rebecca Jones for teaching me qPCR and proof reading the appropriate thesis chapter. I also wish to thank all my friends and colleagues who have made the Maternal and Fetal Health Research Group a friendly and supportive environment and my PhD an enjoyable and productive experience.

Finally, I would like to thank my mum and dad who have supported me through every stage in my academic studies. You have always encouraged me to pursue my own dreams and have shaped me into the person I am today. And last but not least I wish to thank Simon who has never wanted to stand in the way of my career and has supported me in every decision I have made. Although you have taken a back seat on some occasions, we can now start our next chapter together.
DEDICATION

For mum
1. INTRODUCTION

The primary function of the placenta is to allow the exchange of oxygen and nutrients from the mother and remove waste products and metabolites from the fetus. The site of solute transfer is the syncytiotrophoblast layer which surrounds the fetal blood vessels deep within the placental bed and is in direct contact with maternal blood (Benirschke, 1994). Adequate fetoplacental blood flow to the syncytiotrophoblast is essential for delivery of oxygen and nutrient-rich blood to the fetus. Regulation of fetoplacental vascular tone ensures both low vascular resistance and appropriate matching of fetal with maternal blood flow, which maximises nutrient exchange. Throughout gestation of normal pregnancy, fetoplacental vascular resistance decreases which permits the development of a low resistance, low pressure system (Poston et al., 1995). These changes to the fetoplacental circulation are essential to maintain adequate blood flow between the placenta and fetus, which ensures fetal growth. As the fetoplacental vasculature is not innervated, the oxygenation status and circulating humoral vasoactive factors primarily control vascular tone via a direct effect on smooth muscle cells (SMCs) in blood vessels (Poston et al., 1995; Poston, 1997). K⁺ channels located in the SMC membrane are known to respond to vasoactive agents and the surrounding oxygenation to permit control of vascular tone (Jackson, 2000). However, there is currently only indirect evidence implicating a role for K⁺ channels in controlling vascular tone within fetoplacental blood vessels and the precise identity and localisation of these K⁺ channels to SMCs is unknown. In this thesis, it is hypothesised that K⁺ channels are located on the plasma membrane of SMCs in chorionic plate arteries where they modulate vascular tone in response to vasoactive agents and altered oxygenation.

1.1. PLACENTA

1.1.1. Structure and function of the placenta

The unique anatomy of the placenta and the changes in the vascular system that occur throughout gestation ensures efficient nutrient and oxygen transfer between mother and fetus. The human placenta is haemomonochorial which allows maternal blood to directly bathe the single fetal trophoblast cell layer (Benirschke, 1998). This structural
arrangement minimises the diffusion distance for nutrient and oxygen transfer, therefore maximising exchange efficiency (Benirschke, 1994).

The placenta has two distinct surfaces which differ structurally and functionally depending on whether contact is with the fetal (Figure 1-1A) or maternal (Figure 1-1B) interface. The umbilical cord inserts onto the fetal surface of the placenta and contains two arteries and one vein (Gude et al., 2004). The maternal surface of the placenta is embedded within the uterine wall and following delivery of the baby, separates due to contraction of the myometrium (Benirschke, 1998). Cotyledons, which are clearly visible on the maternal side of the placenta, consist of multiple branched blood vessels termed terminal villi, where nutrient and gas exchange occurs (Harding & Bocking, 2001). The large surface area of the placenta, coupled with the extensive branching of the associated blood vessels, ensures maximal growth of the fetus.
Figure 1-1: Term human placenta. (A) Fetal surface with umbilical cord insertion. (B) Maternal surface with fetal membranous sack and clearly visible cotyledons, of which there are typically 10-40.

Adapted from: (Benirschke, 1998)
1.1.2. Fetoplacental vasculature

Development of the fetoplacental vasculature begins at 6-7 days post conception (dpc) (Kaufmann & Scheffen, 1998; Arroyo & Winn, 2008). Vasculogenesis is evident at around 21dpc where mesenchymal stem cells differentiate and give rise to angioblastic cells and eventually endothelial cells (Demir et al., 2007). The endothelial cells form tubes and associate with pericytes, which proliferate and migrate into the vascular smooth muscle (Beck & D'Amore, 1997). Placental vascular growth through angiogenesis begins around 32dpc where existing vessels branch and elongate into new vessels (Arroyo & Winn, 2008). Coordination of endothelial and SMCs is required for the appropriate growth and development of the fetoplacental vasculature.

The fetal heart beat is detectable around week 5 post-conception and requires completion of placental vascular development to ensure continuation of a successful pregnancy (Kaufmann & Scheffen, 1998). The structure of the placenta permits maternal blood derived from the uterine spiral arteries within the endometrium to enter the placental bed and bathe an area termed the intervillous space. This maternal blood is in direct contact with the placental exchange barrier, the syncytiotrophoblast layer, which encapsulates the villous stroma and fetal vessels (Gude et al., 2004). The syncytiotrophoblast is multinucleated and contains multiple microvilli on the maternal facing plasma membrane to increase the surface area for solute exchange (Benirschke, 1998; Kaufmann & Scheffen, 1998).

Fetal blood vessels are derived from the umbilical arteries and vein within the umbilical cord. The umbilical arteries supply deoxygenated blood from the fetus to the placenta, and oxygenated blood from the placenta is carried to the fetus in the umbilical vein (Gude et al., 2004). Following insertion of the umbilical cord onto the placenta, the umbilical vessels branch to form chorionic plate arteries and veins (Gude et al., 2004). Chorionic plate arteries and veins with a diameter <500 µM have similar size characteristics as “resistance” vessels in the systemic circulation and therefore primarily determine fetoplacental vascular resistance (Wareing et al., 2002). Chorionic plate arteries and veins dive down into the placental bed at various points across the surface of the placenta, forming stem villous arteries and veins (Figure 1-2). These stem vessels branch extensively to form intermediate, terminal villous arterioles and venules and eventually the capillary bed where exchange occurs (Fox, 1997; Poston, 1997; Benirschke, 1998).
Figure 1-2: Diagrammatic representation of the vascular supply of the human placenta. The maternal spiral arteries protrude into the intervillous space allowing blood to bathe the villous tree (left). The umbilical vessels branch to form chorionic plate arteries and veins located on the fetal surface of the placenta. These vessels in turn dive into the placental bed to form the villous tree. Magnification of the villous tree (right) illustrates the hierarchy of vessel branching from the stem villous through to intermediate and finally terminal villi.

Adapted from: (Benirschke & Kaufmann, 2000; Gray, 2005)
1.2. CONTROL OF VASCULAR TONE

Blood vessels are an intricate network of tubular channels that transport blood throughout the body and control the amount of blood entering or exiting a tissue or vital organ. Blood flow through a vessel must be tightly regulated to maintain an appropriate blood pressure and distribution to tissues and organs for correct functioning (Jackson, 2000). There are primarily three ways blood flow can be controlled; by changing (1) the blood vessel diameter (2) the vessel length or (3) the blood viscosity (Martini, 2006a). The most physiologically relevant factor that can be altered dynamically to control vascular tone, and therefore systemic vascular resistance, is the diameter of the vessel. According to Pouiseuille’s law which describes the movement of a fluid through a cylindrical tube, the relationship between blood flow and a vessel radius is described by the fourth power \( R \propto (\eta \cdot L)/r^4 \) where; \( R \) = resistance to flow, \( \eta \) = viscosity, \( L \) = vessel length, \( r \) = vessel radius) (Martini, 2006a). This means that a small change in the diameter of a vessel can have a large impact on blood flow and therefore vascular resistance. Blood vessels with a typical internal circumference of <500 µM are termed “resistance vessels” (Mulvany & Aalkjaer, 1990). Resistance vessels primarily control the total peripheral resistance as they are highly abundant in a vascular bed following branching from a large conduit vessel (Mulvany & Aalkjaer, 1990). Therefore, factors that control the diameter of resistance vessels such as circulating vasoactive agonists, neurotransmitters, and metabolic factors, are important in controlling vascular resistance and ultimately blood pressure (Jackson, 2000).

1.2.1. Neuronal Factors

Blood vessels are innervated by the sympathetic nervous system which promotes a decrease in the vessel diameter; a process termed vasoconstriction (Dale, 1913). Sympathetic nerves release noradrenaline which binds to adrenoceptors located on the smooth muscle membrane in a vessel (Guimaraes & Moura, 2001). Binding of noradrenaline to the \( G_\alpha \) linked G-protein coupled adrenoceptor activates phospholipase C which hydrolyses the phospholipid \( \text{PIP}_2 \) into \( \text{DAG} \) and \( \text{IP}_3 \) (Molinoff, 1984; Rang et al., 2003a). \( \text{DAG} \) activates \( \text{PKC} \) which phosphorylates various proteins including many ion channels (Cogolludo et al., 2003). \( \text{IP}_3 \) promotes a rise in intracellular \( \text{Ca}^{2+} \) which
culminates in SMC contraction and therefore vasoconstriction (Akata, 2007). Some blood vessels, such as those supplying gastrointestinal glands or erectile tissues, have neuronal input from the parasympathetic nervous system (Toda & Okamura, 2003). This promotes an increase in the vessel diameter, termed vasodilation. The neurotransmitter released from these nerve terminals is acetylcholine which binds to muscarinic M₃ receptors on endothelial cells to promote nitric oxide release (Furchgott & Zawadzki, 1980). For most blood vessels however, it is the frequency of the action potentials generated in the sympathetic nerves, and therefore the amount of noradrenaline released, that determines the level of constriction and tone of the vessel. This physiological process of transducing an electrical stimulus into a mechanical response is called excitation-contraction coupling (Jackson, 2000).

1.2.2. Humoral Factors

Humoral factors present within the circulation are capable of controlling the local blood flow through a vessel and therefore total peripheral resistance. These factors can act on both the endothelium and SMCs to activate a cascade of intracellular signalling mechanisms, culminating in vasodilation or vasoconstriction. The complex balance between these vasoactive agonists determines the overall level of tone in a vessel.

1.2.2.1. Vasoconstrictors

Several humoral factors within the circulation act upon SMCs to promote vasoconstriction. Many stimuli within the body initiate production of these factors which can then act upon the resistance vessels to alter systemic vascular resistance. For vasoconstrictive agonists, this may include a fall in systemic blood pressure detected by the kidneys or baroreceptors. Under these circumstances, a signal may be generated to increase secretion of renin by the kidneys. Once renin has entered the circulation, it cleaves angiotensinogen into its active metabolite angiotensin II (ATII) via the actions of the angiotensin converting enzyme, ACE (Braun-Menendez et al., 1940; Peart, 1965; von Bohlen und Halbach & Albrecht, 2006). ATII can then bind to angiotensin I receptors expressed on the SMC membrane which are also linked to the
GPCR and promote intracellular Ca\textsuperscript{2+} mobilisation from the sarcoplasmic reticulum (de Gasparo et al., 2000). This increased intracellular Ca\textsuperscript{2+} ultimately results in vasoconstriction (Figure 1-3). ATII can also promote the release of vasopressin (AVP) which amplifies the constriction response as binding of AVP to its receptor increases intracellular Ca\textsuperscript{2+} through the same GPCR-linked pathway as ATII and noradrenaline (Ferguson & Renaud, 1986; Stricker & Sved, 2002).

The signal for local vasoconstriction of blood vessels can also come from noxious stimuli released during local tissue or vessel damage. Increased production of the humoral factors endothelin (ET-1), thromboxane (TxA\textsubscript{2}) and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) occurs under these conditions (Rang et al., 2003b). ET-1 is produced by the endothelium in response to circulating ATII, cytokines, reactive oxygen species and shearing forces on the endothelium following tissue or vessel damage (Marasciulo et al., 2006). TxA\textsubscript{2}, a potent vasoconstrictor derived from the prostaglandin PGH\textsubscript{2}, is released from activated platelets or following damage to the endothelium and is important during clot formation (Kaul et al., 1994; Shen & Tai, 1998). There are many other vasoactive prostaglandins (PGs) that vary functionally to induce both vasodilation and constriction depending on their chemical structure. PGF\textsubscript{2\alpha} and PGE\textsubscript{2} are two types of PGs derived from arachidonic acid that promote vasoconstriction following local tissue damage (Klabunde, 2005b; Matsuoka & Narumiya, 2007). ET-1, TxA\textsubscript{2}, PGF\textsubscript{2\alpha} and PGE\textsubscript{2} bind to their respective G\textsubscript{q}-linked receptor on the SMC membrane which activates phospholipase C to increase formation of IP\textsubscript{3}. This in turn promotes mobilisation of Ca\textsuperscript{2+} from the sarcoplasmic reticulum, which induces constriction (Figure 1-3) (Shen & Tai, 1998; Boron & Boulpaep, 2005).

Vasoconstrictor agonists present within the circulation share a common pathway to induce SMC constriction; they promote an increase in intracellular Ca\textsuperscript{2+} from the sarcoplasmic reticulum via the G\textsubscript{q}-linked GPCR pathway. However, there is increasing evidence that ATII, AVP, ET-1 and TxA\textsubscript{2} elicit vasoconstriction independently of this pathway by inhibiting voltage-gated and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels which promotes membrane depolarisation and Ca\textsuperscript{2+} influx (Scornik & Toro, 1992; Cogolludo et al., 2003; Mackie et al., 2008; Rainbow et al., 2009; Li et al., 2010). Whether these vasoactive agonists directly interact with K\textsuperscript{+} channels or via enzymatic intermediates such as PKC is still under debate. Therefore, agonist stimulation promotes a rise in intracellular Ca\textsuperscript{2+} through activation of several cellular proteins and pathways, which culminates in activation of the contractile filaments and vasoconstriction.
1.2.2.2. Vasodilators

Humoral vasodilators present in the circulation act upon endothelial cells to mediate an increase in the diameter of a blood vessel and therefore blood flow. Many humoral vasodilators promote the release of nitric oxide (NO) from the endothelium which can then act upon the SMCs to elicit vasodilation. These agonists include acetylcholine, bradykinin, and histamine, and are released following inflammation and tissue injury (Toda & Okamura, 2003). Once released into the circulation they bind to their receptor on the endothelial cell membrane and promote a rise in intracellular Ca\(^{2+}\) via the G\(_q\)-linked GPCR pathway (Bruckdorfer, 2005). This in turn activates endothelial nitric oxide synthase (eNOS) to promote the release of NO from the endothelium which can then act upon the SMCs. Once inside the SMCs, NO activates guanylyl cyclase and converts GTP into cGMP (Toda & Okamura, 2003; Bruckdorfer, 2005; Klabunde, 2005a). cGMP has a multitude of biological actions to promote vasodilation including: (1) activating protein kinase G which phosphorylates myosin light chain kinase and the Ca\(^{2+}\) extrusion pump (SERCA); both culminating in a decrease in intracellular Ca\(^{2+}\) and relaxation (2) activating Ca\(^{2+}\)-activated K\(^{+}\) channels leading to hyperpolarisation and (3) inhibiting IP\(_3\) receptors, therefore preventing Ca\(^{2+}\) release from the sarcoplasmic reticulum (Bozotti et al., 1994; Bruckdorfer, 2005). Independent of circulating humoral agents, other factors promote local NO formation and therefore vasodilation. This includes vasoactive peptides such as vascular endothelial cell growth factor (VEGF) and corticotrophin releasing hormone (CRH), and the shearing stresses imposed upon the endothelial cells by the flow of blood (Kimura & Esumi, 2003; Klabunde, 2005a).

In addition to promoting NO formation, many vasodilatory humoral agents including histamine and bradykinin can elicit vasodilation via prostacyclin (PGL\(_2\)) production. The increased intracellular Ca\(^{2+}\) resulting from activation of the histamine H\(_1\) and bradykinin B\(_2\) receptors activates the Ca\(^{2+}\)-dependent enzyme PLA\(_2\). PLA\(_2\) triggers an enzymatic cascade which converts membrane phospholipids into arachidonic acid, PGL\(_2\), and finally PGI\(_2\) (Mombouli & Vanhoutte, 1995; Sato et al., 1999). PGI\(_2\) can diffuse from the endothelial cell into the SM where it activates adenylyl cyclase to increase production of cAMP. cAMP in turn activates protein kinase A which phosphorylates myosin light chain kinase, resulting in relaxation (Boron & Boulpaep, 2005).

Agonist stimulation can also induce vasodilation when both NO and PGI\(_2\) synthesis is inhibited (Chen et al., 1988; Garland & McPherson, 1992). Since this dilatory response requires an intact endothelium, and hyperpolarisation is observed in the SMCs, this NO and PGI\(_2\) independent mechanism of vasodilation is termed endothelium-dependent
hyperpolarisation factor (EDHF) (Chen et al., 1988). Although there is still controversy regarding the identity and precise mechanisms of EDHF-induced vasodilation, K⁺ channels located on the endothelial and SMCs are thought to play a pivotal role in transmitting an agonist-induced signal on the endothelial cells into the hyperpolarisation observed in the SMCs (Edwards et al., 1998).

1.2.3. Metabolic Factors

Blood vessels are sensitive to the local metabolic environment and regulate their tone according to the pO₂, pCO₂, and pH of the interstitial fluid (Boron & Boulpaep, 2005). Changes in these parameters are associated with alterations in metabolic activity. The products of metabolism, CO₂, H⁺ and lactic acid, promote vasodilation to allow their removal and the resulting increase in blood flow and O₂ maintains normal cellular function (Klabunde, 2005b; Deussen et al., 2006).

The pO₂ of the interstitial fluid can directly regulate vascular tone. In the systemic vasculature reductions in O₂ (termed hypoxia) promote vasodilation to maximise blood flow to metabolically active organs (Klabunde, 2005b; Deussen et al., 2006). Vasodilation in the systemic circulation is induced by local mediators, such as adenosine, which are released from the tissue surrounding the vasculature in response to hypoxia. These agents primarily act upon K⁺ channels to promote their opening, and therefore K⁺ efflux, which results in membrane hyperpolarisation and vasodilation (Quayle et al., 1997; Standen & Quayle, 1998; Kinoshita et al., 2003; Park et al., 2007). Hypoxia has been shown to activate K_ATP, K_IR and BK_Ca channels in many systemic vascular beds (see section 1.4 for definitions) including the coronary and cerebral circulations (Noack et al., 1992; Beech et al., 1993; Miller et al., 1993; Kinoshita et al., 2003; Lynch et al., 2006; Park et al., 2007).

Hypoxia, has the opposite effect in the pulmonary circulation and promotes vasoconstriction (Mauban et al., 2005; Moudgil et al., 2005, 2006; Murray et al., 2006; Bonnet & Archer, 2007). In this mechanism termed hypoxic pulmonary vasoconstriction (HPV), the inherent properties of the pulmonary vascular SM is thought to differ from the systemic circulation whereby a reduction in oxygenation increases the concentration of intracellular Ca²⁺ to elicit constriction (McMurtry et al., 1976; Salvaterra & Goldman, 1993; Jabr et al., 1997). This constriction is a physiological mechanism to ensure ventilation-perfusion matching whereby vasoconstriction shunts
blood flow away from a hypoxic region of the lung thus maintaining systemic pO$_2$ without a subsequent increase in pulmonary vascular resistance (Archer et al., 1998). Similar to hypoxic vasodilation in the systemic circulation, K$^+$ channels play a pivotal role in mediating HPV (Post et al., 1992; Archer et al., 1998; Gurney et al., 2002; Joshi et al., 2009; Morecroft et al., 2009). However, hypoxia inhibits K$^+$ channels in pulmonary artery SMCs which promotes membrane depolarisation, Ca$^{2+}$ influx and vasoconstriction. The molecular identity of the K$^+$ channels mediating HPV is under debate; however, potential candidates include Kv1.5, Kv2.1, Kv7 and TASK-1 (see section 1.4 for definitions) as they are thought to have a prominent role in maintaining the resting membrane potential (Archer et al., 1998; Gurney et al., 2002; Joshi et al., 2009; Morecroft et al., 2009).

Hypoxia also regulates vascular tone in the fetal circulation. The fetal pulmonary circulation develops under conditions of hypoxia which is thought to sustain a high vascular resistance (Cornfield, 2010). In fetal pulmonary arterial SMCs it is hypothesised that Kv channels are down-regulated by the chronic exposure to hypoxia which in turn promotes an increase in the expression and activity of Ca$^{2+}$-activated K$^+$ channels (Cornfield et al., 1996; Reeve et al., 1998; Resnik et al., 2006a; Resnik et al., 2006b). This predominance of KCa channels favours a dilatory response to increase pulmonary blood flow following oxygenation of the fetal lung at birth (Cornfield, 2010). In the fetoplacental circulation, hypoxia is also thought to regulate vascular resistance with a potential vasoconstrictor response observed under certain experimental conditions (see section 1.3.4). K$^+$ channels may also play an important role in modulating fetoplacental vascular tone in response to altered oxygenation. However, very little is known about the expression and function of K$^+$ channels per se in the placenta (see section 1.5).

It is evident that the local environment surrounding a vessel can regulate blood flow depending of the metabolic demands of the tissue. Regulation of vascular tone by hypoxia serves as a protective mechanism in both the systemic and pulmonary circulations, to ensure maximal perfusion of metabolically active tissues and an adequate systemic pO$_2$. Similar to many other factors that control vascular resistance, K$^+$ channels serve as the both sensor and effector for the regulation of tone in many vascular beds to changes in oxygenation.
1.2.4. Ion Channels

SMCs are capable of integrating a large number of stimuli to modulate vascular resistance and therefore blood flow (see sections 1.2.1-1.2.3). Regardless of whether the initial stimulus is neuronal, humoral or metabolic, the movement of ions namely K⁺ and Ca²⁺ through their respective ion channel is crucial to driving SMC contraction and relaxation (Figure 1-3 and Figure 1-4).

Muscle contraction requires a rise in intracellular Ca²⁺ from a resting concentration of 0.1 µM to around 10 µM (Hollingworth et al., 1996; Klabunde, 2005a; Cheng & Lederer, 2008). Ca²⁺ ions accumulate in the extracellular milieu and intracellular organelles such as the sarcoplasmic reticulum. The extracellular and sarcoplasmic reticulum Ca²⁺ concentration is approximately 2.5 mM and 100 µM respectively (Shmygol & Wray, 2005; Cheng & Lederer, 2008). Therefore, a large concentration gradient exists which favours the movement of Ca²⁺ from either the extracellular surface or sarcoplasmic reticulum into the cytoplasm where it can bind to the contractile filaments to elicit constriction.

A rise in intracellular Ca²⁺ can be triggered following binding of various neurotransmitters or humoral factors to their respective receptor (Figure 1-3 and sections 1.2.1 and 1.2.2). Activation of these receptors, namely Gq-linked GPCRs, promotes the mobilisation of Ca²⁺ from the sarcoplasmic reticulum via the IP₃ receptor. SMCs express many different Ca²⁺ channels which permit Ca²⁺ influx across the plasma membrane. These channels may be sensitive to changes in the membrane potential (e.g. voltage-gated Ca²⁺ channels; VGCCs), circulating agonists (e.g. receptor-operated Ca²⁺ channels; ROCs), alterations in the ionic composition of intracellular organelles (e.g. store-operated Ca²⁺ channels; SOCs) or membrane stretch (Nelson & Quayle, 1995; Guibert et al., 2008). However, Ca²⁺ entry mediated by VGCCs is the predominant plasma membrane influx pathway during vasoconstriction in SMCs (Jackson, 2000; Akata, 2007). VGCCs open in response to membrane depolarisation to permit Ca²⁺ influx (Jackson, 2000, 2005).

Under basal resting conditions, SMCs and therefore blood vessel tone is maintained in a hyperpolarised, non-excitatory state. This is achieved through the careful regulation of the SMC membrane potential to a value of approximately -60 mV (Casteels et al., 1977). The level of excitation, and therefore degree of SMC contraction, is closely related to the membrane potential. K⁺ channels are responsible for setting the resting
membrane potential as they are open to permit K\textsuperscript{+} efflux, thereby pushing the membrane to the calculated K\textsuperscript{+} Nernst potential (Nelson & Quayle, 1995; Standen & Quayle, 1998; Ko et al., 2008). Closure of these channels in response to circulating vasoactive agonists such as endothelin or thromboxane, or alterations in oxygenation results in K\textsuperscript{+} accumulation within the cytoplasm and therefore membrane depolarisation (Noack et al., 1992; Post et al., 1992; Beech et al., 1993; Miller et al., 1993; Cogolludo et al., 2003; Kinoshita et al., 2003; Lynch et al., 2006; Park et al., 2007; Rainbow et al., 2009; Tang et al., 2009). This in turn shifts the membrane potential to the threshold for VGCC opening to permit Ca\textsuperscript{2+} influx (Nelson & Quayle, 1995). In addition to K\textsuperscript{+} channel closure, membrane stretch can also provide an initial level of membrane depolarisation to open VGCCs and promote vasoconstriction. This is evident in vessels that experience myogenic tone whereby elevations in intraluminal pressure activates stretch-activated channels which promotes non-selective cation influx and depolarisation (Schubert et al., 2008).

Neuronal, humoral and metabolic stimulation provoke a rise in intracellular Ca\textsuperscript{2+} via influx from either the extracellular milieu or sarcoplasmic reticulum. This Ca\textsuperscript{2+} is then able bind to calmodulin which activates myosin light chain kinase to phosphorylate myosin light chain and initiate force contraction (Ogut & Brozovich, 2003). Once contraction has been evoked, the SMCs must relax back to a basal level of tone and the intracellular Ca\textsuperscript{2+} concentration returned to resting values (Figure 1-4). If intracellular Ca\textsuperscript{2+} is not closely buffered it can result in Ca\textsuperscript{2+} overload which is detrimental to normal physiological processes (Akata, 2007). There are various homeostatic mechanisms to permit appropriate buffering of intracellular Ca\textsuperscript{2+} and thereby prevent a continued rise in intracellular Ca\textsuperscript{2+}. The plasma membrane and sarcoplasmic reticulum express the Ca\textsuperscript{2+} ATPase pumps, PMCA and SERCA, which actively extrude Ca\textsuperscript{2+} from the cytoplasm into the extracellular milieu and sarcoplasmic reticulum respectively (Akata, 2007). The vascular SMC membrane also express the Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channel, BK\textsubscript{Ca}, which provides the brake to excessive membrane excitability and therefore Ca\textsuperscript{2+} influx via VGCCs (Figure 1-4). BK\textsubscript{Ca} channels are opened following a rise in intracellular Ca\textsuperscript{2+} and membrane depolarisation to permit K\textsuperscript{+} efflux and subsequently hyperpolarisation (Standen & Quayle, 1998; Wamhoff et al., 2006). Restoration of intracellular Ca\textsuperscript{2+} concentrations to their resting values prevents further binding of Ca\textsuperscript{2+} to the contractile filaments. However, the actin-myosin complex within the contractile apparatus has to also disengage to allow complete relaxation of the muscle to its initial level. This process is initiated following dephosphorylation of myosin light chain by myosin light chain phosphatase (Ogut & Brozovich, 2003; Akata, 2007).
In summary, the intracellular Ca\(^{2+}\) concentration dictates SMC contraction and relaxation and the Ca\(^{2+}\) concentration, among other things, is controlled by K\(^+\) efflux through K\(^+\) channels.
Figure 1-3: Smooth muscle cell ion channel involvement in vasoconstriction. A rise in intracellular Ca\(^{2+}\) is the trigger for vasoconstriction. Ca\(^{2+}\) can enter the cytoplasm from either the sarcoplasmic reticulum (SR) or across the plasma membrane. Circulating humoral agonists and neurotransmitters can bind to their G\(_\text{q}\)-GPCR linked receptor which promotes mobilisation of Ca\(^{2+}\) from the SR and a subsequent rise in intracellular Ca\(^{2+}\). Influx of Ca\(^{2+}\) across the plasma membrane primarily occurs via voltage-gated Ca\(^{2+}\) channels (VGCCs) in smooth muscle cells. VGCCs open in response to membrane depolarisation. Membrane depolarisation occurs following inhibition of K\(^+\) channels that regulate the resting membrane potential or opening of stretch-activated cation channels in response to membrane stretch.

Information collated from (Standen & Quayle, 1998; Jackson, 2000, 2005; Wamhoff et al., 2006; Akata, 2007; Schubert et al., 2008)
Figure 1-4: Smooth muscle cell ion channel involvement in vasodilation. Return of intracellular Ca\(^{2+}\) concentrations back to basal levels promotes vasodilation. The rise in intracellular Ca\(^{2+}\), which occurs during vasoconstriction, activates the Ca\(^{2+}\)-activated K\(^{+}\) channel isoform BK\(_{Ca}\). Opening of this channel promotes K\(^{+}\) efflux and subsequently membrane hyperpolarisation. Hyperpolarisation of the membrane prevents opening of VGCC and further Ca\(^{2+}\) influx. Intracellular Ca\(^{2+}\) is also sequestered back into the SR via the Ca\(^{2+}\)-ATPase pump SERCA. These mechanisms act as the off-switch to return the vessel to a resting state and buffer the intracellular Ca\(^{2+}\) concentration thereby preventing Ca\(^{2+}\) overload.

Information collated from (Standen & Quayle, 1998; Jackson, 2000, 2005; Wamhoff et al., 2006; Akata, 2007)
1.3. CONTROL OF FETOPLACENTAL VASCULAR TONE

The cardiovascular system has to undergo considerable adaptations during pregnancy to accommodate the growing fetus and increased requirements for nutrient and oxygen transfer. Throughout gestation, blood volume increases by approximately 40% which is redistributed to the uterus (Metcalfe & Ueland, 1974). Associated with the increase in blood volume, cardiac output and stroke volume are also increased and total peripheral resistance reduced (Rovinsky & Jaffin, 1966). Despite the increased blood volume to the uterus, the placenta remains a low pressure, low resistance organ. This is achieved through the coordinated regulation of both utero- and fetoplacental vascular tone.

The increased blood supply to the uterus bathes the placental bed via the uterine spinal arteries. The spiral arteries are remodelled during the first trimester of pregnancy which removes the SMCs and the subsequent potential to contract (Pijnenborg et al., 2006). The remodelled vessels become heavily dilated conduits which maximises the delivery of blood to the placenta whilst maintaining a low pressure system. The supply of maternal blood to the placental bed and villous tree structures is heterogeneous (Power et al., 1967; Ragavendra & Tarantal, 2001; Hempstock et al., 2003; Jauniaux et al., 2003). Blood flow through the placental blood vessels needs to be appropriately matched to the maternal blood bathing the placental bed to maximise nutrient and oxygen exchange. The fetoplacental blood vessels are uniquely adapted to regulate their tone under conditions that are not normally observed in other vascular beds. The typical pressure thought to reside within the lumen of fetoplacental resistance vessels is 20 mmHg, some 30 mmHg lower than that observed in systemic vessels of similar size characteristics (Nicolini et al., 1989; Wareing et al., 2002). The placenta also lacks neuronal input; therefore, the regulation of fetoplacental vascular tone is primarily controlled by the local production of vasoactive humoral and metabolic factors (see sections 1.3.2 to 1.3.4). Despite these striking differences between the placental and systemic vasculature, the precise mechanisms controlling fetoplacental vascular tone remain poorly understood.
1.3.1. Experimental methods of assessment

Various in vitro preparations can be used to investigate the mechanisms controlling fetoplacental vascular tone. The impact vasoactive agents have on vascular tone can be monitored at the whole organ, intact vessel, and single cell level.

1.3.1.1. Placental perfusion

The perfused cotyledon is a whole-organ preparation used to investigate macroscopic changes in placental vascular resistance. It achieves this by measuring the perfusion pressure throughout a single or small group of placental cotyledons following cannulation and perfusion of arteries and veins located on the fetal surface of the placenta (Read et al., 1999). Additional perfusion of the maternal side of the placenta produces the dually perfused cotyledon model (Brownbill et al., 2007). The perfused cotyledon preparation gives a good understanding of physiological responses in the isolated organ; however, the precise site of altered perfusion pressure (resistance) cannot be determined.

1.3.1.2. Intact vessels

Studies utilising small isolated blood vessels provide a greater understanding of the physiological pathways controlling vascular tone. Isolated vessels can also be cultured under certain conditions for up to 72 h to study the chronic effects of regulatory factors such as oxygenation on vascular tone or for small-interference RNA experiments (De Mey et al., 1989; Bakker et al., 2000; Manoury et al., 2009; Tai et al., 2009). Myography experiments performed on intact vessels measure changes in the diameter or tension following stimulation with vasoactive agents. Most studies investigating vascular function in the placenta have utilised the wire myography technique which measures changes in isometric tension (Mulvany & Aalkjaer, 1990). The pressure myograph provides a more physiological setting in which to study vascular tone as isolated vessels are subjected to conditions that mimic in vivo flows or pressures within the vessel lumen. The placental vessels most widely studied are isolated chorionic plate and stem villous vessels as they have similar size characteristics to “resistance” vessels and therefore primarily important in controlling fetoplacental vascular tone.
1.3.1.3. Isolated endothelial and smooth muscle cells

Elucidating at the cellular level the signalling pathways and plasma membrane proteins modulated by circulating vasoactive agents requires a single cell model isolated from intact blood vessels. Isolation of both endothelial and SMCs eliminates the contribution of other confounding variables within the whole placenta or intact vessels and can be used in patch clamp electrophysiology, Ca$^{2+}$ imaging, single cell RT-PCR and immunocytochemistry experiments (Hampl et al., 2002; Dong et al., 2005). Single endothelial and SMCs can also be cultured and the physiological environment manipulated to observe how this acutely and chronically affects the underlying mechanisms that regulate vascular reactivity. Many studies have utilised endothelial cells from the fetoplacental vasculature with the recombinant cell line HUVEC, originally derived from the human umbilical vein, a common model to study cardiovascular disease. A model of cultured SMCs from chorionic plate artery explants has been described by Leik et al., (2004). However, a model of single relaxed SMCs freshly isolated from fetoplacental resistance vessels has not been extensively characterised.

1.3.2. Neuronal Factors

The placenta has a unique vascular system which lacks innervation from the autonomic nervous system. It has been demonstrated using various immunohistochemical methods that the umbilical cord and placental vessels do not contain adrenergic, cholinergic, myelineated or non-myelineated nerves (Spivack, 1943; Walker & McLean, 1971; Fox & Khong, 1990; Benirschke & Kaufmann, 2000). Consequently, fetoplacental vessels are weakly responsive to neurotransmitters such as noradrenaline and acetylcholine (Maigaard et al., 1986b; McCarthy et al., 1994; Wareing et al., 2002). The proximal end of the umbilical cord near the abdominal region of the fetus, however, does display evidence of nervous tissue (Lachenmayer, 1971). It is likely that these nerves are extensions from the umbilicus and control fetal vascular tone, or potentially mediate cord constriction following delivery (Benirschke &
Therefore, regulation of fetoplacental blood flow, which determines oxygen and nutrient transfer to the fetus, is primarily governed by local humoral and metabolic factors.

1.3.3. Humoral Factors

Humoral factors present within the fetoplacental circulation, produced from the endothelium or via enzymes located in the placental trophoblast, primarily control fetoplacental vascular tone. This maintains a low vascular resistance to blood flow through the placenta. However, in comparison with the systemic circulation, the potency and sensitivity of the vasoactive agents in the placenta differs markedly.

1.3.3.1. Vasoconstrictors

The placenta has a unique vascular system which responds poorly to humoral agents that are potent vasoconstrictors in the systemic circulation such as catecholamines and oxytocin (Maigaard et al., 1986b; McCarthy et al., 1994; Wareing et al., 2002). Other agents such as angiotensin II (ATII), and vasoconstrictor prostaglandins are present within the fetoplacental circulation. However, evidence is lacking to demonstrate they produce a significant contribution to the control of vascular tone in the placenta.

The placenta has an intact renin-angiotensin system, with ATII receptors and the angiotensin-converting enzyme expressed in fetoplacental blood vessels (Tence & Petit, 1989; Svane et al., 1995). Despite the presence of these factors, ATII induces a relatively weak vasoconstrictor effect in the perfused placenta (Mak et al., 1984; Glance et al., 1986) and isolated fetoplacental vessels (McCarthy et al., 1994; Wareing et al., 2002). Therefore, the physiological contribution of ATII to the regulation of fetoplacental tone is likely to be minimal.

Prostaglandins are produced through the actions of cyclooxygenase enzymes located within the placenta. PGE$_2$ and PGF$_{2\alpha}$ are stable within the fetoplacental vasculature and promote vasoconstriction in the perfused placenta (Mak et al., 1984). These prostaglandins produce a modest constriction of fetoplacental vessels compared with
other humoral agents and therefore, unlikely to significantly control fetoplacental vascular tone (McCarthy et al., 1994).

The predominant vasoconstrictor agonists controlling fetoplacental vascular tone are thromboxane (TxA$_2$), endothelin (ET-1) and vasopressin (AVP). TxA$_2$ is a potent vasoconstrictor of the fetoplacental circulation and produced from circulating platelets, trophoblast, and umbilical vessels (Ritter et al., 1982; Nelson & Walsh, 1989). The TxA$_2$ mimetic U-46619 induces a potent constriction in the perfused placental cotyledon and small resistance chorionic plate vessels (Mak et al., 1984; McCarthy et al., 1994; Read et al., 1999; Wareing et al., 2002). ET-1 is produced by the endothelium and acts on SMCs to produce vasoconstriction through an interaction with ET$_A$ receptors which are expressed in the placental vasculature (Rutherford et al., 1993). ET-1 produces a concentration-dependent increase in fetal perfusion pressure in the perfused placenta and fetoplacental blood vessels (Myatt et al., 1991b; Cooper et al., 2005). AVP is detectable in the umbilical cord blood and causes a sustained constriction of chorionic plate vessels but only a modest constriction in smaller stem villous vessels (Maigaard et al., 1986b, a; Wareing et al., 2002).

1.3.3.2. Vasodilators

Vasodilator humoral agents are important in maintaining a low vascular resistance within the placental circulation. A careful balance between the vasoconstrictor and vasodilator mechanisms ensures the placenta remains a low pressure, low resistance organ thereby maximising perfusion of the placental bed.

There are many agents that promote vasodilation of the fetoplacental circulation. The predominant mechanism by which dilation is elicited is through the release of nitric oxide (NO) from the endothelium. This is evident experimentally as infusion of nitric oxide synthase (NOS) inhibitors increases perfusion pressure in the isolated placental cotyledon (Myatt et al., 1991a). However, the vasoactive peptide neurokinin B promotes vasodilation in the perfused placenta and fetoplacental vessels through an endothelium- and NO-independent mechanism (Brownbill et al., 2003; Laliberte et al., 2004). Other vasoactive peptides including CRH and VEGF induce fetoplacental vasodilation via a NO-dependent mechanism. CRH and VEGFs significantly reduce perfusion pressure in the perfused placenta at physiological concentrations (Clifton et al., 1995; Brownbill et al., 2007).
Increased NO production in response to the endothelial-derived dilators ACh, bradykinin and histamine is the predominant mechanism to promote vasodilation in the systemic circulation (see section 1.2.2.2). However, these agents are ineffective or produce a weak vasodilation in the fetoplacental vasculature. ACh has no effect on the perfusion pressure in the placental cotyledon or isolated fetoplacental vessels (Myatt et al., 1992; McCarthy et al., 1994; Wareing et al., 2002); perhaps due to a lack innervation of the placenta (see section 1.3.2). Bradykinin has conflicting effects on fetoplacental vascular tone, with some studies demonstrating an increase in perfusion pressure of the perfused cotyledon and others reporting no change (de Moura & Lopes, 1995; Read et al., 1995). However, in isolated “resistance” chorionic plate vessels that primarily control fetoplacental vascular resistance, bradykinin failed to produce significant relaxation (McCarthy et al., 1994; Wareing et al., 2002). Similarly, there are conflicting reports regarding the contribution of histamine to fetoplacental vasodilation. In the pre-constricted perfused placenta and stem-villous arteries, histamine induced a relatively weak vasodilation (Myatt et al., 1992; McCarthy et al., 1994). However, histamine significantly dilated pre-constricted chorionic plate arteries (Mills et al., 2007b).

Prostacyclin (PGI₂) is another potent vasodilator in the systemic vasculature and is also synthesised in the placenta where it induces dilation in isolated fetoplacental vessels and the perfused placenta (Mak et al., 1984; Maigaard et al., 1986b). However, due to its short half life and the presence of placental enzymes, the bioactivity of prostacyclin in vivo is low and thus unlikely to play a significant role in the control of fetoplacental vascular tone (Maigaard et al., 1986b).

As NO significantly dilates the fetoplacental circulation but endothelial-derived vasodilators are relatively ineffective, flow-induced shear stress is the predominant mechanism of NO release, and therefore vasodilation in the placenta. This is evident in the perfused placenta (Myatt et al., 1991a) and pressurised chorionic plate arteries (Learmont & Poston, 1996). Flow-induced shear stress is likely to result in a basal production of NO, which maintains the low vascular resistance within the placenta.

1.3.4. *Metabolic Factors*

Oxygen is an important regulator of fetoplacental blood vessel function throughout gestation. During the first trimester of pregnancy, the placenta develops in the absence
of maternal blood flow and therefore in a low oxygen environment (Jauniaux et al., 2000). Once significant blood flow has entered the intervillous space towards the end of the first trimester, there is an increase in the partial pressure of oxygen (pO$_2$) within this area from 3 % at 8 weeks to 8 % at 16 weeks gestation as measured using an oxygen probe under continuous ultrasound guidance (Jauniaux et al., 1999; Jauniaux et al., 2001). This value is then thought to decrease slightly at term to 5 % as placental and fetal oxygen consumption increases, measured from aspirated placental lakes observed during cordocentesis, (Soothill et al., 1986).

Fetoplacental vascular tone is regulated by the surrounding oxygen tension as it directly affects blood vessel constriction and relaxation and regulates the production of vasculogenic, angiogenic and vasoactive factors (Burton et al., 2009). Oxygen gradients exist throughout the placenta during normal pregnancy as the supply of maternal blood across the placental bed is heterogeneous. This is evident from; (1) morphological studies which indicate regional differences in the extent of uterine arteries plugging by invading trophoblasts (Jauniaux et al., 2003), (2) activity of antioxidant enzymes which reflect the prevailing oxygen tension (Hempstock et al., 2003) both performed in human placental tissue, and (3) differential distribution of injected macroaggregates of albumin in the placental bed (Power et al., 1967), (4) echocontrast-enhanced harmonic imaging of the placenta (Ragavendra & Tarantal, 2001) in numerous animal models. The pO$_2$ of the maternal blood directly leaving the spiral arteries and entering the centre of a placental cotyledon is approximately 13 %, 80-100 mmHg, (Jauniaux et al., 2000; Burton & Caniggia, 2001). The maternal blood within the intervillous space then percolates towards the periphery of the cotyledon before draining back into the endometrial veins (Hempstock et al., 2003). As the maternal blood mixes with pre-existing deoxygenated blood within the intervillous space, the pO$_2$ will subsequently reduce towards the periphery with an estimated value of approximately 6 %, 40-50 mmHg, calculated using an oxygen probe in first trimester tissue (Jauniaux et al., 2000; Burton & Caniggia, 2001). Therefore, an oxygen gradient exists between the central and peripheral regions of the placental cotyledon and there are also likely to be pockets within the placenta where blood pO$_2$ widely varies (Figure 1-5). The surrounding pO$_2$ of the intervillous space determines oxygen extraction into the fetoplacental vasculature. The fetal blood is designed to maximise extraction despite low pO$_2$ within the intervillous space as fetal haemoglobin has a greater affinity for oxygen than adult haemoglobin (Carter, 1999). Therefore, the concentration of free dissolved oxygen or pO$_2$ taken from cord umbilical cord blood gas measurements of term placental tissue of the blood is low; typically 2-4 % or 15-30 mmHg (Lackman et al., 2001).
Blood vessels and capillaries within the entire fetoplacental vascular tree are exposed to oxygen gradients of between 2-13 %. As oxygen directly regulates systemic and pulmonary vascular tone (see section 1.2.3), these oxygen gradients are likely to influence the level of tone within the fetoplacental vasculature. This has been demonstrated in the perfused placental cotyledon experiment whereby reductions in pO$_2$ of the maternal perfusate increased basal fetal arterial perfusion pressure (Howard et al., 1987; Byrne et al., 1997; Hampl et al., 2002; Ramasubramanian et al., 2006). The increase in fetal perfusion pressure suggests that a reduction in the oxygenation of the maternal circulation promotes vasoconstriction of the fetoplacental vasculature. This vasoconstriction has been attributed to both a reduction in NO production or bioavailability and a direct effect on vascular SMCs (Byrne et al., 1997; Hampl et al., 2002). The oxygen tensions used in these experiments varied from between 95 % and 21 % for a “normoxic” value and between 8 % and 5 % for a “hypoxic” oxygen tension. Although this reduction in pO$_2$ convincingly produces fetoplacental vasoconstriction, this oxygen gradient is supraphysiological and does not represent the estimated values though to prevail within the placenta in vivo. Over a more physiological oxygen gradient, perfusion of isolated chorionic plate vessels with 2 % pO$_2$ from a normoxic value of 6 % increased basal perfusion pressure in veins but not arteries in pressure myography experiments (Wareing et al., 2006b).

