The influence of the hormonal milieu on functional prostaglandin and oxytocin receptors and their downstream signal pathways in isolated human myometrium

Thesis presented by

Deborah Peninnah Fischer

To the University of Bradford for the degree of

DOCTOR OF PHILOSOPHY

Pharmacy Innovation Research Group

Bradford School of Pharmacy, University of Bradford

2010

Abstract

The influence of the hormonal milieu on functional prostaglandin and oxytocin receptors and their downstream signal pathways in isolated human myometrium

Deborah Peninnah Fischer

Although prostaglandins (PG) and oxytocin are crucial mediators of uterine contractility, their receptor-mediated effects during the menstrual cycle, pregnancy and labour are not fully understood. The aim of this thesis was to elucidate the functional expression of EP, FP, TP and oxytocin receptors in isolated human myometrium relative to myocyte mRNA and signal transduction pathways.

Myometrial samples were obtained from consenting non-pregnant and pregnant donors. Functional techniques were used to determine isometric muscle contractions. Primary uterine myocytes and fibroblasts were cultured at term to identify stimulated changes in calcium (Ca^{2+}), cyclic adenosine monophosphate (cAMP) and mRNA.

Myometrial strips exhibited spontaneous contractions, which were most active mid-cycle under oestrogenic conditions. At this time intrinsic contractility and responsiveness to uterotonins decreased towards the fundus. PGE_2 produced bell-shaped responses with predominant utero-relaxant effects mediated via the EP_2 subtype. Although activity was partially restored by PGE_2 through $EP_{3/1}$ receptors, tissue excitation was more pronounced at FP, TP and oxytocin receptors. Despite high FP mRNA expression, the lower segment uterus was particularly responsive to U46619 and oxytocin at term pregnancy. Even so, Ca^{2+} mobilisation by oxytocin was greater via principal release from intracellular stores. Incubations with atosiban, progesterone and a rho-kinase inhibitor reduced oxytocin-stimulated Ca^{2+} transients. EP_2 also attenuated oxytocic effects but this appeared to be mediated through cAMP rather than Ca^{2+} signalling pathways. With advancing labour, intrinsic myogenic activity declined in parallel with oxytocin desensitisation. However, TP-induced contractions were continued in the lower parturient uterus.

These findings demonstrate that PG and oxytocin receptor expression are regulated in a hormone-dependent temporal and spatial manner. EP_2-mediated cAMP formation appears to promote uterine quiescence, whilst TP receptors may control muscle tonus during parturition. These receptors and their messenger systems represent effective tocolytic targets for uterine hypercontractile disorders, such as dysmenorrhoea and preterm labour.

Keywords: prostaglandins; oxytocin; uterus; menstrual cycle; pregnancy; labour

Contents Page

Section II: Cell culture studies

Published work from thesis

Papers

D.P. Fischer; J.A. Hutchinson; D. Farrar; P.J. O'Donovan; D.F. Woodward; K.M. Marshall (2008) Loss of prostaglandin $F_{2\alpha}$, but not thromboxane, responsiveness in pregnant human myometrium during labour. *J Endocrinol*., 197(1): 171-9.

Presentations

D.P. Fischer; D. Farrar; P.J. O'Donovan; D.F. Woodward; K.M. Marshall (2010) Rho-kinase mediates oxytocin signalling in human myometrial cells. International Congress of Endocrinology, P11-1-7, Kyoto, Japan.

D.P. Fischer; D. Farrar P.J. O'Donovan, K.M. Marshall, D.F. Woodward (2009) Contractions induced by prostaglandins (PG) E_2 and $PGF_{2\alpha}$ are superseded by thromboxane in isolated human myometrium at term pregnancy. Society for Reproduction online, O15, Oxford, UK.

D.P. Fischer; D. Farrar; P.J. O'Donovan; D.F. Woodward; K.M. Marshall (2009) Functional pharmacology of prostaglandin E receptors in isolated human myometrium before and after labour-onset. Endocrine Abstracts online, 19, P301, Harrogate, UK.