In addition to oxygen per se modulating vascular tone, it can also modulate the reactivity of fetoplacental blood vessels to vasoactive humoral factors. In the perfused placenta, a reduction in oxygenation of the perfusate from 60 % to 7 % pO$_2$ did not affect contractile responses to ATII, ET-1, 5-HT or the thromboxane mimetic U-46619 (Read et al., 1995). However, in wire myography experiments utilising isolated chorionic plate arteries, contraction induced by ET-1 and U-46619 were modulated by oxygenation. For ET-1, this was only apparent in vessels that had been normalised under high stretch conditions of approximately 13.3 kPa, which exceeds the physiological pressure of 5.1 kPa observed in these vessels (Cooper et al., 2006). U-46619 constriction in vessels normalised to 5.1 kPa was reduced at 20 % oxygen compared to 6 % or 2 % (Cooper et al., 2006; Wareing et al., 2006c). The effect of altered oxygenation on vasodilatory agents has also been assessed in the perfused placenta. Reduced pO$_2$ does not affect vasodilation elicited by arachidonic acid implying PGI$_2$ production is not modulated by oxygen in the fetoplacental vasculature (Read et al., 1995). However, the actions of vasodilatory agents which act to enhance NO production, such as ATP and bradykinin, were markedly attenuated under low oxygen conditions (Read et al., 1995). Therefore, both basal and stimulated nitric oxide production is regulated by oxygen (Read et al., 1995; Byrne et al., 1997). The
relationship between vasodilation and oxygen has not been investigated in isolated chorionic plate “resistance” vessels.

During normal pregnancy, it is likely that oxygen plays a crucial role in regulating fetoplacental vascular tone to allow the efficient transfer of nutrients and oxygen to the growing fetus. It therefore follows if oxygen delivery to the placenta from the maternal circulation is reduced, or there is a defect within the fetoplacental vasculature that prevents the optimal delivery of oxygen- and nutrient-rich blood to the fetus, fetal growth will be compromised. This is evident in pregnancies at high altitude which are characterised by maternal hypoxia, as there is an average reduction in birth weight by 100 g for every 1000 m gain in altitude (Jensen & Moore, 1997; Giussani et al., 2001). Chronic hypoxia is also a characteristic of the pregnancy complication fetal growth restriction (FGR) (Lackman et al., 2001). FGR is a pathological condition whereby the fetus does not reach its genetic growth potential and affects 3-5 % of all pregnancies (Mandruzzato et al., 2008). FGR not only increases the risk of morbidity and mortality in the perinatal period, but it is also associated with an increased risk of cardiovascular disease in later life (Barker et al., 1990). The chronic hypoxia associated with FGR may result from inadequate spiral artery transformation which reduces the delivery of maternal blood to the placenta (Resnik, 2002; Kinzler & Kaminsky, 2007; Kinzler & Vintzileos, 2008). Chronic hypoxia within the intervillous space is thought to impact upon blood flow through the fetoplacental vasculature and could potentially explain the increased vascular resistance observed in the umbilical artery during diagnosis of the disease with Doppler ultrasound (Kingdom et al., 1997; Mills et al., 2005). In isometric tension studies using isolated chorionic plate vessels from FGR pregnancies, contractile responses to U-46619 were altered compared to vessels isolated from normal pregnancies; an effect that was dependent upon the oxygen concentration (Wareing et al., 2006d). Hypoxia is thought to radiate throughout the entire fetoplacental vascular tree as reduced oxygen levels within the umbilical cord blood are evident in FGR compared to normal pregnancies (Lackman et al., 2001). Whether this is a cause or a consequence of the raised vascular resistance is unknown.

In summary, altered oxygenation within any medium that bathes the fetoplacental vessels, such as the intervillous space or amniotic fluid, has the potential to modulate basal and agonist-induced tone during normal pregnancy. Fetoplacental blood flow needs to be tightly regulated to ensure optimal transfer of oxygen and nutrients to the growing fetus. Any alteration in the delivery of blood to the placenta can produce chronic changes in the intervillous space pO₂ which modulates fetoplacental vascular tone and therefore impacts upon blood flow to the fetus. Understanding further how
oxygen can modulate fetoplacental vascular tone may provide insights into how these mechanisms are dysregulated in pregnancy complications associated with aberrant blood flow and chronically altered oxygenation.
Figure 1-5: Oxygen gradients within the placental cotyledon. Maternal blood enters the intervillous space within the central region of the placental cotyledon via the spiral arteries. The partial pressure of oxygen (pO$_2$) within these vessels is thought to mimic the values present within the systemic circulation, approximately 13% (80-100 mmHg). The maternal blood percolates to the periphery of the cotyledon and mixes with deoxygenated blood before draining back into the endometrial veins which have a predicted pO$_2$ of 6% (45 mmHg). This results in an oxygen gradient between the central and peripheral regions of the cotyledon. Therefore, the villous blood vessels and capillaries supplying these regions are exposed to varying oxygen tensions. Similarly the chorionic plate vessels, which converge to form the umbilical vessels, are exposed to low oxygen tensions typically between 2-4% (15-25 mmHg).

Image adapted from: (Jauniaux et al., 2000; Jauniaux et al., 2001; Lackman et al., 2001; Hempstock et al., 2003; Jauniaux et al., 2003).
1.3.5. Ion Channels

In contrast with the systemic vasculature, the mechanisms underlying the regulation of SMC constriction and relaxation by ion channels in the fetoplacental vasculature is poorly understood. K⁺ channels control the membrane potential of SMCs and therefore regulate their excitability to vasoactive agents. Microelectrode impalement studies have measured the resting membrane potential within the range of -32 mV to -48 mV for chorionic plate arteries and -28 mV to -44 mV for veins (Ibrahim et al., 1996). Most studies have indirectly demonstrated that ion channels are responsible for maintaining the resting membrane potential and modulating fetoplacental vascular tone. The expression and localisation of these channels to a single vascular cell type is unknown.

K⁺ channels have been shown to regulate the resting membrane potential, basal and agonist-induced tone in fetoplacental vessels and will be discussed further in section 1.5.

Despite Ca²⁺ influx into the SMC being the crucial step in eliciting vasoconstriction, the identity of the pathways and mechanisms that permit a rise in intracellular Ca²⁺ in the fetoplacental vasculature is poorly understood. Similar to the systemic vasculature, VGCCs are likely to be the primary pathway to permit Ca²⁺ influx into the SMCs within the placental vessels. VGCCs are expressed and functional in SMCs isolated from umbilical arteries (Cairrao et al., 2009). These channels also contribute to KCl and 5-HT induced constriction in chorionic plate resistance arteries as application of the VGCC channel blocker nifedipine reduced constriction (Maigaard et al., 1984; Marin et al., 1990). However, nifedipine did not completely abolish agonist-induced constriction suggesting other Ca²⁺ entry pathways permit Ca²⁺ influx to allow vasoconstriction. In common with the systemic vasculature where Ca²⁺ influx is also mediated by stretch-activated, receptor- and store operated Ca²⁺ channels, they may also contribute to agonist-induced vasoconstriction in fetoplacental vessels. However, this has not been investigated and remains to be determined. Interestingly, electron micrographs of SMCs within sections of chorionic plate arteries did not demonstrate any structures resembling a sarcoplasmic reticulum (Sweeney et al., 2006). Although this has not been demonstrated functionally by using pharmacological agents to deplete the sarcoplasmic reticulum and measuring intracellular Ca²⁺, this may impact upon the mechanisms and pathways that permit Ca²⁺ influx and therefore constriction.
1.3.6. Summary

The placenta has a unique vascular system consisting of arteries which carry deoxygenated blood from the fetus to the placenta and veins which carry oxygenated blood to the fetus. These vessels are adapted to maintain a low pressure, low resistance system to permit maximal exchange of oxygen and nutrients to the developing fetus. This function is achieved through the careful regulation of factors that control the level of vasoconstriction and vasodilation in the fetoplacental blood vessels. As the placenta is not innervated, neurotransmitters such as acetylcholine and catecholamines do not modulate fetoplacental vascular tone. Humoral vasoactive agonists and the surrounding metabolic environment are therefore the primary determinants of vascular resistance within the placenta. However, not all vasoactive agents potently modulate fetoplacental vascular responses under physiological conditions. The primary agonists that promote vasoconstriction of placental blood vessels are thromboxane, endothelin and vasopressin. The main mechanism to elicit vasodilation in the placenta is through nitric oxide release. However, endothelial-derived vasodilators that promote nitric oxide release are relatively ineffective in the placenta. Therefore, flow-induced nitric oxide release is the primary mechanism to promote vasodilation of the fetoplacental vasculature. The surrounding metabolic environment, including the level of oxygenation, also controls fetoplacental vascular tone through the regulation of both basal and agonist-induced responses. Integrating all the humoral and metabolic factors into a change in fetoplacental vascular tone are ion channels. Ion channels are important regulators of vascular tone in the systemic and pulmonary circulations; however, their role has been relatively unexplored in the placenta. Understanding the identity and function of these channels in the fetoplacental vasculature will improve our knowledge of the factors that control blood flow through the placenta during normal pregnancy. This in turn will help identify potential therapeutic targets to improve blood flow in pregnancy complications associated with raised fetoplacental vascular resistance and reduced fetal growth.
1.4. **K⁺ CHANNELS: STRUCTURE AND FUNCTION**

There are over 150 K⁺ channels expressed in many different tissues within the body. They perform a diverse array of physiological functions including controlling the action potential duration, inducing hormone secretion and regulating vascular tone (Korn & Trapani, 2005). K⁺ channels are capable of performing many functions as they are regulated by various stimuli including pH, temperature, membrane stretch, oxygen, calcium, ATP and vasoactive humoral factors. The diversity in K⁺ channel function is achieved through the large number of genes that encode the pore forming α-subunits, the way in which they assemble, and their association with auxiliary β-subunits.

1.4.1. **Structure**

Functional K⁺ channels are composed of α- and β-subunits. The α-subunits are the pore forming unit with allow the passage of K⁺ ions. Assembly of a functional channel requires four α-subunits combining together to form a tetramer. These α-subunits can either be the same gene product, forming a homotetramer, or comprise of different subunits, forming a heterotetramer (Coetzee et al., 1999). The formation of a homotetramer or a heterotetramer modifies the biophysical properties of the channel including activation, inactivation and gating. It can also determine the localisation of the channel to specific tissues and therefore permit a specified physiological function. Some K⁺ channels can associate with auxiliary β-subunits which modifies the rate of channel activation, inactivation and expression levels or tissue distribution (Standen & Quayle, 1998; Coetzee et al., 1999).

K⁺ channels can be classified into four groups according to the structure of their α-subunit; voltage-gated, Ca²⁺-activated, inwardly rectifying, and twin-pore channels (Figure 1-6). Although these four groups are functionally distinct, the channels all mediate K⁺ efflux from a cell down the K⁺ concentration gradient under physiological conditions ([K⁺]ᵢ= 140 mM; [K⁺]ₑ= 5 mM).
1.4.1.1. Voltage-Gated K⁺ Channels

The pore forming α-subunit of voltage-gated K⁺ channels (Kᵥ) contains 6 transmembrane domains with hydrophobic residues that span the phospholipid plasma membrane (Figure 1-6). These domains are labelled S1 through to S6. S1 is nearest the amino (N)-terminal domain and S6 nearest the carboxy (C)-terminal domain which both have an intracellular localisation (Korn & Trapani, 2005). The fourth transmembrane domain S4, acts as the voltage sensor and contains a series of positively charged amino acid residues, namely arginine (Aggarwal & MacKinnon, 1996). Upon membrane depolarisation, S4 moves outwards, which enables opening of the channel gate to permit channel activation and ion flux (Aggarwal & MacKinnon, 1996; Korn & Trapani, 2005). Ions pass through the channel via a pore that is formed by the residues between S5 and S6 termed the P-loop. The P-loop acts as the channels selectivity filter to permit the passage of K⁺ ions rather than other cations such as Na⁺ or Ca²⁺ (Korn & Trapani, 2005). Four P-loops are required to form a functional pore and therefore channel, explaining why K⁺ channels are tetrameric. The N-terminus of several Kᵥ channels contains an N-inactivation ball which enters the channel pore following membrane depolarisation and inactivates the channel, preventing further efflux (Korn & Trapani, 2005).

Kᵥ channels are a diverse group of channels with genes encoding 12 Kᵥ families; Kᵥ1-Kᵥ12 (Gutman et al., 2005). Within each family there are several subfamilies denoted Kᵥ1.X, Kᵥ2.X, Kᵥ3.X etc (Table 1-1). The large number of Kᵥ channels is increased further when it is considered that many of these channels are able to form heterotetramers. The Kᵥ2 group consisting of Kᵥ2.1 and Kᵥ2.2 are able to form homotetramers and can also associate and form heterotetramers with many other Kᵥ subunits which modifies the biophysical properties of the channel including current amplitude and steady-state activation (Coetzee et al., 1999). Many of the subunits that Kᵥ2 channels associate with do not form functional homotetrameric channels. This includes the Kᵥ9.3 subunit which is electrically silent when expressed in recombinant systems, but can combine with Kᵥ2.1 channels where it has an important physiological role in controlling the reactivity of pulmonary arterial SMCs to changes in oxygenation (Patel et al., 1997). Other Kᵥ channel subunits can only form functional heterotetrameric channels with members of their own sub-family. This includes the Kᵥ1 channels which readily coassemble with other Kᵥ1.X channels but not with Kᵥ2-Kᵥ12 families (Gutman et al., 2005). The multitude of possible permutations for Kᵥ channel assembly, which determines the biophysical properties of the channels, produces a diverse array of physiological functions. This in turn enables localisation of a specific...
channel combination that can be potentially targeted and modulated by a therapeutic intervention.

1.4.1.2. Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Channels

The family of Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Channels (K\textsubscript{Ca}) comprise of 3 main channels; BK\textsubscript{Ca} (K\textsubscript{Ca}1.1), SK\textsubscript{Ca} (K\textsubscript{Ca}2.1-2.3) and IK\textsubscript{Ca} (K\textsubscript{Ca}3.1; Table 1-1). Unlike the K\textsubscript{v} family where all members have the same macroscopic structure, the K\textsubscript{Ca} family are structurally distinct which determines how they are regulated (Figure 1-6). However, their common feature is that they are all activated to a greater or lesser extent by a rise in intracellular Ca\textsuperscript{2+}. With the exception of the large-conductance K\textsubscript{Ca} channel BK\textsubscript{Ca}, K\textsubscript{Ca} channels contain 6 transmembrane domains denoted S1 to S6 (Figure 1-6). The channel pore, or P-loop is localised between S5 and S6. BK\textsubscript{Ca} has an additional transmembrane domain, S0, which precedes S1 and therefore results in an extracellular localisation of the N-terminal domain (Korn & Trapani, 2005). The function of S0 is thought to enable interaction and regulation with auxiliary \(\beta\)-subunits (Meera \textit{et al.}, 1997). BK\textsubscript{Ca} channels are activated by membrane depolarisation and an increase in intracellular Ca\textsuperscript{2+} during depolarisation (Korn & Trapani, 2005). Because changes in the membrane potential influences channel opening, BK\textsubscript{Ca} requires a voltage sensor which is localised to the S4 domain (Korn & Trapani, 2005). The C-terminus of BK\textsubscript{Ca} contains four additional hydrophobic regions, S7-S10, which are potentially membrane spanning and participate in Ca\textsuperscript{2+} sensing (Meera \textit{et al.}, 1997). BK\textsubscript{Ca} channels are thought to bind Ca\textsuperscript{2+} directly via three negatively charged residues preceding S10 termed the “Ca\textsuperscript{2+} bowl” (Meera \textit{et al.}, 1997; Wei \textit{et al.}, 2005). SK\textsubscript{Ca} and IK\textsubscript{Ca} are not activated by membrane depolarisation and as such do not contain a S4 voltage sensor. In addition, Ca\textsuperscript{2+} does not directly activate SK\textsubscript{Ca} and IK\textsubscript{Ca}; rather, the C-terminus contains a calmodulin motif which binds Ca\textsuperscript{2+}, resulting in a conformational change in the channel structure and activation (Schumacher \textit{et al.}, 2001).

K\textsubscript{Ca} channels are homotetrameric and do not coassemble with other K\textsubscript{Ca} sub-family members. Functional diversity is achieved through association with auxiliary \(\beta\)-subunits and modulation of intracellular residues by protein kinases (Standen & Quayle, 1998). BK\textsubscript{Ca} channels do however, show an additional level of functional diversity as they have multiple regions that can be alternatively spliced (Coetzee \textit{et al.}, 1999). Coassembly of the BK\textsubscript{Ca} spliced variants will modulate the biophysical properties of the channel and increases the possible permutations for physiological functions.
1.4.1.3. Inwardly-Rectifying K⁺ Channels

The structure of the inwardly-rectifying K⁺ Channels (Kᵢᵣ) suggests they are the most evolutionary primitive of all the K⁺ channel families. They consist of 2 transmembrane domains connected by the P-loop which forms the channel pore. Similar to the other K⁺ channel families, functional Kᵢᵣ channels require tetramer formation. Kᵢᵣ channels have a similar hierarchy for channel assembly as Kᵥ channels. They comprise of 7 families, Kᵢᵣ1- Kᵢᵣ7 and within each of these families there are several sub-families (Table 1-1). In common with Kᵥ and KₐCa, Kᵢᵣ channels can associate with auxiliary β-subunits, form heterotetramers within and between sub-family members, and produce splice variants (Coetzee et al., 1999; Kubo et al., 2005).

Association with β-subunits is particularly important for the Kᵢᵣ6 channel family which are also termed the KₐATP channels. KₐATP channels are sensitive to intracellular ATP that is conferred upon the channel by association with a β-subunit which has important physiological implications (see section 1.4.2.3). Functional KₐATP channels require an octomeric complex consisting of four KₐATP α-subunits and four β-subunits. The β-subunits are members of the ATP-binding cassette (ABC) family of proteins termed sulphonyl-urea proteins (SUR) (Ko et al., 2008). Depending on which Kᵢᵣ6 subunit (Kᵢᵣ6.1, Kᵢᵣ6.2) combines with each SUR subunit (SUR1, SUR2A, SUR2B) determines the tissue localisation of the channel and physiological function.

1.4.1.4. Twin-Pore K⁺ Channels

Twin-pore K⁺ channels (K₂P) are the most recently discovered group of K⁺ channels. They have a unique structure consisting of two P-loops in each α-subunit (Figure 1-6). As a functional K⁺ channel requires four P-loops, only two K₂P α-subunits are required to coassemble where they form a dimer (Goldstein et al., 2005). There are 15 mammalian genes that encode K₂P channels, however, due to their recent discovery, not all the genes encode a functional channel where a physiological function has been assigned. K₂P channels can be classified into six subfamilies on the basis of structural and functional similarity; TWIK, TASK, TREK, TALK, THIK, and TRESK (see Table 1-1). The TWIK subfamily consists of TWIK-1 and TWIK-2 isoforms; the TASK subfamily consists of TASK-1, TASK-3 and TASK-5 isoforms; the TREK subfamily consists of TREK-1, TREK-2 and TRAAK; the TALK subfamily consists of TALK-1, TALK-2 and TASK-2 isoforms; the THIK subfamily consists of THIK-1 and THIK-2
isoforms; and the **TRESK** subfamily consists of TRESK-1 isoform (Enyedi & Czirjak, 2010). Homodimers are the common functional subunit configuration for $K_{2P}$ channels. Although $K_{2P}$ channels were not initially thought to form heteromeric channels (Coetzee *et al.*, 1999), there is now emerging evidence that they can with important functional implications for pH sensing in the brain (Berg *et al.*, 2004).
Figure 1-6: Structural Classification of K⁺ Channels. K⁺ Channels can be classified into four groups based on their structure, each having different functional properties. Functional K⁺ channels require assembly of four pore forming α-subunits. The combination of four identical subunits results in the formation of a homotetrameric channel, whereas four different subunits within the same family can combine to form a heterotetrameric channel. Voltage-gated K⁺ Channels (Kᵥ) structurally consist of six transmembrane domains (S1-S6). S4 acts as the voltage sensor and the ionic pore is formed by the P-loop between S5 and S6. Ca²⁺-activated K⁺ Channels (KCa) are structurally similar to Kᵥ channels, having six transmembrane domains. However, the KCa isoform, BKCa, has an additional transmembrane domain S0 which precedes S1. In addition, this channel is voltage sensitive with S4 functioning as the voltage sensor. The C-terminus of all KCa channels contain a Ca²⁺-sensing domain which modulates channel opening. Inwardly rectifying K⁺ channels (Kir) are the most evolutionary primitive channel, consisting of two transmembrane domains connected by a P-loop. Twin-pore K⁺ Channels (K₂P) contain two P-loops within their primary structure and therefore require only two α-subunits to form a functional channel.

Information collated from (Gutman et al., 2005; Korn & Trapani, 2005; Kubo et al., 2005; Wei et al., 2005)
### Table 1: K⁺ channel Groups

Many genes encode K⁺ channels which form 4 main groups: voltage-gated (Kᵥ), Ca²⁺-activated (KᵥCa), inwardly-rectifying (KᵢR) and two-pore domain (K₂P). Each group can be classified into families and then subclassified into sub-families. Kᵥ channels contain 12 families Kᵥ₁ to Kᵥ₁₂. KᵥCa channels contain 3 families, BKᵥCa, SKᵥCa, and IKᵥCa. KᵢR channels consist of 7 families, KᵢR₁ to KᵢR₇, and K₂P channels contain 15 families K₂P₁ to K₂P₁₈.

<table>
<thead>
<tr>
<th>Voltage-gated K⁺ channels: Kᵥ</th>
<th>Ca²⁺-activated K⁺ channels: KᵥCa</th>
<th>Inwardly-Rectifying K⁺ channels: KᵢR</th>
<th>Twin-pore domain K⁺ channels: K₂P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kᵥ₁</td>
<td>Kᵥ₁, Kᵥ₁, Kᵥ₁, Kᵥ₁, Kᵥ₁, Kᵥ₁₂, Kᵥ₁, Kᵥ₁, Kᵥ₁, Kᵥ₁, Kᵥ₁, Kᵥ₁₂</td>
<td>Kᵥ₁ Ca¹</td>
<td>KᵢR₁</td>
</tr>
<tr>
<td>Kᵥ₂</td>
<td>Kᵥ₂, Kᵥ₂, Kᵥ₂</td>
<td>Kᵥ₂ Ca¹ (SKᵥCa)</td>
<td>KᵢR₂</td>
</tr>
<tr>
<td>Kᵥ₃</td>
<td>Kᵥ₃, Kᵥ₃, Kᵥ₃, Kᵥ₃, Kᵥ₃</td>
<td>Kᵥ₃ Ca¹ (IKᵥCa)</td>
<td>KᵢR₃</td>
</tr>
<tr>
<td>Kᵥ₄</td>
<td>Kᵥ₄, Kᵥ₄, Kᵥ₄, Kᵥ₄</td>
<td>KᵢR₄</td>
<td>KᵢR₄, KᵢR₄</td>
</tr>
<tr>
<td>KᵢR₅</td>
<td>KᵢR₅</td>
<td>KᵢR₅</td>
<td></td>
</tr>
<tr>
<td>KᵢR₆</td>
<td>KᵢR₆ (KᵢCa₁)</td>
<td>KᵢR₆, KᵢR₆, KᵢCa₁</td>
<td></td>
</tr>
<tr>
<td>KᵢR₇</td>
<td>KᵢR₇</td>
<td>KᵢR₇, KᵢR₇</td>
<td></td>
</tr>
<tr>
<td>K₂P₁</td>
<td>K₂P₁</td>
<td>K₂P₁, K₂P₁</td>
<td></td>
</tr>
<tr>
<td>K₂P₂</td>
<td>K₂P₂</td>
<td>K₂P₂, K₂P₂</td>
<td></td>
</tr>
<tr>
<td>K₂P₃</td>
<td>K₂P₃</td>
<td>K₂P₃, K₂P₃</td>
<td></td>
</tr>
<tr>
<td>K₂P₄</td>
<td>K₂P₄</td>
<td>K₂P₄, K₂P₄</td>
<td></td>
</tr>
<tr>
<td>K₂P₅</td>
<td>K₂P₅</td>
<td>K₂P₅, K₂P₅</td>
<td></td>
</tr>
<tr>
<td>K₂P₆</td>
<td>K₂P₆</td>
<td>K₂P₆, K₂P₆</td>
<td></td>
</tr>
<tr>
<td>K₂P₇</td>
<td>K₂P₇</td>
<td>K₂P₇, K₂P₇</td>
<td></td>
</tr>
<tr>
<td>K₂P₈</td>
<td>K₂P₈</td>
<td>K₂P₈, K₂P₈</td>
<td></td>
</tr>
<tr>
<td>K₂P₉</td>
<td>K₂P₉</td>
<td>K₂P₉, K₂P₉</td>
<td></td>
</tr>
<tr>
<td>K₂P₁₀</td>
<td>K₂P₁₀</td>
<td>K₂P₁₀, K₂P₁₀</td>
<td></td>
</tr>
<tr>
<td>K₂P₁₁</td>
<td>K₂P₁₁</td>
<td>K₂P₁₁, K₂P₁₁</td>
<td></td>
</tr>
<tr>
<td>K₂P₁₂</td>
<td>K₂P₁₂</td>
<td>K₂P₁₂, K₂P₁₂</td>
<td></td>
</tr>
<tr>
<td>K₂P₁₃</td>
<td>K₂P₁₃</td>
<td>K₂P₁₃, K₂P₁₃</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1-1: K⁺ channel Groups.** Many genes encode K⁺ channels which form 4 main groups: voltage-gated (Kᵥ), Ca²⁺-activated (KᵥCa), inwardly-rectifying (KᵢR) and two-pore domain (K₂P). Each group can be classified into families and then subclassified into sub-families. Kᵥ channels contain 12 families Kᵥ₁ to Kᵥ₁₂. KᵥCa channels contain 3 families, BKᵥCa, SKᵥCa, and IKᵥCa. KᵢR channels consist of 7 families, KᵢR₁ to KᵢR₇, and K₂P channels contain 15 families K₂P₁ to K₂P₁₈.
1.4.2. Function: Control of Vascular Tone

K⁺ channels participate in a multitude of physiological processes. They have a prominent role in the cardiovascular system where they are important in controlling cardiac contraction and systemic vascular resistance. This includes controlling the activation of vascular endothelial and SMCs, and therefore contraction and relaxation of blood vessels. The excitability of these cells is determined by the membrane potential; which at rest is maintained in a quiescent state by the efflux of K⁺ through ion channels (Ko et al., 2008). The vasculature expresses many different isoforms from all four K⁺ channel groups, which in addition to maintaining the resting membrane potential, also respond to a variety of different stimuli to modulate vascular tone.

1.4.2.1. Voltage-Gated K⁺ Channels

Voltage-gated K⁺ channels (Kv) are expressed in both the endothelium and SMCs of the vasculature. In both cell types, Kv channels are important in maintaining the resting membrane potential, ensuring this stays within physiological constraints, and modulating vascular tone in response to circulating vasoactive agents (Jackson, 2005). The primary stimulus for activation of Kv channels is a change in the charge distribution across the plasma membrane; commonly referred to as membrane depolarisation. The voltage threshold for Kv activation differs for each Kv isoform (Gutman et al., 2005).

Kv channels have a prominent role in maintaining resting tone within vascular SM; however, the Kv isoform(s) responsible for this are under debate (Archer et al., 1998; Gurney et al., 2002; Korn & Trapani, 2005). Kv2.1 channels have been implicated in maintaining the resting membrane potential in SMCs including the pulmonary vasculature (Archer et al., 1998). However, it has been argued that the voltage-threshold for activation of this channel is more depolarised than the apparent resting membrane potential of between -40 mV and -60 mV in vascular SMCs (Nelson & Quayle, 1995; Coetzee et al., 1999; Gurney et al., 2002; Gutman et al., 2005). The K⁺ channel(s) mediating the resting membrane potential in vascular SMCs must be active between -40 mV and -60 mV and display little inactivation to maintain efflux (Evans et al., 1996; Greenwood & Ohya, 2009). Other Kv channels have been implicated including the Kv7 family which have biophysical properties that satisfy the criteria for maintaining the SMC resting membrane potential (Mackie et al., 2008; Yeung et al., 2008; Joshi et al., 2009).
Many 
Kv channels are functional in vascular SMCs where they are important in controlling vascular tone. This includes the Kv1 family (Halliday et al., 1995; Archer et al., 2004; Plane et al., 2005), Kv2 family (Archer et al., 2004; Zhong et al., 2010), Kv4 family (Yeung et al., 2006), Kv7 family (Yeung et al., 2007; Mackie et al., 2008; Joshi et al., 2009) and the Kv11 family, more commonly identified as the hERG channel (Yeung & Greenwood, 2007). These channels open in response to membrane depolarisation and permit K+ efflux, thereby returning the membrane potential to a hyperpolarised and quiescent state (Ko et al., 2008). Therefore, they act as a braking mechanism to avoid excessive depolarisation, Ca2+ influx and contraction.

Kv channels localised to the SMC membrane participate in the mechanisms of vasodilation and vasoconstriction as they are modulated by a variety of different stimuli (Jackson, 2005). Vasoconstrictor agonists that act via the Gq-linked GPCR pathway, such as ATII, ET-1, AVP and TxA2, inhibit Kv channels as part of their mechanism to elicit constriction (Scornik & Toro, 1992; Cogolludo et al., 2003; Mackie et al., 2008; Rainbow et al., 2009; Li et al., 2010). Similarly, vasodilator agents acting via the cAMP signalling cascade, such as histamine, open Kv channels as part of their mechanism to elicit dilation (Ishikawa et al., 1993; Jackson, 2000; Neylon, 2002).

Some Kv channel isoforms are also regulated by changes in oxygen, which in turn modulates vascular tone. These channels are collectively classified as “O2-sensitive” and include the Kv1.2, Kv1.5, Kv2.1, Kv3.1b and Kv9.3 isoforms (Patel et al., 1997; Archer et al., 1998; Osipenko et al., 2000). Kv channels are deemed O2-sensitive if they contain key cysteine and methionine residues that can be oxidised or reduced in response to altered oxygenation which changes the conformation of the channel pore and therefore ion flux (Moudgil et al., 2005). Whether modulation of these key residues is via a direct effect of oxygen or following production of free radicals is unclear. Reduced oxygenation, either resulting in an increase or decrease in free radical production (Archer et al., 1986; Waypa et al., 2001), has been shown to inhibit opening of O2-sensitive Kv channels localised to the SMC membrane. This in turn results in membrane depolarisation, Ca2+ influx and subsequently vasoconstriction in pulmonary SMCs (Post et al., 1992). The identity of the O2-sensitive Kv channel(s) inhibited by reduced oxygenation is currently debated with strong evidence implicating Kv1.5 and Kv2.1 channels (see section 1.2.3.) (Archer et al., 1998).

Relative to the vascular SMC literature, little is known about the expression and function of Kv channels in endothelial cells. The Kv1 subfamily is expressed in endothelial cells of the microvasculature where they are thought to be important in
maintaining the resting membrane potential (Nelson & Quayle, 1995; Dittrich & Daut, 1999; Jackson, 2005). The endothelial cell membrane potential will influence the release of endothelial-derived vasodilators. For example, $K_v$ channel opening and the subsequent $K^+$ efflux will produce an electrical gradient that favours $Ca^{2+}$ influx and an increased production of vasodilators (Jackson, 2005).

To summarise, $K_v$ channels are expressed in the endothelium and vascular SM where they function to control vascular tone. As $K_v$ channel isoforms have variable kinetic properties and can be regulated at several levels by a multitude of different proteins, it enables the fine control of vascular resistance according to the conditions of the extracellular milieu.

1.4.2.2. Ca$^{2+}$-Activated K$^+$ Channels

Ca$^{2+}$-activated K$^+$ channels ($K_{Ca}$) are activated by increases in intracellular Ca$^{2+}$, which results from either influx across the plasma membrane by specific channels, or via the release from intracellular stores. The three $K_{Ca}$ channel isoforms $BK_{Ca}$, $SK_{Ca}$ and $IK_{Ca}$ are expressed and functional within the vascular SM and/or endothelium where they participate primarily in vasodilation (Jackson, 2005).

Within vascular SM, $BK_{Ca}$ channels are the predominant $K_{Ca}$ isoform which open in response to membrane depolarisation and increased intracellular Ca$^{2+}$ (Korn & Trapani, 2005; Ko et al., 2008). $BK_{Ca}$ channels are not thought to regulate the membrane potential in adult SMCs as their activation threshold requires a high concentration of Ca$^{2+}$ which is not thought to exist under resting physiological conditions (Jackson, 2005). However, $BK_{Ca}$ is thought to maintain the resting membrane potential in fetal pulmonary vascular SMCs where Ca$^{2+}$ may be raised during vasculogenesis (Reeve et al., 1998; Resnik et al., 2006a). The primary role of these channels in adult vascular SMCs is to act as a braking mechanism to prevent excessive depolarisation by opening in response to raised intracellular Ca$^{2+}$ thereby inducing hyperpolarisation and vasodilation (Ledoux et al., 2006). $BK_{Ca}$ channels are activated by various vasodilatory agents including NO, and arachidonic acid either directly or via protein kinases (see section 1.2.2.2) (Bolotina et al., 1994). $BK_{Ca}$ channels are also modulated by oxygen whereby hypoxia increases channel opening and alters expression levels in fetal vascular SMCs (Gebremedhin et al., 1994; Resnik et al., 2006a). A role for $BK_{Ca}$ channels in vascular endothelial cells is emerging whereby opening of these channels,
and subsequent hyperpolarisation, would enhance endothelial-dependent vasodilation (Faehling et al., 2001; Ledoux et al., 2006; Hughes et al., 2010; Vang et al., 2010). However, this physiological role for BK\textsubscript{Ca} channels in the endothelium is contentious as there are some reports which fail to observe expression or function of these channels in certain vascular endothelial cells (Gauthier et al., 2002). Further studies are necessary to determine whether species variability or heterogeneity amongst vascular beds can account for these contradictory reports.

The predominant K\textsubscript{Ca} channels expressed in the endothelium are SK\textsubscript{Ca} and IK\textsubscript{Ca} channels. These channels are targets for mediating endothelial-dependent vasodilation induced by the humoral vasodilators histamine, acetylcholine, substance P, bradykinin and endothelial-derived hyperpolarisation factor (EDHF) (Edwards et al., 1998; Jackson, 2005; Ledoux et al., 2006). Opening of SK\textsubscript{Ca} and IK\textsubscript{Ca} channels provide the principal hyperpolarising stimulus for Ca\textsuperscript{2+} influx into endothelial cells that drives NO production, thereby promoting vasodilation (Ledoux et al., 2006). SK\textsubscript{Ca} and IK\textsubscript{Ca} channels are not thought to localise to the SMCs in the systemic vasculature where they would potentially modulate vasoactive responses in the intact vessel (Edwards et al., 1999; Walker et al., 2001). However, these channels are important in regulating SMC proliferation and migration as they maintain the resting membrane potential and control Ca\textsuperscript{2+} entry, which activates many growth factors that drive these processes (Owens, 1995; Neylon, 2002; Owens et al., 2004; Rensen et al., 2007). SMCs that primarily function to control proliferation are said to have a “synthetic phenotype”, rich in extracellular matrix proteins and organelles important in protein synthesis (Owens, 1995; Rensen et al., 2007). This is in contrast to SMCs with a “contractile” phenotype which control vascular tone and contain an abundance of contractile filaments (Owens, 1995; Rensen et al., 2007). Changes in the phenotype of SMCs is associated with a change in the predominant K\textsuperscript{+} channel family expressed, with IK\textsubscript{Ca} channels most abundant in “synthetic”, proliferative SMCs (Neylon, 2002). SMC proliferation has an important physiological role in vasculogenesis and expression of IK\textsubscript{Ca} channels is high in fetal vascular SMCs when this process predominates over the control of blood vessel tone (Comfied et al., 1996; Snetkov et al., 1996; Reeve et al., 1998; Lin et al., 2003; Resnik et al., 2006a; Resnik et al., 2006b). This prominence of IK\textsubscript{Ca} channels is thought to be linked to the oxygenation status as the process of vasculogenesis is inherently associated with hypoxia which in itself promotes K\textsubscript{Ca} expression (Gebremedhin et al., 1994; Reeve et al., 1998; Resnik et al., 2006a; Marino et al., 2007). This produces developmental differences in K\textsubscript{Ca} expression as the function of blood vessels switches from predominantly proliferative and migratory during the fetal period to a mature contractile state in adulthood.
In summary, $K_{Ca}$ channel isoforms differentially distribute between the blood vessel cellular layers; with $BK_{Ca}$ predominating in the SM and $SK_{Ca}$ and $IK_{Ca}$ localised to the endothelium. When expressed in their respective cell type, all $K_{Ca}$ channel isoforms mediate vasodilation. However, as a greater understanding of each cell type develops and different phenotypes are discovered, a cross-over in $K_{Ca}$ isoform expression between the SM and endothelium is emerging. This in turn will impact upon the function of each cell type as the different channel isoforms are activated, regulated and post-translationally modified by a repertoire of different extracellular and intracellular factors.

1.4.2.3. Inwardly-Rectifying K$^+$ Channels

Inwardly-rectifying K$^+$ channels ($K_{IR}$) are expressed in vascular endothelial and SMCs; however, their precise physiological role is uncertain. Evidence suggests $K_{IR}$ channels may be important in maintaining the resting membrane potential in some SMCs as they are inherently inwardly-rectifying. This property implies that at hyperpolarised membrane potentials, K$^+$ flux is primarily inward due to the presence of intracellular blocking particles which occlude the channel pore at depolarised potentials (Ishihara et al., 1989). This block is not absolute; therefore, across the range of physiological membrane potentials, there is a small but influential K$^+$ efflux that helps maintain the resting potential (Nelson & Quayle, 1995; Korn & Trapani, 2005). Functional expression of $K_{IR}2.1$, $K_{IR}2.2$ and $K_{IR}2.4$ channels are evident in several vascular SMCs isolated from coronary, cerebral and pulmonary arteries (Tennant et al., 2006; Wu et al., 2007).

$K_{IR}$ channels are also thought to play a physiological role in mediating vasodilation. In both endothelial and SMCs, $K_{IR}$ channels are activated following moderate increases in extracellular K$^+$, which amplifies the hyperpolarising stimulus initiated by $K_{Ca}$ channel opening (Nelson & Quayle, 1995; Korn & Trapani, 2005; Kubo et al., 2005; Ko et al., 2008). $K_{IR}$ channel function is modulated by humoral vasoactive agents which in turn alters vascular tone. Vasoconstrictor agonists such as ATII, vasopressin, ET-1 and histamine have been reported to inhibit $K_{IR}$ channels localised to vascular endothelial cells through a G-protein dependent mechanism (Hoyer et al., 1991; Nilius et al., 1993; Nilius & Droogmans, 2001). Conversely, vasodilatory agents such as bradykinin may activate $K_{IR}$ channels in SMCs to aid propagation of the hyperpolarising stimuli throughout the vessel (Rivers et al., 2001).
A physiological role for the K_{IR}6 family (K_{ATP}) is well established in the vasculature as a metabolic sensor that can regulate blood flow according to the surrounding metabolic environment (Nelson & Quayle, 1995). K_{ATP} channels are sensitive to the intracellular concentration of ATP due to the presence of the auxiliary β-subunit SUR (see section 1.4.1.3). An increase in the intracellular concentration of ATP inhibits channel opening resulting in membrane depolarisation (Ko et al., 2008). However, K_{ATP} channels are also regulated by ADP, H^+, Ca^{2+}, and vasoactive humoral agonists (Nilius & Droogmans, 2001). Vasodilators acting through PKA such as PGI_2 and NO may exert their dilatory effect through activation of SMC localised K_{ATP} channels (Nelson & Quayle, 1995). K_{ATP} channels are also a target for vasoconstrictors acting through PKC such as noradrenaline, AVP, ET-1 and ATII which elicit constriction in part through K_{ATP} inhibition (Miyoshi & Nakaya, 1991; Kontani et al., 1992; Miyoshi et al., 1992; Jackson, 2005). K_{ATP} are also inherently regulated by oxygenation given the association between oxygen availability and metabolic rate. For example, during periods of reduced oxygenation, the rate of metabolism decreases resulting in reduced ATP production that permits K_{ATP} channels opening (Standen & Quayle, 1998; Brayden, 2002). Vascular endothelial cells may also express K_{ATP} channels which are implicated in mediating vasodilation in response to osmotic changes (Ishizaka & Kuo, 1997; Jackson, 2005).

In summary, K_{IR} channels, including the K_{ATP} isoforms, are localised to the endothelium and SMCs in many vascular beds. These channels are targets for humoral vasoactive agonists and thereby regulate vasoconstriction and vasodilation. In addition, K_{IR} channels are able to sense changes in the surrounding metabolic environment and translate this to altered channel function and ultimately vascular tone.

1.4.2.4. Twin-Pore K^+ Channels

Twin-pore K^+ channels (K_{2P}) are referred to as "leak" channels as they are voltage-independent and open at negative membrane potentials (Korn & Trapani, 2005). This makes K_{2P} channels suitable candidates for setting the resting membrane potential (Patel & Honore, 2001; Gurney & Manoury, 2009). K_{2P} channels are also regulated by many different stimuli including membrane stretch, vasoactive agonists, pH and oxygenation (Enyedi & Czirjak, 2010).
The six \( \text{K}_{\text{2p}} \) subfamilies TWIK, TASK, TREK, TALK, THIK, and TRESK are categorised according to structural homology (see section 1.4.1.4). As these channels are studied in more detail, the repertoire of physiological stimuli that regulate their function is increasing. This has resulted in considerable crossover between the six \( \text{K}_{\text{2p}} \) subfamilies regarding the stimuli that regulate their function. Table 1-2 illustrates the physiological stimuli that modulate \( \text{K}_{\text{2p}} \) channel function in the body.