D.P. Fischer; K.M. Marshall; D. Farrar; P.J. O'Donovan; D.F. Woodward (2009) Steroidogenic effects on oxytocin signalling in human myocytes at term pregnancy. Society for Fertility online, O30, Edinburgh, UK.

D.P. Fischer; D. Farrar; P.J. O'Donovan; D.F. Woodward; K.M. Marshall (2008) Predominant prostaglandin EP_2 receptor function in isolated human myometrium at term pregnancy. Proceedings of the British Pharmacological Society, P71, Brighton, UK.

D.P Fischer; D. Farrar; P.J. O'Donovan; D.F. Woodward; K.M. Marshall (2008) Cyclic AMP attenuates oxytocic effects in myometrium from term pregnant women. Society for Reproduction online, P85, Edinburgh, UK.

D.P. Fischer; P.J. O'Donovan; D. Farrar; D.F. Woodward; K.M. Marshall (2008) Topographical effects of functional prostanoid and oxytocin receptors on midcycle contractions in isolated human myometrium, European Congress of Endocrinology, P637, Berlin, Germany.

D.P. Fischer; D. Farrar; P.J. O'Donovan; D.F. Woodward; K.M. Marshall (2008) Desensitisation of oxytocin receptors with labour-onset in isolated human myometrium. Endocrine Abstracts online, P293, Harrogate, UK.

J.H. Durn; **D.P. Fischer**; K.M. Marshall; A. Nicolaou; D. Farrar; P.J. O'Donovan; D.F. Woodward (2007) A profile of prostanoids released from non-labouring human myometrial tissues at term using liquid chromatography coupled with electrospray ionisation mass spectrometry (ESI-LC-MS). Bioactive Lipids in Cancer, Inflammation and Related Diseases, P177. Montreal, Canada.

D.P. Fischer; D. Farrar; P.J. O'Donovan; D.F. Woodward; K.M. Marshall (2007) Oxytocin, prostaglandin $F_{2\alpha}$ and thromboxane-induced calcium mobilisation and contractility of isolated human myometrium from term pregnant donors. Bioactive Lipids in Cancer, Inflammation and Related Diseases, P141. Montreal, Canada.

D.P. Fischer; D. Farrar; P.J. O'Donovan; K.M. Marshall; D.F. Woodward (2007) Calcium-mediated oxytocic effects in human uterine myocytes. Society for Reproduction online, O35. York, UK.

D.P. Fischer; K.M. Marshall; N. Duckworth; D. Farrar; P.J. O'Donovan; D.F. Woodward (2007) Prostaglandin EP_2-mediated reduction of oxytocin-induced activity in isolated human myometrium. Endocrine Abstracts online, 13, P263. Birmingham, UK.

D.P. Fischer; K.M. Marshall; J.A. Hutchinson; D. Farrar; P.J. O'Donovan; D.F. Woodward (2006) Thromboxane mediated effects in isolated human myometrium at term labour. Proceedings of the British Pharmacological Society. Oxford, UK.

D.P. Fischer; J.A. Hutchinson; D. Farrar; P.J. O'Donovan; D.F. Woodward; K.M. Marshall (2006) Functional pharmacology of prostaglandin E receptor agonists in isolated human myometrium from term pregnant donors. Endocrine Abstracts online, 12, P112. London, UK.

D.P. Fischer; D. Farrar; P.J. O'Donovan; J. Senior; K.M. Marshall (2005) The effect of the hormonal milieu on the response of the isolated human myometrium to PGE_2. Endocrine Abstracts online, 10, OC23. London, UK.

K.M. Marshall; **D.P. Fischer**; J.A. Hutchinson; J. Senior; D. Farrar; P.J. O'Donovan; J. Chen; D.F. Woodward (2005) The effect of $PGF_{2\alpha}$, PGE_2 and U46619 on human myometrium from pregnant, non-labouring and labouring women. Prostaglandins & other Lipid Mediators, P138. San Francisco, USA.