The \( \text{K}_{\text{2p}} \) subfamilies TASK, TREK, TWIK and THIK are expressed in the vasculature (Gardener et al., 2004). These channels modulate vascular tone as they are regulated by humoral agonists such as arachidonic acid and PGI\(_2\) (see Table 1-2). When applied to blood vessels, arachidonic acid and PGI\(_2\) promotes vasodilation through a mechanism that may involve TASK-1, TREK-1, TREK-2, TWIK-2, TRAAK and THIK-1 channels (Bryan et al., 2006; Olschewski et al., 2006; Gurney & Manoury, 2009). The TASK subfamily are thought to have an important role in contributing to the voltage-independent component of the resting membrane potential in several vascular beds including the pulmonary and cerebral circulations (Evans et al., 1996; Gurney et al., 2002; Gurney et al., 2003; Blondeau et al., 2007). TASK-1 is likely to be one of the molecular correlates that contribute to the resting membrane potential in pulmonary vascular SMCs (Gurney et al., 2003). TASK-1 channels are sensitive to changes in pH and oxygenation, both of which are known to affect the resting membrane potential (Post et al., 1992; Gurney et al., 2003).

Recent studies suggest \( \text{K}_{\text{2p}} \) channels including the TREK-1 isoform are expressed in the endothelium (Garry et al., 2007). Similar to other endothelial \( \text{K}^+ \) channels, \( \text{K}_{\text{2p}} \) channels may be important in setting the endothelial resting membrane potential (Garry et al., 2007). However, reports in other vascular beds fail to demonstrate \( \text{K}_{\text{2p}} \) channel expression in the endothelium in isolated blood vessels, indicating a possible heterogeneity between vascular beds (Bryan et al., 2006). Elucidating the functional expression profiles of \( \text{K}_{\text{2p}} \) channels in both endothelial and SMCs will help understand their physiological significance and potential to be altered in certain disease states.
<table>
<thead>
<tr>
<th>K_{2p} Subfamily</th>
<th>K_{2p} isoforms</th>
<th>Activators</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWIK</td>
<td>TWIK-1, TWIK-2</td>
<td>Alkaline pH_e</td>
<td></td>
</tr>
<tr>
<td>TASK</td>
<td>TASK-1, TASK-3, TASK-5</td>
<td>Alkaline pH_e, Volatile anaesthetics</td>
<td>Acidic pH_e, Hypoxia</td>
</tr>
<tr>
<td>TREK</td>
<td>TREK-1, TREK-2, TRAAK</td>
<td>Membrane stretch, Arachidonic acid, Fatty acids, Acidic pH_i, Volatile anaesthetics</td>
<td></td>
</tr>
<tr>
<td>TALK</td>
<td>TALK-1, TALK-2, TASK-2</td>
<td>Alkaline pH_e, Nitric oxide, Reactive oxygen species</td>
<td>Volatile anaesthetics, Hypoxia</td>
</tr>
<tr>
<td>THIK</td>
<td>THIK-1, THIK-2</td>
<td>Arachidonic acid</td>
<td>Fatty acids, Acidic pH_e</td>
</tr>
<tr>
<td>TRESK</td>
<td>TRESK-1</td>
<td>Ca^{2+}_i, Volatile anaesthetics</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-2: Functional roles for K_{2p} channels. A large cross-over exists within K_{2p} channel subfamilies regarding the stimuli that regulate their function. pH, oxygen, fatty acids, arachidonic acid and volatile anaesthetics regulate K_{2p} channels. As the molecular and functional identity is explored in greater detail, novel activators and inhibitors of K_{2p} channels will be discovered.

Information collated from: (Duprat et al., 2005; Goldstein et al., 2005; Gurney & Manoury, 2009; Enyedi & Czirjak, 2010)
1.5. **K⁺ CHANNEL EXPRESSION AND FUNCTION IN THE FETOPLACENTAL VASCULATURE**

Given the important role K⁺ channels play in controlling vascular tone in systemic and pulmonary blood vessels (see section 1.4), it is conceivable that they are implicated in modulating fetoplacental vascular tone. Interest in fetoplacental K⁺ channels has increased over recent years; however, knowledge of their expression and function in placental “resistance” vessels, which primarily regulate vascular tone is lacking. The advent of techniques to assess ion channel expression and function at the single cell layer, such as single cell RT-PCR and electrophysiology has resulted in a substantial increase in the number of publications investigating vascular K⁺ channels. However, this sparked interest in K⁺ channels and vascular physiology has not translated to the placenta. As blood flow through the placenta affects nutrient and oxygen delivery to the growing fetus, understanding the mechanisms that control fetoplacental vascular tone has important physiological consequences. In addition, aberrant fetal growth is associated with adulthood cardiovascular and metabolic complications which has important long-term health and cost implications for the ageing population (Barker et al., 1990). Therefore, a greater understanding of the mechanisms controlling fetoplacental blood flow is necessary. Focusing investigations on the regulation of fetoplacental blood flow by K⁺ channels may aid in the discovery of therapeutic targets to improve placental blood flow and ultimately fetal growth in many pregnancy complications.

1.5.1. **Expression**

The placenta expresses members from all four K⁺ channel groups; Kv, KCa, Kir and K2P (Hampl et al., 2002; Sand et al., 2006; Wareing et al., 2006a; Corcoran et al., 2008). However, very few studies have demonstrated that these channels are expressed specifically in the fetoplacental “resistance” vessels and currently it is not known whether they are localised to the endothelium or SM.
1.5.1.1. Voltage-Gated K⁺ Channels

Studies investigating Kv channel expression have mainly focused on the O₂-sensitive isoforms. Using whole placental homogenate which contains many different cell types, mRNA was observed for the Kv1.5, Kv2.1, and Kv9.3 isoforms but not Kv1.2 (Hampl et al., 2002; Wareing et al., 2006a). Protein expression of Kv1.5, Kv2.1, and Kv3.1 was similarly observed in placental homogenate (Hampl et al., 2002; Wareing et al., 2006a). Within isolated placental chorionic plate arteries and veins, mRNA for the Kv2.1 and Kv9.3 isoforms was detected (Wareing et al., 2006a; Corcoran et al., 2008). Protein expression was only assessed in these vessels for Kv1.5, Kv2.1 and Kv7.4 channels using western blotting and immunohistochemistry (Wareing et al., 2006a; Corcoran et al., 2008; Mills et al., 2009b; Sampson et al., 2010). Expression of the O₂-sensitive Kv isoforms, or other members of the Kv family, have not been localised to the vascular SMCs where they would directly modulate vasoactive responses.

1.5.1.2. Ca²⁺-Activated K⁺ Channels

Expression of the KCa isoform BKCa is present at the mRNA and protein level in placental homogenate and chorionic plate arteries and veins (Hampl et al., 2002; Sand et al., 2006; Wareing et al., 2006a; Corcoran et al., 2008). Using immunohistochemistry, Sand et al., (2006) demonstrated localisation of BKCa to the endothelium and SM in chorionic plate artery sections. SKCa and IKCa expression has not been extensively studied in the placenta despite initial cloning of IKCa from this organ (Ishii et al., 1997).

1.5.1.3. Inwardly-Rectifying K⁺ Channels

The Kir isoform Kgr6.1, which forms a functional KATP channel when combined with SUR2A, has been identified at the mRNA and protein level in placental homogenate and isolated chorionic plate arteries and veins (Wareing et al., 2006a; Corcoran et al., 2008). It is unknown whether the KATP α-subunit is localised to the endothelial or SMCs. Expression of other Kir channels in the placenta has not been studied.
1.5.1.4. Twin-Pore K⁺ Channels

TASK-1 is the only $K_{2P}$ channel that has been targeted for expression studies in the placenta. mRNA for this channel is present in isolated chorionic plate arteries and veins but it is unknown which cell type it is localised to (Wareing et al., 2006a).

1.5.2. Function: Control of Fetoplacental Vascular Tone

There are a limited number of functional studies investigating the contribution of K⁺ channels to the control of fetoplacental vascular tone (Guiet-Bara et al., 1999; Guiet-Bara & Bara, 2002; Hampl et al., 2002; Sand et al., 2006; Wareing et al., 2006a; Wareing et al., 2006e; Jewsbury et al., 2007; Mills et al., 2009a). Initial evidence for the involvement of K⁺ channels in fetoplacental vascular responses came following an observed change in the membrane potential of chorionic plate arterial SMCs in proportion to the external K⁺ concentration (Ibrahim et al., 1998). Extensions of these early microelectrode studies have aimed at elucidating the identity of these K⁺ channels and their contribution to fetoplacental vascular tone using the whole organ, intact blood vessels and isolated cells. These studies have demonstrated, for the most part indirectly, that the four major K⁺ channel families are functional within the fetoplacental vasculature. However, to date only one study has attempted to functionally identify K⁺ channels in SMCs that could control fetoplacental vascular tone using patch clamp electrophysiology of SMCs isolated from larger diameter (>500 μm) chorionic plate arteries.

1.5.2.1. Voltage-Gated K⁺ Channels

Initial functional evidence for the involvement of $K_v$ channels in the control of fetoplacental vascular tone was obtained in the perfused placenta whereby addition of the $K_v$ channel blocker, 4-aminopyridine (4-AP), increased fetal perfusion pressure (Hampl et al., 2002; Bisseling et al., 2005). The raised perfusion pressure suggests vasoconstriction of fetoplacental blood vessels in response to $K_v$ inhibition. However, it does not give any indication whether this is a global constriction or a localised response within the “resistance vessels” which primarily determine vascular resistance and therefore perfusion pressure. 4-AP increased fetal perfusion pressure to a similar
magnitude as hypoxia (Hampl et al., 2002), suggesting $K_v$ inhibition may prove the mechanistic link to this response. However, as illustrated in section 1.3.4, this level of hypoxia (8 % $O_2$; 60 mmHg) exceeds the predicted normoxic, or “normal”, oxygenation observed within the fetoplacental vasculature questioning the physiological significance of this observation.

Myography studies utilising isolated chorionic plate vessels with size characteristics of “resistance vessels”, have attempted to determine the location of the enhanced fetal perfusion pressure in the perfused placenta following 4-AP application. In these vessels, $K_v$ channels are important in maintaining basal tone as 4-AP promoted constriction under resting conditions on the wire myograph (Sand et al., 2006; Wareing et al., 2006a); an effect that was dose-dependent when assessed in the pressure myograph where flow was present and $pO_2$ 6 % (Mills et al., 2009a).

$K_v$ channels also have the capacity to modulate contractile responses to vasoactive agonists including the thromboxane mimetic U-46619. Preincubation of both chorionic plate arteries and veins with 4-AP (10 min) produced a parallel upward shift in the concentration-response curve to U-46619 (Wareing et al., 2006a). Whether these alterations in basal- and agonist-induced tone in the isolated vessels following inhibition with 4-AP is modulated by extremes of oxygenation are not known. This is an important physiological consideration given the diverse oxygen gradients observed in the placenta and the important impact this has on the control of fetoplacental vascular tone (see section 1.3.4).

Enhanced constriction following inhibition of $K_v$ channels, either under resting or agonist-induced conditions, implicates localisation of these channels to the SMCs where they can directly control constriction. There are a limited number of studies investigating $K_v$ channel function in the individual cellular layers of fetoplacental blood vessels. Microelectrode studies implicate $K_v$ channels as an important determinant of the resting membrane potential in both chorionic plate arterial endothelial and SMCs given the membrane depolarisation elicited by TEA application (Guiet-Bara et al., 1999; Guiet-Bara & Bara, 2002). Electrophysiology recordings performed on SMCs isolated from larger diameter (>500 µM) chorionic plate arteries demonstrate the presence of functional $K_v$ channels as application of 4-AP (5 mM) decreased whole-cell $K^+$ currents (Hampl et al., 2002). The channels contributing to the whole-cell $K^+$ currents in these cells were also demonstrated to be sensitive to hypoxia (Hampl et al., 2002). Although this implicates the functional expression of “$O_2$-sensitive” $K_v$ channels in the isolated SMCs, the level of hypoxia (6 %; 38 mmHg) is not physiologically representative of a
low O$_2$ state within the placenta. Therefore, it remains to be determined whether K$_v$ channels are sensitive to physiological oxygen tensions experienced by these placental arteries.

Following the generalised observation that blockade of the K$_v$ group with 4-AP (1-5 mM) has the potential to modulate fetoplacental vascular tone in the perfused placenta, intact blood vessels and isolated SMCs, elucidating which K$_v$ channel isoforms are responsible for these responses requires the use of more specific K$_v$ channel modulators. Studies using intact blood vessels and the wire myography technique have demonstrated the functional expression of various putative O$_2$-sensitive K$_v$ channel isoforms. Margatoxin and stromatoxin which inhibit K$_v$1.2 and K$_v$2.1 channels respectively (Gutman et al., 2005), enhanced agonist (U-46619) induced constriction in chorionic plate vessels (Kiernan et al., 2010). This implicates functional K$_v$1.2 and K$_v$2.1 channels in these vessels. In similar experiments, correolide enhanced U-46619 induced constriction in chorionic plate veins but not arteries (Kiernan et al., 2010). Correolide was initially thought to specifically inhibit K$_v$1.3 and K$_v$1.5 channels (Felix et al., 1999; Archer et al., 2004) but now appears to cross-react with all members of the K$_v$1 family (Hanner et al., 1999). Therefore, to elucidate further the role individual K$_v$1.X isoforms play in controlling chorionic plate vascular tone requires the use of more specific channel modulators. There is also evidence that K$_v$7 channels are important in modulating vascular responses in fetoplacental vessels. Inhibition of these channels with linopiridine enhanced basal resting tone and flupirtine, a K$_v$7 opener, blunted AVP-induced contraction (Mills et al., 2009b). To date, there are no functional studies utilising specific K$_v$ isoform channel modulators to assess their effects on whole-cell K$^+$ currents in isolated chorionic plate SMCs.

1.5.2.2. Ca$^{2+}$-Activated K$^+$ Channels

A few studies have examined the function of BK$_{Ca}$ channels in the fetoplacental vasculature. In the perfused placenta, blockade of BK$_{Ca}$ channels with iberiotoxin did not increase fetal perfusion pressure (Hampl et al., 2002). However, in isolated chorionic plate arteries iberiotoxin increased the maximal U-46619-induced constriction (Wareing et al., 2006a). BK$_{Ca}$ channels are also thought to be important in the mechanism of NO-induced relaxation in these vessels as inhibition of these channels with charybdotoxin prevented relaxation to the NO donor SNAP (Sand et al., 2006). In electrophysiology experiments performed on SMCs isolated from large diameter
chorionic plate arteries, application of iberiotoxin slightly reduced whole-cell K$^+$ currents (Hampl et al., 2002). Iberiotoxin was applied in combination with barium and glyburide to block K$\text{IR}$ and K$\text{ATP}$ channels respectively. Therefore, it is not known whether the reduced K$^+$ current was specifically due to BK$\text{Ca}$ inhibition. The functional contribution of BK$\text{Ca}$ channels to whole-cell K$^+$ currents and therefore the regulation of fetoplacental vascular tone remains to be resolved. There is currently only limited evidence implicating SK$\text{Ca}$ and IK$\text{Ca}$ channel isoforms in the control fetoplacental vascular tone as inhibition of these channels with apamin and charybotoxin slightly enhanced perfusion pressure in the isolated placental cotyledon (Bisseling et al., 2005). It is not known whether these channels are functional in intact chorionic plate vessels or isolated endothelial or SMCs.

1.5.2.3. Inwardly-Rectifying K$^+$ Channels

Studies investigating K$\text{IR}$ channel function have focused on the K$\text{IR}6.1$ (K$\text{ATP}$) isoform and produced conflicting results. In the perfused placenta, inhibition of K$\text{ATP}$ channels with glibenclamide increased fetal perfusion pressure implicating vasoconstriction. Experiments using intact blood vessels demonstrate functional K$\text{ATP}$ channels important in basal tone and agonist-induced responses in chorionic plate vessels. The K$\text{ATP}$ channel opener pinacidil induced relaxation following pre-constriction with U-46619 in intact chorionic plate vessels; an effect that was partially reversed by glibenclamide (Wareing et al., 2006a; Wareing et al., 2006e; Jewsbury et al., 2007). However, whole-cell patch clamp recordings on placental SMCs were unable to identify K$\text{ATP}$ currents (Hampl et al., 2002). The reason for this discrepancy in the isolated placental SMCs is unknown but may relate to heterogeneity in vessel size and therefore K$^+$ channel expression/function as the vessels used in the electrophysiology experiments (<500 µM) were larger than the vessels studied in the myograph (<300 µm). Alternatively, the electrophysiology solutions utilised may not permit K$\text{ATP}$ opening under basal conditions due to the absence of Mg$^{2+}$-nucleotides in the pipette solution.

Elucidating the contribution of K$\text{ATP}$ channels to basal tone has also produced conflicting data. Wire myography experiments suggest K$\text{ATP}$ channels are closed at rest as glibenclamide was without an effect on basal tone (Wareing et al., 2006e). Similarly, the K$\text{ATP}$ channel openers pinacidil and KRN2391 significantly reduced basal tone in similar experiments (Wareing et al., 2006e; Jewsbury et al., 2007). Conversely, microelectrode experiments suggest K$\text{ATP}$ channels are open and contribute to the
resting membrane potential (Guiet-Bara et al., 1999). Microelectrode experiments measure membrane potential changes directly in SMCs which eliminates the influence from metabolic and endothelium-derived factors that can potentially regulate K\textsubscript{ATP} channels. This may explain the apparent discrepancies. Alternatively, a basal production of ATP may be present within the whole vessel thereby closing K\textsubscript{ATP} channels, explaining the wire myography results. Given the myography results implicating K\textsubscript{ATP} channels as important regulators of placental vascular tone, further detailed experiments using a combination of different K\textsubscript{ATP} modulators are necessary to determine if they are functionally present within the endothelium or SM of fetoplacental vessels.

1.5.2.4. Twin-Pore K\textsuperscript{+} Channels

The role K\textsubscript{2P} channels play in controlling fetoplacental vascular tone is poorly understood. TASK-1 channels were targeted for functional experiments given the presence of mRNA for this channel in chorionic plate vessels (see section 1.5.1.4.). Addition of anandamide, which inhibits TASK-1, increased basal and agonist-induced tone in intact chorionic plate vessels on the wire myograph (Wareing et al., 2006a). There is currently no functional evidence for the involvement of other K\textsubscript{2P} channels in fetoplacental vessels. Similarly, it is not known whether K\textsubscript{2P} channels contribute to whole-cell K\textsuperscript{+} currents in isolated chorionic plate SMCs.

1.5.3. Summary

Collating existing functional and expression data, K\textsubscript{v}, K\textsubscript{Ca}, K\textsubscript{IR} and K\textsubscript{2P} channels regulate fetoplacental vascular tone. Manipulation of these channels with pharmacological agents modulates both basal and agonist-induced responses in the whole placenta and intact blood vessels. Oxygenation has been an area of interest in fetoplacental K\textsuperscript{+} channel research given its important role in controlling fetoplacental vascular tone. There appears to be some cross-over between the responses of chorionic plate vessels to K\textsuperscript{+} channel manipulation and altered oxygenation. Although “O\textsubscript{2}-sensitive” K\textsuperscript{+} channels are expressed in these vessels, it remains to be resolved whether they are modulated over a physiologically relevant oxygen gradient and exposure period experienced by the placenta in vivo. It is also unclear whether the alterations in
vascular tone following K⁺ channel and oxygenation manipulation are the result of “O₂-sensitive” K⁺ channels localised to the SMCs where they would directly control constriction and relaxation. This is partly due to the lack of an in vitro model of chorionic plate arterial SMCs. Given the paucity of data assessing K⁺ channel function in chorionic plate arterial SMCs, detailed electrophysiological studies are necessary to identify the K⁺ channel isoforms contributing to whole-cell K⁺ currents in chorionic plate arterial SMCs. Understanding the K⁺ channel subset within these cells may help identify potential new drug targets to promote vasodilation and improve blood flow in pregnancy conditions associated with increased vascular resistance.
1.6. AIMS AND HYPOTHESES

This project tests the overall hypothesis that oxygen-sensitive K⁺ channels are expressed in smooth muscle cells of human placental chorionic plate resistance arteries and modulate arterial constriction in response to altered oxygenation.

The aims of the study are:

1. **Determine whether oxygen alters agonist-induced chorionic plate artery constriction and whether effects of oxygen are modulated by K⁺ channel blockers**
   i. Using wire myography, investigate the effect of acute and chronic (24 and 48 h) exposure to placental hyperoxia, normoxia and hypoxia on basal tone and U-46619-induced constriction of chorionic plate arteries
   ii. Investigate the effect of 4-AP (Kv channel blocker) and DPO-1 (blocker of the oxygen-sensitive Kv channel Kv1.5) on basal tone and U-46619-induced constriction of chorionic plate arteries acutely and chronically (24 and 48 h) exposed to placental hyperoxia, normoxia and hypoxia.

2. **Determine the expression and activity of K⁺ channels in SMCs isolated from chorionic plate arteries**

   a) **Evaluate methods to isolate viable SMCs:**
      i. Characterise the phenotype of SMC outgrowths from chorionic plate arteries in explant culture (immunostaining for markers of contractile and synthetic SMC)
      ii. Characterise the phenotype of SMCs freshly isolated from chorionic plate arteries

   b) **Determine K⁺ channel protein expression and whole cell K⁺ currents in freshly isolated SMCs:**
      iii. Determine the protein expression of Kᵥ, Kᵥ1.5 and other K⁺ channel isoforms in freshly isolated chorionic plate arterial SMCs (immunostaining)
      iv. Determine the mRNA (qPCR) and protein expression and localisation (immunostaining) of Kᵥ, Kᵥ1.5 and other K⁺ channel isoforms in chorionic plate arteries
      v. Identify and characterise K⁺ channels that contribute to whole-cell K⁺ currents using a range of K⁺ channel modulators targeting Kᵥ, Kᵥ1.5 and other K⁺ channel isoforms (patch clamp).
2. ROLE OF OXYGEN-SENSITIVE $K_v$ CHANNEL BLOCKERS IN REGULATING CHORIONIC PLATE ARTERIAL TONE

2.1. INTRODUCTION

Oxygen and humoral vasoactive agents are important regulators of blood flow through the fetoplacental vasculature as the placenta lacks innervation (Spivack, 1943; Walker & McLean, 1971; Fox & Khong, 1990; Benirschke & Kaufmann, 2000). The placenta is exposed to different oxygenation gradients throughout gestation. During the first trimester of pregnancy, the placenta develops in a low oxygen environment due to the absence of maternal blood flow (Jauniaux et al., 2000). At term when the utero- and fetoplacental circulations are established, the placenta is exposed to diverse oxygen gradients as perfusion with maternal blood across a single placental cotyledon is heterogeneous (Power et al., 1967; Jauniaux et al., 1999; Jauniaux et al., 2000; Jauniaux et al., 2001; Ragavendra & Tarantal, 2001; Hempstock et al., 2003; Jauniaux et al., 2003). These oxygen gradients will affect many of the physiological mechanisms important in sustaining adequate nutrient and gas exchange across the placenta which is necessary for a successful pregnancy.

Placental “normoxia” is considered an oxygen tension equivalent to 6 % or 40 - 50 mmHg; a value thought to prevail within the intervillous space at term (Jauniaux et al., 1999; Burton & Caniggia, 2001; Jauniaux et al., 2001). The chorionic plate and umbilical vessels are exposed to lower oxygen tensions of between 2-4 % or 15-30 mmHg, with oxygenations of less than 2 % deemed as placental “hypoxia” (Lackman et al., 2001). Under physiological conditions, the partial pressure of oxygen found in the systemic circulation is approximately 21 % (85-147 mmHg) (Martini, 2006b). However, this oxygenation is termed “hyperoxic” for the placenta despite very discrete regions of the placental bed that lie within close proximity to the maternal spiral arteries, being exposed to these conditions (Jauniaux et al., 2000; Burton & Caniggia, 2001). Therefore, the fetoplacental blood vessels and capillaries are exposed to oxygen gradients of between 2-21 % which will directly influence their level of tone and ultimately nutrient and oxygen exchange to the fetus.

Previous studies have demonstrated that altered oxygenation affects fetoplacental vascular tone (Howard et al., 1987; Hampl et al., 2002; Wareing et al., 2006b; Wareing et al., 2006c). Vasoconstriction of the fetoplacental vasculature is evident following a
reduction in pO$_2$ from placental hyperoxia (95-21 %) to normoxia (8-5 %) (Howard et al., 1987; Byrne et al., 1997; Hampl et al., 2002; Ramasubramanian et al., 2006). This observation, termed hypoxic fetoplacental vasoconstriction (HFPV), is likened to hypoxic pulmonary vasoconstriction (HPV). HFPV is hypothesised to be physiologically beneficial by enabling matching of fetoplacental blood flow with the heterogeneous supply of maternal blood across the placental bed. This vasoconstriction of the fetoplacental vasculature in response to reduced oxygenation would in turn divert blood flow to highly perfused regions of the placenta rich in oxygen, thereby maximising nutrient and gas exchange to the developing fetus (Howard et al., 1987; Hampl et al., 2002). The effect “true” placental hypoxia (2 %) has on fetoplacental vascular resistance has not been examined in the perfused placenta; however exposure to 2 % O$_2$ did not modulate vascular tone in pressurised chorionic plate arteries (Wareing et al., 2006b).

If oxygen delivery to the placenta from the maternal circulation is impaired, the placenta will be exposed to chronic periods of reduced oxygenation. Chronic hypoxia and increased vascular resistance within the fetoplacental circulation is a characteristic of the pregnancy complication fetal growth restriction (FGR) (Kingdom et al., 1997; Mills et al., 2005). Chronic hypoxia in FGR may result in sustained constriction of the fetoplacental vasculature, accounting for raised vascular resistance, reduced transfer of oxygen and nutrients across the placenta and ultimately reduced fetal growth. However, the effect chronic exposure to altered oxygenation has on the reactivity of the fetoplacental arteries that primarily determine fetoplacental vascular resistance (chorionic plate arteries), has not been determined.

Similar to HPV observed in the lung, a prominent role for voltage-gated K$^+$ channels (K$_v$) has been attributed to mediating hypoxic vasoconstriction in the placenta (Hampl et al., 2002). Inhibition of this channel family with 4-AP (5 mM) increased fetal perfusion pressure in the placental cotyledon, and inhibited whole-cell K$^+$ currents in isolated chorionic plate arterial SMCs to a similar magnitude as seen following a reduction in oxygen from hyperoxia (20 %) to normoxia (8-5 %) (Hampl et al., 2002). Therefore, constriction of fetoplacental blood vessels in response to reduced oxygenation has been hypothesised to reflect inhibition of K$_v$ channels in chorionic plate arterial SMCs (Hampl et al., 2002). Whether K$_v$ channels are functional at oxygenations simulating placental hyperoxia and hypoxia is not known.

A key role for the oxygen-sensitive K$_v$1.5 isoform of the K$_v$ channel family has been implicated in mediating HPV. The pore forming K$_v$1.5 $\alpha$-subunit is expressed in
pulmonary resistance arteries and isolated SMCs at both the mRNA and protein level (Archer et al., 1998; Archer et al., 2004). It has been demonstrated that direct inhibition of this channel with specific $K_v$1.5 antibodies; significantly reduced current-voltage relationships in isolated SMCs, increased pulmonary artery perfusion pressure in the isolated lung, and inhibited the rise in intracellular $Ca^{2+}$ induced by hypoxia (Archer et al., 1998). Gene-targeted deletion of $K_v$1.5 in the mouse does not result in increased pulmonary artery perfusion pressure in the isolated lung or an increase in tone in pulmonary rings following hypoxic stimulation (Archer et al., 2001). In addition, isolated pulmonary arterial SMCs from these mice were less sensitive to hypoxia, displaying a modest reduction in whole-cell currents compared to wild type SMCs (Archer et al., 2001). These studies suggest that hypoxia inhibits $K_v$1.5 channels localised to the SMCs of pulmonary resistance arteries; resulting in membrane depolarisation, opening of voltage-gated $Ca^{2+}$ channels, a rise in intracellular $Ca^{2+}$ and ultimately vasoconstriction. Within the placenta, $K_v$1.5 channels are expressed at the mRNA and protein level (Hampl et al., 2002). However, whether they are functional, modulate fetoplacental vascular tone, and are regulated by altered oxygenation is not known. $K_v$1.5 channels are also regulated by prolonged oxygenation changes typically experienced during assent to high altitude or in diseases associated with chronic hypoxia such as pulmonary hypertension (Remillard & Yuan, 2005; Bonnet & Archer, 2007). Chronic periods of hypoxia are associated with downregulation of mRNA and protein expression for $K_v$1.5 channels (Wang et al., 1997; Platoshyn et al., 2001; Hong et al., 2004). Animal models of chronic hypoxia and pulmonary hypertension display enhanced pulmonary vascular resistance and reduced hypoxia-sensitive whole-cell $K^+$ currents (McMurtry et al., 1978; Pozeg et al., 2003). Adenoviral gene transfer of $K_v$1.5 cloned from humans into the chronically hypoxic rat ameliorates these common characteristics of hypoxic vasoconstriction (Pozeg et al., 2003). Chronic hypoxia and raised vascular resistance evident in FGR may be associated with aberrant expression and/or function of oxygen-sensitive $K_v$ channels. However, it is yet to be investigated whether chorionic plate arteries and $K_v$ channels can be modulated by chronic oxygenation changes.

This study tested the hypothesis that chorionic plate arterial constriction, and $K_v$ and $K_v$1.5 function is modulated by acute and chronic oxygenation changes. The effect of acute and chronic (24 h and 48 h) exposure to placental hyperoxia, normoxia, and hypoxia on chorionic plate artery constriction was examined. The ability of $K_v$ and $K_v$1.5 channels to modulate chorionic plate arterial constriction in response to acute and chronic alterations in oxygenation was also investigated. $K^+$ channel function was
assessed using 4-AP and DPO-1; inhibitors of the K, family and K,1.5 isoform respectively (Gutman et al., 2005; Stump et al., 2005; Lagrutta et al., 2006; Regan et al., 2006).
2.2. MATERIALS AND METHODS

2.2.1. Vessel Preparation

Control placentas (N = 28) were obtained following vaginal delivery (n=8) or elective Caesarean section (n=20) at term (37-42 weeks gestation) from women with uncomplicated pregnancies (no evidence of hypertension, fetal growth restriction or other medical disorders, BMI 20-35. The patients had a mix of ethnicities with the majority white Caucasian. Patient demographics were not taken into account when analysing the data because patient numbers were too small for statistical analysis of differences between groups (eg ethnicity) and the effects of drugs and treatments were compared to matched controls obtained from the same placenta; any variation detected between experimental groups must be due to drug application. Within 30 minutes of delivery of the placenta, the umbilical artery was located and traced along the chorionic plate to the perimeter where “resistance” chorionic plate arteries (150-500 µm internal diameter) are localised. These vessels were chosen as they are the likely site of resistance within the fetoplacental vasculature. Small sections of the chorionic plate containing these vessels were cut away from the placenta and underlying villous tissue and placed in ice-cold physiological salt solution (PSS in mM; 119 NaCl, 25 NaHCO₃, 4.69 KCl, 2.4 MgSO₄, 1.6 CaCl₂, 1.18 KH₂PO₄, 6.05 glucose, 0.034 EDTA; pH 7.4). Small chorionic plate arteries (internal diameter 270 ± 5 µM, mean ± SEM; n = 292, N = 28) were dissected away from the surrounding connective tissue and cut into 2–3 mm lengths. Vessels isolated from a single placenta were randomly placed into three experimental groups; (1) acute, (2) chronic 24 h culture and (3) chronic 48 h culture. For tissue culture experiments, 5-6 vessel segments were placed in a P35 culture dish (Nunc; Thermo Fisher Scientific Inc) containing 2 ml culture media (DMEM containing 4.5 g/l glucose, 10 % fetal calf serum, 1 % PSG; penicillin, streptomycin, glutamine, Sigma-Aldrich) and maintained in a humidified incubator (37 °C) at 20 % pO₂ (5 % CO₂ in air), 6 % pO₂ (5 % CO₂ in 5 % O₂) and 2 % pO₂ (5 % CO₂ in N₂) for 24 h and 48 h.

2.2.2. Wire Myography

Functional wire myography experiments were performed on chorionic plate arteries normalised and equilibrated at three different oxygen tensions; 5 % CO₂ in air (termed
20 % HYPEROXIA, BOC special gas, British Oxygen Company, UK), 5 % CO₂ in 5 % oxygen (final dissolved oxygen content of 4.8 – 6.0 %; termed 6 % NORMOXIA), or 5 % CO₂ in nitrogen (final dissolved oxygen content of 0.8–1.0 %; termed 2 % HYPOXIA). Using identical solution gassing conditions and myography experimental protocols, oxygenation has been previously measured in individual myograph chambers adjacent to the tissue (described in Wareing et al., (2006c)) using a probe that measures dissolved oxygen (World Precision Instruments, Sarasota, FL; probe measurement accuracy ± 1 %). “Acute” and “chronic” myography experiments were performed on chronic plate arteries at the three oxygen tensions. “Acute” experiments were performed immediately following vessel dissection. “Chronic” experiments were performed on vessels cultured for both 24 h and 48 h. Acute and chronic experiments were performed on vessels isolated from the same placenta at either hyperoxia (20 % pO₂), normoxia (6 % pO₂), or hypoxia (2 % pO₂).

Chorionic plate arterial segments were mounted onto 40 µM steel wires on a 610M -wire myograph (Danish Myotech, Aarhus, Denmark) and bathed in 5 ml PSS warmed to 37 °C. Vessels were normalised to 0.9 of the vessel diameter in vivo if subjected to a transmural pressure of 5.1 kPa, mimicking a physiological resting tension of 25 mmHg, using the classical normalisation (CN) procedure (Wareing et al., 2002; Cooper et al., 2005, 2006). The CN procedure involved sequential stretching of the vessel and passive tension recorded until this value exceeded 5.1 kPa and then tension returned to 0.9 of the 5.1 kPa value. Post-normalisation, vessels were allowed to equilibrate for 30 min.

Protocols investigating vasoconstriction in these vessels were identical regardless of culture duration or pO₂; an example trace is shown in Figure 2-1. The contractile ability of placental vessels was assessed at the beginning and end of the experiment using 120 mM KCl PSS applied for 5 min or until a plateau had been reached. Following wash out with PSS and return to a stable baseline, concentration-response curves were constructed to the thromboxane mimetic U-46619 (0.1–2,000 nM in 2 min increments / 5 min plateau). These were performed before and following a 10 min incubation period with vehicle (DMSO 0.001 %) or drug (1 mM 4-aminopyridine and 3 µM DPO-1). 1 mM 4-AP was used in line with previous studies in chorionic plate arteries (Wareing et al., 2006a) and 3 µM DPO-1 represented a submaximal (10 x IC₅₀) dose (Lagutta et al., 2006). Vessels greater than 500 µm in diameter were excluded from the study. All measurements were taken at the peak constriction value following each drug application.
Figure 2-1: Wire Myography Protocol. Following vessel normalisation (approximately 40 mins from mounting), two 120 mM KPSS constrictions were performed. Upon return to a stable baseline, increasing concentrations of U-46619 (0.1 – 2,000 nM in 2 min increments) was applied and washout with PSS performed following a 5 min plateau at the highest concentration. Vehicle (as in the example above) or drug was applied for 15 min and in the continued presence, another U-46619 dose-response curve performed. A final 120 mM KPSS constriction was performed following washout and return to a stable baseline. The entire experiment lasted approximately 3.5 h.

2.2.3. Chemicals and Statistical Analyses

General chemicals were purchased from Sigma-Aldrich, Poole, Dorset, UK. 4-AP (Cat No; 275875; Sigma-Aldrich) was dissolved in dH₂O at a concentration of 0.5 M, pH 7.4 with H₂SO₄, and stored at room temperature (22-25 °C). DPO-1 (Cat No; D7443; Sigma-Aldrich) was dissolved in DMSO at a concentration of 3 mM and stored in aliquots at -20 °C.

Vessel tension was expressed as active effective pressure in kilopascals (kPa) to standardise for variation in diameter between individual vessels. Active effective pressure was calculated using the Laplace relationship where internal diameter is calculated by dividing the internal circumference (μm) by π;

\[
\text{Active effective pressure} = \frac{\text{Wall tension (mN)} \times 2\pi}{\text{Internal diameter (μm)}}
\]
All data were tested for conformation to a normal distribution using the Kolmogorov-Smirnov, D'Agostino and Pearson, and Shapiro-Wilk normality tests. Maximal constriction ($V_{\text{max}}$) and sensitivity ($EC_{50}$) were calculated using a sigmoidal dose-response curve fitted using the GraphPad Prism package (version 5.01; GraphPad software, San Diego, CA, USA). $V_{\text{max}}$, $EC_{50}$ and depolarisation-induced constriction (120 mM KPSS) were compared with Wilcoxon signed rank test and Kruskall-Wallis where appropriate. The mean value for the two KPSS constrictions at the beginning of the experiments were analysed unless otherwise stated. For $V_{\text{max}}$ and $EC_{50}$ comparisons of the effect of control vs. drug, Kruskall-Wallis was performed as control was identical regardless of the drug applied. All statistics were non-parametric and paired. The data were not normally distributed (as determined by each test) and the observations (control vs. treatment) were obtained from the same placenta. Data are expressed as median ± IQR unless otherwise stated and concentration-response curves expressed as mean ± SEM. Statistics were performed on $N =$ no. of placentas; each $N$ was calculated as an average of $n =$ 1-4 vessels per placenta depending on the experimental scenario.

A $P<0.05$ was deemed to be indicative of statistical significance at the start of the study. A power calculation was performed using data from previous studies carried out in the laboratory as outlined below. Placental chorionic plate arteries were exposed to the thromboxane–mimetic U46619 in 20 % oxygenation; this elicited a maximal contraction (measured as active effective pressure in kPa) of $7.86 \pm 1.96$ kPa. When oxygenation was performed at 6 %, the maximal contraction to U-46619 was $11.82 \pm 3.59$ kPa. The smallest difference in between these mean values that is significant is 11.82 - 7.86 = 3.96 kPa. The assumed standard deviation from these groups was $(1.96 + 3.59) / 2 = 2.775$ kPa. This corresponds to a standardised difference of $3.96 / 2.775 = 1.427$. Using Altman's nomogram, a total of 16 patients (8 control and 8 test) would be required for the study to detect a similar difference in means with a power of 90 % at the 5 % level of significance.
2.3. RESULTS

2.3.1. Effect of acute exposure to different oxygen tensions on contractile responses

The thromboxane mimetic U-46619 consistently produced a concentration-dependent constriction of chorionic plate arteries at all oxygen tensions tested. Acute exposure to different oxygen tensions did not affect the sensitivity (EC$_{50}$; Figure 2-2B) or maximal constriction (V$_{max}$; Figure 2-2C) to U-46619 (P>0.05; Kruskal-Wallis). Depolarisation-induced constriction following addition of 120 mM K$^+$ containing physiological salt solution did not significantly differ in experiments performed under acute hyperoxic, normoxic or hypoxic conditions (Figure 2-2D; P>0.05; Kruskal-Wallis).
Figure 2-2: Effect of acute exposure to different oxygen tensions on chorionic plate artery constriction. U-46619 dose-response curves were performed on vessels acutely maintained under hyperoxic (20 % O₂), normoxic (6 % O₂) or hypoxic (2 % O₂) oxygen tensions (A). EC₅₀ (B) or V_max (C) values were not significantly different between these oxygenation gradients (P>0.05; Kruskal-Wallis). Depolarisation-induced constriction with 120 mM KPSS solution (D) was not different between experiments performed under hyperoxic, normoxic or hypoxic oxygenations (P>0.05; Kruskal-Wallis). All data in (A) are mean ± SEM of N placentas (n = no. of vessels); (B-D) each point represents a mean of between n = 3 - 4 vessels from a single placenta with the median denoted by the line.
2.3.2. Effect of chronic exposure to different oxygenations on contractile responses

The thromboxane mimetic U-46619 consistently produced a concentration-dependent constriction of chorionic plate arteries that had been cultured for 24 h (Figure 2-3A) and 48 h (Figure 2-4A) under hyperoxia (20 % O₂), normoxia (6 % O₂) and hypoxia (2 % O₂).

Chronic exposure to hyperoxia (●), normoxia (○) or hypoxia (●) for 24 h did not significantly affect the sensitivity (EC₅₀; Figure 2-3B) or maximal constriction (Vₘₐₓ; Figure 2-3C) to U-46619 (P>0.05; Kruskal-Wallis). Depolarisation-induced constriction following addition of a 120 mM K⁺ physiological salt solution did not significantly differ in experiments performed in vessels cultured for 24 h under hyperoxia, normoxia or hypoxia (Figure 2-3D; P>0.05; Kruskal-Wallis).

Chronic exposure to hyperoxia (●), normoxia (○) or hypoxia (●) for 48 h did not affect the sensitivity (EC₅₀; Figure 2-4B) or maximal constriction (Vₘₐₓ; Figure 2-4C) to U-46619 at all oxygenations tested (P>0.05; Kruskal-Wallis). Depolarisation-induced constriction following addition of a 120 mM K⁺ physiological salt solution did not significantly differ in experiments performed in vessels cultured for 48 h under hyperoxia, normoxia or hypoxia (Figure 2-4D; P>0.05; Kruskal-Wallis).
Figure 2-3: Effect of chronic (24 h) exposure to different oxygen tensions on chorionic plate artery constriction. U-46619 dose-response curves were performed on vessels cultured for 24 h under hyperoxic (20 % O₂), normoxic (6 % O₂) or hypoxic (2 % O₂) oxygen tensions (A). EC₅₀ (B) or Vₘₐₓ (C) values remained unaltered (P>0.05; Kruskal-Wallis). Depolarisation-induced constriction with 120 mM KPSS solution (D) was not different between experiments performed on vessels cultured for 24 h under hyperoxia, normoxia or hypoxia (P>0.05; Kruskal-Wallis). All data in (A) are mean ± SEM of N placentas (n = no. of vessels); (B-D) each point represents a mean of between n = 3 - 4 vessels from a single placenta with the median denoted by the line.
Figure 2-4: Effect of chronic (48 h) exposure to different oxygen tensions on chorionic plate artery constriction. U-46619 dose-response curves were performed on vessels cultured for 48 h under hyperoxic (20% O₂), normoxic (6% O₂) or hypoxic (2% O₂) oxygen tensions (A). EC₅₀ (B) or Vₘₐₓ (C) values remained unaltered (P>0.05; Kruskal-Wallis). Depolarisation-induced constriction with 120 mM KPSS solution (D) was not different between experiments performed on vessels cultured for 48 h under hyperoxia, normoxia or hypoxia (P>0.05; Kruskal-Wallis). All data in (A) are mean ± SEM of N placentas (n = no. of vessels); (B-D) each point represents a mean of between n = 3 - 4 vessels from a single placenta with the median denoted by the line.
2.3.3. Effect of hyperoxia on the ability of $K_v$ channel blockers to regulate chorionic plate arterial tone

U-46619 produced a concentration-dependent increase in constriction chorionic plate arteries cultured under hyperoxia for 24 h (○) or 48 h (●) either prior to (Figure 2-5A) or following (Figure 2-5B) vehicle incubation. Vessel culture did not significantly affect the sensitivity (EC$_{50}$) of U-46619 dose-response curves either prior to (acute 46.6 ± 13.6 nM vs. 24 h 40.3 ± 23.4 nM vs. 48 h 41.5 ± 15.0 nM; median ± IQR; P>0.05; Kruskal-Wallis) or following vehicle incubation (acute 49.7 ± 18.4 nM vs. 24 h 58.9 ± 16.9 nM vs. 48 h 54.5 ± 11.6 nM; median ± IQR; P>0.05; Kruskal-Wallis). Similarly, vessel culture for 24 h (○) or 48 h (●) did not significantly affect maximal constriction ($V_{\text{max}}$) either prior to (acute 12.1 ± 5.7 kPa vs. 24 h 13.7 ± 6.3 kPa vs. 48 h 13.4 ± 7.0 kPa; median ± IQR; P>0.05; Kruskal-Wallis) or following vehicle incubation (acute 15.0 ± 8.3 kPa vs. 24 h 15.2 ± 7.0 nM vs. 48 h 15.1 ± 5.0 kPa; median ± IQR; P>0.05; Kruskal-Wallis). Depolarisation-induced constrictions obtained under acute, chronic 24 h or 48 h hyperoxic conditions were not significantly different (Figure 2-5C; P>0.05; Kruskal-Wallis). The contractile capacity of all vessels was sustained throughout the duration of the experiment indicated by no change in depolarisation-induced constrictions at the beginning (Pre) or end (Post) of the experiment (Figure 2-6; P>0.05; Wilcoxon signed-rank test).

To assess the effect of $K_v$ channel blockers on chorionic plate artery constriction, drug treated groups were compared to time-matched controls as EC$_{50}$ and $V_{\text{max}}$ values were significantly different following vehicle incubation in chronic 48 h (PRE 41.6 ± 19.5 nM vs. POST 54.5 ± 26.7 nM; median ± IQR; P<0.05; Wilcoxon matched-pairs; Figure 2-8E) and acute conditions (PRE 14.1 ± 7.6 kPa vs. POST 15.0 ± 8.3 kPa; median ± IQR; P<0.05; Wilcoxon matched-pairs; Figure 2-8B) respectively.

Preincubation with 4-AP (10 min) to inhibit $K_v$ channels had no significant effect on the sensitivity (EC$_{50}$) of U-46619 dose-response curves performed on vessels acutely exposed to hyperoxic conditions (control 50.7 ± 2.2 nM vs. 4-AP 46.9 ± 28.1 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-9A) or chronically exposed to hyperoxia for 24 h (control 58.9 ± 16.9 nM vs. 4-AP 55.7 ± 45.1 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-9B) or 48 h (control 54.5 ± 11.6 nM vs. 4-AP 34.2 ± 30.0 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-9C). 4-AP had no significant effect on the maximal U-46619 constriction ($V_{\text{max}}$) of chorionic plate arteries acutely exposed to hyperoxic conditions (control 14.9 ± 6.3 kPa vs. 4-AP 13.1 ± 5.5 kPa;
median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-9A) or chronically exposed to hyperoxia for 24 h (control 15.2 ± 6.7 kPa vs. 4-AP 13.9 ± 5.4 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-9B) or 48 h (control 15.7 ± 5.3 kPa vs. 4-AP 13.4 ± 10.8 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-9C). Passive resting tension was significantly increased following 10 min incubation with 4-AP in acute (Figure 2-10A) experiments, and experiments performed on vessels cultured for 24 h (Figure 2-10B) and 48 h (Figure 2-10C) under hyperoxic conditions (P<0.05; Wilcoxon-signed rank test).

Preincubation with DPO-1 (10 min) to inhibit the oxygen-sensitive Kᵥ channel Kᵥ1.5 had no significant effect on the sensitivity of (EC₅₀) U-46619 dose-response curves performed on vessels acutely exposed to hyperoxic conditions (control 50.7 ± 2.2 nM vs. DPO-1 52.6 ± 55.7 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-11A) or chronically exposed to hyperoxia for 24 h (control 58.9 ± 16.9 nM vs. DPO-1 51.0 ± 33.8 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-11B) or 48 h (control 53.1 ± 3.8 nM vs. DPO-1 52.5 ± 9.4 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-11C). DPO-1 had no significant effect on the maximal U-46619 constriction (Vₘₐₓ) of chorionic plate arteries acutely exposed to hyperoxic conditions (control 14.9 ± 6.3 kPa vs. DPO-1 11.4 ± 4.7 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-11A) or chronically exposed to hyperoxia for 24 h (control 15.2 ± 6.7 kPa vs. DPO-1 13.5 ± 8.2 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-11B) or 48 h (control 54.5 ± 11.6 nM vs. DPO-1 58.0 ± 41.8 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-11C). Similarly, passive resting tension was not significantly affected following 10 min incubation with DPO-1 under all conditions tested (Figure 2-12; P>0.05; Wilcoxon-signed rank test).
Figure 2-5: Effect of chronic exposure to hyperoxia on chorionic plate artery constriction. The sensitivity and maximal constriction to U-46619 was not significantly affected by 24 h or 48 h vessel culture compared to acute exposure to hyperoxic conditions either prior to (A) or following (B) vehicle incubation (P>0.05; Kruskal-Wallis). Similarly, depolarisation-induced constriction (C) with 120 mM KPSS solution was not different following acute or chronic (24h or 48h) hyperoxic exposures (P>0.05; Kruskal-Wallis). All data in (A-B) are mean ± SEM of N placentas (n = no. of vessels); (C) each point represents a mean of between n = 2 - 4 vessels from a single placenta with the median denoted by the line.
Figure 2-6: Depolarisation-induced constriction of chorionic plate arteries under hyperoxia. Depolarisation-induced constriction with 120 mM KPSS solution was not significantly different at the beginning (Pre) or end (Post) of the experiment following acute (A), chronic 24 h (B) or 48 h (C) hyperoxic exposures (P>0.05; Wilcoxon signed rank test). Line represents median and each point corresponds to the mean of between n = 2 - 4 vessels from a single placenta.
Figure 2-7: Effect of vehicle incubation on chorionic plate artery constriction under hyperoxia. U-46619 dose response curves were performed both prior to (PRE) and following (POST) vehicle incubation (DMSO 0.001 %) in acute experiments (A) and vessels chronically cultured under hyperoxic conditions for 24 h (B) and 48 h (C). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-8: Effect of vehicle incubation on chorionic plate artery sensitivity to U-46619 and maximal constriction under hyperoxia. The sensitivity (EC$_{50}$) of chorionic plate arteries to U-46619 dose-response curve either prior to (Pre) or following (Post) vehicle incubation in vessels acutely exposed to hyperoxia (A) or cultured for 24 h (C) or 48 h (E). Similarly, maximal constriction (V$_{max}$) to U-46619 was compared either prior to (Pre) or following (Post) vehicle incubation in vessels acutely exposed to hyperoxia (B) or cultured for 24 h (D) or 48 h (F). EC$_{50}$ and V$_{max}$ values were significantly different following vehicle incubation in chronic 48 h and acute hyperoxic conditions respectively (P<0.05; Wilcoxon matched-pairs). Line represents median and each point corresponds to the mean of between n = 2 - 4 vessels from a single placenta.
Figure 2-9: Effect of 4-AP on agonist-induced constriction in chorionic plate arteries under hyperoxia. U-46619 dose response curves in the absence and presence of 4-AP under acute conditions (A) and in vessels cultured for 24 h (B) or 48 h (C) at hyperoxia. 4-AP did not significantly affect EC$_{50}$ and V$_{max}$ values under acute conditions or following chronic exposure to hyperoxia for 24 h and 48 h (P>0.05; Kruskal-Wallis; see text). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-10: Effect of 4-AP on basal tone in chorionic plate arteries under hyperoxia. Incubation with 4-AP (10 min; Post) significantly increased basal tension under acute (A) hyperoxic conditions and in vessels cultured for 24 h (B) or 48 h (C) at 21 % O$_2$ (P<0.05; Wilcoxon signed rank test). Each point corresponds to the mean of between n = 1 - 2 vessels from a single placenta.
Figure 2-11: Effect of DPO-1 on agonist-induced constriction in chorionic plate arteries under hyperoxia. U-46619 dose response curves in the absence and presence of DPO-1 under acute conditions (A) and in vessels cultured for 24 h (B) or 48 h (C) at hyperoxia. DPO-1 did not significantly affect EC$_{50}$ and V$_{max}$ values under acute conditions or following chronic exposure to hyperoxia for 24 h and 48 h (P>0.05; Kruskal-Wallis; see text). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-12 Effect of DPO-1 on basal tone in chorionic plate arteries under hyperoxia. Basal tension remained unaffected following incubation with DPO-1 (10 min; Post) under acute (A) hyperoxic oxygenations or in vessels cultured for 24 h (B) or 48 h (C) at 20 % O₂ (P>0.05; Wilcoxon signed rank test). Each point corresponds to the mean of between n = 1 - 2 vessels from a single placenta.
2.3.4. Effect of normoxia on the ability of $K_v$ channel blockers to regulate chorionic plate arterial tone

U-46619 produced a concentration-dependent increase in constriction chorionic plate arteries cultured under normoxia for 24 h (○) or 48 h (△) either prior to (Figure 2-13A) or following (Figure 2-13B) vehicle incubation. Vessel culture did not significantly affect sensitivity ($EC_{50}$) either prior to (acute $30.6 \pm 17.7$ nM vs. 24 h $31.6 \pm 28.7$ nM vs. 48 h $21.2 \pm 17.1$ nM; median ± IQR; $P>0.05$; Kruskal-Wallis) or following vehicle incubation (acute $38.7 \pm 20.0$ nM vs. 24 h $45.4 \pm 33.9$ nM vs. 48 h $53.3 \pm 24.9$ nM; median ± IQR; $P>0.05$; Kruskal-Wallis). Similarly, vessel culture for 24 h (○) or 48 h (△) did not significantly affect maximal constriction ($V_{\text{max}}$) either prior to (acute $11.4 \pm 4.4$ kPa vs. 24 h $12.1 \pm 2.9$ kPa vs. 48 h $11.4 \pm 4.6$ kPa; median ± IQR; $P>0.05$; Kruskal-Wallis) or following vehicle incubation (acute $11.9 \pm 4.2$ kPa vs. 24 h $13.7 \pm 5.5$ kPa vs. 48 h $12.2 \pm 5.4$ kPa; median ± IQR; $P>0.05$; Kruskal-Wallis). Depolarisation-induced constrictions obtained under acute, chronic 24 h or 48 h normoxic conditions were not significantly different (Figure 2-13C; $P>0.05$; Kruskal-Wallis). The contractile capacity of all vessels was sustained throughout the duration of the experiment indicated by no change in depolarisation-induced constrictions at the beginning (Pre) or end (Post) of the experiment (Figure 2-14; $P>0.05$; Wilcoxon signed-rank test).

To assess the effect of $K_v$ channel blockers on chorionic plate artery constriction, drug treated groups were compared to time-matched controls as $EC_{50}$ and $V_{\text{max}}$ values were significantly different following vehicle incubation in chronic 48 h (PRE $19.2 \pm 15.0$ nM vs. POST $53.2 \pm 24.9$ nM; median ± IQR; $P<0.05$; Wilcoxon matched-pairs; Figure 2-16E) and acute conditions (PRE $10.7 \pm 4.4$ kPa vs POST $11.9 \pm 4.9$ kPa; median ± IQR; $P<0.05$; Wilcoxon matched-pairs; Figure 2-16B) respectively.

Preincubation with 4-AP (10 min) to inhibit $K_v$ channels had no significant effect on the sensitivity ($EC_{50}$) of U-46619 dose-response curves performed on vessels acutely exposed to normoxic conditions (control $43.4 \pm 20.0$ nM vs. 4-AP $20.0 \pm 15.5$ nM; median ± IQR; $P>0.05$; Kruskal-Wallis; Figure 2-17A) or cultured for 24 h (control $49.4 \pm 37.2$ nM vs. 4-AP $43.7 \pm 21.4$ nM; median ± IQR; $P>0.05$; Kruskal-Wallis; Figure 2-17B) or 48 h (control $53.3 \pm 24.9$ nM vs. 4-AP $53.3 \pm 20.5$ nM; median ± IQR; $P>0.05$; Kruskal-Wallis; Figure 2-17C) under normoxia. 4-AP had no significant effect on the maximal U-46619 constriction ($V_{\text{max}}$) of chorionic plate arteries acutely exposed to normoxic conditions (control $11.8 \pm 4.9$ kPa vs. 4-AP $12.2 \pm 11.6$ kPa; median ± IQR;
P>0.05; Kruskal-Wallis; Figure 2-17A) or chronically exposed to normoxia for 24 h (control 13.9 ± 6.7 kPa vs. 4-AP 12.2 ± 4.5 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-17B) or 48 h (control 12.4 ± 5.2 kPa vs. 4-AP 10.1 ± 4.6 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-17C). Passive resting tension was significantly increased following 10 min incubation with 4-AP in acute experiments (Figure 2-18A; P<0.05; Wilcoxon-signed rank test), but not in experiments using vessels cultured for 24 h (Figure 2-18B) or 48 h (Figure 2-18C) under normoxic conditions (P>0.05; Wilcoxon-signed rank test).

Preincubation with DPO-1 (10 min) to inhibit the oxygen-sensitive K_v channel K_v1.5 had no significant effect on the sensitivity (EC_{50}) of U-46619 dose-response curves performed on vessels acutely exposed to normoxic conditions (control 43.4 ± 20.0 nM vs. DPO-1 22.7 ± 26.4 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-19A) or chronically exposed to hyperoxia for 24 h (control 49.4 ± 37.2 nM vs. DPO-1 62.7 ± 33.0 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-19B) or 48 h (control 53.3 ± 24.9 nM vs. DPO-1 63.1 ± 58.7 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-19C). DPO-1 had no significant effect on the maximal U-46619 constriction (V_{max}) of chorionic plate arteries acutely exposed to normoxic conditions (control 11.8 ± 4.9 kPa vs. DPO-1 13.5 ± 2.3 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-19A) or chronically exposed to normoxia for 24 h (control 13.9 ± 6.7 kPa vs. DPO-1 12.2 ± 8.3 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-19B) or 48 h (control 12.4±5.2 kPa vs. DPO-1 10.9 ± 5.5 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-19C). Passive resting tension was not significantly affected following 10 min incubation with DPO-1 under all conditions tested (Figure 2-20; P>0.05; Wilcoxon-signed rank test).
Figure 2-13: Effect of chronic exposure to normoxia on chorionic plate artery constriction. The sensitivity and maximal constriction to U-46619 was not significantly affected by 24 h or 48 h vessel culture compared to acute exposure to normoxic conditions either prior to (A) or following (B) vehicle incubation (P>0.05; Kruskal-Wallis). Similarly, depolarisation-induced constriction (C) with 120 mM KPSS solution was not different following acute or chronic (24 h or 48 h) normoxic exposures (P>0.05; Kruskal-Wallis). All data in (A-B) are mean ± SEM of N placentas (n = no. of vessels); (C) each point represents a mean of between n = 2 - 4 vessels from a single placenta with the median denoted by the line.
Figure 2-14: Depolarisation-induced constriction of chorionic plate arteries under normoxia. Depolarisation-induced constriction with 120 mM KPSS solution was not significantly different at the beginning (Pre) and end (Post) of the experiment following acute (A), chronic 24 h (B) or 48 h (C) normoxic exposures (P>0.05; Wilcoxon signed rank test). Line represents median and each point corresponds to the mean of between \( n = 2 - 4 \) vessels from a single placenta.
Figure 2-15: Effect of vehicle incubation on chorionic plate artery constriction under normoxia. U-46619 dose response curves were performed both prior to (PRE) and following (POST) vehicle incubation (DMSO 0.001 %) in acute experiments (A) and vessels chronically cultured under normoxic conditions for 24 h (B) and 48 h (C). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-16: Effect of vehicle incubation on chorionic plate artery sensitivity to U-46619 and maximal constriction under normoxia. The sensitivity (EC$_{50}$) of chorionic plate arteries to U-46619 dose-response curve either prior to (Pre) or following (Post) vehicle incubation in vessels acutely exposed normoxia (A) or cultured for 24 h (C) or 48 h (E). Similarly, maximal constriction (V$_{\text{max}}$) to U-46619 was compared either prior to (Pre) or following (Post) vehicle incubation in vessels acutely exposed to normoxia (B) or cultured for 24 h (D) or 48 h (F). EC$_{50}$ and V$_{\text{max}}$ values were significantly different following vehicle incubation in chronic 48 h and acute normoxic conditions respectively (P<0.05; Wilcoxon matched-pairs). Line represents median and each point corresponds to the mean of between n = 2 - 4 vessels from a single placenta.
Figure 2-17: Effect of 4-AP on agonist-induced constriction in chorionic plate arteries under normoxia. U-46619 dose response curves in the absence and presence of 4-AP under acute conditions (A) and in vessels cultured for 24 h (B) or 48 h (C) at normoxia. EC$_{50}$ and V$_{max}$ values were unaffected by 4-AP application under acute conditions or following chronic exposure to normoxia for 24 h and 48 h (P>0.05; Kruskal-Wallis; see text). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-18: Effect of 4-AP on basal tone in chorionic plate arteries under normoxia. Incubation with 4-AP (10 min; Post) significantly increased basal tension in acute (A) normoxic conditions (P<0.05; Wilcoxon signed rank test). Basal resting tension in vessels cultured for 24 h (B) or 48 h (C) at 6 % O₂ remained unaffected by 4-AP (P<0.05). Each point corresponds to the mean of between n = 1 - 2 vessels from a single placenta.
Figure 2-19: Effect of DPO-1 on agonist-induced constriction in chorionic plate arteries under normoxia. U-46619 dose response curves in the absence and presence of DPO-1 under acute conditions (A) and in vessels cultured for 24 h (B) or 48 h (C) at normoxia. DPO-1 did not significantly affect EC\textsubscript{50} and V\textsubscript{max} values under acute conditions or following chronic exposure to normoxia for 24 h and 48 h (P>0.05; Kruskal-Wallis; see text). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-20: Effect of DPO-1 on basal tone in chorionic plate arteries under normoxia. Basal tension remained unaffected following incubation with DPO-1 (10 min; Post) under acute (A) normoxic oxygenations or in vessels cultured for 24 h (B) or 48 h (C) at 6 % O₂ (P>0.05; Wilcoxon signed rank test). Each point corresponds to the mean of between n = 1 - 2 vessels from a single placenta.
2.3.5. **Effect of hypoxia on the ability of \( K_v \) channel blockers to regulate chorionic plate arterial tone**

U-46619 produced a concentration-dependent increase in constriction chorionic plate arteries cultured under hypoxia for 24 h (○) or 48 h (●) either prior to (Figure 2-21A) or following (Figure 2-21B) vehicle incubation. Vessel culture did not significantly affect sensitivity (EC_{50}) either prior to (acute 39.7 ± 28.6 nM vs. 24 h 55.0 ± 14.9 nM vs. 48 h 42.1 ± 32.9 nM; median ± IQR; \( P>0.05; \) Kruskal-Wallis) or following vehicle incubation (acute 48.3 ± 38.6 nM vs. 24 h 71.9 ± 45.6 nM vs. 48 h 61.3 ± 30.5 nM; median ± IQR; \( P>0.05; \) Kruskal-Wallis). Similarly, vessel culture for 24 h (○) or 48 h (●) did not significantly affect maximal constriction (\( V_{\text{max}} \)) either prior to (acute 11.4 ± 3.5 kPa vs. 24 h 9.2 ± 5.2 kPa vs. 48 h 10.0 ± 4.5 kPa; median ± IQR; \( P>0.05; \) Kruskal-Wallis) or following vehicle incubation (acute 11.2 ± 4.3 kPa vs. 24 h 12.2 ± 9.7 kPa vs. 48 h 12.1 ± 3.6 kPa; median ± IQR; \( P>0.05; \) Kruskal-Wallis). Depolarisation-induced constrictions obtained under acute, chronic 24 h or 48 h hypoxic conditions were not significantly different (Figure 2-21C; \( P>0.05; \) Kruskal-Wallis). The contractile capacity of all vessels was sustained throughout the duration of the experiment indicated by no change in depolarisation-induced constrictions at the beginning (Pre) or end (Post) of the experiment (Figure 2-22; \( P>0.05; \) Wilcoxon signed-rank test).

To assess the effect of \( K_v \) channel blockers on chorionic plate artery constriction, drug treated groups were compared to time-matched controls as \( V_{\text{max}} \) values were significantly different following vehicle incubation in acute experiments (PRE 10.3 ± 4.5 kPa vs. POST 11.2 ± 4.3 kPa; median ± IQR; \( P<0.05; \) Wilcoxon matched-pairs; Figure 2-24B).

Preincubation with 4-AP (10 min) to inhibit \( K_v \) channels significantly enhanced the sensitivity (EC_{50}) of chorionic plate arteries to U-46619 under acute hypoxic conditions (control 48.3 ± 38.6 nM vs. 4-AP 15.8 ± 14.4 nM; median ± IQR; \( P<0.05; \) Kruskal-Wallis; Figure 2-25A) but not in vessels cultured for 24 h (control 58.9 ± 45.3 nM vs. 4-AP 57.7 ± 68.9 nM; median ± IQR; \( P>0.05; \) Kruskal-Wallis; Figure 2-25B) or 48 h (control 61.7 ± 30.5 nM vs. 4-AP 64.7 ± 57.9 nM; median ± IQR; \( P>0.05; \) Kruskal-Wallis; Figure 2-25C) under hypoxia. 4-AP had no significant effect on the maximal U-46619 constriction (\( V_{\text{max}} \)) of chorionic plate arteries acutely exposed to hypoxic conditions (control 11.3 ± 5.2 kPa vs. 4-AP 12.8 ± 7.0 kPa; median ± IQR; \( P>0.05; \) Kruskal-Wallis; Figure 2-25A) or chronically exposed to hypoxia for 24 h (control 12.1 ±
9.0 kPa vs. 4-AP 8.6 ± 5.6 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-25B) or 48 h (control 12.7 ± 4.2 kPa vs. 4-AP 10.6 ± 3.4 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-25C). Passive resting tension was significantly increased following 10 min incubation with 4-AP in acute experiments (Figure 2-26; P<0.05; Wilcoxon-signed rank test), but not in experiments using vessels cultured for 24 h (Figure 2-26B) or 48 h (Figure 2-26C) under hypoxia (P>0.05; Wilcoxon-signed rank test).

Preincubation with DPO-1 (10 min) to inhibit the oxygen-sensitive K<sub>v</sub> channel K<sub>v</sub>1.5 had no significant effect on the sensitivity (EC<sub>50</sub>) of U-46619 dose-response curves performed on vessels acutely exposed to hypoxic conditions (control 48.3 ± 38.6 nM vs. DPO-1 67.7 ± 29.7 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-27A) or chronically exposed to hypoxia for 24 h (control 58.9 ± 45.3 nM vs. DPO-1 73.7 ± 29.3 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-27B) or 48 h (control 61.7±30.5 nM vs. DPO-1 83.8 ± 26.8 nM; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-27C). DPO-1 had no significant effect on the maximal U-46619 constriction (V<sub>max</sub>) of chorionic plate arteries acutely exposed to hypoxic conditions (control 11.3 ± 5.2 kPa vs. DPO-1 11.4 ± 4.1 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-27A) or chronically exposed to hypoxia for 24 h (control 12.1 ± 9.0 kPa vs. DPO-1 11.9 ± 8.7 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-27B) or 48 h (control 12.7 ± 4.2 kPa vs. DPO-1 11.4 ± 8.0 kPa; median ± IQR; P>0.05; Kruskal-Wallis; Figure 2-27C). Passive resting tension was not significantly affected following 10 min incubation with DPO-1 under all conditions tested (Figure 2-28; P>0.05; Wilcoxon-signed rank test).
Figure 2-21: Effect of chronic exposure to hypoxia on chorionic plate artery constriction. The sensitivity and maximal constriction to U-46619 was not significantly affected by 24 h or 48 h vessel culture compared to acute exposure to hypoxic conditions either prior to (A) or following (B) vehicle incubation (P>0.05; Kruskal-Wallis). Similarly, depolarisation-induced constriction (C) with 120 mM KPSS solution was not different following acute or chronic (24 h or 48 h) hypoxic exposures (P>0.05; Kruskal-Wallis). All data in (A-B) are mean ± SEM of N placentas (n = no. of vessels); (C) each point represents a mean of between n = 2 - 4 vessels from a single placenta with the median denoted by the line.
Figure 2-22: Depolarisation-induced constriction of chorionic plate arteries under hypoxia. Depolarisation-induced constriction with 120 mM KPSS solution was not significantly different at the beginning (Pre) and end (Post) of the experiment following acute (A), chronic 24 h (B) or 48 h (C) hypoxic exposures (P>0.05; Wilcoxon signed rank test). Line represents median and each point corresponds to the mean of between n = 2 - 4 vessels from a single placenta.
Figure 2-23: Effect of vehicle incubation on chorionic plate artery constriction under hypoxia. U-46619 dose response curves were performed both prior to (PRE) and following (POST) vehicle incubation (DMSO 0.001 %) in acute experiments (A) and vessels chronically cultured under hypoxic conditions for 24 h (B) and 48 h (C). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-24: Effect of vehicle incubation on chorionic plate artery sensitivity to U-46619 and maximal constriction under hypoxia. The sensitivity (EC$_{50}$) of chorionic plate arteries to U-46619 dose-response curve either prior to (Pre) or following (Post) vehicle incubation in vessels acutely exposed hypoxia (A) or cultured for 24 h (C) or 48 h (E). Similarly, maximal constriction (V$_{max}$) to U-46619 was compared either prior to (Pre) or following (Post) vehicle incubation in vessels acutely exposed to hypoxia (B) or cultured for 24 h (D) or 48 h (F). EC$_{50}$ and V$_{max}$ values were significantly different following vehicle incubation in chronic 48 h and acute hypoxic conditions respectively (P<0.05; Wilcoxon matched-pairs). Line represents median and each point corresponds to the mean of between n = 2 - 4 vessels from a single placenta.
Figure 2-25: Effect of 4-AP on agonist-induced constriction in chorionic plate arteries under hypoxia. U-46619 dose response curves in the absence and presence of 4-AP under acute conditions (A) and in vessels cultured for 24 h (B) or 48 h (C) at hypoxia. 4-AP significantly enhanced the sensitivity (EC$_{50}$) to U-46619 under acute conditions only (P<0.05; Kruskal-Wallis; see text). V$_{max}$ values were unaffected by 4-AP application under acute conditions or following chronic exposure to hypoxia for 24 h and 48 h (P>0.05; see text). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-26: Effect of 4-AP on basal tone in chorionic plate arteries under hypoxia. Incubation with 4-AP (10 min; Post) significantly increased basal tension in acute (A) hypoxic conditions (P<0.05; Wilcoxon signed rank test). Basal resting tension in vessels cultured for 24 h (B) or 48 h (C) at 2 % O₂ remained unaffected by 4-AP (P<0.05). Each point corresponds to the mean of between n =1 - 2 vessels from a single placenta.
Figure 2-27: Effect of DPO-1 on agonist-induced constriction in chorionic plate arteries under hypoxia. U-46619 dose response curves in the absence and presence of DPO-1 under acute conditions (A) and in vessels cultured for 24 h (B) or 48 h (C) at hypoxia. DPO-1 did not significantly affect EC$_{50}$ and V$_{max}$ values under acute conditions or following chronic exposure to hypoxia for 24 h and 48 h (P>0.05; Kruskal-Wallis; see text). Data are mean ± SEM for N = no. of placentas (n = no. of vessels).
Figure 2-28: Effect of DPO-1 on basal tone in chorionic plate arteries under hypoxia. Basal tension remained unaffected following incubation with DPO-1 (10 min; Post) under acute (A) hypoxic oxygenations or in vessels cultured for 24 h (B) or 48 h (C) at 2 % O₂ (P>0.05; Wilcoxon signed rank test). Each point corresponds to the mean of between n = 1 - 2 vessels from a single placenta.
2.3.6. Summary

Agonist- and depolarisation-induced constrictions in chorionic plate arteries were unaffected by acute or chronic 24 h and 48 h exposure to hyperoxia (20 % O₂), normoxia (6 % O₂) or hypoxia (2 % O₂) (Table 2-1).

4-AP significantly enhanced the sensitivity of chorionic plate arteries to U-46619 in vessels acutely exposed to hypoxia but this was abolished following culture for 24 h and 48 h (Table 2-2). 4-AP increased basal resting tone under acute conditions at all oxygenations; however, this was only maintained following vessel culture under hyperoxic conditions (Table 2-2).

DPO-1 had no effect on basal tone or U-46619 dose-response curves in chorionic plate arteries acutely exposed to hyperoxia, normoxia and hypoxia or following 24 h and 48 h culture under these oxygenations (Table 2-3).
Table 2-1: Summary- Effect of different oxygenations on agonist- and depolarisation-induced constriction.

<table>
<thead>
<tr>
<th></th>
<th>Agonist-induced constriction</th>
<th>Depolarisation-induced constriction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyperoxia</td>
<td>Normoxia</td>
</tr>
<tr>
<td></td>
<td>EC$_{50}$</td>
<td>V$_{max}$</td>
</tr>
<tr>
<td>Acute</td>
<td>↔ ↔ ↔ ↔ ↔</td>
<td>↔ ↔ ↔ ↔ ↔</td>
</tr>
<tr>
<td>Chronic: 24 h</td>
<td>↔ ↔ ↔ ↔ ↔</td>
<td>↔ ↔ ↔ ↔ ↔</td>
</tr>
<tr>
<td>Chronic: 48 h</td>
<td>↔ ↔ ↔ ↔ ↔</td>
<td>↔ ↔ ↔ ↔ ↔</td>
</tr>
</tbody>
</table>

Table 2-2: Summary- Effect of different oxygenations on the ability of 4-AP to modulate agonist- induced constriction and basal tone.

<table>
<thead>
<tr>
<th></th>
<th>4-AP: Agonist-induced constriction</th>
<th>4-AP: Basal Tone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyperoxia</td>
<td>Normoxia</td>
</tr>
<tr>
<td></td>
<td>EC$_{50}$</td>
<td>V$_{max}$</td>
</tr>
<tr>
<td>Acute</td>
<td>↔ ↔ ↔ ↔ ↑</td>
<td>↑ ↔ ↔ ↑</td>
</tr>
<tr>
<td>Chronic: 24 h</td>
<td>↔ ↔ ↔ ↔ ↑</td>
<td>↑ ↔ ↔ ↔</td>
</tr>
<tr>
<td>Chronic: 48 h</td>
<td>↔ ↔ ↔ ↔ ↑</td>
<td>↑ ↔ ↔ ↔</td>
</tr>
</tbody>
</table>

Table 2-3: Summary- Effect of different oxygenations on the ability of DPO-1 to modulate agonist-induced constriction and basal tone.
2.4. Discussion

This study is the first to examine (1) the effect of acute and chronic exposure to different oxygenations on chorionic plate arterial constriction and (2) the effect of acute and chronic exposure to different oxygenations on the ability of the oxygen-sensitive $K_v$ channel $K_v1.5$ to modulate chorionic plate arterial constriction.

2.4.1. Effect of acute exposure to different oxygenations on contractile responses

An acute reduction in oxygenation from hyperoxia to normoxia promotes vasoconstriction of the fetoplacental vasculature in the perfused placental cotyledon and isolated chorionic plate arteries (Howard et al., 1987; Byrne et al., 1997; Hampl et al., 2002; Ramasubramanian et al., 2006). However, when oxygenation is reduced from normoxia to hypoxia, constriction of isolated chorionic plate arteries is not observed (Wareing et al., 2006b). This suggests hypoxic fetoplacental vasoconstriction (HFPV) does not occur over a physiologically relevant oxygen gradient.

Under physiological conditions in vivo, the fetoplacental vasculature would be exposed to basal levels of humoral vasoactive agents within the circulation that would influence resting tone and responses to altered oxygenation. In the lung, hypoxic constriction is more pronounced when there is a small degree of pretone in the pulmonary arteries induced by a vasoconstrictor such as PGF$_{2\alpha}$ (Ward & Robertson, 1995). Therefore, investigating the effect of altered oxygenation on vascular reactivity in the presence of vasoactive agonists may reveal a physiological role for hypoxic constriction of the fetoplacental vasculature at relevant oxygen tensions. However, previous studies investigating the effect of acute exposure to different oxygenations on agonist-induced constrictions in chorionic plate arteries failed to demonstrate any difference in U-46619 dose-response curves performed under normoxia and hypoxia; moreover, hyperoxia significantly reduced U-46619 constriction (Wareing et al., 2006c). These series of experiments were replicated in the current study but no differences in the sensitivity (Figure 2-2B) or maximal constriction (Figure 2-2C) to U-46619 were observed under the three oxygen tensions. The reason for the discrepancy between the current study and Wareing et al., (2006c) is not known. There is a trend for an increase in U-46619 constriction under hyperoxia in chorionic plate arteries isolated from placentas delivered by caesarean section compared to normal vaginal delivery (Mills et al.,
However, it is unlikely that mode of delivery accounts for the differences between the current study and that of Wareing et al., (2006c) as in both studies the proportion of women delivering by Caesarean section were similar. In summary, the results from the current study suggest that vasoconstriction of chorionic plate arteries to the thromboxane mimetic U-46619 are unaffected by acute exposures to different oxygenations. This consolidates previous observations (Wareing et al., 2006a; Wareing et al., 2006b; Wareing et al., 2006c) suggesting that acute exposure to true placental hypoxia of less than 2 % does not promote vasoconstriction of chorionic plate arteries under agonist-induced conditions.

2.4.2. Effect of chronic exposure to different oxygenations on contractile responses

Increasing the duration of exposure to different oxygenations may reveal a constrictive response to hypoxia in chorionic plate arteries. Chronic exposure to hypoxia is associated with alterations in the expression and function of numerous ion channels that control excitation-contraction coupling in vascular smooth muscle including voltage-gated K⁺ and Ca²⁺ channels, and voltage-independent Ca²⁺ channels (Shimoda, 2010). Similar to acute hypoxia (section 1.2.3), chronic hypoxia has differential effects on vascular tone depending on the vascular bed in question. Vessels isolated from the systemic circulation respond to chronic hypoxia with a reduced ability to contract to vasoactive agonists (Auer & Ward, 1998; Zacour et al., 2002). Conversely, chronic hypoxia enhances constriction in pulmonary resistance arteries (McMurtry et al., 1978; Pozeg et al., 2003). If, as suggested by many researchers (Howard et al., 1987; Read et al., 1995; Hampl et al., 2002; Jakoubek et al., 2006; Ramasubramanian et al., 2006), the placental vasculature behaves like the pulmonary circulation, chronic hypoxia would be expected to enhance constriction. However, the results from the current study suggest chronic hypoxia for 24 h and 48 h had no significant effect on the sensitivity (Figure 2-3B and Figure 2-4B) or maximal constriction (Figure 2-3C and Figure 2-4C) to U-46619. Furthermore, the U-46619 dose-response curve (Figure 2-3A) reveals a trend towards a reduction in constriction in response to chronic hypoxia. These data, along with the lack of effect of acute changes in pO₂, suggest that both short and long term hypoxia does not increase the contractile responsiveness of CPAs and refutes the existence of a hypoxic vasoconstrictor response in these vessels, albeit in response to one agonist.
2.4.3. Effect of acute exposure to different oxygenations on the ability of $K_v$ channels to modulate chorionic plate arterial constriction

Inhibition of $K_v$ channels with 4-AP increased basal resting tone at all oxygenations and enhanced the sensitivity of chorionic plate arteries to U-46619 under hypoxic oxygenations (Table 2-2). This consolidates previous studies and suggests that $K_v$ channels are functional in chorionic plate arteries where they contribute to both basal- and agonist-induced tone (Wareing et al., 2006a).

These results also demonstrate that the ability of $K_v$ channels to modulate agonist-induced tone, but not basal tone, is altered by the prevailing oxygenation. $K_v$ channel inhibition enhances the sensitivity of chorionic plate arteries to U-46619 under hypoxia oxygenations only despite 4-AP increasing basal tone under all oxygenations. This suggests firstly, that under resting conditions oxygen does not markedly affect the ability of $K_v$ channels to modulate chorionic plate arterial tone. Secondly, the enhanced sensitivity to U-46619 may reflect alterations in the downstream effectors of the contractile pathway which either increase the excitability under hypoxia or dampen down excitability under hyperoxia and normoxia. It is also plausible that the increase in basal tone under hypoxia primes the vessel to a level of depolarisation which surpasses a threshold to permit an enhanced sensitivity following U-46619 application. This may be true in this study as qualitatively, the magnitude of the increase in basal tone by 4-AP is greater under hypoxia than hyperoxia and normoxia.

The effect of a change in oxygen on chorionic plate arterial tone was not investigated in the current study, which is hypothesised to underlie hypoxic fetoplacental vasoconstriction (Howard et al., 1987; Hampl et al., 2002). If a reduction in oxygenation promoted an increase in tone reflecting $K_v$ inhibition (Hampl et al., 2002), a higher level of resting tone would be expected in chorionic plate arteries acutely exposed to hypoxia compared to hyperoxia or normoxia. This could not be formally assessed in the wire myograph setting as resting tone is pre-determined by the normalisation procedure irrespective of the physiological tone the vessel would acquire under different oxygenations. However, when this was investigated in pressurised chorionic plate arteries, a switch in the prevailing oxygenation from normoxia to hypoxia did not promote vasoconstriction (Wareing et al., 2006b). Together with the observations in the current study that acutely, 4-AP enhanced basal tone under all oxygenations including hypoxia, this argues against chorionic plate arteries acting as the site of hypoxic fetoplacental vasoconstriction through a $K_v$ channel dependent mechanism over a
physiologically relevant oxygen gradient. Other vessels such as chorionic plate veins or stem villous vessels may be the site of such a response and confirmation of this requires simultaneous measurement of K⁺ current in isolated smooth muscle cells following physiological reductions in oxygenation. However, the question remains whether this phenomenon is observed in the intact placental cotyledon in response to true placental hypoxia (see section 1.3.4).

2.4.4. Effect of chronic exposure to different oxygenations on the ability of Kᵥ channel blockers to modulate chorionic plate arterial constriction

If, in common with the pulmonary vasculature, a chronic reduction in oxygenation promotes an increase in tone through down-regulation of Kᵥ channels in chorionic plate arteries, a higher level of resting tone would be expected in vessels exposed to chronic hypoxia. Again, this has not been formally assessed in the current study due to the limitations of the wire myography technique. However, the activity of Kᵥ channels under chronic conditions does appear to be modulated by the oxygen availability. The ability of 4-AP to increase basal tone indicates that Kᵥ channels are open and could contribute to the resting membrane potential following 48 h exposure to hyperoxia (Figure 2-10), but not in vessels exposed for 24 h or 48 h to normoxia and hypoxia (Figure 2-18 and Figure 2-26 respectively). The abolition of an increase in basal tone under 24 h and 48 h exposure to normoxia and hypoxia may explain why 4-AP application had no effect on the sensitivity of chorionic plate arteries to U-46619. Taken together, these observations suggest that either the expression/function of 4-AP sensitive K⁺ channels is modulated by vessel culture and/or oxygen availability. To summarise, this data furthermore argues against vasoconstriction in the placenta in response to chronic reductions in oxygenation and any involvement of Kᵥ channels as physiologically relevant levels of hypoxia failed to enhance basal- or agonist-induced tone in chorionic plate arteries.
2.4.5. Effect of acute exposure to different oxygenations on the ability of the oxygen-sensitive $K_v$ channel $K_v1.5$ blocker to modulate chorionic plate arterial constriction

The differences in chorionic plate arterial reactivity to 4-AP under hyperoxia, normoxia and hypoxia observed above may be due to a direct effect of oxygen on oxygen-sensitive $K_v$ channels. Many $K_v$ channel isoforms are sensitive to oxygen including $K_v1.2$, $K_v1.5$, $K_v2.1$, $K_v3.1b$ and $K_v9.3$ (Patel et al., 1997; Archer et al., 1998; Osipenko et al., 2000). These channels contain key cysteine and methionine residues that can be oxidised or reduced in response to altered oxygenation which changes the conformation of the channel pore and therefore ion flux (Moudgil et al., 2005). Whether modulation of these key residues is via a direct effect of oxygen or following production of reactive oxygen species (ROS) is unclear. Acute reductions in oxygen, either resulting in an increase or decrease in ROS production (Archer et al., 1986; Waypa et al., 2001), has been shown to inhibit opening of the oxygen-sensitive $K_v$ channel $K_v1.5$ localised to the SMC membrane resulting in vasoconstriction (Archer et al., 1998; Archer et al., 2001; Archer et al., 2004). Given the prominent role for $K_v1.5$ in hypoxic pulmonary vasoconstriction, it has been hypothesised that $K_v1.5$ may also mediate this response in the fetoplacental vasculature (Hampl et al., 2002). Although the current study consolidates previous observations suggesting that over a physiologically relevant oxygen gradient hypoxic constriction does not occur in the fetoplacental vasculature (Wareing et al., 2006b; Wareing et al., 2006c), it is not known whether $K_v1.5$ channels are functional in the placenta and contribute to vascular reactivity over a relevant range of oxygen tensions. In this study $K_v1.5$ channels were targeted with the inhibitor DPO-1 which selects for these channels in heterologous expression systems and primary cardiac myocytes (Gutman et al., 2005; Stump et al., 2005; Lagrutta et al., 2006; Regan et al., 2006).

Under all oxygenations tested, inhibition of $K_v1.5$ channels with DPO-1 did not affect basal tone (Figure 2-12A, Figure 2-20A, Figure 2-28A). These results suggest that $K_v1.5$ channels do not contribute to maintaining the resting membrane potential in chorionic plate arteries under placental hyperoxia, normoxia and hypoxia. This is in agreement with studies in the pulmonary vasculature that exclude a role for $K_v1.5$ in maintaining the resting membrane potential as the voltage-threshold for activation of these channels is more depolarised than the apparent resting membrane potential of between -40 mV and -60 mV in vascular SMCs (Nelson & Quayle, 1995; Coetzee et al., 1999; Gurney et al., 2002; Gutman et al., 2005). Other channels such as $K_v2.1$ (Archer et al., 1998; Archer et al., 2001), $K_v7$ (Joshi et al., 2009), and TASK-1 (Gurney...
et al., 2003) are likely candidates for maintaining the resting membrane potential in pulmonary arterial SMCs and may also play a role in chorionic plate arterial SMCs.

The effect of acute exposure to different oxygenations on the ability of K\(_v\)1.5 channels to modulate U-46619 constriction was investigated in this study. DPO-1 caused a small potentiation of chorionic plate arterial constriction under acute normoxic conditions evident by an upward shift in the U-46619 dose-response curve which did not reach statistical significance in the form of a change in EC\(_{50}\) or V\(_{\text{max}}\) values but may have biological significance (Figure 2-19A). A recent study failed to demonstrate a role for functional K\(_v\)1.5 channels in U-46619 constriction in chorionic plate arteries, evident from a lack of an effect of the K\(_v\)1.X inhibitor correolide (Kiernan et al., 2010). However, the upward shift in the U-46619 dose-response curve following DPO-1 application observed in this study (Figure 2-19A) suggests that K\(_v\)1.5 channels may make a small contribution to fetoplacental vascular tone but any marked increase in constriction is masked by other whole vessel influences such as the endothelium. Therefore, elucidating the role K\(_v\)1.5 channels play in modulating chorionic plate arterial tone requires further clarification using isolated chorionic plate arterial SMCs to assess K\(_v\)1.5 expression/function and other K\(_v\)1.5 channel modulators in both wire myography and pressure myography experiments under luminal flow and pressure.

2.4.6. Effect of chronic exposure to different oxygenations on the ability of the oxygen-sensitive K\(_v\)1.5 blocker to modulate chorionic plate arterial constriction

Chronic reductions in oxygenation are associated with down-regulation in the expression and function of K\(_v\)1.5 channels in pulmonary resistance arteries, which may account for raised vascular resistance in pulmonary hypertension where chronic hypoxia is common (McMurtry et al., 1978; Wang et al., 1997; Platoshyn et al., 2001; Pozeg et al., 2003; Hong et al., 2004). Given the observation of chronic hypoxia (Kingdom et al., 1997) and increased fetoplacental vascular resistance in fetal growth restriction (Mills et al., 2005), the current study aimed to determine whether the ability of K\(_v\)1.5 channels to modulate agonist-induced constriction in chorionic plate arteries could be affected by chronic exposure to different oxygenations.

DPO-1 failed to enhance basal- or agonist-induced tone for chronic 24 h and 48 h exposure to hyperoxia, normoxia or hypoxia (Table 2-3). However, the upward shift in the U-46619 dose-response curve observed when DPO-1 is applied to acutely isolated chorionic plate arteries under normoxia is abolished 24 h and 48 h culture. This may
reflect down-regulation of K_v 1.5 channels as it has been shown previously that mRNA for this channel is reduced in pulmonary arteries following 48 h culture (Manoury et al., 2009). Despite no observed difference in K_v 1.5 protein expression in the smooth muscle of chorionic plate arterial sections with immunohistochemistry (Sampson et al., 2010), a more quantitative analytical technique such as Western blotting or QPCR may reveal reduced expression following vessel culture. In summary, vessel culture may modulate K_v 1.5 expression and/or function and therefore the ability of these channels to affect U-46619 constriction.

2.4.7. Conclusion

K_v channels modulate basal tone and may therefore contribute to maintaining the resting membrane potential. K_v channels also alter the sensitivity of chorionic plate arteries to the thromboxane mimetic U-46619 in an oxygen-dependent manner. However, these effects do not appear to be specifically mediated by the oxygen-sensitive isoform K_v 1.5 as inhibition of these channels with DPO-1 did not significantly alter basal- or agonist-induced tone. Acute and chronic exposure of chorionic plate arteries to placental hyperoxia, normoxia and hypoxia did not affect vasoconstriction following membrane depolarisation or application of the thromboxane mimetic U-46619. Therefore, the contractile status of fetoplacental resistance arteries appears relatively unaffected by alterations in oxygen. These data do not support a role for hypoxic chorionic plate artery constriction under physiologically relevant oxygen tensions and a potential involvement from K_v channels.
3. **K⁺ CHANNEL EXPRESSION AND FUNCTION IN CHORIONIC PLATE ARTERIES**

3.1. **PROTEIN EXPRESSION OF K⁺ CHANNELS IN CHORIONIC PLATE ARTERIAL SMOOTH MUSCLE CELLS**

3.1.1. **Introduction**

Determining whether an ion channel directly controls vasoconstriction and vasodilation requires the study of single vascular SMCs isolated from the intact blood vessel. Using immunocytochemistry and patch clamp electrophysiology, the expression and function of these channels can be studied without the influence of other cells that constitute the intact vessel. These techniques have proved central to our current understanding of the excitation-contraction coupling mechanism in vascular SM in the systemic and pulmonary circulations (Beech & Bolton, 1989; Clapp & Gurney, 1991; Post et al., 1992; Snetkov et al., 1996; Reeve et al., 1998; Neylon et al., 1999; Tang & Wang, 2001; Gurney et al., 2002; Smirnov et al., 2002; Gurney et al., 2003; Milesi et al., 2003; Tammaro et al., 2004; Miguel-Velado et al., 2005). Developing a method to isolate SMCs from chorionic plate arteries will help determine which ion channels, including K⁺ channels, directly control basal- and agonist-induced tone in the fetoplacental vasculature.

Isolation of SMCs from intact blood vessels most commonly requires the use of digestive enzymes in a multi-step procedure. SMCs isolated from the vessel in this way can be placed in culture or used for acute experiments performed within a few hours of isolation (Chamley-Campbell et al., 1979; Clapp & Gurney, 1991; Jackson et al., 1997). One model of SMCs in culture requires the placement of small sections of blood vessels endothelial cell surface down onto a Petri dish which permits outgrowth of SMCs from the vessel explant that can be later passaged (Chamley-Campbell et al., 1979). Determining whether a cultured or acutely isolated SMC model is required for the study of ion channel expression and function is an important consideration as SMCs are not terminally differentiated like skeletal or cardiac muscle (Owens, 1995). SMCs retain a high degree of plasticity and change their phenotype when placed in culture and in vivo in response to growth factors, oxygen and vascular injury (Owens,
1995; Owens et al., 2004; Rensen et al., 2007). The process whereby the phenotype of a SMC changes either in vitro or in vivo is referred to as “phenotypic modulation” and results in morphological, functional and biochemical alterations in the SMC (Owens et al., 2004). Models of SMCs in culture are used when regulatory factors such as hormones, growth factors and oxygen are studied to determine their impact on SMC function over a long time period. However, as SMC culture is associated with phenotypic modulation and changes in ion channel expression/function (Tang & Wang, 2001), caution must be employed when interpreting results and extrapolating the physiological relevance back to the intact blood vessel or animal. A more commonly used SMC model is one where SMCs are acutely isolated from the vessel as the phenotype of the isolates are unlikely to be significantly different from the native tissue given the short time course from isolation to experimentation. Therefore, this would be a more useful model to study ion channel expression/function as it allows more appropriate comparisons to be drawn to the native vessel. However, a model of SMCs in culture may provide valuable insight into the long term regulation of ion channels per se by exogenous regulatory factors.

In other vascular beds, SMC phenotype is known to influence which ion channels, in particular K+ channels, are expressed and functional in these cells and enables SMCs to perform a variety of different functions in vivo (Snetkov et al., 1996; Tang & Wang, 2001; Neylon, 2002; Miguel-Velado et al., 2005; Beech & Cheong, 2006; Beech, 2007; Berra-Romani et al., 2008; Tanaka et al., 2008). SMCs can have a range of phenotypes with “contractile” and “synthetic” phenotypes representing the extremes in a spectrum (Rensen et al., 2007). Contractile SMCs predominantly express voltage-gated (Kv) and the large-conductance Ca2+-activated (BKCa) K+ channel (Scornik & Toro, 1992; Bolotina et al., 1994; Archer et al., 1998; Edwards et al., 1998; Cogolludo et al., 2003; Jackson, 2005; Ko et al., 2008; Mackie et al., 2008; Joshi et al., 2009; Rainbow et al., 2009; Li et al., 2010). These channels regulate the contractile machinery, control the resting membrane potential, are activated by many humoral vasoactive agents and therefore allow contractile SMCs to primarily mediate contraction and relaxation in mature adult blood vessels (Owens et al., 2004). In SMCs with a synthetic phenotype, Ca2+-activated K+ channels predominate; however, very few BKCa channels are expressed and IKCa is the predominant isoform (Neylon et al., 1999; Neylon, 2002; Beech & Cheong, 2006). IKCa channels regulate the membrane potential and are highly sensitive to changes in intracellular Ca2+ which allows synthetic SMCs to undergo proliferation and migration in response to circulating growth factors during vasculogenesis and angiogenesis in fetal blood vessels (Owens, 1995; Neylon et al., 1999; Neylon, 2002; Beech & Cheong, 2006). Morphological analyses reveal the
presence of both contractile and synthetic SMCs in fetoplacental vessels, including intact chorionic plate arteries when visualised by electron microscopy (Sweeney et al., 2006). Therefore, a wide variety of different $K^+$ channels may be expressed in chorionic plate arterial SMCs which are important in controlling excitation-contraction coupling and vasculogenesis. However, direct studies assessing $K^+$ channel expression in chorionic plate arterial SMCs and the phenotype of the isolated SMCs have not been performed.

The overall aim of this study (Chapter 3) is to develop a suitable \textit{in vitro} model to examine $K^+$ channel expression and function in chorionic plate arterial SMCs. The initial part of the study (section 3.1) tested the hypothesis that chorionic plate arterial SMCs express different $K^+$ channels which reflects the expression profile and phenotype of SMCs \textit{in situ}. Two models of isolated chorionic plate arterial SMCs were developed; (1) a model of SMCs in culture and (2) an acutely isolated SMC model. Expression of candidate $K^+$ channels typically localised to contractile and synthetic SMCs was assessed using immunocytochemistry in both chorionic plate arterial SMCs and the intact vessel. The phenotype of chorionic plate arterial SMCs was characterised by determining the expression of phenotypic marker proteins in the isolates and comparing this to the phenotype that exists \textit{in situ}. 
3.1.2. **Materials and Methods**

3.1.2.1. Cultured cell isolation protocol

Chorionic plate arteries were dissected as described in section 2.2.1 under sterile conditions and cultured using the technique described by (Leik et al., 2004). Vessels were cut longitudinally into 3 mm lengths and placed endothelial cell surface side down onto P35 culture dishes. To aid attachment of the vessel section to the dish and ensure optimal visualisation for future experiments, a light scratch was made in the centre of the dish with a sterile surgical blade. Sterile culture media (50 % DMEM containing 4.5g/l glucose, 50 % HAM F12, 10 % fetal calf serum, 1 % PSG; penicillin, streptomycin glutamine, 0.1 % gentamicin, Sigma-Aldrich) was carefully pipetted into the culture dishes to avoid disruption of the vessel explant. The explants were maintained in a humidified incubator at 95 % air/ 5 % CO₂ (pO₂= 20 %) and following 2 weeks in culture, half of the cultures with vessels that remained in the centre of the dish were randomly selected and fixed in methanol for 35 minutes at -20 °C. Cultures with vessels that were at the perimeter of the dish were discarded for experiments as visualisation of the outgrowths was impaired. The media was replenished on the remaining explants at 2 weeks and the fixation process repeated following a total of 4 weeks in culture.

3.1.2.2. Acute cell isolation protocol

A protocol aimed at freshly isolating SMCs from chorionic plate arteries was optimised from several methods described in the literature (Table 3-1 - Table 3-3). The attempted protocols can be categorised as follows; (1) Methods described in human placental arteries (Table 3-1), (2) Methods described in animal arteries performed under Ca²⁺ free conditions (Table 3-2), (3) Methods described in animal arteries performed under low Ca²⁺ conditions (Table 3-3).
(1) Methods described in human placental arteries

The method described by Hampl et al., (2002) to isolate SMCs from large diameter (>500 μm) chorionic plate arteries was replicated. Small resistance chorionic plate arteries (<500 μM) with a length totalling approximately 5 cm were dissected free of connective tissue and the adventitia removed by gentle stripping using fine dissecting forceps. Vessel sections were cut into approximately ten 5 mm strips and placed in cold (4 °C) Ca²⁺ free-HBSS (Hank’s buffered salt solution) containing (mM); NaCl 140, KCl 4.2, KH₂PO₄ 1.2, MgCl₂ 0.5, HEPES 1.0 and EGTA 0.1, for 10 minutes. The vessel sections were then transferred to a tube containing 1 mg/ml papain, 0.75 mg/ml DTT, 0.8 mg/ml BSA in 1 ml Ca²⁺ free-HBSS without EGTA and incubated at 4°C for 20 minutes. This solution was supplemented with 0.8 mg/ml collagenase type 1A and transferred to a water bath warmed to 37°C for 10 minutes. The vessel sections were washed in 1 ml of Ca²⁺ free-HBSS containing 1 mM glucose. Following gentle dispersion with a fine tip Pasteur pipette, no cells were present (n=4 attempts). Following advice and personal communication with the corresponding author, the above protocol was modified as follows (Table 3-1); (1) the initial incubation of vessels in Ca²⁺ free-HBSS was performed at room temperature (22-24°C) for 20 minutes, (2) the first enzyme incubation step contained the addition of collagenase type 4 0.8 mg/ml and was incubated at 4°C for 15 minutes, then transferred to 37°C for 10 minutes. This adapted protocol did not yield any cells.

Salemme et al., (2007) isolated SMCs from human umbilical arteries using a dissociation media (DM) containing (mM); NaCl 140, KH₂PO₄ 5, MgCl₂ 5, glucose 20, HEPES 5, pH 7.4. Vessel sections were incubated in 2 mg/ml collagenase type 1A at 37°C for 25 minutes then washed in fresh dissociation media and triturated with a fire-polished Pasteur pipette. When repeated using small resistance chorionic plate arteries, this protocol did not yield any cells.

(2) Methods described in animal arteries performed under Ca²⁺ free conditions

Isolation protocols documented to successfully isolate animal SMCs were next adopted in an attempt to liberate SMCs from chorionic plate arteries (Table 3-2). The method described by Harhun et al., (2009) successfully isolated SMCs from rat middle cerebral arteries using a DM containing (mM); NaCl 120, KCl 6, glucose 10, HEPES 10, MgCl₂ 1.2. Vessel sections were incubated in Ca²⁺-free DM containing 2 mg/ml collagenase type 1A and 1 mg/ml protease type X for 15 minutes at 37 °C. Vessel sections were washed three times in warmed Ca²⁺-free dissociation media and titrated with a fire-
polished Pasteur pipette. Using this protocol, some SMCs were released from the chorionic plate arteries; however, membrane blebbing (an indicator of apoptosis) suggested that this methodology was over-digesting and damaging cells.

The isolation protocol described by Joshi et al., (2009) utilised three incubation steps. Using this method, chorionic plate arteries with a length of approximately 5cm were dissected free from the chorionic plate, slit longitudinally, cut into 4 sections and maintained in DM containing (mM); NaCl 110, KCl 5, HEPES 10, KH₂PO₄ 0.5, NaH₂PO₄ 0.5, NaHCO₃ 10, Taurine 10, EDTA 5, Glucose 10, MgCl₂ 2. The vessel sections were incubated for 60 minutes at 4 °C in 1 ml of DM containing 1.5 mg/ml papain. To this solution, 1 mg/ml DTT was added and placed in a water bath warmed to 37 °C for 6 minutes. Following this incubation step, the vessel was transferred to 1 ml of fresh DM containing 1.4 mg/ml collagenase type 1A and warmed at 37°C for 5 minutes. The vessel was carefully transferrd through three eppendorf tubes containing 1 ml DM and gently titrated in the final tube using a Pasteur pipette. Relaxed SMCs were liberated from the chorionic plate arteries; however, the yield was variable between preparations and cells did not adhere sufficiently to a substrate for electrophysiology or immunostaining experiments.

This protocol was subsequently modified to improve cell quality, membrane integrity and therefore adherence. Replacement of the digestive enzyme collagenase type 1A with collagenase type F did not liberate any cells from chorionic plate arteries. Therefore, collagenase type 1A is a critical enzyme in this protocol. Prolongation of the papain and DTT incubation step to 12 minutes, and collagenase type 1A incubation step to 7.5, 10, 12 and 15 minutes, were both unsuccessful in increasing yield, cell quality or improving adhesion. Poor adhesion may result from inadequate digestion of the vessel which produces single SMCs with collagen and connective tissue deposits on their membranes which in turn prevents adhesion. Therefore, the concentrations of collagenase type 1A and papain were increased to 3.4 mg/ml and 3.0 mg/ml respectively. Under both conditions, yield was low and cells were over-digested and displayed membrane blebbing indicative of apoptosis.

As modification of enzyme type, concentration and incubation duration did not improve cell quality or adherence, these modifications were not adopted and the original protocol was regarded as "optimal". Using this protocol (1.5 mg/ml papain at 4 °C for 60 min; 1.5 mg/ml papain and 1 mg/ml DTT at 37 °C for 6 min; 1.4 mg/ml collagenase type 1A at 37 °C for 5 min) different coated substrates were next used in an attempt to improve adhesion. The following substrates were utilised (n=X attempts); plastic culture
dishes (n=18), glass coverslips (n=23), acetone-dried coverslips (n=5), membrane-coated slides (n=1), glass chamber slides (n=1), super frosted slides (n=1), poly-L-lysine coated plastic and glass coverslips (n=4), serum coated glass-coverslips (n=1), gelatine coated plastic and glass coverslips (n=12), collagen coated plastic and glass coverslips (n=10), Cell-Tak™ coated plastic and glass coverslips (n=4). Regardless of substrate used, adhesion was not improved and less than 10% of the cells remained following gentle perfusion with extracellular solution during electrophysiology or methanol fixation during immunostaining experiments.

(3) Methods described in animal arteries performed under low Ca\(^{2+}\) conditions

The above protocols all utilise a Ca\(^{2+}\)-free DM. As Ca\(^{2+}\) has been suggested to improve cell adhesion (Heimark, 1991; Braun et al., 1999), protocols specifying a low Ca\(^{2+}\)-containing DM were employed (Table 3-3) (Plane et al., 2005; Ayon et al., 2009; Greenwood et al., 2009). These methods are described as a two step process; the first step is performed in Ca\(^{2+}\)-free DM containing (mM); NaCl 120, NaHCO\(_3\) 25, KCl 4.2, KH\(_2\)PO\(_4\) 0.6, MgCl\(_2\) 1.2, Glucose 11, pH 7.4; and the second step in low Ca\(^{2+}\) (0.01 mM)-containing DM. As described by Plane et al., (2005) in rat middle cerebral artery, chorionic plate arteries were initially incubated in Ca\(^{2+}\)-free DM containing (mg/ml); 0.25 papain, 1.0 DTT and 1.0 BSA for 15 minutes at 37°C, followed by a second incubation step in 0.01 mM Ca\(^{2+}\)-DM containing (mg/ml); 1.0 BSA, 0.7 collagenase type 1A and 0.3 collagenase type F. This protocol yielded few SMCs that appeared over-digested, displaying membrane blebbing.

A gentle overnight digestion protocol was next employed, utilising fewer digestive enzymes in an attempt to produce healthy SMCs. As described by Ayon et al., (2009) in rat pulmonary artery, vessel sections were incubated at 4°C overnight (~16 hours) in Ca\(^{2+}\)-free DM containing (mg/ml); 2.0 BSA, 0.14 DTT, and 0.3 papain. The vessels were next incubated in this enzyme mixture prepared in 0.01 mM Ca\(^{2+}\)-DM for 5 minutes at 37°C and then washed and triturated using a fire-polished Pasteur pipette. No cells were present using this protocol suggesting chorionic plate arteries require a strong digestion protocol to release single relaxed SMCs.

The protocol described by Greenwood et al., (2009) in mouse uterus is a two step process which utilises higher concentrations of digestive enzymes and performed for longer incubation periods at 3 7°C. For this method, chorionic plate arteries were dissected free of the placental chorionic plate and excess connective tissue removed.
with gentle stripping using fine dissecting forceps. Intact vessels were maintained in Ca$^{2+}$ free-DM containing (mM): 120 NaCl, 25 NaHCO$_3$, 4.2 KCl, 0.6 KH$_2$PO$_4$, 1.2 MgCl$_2$, 11 Glucose; pH 7.4 for 5 minutes. The vessels were incubated in 1 ml DM containing (mg/ml): 1.0 papain, and 1.0 DTT for 20 minutes at 37 °C. The vessels were washed three times in ice-cold Ca$^{2+}$ free-DM and then transferred to 0.01 mM Ca$^{2+}$-DM containing (mg/ml): 1.0 collagenase type 1A and 1.0 collagenase type F and incubated at 37 °C for 10 minutes. The vessels were washed three times in ice-cold 0.01 mM Ca$^{2+}$-DM and triturated with a fire-polished glass Pasteur pipette. In the majority (approximately 60 %) of isolations, this protocol produced a high yield of partially contracted cells which had clear defined membranes and adhered to a glass or plastic substrate within 10 minutes. This protocol was then used for subsequent immunostaining and electrophysiology experiments.
<table>
<thead>
<tr>
<th>Protocol</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Result</th>
</tr>
</thead>
</table>
| Hampl et al., (2002): 1  | **ENZYMES (mg/ml): N/A**  
                          | **TEMPERATURE: 4°C**  
                          | **DURATION: 10 min**  | **ENZYMES (mg/ml): 1.0 papain, 0.75**  
                          |  
                          | DTT, 0.85 BSA  
                          | **TEMPERATURE: 4°C**  
                          | **DURATION: 20 min**  | **ENZYMES (mg/ml): 1.0 papain, 0.75**  
                          |  
                          | DTT, 0.85 BSA, 0.8 collagenase type 1A  
                          | **TEMPERATURE: 37°C**  
                          | **DURATION: 10 min**  | - No cells obtained |
| Hampl et al., (2002): 2  | **ENZYMES (mg/ml): N/A**  
                          | **TEMPERATURE: 22-24°C**  
                          | **DURATION: 20 min**  | **ENZYMES (mg/ml): 1.0 papain, 0.75**  
                          |  
                          | DTT, 0.85 BSA, 0.8 collagenase type 4  
                          | **TEMPERATURE: 4°C**  
                          | **DURATION: 15 min**  | **ENZYMES (mg/ml): 1.0 papain, 0.75**  
                          |  
                          | DTT, 0.85 BSA, 0.8 collagenase type 4  
                          | **TEMPERATURE: 37°C**  
                          | **DURATION: 10 min**  | - No cells obtained |
| Salemme et al., (2007)   | **ENZYMES (mg/ml): 2.0 collagenase type 1A** | **TEMPERATURE: 37°C**  
                          | **DURATION: 25 min**  |                                                |                                                |                                                | - No cells obtained |

**Table 3-1**: Summary of isolation adaptations based on methods described in human placental arteries.
<table>
<thead>
<tr>
<th>Protocol</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harhun et al., (2009)</td>
<td>ENZYMES (mg/ml): 2.0 collagenase type 1A, 1.0 protease type X</td>
<td>ENZYMES (mg/ml): 1.5 papain</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>Few cells obtained, Membrane blebbing, Over-digested</td>
</tr>
<tr>
<td></td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DURATION: 15 min</td>
<td>DURATION: 6 min</td>
<td>DURATION: 5 min</td>
<td></td>
</tr>
<tr>
<td>Joshi et al., (2009): 1</td>
<td>ENZYMES (mg/ml): 1.5 papain, 1.0 DTT</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>ENZYMES (mg/ml): 3.8 collagenase type 1A</td>
<td>Variable yield of relaxed SMCs, Poor adhesion to substrate</td>
</tr>
<tr>
<td></td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DURATION: 6 min</td>
<td>DURATION: 7.5, 10, 12 or 15 min</td>
<td>DURATION: 6 min</td>
<td></td>
</tr>
<tr>
<td>Joshi et al., (2009): 2</td>
<td>ENZYMES (mg/ml): 1.5 papain, 1.0 DTT</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>No cells obtained</td>
</tr>
<tr>
<td></td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DURATION: 6 min</td>
<td>DURATION: 5 min</td>
<td>DURATION: 5 min</td>
<td></td>
</tr>
<tr>
<td>Joshi et al., (2009): 3</td>
<td>ENZYMES (mg/ml): 1.5 papain, 1.0 DTT</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>Variable yield of relaxed SMCs, Poor adhesion to substrate</td>
</tr>
<tr>
<td></td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DURATION: 6 min</td>
<td>DURATION: 7.5, 10, 12 or 15 min</td>
<td>DURATION: 6 min</td>
<td></td>
</tr>
<tr>
<td>Joshi et al., (2009): 4</td>
<td>ENZYMES (mg/ml): 1.5 papain, 1.0 DTT</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>Few cells obtained, Membrane blebbing, Over-digested</td>
</tr>
<tr>
<td></td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DURATION: 6 min</td>
<td>DURATION: 5 min</td>
<td>DURATION: 5 min</td>
<td></td>
</tr>
<tr>
<td>Joshi et al., (2009): 5</td>
<td>ENZYMES (mg/ml): 1.5 papain, 1.0 DTT</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>Variable yield of relaxed SMCs, Poor adhesion to substrate</td>
</tr>
<tr>
<td></td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DURATION: 6 min</td>
<td>DURATION: 5 min</td>
<td>DURATION: 5 min</td>
<td></td>
</tr>
<tr>
<td>Joshi et al., (2009): 6</td>
<td>ENZYMES (mg/ml): 3.0 papain, 1.0 DTT</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>ENZYMES (mg/ml): 1.4 collagenase type 1A</td>
<td>Few cells obtained, Membrane blebbing, Over-digested</td>
</tr>
<tr>
<td></td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td>TEMPERATURE: 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DURATION: 6 min</td>
<td>DURATION: 5 min</td>
<td>DURATION: 5 min</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-2: Summary of isolation adaptations based on methods performed under Ca\(^{2+}\) free conditions.
<table>
<thead>
<tr>
<th>Protocol</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plane et al., (2005)</td>
<td><strong>SOLUTION:</strong> 0 mM Ca(^{2+}) Dissociation media&lt;br&gt;<strong>ENZYMES (mg/ml):</strong> 0.25 papain, 1.0 DTT, 1.0 BSA&lt;br&gt;<strong>TEMPERATURE:</strong> 37°C&lt;br&gt;<strong>DURATION:</strong> 15 min</td>
<td><strong>SOLUTION:</strong> 0.01 mM Ca(^{2+}) Dissociation media&lt;br&gt;<strong>ENZYMES (mg/ml):</strong> 1.0 BSA, 0.7 collagenase type 1A, 0.3 collagenase type F&lt;br&gt;<strong>TEMPERATURE:</strong> 37°C&lt;br&gt;<strong>DURATION:</strong> 10 min</td>
<td>- Few cells obtained,&lt;br&gt;- Membrane blebbing&lt;br&gt;- Over-digested</td>
</tr>
<tr>
<td>Ayon et al., (2009)</td>
<td><strong>SOLUTION:</strong> 0 mM Ca(^{2+}) Dissociation media&lt;br&gt;<strong>ENZYMES (mg/ml):</strong> 2.0 BSA, 0.14 DTT, 0.3 papain&lt;br&gt;<strong>TEMPERATURE:</strong> 4°C&lt;br&gt;<strong>DURATION:</strong> 16h</td>
<td><strong>SOLUTION:</strong> 0.01 mM Ca(^{2+}) Dissociation media&lt;br&gt;<strong>ENZYMES (mg/ml):</strong> 2.0 BSA, 0.14 DTT, 0.3 papain&lt;br&gt;<strong>TEMPERATURE:</strong> 37°C&lt;br&gt;<strong>DURATION:</strong> 5 min</td>
<td>- No cells obtained</td>
</tr>
<tr>
<td>Greenwood et al., (2009)</td>
<td><strong>SOLUTION:</strong> 0 mM Ca(^{2+}) Dissociation media&lt;br&gt;<strong>ENZYMES (mg/ml):</strong> 1.0 papain, 1.0 DTT,&lt;br&gt;<strong>TEMPERATURE:</strong> 37°C&lt;br&gt;<strong>DURATION:</strong> 20 min</td>
<td><strong>SOLUTION:</strong> 0.01 mM Ca(^{2+}) Dissociation media&lt;br&gt;<strong>ENZYMES (mg/ml):</strong> 1.0 collagenase type 1A, 1.0 collagenase type F&lt;br&gt;<strong>TEMPERATURE:</strong> 37°C&lt;br&gt;<strong>DURATION:</strong> 10 min</td>
<td>- Consistently high yield of partially contracted SMCs,&lt;br&gt;- Clean membranes, no blebbing,&lt;br&gt;- Adherent to substrate</td>
</tr>
</tbody>
</table>

Table 3-3: Summary of isolation adaptations based on methods performed under low Ca\(^{2+}\) conditions.
3.1.2.3. Immuncytochemistry

Chorionic plate sections (3cm x 2cm, N=4 placentas) containing arteries were dissected, washed in PBS, and fixed in 10 % neutral buffered formalin for 24 h at 4 °C. Tissues were embedded in paraffin wax and orientated to allow cross sectioning through the arteries. Paraffin sections (5 µM thickness) were dewaxed, rehydrated, and microwaved for antigen retrieval in 0.01M sodium citrate (pH 6.0). Endogenous peroxidase activity was quenched with 3 % H₂O₂ for 10 min at room temperature, followed by two washes in TBS containing 0.6 % Tween 20 and a final wash in TBS (mM; 5 Trisma Base, 300 NaCl pH 7.6). Non-specific binding was prevented by incubation for 30 min at room temperature with TBS containing non-immune block (10 % goat or swine serum, 2 % human serum, and 0.1 % Tween 20). Primary antibodies (Table 3-4) were optimised and diluted in non-immune block and incubated with the tissue sections overnight at 4 °C. Negative controls were performed following substitution of the primary antibody with the corresponding concentration of non-immunised IgG. Following wash and removal of the primary antibody with TBS containing 0.6 % Tween 20, biotinylated secondary antibodies (Table 3-4) were diluted in non-immune block and applied for 30 min and the washing procedure repeated. The tissue sections were next incubated for 30 minutes with avidin peroxidase (100 µg/ml in TBS) at room temperature for 30 minutes. Staining was developed using diaminobenzidine (0.75 % solution in TBS) and counterstained with Harris's haematoxylin, dehydrated and then mounted. Initially, immunofluorescence was performed on the isolated SMCs but visualisation difficulties prevented further use of this technique (see Appendix 1). Subsequently, immunocytochemistry was performed on the isolates utilising the above protocol without dewax, rehydration and antigen retrieval steps.

3.1.2.4. Chemicals

General chemicals were purchased from Sigma-Aldrich, Poole, Dorset, UK. Bovine serum albumin (BSA; Cat No; A0281), protease type X (Cat No; P1512), dithiothreitol (DTT; Cat No; D0632), collagenase type F (Cat No; C7926), collagenase type 1A (Cat No; C9891), papain (Cat No; 76218), goat serum (Cat No; G6767), avidin peroxidise (Cat No; A3151), Tween-20 (Cat No; P1379), 3,3′-Diaminobenzidine tetrahydrochloride hydrate (DAB; Cat No; 261890) and Harris’s haematoxylin (Cat No; HHS16) were obtained from Sigma-Aldrich, Poole, Dorset, UK. Swine serum (Cat No; S-4000) was obtained from Vector Laboratories Inc., Burlingame, CA, USA.
<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
<th>Source</th>
<th>Secondary Antibodies</th>
<th>Stock Concentration</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>monoclonal mouse anti-α-smooth muscle actin</td>
<td>6.1 mg/ml</td>
<td>6.1 µg/ml (IF) 15.25 µg/ml (IHC)</td>
<td>Sigma-Aldrich (A2547)</td>
<td>goat anti-mouse IgG FITC</td>
<td>1.1 mg/ml</td>
<td>11 µg/ml</td>
<td>Sigma-Aldrich (F2012)</td>
</tr>
<tr>
<td>monoclonal mouse anti-h-caldesmon</td>
<td>15.4 mg/ml</td>
<td>7.7 µg/ml (IHC)</td>
<td>Sigma-Aldrich (C4562)</td>
<td>Biotinylated goat anti-mouse IgG</td>
<td>0.8 mg/ml</td>
<td>4 µg/ml</td>
<td>DAKO (E0433)</td>
</tr>
<tr>
<td>monoclonal mouse anti-smooth muscle myosin heavy chain 2</td>
<td>Concentration not determined</td>
<td>1:250 (IHC)</td>
<td>Abcam (ab683)</td>
<td>Biotinylated swine anti-rabbit IgG</td>
<td>0.4 mg/ml</td>
<td>2 µg/ml</td>
<td>DAKO (E0431)</td>
</tr>
<tr>
<td>monoclonal mouse anti-non-muscle heavy chain myosin-B</td>
<td>Concentration not determined</td>
<td>1:1000 (IHC)</td>
<td>Abcam (ab684)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>monoclonal mouse anti-CD31</td>
<td>0.5 mg/ml</td>
<td>25 µg/ml</td>
<td>DAKO (M0823)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>polyclonal rabbit anti-Kv1.5</td>
<td>0.2 mg/ml</td>
<td>8 µg/ml (IHC)</td>
<td>Santa Cruz (sc-25681)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>polyclonal rabbit anti-Kv2.1</td>
<td>0.2 mg/ml</td>
<td>10 µg/ml (IHC)</td>
<td>Santa Cruz (sc-28633)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>polyclonal rabbit anti-Kv6.1</td>
<td>0.2 mg/ml</td>
<td>8 µg/ml (IHC)</td>
<td>Santa Cruz (sc-20808)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>polyclonal rabbit anti-BKCa</td>
<td>0.4 mg/ml</td>
<td>10 µg/ml (IHC)</td>
<td>Aломоне Labs (APC-107)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>polyclonal rabbit anti-IKCa</td>
<td>0.2 mg/ml</td>
<td>8 µg/ml (IHC)</td>
<td>Aломоне Labs (APC-064)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3-4: Primary and secondary antibodies used for immunostaining studies.
3.1.3. Results

3.1.3.1. Characterisation of cultured cell phenotype

SMC explant cultures from chorionic plate arteries (n=466 vessel sections) were established from N=33 placentas, and grown in culture for up to 4 weeks. Of the total number of vessels, 248 (53%) were suitable for experiments. In 56 cases (12%), the vessel failed to adhere to the dish and in 77 cases (17%) the vessel explant attached close to the perimeter of the dish. The remaining cultures became infected (85 explants; 18%).

Phase contrast images of fixed SMCs derived from arterial explants were taken following 2 and 4 weeks in culture for N=4 placentas. Cell morphology was assessed close to the vessel explant and in the middle and edge of the outgrowth colonies. Figure 3-1 illustrates representative phase contrast images of SMC outgrowths from vessels isolated from the same placenta at the two culture time points.

Cell morphology was not homogenous in all explant outgrowths; displaying both long, spindly (Figure 3-1A, D, E) and large, flat cells (Figure 3-1B, C) each with varying growth rates. Morphology also differed in the same dish of cells in the vicinity of the vessel explant. As illustrated in Figure 3-1A, cell density is high on one side of the explant displaying the typical “hill and valley” orientation. In contrast, the other side of the same explant (Figure 3-1B), has reduced cell number with larger, flatter cells. A similar pattern was observed at 4 weeks in culture; however, as illustrated in Figure 3-1E and F, cell growth can even be absent on the adjacent side of a confluent vessel explant. In some cultures, highly confluent cells displayed growth directed towards a central point, forming a raised mound (Figure 3-1E).
Figure 3-1: Phase contrast images of arterial smooth muscle outgrowths close to the vessel explant with time in culture. (A-F) Representative examples of SMCs derived from chorionic plate arteries taken from one placenta following 2 and 4 weeks in culture. x200 magnification.

E = vessel explant, F = large flat cells, H&V = hill and valley, M = mound, S = long spindly cells
Cells located away from the vessel explant also displayed various morphologies with some similarities apparent between cultures. Monolayer sheets of SMCs forming the “hill and valley” growth pattern were evident in highly confluent cultures, similar to those situated close to the explant (Figure 3-2A, F). These long spindly cells were densely packed into organised, parallel sheets. When growth of two adjacent sheets was directed towards each other, ridges were observed (Figure 3-2A). The mound formation described above also occurred in the confluent monolayer sheets away from the vessel explant. When left in culture longer, the outgrowths from these mound structures aligned to form ring structures (Figure 3-2F). In some instances, single flatter cells were present in the centre of this ring.

Cell morphology and density differed within the same culture (Figure 3-2A, B and 3.2E, F). Cells located at the edge of the outgrowth colonies differed in appearance compared to other SMCs present in the culture. In general, these cells were flatter cobblestone shaped with numerous processes, contained more cytoplasmic material and could be multinucleate in some instances (Figure 3-2B, C, D). These patterns were observed at both 2 and 4 weeks in culture.
Figure 3-2: Phase contrast images of arterial smooth muscle cells in the centre and edge of the outgrowth colonies with time in culture. (A-F) Representative examples of SMCs derived from chorionic plate arteries taken from one placenta following 2 and 4 weeks in culture. x200 magnification.

C = cobblestone shaped cells, F = large flat cells, H&V = hill and valley, M = mound, Mu = multinucleated cell, P = processes, Rd = ridge, Rg = ring.
Characterisation of the SM cultures utilised markers of SMC origin and phenotype. Antibodies directed towards the generic SMC marker \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) demonstrated intense staining in arterial outgrowths from \(N=5\) placentas at various time points between 2 and 4 weeks in culture. Figure 3-3 illustrates immunofluorescent images and the corresponding phase contrast image from cells originating from the same artery at both 2 and 4 weeks. Staining was observed in both long, spindly sheets of cells, and cobblestone shaped cells with processes. Staining was throughout the cytoplasm in a filamentous pattern.

Assessing the use of more specific phenotypic marker proteins in the SM cultures was not performed due to difficulties resolving positive staining from non-specific background fluorescence (see Appendix 1). This, coupled with the difficulties assessing \(K^+\) channel function with electrophysiology (see Appendix 2), prevented further characterisation of this model's phenotype.
Figure 3-3: Immunofluorescence staining of arterial cells utilising an antibody to α-smooth muscle actin. (A, B) Representative examples of immunofluorescent staining for nuclei (blue) and α-SMA (green) in cells derived from the same vessel at 2 and 4 weeks in culture and the corresponding phase contrast image. (C.) Negative control: goat anti-mouse IgG FITC with omission of α-SMA primary antibody. Magnifications x100 or x400 as indicated.

C= cobblestone shaped cells, S= long spindly cells
3.1.3.2. Characterisation of acutely isolated cell phenotype

Freshly isolated SMCs were imaged (phase contrast) following enzyme dissociation of chorionic plate arteries. Most cell suspensions contained relaxed viable cells which were oval shaped with clear defined membranes (Figure 3-4A). Other stellate shaped cells were present in the cell suspension (Figure 3-4B). Some cells displayed membrane blebbing and over digested cells were identified as having a poorly defined membrane (Figure 3-4B).

Figure 3-4: Phase contrast images of freshly isolated arterial smooth muscle cells. SMCs enzymatically dissociated from intact vessels are oval shaped when in the relaxed configuration but other stellate shaped cells are present. x400 magnification.

B= blebbing membrane, D= over digested, O= oval shaped, St= stellate
Isolated cells were smooth muscle evident with positive immunostaining for SMC markers (N=5 placentas; Figure 3-5A, C-E) and the absence of staining for the endothelial cell marker CD31 (N=3; Figure 3-5B). All SMCs stained positive for the SMC markers α-smooth muscle actin (α-SMA; N=4; Figure 3-5A) and myosin heavy chain-2 (MHC-2; N=3; Figure 3-5C). Staining for the contractile and synthetic SMC phenotypic markers h-caldesmon (N=3; Figure 3-5D) and non-muscle myosin heavy chain-B (NMMHC-B; N=3; Figure 3-5E) respectively, was variable. Cells displayed both positive and negative staining for MHC-2 and NMMHC-B, with a spectrum of staining intensities between these extremities apparent in the same field of vision.

Confirmation that the isolation contained SMCs with characteristics of both contractile and synthetic cells, reminiscent of the native SM of chorionic plate arteries, was apparent following positive immunostaining in the intact chorionic plate arterial sections for α-SMA (N=4; Figure 3-6A), MHC-2 (N=4; Figure 3-6B), h-caldesmon (N=4; Figure 3-6C) and NMMHC-B (N=4; Figure 3-6D). Substitution of primary antibody with non-immune IgG at an equivalent concentration did not produce non-specific staining in either the isolated SMCs (N=4; Figure 3-5E) or chorionic plate arterial sections (N=4; Figure 3-6F).
Figure 3-5: Phenotype characterisation of acutely isolated chorionic plate arterial SMCs. Representative examples of immunocytochemistry (A) α-smooth muscle actin (α-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).
Figure 3-6: Phenotype characterisation of chorionic plate arterial sections. Representative examples of immunohistochemistry (A) α-smooth muscle actin (α-SMA), (B) myosin-heavy chain-2 (MHC-2) (C) h-caldesmon, (D) non-muscle myosin heavy chain-B (NMMHC-B), (E) CD31 and (F) negative control; non-immune IgG. Positive immunostaining (DAB; brown) and nuclei (haematoxylin; blue). SMC; smooth muscle cell, L; lumen.
3.1.3.3. K⁺ channel protein expression

K⁺ channel expression was not explored in the SMC cultures as they were difficult to study by patch clamp electrophysiology (see Appendix 2). Therefore, activity and expression of channels in cultured SMCs could not be matched.

Expression of K⁺ channels was assessed in the acutely isolated chorionic plate arterial cells using immunocytochemistry. Representative examples of K⁺ channels typically localised to contractile (Kᵥ1.5; N=3; Figure 3-7A, Kₐ₅P; N=4; Figure 3-7B and BKₖ; N=3; Figure 3-7C) and synthetic SMCs (IKₖ; N=3; Figure 3-7D) were expressed in the isolated SMCs.

Confirmation that the isolation procedure did not affect expression of K⁺ channels was apparent following positive immunostaining in the intact chorionic plate arterial sections for Kᵥ1.5 (N=4; Figure 3-8B), Kₐ₅P (N=4; Figure 3-8C), BKₖ (N=4; Figure 3-8D) and IKₖ (N=4; Figure 3-8E). Substitution of primary antibody with non-immune IgG at an equivalent concentration did not produce non-specific staining in either isolated SMCs (N=4; Figure 3-7E) or chorionic plate arterial sections (N=4; Figure 3-8F).
Figure 3-7: K⁺ channel expression in acutely isolated SMCs. Representative examples of immunocytochemistry (A) Kv1.5, (B) K$_{ATP}$, (C) BK$_{Ca}$, (D) IK$_{Ca}$, and (E) negative control; non-immune IgG. Positive immunostaining (DAB; brown) and nuclei (haematoxylin; blue).
Figure 3-8: K⁺ channel expression in chorionic plate arterial sections. Representative examples of immunohistochemistry (A) α-smooth muscle actin, (B) Kv1.5, (C) KᵥATP, (D) BKCa, (E) IKCa and (F) negative control; non-immune IgG. Positive immunostaining (DAB; brown) and nuclei (haematoxylin; blue). SMC; smooth muscle cell, L; lumen.
3.1.4. Discussion

In the current study, two in vitro models of SMCs isolated from chorionic plate arteries were established to study K⁺ channel expression and function; (1) a model of SMCs in culture and (2) an acutely isolated SMC model. The model of SMCs in culture was adopted from the smooth muscle explant method described by Leik et al., (2004) in chorionic plate arteries. However, these cells proved unsuitable for electrophysiology experiments to assess K⁺ channel function (see Appendix 2) and were not extensively characterised. The acutely isolated SMC model was developed following extensive optimisation from numerous animal SMC isolation protocols and can be used to assess K⁺ channel expression/ function and cellular phenotype.

3.1.4.1. Characterisation of SMC culture model

Cellular outgrowths from chorionic plate arterial explants were characterised using morphological and immunocytochemical techniques. Phase contrast images of the cultured cells indicate the presence of SMCs with a contractile (Figure 3-1A) and synthetic phenotype (Figure 3-1B). Contractile SMCs are typically elongated, spindle shaped and when confluency is reached align to form the “hill and valley” orientation (Rensen et al., 2007). Synthetic SMCs are larger, flatter and typically cobblestone shaped, containing an abundance of rough endoplasmic reticulum and golgi apparatus in the cytoplasm (Owens, 1995; Rensen et al., 2007). Some of the synthetic cells in the cultures were multinucleated (Figure 3.2B and 3.4B) which may indicate that at the point of fixation, they were undergoing mitosis.

Confluency was influential in determining the morphology of the cell outgrowths. In general, highly confluent cells were long and spindly, and aligned to form parallel sheets in the “hill and valley” orientation (Figure 3-1A). When cell growth and number was reduced, the cells were flatter, less densely packed and lost their polarised orientation, resulting in disorganised growth patterns (Figure 3-1B). Sufficient cell-cell interactions between highly confluent SMCs may help retain an organised contractile morphology (Sobue et al., 1999). Loss of these interactions when growth was reduced may explain why the cells were flatter and grew in a disorganised manner. Why cell growth was heterogeneous between cultures isolated from the same vessel is not known. However, adequate adhesion of the vessel explant to the culture dish may
contribute, as is was apparent in 12 % of cultures where the vessel did not adhere at all, and in 17 % of cultures where the vessel adhered to the perimeter of the dish.

Ridges, ring and mound structures were observed in the SMC outgrowths. Ridges appeared to result from the collision of two adjacent sheets of SMCs forming a raised surface along the point of impact. These structures were present in most confluent cultures (Figure 3-2A). The formation of ring-like, microvascular structures has been previously observed in bovine and human capillary endothelial cultures isolated from the adrenal gland (Folkman & Haudenschild, 1980; Chrobak et al., 2006). One study has shown that culturing rat aortic SMCs via the explant method resulted in ring formation 24 to 48 hours following reseeding (Bonanno & Nicosia, 1992). In the current study where immunostaining confirmed that the isolated cells were SM, ring-like structures were observed at both 2 and 4 weeks in culture (Figure 3-2F). The presence of these structures may suggest that cultured SMCs are able to form microvascular structures in the absence of an influence from the endothelium. However, ring formation in cultured SMCs is poorly documented and therefore its significance remains unclear.

In confluent arterial SMCs following 2 and 4 weeks in culture, mound structures were present (Figure 3-1E and Figure 3-2F). Visually, it appeared that these structures arose when SMC growth was directed towards a central point resulting in a raised surface. These structures have been likened to atherosclerotic plaque-like mounds when observed in various cultured SMCs (Gimbrone & Cotran, 1975; May et al., 1975). The central core of the mound structure is believed to contain cellular debris and extracellular material covered by a cap of mature and modified SMCs which have incorporated lipid (May et al., 1975; Chamley-Campbell et al., 1979). In the present study, these formations were commonly found at the perimeter of a ring structure. Therefore, these mound structures may have incorporated cellular material, resulting in a clearance from the confluent SMC sheet. The remaining cells are then unable to grow over this area and re-align forming a ring structure. Interestingly, mound structures were observed in bovine aortic cultures following incubation in hypoxic conditions but not when cultured under normoxia (May et al., 1975). In common with the current study, similar structures were observed in SMCs isolated from human umbilical veins under normoxic conditions (Gimbrone & Cotran, 1975). The reasoning behind this discrepancy is unknown; however, the environment that the cells are exposed to in vivo, in particular the level of oxygenation and whether the vessel studied is an artery or vein, may be influential. Alternatively, the disparity between pO₂ and
mound formation could reflect possible inter-species differences in the behaviour of SMCs in culture.

Cellular outgrowths from the chorionic plate arterial explants were confirmed as SM due to the presence of positive immunofluorescence staining with α-SMA in long straight fibrils located throughout the cytoplasm (Figure 3-3). This is in common with Leik et al., (2004) who demonstrated α-SMA expression in chorionic plate arterial outgrowths by Western blotting and immunofluorescence. Staining was present both at 2 and 4 weeks in culture indicating that SMC origin is preserved throughout multiple cellular divisions. α-SMA is the most abundant protein in SMCs and is a component of the contractile machinery important in the development of force within the cell (Owens et al., 2004). It is the first known protein expressed during development of a differentiated SMC (Sobue et al., 1999; Owens et al., 2004). The primary objective of this study was to develop a model that could be utilised to study the long term effect of exogenous regulators on K⁺ channel expression and function using patch clamp electrophysiology. As the SMC outgrowths were unsuitable for this technique (see Appendix 2), the K⁺ channel expression and cellular phenotype were not explored further.

In summary, explant cultures derived from chorionic plate arteries using the method described in section 3.1.2.1 are smooth muscle as they display positive immunostaining for α-SMA. The cultures contain both long, spindly cells, and large, flat cobblestone shaped cells implicating the presence of SMCs with both contractile and synthetic phenotypes respectively. However, due to the large flat morphology of the single cultured cells, establishment of a high resistance seal was difficult. Therefore, this in vitro model of cultured chorionic plate arterial SMCs may not be suitable for the routine exploration of K⁺ channel function using whole-cell electrophysiology methods.

3.1.4.2. Acutely isolated cell model characterisation

The morphology of acutely isolated chorionic plate arterial SMCs using the final protocol described in section 3.1.2.2 was generally long and oval shaped (Figure 3-4). This is typical of many relaxed arterial SMCs isolated from other species (Clapp & Gurney, 1991; Jackson et al., 1997; Ng et al., 2008; Cairrao et al., 2009).
Isolation of viable cells that adhered to a substrate sufficiently for immunostaining and electrophysiology experiments proved problematic. In most isolations, a high proportion of cells had poorly defined membranes and only 10-20% of the total yield adhered. Modifying SMC isolation protocols described in human placental arteries and animal blood vessels, and utilising a variety of different substrates were measures that proved unsuccessful in obtaining a high yield of adherent, viable cells from chorionic plate arteries. The optimal protocol for isolating chorionic plate arterial SMCs was originally described in mouse myometrium and utilised a low Ca²⁺-dissociation media with increased enzyme concentrations, incubation temperatures and durations (Greenwood et al., 2009). These conditions appeared to permit adequate digestion of the fibrous connective tissue surrounding chorionic plate arteries, which liberated relaxed SMCs. However, even under these “optimal” conditions, some isolations produced a poor yield of cells that did not adhere for subsequent experiments. This was accepted as the innate biological variability which may be exacerbated by factors inherent to the collection of human placental tissue such as time from delivery to lab bench and mode of delivery.

Adhesion and fixation of chorionic plate arterial SMCs permitted characterisation of the isolates with immunocytochemistry. Initial characterisation confirmed the isolates were SM, demonstrated by positive immunostaining of the SMC marker α-SMA (Figure 3-5A) and the absence of staining for the endothelial cell marker CD31 (Figure 3-5B). Additional use of other SMC markers such as myosin heavy chain-2 (MHC-2), h-caldesmon and non-muscle myosin heavy chain-B (NMMHC-B) enabled further confirmation that the isolates were SM and permitted identification of the SMC phenotype.

As described in section 3.1.1, SMCs can have a range of different phenotypes which determines their tissue localisation, function and ion channel expression profiles. In an attempt to further elucidate the phenotype of the acutely isolated chorionic plate arterial SMCs, the use of antibodies directed against proteins specific to contractile and synthetic phenotypes were used. Myosin heavy chains (MHC), which are essential components of the contractile apparatus, is documented to show the highest specificity to SMCs with a contractile phenotype (Owens, 1995). Two isoforms are present within vascular SMCs, MHC-1 and MHC-2, whose expression are differentially regulated during development (Kuro-o et al., 1989). MHC-1 is exclusively expressed in fetal SMCs during early gestational development (Aikawa et al., 1993). Conversely, MHC-2 expression is significantly up regulated during late fetal and postnatal development. Positive immunostaining for MHC-2 was present in the isolated chorionic plate arterial
SMCs (Figure 3-5C). Immunostaining was performed on the isolated SMCs for the protein h-caldesmon which is expressed in SMCs with a contractile and synthetic phenotype (Owens, 1995; Rensen et al., 2007). H-caldesmon is a component of the contractile machinery that binds to- and inhibits actin’s ability to stimulate myosin, therefore preventing contraction. Binding of the Ca$^{2+}$-calmodulin complex to caldesmon relieves this inhibition and promotes contraction (Huber, 1997). In common with Leik et al., (2004) who showed h-caldesmon staining by Western blotting in cultured chorionic plate arterial SMCs, positive staining for h-caldesmon was present in the acutely isolated SMCs (Figure 3-5D). However, the staining intensity was variable between the isolates, with some cells displaying strong staining, and others displaying negative staining. A similar staining pattern was observed for the synthetic SMC marker NMMHC-B (Figure 3-5E). Vascular SMCs express two variants of NMMHCs (Sobue et al., 1999). NMMHC-B is predominantly expressed in non-muscle cells, developing fetal vascular SMCs and cultured synthetic SMCs (Kuro-o et al., 1991; Arens et al., 1998).

The presence of variable staining intensities for NMMHC-B in the isolates suggests that some cells may display a greater synthetic phenotype than others, thereby explaining why h-caldesmon expression shows a similar staining pattern. The observed expression of MHC-2, h-caldesmon and NMMHC-B suggests that chorionic plate arterial SMCs have a mixed phenotype with both contractile and synthetic characteristics.

Confirmation that the isolation protocol did not alter the SMC phenotype and was maintained in situ, was evident with positive staining for α-SMA (Figure 3-6A), h-caldesmon (Figure 3-6B), MHC-2 (Figure 3-6C) and NMMHC-B (Figure 3-6D) in serial sections of intact chorionic plate arteries. These observations of both muscle (MHC-2) and non-muscle (NMMHC-B) myosin variants in the placental vasculature is in common with a recent study which assessed the expression of numerous myosin isoforms in the placenta (Matsumura et al., 2011). This study concluded that the human placenta predominantly expresses non-muscle myosin variants including NMMHC-B, a marker of fetal vascular SMCs. Expression of both MHC-2 and NMMHC-B myosin isoforms were localised to the fetoplacental blood vessels and extravascular stromal cells (Matsumura et al., 2011). A similar staining pattern for these SMC marker proteins were observed in the current study in chorionic plate arterial sections (Figure 3-6).

The results from the current study confirmed previous observations in the chorionic plate artery in situ (Sweeney et al., 2006), that SMCs isolated from these vessels are a mixed population with both contractile and synthetic characteristics. As the placenta is derived from the fetus during embryonic development, this may explain why
fetoplacental blood vessels retain a fetal/synthetic phenotype at term evident by continued NMMHC-B expression. The presence of SMCs with a contractile and synthetic phenotype may have important functional implications, allowing SMCs to have a dual role in normal pregnancy to control both vascular resistance and vascular growth during vasculogenesis. This in turn will impact upon the K⁺ channel expression and functional profiles in these cells.

3.1.4.3. K⁺ channel expression in chorionic plate arterial SMCs

K⁺ channels control many aspects of SMC physiology including vasoconstriction, vasodilation and proliferation (Neylon, 2002; Ko et al., 2008). As SMC phenotype is closely related to these different functions, the phenotype will also influence the K⁺ channel expression profiles in these cells. As described in section 3.1.1, SMCs with a contractile phenotype predominantly express the voltage-gated (Kᵥ) family and large-conductance Ca²⁺-activated (BKᵥ) K⁺ channels, whereas SMCs with a synthetic phenotype predominantly express the Ca²⁺-activated K⁺ channel isoform IKᵥCa (Scornik & Toro, 1992; Bolotina et al., 1994; Archer et al., 1998; Edwards et al., 1998; Neylon et al., 1999; Neylon, 2002; Cogolludo et al., 2003; Jackson, 2005; Ko et al., 2008; Mackie et al., 2008; Joshi et al., 2009; Rainbow et al., 2009; Li et al., 2010). The expression of representative examples of K⁺ channels localised to SMCs with a contractile and synthetic phenotype were assessed in the chorionic plate arterial isolates. The K⁺ channels isoforms Kᵥ1.5, KᵢR₆.1 (Kارة ATP) and BKᵥCa are well documented to control excitation-contraction coupling in the systemic and pulmonary vasculatures (Jackson, 2000; Ko et al., 2008). Expression of these isoforms have been demonstrated by PCR and/or Western blot in the placenta, and pharmacological manipulation of Kᵥ, KᵢR and BKᵥCa channels modulates vascular reactivity in the perfused placental cotyledon and isolated chorionic plate arteries (Hampel et al., 2002; Bisseling et al., 2005; Sand et al., 2006; Wareing et al., 2006a; Wareing et al., 2006c; Wareing et al., 2006e; Jewsbury et al., 2007; Corcoran et al., 2008). However, expression of these channels has not been localised to SMCs isolated from chorionic plate arteries. The current study provides the first evidence that Kᵥ1.5 (Figure 3-7A), KᵢR (Figure 3-7B) and BKᵥCa channels (Figure 3-7C) are expressed in chorionic plate arterial SMCs where they have the potential to mediate excitation-contraction coupling. IKᵥCa channels, which are important in mediating proliferation and maintaining the resting membrane potential in SMCs with a synthetic phenotype, were also expressed in the acutely isolated chorionic plate arterial SMCs (Figure 3-7D). Although originally cloned from the human placenta (Ishii et al., 2009).
IK_{Ca} channels have not been localised to the fetoplacental vasculature. The previous study (section 3.2) is the first to demonstrate IK_{Ca} mRNA expression in chorionic plate arteries which supports the data in this study showing localisation of IK_{Ca} protein to the SMCs. Expression of Kv1.5, K\textsubscript{ATP}, BK\textsubscript{Ca} and IK\textsubscript{Ca} channel protein is evident in the SMCs \textit{in situ} and therefore not altered by the isolation procedure (Figure 3-8).

To summarise, chorionic plate arterial SMCs express Kv1.5, K\textsubscript{ATP}, BK\textsubscript{Ca} and IK\textsubscript{Ca} protein. Assessment of the isolated SMC phenotype supports previous observations that these cells have both contractile and synthetic characteristics \textit{in situ}. This mixed phenotype may account for the expression of diverse K\textsuperscript{+} channel isoforms which reside in SMCs with different phenotypes. Definitive proof that K\textsuperscript{+} channel expression correlates with phenotype would necessitate dual staining for channel protein and SMC phenotypic markers. Further studies are required to determine whether the specific K\textsuperscript{+} channel isoforms underlie the physiological functions of contractile and synthetic SMCs. However, initial patch clamp studies are needed to confirm functional expression of these K\textsuperscript{+} channels in isolated chorionic plate arterial SMCs. If these initial findings in the current study are substantiated in future experiments, this mixed phenotype, and the associated K\textsuperscript{+} channels, may represent a dual function of fetoplacental SMCs to control both vascular resistance and promote vasculogenesis during pregnancy.
3.2. mRNA EXPRESSION OF K⁺ CHANNELS IN CHORIONIC PLATE ARTERIES

3.2.1. Introduction

The expression profile of K⁺ channels in the fetoplacental vasculature is poorly understood. The previous study (see section 3.1) is the first to demonstrate protein expression of Kv1.5, BKCa, KATP, and IKCa channels to intact chorionic plate arteries and the isolated SMCs. The current study aimed to consolidate these findings and tested the hypothesis that mRNA for these K⁺ channels is expressed in the intact chorionic plate artery. Furthermore, the expression of these isoforms was assessed using quantitative PCR (qPCR) in vessels cultured for 48 h under conditions mimicking placental normoxia given the previous observations that K⁺ channel mRNA expression is altered in culture (Dreja et al., 2001; Bergdahl et al., 2005; Manoury et al., 2009; Tai et al., 2009).

3.2.2. Materials and Methods

3.2.2.1. Vessel Preparation

Control placentas (N=10) were obtained following vaginal delivery or elective caesarean section at term (37-42 weeks gestation) from women with uncomplicated pregnancies (no evidence of hypertension, fetal growth restriction or other medical disorders). Within 30 minutes of delivery of the placenta, the umbilical artery was located and traced along the chorionic plate to the perimeter where “resistance” chorionic plate arteries (150-500 μm internal diameter) are localised. Small sections of the chorionic plate containing these vessels were cut away from the placenta and underlying villous tissue and placed in ice-cold physiological salt solution (PSS- mM; 119 NaCl, 25 NaHCO₃, 4.69 KCl, 2.4 MgSO₄, 1.6 CaCl₂, 1.18 KH₂PO₄, 6.05 glucose, 0.034 EDTA; pH 7.4). Small chorionic plate arteries (approximately 3cm lengths; diameter <500 μm) were dissected away from the surrounding connective tissue, opened longitudinally and cut into 2- to 3-mm lengths. Vessels isolated from a single placenta were separated into two groups; (1) 5-6 vessel segments snap frozen immediately in liquid nitrogen following isolation and stored at -80 °C, and (2) 5-6 vessel segments placed into culture at 6 % pO₂ (5 % CO₂ in 5 % O₂) and snap frozen following 48 h culture. For tissue culture experiments, vessels were placed in a P35
culture dish (Nunc; Thermo Fisher Scientific Inc) containing 2 ml culture media (DMEM containing 4.5 g/l glucose, 10 % fetal calf serum, 1 % PSG; penicillin, streptomycin, glutamine, Sigma-Aldrich) and maintained in a humidified incubator (37°C) at 6 % pO$_2$ (5 % CO$_2$ in 5 % O$_2$) for 48 h.

3.2.2.2. RNA extraction

RNA was extracted from chorionic plate arteries using the RNeasy® Fibrous Tissue Mini Kit according to the manufacturer’s instructions (QIAGEN®, Crawley, UK). Each tissue sample was homogenised with 3 μl β-Mercaptoethanol in 300 ml Buffer RLT using a pellet pestles cordless homogeniser (Sigma-Aldrich). Once the vessels were homogenised the lysate was transferred to a new tube containing 590 μl RNase-free water and 10 μl proteinase K. This mixture was then incubated at 55°C for 10 minutes and centrifuged for 3 minutes at 10,000g in a Denville 260D microcentrifuge (Scientific Inc., location). The supernatant was transferred to a new tube containing 450 μl of 100 % ethanol and applied to an RNeasy® Mini spin column placed in a 2 ml collection tube and centrifuged at 20-25°C for 30 seconds at 13,000 g. The flow-through was discarded. To the RNeasy® spin column, 350 μl of Buffer RW1 was added and centrifuged for 30 seconds at 13,000 g. Following disposal of the flow-through, 10 μl of DNase I in 70 μl Buffer RDD was added to the spin column and incubated at room temperature for 15 minutes to remove contaminating DNA. Once this time had elapsed, 350 μl of Buffer RW1 was added and centrifuged for 30 seconds at 13,000 g. Again, once flow-through was discarded, 500 μl of Buffer RPE was added and centrifuged at 20-25°C for 30 seconds at 13,000 g. The Buffer RPE wash step was then repeated but centrifugation lasted 2 minutes. The final step in the extraction process required elution of the RNA in 30 μl RNase-free water by centrifugation for 1 minute at 13,000 g. This procedure was performed on each sample of chorionic plate arteries; no pooling of tissue was performed.

3.2.2.3. Spectrophotometry

Spectrophotometry was performed to provide an estimation of the concentration and purity of extracted RNA. RNA samples were diluted 1:25 in RNase-free water and transferred to an Eppendorf UVette®. The sample was then placed into an Eppendorf Biospectrophotometer (Eppendorf, Cambridge, UK) and the absorbance of the diluted
RNA measured at 230 nm, 260 nm, and 280 nm wavelengths. The 260 nm wavelength measurement corresponds to the concentration of RNA whereby a wavelength of 1 equates to an RNA concentration of 40 μg/ml. The 280 nm wavelength measures protein contamination. The 260/280 ratio is used to assess RNA purity, with a ratio of 1.8-2.0 indicative of highly pure RNA. The 230 nm wavelength measures ethanol and phenol impurities from the extraction process and the 230/280 ratio should be less than 1. From the spectrophotometry process, the calculated concentration of RNA for the chorionic plate arteries ranged from 5 – 191 ng/μl.

3.2.2.4. Ribogreen Assay

The RNA from each sample was quantified using a Quant-iT™ Ribogreen® RNA assay kit (Molecular Probes, Invitrogen). A standard curve was constructed using rRNA diluted in Tris-EDTA (TE) buffer with a range of 0-1000 ng/ml. The stock rRNA (100 μg/μl) was diluted 1:100 to obtain 1000 ng/ml and then doubling dilutions performed to obtain standards of 500, 125, 62.5, 31.25, 15.63 ng/ml. TE buffer was used as a blank. The Ribogreen assay for all standard and samples were performed in duplicate. RNA samples were diluted to ensure fit within the standard curve using the values obtained from the spectrophotometer. Samples with a spectrophotometry concentration >40 ng/μl were diluted 1:250 and samples with a concentration <40 ng/μl were diluted 1:100 in RNase-free water. 100 μl of each standard and sample was pipetted in duplicate into wells of a sterile 96 well plate (Corning, NY, USA). To each well 100 μl of Ribogreen reagent was added (diluted 1:500 in TE buffer). Following gentle mixing, the 96 well plate was placed in a fluorescent plate reader (Spectra Max Gemini XS, Molecular Devices, Wokingham, UK) and the samples excited at 490 nm and fluorescence emission read at 560 nm. A standard curve was constructed using GraphPad Prism package (version 5.01; GraphPad software, San Diego, CA, USA) and sample RNA concentrations determined by extrapolation from the standard curve.

3.2.2.5. Reverse transcription

Reverse transcription converted the extracted RNA to cDNA using a Stratagene Affinity Script Multi-temperature cDNA synthesis kit (Agilent, Stockport, UK). A master mix of reagents was prepared which contained Affinity Script RT buffer, 25 mM of dNTP (dATP, dCTP, dGTP, dTTP), RNase Block RNase inhibitor and 1 μl AffinityScript
RTase. For each sample, 25 ng of RNA and 0.3 μg of random hexameric primers were combined and incubated at 65 °C for 5 minutes. 4.3 μl of the master mix was added to each sample and performed in duplicate to ensure the efficiency of the reaction. This mixture was incubated at numerous temperatures: (1) 25°C for 10 minutes, (2) 42°C for 60 minutes and (3) 70 °C for 15 minutes. A PCR reaction examining expression of the housekeeping gene (YWHAZ) was performed on all the duplicate samples to confirm the presence of cDNA. The duplicate samples were subsequently pooled for use in the qPCR reactions as standards. 1μg of reference RNA and placental reference RNA were included and reverse transcribed. All cDNA samples were stored at -20 °C.

3.2.2.6. Quantitative Real Time Polymerase Chain Reaction (qPCR)

Quantitative PCR (qPCR) was the method of choice for this study to firstly determine whether mRNA for various K+ channels were expressed in chorionic plate arteries, and secondly whether their expression altered following culture for 48 h under normoxic conditions. qPCR enables the real-time monitoring of the amount of DNA generated during the exponential phase of the amplification phase of each PCR reaction. Primarily two methods are utilised to measure DNA amplification using fluorescence dyes: (1) double-stranded DNA binding dyes, (2) fluorescence reporter probes. Double-stranded DNA (ds-DNA) binding dyes, such as SYBR green, binds to all ds-DNA which will include the amplified PCR product of choice and other non-specific products (Bustin, 2000). Fluorescence reporter probes are designed to bind to a specific sequence in the mid-portion of the amplicon, which is advantageous when the primers are not specific (Bustin, 2002). SYBR green was selected for this project as it is cost-effective and the primers were specific for the genes of interest as determined by BLAST searches performed on the NCBI website. Moreover, following optimisation of the amplification conditions, all PCRs produced a single amplicon of the predicted length providing reassurance of their specificity.

Two methods are commonly used to quantify the amount of DNA detected using the fluorescence methods described above: (1) relative and (2) absolute. Relative quantification involves construction of a cDNA standard curve using RNA derived from a cell line or tissue which abundantly expresses the gene of interest. The cDNA is serially diluted and subjected to PCR amplification alongside the samples and a standard curve is constructed by plotting fluorescence against Ct value. The relative expression of the target gene between treatments or conditions is then extrapolated
from the standard curve (Bustin, 2000). Absolute quantification requires the exact concentration of a standard to be determined by performing PCR on the cDNA standard, followed by purification of the DNA by gel electrophoresis, band excision and gel purification. The concentration of the resultant purified DNA standard is then measured by spectrophotometry. This standard is then serially diluted and amplified as above, but with an exact number concentration range utilised. This enables calculation of the exact concentration of the sample target DNA by extrapolating to the standard curve (Bustin, 2000, 2002). This method is used when it is important to assess the exact concentration of the K⁺ channels in the different conditions. As the primary aim of this study was to determine whether K⁺ channels were expressed in chorionic plate arteries and whether their expression level was altered by culture, then it was acceptable to examine the relative expression levels.

Quantitative PCR analysis was performed for Kv1.5, BKCa, Kir6.1, SKCa2, SKCa3 and IKCa channels using primers either previously used in the laboratory or obtained from PrimerBank and BLAST searched for specificity (Table 3-5). Each primer set was optimised under different annealing temperatures to ensure a single product was amplified and a single band of an agarose gel. Further confirmation of the specificity of the primers would necessitate sequencing of the PCR products, which was not routinely performed as the dissociation curve for each primer set contained a single peak, a single band of the correct size was obtained on a gel, and the BLAST search did not reveal cross-contamination with other non-specific gene products.

Expression of Kv1.5, BKCa, Kir6.1, SKCa2, SKCa3 and IKCa channels was compared in chorionic plate arteries freshly isolated from the placenta with vessels isolated from the same placenta but placed in culture for 48 h at 6 % O₂. A standard curve of reference cDNA (see Table 3-5 for details for each primer set) was made by serial dilutions (x-5). All the cDNA samples and standards were diluted 1:10 in RNase and DNase-free water from the original stock. A PCR master mix was prepared containing; RNase and DNase-free water (1.6 μl), Brilliant II SYBR® Green Master Mix (6.3 μl, Stratagene), 0.25 μM upstream primer (0.3 μl) and 0.25 μM downstream primer (0.3 μl) per reaction. 8.5 μl of this master mix was combined with 4 μl of cDNA in RNase-free PCR strip tubes (Sarstedt, Germany). All PCR reactions were performed in duplicate using the Stratagene MX3000P system (Agilent). Cycling parameters were: 1 cycle at 95 °C for 10 minutes, 40 cycles at 95 °C for 30 seconds (denature), X °C for 1 minute (X denotes the annealing temperature specific for each primer set; see Table 3-5) and 72 °C for 1 minute (extension). Fluorescence measurements were calculated after each anneal and extension step and each sample extrapolated off the standard curve to obtain the
relative expression of each channel. The standard curves for each PCR experiment had efficiencies of between 80 % and 120 % which allowed comparisons of the relative abundance of the K\(^+\) channels using Ct values, thus comparing the number of cycles required to detect the channel amplicon at the level of the threshold (set at the lowest point of the exponential part of the amplification curve). This approach was adopted as it permits a comparison between the relative expression level of different K\(^+\) channels; this would not be possible using the standard curve method given the variable concentrations of each channel in the standard cDNA. A dissociation curve was included in every run to ensure specificity of the amplification and a single peak, representative of a single PCR product, was observed for each primer set (Figure 3-9). K\(^+\) channel expression levels were normalised to the housekeeping gene RPL13 whose expression was unaltered following culture. PCR data were exported to Microsoft Excel and the expression level of each K\(^+\) channel extrapolated from the standard curve.

The PCR products were resolved by 2 % agarose gel electrophoresis to confirm that the single PCR product was the amplicon of interest. The gel was prepared by dissolving 3 g agarose (Cat No: A9539, Sigma) in 150 ml 1xTAE buffer (50x stock TAE buffer; pH; 8.3) and boiling for 3-4 minutes. Following addition of 5 μl / 100 ml ethidium bromide, the gel was poured into a tank and left to set. 5 μl of 100bp ladder was added in one lane and 5 μl of xylene cyanol was mixed with each sample and loaded into the subsequent wells. For each gel, products from a chorionic plate artery sample, a corresponding standard and a negative were resolved. The gel was run at 120V from negative to positive for 1 h until the blue marker was half way down the gel. The gel was imaged using Genesnap (Syngene, Cambridge, UK).
<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Sequence</th>
<th>Annealing Temperature (°C)</th>
<th>Amplicon Length (bp)</th>
<th>Standard</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Kv1.5      | FORWARD: GAAAGACGGGCACTCAGA  
| BKCa       | FORWARD: AAGCAACGGGATGGAGGCAT  
| Ks6.1      | FORWARD: CACAGAAGGGCAGAAACTTTGATT  
| SKCa2      | FORWARD: TGGTAGCTGTAGTGCAAGGA  
REVERSE: TGTTCCCTGAGTACATTGGC | 60 | 125 | Human reference RNA | PrimerBank ID: 25777644b1 |
| SKCa3      | FORWARD: GCGCTGATAGCCATGACCTAC  
REVERSE: CGTGCGTCCAGAAGAACCTT | 61 | 106 | Chorionic plate artery reference RNA | PrimerBank ID: 116805329b1 |
| IKCa       | FORWARD: GCTGCTGCCTCTCTACCTCTG  
REVERSE: AAGCGGACTTGATTTGAGAGCG | 61 | 102 | Human reference RNA | PrimerBank ID: 4504859a1 |
| RPL13      | FORWARD: CGAGTGGCTGGAGACCTTACC  
REVERSE: CTTCTCGCCCTGTTCGCAGTAG | 59 | 121 | Human reference RNA | PrimerBank ID: 6912634a1 |

Table 3-5: Primers and experimental conditions used for qPCR.
Figure 3-9: qPCR performed on candidate K⁺ channel. Representative example of (A) dissociation curve (B) amplification plot and (C) standard curve for the BKₖCa channel.
3.2.3. Results

3.2.3.1. K⁺ channel mRNA expression in chorionic plate arteries

Amplicons corresponding to the Kv1.5 (Figure 3-10A), BKCa (Figure 3-10B), Kir6.1 (Figure 3-10C), IKCa (Figure 3-10D) and SKCa3 (Figure 3-10E) genes were amplified in all the chorionic plate arteries freshly isolated from 10 placentas. For SKCa2, no detectable signal was recorded after 40 cycles (no CT value) in any of the freshly isolated chorionic plate arteries. Therefore the band present in Figure 3-10E is obtained from chorionic plate arteries cultured for 48h. Resolving the PCR product on an agarose gel obtained a single band of approximately; Kv1.5 100bp, BKCa 150bp, Kir6.1 100bp, IKCa 100bp, SKCa2 120, and SKCa3 100bp. This is in line with the predicted amplicon lengths of 103 (Kv1.5), 147 (BKCa), 92 (Kir6.1), 102 (IKCa), 125 (SKCa2), and 106 (SKCa3).

Assessment of the relative abundance of the K⁺ channels in freshly isolated chorionic plate arteries was performed through comparison of the cycle threshold (CT) values. These analyses were appropriate as for all primer pairs the amplification efficiency was within the acceptable constraints of between 80 % and 120 %. The comparisons predicted that Kv1.5 channels were the most abundant due to the low CT value, whereas IKCa channels displayed the lowest expression indicated by a high CT value (Figure 3-11). Kv1.5 expression was significantly greater than BKCa (P<0.05), SKCa3 (P<0.01) and IKCa (P<0.001). SKCa3 expression was significantly greater than Kir6.1 (P<0.05). Kir6.1 expression was significantly greater than IKCa (P<0.001; Friedman’s test with Dunn’s multiple comparison post hoc test).
Figure 3-10: K⁺ channel transcripts in chorionic plate arteries. RT-PCR detection of (A) Kv1.5 (B) BKCa (C) Kir6.1 (D) IKCa (E) SKCa3 and (F) SKCa2 transcripts in human reference cDNA (Std), chorionic plate artery (CPA). SKCa2 was not expressed in chorionic plate arteries isolated from 10 placentas; therefore, the band in (D) is from chorionic plate arteries cultured for 48h at 6 % O₂. Negative (Neg) is omission of cDNA. Amplicon lengths; Kv1.5 93, BKCa 147, Kir6.1 92, IKCa 102, SKCa3 106 and SKCa2 125.
Figure 3-11: Relative abundance of K⁺ channels in freshly isolated chorionic plate arteries. Relative abundance of K⁺ channels was assessed using cycle threshold (CT) values. Kv1.5 expression was significantly greater than BKCa (*P<0.05), SKCa3 (**P<0.01) and IKCa (***P<0.001). SKCa3 expression was significantly greater than KIR6.1 (*P<0.05). KIR6.1 expression was significantly greater than IKCa (***P<0.001; Friedman’s test with Dunn’s multiple comparison post hoc test). Horizontal line corresponds to the median with each point representing the number of placentas.
3.2.3.2. Effect of 48h culture on K\textsuperscript{+} channel mRNA expression

Quantitative PCR demonstrated that Kv1.5 (Figure 3-12B; P<0.01; Wilcoxon matched-pairs test) and Kv6.1 (Figure 3-12C; P<0.01) channel mRNA expression was significantly decreased by 48 h culture under 6 % O\textsubscript{2}. BK\textsubscript{Ca} (Figure 3-12D; P<0.01), IK\textsubscript{Ca} (Figure 3-12E; P<0.05) and SK\textsubscript{Ca}2 (Figure 3-12F; P<0.01) mRNA expression was significantly increased following 48 h culture under 6 % O\textsubscript{2} (Wilcoxon matched-pairs test). SK\textsubscript{Ca}2 was not expressed in any of the freshly isolated chorionic plate arteries following 40 cycles. SK\textsubscript{Ca}3 mRNA expression was not significantly altered by culture (Figure 3-12G; P>0.05). K\textsuperscript{+} channel mRNA expression was normalised to the housekeeping gene RPL13 as expression of this PCR product was unaltered by culture (Figure 3-12A).

Assessing the relative abundance of the K\textsuperscript{+} channels in chorionic plate arteries cultured for 48 h at 6 % O\textsubscript{2} was performed through comparison of the cycle threshold (C\textsubscript{T}) values. The comparisons predicted that BK\textsubscript{Ca} and Kv6.1 channels were the most abundant due to their low C\textsubscript{T} value (Figure 3-13). BK\textsubscript{Ca} expression was significantly greater than Kv1.5 (P<0.05) and IK\textsubscript{Ca} (P<0.001; Friedman’s test with Dunn’s multiple comparison post hoc test).
Figure 3-12: Relative change in K⁺ channel mRNA expression in freshly isolated and cultured chorionic plate arteries. K⁺ channel mRNA expression was normalised to the housekeeping gene RPL13 (A) whose expression was not significantly altered by culture (P>0.05). Kv1.5 (B; **P<0.01) and Kv6.1 mRNA expression was significantly decreased by 48 h culture under 6% O₂ (C; **P<0.01). BKCa (D; **P<0.01), IKCa (E; *P<0.05) and SKCa2 (F; **P<0.01) mRNA expression was significantly increased following 48 h culture under 6% O₂. SKCa2 was not expressed in any of the freshly isolated chorionic plate arteries following 40 cycles. SKCa3 mRNA expression was not significantly altered by culture (G; P>0.05; Wilcoxon matched-pairs signed rank test).
Figure 3-13: Relative abundance of K⁺ channels in chorionic plate arteries cultured for 48h at 6 % O₂. Relative abundance of K⁺ channels was assessed using cycle threshold (CT) values. BK⁺Ca expression was significantly greater than Kv1.5 (*P<0.05) and IK⁺Ca (**P<0.001; Friedman’s test with Dunn’s multiple comparison post hoc test). Horizontal line corresponds to the median with each point representing the number of placentas.
3.2.4. Discussion

This study characterised the mRNA expression of 5 K^+ channel isoforms in human placental arteries. Channels that have been previously suggested to be important in the control of placental tone (by their protein expression or by functional assays) were chosen. These included Kv1.5, BK_{Ca}, Kir6.1 (K_{ATP}), IK_{Ca} and SK_{Ca} (see section 3.3) (Hampl et al., 2002; Sand et al., 2006; Wareing et al., 2006a; Wareing et al., 2006e; Jewsbury et al., 2007; Corcoran et al., 2008).

This study is the first to localise Kv1.5 mRNA to chorionic plate resistance arteries (Figure 3-10A). This consolidates previous observation of protein expression of this isoform in placental homogenate (Hampl et al., 2002), intact chorionic plate arteries and isolated chorionic plate arterial SMCs (see section 3.1), and functional Kv1.5 channels in chorionic plate arterial SMCs (section 3.3).

BK_{Ca} channels are expressed at the mRNA level in chorionic plate arteries (Wareing et al., 2006a; Corcoran et al., 2008), which is confirmed in the present study (Figure 3-10B). BK_{Ca} protein is also expressed in placental homogenate (Hampl et al., 2002; Sand et al., 2006), chorionic plate arteries (section 3.1.3.3) (Sand et al., 2006) and SMCs isolated from chorionic plate arteries (section 3.1.3.3). Functional BK_{Ca} channels modulate chorionic plate arterial tone (Hampl et al., 2002; Sand et al., 2006; Wareing et al., 2006a) and is the predominant K^+ current in SMCs isolated from these vessels (section 3.3.3.3).

Kir6.1 (K_{ATP}) channels are well characterised in the fetoplacental vasculature. mRNA for this channel is present in chorionic plate arteries (Figure 3-10C) (Wareing et al., 2006a; Corcoran et al., 2008) and protein has been demonstrated in intact chorionic plate arteries and localised specifically to the SMCs (section 3.1.3.3). K_{ATP} channels also modulate vasoconstriction and vasodilation in chorionic plate arteries (Wareing et al., 2006e; Jewsbury et al., 2007). However, no K_{ATP} currents have been recorded in the isolated SMCs (section 3.3.3) (Hampl et al., 2002) but this may be an artefact of the electrophysiology solutions used in these experiments.

IK_{Ca} channels were originally cloned from the human placenta given its high abundance here compared to other tissues (Ishii et al., 1997; Joiner et al., 1997). Localisation of this channel was thought to be predominantly within the placental epithelial layer given the existence of a charybdotoxin-sensitive and iberiotoxin-insensitive K^+ efflux pathway
in isolated placental trophoblast cells (Clarson et al., 2002). This study is the first to demonstrate protein expression of $\text{IK}_{\text{Ca}}$ to chorionic plate arteries and SMCs isolated from these vessels where they make a significant contribution to whole-cell $K^+$ currents (section 3.3.3.3). Localisation of $\text{IK}_{\text{Ca}}$ to the fetoplacental vasculature is confirmed in the present study whereby mRNA transcripts are amplified in chorionic plate arteries (Figure 3-10D).

An apamin-sensitive $\text{SK}_{\text{Ca}}$ channel has been identified that contributes to whole-cell $K^+$ currents in chorionic plate arterial SMCs (section 3.3.3.3). The precise $\text{SK}_{\text{Ca}}$ isoform responsible for these currents appears to be $\text{SK}_{\text{Ca}}^3$ as $\text{SK}_{\text{Ca}}^2$ transcripts were not amplified following 40 cycles in chorionic plate arteries freshly isolated from 10 placentas (Figure 3-10E, F). $\text{SK}_{\text{Ca}}^3$ channels appear to be the predominant isoform in the vasculature, in particular the endothelium (Edwards et al., 1998; Gutman et al., 2005; McNeish et al., 2006; Garland et al., 2010). It cannot be deduced from these PCR experiments whether the $\text{SK}_{\text{Ca}}^3$ mRNA signal is solely due to expression in the SMCs. Although it is likely that this channel is also expressed in the endothelium, the observation that the endothelium degenerates in chorionic plate arteries following culture and of prominent apamin-sensitive currents in the isolated SMCs (Sampson et al., 2010), suggests that the mRNA signal is likely to be predominantly from the SM. $\text{SK}_{\text{Ca}}^2$ channels are expressed in the nervous system (Stocker & Pedarzani, 2000; Gutman et al., 2005); therefore, it is encouraging that it was not found in chorionic plate arteries.

Chorionic plate artery culture for 48 h under placental normoxia is associated with a non-significant, but potentially biologically significant, reduction in U-46619 constriction. The results from this study suggest that vessel culture is associated with alterations in mRNA expression of several $K^+$ channels which may account for the small non-significant reduction in constriction to U46619 observed following the same conditions of culture (Figure 2-4) The results from this study suggest that vessel culture is associated with alterations in mRNA expression of numerous $K^+$ channels which may account for the reduced constriction. Culture for 48 h under conditions mimicking placental normoxia decreased $K_v 1.5$ mRNA expression (Figure 3-12). This is in accordance with previous studies in pulmonary resistance arteries demonstrating a similar reduction in $K_v 1.5$ mRNA following vessel culture (Manoury et al., 2009). Reduced expression of $K_v 1.5$ channels may be expected to result in sustained membrane depolarisation, hyperexcitability and increased constriction under both basal conditions and following agonist stimulation (Bakker et al., 2000; Eskesen & Edvinsson, 2006; Lee et al., 2006; Nilsson et al., 2008; Manoury et al., 2009; Tai et al., 2009).
However, in chorionic plate arteries U-46619 induced constriction was not significantly affected by 48 h culture under 6 % O₂; furthermore, there was an apparent reduction in constriction. Interestingly, mRNA for K⁺ channels important in inducing relaxtion, including BK<sub>Ca</sub>, K<sub>ATP</sub> and IK<sub>Ca</sub>, is increased in vessels cultured for 48 h under normoxia (Figure 3-12); this may explain the trend towards reduced constriction under these conditions in the wire myograph (Figure 2-4). It is interesting why culture permits an increase in the neuronal SK<sub>Ca</sub>2 channel in the fetoplacental vasculature particularly when this vascular bed lacks innervation (Spivack, 1943; Walker & McLean, 1971; Fox & Khong, 1990; Benirschke & Kaufmann, 2000). Expression of other channels is known to be modulated by culture including voltage-gated and store-operated Ca<sup>2+</sup> channels which are down-regulated and up-regulated respectively (Dreja et al., 2001; Bergdahl et al., 2005; Tai et al., 2009). Why culture alters ion channel expression is unknown. However, it has been suggested that an inadequate supply of oxygen to the inner layers of the vessel media is thought to contribute, particularly with respect to oxygen-sensitive ion channels (Manoury et al., 2009). Regardless of the biological rationale for this alteration in ion channel expression, it is clear that this will impact on the ability of the vessels to regulate vascular tone and the underlying mechanisms that control these processes. This is an important consideration when utilising this model to study the chronic effect of altered oxygenation on K⁺ channel function in chorionic plate arteries.

In summary, this study confirms Kv<sub>1.5</sub>, BK<sub>Ca</sub>, K<sub>ATP</sub>, IK<sub>Ca</sub> and SK<sub>Ca</sub>3 mRNA expression in chorionic plate arteries and consolidates previous observations of protein expression of these isoforms in the intact artery and isolated SMCs.
3.3. FUNCTION OF K⁺ CHANNELS IN CHORIONIC PLATE ARTERIAL SMOOTH MUSCLE CELLS

3.3.1. Introduction

The resting membrane potential of SMCs is an important regulator of vascular tone as changes by only a few millivolts cause significant changes in blood vessel diameter (Brayden & Nelson, 1992; Nelson & Quayle, 1995). SMCs express K⁺ channels which determine the resting membrane potential whereby K⁺ efflux maintains SMCs in a quiescent state at rest (Ko et al., 2008). Many stimuli, including circulating vasoactive agents and oxygen, regulate the opening of K⁺ channels which in turn alters the membrane potential and ultimately vascular tone. This process of excitation-contraction coupling in the vasculature requires localisation of K⁺ channels to the SM.

In the fetoplacental vasculature, direct evidence localising K⁺ channels to the SM of chorionic plate “resistance” arteries where they would potentially control the resting membrane potential and respond to circulating vasoactive agents, is lacking. Microelectrode impalement studies predict the resting membrane potential in chorionic plate arterial SMCs to be around -38 mV with a possible involvement of K⁺ channels given the propensity for this value to fluctuate in proportion to the external K⁺ concentration (Ibrahim et al., 1998). Previous studies in intact placental chorionic plate arteries have provided indirect evidence that K⁺ channels, including Kv, BKCa and KATP isoforms, modulate vascular reactivity and basal tone through effects on vascular SM (Hampl et al., 2002; Sand et al., 2006; Wareing et al., 2006a; Wareing et al., 2006e; Jewsbury et al., 2007; Corcoran et al., 2008; Mills et al., 2009a; Mills et al., 2009b; Kiernan et al., 2010). The first direct evidence to confirm expression of various K⁺ channels including Kv1.5, BKCa, IKCa and KATP in single SMCs isolated from chorionic plate arteries has been provided in section 3.1. Studies characterising the functional K⁺ channel profile in fetoplacental arterial SMCs are lacking, with current evidence confirming 4-AP-sensitive Kv channels predominantly contribute to the whole-cell currents in SMCs isolated from large (>500 μm diameter) chorionic plate arteries (Hampl et al., 2002). Extensive electrophysiology experiments have not been performed in SMCs isolated from small resistance (<500 μm) chorionic plate arteries, to identify which ion channels, including K⁺ channels, are functional in these cells and therefore have the potential to mediate excitation-contraction coupling.
The current study aimed to characterise the functional $K^+$ channel profile in SMCs isolated from small resistance chorionic plate arteries using whole-cell electrophysiology. Given previously documented expression of $K_{v1.5}$, $BK_{Ca}$, $IK_{Ca}$ and $K_{ATP}$ in these cells (section 3.1), and modulation of basal and agonist-induced tone in chorionic plates arteries by $K_{v}$ channels (section 0), $BK_{Ca}$ (Sand et al., 2006; Wareing et al., 2006a), and $K_{ATP}$ channel openers/ blockers (Wareing et al., 2006a; Wareing et al., 2006e; Jewsbury et al., 2007), this study tested the hypothesis that numerous $K^+$ channel isoforms are functional and contribute to whole-cell currents in chorionic plate arterial SMCs.

3.3.2. Materials and Methods

3.3.2.1. SMC Isolation Procedure

Patch clamp experiments were performed on acutely isolated vascular SMCs according to the final protocol described in section 3.1.2.2. Briefly, small (<500μm) chorionic plate arteries were dissected from normal placentas delivered at term (N=24) using fine dissecting forceps. Intact vessels were maintained in Ca$^{2+}$ free-dissociation media (DM) containing (mM): 120 NaCl, 25 NaHCO$^3$, 4.2 KCl, 0.6 KH$_2$PO$_4$, 1.2 MgCl$_2$, 11 Glucose; pH 7.4 for 5 minutes. The vessels were incubated in 1 ml DM containing (mg/ml); 1.0 papain, and 1.0 DTT for 20 minutes at 37°C. The vessels were washed three times in ice-cold Ca$^{2+}$ free-DM and then transferred to 0.01 mM Ca$^{2+}$-DM containing (mg/ml); 1.0 collagenase type 1A and 1.0 collagenase type F and incubated at 37°C for 10 minutes. The vessels were washed three times in ice-cold 0.01 mM Ca$^{2+}$-DM and triturated with a fire-polished glass Pasteur pipette.

3.3.2.2. Whole-cell Electrophysiology

Only spindle-shaped, relaxed SMCs obtained within 4 hours of isolation were used in electrophysiology experiments. Aliquots of the cell suspension (50 μl) were left to settle and attach to the bottom of P35 culture dishes for 20-30 minutes before addition of extracellular solution (2 ml). All recordings were made using the whole-cell patch-clamp
technique (Hamill et al., 1981). Haematocrit glass patch pipettes of resistance 3-6MΩ 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. Only those cells for which a stable giga-Ohm resistance seal was established and an access resistance of <20 MΩ in the whole-cell configuration were selected for experiments to ensure the quality of recordings. Clamped cells were allowed to reach a steady state, evident from stable current-voltage relationship curves, in extracellular solution for approximately 3 minutes. A 500 ms depolarising pulse from -80 mV to +50 mV was performed in control conditions for 1 minute and then the drug added to the extracellular solution with microinjection. Once deviations in the current pulse were observed implicating a drug effect, the current-voltage protocols were recorded until a new steady state was reached in the presence of the applied drug. All recordings were performed at room temperature (22-25°C).

3.3.2.3. Solutions

Cells were bathed with extracellular solution containing (mM): NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, HEPES 5, Mannitol 10 and Glucose 5, (pH 7.3 with NaOH). Patch pipettes were filled with (mM): K-aspartate 120, KCl 20, MgCl₂ 1, EGTA 0.5, Mannitol 35 and HEPES 5 (pH 7.2 with KOH). The theoretical reversal potential as calculated by the Nernst equation for K⁺ and Cl⁻ in K-aspartate containing intracellular solution was -84 mV and -48 mV respectively. K⁺ channel function was assessed by extracellular application of; 4-aminopyridine (4-AP; Kᵥ inhibitor; 5 mM), DPO-1 (Kᵥ1.5 inhibitor; 3 μM), tetraethylammonium (TEA; KᵥCa inhibitor; 5 mM), charybdotoxin (ChTx; BKᵥCa and IKᵥCa inhibitor 100nM), iberiotoxin (IbTx; BKᵥCa inhibitor; 100 nM), TRAM-34 (IKᵥCa inhibitor; 10 μM), apamin (SKᵥCa inhibitor; 100 nM) and 1-EBIO (IKᵥCa and SKᵥCa opener; 100 μM). The concentration of 4-AP and TEA were chosen as a maximal dose known to inhibit most Kᵥ and KᵥCa isoforms respectively (Gutman et al., 2005; Wei et al., 2005). DPO-1 was applied at a submaximal dose (IC₅₀ 0.3 μM) (Lagrutta et al., 2006). ChTx, TRAM-34, IbTx, apamin and 1-EBIO were applied at concentrations used in previous studies in vascular SMCs (Beech & Bolton, 1989; Archer et al., 1996; Gollasch et al., 1996; Edwards et al., 1998; Sones et al., 2009). TEA and 4-AP were dissolved in
extracellular solution and dH₂O respectively at a concentration of 0.5M and stored at room temperature (22-25°C). DPO-1 was dissolved in DMSO at a stock concentration of 3 mM and stored in aliquots at -20°C. Charybdotoxin, iberiotoxin, TRAM-34 and apamin were dissolved in Dubecoo's phosphate buffered solution (PBS) containing CaCl₂ and MgCl₂ (Cat No; D8662; Sigma-Aldrich, Poole, Dorset, UK) at a stock concentration 1000 times greater than the working concentration and stored in aliquots at -20 °C. Dilution of the stock solution of each drug with extracellular solution was made immediately before use.

3.3.2.4. Chemicals and statistical analysis

General chemicals were purchased from Sigma-Aldrich, Poole, Dorset, UK. 4-AP, TEA, DPO-1, 1-EBIO and TRAM-34 were purchased from Sigma-Aldrich, Poole, Dorset, UK. Charybdotoxin, iberiotoxin and apamin were purchased from Alomone Labs, Jerusalem, Israel. Whole-cell currents were normalised to cell capacitance and results represented as mean ± S.E.M (n= no. of cells; N= no. of placentas). Current-voltage relationships were constructed from currents measured at the end of the 500ms voltage step and drug effects compared using Two-way ANOVA. Drug effects on currents obtained at a single voltage step were compared with Wilcoxon matched-pairs signed rank test. Statistical significance was considered at the P<0.05 level.
3.3.3. Results

3.3.3.1. Characterisation of passive membrane properties and whole-cell current profiles

The capacitance of chorionic plate arterial SMCs ranged from 11.0 pF to 41.8 pF, with a mean value of 23.2±7.6 pF (mean±SEM; n=66; N=24; Figure 3-14A). A large distribution in the maximum current at +80 mV was recorded between cells, with currents ranging from 10 pA to 2500 pA (356±51 pA; n=66, N=24; Figure 3-14B). The maximum current was related to cell capacitance, a proxy measure of cell size, with smaller cells displaying larger currents (P<0.05; r²=0.07; linear regression; Figure 3-14C).

Step depolarisation of chorionic plate arterial SMCs from a holding potential of -60 mV to a series of test potentials between -70 mV and +80 mV elicited outward currents (I_K) with a threshold potential of activation between -40 mV and -10 mV. Current was predicted to be carried predominantly by K⁺ ions based on the composition of the intra- and extracellular solutions. Two distinct families of whole-cell current profiles were evident in chorionic plate arterial SMCs (Figure 3-15). In the majority of cells (56/66; 85 %), currents displayed time-dependent activation, were outwardly rectifying at potentials greater than +20 mV, had a high current amplitude (401±58 pA at +80 mV) and exhibited noisy currents at +80 mV characteristic of spontaneously transient outward currents (STOCs) observed in other SMCs (Beech & Bolton, 1989; Clapp & Gurney, 1991; Smirnov & Aaronson, 1992; Halliday et al., 1995; Archer et al., 1996; Gollasch et al., 1996). STOCs were superimposed on a linear, time-independent current. A minority of cells (10/66; 15 %; Figure 3-15) contained only the linear, time-independent current of smaller amplitude. These recordings could be further subclassified according to maximal current at +80 mV and reversal potential; (1) maximal current <100 pA and a reversal potential between approximately -10 mV and 0 mV (n=8; N=5), (2) maximal current >100 pA and a reversal potential approximating -30 mV (n=2; N=2; Figure 3-15).

Extensive kinetic analyses were not performed on the majority of recordings which contained STOCs as the number, magnitude and time course of STOCs was highly variable between cells.
Figure 3-14: Properties of chorionic plate arterial SMCs. (A) Membrane capacitance (pF) is plotted as a function of cell number (n=66; N=24). (B) Maximum current obtained at +80 mV is plotted as a function of cell number (n=66; N=24). (C) Negative relationship between maximum current at +80 mV and membrane capacitance (P<0.05; r²=0.07; linear regression).
Whole-cell recordings
(n=66 cells; N=24 placentas)

STOCs
n=56; N=17
• Outward Rectifiers
• Time-dependent activation

No STOCs
n=10; N=7
• Linear IV-relationship

Figure 3-15: Characterisation of whole-cell current recordings in chorionic plate arterial SMCs. Current-voltage relationships were recorded from chorionic plate arterial SMCs (n=66 cells; N=24 placentas). Whole-cell recordings were categorised according to the presence (n=56; N=17) or absence (n=10; N=7) of spontaneously transient outward currents (STOCs) at depolarised potentials. Recordings containing STOCs were outwardly rectifying and exhibited time-dependent activation. The magnitude and number of potentials displaying STOCs varied between cells. Recordings which displayed no STOCs had a linear current voltage relationship and could be further classified according to maximum current and reversal potential; n=8 recordings (N=5) had a maximum current at +80 mV of less than 100 pA and a reversal potential ranging between 0 mV and -10 mV, n=2 recordings had a maximum current greater than 100 pA and a reversal potential of approximately -30 mV.
3.3.3.2. Voltage-gated K⁺ Channel Pharmacology (Kᵥ)

Inhibition of Kᵥ channels with 4-AP (5 mM) reduced whole-cell currents in cells where STOCs were both present at +80 mV (Figure 3-11A) and absent (Figure 3-11B). 4-AP did not abolish STOCs (Figure 3-11A) but predominantly inhibited the linear current at potentials between 0 mV and +40 mV (at +40 mV: control 4.7± 0.8 pA/pF; 4-AP 2.9±0.4 pA/pF; P<0.05; Wilcoxon matched-pairs signed rank test; n=11, N=8). Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence (●) and presence (○) 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 3-11C). 4-AP application produced a rightward shift in the current-voltage relationship (Figure 3-11C) and appeared to predominantly exert its effects through inhibition of channels permitting K⁺ efflux as the 4-AP sensitive current obtained from the mean current-voltage relationship was shifted to the left, approaching the theoretical reversal potential for K⁺ (-84 mV) as calculated by the Nernst equation (Figure 3-11D).

Specific inhibition of the Kᵥ1.5 channel isoform with DPO-1 (3 μM) produced similar effects on whole-cell currents as 4-AP. DPO-1 reduced currents in cells where STOCs were both present at +80 mV (Figure 3-17A) and absent (Figure 3-17B). DPO-1 did not abolish STOCs (Figure 3-17A) but predominantly inhibited the linear current at potentials between 0 mV and +40 mV (at +40 mV: control 2.8± 0.6 pA/pF, DPO-1 1.5±0.5 pA/pF; P<0.05; n=9, N=7). Current-voltage relationships measured at the end of the 500ms voltage step were obtained in the absence (●) and presence (○) of 3 μM DPO-1 with significant inhibition of currents evident at potentials depolarised to +30 mV (P<0.05; Two-way-ANOVA followed by Bonferroni Post Hoc Test; Figure 3-17C). DPO-1 application produced a rightward shift in the current-voltage relationship (Figure 3-17C) and appeared to predominantly exert its effects through inhibition of channels permitting K⁺ efflux as the DPO-1 sensitive current obtained from the mean current-voltage relationship was shifted to the left (Figure 3-17D).
Figure 3-16: Effect of inhibition of voltage-gated K⁺ channels with 4-aminopyridine (4-AP) on whole-cell currents in chorionic plate arterial SMCs. 4-AP (5 mM) inhibited the linear current irrespective of whether STOCs were present at +80 mV (A) or absent (B) in extracellular solutions. (C) Mean current-voltage relationships measured at the end of the 500ms voltage step ranging from -70 mV to +80 mV were obtained in the absence (●) and presence (○) of 5 mM 4-AP (*P<0.05; Two-way-ANOVA followed by Bonferroni Post Hoc Test; mean±SEM; n=11; N=8). (D) 4-AP sensitive current calculated from mean current voltage relationships obtained in (C).
Figure 3-17: Effect of inhibition of the voltage-gated K+ channel isoform Kv1.5 with DPO-1 on whole-cell currents in chorionic plate arterial SMCs. DPO-1 (3 μM) inhibited the linear current irrespective of whether STOCs were present at +80 mV (A) or absent (B) in extracellular solutions. (C) Mean current-voltage relationships measured at the end of the 500ms voltage step ranging from -70 mV to +80 mV were obtained in the absence (●) and presence (○) of 3 μM DPO-1 (*P<0.05; Two-way-ANOVA followed by Bonferroni Post Hoc Test; mean±SEM; n=9; N=7). (D) DPO-1 sensitive current calculated from mean current voltage relationships obtained in (C).
3.3.3.3. Ca^{2+}-activated K⁺ Channel Pharmacology (K\textsubscript{Ca})

Inhibition of K\textsubscript{Ca} channels with TEA (5 mM) abolished STOCs and the majority of outward current; but had little effect on the linear current which was selectively inhibited by 4-AP (Figure 3-18A, B, C). TEA preferentially inhibited whole-cell K⁺ currents at potentials positive to +50 mV (Figure 3-18D), with a 66±5 % decrease observed at +80 mV (mean±SEM; n=11; N=6). Current-voltage relationships measured at the end of the 500ms voltage step were obtained in the absence (●) and presence (○) of 5 mM TEA 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 3-18E). The TEA-sensitive current displayed strong outward rectification (Figure 3-18F).

Inhibition of the Ca^{2+}-activated K⁺ channel isoforms BK\textsubscript{Ca} and IK\textsubscript{Ca} with charybdotoxin (ChTx; 100 nM) abolished STOCs and the majority of the outward current (n=4; N=2; Figure 3-19). Selective blockade of IK\textsubscript{Ca} channels with TRAM-34 (10 μM) was without effect on outward currents and STOCs (n=3, N=2; Figure 3-20). Conversely, inhibition of BK\textsubscript{Ca} channels with iberiotoxin (IbTx; 100 nM) abolished STOCs and outward currents at +80 mV by 61±12 % in common with TEA and ChTx; but had little effect on the linear current (mean±SEM; n=10, N=4; Figure 3-21A, B). Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence (●) and presence (○) of 100 nM IbTx with significant inhibition evident at potentials depolarised to +70 mV (P<0.05; Two-way-ANOVA followed by Bonferroni Post Hoc Test; Figure 3-21C). The IbTx-sensitive current displayed strong outward rectification (Figure 3-21D).

Further analysis of the contribution of K\textsubscript{Ca} channels to whole-cell currents in chorionic plate arterial SMCs was performed with the IK\textsubscript{Ca} and SK\textsubscript{Ca} channel activator 1-EBIO (100 μM). Extracellular application of 1-EBIO increased whole-cell currents in recordings where STOCs were both present at +80 mV (Figure 3-22A) and absent (Figure 3-22B) under basal conditions (control: 6.6±2.1 pA/pF; 1-EBIO: 29.1±6.9 pA/pF at +80 mV; P<0.05; n=22, N=10; Wilcoxon matched-pairs signed rank test). Current-voltage relationships measured at the end of the 500 ms voltage step were obtained in the absence (●) and presence (○) of 100 μM 1-EBIO with significant activation evident at potentials depolarised to +70 mV (P<0.05; Two-way-ANOVA followed by Bonferroni Post Hoc Test; Figure 3-22D). The 1-EBIO-sensitive current displayed strong outward rectification (Figure 3-22D). The percentage change in whole-cell K⁺ currents following 1-EBIO application demonstrated a significant increase in current at depolarised
potentials, with a 431±62 % increase observed at +80 mV (P<0.05; Wilcoxon-signed rank test; Figure 3-22F). Abolition of the 1-EBIO response with TEA confirmed this increase in whole-cell currents was mediated by a K<sub>Ca</sub> channel (Figure 3-23). Determining the precise K<sub>Ca</sub> isoform responsible for the 1-EBIO-sensitive current excluded a role for BK<sub>Ca</sub> channels as selective blockade of these channels with IbTx (100 nM; n=3, N=3), in the continued presence of 1-EBIO, had no effect on the magnitude of the outward currents (1-EBIO: 15.2±11.2 pA/pF; 1-EBIO + IbTx: 14.2±9.0 pA/pF at +80 mV; P>0.05; Wilcoxon matched-pairs signed rank test; n=3, N=3; Figure 3-24). Inhibition of IK<sub>Ca</sub> isoforms with TRAM-34 (10 μM; n=7, N=4) abolished the 1-EBIO sensitive currents (1-EBIO: 34.5±10.0 pA/pF; 1-EBIO + TRAM-34: 7.1±2.7 pA/pF at +80 mV; P<0.05; Wilcoxon matched-pairs signed rank test; n=7, N=4; Figure 3-25A). 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 (●) (Figure 3-25C). Addition of TRAM-34 following 1-EBIO application returned the current voltage relationship to control levels (Figure 3-25C). SK<sub>Ca</sub> channels produced a small but significant contribution to 1-EBIO sensitive currents as selective blockade of this isoform with apamin (100 nM) reduced currents at +80 mV from 17.5±5.0 pA/pF to 10.7±2.4 pA/pF (P<0.05; Wilcoxon matched-pairs signed rank test; n=7; N=3 Figure 3-26).
Figure 3-18: Effect of inhibition of Ca\(^{2+}\)-activated K\(^+\) channels with tetraethylammonium (TEA) on whole-cell currents in chorionic plate arterial SMCs. (A-C) Representative examples of records where TEA (5 mM) inhibited STOCs at depolarised voltage steps. (B-C) 4-AP inhibited the linear currents at relatively negative potentials but has no effect on STOCs. (D) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (-) and following application of TEA (-). (E) 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 (●) and presence (○) of 5 mM TEA (*P<0.05; Two-way-ANOVA followed by Bonferroni Post Hoc Test; mean ±SEM; n=11; N=6). (F) TEA sensitive current calculated from mean current voltage relationships obtained in (E).
Figure 3-19: Effect of inhibition of the Ca\(^{2+}\)-activated K\(^+\) channel isoforms BK\(_{Ca}\) and IK\(_{Ca}\) with charybdotoxin (ChTx) on whole-cell currents in chorionic plate arterial SMCs. (A) Representative example of a record where ChTx (100 nM) inhibited STOCs obtained at depolarised voltage steps (n=4; N=2). (B) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (-) and following application of ChTx (-).

Figure 3-20: Effect of inhibition of the Ca\(^{2+}\)-activated K\(^+\) channel isoform IK\(_{Ca}\) with TRAM-34 on whole-cell currents in chorionic plate arterial SMCs. (A) Representative example of a record where TRAM-34 (10 μM) had no effect on STOCs (n=3; N=2). (B) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (-) and following application of TRAM-34 (-).
Figure 3-21: Effect of inhibition of the Ca\(^{2+}\)-activated K\(^+\) channel isoform BK\(_{\text{Ca}}\) with iberiotoxin (IbTx) on whole-cell currents in chorionic plate arterial SMCs. (A) Representative example of a record where IbTx (100 nM) inhibited STOCs obtained at depolarised voltage steps. (B) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (●) and following application of IbTx (○). (C) 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 (●) and presence (○) of 100 nM IbTx (*P<0.05; Two-way-ANOVA followed by Bonferroni Post Hoc Test; mean±SEM; n=8; N=4). (D) IbTx sensitive current calculated from mean current voltage relationships obtained in (C).
Figure 3-22: Effect of activation of the Ca^{2+}-activated K^{+} channel isoforms IK_{Ca} and SK_{Ca} with 1-EBIO on whole-cell currents in chorionic plate arterial SMCs. 1-EBIO (100 μM) increased outwards currents irrespective of whether STOCs were present at +80 mV (A) or absent (B) in extracellular solutions. (C) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (-) and following application of 1-EBIO (-). (D) Mean current-voltage relationships obtained at the end of the 500 ms voltage step in the absence and presence of 1-EBIO (*P<0.05; Two-way-ANOVA followed by Bonferroni Post Hoc Test; mean±SEM; n=22; N=10). (E) 1-EBIO sensitive current calculated from mean current voltage relationships obtained in (D). (F) Percentage change in currents following application of 1-EBIO as a function of the applied voltage step (*P<0.05; Wilcoxon-signed rank test; median±IQR; n=22; N=10).
Figure 3-23: Effect of inhibition of Ca\(^{2+}\)-activated K\(^+\) channels with tetraethylammonium (TEA) on 1-EBIO-sensitive currents in chorionic plate arterial SMCs. (A) Representative example of a record where the 1-EBIO (100 \(\mu\)M) sensitive current was inhibited by TEA (5 mM; n=2; N=2). (B) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (-) and following application of 1-EBIO (-), and TEA (-) in the continued presence of 1-EBIO.
Figure 3-24: Effect of inhibition of the Ca\(^{2+}\)-activated K\(^+\) channel isoform BK\(_{Ca}\) with iberiotoxin (IbTx) on 1-EBIO-sensitive currents in chorionic plate arterial SMCs. (A) Representative example of a record where the 1-EBIO (100 μM) sensitive current was unaffected by IbTx (100 nM; n=3; N=3). (B) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (-) and following application of 1-EBIO (-), and IbTx (-) in the continued presence of 1-EBIO.
Figure 3-25: Effect of inhibition of the Ca²⁺-activated K⁺ channel isoform IKₓ with TRAM-34 on 1-EBIO-sensitive currents in chorionic plate arterial SMCs. (A) Representative example of a record where the 1-EBIO (100 μM) sensitive current was inhibited by TRAM-34 (10 μM). (B) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (-) and following application of 1-EBIO (-), and TRAM-34 (-) in the continued presence of 1-EBIO. (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 TRAM-34 in the continued presence of 1-EBIO (mean±SEM; n=7; N=4).
Figure 3-26: Effect of inhibition of the Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel isoform SK\textsubscript{Ca} with apamin on 1-EBIO-sensitive currents in chorionic plate arterial SMCs. (A) Representative example of a record where the 1-EBIO (100 \(\mu\text{M}\)) sensitive current was inhibited by apamin (100 nM) and TRAM-34 (10 \(\mu\text{M}\)). (B) Sample trace showing outward currents recorded following step depolarisation from -70 mV to +50 mV for 500 ms in extracellular solutions (-) and following application of 1-EBIO (-), and apamin (-) in the continued presence of 1-EBIO. (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 (mean±SEM; \(n=7; N=3\)).
3.3.4. Discussion

This current study is the first to extensively characterise the electrophysiological and pharmacological properties of $K^+$ channels in SMCs isolated from small diameter chorionic plate resistance arteries. Conclusive evidence is provided which demonstrates functional $K_v$ and $K_{Ca}$ channel isoforms in these cells that have the potential to mediate excitation-contraction coupling in the fetoplacental vasculature.

3.3.4.1. Characterisation of whole-cell currents

Various electrophysiological parameters were recorded under whole-cell conditions and characterised in chorionic plate arterial SMCs for comparison with other vascular SMCs. One such parameter was cell capacitance, which is proportional to the membrane surface area and calculated from the area under the capacitive transient under whole-cell conditions. A large range in capacitance values were observed in chorionic plate arterial SMCs (11.0 pF to 41.8 pF; 23.2 ± 7.6 pF mean ± SEM; n=66, N=24; Figure 3-14A). Similar values are described in other vascular SMCs including; mouse pulmonary artery SMCs (9.4 pF) (Ko et al., 2007), fetal rat pulmonary resistance arteries (15.9 pF) (Reeve et al., 1998), human coronary artery SMCs (32.1 pF) (Gollasch et al., 1996) and human mesenteric arterial SMCs (46 pF) (Smirnov & Aaronson, 1992). Together with the observed similarities in morphology and phenotype of the chorionic plate arterial isolates with other freshly isolated vascular SMCs and the expression of $\alpha$-smooth muscle actin (but not CD31; see section 3.1.3.2), this provides additional confirmation that the devised isolation protocol yields vascular SMCs.

Maximal current recorded at +80 mV in chorionic plate arterial SMCs was similar to other vascular SMCs including those isolated from large diameter chorionic plate arterial SMCs (Clapp & Gurney, 1991; Hampl et al., 2002; Smirnov et al., 2002; Ko et al., 2007). However, a large range in currents were observed at this potential in SMCs isolated from chorionic plate resistance arteries (10 pA to 2500 pA; 356 ± 51 pA; mean ± SEM; n=66, N=24; Figure 3-14B). This variation correlated with capacitance, whereby cells with a larger surface area exhibited smaller currents (Figure 3-14C); although, the predictive value of this relationship was poor as indicated by the low $r^2$ value. However, the significant negative relationship between current magnitude and capacitance implies that increased insertion of active channels in the membrane does
not occur in parallel with an increase in the surface area of chorionic plate arterial SMCs. No such relationship was documented in the previous study performed in larger diameter chorionic plate arteries (Hampl et al., 2002). However, maximal current amplitude did correlate with vessel diameter whereby SMCs isolated from the largest vessels (>1 mm) had smaller currents compared with SMCs from small diameter vessels (>500 μm) (Hampl et al., 2002). Taken together with the results from the current study, this suggests that K⁺ channel activity, assessed by current amplitude, is heterogeneous in SMCs within the same vessel and along the fetoplacental vascular tree. This heterogeneity will in turn impact upon the capacity of the SMCs to control excitation-contraction coupling and therefore regulate fetoplacental vascular tone.

Heterogeneity in K⁺ channel activity within SMCs isolated from chorionic plate resistance arteries was further substantiated with the observation of two distinct families of whole-cell currents (Figure 3-15). Whole-cell currents appeared to consist of two components; (1) a linear, time-independent current, and (2) an outwardly rectifying current characterised by the presence of spontaneously transient outward currents (STOCs) described in other vascular SMCs (Beech & Bolton, 1989; Clapp & Gurney, 1991; Smirnov & Aaronson, 1992; Halliday et al., 1995; Archer et al., 1996; Gollasch et al., 1996; Smetkov et al., 1996; Smetkov et al., 1998). All cells contained the linear current. However, the majority of cells (85 %) exhibited STOCs which were superimposed upon the linear current. The number of voltage steps displaying STOCs varied, with STOCs observed at only +80 mV in some cells and from +10 mV in other cells.

In all recordings, outward currents were predicted to be predominantly K⁺ given the composition of the extracellular and intracellular solutions. Confirmation of the K⁺ selectivity of the currents would require manipulation of the extracellular solution to obtain an equimolar K⁺ concentration across the membrane that produces a rightward shift in the reversal potential to approximately 0 mV (Clarson et al., 2001). However, extensive and prolonged changes of the bath solution requires adequate adhesion of the cells to the culture dish and patent seals which often proved problematic in chorionic plate arterial SMCs (see section 3.1.2.2). Another approach to confirm the selectivity of whole-cell currents to K⁺ is to perform tail-current analyses, allowing quantification of the reversal potential. Although the voltage profile used in the current study included a step repolarisation to -40 mV following the 500 ms voltage steps, tail currents were not observed under control conditions. Therefore, further experiments are required utilising a different protocol whereby cells are step repolarised to various potentials to record more distinct tail currents. Despite the lack of a precise reversal
potential, estimation of where the line crossed the x axis for each recording obtained a range in values between -40 mV and -10 mV. This is considerably more depolarised than predicted for a purely K⁺ selective current (-84 mV). This implies a varying contribution of currents through channels other than K⁺ channels, shifting these values to a greater or lesser extent to more depolarised potentials. These currents may predominantly consist of cations (Ca²⁺ or Na⁺) or anions (Cl⁻) entering the cell and the use of additional pharmacological modulators would be necessary to confirm the identity of the channels mediating these currents. Given the variable number of voltage steps displaying STOCs and contribution from “contaminating currents”, extensive quantitative analyses to assess the time dependency, conductance and activation kinetics were not performed. For this reason, a pharmacological approach was pursued utilising broad spectrum K⁺ channel blockers in the first instance, to identify the channels responsible for STOCs and linear currents and ultimately identify which K⁺ channels are functional in chorionic plate arterial SMCs.

3.3.4.2. Voltage-gated K⁺ channels (Kᵣ)

Kᵣ channels modulate fetoplacental vascular tone, evident in studies performed in the perfused placenta (Hampl et al., 2002), intact chorionic plate resistance arteries (section 0) (Wareing et al., 2006a; Mills et al., 2009a; Mills et al., 2009b), and SMCs isolated from large diameter chorionic plate arteries (Hampl et al., 2002). Given expression of the Kᵣ channel isoform Kᵣ1.5 in SMCs isolated from small resistance chorionic plate arteries and function in the intact vessel (section 0, 3.1 and 3.2), the current study investigated the contribution of Kᵣ and specifically Kᵣ1.5 channels to the different components of whole-cell K⁺ current in chorionic plate arterial SMCs.

Kᵣ channels are thought to be important in maintaining the resting membrane potential in vascular SMCs (Archer et al., 1998; Joshi et al., 2009). Although the resting membrane potential of chorionic plate arterial SMCs is thought to be around -38 mV from microelectrode impalement studies in the intact vessel (Ibrahim et al., 1998), it is unknown what the resting membrane potential is in the isolated cells. Indirect evidence suggests Kᵣ channels contribute to maintaining the resting potential in fetoplacental arterial SMCs as inhibition of these channels with 4-AP increased basal tone in the intact vessel (see section Figure 2-10) (Wareing et al., 2006a; Mills et al., 2009a). The present study did not calculate the resting membrane potential in chorionic plate arterial SMCs. However, at a concentration that inhibits most isoforms of the Kᵣ family (Gutman et al., 2005), 4-AP predominantly reduced currents at more negative
potentials (see below and Figure 3-16). This suggests that \( K_v \) channels may be open and contribute to the resting membrane potential. Confirmation of this would necessitate perforated patch or current clamp experiments.

Blockade of \( K_v \) channels with 4-AP predominantly inhibited the linear current and had little effect on STOCs (Figure 3-16A, B). This has been similarly observed in rabbit aortic SMCs (Halliday et al., 1995) and human coronary artery SMCs (Gollasch et al., 1996) whereby 4-AP preferentially inhibited low-noise currents at relatively negative potentials, but had little effect on the noisy STOCs at depolarised potentials. Mean current-voltage relationships of all recordings treated with 4-AP do however, demonstrate significant inhibition of currents at +70 mV and +80 mV where STOCs would predominate (Figure 3-16C). This may be due to a small contribution of \( K_v \) channels at the most depolarised potentials or reflect a reduction in the linear current which ultimately impacts upon the magnitude of the maximal current. The 4-AP sensitive current (Figure 3-16D) obtained from the mean current-voltage relationship, is very weakly rectifying and the leftward shift in the reversal potential suggests this current is mediated by \( K^+ \) efflux.

Recordings displaying the linear current only, were categorised into two groups according to maximal current at +80 mV and the predicted reversal potential (Figure 3-15). As 4-AP-sensitive \( K_v \) channels inhibited the linear current only, the recordings with larger currents and a reversal potential shifted more towards \( E_K \), may have an increased contribution from \( K_v \) channels compared to cells with small currents and \( V_{\text{rev}} \) of approximately 0 mV. However, there were insufficient numbers of recordings displaying only the linear current in each group to assess this formally. The residual linear current in the presence of 4-AP may be mediated by background \( K^+ \) currents (e.g. TASK channels) or a non-selective cation channel as described in human fetal airway SMCs (Snetkov et al., 1998). 4-AP-insensitive \( K_v \) channels such as \( K_{v,2.1} \) and \( K_{v,7.X} \), may contribute to some of the residual current (Coetzee et al., 1999; Gutman et al., 2005). However, the contribution from these isoforms is likely to be minimal given the high concentrations of 4-AP used in this study (5 mM) (Yeung & Greenwood, 2005). Similarly, \( K_{\text{ATP}} \) channels are unlikely to contribute to the residual current as \( Mg^{2+} \)-nucleotides are required for channel activation which were not present in the pipette solution in these experiments (Yokoshiki et al., 1998). However, it would be interesting to manipulate the recording solutions to investigate the functional role of \( K_{\text{ATP}} \) channels in chorionic plate arterial SMCs given the observed expression in these cells (see section 3.1.3.3), and important role in controlling basal and agonist-induced tone in the intact artery (Wareing et al., 2006e; Jewsbury et al., 2007).
In non-placental vascular SMCs the predominant K⁺ channel contributing to whole-cell currents is a delayed rectifier K⁺ channel which is rapidly activating, slowly decaying and sensitive to 4-AP (Archer et al., 1996; Gollasch et al., 1996; Platoshy et al., 2001; Hampl et al., 2002; Smirnov et al., 2002; Ko et al., 2007). This current did not predominate in chorionic plate arterial SMCs under the recording conditions of these experiments. However, the ability of 4-AP to modulate vascular tone in intact chorionic plate arteries may result from K⁺ channels localised to the SMCs as 4-AP sensitive K⁺ channels makes a small but significant contribution to the whole-cell currents, implicating the presence of a delayed rectifier K⁺ channel in these cells.

There are numerous candidates for the delayed rectifier in vascular SMCs including K⁺₁.₅ channels (Archer et al., 1998; Platoshy et al., 2001). This thesis has previously suggested that K⁺₁.₅ channels may be functional in chorionic plate arteries given the observed expression of K⁺₁.₅ protein in SMCs isolated from these vessels (see section 3.1.3.3). However, specific inhibition of this isoform is insufficient to significantly modulate constriction which may reflect confounding whole-vessel influences (see section 2.3.4). In this study it was investigated whether K⁺₁.₅ channels are functional in SMCs isolated from intact chorionic plate arteries explaining the potentially biologically relevant, but statistically insignificant, alteration in U-46619 constriction. To investigate this, the K⁺₁.₅ channel blocker DPO-1 was applied at a submaximal dose (3 μM; IC₅₀ 0.3 μM) to chorionic plate arterial SMCs (Lagrutta et al., 2006). DPO-1 inhibited currents in a similar manner as 4-AP. DPO-1 reduced the linear current but had little effect on STOCs (Figure 3-17A, B) and significantly inhibited mean current-voltage relationships between +30 mV and +80 mV (Figure 3-17C). In common with 4-AP, this effect of DPO-1 at depolarised potentials may reflect inhibition of the linear current which impacts upon the magnitude of currents at +80 mV. It is clear from the representative traces in Figure 3-17 that DPO-1 has no effect on STOCs which must be mediated by a channel other than K⁺₁.₅. The observed similarities between the inhibitory profiles of 4-AP and DPO-1 may question the specificity of DPO-1 for K⁺₁.₅.

The differential effect of 4-AP and DPO-1 on vascular tone in chorionic plate arteries (see section 2.3.6) suggests these drugs act on different subsets of K⁺ channels. However, the same concentration of DPO-1 was applied to both the isolated SMCs and intact vessel. Therefore, DPO-1 may have reached saturation for specific inhibition of K⁺₁.₅ in the isolated SMCs under patch clamp experiments resulting in non-specific effects on other K⁺ channels. Further studies are required utilising a range of DPO-1 concentrations to construct a concentration response curve in isolated chorionic plate arterial SMCs. Application of DPO-1 at 3 μM to other vascular SMCs where K⁺₁.₅ makes a significant contribution to whole-cell K⁺ currents, such as pulmonary
resistance arterial SMCs, may help determine the specificity of this drug and chosen concentration. However, DPO-1 (1 μM) specifically inhibits Kv1.5 channels in heterologous systems and atrial myocytes where this channel is most abundant. Therefore, this study provides the first functional evidence for Kv1.5 channels in placental arterial SMCs and suggests that Kv1.5 channels localised to the SMC membrane have the potential to modulate fetoplacental vascular tone or other physiological processes.

To summarise, Kv and Kv1.5 channels make a small but significant contribution to whole-cell currents where they predominantly contribute to the linear current which is present in all chorionic plate arterial SMCs. The lack of a mark effect on whole-cell currents may explain why modulation of these channels in the intact vessel did not have a marked effect on basal- and agonist- induced tone. To conclude, this study is the first to demonstrate functional Kv and Kv1.5 channels in chorionic plate arterial SMCs.

3.3.4.3. Ca$^{2+}$-activated K$^+$ channels (K$_{Ca}$)

The suggestion that STOCs and the linear current are two independent events is supported by the above pharmacological studies which show that the Kv channel blocker 4-AP inhibits the linear current but has no effect on STOCs. In non-placental vascular SMCs, STOCs are mediated by the K$_{Ca}$ channel, BK$_{Ca}$ which has a large single-channel conductance and accounts for the characteristic noise seen in these currents at depolarised potentials (Beech & Bolton, 1989; Clapp & Gurney, 1991; Smirnov & Aaronson, 1992; Halliday et al., 1995; Archer et al., 1996; Gollasch et al., 1996; Snetkov et al., 1996; Snetkov et al., 1998). BK$_{Ca}$ channels are expressed at the mRNA and protein level in chorionic plate vessels where they modulate fetoplacental vascular tone in the perfused placenta and intact artery (see section 3.1 and 3.2) (Hampl et al., 2002; Sand et al., 2006; Wareing et al., 2006a; Corcoran et al., 2008). This thesis has also demonstrated expression and localisation of BK$_{Ca}$ to the SM using both immunohistochemistry of chorionic plate arterial cross-sections and immunocytochemistry of SMCs isolated from these vessels (see section 3.1). BK$_{Ca}$ channels are functional in SMCs isolated from large diameter chorionic plate arteries using electrophysiology, as the specific BK$_{Ca}$ inhibitor iberiotoxin, slightly reduced whole-cell K$^+$ currents (Hampl et al., 2002). The presence of currents that resemble STOCs seen in other vascular SMCs, suggests BK$_{Ca}$ channels are functional in SMCs.
isolated from small diameter chorionic plate arteries which are thought to primarily
determine fetoplacental vascular resistance.

To confirm the contribution of $K_{Ca}$ channels to STOCs, the broad spectrum $K_{Ca}$ inhibitor
TEA (5 mM) was applied to whole-cell currents at a concentration known to inhibit all
$K_{Ca}$ isoforms (Wei et al., 2005). Under these conditions STOCs were abolished (Figure
3-18A-D). This inhibition was significant at potentials positive to +60 mV as illustrated
in the mean current-voltage plot (Figure 3-18E). Confirmation that STOCs determine
the degree of outward rectification is evident following TEA application whereby the
mean current-voltage plot in the presence of TEA is linear and the TEA-sensitive
current is outwardly rectifying (Figure 3-18E, F). Therefore, the majority of whole-cell
current in chorionic plate arterial SMCs is mediated by an outwardly rectifying $K_{Ca}$
channel. To determine specifically which $K_{Ca}$ channel isoform was responsible for
STOCs, the specific $BK_{Ca}$ and $IK_{Ca}$ inhibitor charybdotoxin (100 nM) was applied to
whole-cell currents. Charybdotoxin abolished STOCs suggesting $BK_{Ca}$ or $IK_{Ca}$ is the
channel responsible for these currents (Figure 3-19). The specific $IK_{Ca}$ inhibitor TRAM-
34 (10 μM; Figure 3-20) had no effect on outward currents, but they were abolished by
the specific $BK_{Ca}$ inhibitor iberiotoxin (100 nM; Figure 3-21) suggesting that, in common
with other vascular SMCs, STOCs in chorionic plate arterial SMCs are mediated by
$BK_{Ca}$ channels. $BK_{Ca}$ channels localised to the SMC membrane modulate vascular
reactivity in the intact chorionic plate artery (Bisseling et al., 2005; Sand et al., 2006;
Wareing et al., 2006a). Inhibition of $BK_{Ca}$ channels with iberiotoxin only slightly
increased perfusion pressure in the perfused placenta and enhanced U-46619
constriction in chorionic plate arteries on the wire myograph. The observation in the
present study that $BK_{Ca}$ channels are the predominant current in SMCs isolated from
small resistance chorionic plate arteries would potentially implicate a larger effect on
vascular tone following iberiotoxin application. However, $BK_{Ca}$ channels may have a
more prominent role in mediating vasodilation in vivo and therefore act as a therapeutic
target to improve blood flow within the fetoplacental vasculature.

$BK_{Ca}$ channels are activated by either membrane depolarisation or micromolar
concentrations of intracellular $Ca^{2+}$ but these factors can act synergistically in a
physiological setting to facilitate channel opening at more hyperpolarised potentials
(Schubert & Nelson, 2001; Magleby, 2003). The observation of active $BK_{Ca}$ channels in
chorionic plate arterial SMCs was unexpected given the composition of the
extracellular and intracellular solutions. The predicted intracellular $Ca^{2+}$ concentration is
low (~10 nM) due to buffering with EGTA (0.5 mM). In arterial SM, localised increases
in intracellular $Ca^{2+}$ through ryanodine receptors on the sarcoplasmic reticulum activate
BK$_{Ca}$ rather than influx across the membrane via voltage-gated Ca$^{2+}$ channels (Nelson et al., 1995). Electron microscopy studies of the vascular wall of chorionic plate arteries in situ, failed to identify structures resembling the sarcoplasmic reticulum in the SMC cytoplasm (Sweeney et al., 2006). Therefore, unless intracellular Ca$^{2+}$ release in the placental SMCs is mediated through other Ca$^{2+}$ stores such as lysosomes (Evans, 2010; Patel & Docampo, 2010), activation of BK$_{Ca}$ is likely to result from membrane depolarisation elicited during the voltage step protocol. BK$_{Ca}$ may also be active under low Ca$^{2+}$ recording conditions due to uncoupling of endogenous BK$_{Ca}$ inhibitors following dialysis of the intracellular fluid during establishment of the whole-cell configuration. For example, BK$_{Ca}$ channels in arterial SM are inhibited by protein kinase C (PKC) induced phosphorylation of the channel (Schubert et al., 1999). Therefore, unless BK$_{Ca}$ and PKC are tethered in a signalosome complex such as caveolae, dialysis of the intracellular fluid may prevent close association of these molecules and ultimately a reduction in PKC-mediated phosphorylation, which would account for the increased propensity for BK$_{Ca}$ to be open under low Ca$^{2+}$ conditions. Regardless of the underlying mechanisms that permit BK$_{Ca}$ activation in the current study, the observation that BK$_{Ca}$ is the predominant K$^+$ current in chorionic plate arterial SMCs in spite of low intracellular Ca$^{2+}$ suggests these channels are crucially important in controlling fetoplacental vascular tone under physiological conditions.

Differences in BK$_{Ca}$ activity between chorionic plate arterial SMCs is evident from variations in the number of voltage-steps displaying STOCs. This may reflect subtle fluctuations in the intracellular Ca$^{2+}$ concentration, BK$_{Ca}$ expression or levels of endogenous signalling molecules that modulate channel activity. A similar variation in outward current was observed in fetal airway SMCs resulting from differences in the number of BK$_{Ca}$ channels per cell (Snetkov et al., 1998). In this study, it was concluded that the wide fluctuation in current was unlikely to be related to the phosphorylation status of the channel, as PKA or PKC regulation of BK$_{Ca}$ open probability is minor. The authors hypothesise that the wide variation in maximal current is due to heterogeneity in BK$_{Ca}$ expression, which is in turn related to the immature SMC phenotype, as the vessels are derived from the fetus (Snetkov et al., 1998). Therefore, variation in STOCs and maximal current in the present study may be related to the SMC phenotype as chorionic plate arteries are fetal in origin.

A close relationship exists between the development and phenotype of SMCs, and the K$^+$ channel families they express (Neylon, 2002; Beech & Cheong, 2006; Beech, 2007). Fetal pulmonary vascular SMCs predominantly express K$_{Ca}$ channels, including the BK$_{Ca}$ isoform, where they make a significant contribution to whole-cell currents and are
important in maintaining the resting membrane potential (Reeve et al., 1998; Snetkov et al., 1998; Rhodes et al., 2001). During the postnatal period, there is a shift in $K^+$ channel expression from $BK_{Ca}$ to predominantly 4-AP sensitive $K_v$ channels in adult pulmonary vascular SMCs (Beech & Bolton, 1989; Clapp & Gurney, 1991; Smirnov & Aaronson, 1992; Halliday et al., 1995; Archer et al., 1996; Gollasch et al., 1996; Smirnov et al., 2002; Platoshyn et al., 2004; Ko et al., 2007). Changes in oxygenation are thought to underlie the maturational shift in vascular SMC $K^+$ channel expression and function whereby hypoxia within the fetal lung favours $BK_{Ca}$ channels through a hypoxia inducible factor-1α (HIF-1α) mediated mechanism (Resnik et al., 2006a; Marino et al., 2007). Under physiological conditions in vivo, the fetoplacental vasculature is exposed to reduced oxygen tensions which are deemed relatively hypoxic compared with oxygenations observed in the adult systemic and pulmonary circulations (see section 1.3.4) (Soothill et al., 1986; Jauniaux et al., 1999; Jauniaux et al., 2000; Jauniaux et al., 2001; Lackman et al., 2001). Therefore, it is plausible that the fetal phenotype of the chorionic plate arterial SMCs, coupled with the relatively low oxygen tensions the fetoplacental vasculature is exposed to in vivo, explains the prominence of $BK_{Ca}$ currents over other $K^+$ channels in these cells.

In fetal vascular SMCs, the $BK_{Ca}$ isoform is crucial in controlling contraction and relaxation (Reeve et al., 1998; Snetkov et al., 1998; Rhodes et al., 2001). However, other $K_{Ca}$ isoforms, including $IK_{Ca}$, are also expressed in fetal and adult vascular SMCs which have a synthetic rather than a contractile phenotype (Neylon, 2002; Beech & Cheong, 2006). Synthetic SMCs are highly proliferative and have an important role in vasculogenesis in fetal blood vessels and the vascular remodelling process seen in atherosclerosis in the adult (Owens, 1995; Owens et al., 2004; Rensen et al., 2007).

Given the finding that chorionic plate arterial SMCs have a mixed phenotype and express proteins specific to both contractile and synthetic SMCs, it was investigated whether $K^+$ channels localised to synthetic SMCs, namely $IK_{Ca}$, were functional in these cells. Activation of $IK_{Ca}$ channels with the pharmacological agent 1-EBIO at a concentration used in previous studies (Edwards et al., 1999; Sones et al., 2009), produced a significant increase in outward currents (Figure 3-22). 1-EBIO increased currents in all cells tested irrespective of whether STOCs were present or absent under basal conditions suggesting that 1-EBIO is not activating $BK_{Ca}$ channels which mediate STOCs. This was later confirmed following application of the $BK_{Ca}$ inhibitor iberiotoxin which had no effect on 1-EBIO sensitive currents (Figure 3-24). The increase in current was confirmed to be predominantly due to activation of $IK_{Ca}$ channels as the 1-EBIO sensitive current was abolished by TRAM-34 (Figure 3-25). TRAM-34 application alone had no effect on whole-cell currents suggesting $IK_{Ca}$ channels are closed under the
control conditions used in the present study (Figure 3-20). Therefore, IK$_{Ca}$ channels require activation with 1-EBIO to permit K$^+$ efflux as activation of these channels by intracellular Ca$^{2+}$ requires concentrations to reach 0.1 µM (Ishii et al., 1997; Joiner et al., 1997), and the estimated value in the pipette solution is 10 nM.

Expression and function of IK$_{Ca}$ channels in chorionic plate arterial SMCs (see section 3.1.4.3 and Figure 3-25), is a novel finding for both the fetoplacental and systemic vasculature. The general consensus in vascular physiology is that IK$_{Ca}$ channels are localised to the endothelium as both immunohistochemical and electrophysiological studies fail to observe IK$_{Ca}$ expression or function in the SM (Edwards et al., 1998; Edwards et al., 1999; Busse et al., 2002; Sones et al., 2009; Garland et al., 2010). Similar electrophysiology experiments performed in rat hepatic arterial myocytes (Edwards et al., 1999) and mouse portal vein SMCs (Sones et al., 2009), failed to observe any effect of 1-EBIO application on whole-cell K$^+$ currents. This contrasts the present study whereby 1-EBIO increased currents in chorionic plate arterial SMCs at +80 mV by over 400 % (Figure 3-22F). Although IK$_{Ca}$ channels are not thought to reside in SMCs from systemic vessels that primarily function to control vascular resistance, IK$_{Ca}$ channels are expressed in cultured SMCs with a synthetic phenotype and in injured vessels undergoing vascular remodelling (Neylon et al., 1999; Neylon, 2002; Kohler et al., 2003; Beech & Cheong, 2006; Beech, 2007). Therefore, it is conceivable that the synthetic phenotype of chorionic plate arterial SMCs, coupled with their fetal origin, favours the functional expression of IK$_{Ca}$ channels. The physiological significance of IK$_{Ca}$ channels in chorionic plate arterial SMCs is unknown but they may play an important role in placental vasculogenesis throughout gestation, becoming redundant in this role near to term when placental growth is essentially complete. However, it remains to be determined whether chorionic plate arterial SMCs can proliferate per se and whether this is mediated by IK$_{Ca}$ channels. This would provide functional confirmation of the synthetic phenotype of chorionic plate arterial SMCs.

It is evident that the majority of the 1-EBIO sensitive current is due to activation of IK$_{Ca}$ channels. However, a small but significant component can be attributed to opening of SK$_{Ca}$ channels as the 1-EBIO sensitive current was reduced following addition of the SK$_{Ca}$ inhibitor apamin at a submaximal concentration used in previous studies (Figure 3-26) (Edwards et al., 1998; McNeish et al., 2006). In common with IK$_{Ca}$ channels, SK$_{Ca}$ are thought to be predominantly localised to the endothelium where they participate in the EDHF response (Edwards et al., 1998; McNeish et al., 2006; Garland et al., 2010). Therefore, functional SK$_{Ca}$ channels in the chorionic plate arterial SMCs was unexpected and likely to be due to the SK$_{Ca}$3 isoform given mRNA expression for
the pore forming α-subunit in the intact artery (see section 3.2.3.1). The functional significance of SKCa3 channels in chorionic plate arterial SMCs is not known but they may be important in dampening excitation and promoting vasodilation following a rise in intracellular Ca²⁺ in a similar manner as BKCa channels.

In summary, chorionic plate arterial SMCs are a heterogeneous population of cells, with electrically distinct properties implicating the presence of different populations of K⁺ channels. Recordings are categorised into two groups according to the characteristics of the whole-cell currents; (1) recordings displaying STOCs, and (2) recordings containing a linear time-independent current only. STOCs and linear currents are mediated by two independent events and therefore channels. BKCa channels are responsible for STOCs and the linear current by voltage-gated K⁺ channels including the Kv1.5 isoform. Localisation of Kv1.5 and BKCa channels to the SM enables them to directly control excitation-contraction coupling, extrapolating to an alteration in fetoplacental vascular tone in the intact chorionic plate artery (see section 2.3.4) (Sand et al., 2006; Wareing et al., 2006a). KCa are the predominant channel family in chorionic plate arterial SMCs as the majority of cells displayed STOCs. STOCs were highly variable between cells suggesting a possible heterogeneity in BKCa channel activity and/or expression in the SMCs within the vessel wall. This will ultimately impact upon the ability of chorionic plate arterial SMCs to act in a coordinated manner to regulate fetoplacental vascular tone. In addition to BKCa, both IKCa and SKCa channels are functional in chorionic plate arterial SMCs, which was an unexpected finding and might relate to SMC phenotype. The physiological significance of IKCa and SKCa within the SMCs is unclear but implicates a potential novel function for vascular SMCs, and the underlying K⁺ channels, to control both vasculogenesis and vascular resistance in the placenta. Other K⁺ channels identified by immunostaining or shown to modulate chorionic plate artery reactivity, including KATP or K2P isoforms, may contribute to whole-cell currents in chorionic plate arterial SMCs under the correct recording conditions (see section 3.1.3.3) (Wareing et al., 2006a; Wareing et al., 2006e; Jewsbury et al., 2007). In conclusion, this study provides the first direct evidence for functional Kv1.5, BKCa, IKCa and SKCa channels localised to chorionic plate arterial SMCs where they participate in many aspects of SMC physiology.
4. GENERAL DISCUSSION

Blood flow through the fetoplacental vasculature needs to be appropriately regulated to ensure maximal exchange of nutrients and oxygen to the growing fetus. The diameter of a blood vessel primarily determines the amount of blood that can pass along its length. Vessel diameter is altered by the level of constriction and relaxation of the vascular smooth muscle (SM) which is in turn controlled by the movement of ions across the SMC membrane through channels. $K^+$ channels are particularly important in determining the excitability of SMCs, as they are responsible for maintaining the resting membrane potential. Modulation of $K^+$ channel opening by circulating vasoactive agents and the prevailing oxygenation underlies SMC constriction and relaxation. Excitation-contraction coupling requires localisation of $K^+$ channels to the SMC membrane. In the fetoplacental vasculature, altered oxygenation and $K^+$ channels are known to modulate vascular tone. However, studies confirming both expression and function of these channels to the SMCs are lacking. This thesis aimed to determine the $K^+$ channel profile in chorionic plate arterial SMCs which have the potential to regulate vascular tone in response to altered oxygenation. Using an integrated approach and various experimental techniques, this thesis has identified several $K^+$ channels that are expressed and functional in chorionic plate arteries including isoforms that are not thought to reside in systemic vascular SMCs in vivo under physiological conditions. The $K^+$ channel isoforms identified may serve as therapeutic targets to improve fetoplacental blood flow, and therefore nutrient and oxygen delivery to the fetus, in pregnancy complications associated with raised vascular resistance.

4.1. Summary of Findings

Expression of the candidate “$O_2$-sensitive $K^+$ channel” $K_v1.5$, has been demonstrated at both the mRNA and protein level in chorionic plate arteries. $K_v1.5$ channels are localised to the SMCs of chorionic plate arteries as demonstrated using immunocytochemical and electrophysiological methods. In the intact artery, inhibition of $K_v1.5$ channels did not markedly modulate vasoconstrictor responses to the thromboxane mimetic U-46619. There is a trend towards an enhanced constriction following DPO-1 application under acute normoxic conditions which does not reach statistical significance potentially due to confounding effects within the intact vessel. It
is possible that \( K_v1.5 \) channels do not have a prominent role in regulation of chorionic plate arterial tone but are involved in other aspects of vascular SMC physiology.

A prominent role for \( \text{Ca}^{2+} \)-activated \( K^+ \) channels (\( K_{\text{Ca}} \)) in chorionic plate arteries has also been highlighted in this thesis. The majority of whole-cell \( K^+ \) current in chorionic plate arterial SMCs is mediated by the \( \text{BK}_{\text{Ca}} \) channel, and \( \text{BK}_{\text{Ca}} \) channel protein is also expressed in these isolates. Consolidation of this data confirmed both mRNA and protein expression for the \( \text{BK}_{\text{Ca}} \alpha \)-subunit in the intact vessel. Other members of the \( K_{\text{Ca}} \) family, \( \text{IK}_{\text{Ca}} \) and \( \text{SK}_{\text{Ca}} \), can also be activated and contribute to whole-cell \( K^+ \) currents in chorionic plate arterial SMCs. This has not been previously observed in SMCs freshly isolated from non-placental vessels. \( \text{SK}_{\text{Ca}} \) (\( \text{SK}_{\text{Ca}3} \)) and \( \text{IK}_{\text{Ca}} \) mRNA transcripts are expressed \textit{in situ} in chorionic plate arteries. In addition, \( \text{IK}_{\text{Ca}} \) channel protein is localised to the isolated SMCs and SMCs in the intact artery.

\( \text{KIR}6.1 \) (\( K_{\text{ATP}} \)) channels are expressed at both the mRNA and protein level in chorionic plate arteries and the isolated SMCs. Opening \( K_{\text{ATP}} \) channels by pharmacological means has a profound vasodilatory effect on chorionic plate arteries and are likely to be functional in the isolated SMCs (Jewsbury et al., 2007). Contribution of these channels to basal whole-cell currents was not expected under the recording conditions used in the present study, as \( \text{Mg}^{2+} \)-nucleotides were absent from the intracellular solution which are required for channel activation. However, \( K_{\text{ATP}} \) channel openers may elicit an effect but this was not explored in the current study.

The results from this thesis demonstrating functional \( K_v1.5, \text{BK}_{\text{Ca}}, \text{IK}_{\text{Ca}} \) and \( \text{SK}_{\text{Ca}} \) channels localised to the chorionic plate arterial SM has two major implications for fetoplacental vascular physiology. It firstly questions the worth of the common analogy of the fetoplacental vasculature with the pulmonary circulation, particularly with respect to the predominance of \( K_v \) channels and their direct regulation by oxygen. Secondly, this study provides evidence for a novel role for chorionic plate arterial SMCs during normal pregnancy. Underpinning this hypothesis is the observation of multiple SMC phenotypes, which are potentially regulated by \( K^+ \) channels in chorionic plate arteries. The significance of these findings may have particularly important implications when extrapolated to pregnancy pathologies, such as fetal growth restriction, where alterations in both oxygenation and fetoplacental vascular resistance are observed.
4.2. Physiological Relevance and Interpretation

4.2.1. Fetoplacental vasculature Vs. Pulmonary vasculature: Regulation by oxygen

For many years the placenta has been described as the “fetal lung” as it is the site of gaseous exchange for the fetus throughout gestation. This analogy is strengthened when the similarities between the anatomy of the placental and pulmonary vasculature are considered. In both the lung and placenta, the arterial and venous systems carry deoxygenated and oxygenated blood respectively, and oxygen exchange occurs within the small capillaries. This has led to the suggestion that fetoplacental vascular tone is regulated in a similar manner as the lung. In particular, the important role oxygen plays in controlling pulmonary vascular tone has been adopted for the placenta given its lack of innervation and reliance on oxygen and vasoactive agents to determine fetoplacental vascular resistance.

The mechanism of hypoxic fetoplacental vasoconstriction (HFPV) has been proposed in the placenta as a means by which oxygen can regulate placental blood flow to ensure optimal nutrient and oxygen transfer to the fetus. A HFPV response would divert blood flow to highly perfused areas of the placenta rich in oxygen to facilitate appropriate matching of fetoplacental blood flow with the maternal circulation (Howard et al., 1987; Hampl et al., 2002). This is analogous to ventilation-perfusion matching in the lung and hypoxic pulmonary vasoconstriction (HPV) whereby pulmonary vessels supplying a poorly ventilated and therefore hypoxic lobule constrict (Sweeney & Yuan, 2000; Moudgil et al., 2005; Remillard & Yuan, 2005; Murray et al., 2006). As discussed previously (section 1.3.4), the physiological significance of a HFPV mechanism has been questioned as only a small change in perfusion pressure has been observed following a large alteration in oxygenation, over a range of pO$_2$ that is not thought to reside in the placenta in vivo. The results from the current study support this view as short term exposure to a range of oxygenations thought to exist in the placenta throughout normal pregnancy (20 %, 6 % and 2 % O$_2$), did not alter constriction to the thromboxane mimetic U-46619 in chorionic plate resistance arteries. If the site of HFPV resided within these vessels that primarily determine fetoplacental vascular resistance, enhanced constriction may have been expected as oxygenation decreased. The absence of an acute HFPV response in chorionic plate arteries over a physiological oxygenation gradient is supported by previous studies using both wire and pressure myography (Wareing et al., 2006b; Wareing et al., 2006c). In the lung, prolonged
exposure to hypoxia induces a chronic HPV response that results in sustained constriction, raised pulmonary vascular resistance and ultimately pulmonary hypertension (McMurtry et al., 1978; Pozeg et al., 2003; Remillard & Yuan, 2005; Bonnet & Archer, 2007). In this thesis, chronic (48h) exposure of chorionic plate arteries to placental hypoxia did not increase constriction. Therefore, mounting evidence from this thesis and others suggests fetoplacental resistance arteries are dissimilar to the pulmonary vasculature as both acute and chronic exposure to a level of hypoxia that is physiologically relevant for the placenta, does not promote vasoconstriction.

In pulmonary resistance arteries, HPV is mediated by K* channels localised to the SMCs. Hypoxia is thought to inhibit specifically a Kv channel which results in membrane depolarisation, Ca2+ influx and vasoconstriction (Post et al., 1992; Archer et al., 1998; Gurney et al., 2002; Joshi et al., 2009; Morecroft et al., 2009). The identity of the Kv channel that mediates HPV is under debate; however, there is strong evidence supporting a role for the "O2-sensitive K* channels", Kv1.5 and Kv2.1 (Archer et al., 1998; Archer et al., 2001; Pozeg et al., 2003; Archer et al., 2004). In the fetoplacental vasculature, hypoxia has been shown to inhibit Kv channels yet the hypoxic stimulation used in these experiments is not physiologically relevant for the placenta (Hampl et al., 2002). In this thesis, broad spectrum inhibition of Kv channels with 4-AP enhanced basal tension in chorionic plate arteries irrespective of whether the experiment was performed under placental normoxia or hypoxia. These results imply that Kv channels in these vessels are open under these oxygenations and susceptible to block by 4-AP. Therefore, in contrast with the lung, placental hypoxia per se does not inhibit Kv channels and therefore increase chorionic plate artery constriction. Similarly, this study does not support a role for Kv1.5 channels in mediating a potential hypoxic constriction response in placental resistance arteries as inhibition of this isoform with DPO-1 had no effect on basal- and agonist-induced tone at all oxygenations.

The electrophysiological properties of chorionic plate arterial SMCs have been compared to pulmonary resistance arterial SMCs, suggesting a predominance of Kv channels to whole-cell K* currents (Hampl et al., 2002). However, the patch clamp data performed in this thesis using SMCs isolated from small resistance (<500 μM) chorionic plate arteries suggest Kv, and in particular Kv1.5 channels, only make a small contribution to whole-cell currents. Inhibition of Kv channels with 4-AP significantly inhibited currents at relatively negative potentials but had no effect on the majority of the whole-cell current. The predominant channel responsible for currents in freshly isolated chorionic plate arterial SMCs was BKCa as outward currents were abolished by
TEA, charybdotoxin and iberiotoxin. Again, this provides further evidence that the analogy of placenta and lung is incorrect when considering the response of the fetoplacental vasculature to $K^+$ channels and oxygenation.

The predominance of $BK_{Ca}$ channel currents in chorionic plate arterial SMCs may be an important clue for identifying other vascular systems which have a comparatively similar vascular physiology to the placenta. Studies in vascular and non-vascular SMCs isolated from the fetal lung demonstrate the dominance of $BK_{Ca}$ over $K_v$ currents in whole-cell recordings (Cornfield et al., 1996; Reeve et al., 1998; Snetkov et al., 1998; Resnik et al., 2006a; Resnik et al., 2006b). In fetal pulmonary SMCs, $BK_{Ca}$ channels serve as regulators of the resting membrane potential (Cornfield et al., 1996; Reeve et al., 1998; Snetkov et al., 1998); a role which is favoured by $K_v$ channels in mature adult pulmonary SMCs (Snetkov et al., 1996; Archer et al., 1998; Gurney et al., 2002; Joshi et al., 2009). An increased contribution from $BK_{Ca}$ channels in fetal pulmonary SMCs is thought to be related to the prevailing oxygenation as oxygen regulates both expression and activity of this channel (Miller et al., 1993; Gebremedhin et al., 1994; Resnik et al., 2006a; Zhao et al., 2007). The fetal lung develops under conditions of prolonged hypoxia which is hypothesised to down-regulate $K_v$ channel expression and favour increased $BK_{Ca}$ (Resnik et al., 2006a; Resnik et al., 2006b). During maturation of the lung following delivery of the fetus, inspiration of oxygen in the lung promotes $K_v$ channel upregulation and a switch in the predominant $K^+$ channel in adult pulmonary vascular SMCs to $K_v$ (Reeve et al., 1998). These observations may be of particular relevance to the fetoplacental vasculature as it is known that throughout gestation the placenta is exposed to a low oxygen environment (Jauniaux et al., 2000; Burton & Caniggia, 2001; Lackman et al., 2001; Martini, 2006a). The demonstration in this thesis that $BK_{Ca}$ channels are the predominant current in chorionic plate arterial SMCs, may therefore be a direct consequence of the reduced oxygenations experienced by the placenta in vivo compared to non-placental vascular beds. The relative hypoxia within the placenta may favour $BK_{Ca}$ channels and reduce the contribution from $K_v$ channels to whole-cell currents. Therefore, the fetal pulmonary system may be a more physiologically relevant vascular bed to drawn comparisons with the placenta, particularly in terms of oxygenation and $K^+$ channel expression/function.
4.2.2. Smooth muscle cell phenotype: Implications on fetoplacental vascular physiology

Characterisation of the SMCs isolated from chorionic plate arteries in this thesis led to the discovery that they display a mixed phenotype with both contractile and synthetic properties. This observation strengthens the proposal that the fetoplacental vasculature has many similarities with the fetal pulmonary system, as SMC phenotype is determined in part by the maturational status of the vessel (Owens, 1995; Owens et al., 2004). SMCs within fetal blood vessels, including those within the fetal lung, have a synthetic phenotype and are highly proliferative and migratory (Frid et al., 1994; Mironov et al., 1995; Snetkov et al., 1998). This enables fetal vascular SMCs to participate in vasculogenesis and angiogenesis during embryonic growth and development (Sobue et al., 1999; Rensen et al., 2007). SMCs within adult blood vessels primarily function to control vascular resistance and therefore blood flow. These cells display a contractile phenotype as the cytoplasm contains the contractile machinery necessary for the regulation of tone (Owens, 1995; Rensen et al., 2007).

In this thesis, chorionic plate arteries and SMCs isolated from these vessels expressed markers for both contractile and synthetic phenotypes. The contractile phenotype of these cells permits contraction and relaxation of chorionic plate arteries and regulation of fetoplacental blood flow. This is necessary for maximal nutrient and oxygen transfer to the fetus and therefore adequate fetal growth. A synthetic SMC phenotype would be of physiological relevance during early pregnancy where vasculogenesis and angiogenesis are prominent (Benirschke, 1998; Benirschke & Kaufmann, 2000; Gude et al., 2004; Arroyo & Winn, 2008). During the third trimester of pregnancy, a synthetic phenotype may enable the SMCs to participate in the final stages of angiogenesis, thereby allowing the continued enlargement of chorionic plate arteries to sustain the increasing demands from the fetal heart. Maintenance of a synthetic phenotype throughout gestation may also be the result of the composition and architecture of the vessel wall. Within the chorionic plate arterial wall there is an abundance of extracellular matrix and collagen between the SMCs (Sweeney et al., 2006). SMC phenotype is highly plastic and sensitive to environmental cues; therefore, the excess connective tissue surrounding the SMCs may release factors or signalling molecules that preserves a synthetic phenotype (Rensen et al., 2007). The large spaces and apparent lack of organisation between chorionic plate arterial SMCs resulting from excess connective tissue, coupled with a synthetic phenotype, may explain why the placental vasculature is relatively unresponsive to many of the vasoactive agents that are potent in the systemic circulation. Taken together, the results from this thesis...
implicates a dual role for chorionic plate arterial SMCs to control both vascular growth and fetoplacental vascular resistance throughout pregnancy.

The speculation that a mixed SMC phenotype is significant for fetoplacental vascular physiology is strengthened with the observation of diverse K⁺ channel families localised to these cells. SMC phenotype influences K⁺ channel expression, which in turn allows SMCs to perform a variety of functions. Voltage-gated K⁺ channels (Kᵥ), ATP-sensitive K⁺ channels (KᵥATP) and the Ca²⁺-activated K⁺ channel isoform BKᵥCa, participate in excitation-contraction coupling and therefore predominate in SMCs with a contractile phenotype (Ko et al., 2008). The main K⁺ channel expressed in SMCs with a synthetic phenotype is the Ca²⁺-activated K⁺ channel isoform IKᵥCa (Neylon et al., 1999). IKᵥCa channels maintain the resting membrane potential in synthetic SMCs and promote Ca²⁺ entry and consequently proliferation during vessel growth (Neylon et al., 1999; Neylon, 2002). This thesis has identified Kᵥ1.5, BKᵥCa, KᵥATP, IKᵥCa and SKᵥCa channels in chorionic plate arterial SMCs, and therefore K⁺ channels which are localised to SMCs with both a contractile and synthetic phenotype. This suggests a possible synergy between phenotype and K⁺ channel expression/ function in chorionic plate arterial SMCs.

In the fetoplacental vasculature, localisation of Kᵥ1.5, BKᵥCa, and KᵥATP channels to the vascular SM are likely to be important mediators of excitation-contraction coupling following stimulation by vasoactive agents and altered oxygenation (see sections 2.3.4, 3.2.3.1, 3.1.3.3 and 3.3.3) (Wareing et al., 2006a; Wareing et al., 2006e; Jewsbury et al., 2007). As discussed in section 3.3.4, the predominance of BKᵥCa over Kᵥ channels in whole-cell recordings most likely reflects the fetal origin of chorionic plate arteries and exposure to reduced oxygen tensions, as has been suggested in the fetal lung. Although inhibition of BKᵥCa channels with iberiotoxin significantly increased U-46619 constriction in chorionic plate arteries (Sand et al., 2006; Wareing et al., 2006a), the magnitude of this response may have been predicted to be greater given the dominance of BKᵥCa to total K⁺ currents. However, BKᵥCa channels may have a more prominent role in mediating vasodilation following stimulation by endogenous vasoactive agents or potential therapeutic agents; this remains to be explored.

Localisation of IKᵥCa channels to chorionic plate arteries suggests a potential involvement in vascular growth during early pregnancy. By the end of the second trimester, vasculogenesis and angiogenesis are largely complete with only minor adaptations occurring in the third trimester to meet the increasing demand of the fetal circulation (Benirschke, 1998; Gude et al., 2004; Arroyo & Winn, 2008). Continued expression and function of IKᵥCa channels in chorionic plate arterial SMCs at term may
suggest an involvement in these processes. Alternatively, these channels may be functionally redundant with sustained expression reflecting the mixed SMC phenotype with synthetic characteristics. Further studies are required to determine the physiological significance of $\text{IK}_{\text{Ca}}$ channels in the fetoplacental vasculature at term.

Confirmation that a relationship between SMC phenotype and $K^+$ channel expression/function exists in the placental vasculature is apparent in the vessel culture studies performed in section 3.2. Vessel culture promotes alterations in the structure, function and composition of the arterial wall (De Mey et al., 1989; Lindqvist et al., 1999; Guo et al., 2008). Addition of fetal calf serum to the culture media is thought to promote SMC proliferation and down-regulation of the contractile filaments; an effect that mimics modulation into a synthetic phenotype (Guo et al., 2008; Zheng et al., 2010). Vessel culture is also associated with alterations in $K^+$ channel expression, which may be the result of SMC phenotypic modulation and/or reduced oxygen availability within the vessel wall (Manoury et al., 2009). Chorionic plate artery culture promoted changes in the relative abundance of $K^+$ channels compared to freshly isolated arteries (see section 3.2.3.2). $K^+$ channels predominantly localised to SMCs with a contractile phenotype, $K_{v1.5}$, $K_{\text{ATP}}$, and $\text{BK}_{\text{Ca}}$, were most abundant in freshly isolated chorionic plate arteries (see Figure 3-11). However, culture of chorionic plate arteries for 48 h under conditions of placental normoxia down-regulated expression of $K_{v1.5}$, and $K_{\text{ATP}}$ and increased expression of the $\text{IK}_{\text{Ca}}$ channel which predominates in SMCs with a synthetic phenotype (see Figure 3-12). Expression of $\text{BK}_{\text{Ca}}$ channels was increased following vessel culture (see Figure 3-12). If modulation to a synthetic SMC phenotype occurs in chorionic plate arteries in culture, down-regulation of $\text{BK}_{\text{Ca}}$ channels may be expected as they are localised to SMCs with a contractile phenotype where they control excitation-contraction coupling. However, the thickness of the vessel wall and subsequent reduced oxygen supply to the inner SMC layers of the cultured vessels may favour $\text{BK}_{\text{Ca}}$ expression (Miller et al., 1993; Gebremedhin et al., 1994; Resnik et al., 2006a; Zhao et al., 2007). This may explain why depolarisation- and agonist-induced constriction was maintained in the cultured vessels (see section 2.3.2). Although it remains to be determined whether alterations in the expression of contractile and synthetic phenotypic markers occur following 48 h culture under placental normoxia, these data suggest that $K^+$ channel expression may also be closely related to SMC phenotype in chorionic plate arteries.

In summary, chorionic plate arterial SMCs display a mixed phenotype with both contractile and synthetic characteristics. Underlying these phenotypes is the demonstration of $K^+$ channel isoforms specific to contractile and synthetic SMCs. $K_{v1.5}$,
BKCa and KATP channels may control excitation-contraction coupling and underlie the contractile phenotype of chorionic plate arterial SMCs. Conversely, the ability of chorionic plate arterial SMCs with a synthetic phenotype to control proliferation and migration during vasculogenesis may be mediated in part by IKCa channels. Taken together, the results from this thesis support the notion that chorionic plate arterial SMCs express distinct K⁺ channel isoforms, which presents them with a dual function to control both vascular resistance and vessel growth during normal pregnancy.

4.2.3. Implications for pregnancy pathologies: Fetal Growth Restriction

A dual role for chorionic plate arterial SMCs during normal pregnancy has important implications in pregnancy pathologies such as fetal growth restriction (FGR). FGR affects 3-5 % of all pregnancies and is a pathological condition whereby the fetus does not reach its genetic growth potential (Mandruzzato et al., 2008). The fetus has an increased risk of perinatal morbidity and mortality and developing cardiovascular disease in later life (Barker et al., 1990). Abnormal umbilical artery Doppler ultrasound measurements are common in FGR pregnancies, indicative of increased vascular resistance within the fetoplacental vasculature (Kingdom et al., 1997; Mills et al., 2005). Increased resistance on the fetal side can result from altered angiogenesis and/or vascular function in the fetoplacental vessels (Resnik, 2002; Kinzler & Vintzileos, 2008). Therefore, abnormalities in the ability of chorionic plate arterial SMCs to control both vascular tone and/or vessel growth, may account for aberrant blood flow in FGR.

Inadequate development and branching of the fetoplacental vessels is evident in FGR placentas (Krebs et al., 1996; Todros et al., 1999; Kingdom et al., 2000). Oxygen is an important modulator of vascular development as vasculogenesis and angiogenesis are stimulated by hypoxia (Arroyo & Winn, 2008). In cases of FGR characterised by impaired spiral artery remodelling, uteroplacental hypoxia exists within the intervillous space (Chang et al., 1994). This hypoxia in turn promotes an adaptive mechanism whereby angiogenesis is stimulated to produce a highly vascularised villous tree (Burton et al., 1996). This may explain why these cases of FGR are not associated with abnormalities in umbilical artery Doppler waveforms as impedance to blood flow is reduced (Kingdom et al., 1997; Mayhew et al., 2007). In early-onset FGR characterised by abnormal umbilical artery Doppler waveforms, fetoplacental vascular development is perturbed as non-branching angiogenesis predominates, resulting in long capillaries
within the villous tree and impaired blood flow (Krebs et al., 1996; Kingdom et al., 1997; Kingdom et al., 2000; Mayhew et al., 2004; Mayhew et al., 2007). Hypoxia is known to promote modulation of SMCs into a highly proliferative synthetic phenotype, which control vasculogenesis and angiogenesis (Frid et al., 1994; Wohrley et al., 1995; Zhou et al., 2007). This thesis has demonstrated that SMCs within the chorionic plate arteries at term display a mixed phenotype with synthetic characteristics. If this phenotype is extrapolated to the stem villous vessels, the changes in fetoplacental vascular development in FGR may reflect differences in the activation and function of synthetic SMCs in accordance with the prevailing oxygenation. Analysis of chorionic plate arterial SMCs earlier in gestation may provide further information on their role in vascular development both in normal pregnancy and FGR.

Vascular resistance can also be increased in a vessel that has undergone structural remodelling and subsequent occlusion of the lumen (Durmowicz & Stenmark, 1999). Growth, proliferation and migration of SMCs with a synthetic phenotype underlies vascular remodelling (Regan et al., 2000; Remillard & Yuan, 2005; Moudgil et al., 2006). If the local environment surrounding the fetoplacental SMCs in FGR is altered to favour a synthetic over a contractile phenotype, SMC proliferation may be initiated and subsequently the remodelling process stimulated. This in turn may account for the raised vascular resistance seen in this pregnancy complication. Preliminary evidence suggests alterations in the lumen:wall ratio and SMC proliferation rate in chorionic plate arteries is unaltered in FGR compared to normal pregnancy (Sampson et al., 2010). However, it must be noted that this study utilised FGR samples diagnosed at term where abnormal umbilical artery Doppler measurements were not always observed. Therefore, vascular remodelling may be evident in early-onset severe FGR where raised vascular resistance is apparent from Doppler ultrasound.

$K^+$ channels control many aspects of SMC physiology including vascular tone and vessel growth. Raised fetoplacental vascular resistance in FGR may result from altered expression and/or function of $K^+$ channels, which mediate excitation-contraction coupling and SMC proliferation. The mechanisms underlying vasculogenesis and angiogenesis in non-placental vascular beds involve activation of $IK_{Ca}$ channels in SMCs with a synthetic phenotype (Neylon et al., 1999; Neylon, 2002). $IK_{Ca}$ channel activation promotes membrane hyperpolarisation which favours $Ca^{2+}$ influx and therefore activation of many $Ca^{2+}$-dependent growth factors (Neylon, 2002; Beech & Cheong, 2006). The demonstration of functional $IK_{Ca}$ channels in chorionic plate arterial SMCs isolated from term placertas suggests that these channels may play a role in vascular development throughout gestation and their function perturbed in FGR.
**K⁺ channels** may also serve as a therapeutic target to improve fetoplacental blood flow in FGR in the third trimester. K⁺ channels that control excitation-contraction coupling may be exploited as a strategy to induce dilation of the fetoplacental vasculature and therefore increase oxygen and nutrient delivery to the severely compromised fetus. The K⁺ channel isoforms Kᵥ1.5, KᵥATP and BKᵥCa which have been identified in chorionic plate arterial SMCs may serve as potential targets for promoting vasodilation. Although these isoforms are localised in non-placental vascular beds, targeting these channels with vasodilatory agents may serve as a final attempt to increase blood flow to the placenta in severe cases of FGR where there is a high risk of fetal mortality. Ideally, selectively targeting a K⁺ channel within the fetoplacental vascular SM would minimise any potential cross-reactions with non-placental vascular beds. With this mind, IKᵥCa channels may be a likely target as pharmacological agents that open these channels had a profound effect on whole-cell K⁺ currents in chorionic plate arterial SMCs, but are ineffective in SMCs isolated from the systemic circulation. The large increase in K⁺ efflux elicited following IKᵥCa opening in placental SMCs should promote vasodilation as these cells also display a contractile phenotype and therefore potential to mediate excitation-contraction coupling. It is currently unknown whether this increase in K⁺ efflux extrapolates to vasodilation in the intact vessel or perfused placental cotyledon. However, it may prove an effective strategy to reduce vascular resistance in FGR given the significant contribution from IKᵥCa currents in SMCs isolated from fetoplacental resistance vessels.
4.3. Future Work

Future work arising from this thesis can be subdivided according to the two main findings that (1) chorionic plate arterial SMCs display a mixed phenotype and (2) chorionic plate arterial SMCs express diverse $K^+$ channel isoforms. Further studies can also aim at understanding how these findings are applicable to fetal growth restriction;

4.3.1. Chorionic plate arterial SMCs: Phenotype

- Assess mRNA expression of phenotypic markers in single chorionic plate arterial SMCs using laser dissection and single-cell PCR to determine if expression differs between cells

- Assess mRNA expression of phenotypic markers in freshly isolated chorionic plate arteries and compare with expression in vessels cultured for 48h under placental normoxia to determine whether culture promotes phenotypic modulation

- Assess the proliferative potential of chorionic plate arterial SMCs to determine what the functional implications are of the synthetic phenotype

- Isolate SMCs from chorionic plate arteries earlier in gestation and characterise their phenotype to determine whether gestation affects SMC phenotype

4.3.2. Chorionic plate arterial SMCs: $K^+$ channel expression and function

- Assess mRNA expression of $K_v 1.5$, $BK_{Ca}$, $K_{ATP}$, $IK_{Ca}$, $SK_{Ca3}$ in single chorionic plate arterial SMCs

- Assess function of $K_v 1.5$ channels utilising different channel modulators in chorionic plate arteries and isolated SMCs using wire myography and electrophysiology
• Assess function of $K_{ATP}$ channels in chorionic plate arterial SMCs using electrophysiology.

• Assess function of $I_{K_{Ca}}$ channels in intact chorionic plate arteries with wire myography to determine whether $I_{K_{Ca}}$ opening with 1-EBIO mediates vasodilation.

• Assess function of $I_{K_{Ca}}$ channels in the perfused placental cotyledon to determine whether $I_{K_{Ca}}$ opening with 1-EBIO reduces fetal perfusion pressure.

• Assess the functional role of $I_{K_{Ca}}$ channels in chorionic plate arterial SMCs by applying channel modulators to determine their effect on cell proliferation.

4.3.3. Fetal Growth Restriction

• Assess mRNA and protein expression of phenotypic markers in chorionic plate arterial SMCs obtained from FGR placentas.

• Assess wall:lumen ratio in chorionic plate arteries from FGR placentas with abnormal umbilical artery Doppler measurements to determine if there is any evidence of vascular remodelling.

• Characterise whole-cell $K^+$ currents from chorionic plate arterial SMCs isolated FGR placentas and determine the contribution from $I_{K_{Ca}}$ channels.

• Apply $I_{K_{Ca}}$ channel opener 1-EBIO to an animal model of FGR to determine whether the resulting vasodilation improve placental blood flow and fetal growth.
4.4. Conclusion

This thesis has advanced our current understanding of the function of chorionic plate arterial SMCs in normal pregnancy and their regulation by K$^+$ channels. Chorionic plate arterial SMCs have a mixed phenotype with both contractile and synthetic characteristics which implicates a potential dual role for these cells throughout gestation to control both fetoplacental vascular resistance and vessel growth. These physiological functions may be regulated by the expression and function of distinct K$^+$ channel isoforms localised to SMCs with contractile and synthetic phenotypes. Understanding further the mechanisms underlying phenotypic modulation of SMCs in chorionic plate arteries, and how it is regulated by K$^+$ channels in both normal pregnancy and FGR, may highlight potential pathways that can be targeted to improve blood flow through the placenta and therefore nutrient and oxygen delivery to the fetus.
5. References


Sones WR, Leblanc N & Greenwood IA. (2009). Inhibition of vascular calcium-gated chloride currents by blockers of K\(_{\text{Ca}}\)1.1, but not by modulators of K\(_{\text{Ca}}\)2.1 or K\(_{\text{Ca}}\)2.3 channels. *Br J Pharmacol* **158**, 521-531.


6. APPENDIX

Immunofluorescence staining for SMC phenotypic markers (h-caldesmon, MHC-2, NMMHC-B) proved difficult when attempting to resolve positive staining above background or non-specific signal. Choosing the correct negative staining for comparison with the positive tissue is crucial when determining expression of the marker of interest. Substitution of primary antibody with non-immune IgG (Appendix 1B) resulted in non-specific binding of the secondary antibody and a background signal with similar intensity and pattern as the positive tissue (Appendix 1A). This was irrespective of the cell type, concentration of non-immune IgG or secondary fluorophore (FITC conjugated antibodies produced greater non-specific fluorescence than Alexa-568). The non-specific background fluorescence could be adequately compensated when antibodies are directed against a protein that is more abundantly expressed and therefore the staining intensity is markedly greater (e.g. α-SMA). Therefore, immunofluorescent studies were not suitable for assessing the expression of SMC phenotypic markers whose expression may be relatively low in the cell.

Appendix 1: Non-specific staining for SMC phenotypic markers. (A) Immunofluorescent staining for nuclei (blue; DAPI) and h-caldesmon (10μg/ml; red; Goat anti-Mouse IgG Alexa Fluor 568, A12380) in cultured SMCs. (B) Negative control in BeWo choriocarcinoma cell line: Goat anti-Mouse IgG Alexa Fluor 568 with substitution of primary antibody for non-immune IgG at 2.5μg/ml. (C) Negative control in BeWo choriocarcinoma cell line; Goat anti-Mouse IgG Alexa Fluor 568 only. Magnification x400.
Electrophysiology experiments were performed in morphologically distinct SMCs (n=12 arterial explant cultures) between 2 and 4 weeks in culture. High resistance, giga ohm seals were not obtained in any of the patched cells, with the maximum seal resistance achieved being approximately 200MΩ. In most cells tested, seal resistances would not increase further and as such, the whole-cell configuration could not be obtained. However, in 10 cells (N=3 cultures) the membrane spontaneously ruptured upon approach to the giga ohm seal and whole-cell currents were measured. Mean cell capacitance in these cells was 59.9 ± 6.48 pF but large leak currents were recorded (820 ± 26.3pA). Whole-cell patch clamp experiments initially utilised a KCl-rich intracellular solution (see solutions 3.3.2.3). Under these conditions, the mean current-voltage plot demonstrated a linear relationship with a reversal potential close to 0 mV (Appendix 2). This depolarised reversal potential was hypothesised to result from outward chloride current due to the presence of high intracellular chloride in the intracellular pipette solution (140 mM). Therefore, the intracellular solution was changed to one containing K-aspartate in an attempt to reduce contaminating chloride currents ([Cl] = 22 mM). Under these conditions, the mean current-voltage relationship in these cells (n=3) showed slight rectification and a reversal potential of approximately -10 mV (Appendix 2). Recordings in the cultured SMCs under all conditions were unstable, resulting in large leak currents as high resistance seals were not obtained. Therefore, it is not clear whether the currents measured are the result of ionic movement through membrane localised channels, or are a component of the leak current. This may explain the depolarised reversal potential and slightly rectified current-voltage relationships. Why high resistance seals could not be obtained in the cultured SMCs is unclear. However, the flat morphology of the single cells located at the edge of the colony outgrowths may not permit sufficient access of the patch pipette with the plasma membrane to establish a tight, high resistance seal. Passaging the cultured SMCs may slightly round up the cells as a result of the trypsin treatment. However, as SMC culture is associated with concomitant changes in the expression of many ion channels, including K⁺ channels, compared to the native tissue (Neylon et al., 1999; Tang & Wang, 2001; Cui et al., 2002; Miguel-Velado et al., 2005; Tharp et al., 2006), further optimisation of this cellular model was not performed.
Appendix 2: Mean current-voltage relationship for cultured smooth muscle cells. Recordings were made using both KCl (●) and K-aspartate (○) rich intracellular solutions. Mean (n = no. of cells; ±SEM for) current-voltage protocols demonstrate a near linear relationship, reversing close to 0 mV under control conditions. Cells were step depolarised for 100 ms from a holding potential of -60 mV to potentials between -120 and +90 mV in 10 mV increments.