Acknowledgements

Most of all I would like to thank Prof. Kay Marshall for her expert supervision, advice, support and ample encouragement throughout my time at Bradford. Her scientific input and critical appraisals of both my research and thesis have been invaluable. I am especially grateful for the wonderful opportunities to visit and gain experience at Allergan Inc. in sunny California as well as attending a number of conferences in the UK and abroad. I thank Allergan Inc. for funding my research and for their generous supply of compounds.

I wish to give special thanks to Dr. David Woodward from Allergan and Dr. Judith Senior whose advice and help with all things prostaglandins I much appreciate. I am also grateful to the Bradford School of Pharmacy for the provision of excellent research facilities within the department and to Jayne for all her help.

This research would not have been possible without the supply of human uterine muscle from the Bradford Royal Infirmary and Yorkshire Clinic. I would particularly like to thank Prof. Peter O'Donovan for collecting the samples from non-pregnant women and to Diane Farrar for obtaining consent from all of the labouring donors. I also wish to thank the very organised and friendly staff at the Maternity Unit and Nucleus Theatre for their large contribution of samples and support for our research at the University as well as the women of Bradford for their altruism.

For my functional studies, I would like to thank Rick, Mark and Darren for their technical expertise with the *in vitro* equipment. I am very grateful for the help and kind hospitality that I received from Jenny, Elaine, Nancy, Susan and Ta at Allergan. I would also like to thank Anna and Jon in lab M9c and Abba D, Wayne and Annie in lab K11 for their science-related chats, encouragement and great friendship. My past and present office friends, especially Anna, Natster, Jo and Uzmah, have given me much support and daily amusement. Also Jenny, Lisa, Nick-Nox and Stace have been excellent friends and Josh, from me to uteri.

Finally, I wish to thank my parents, Naomi & Yossi and Robs for their unfailing support and encouragement.

Abbreviations

Adenosine-5'-trisphosphate (ATP)
Adenylyl cyclase (AC)
Antibody (Ab)
Bovine serum albumen (BSA)
Calcium (Ca^{2+})
Calmodulin (Cal)
Carbon dioxide (CO_2)
Chemoattractant receptor-homologous molecule expressed on T helper 2 cells ($CRTH_2$)
Complementary DNA (cDNA)
Connexin (Cx)
Contractile-associated proteins (CAPs)
Cyclic adenosine monophosphate (cAMP)
Cyclooxygenase (COX)
Deoxyribonuclease I (DNase I)
Diacylglycerol (DAG)
Diethyl pyrocarbonate (DEPC)
Dimethyl sulfoxide (DMSO)
Distilled water (dH_2O)
Dulbecco's modified eagles medium (DMEM)
Early growth response factor-1 (ERG-1)
Enzyme acceptor (EA)
Enzyme donor (ED)
Enzyme fragment complementation (EFC)
Escherichia coli β-galactosidase (β-gal)
Ethylendiamine tetra-acetic acid (EDTA)
Extracellular signal-regulated kinase (ERK)
Fluorometric imaging plate reader (FLIPR-Tetra)
Fluorescent light units (FLU)
Foetal calf serum (FCS)
Follicle-stimulating hormone (FSH)
Glycogen kinase-3 (GSK-3)
Guanine nucleotide binding (G)-proteins
Guanosine-5'-trisphosphate (GTP)
Hanks' balanced salt solution (HBSS)
Inositol, 1,4,5-trisphosphate (IP_3)
Isobutylmethylxanthine (IBMX)
Luteinising hormone (LH)
Maloney murine leukaemia virus (M-MLV)

Messenger ribonucleic acid (mRNA)
Mitogen-activated protein (MAP) kinase
MLC_{20} phosphatase (MLCP)
Molar (M)
Moles (mol)
Myosin light chain (MLC_{20})
Myosin light chain kinase (MLCK)
Non-steroidal anti-inflammatory drugs (NSAIDs)
Nuclear factor kappa B (NF-κB)
17-β Oestradiol (E_2)
Oestrogen receptor (ER)
Optical densities (OD)
Optimal cutting temperature (OCT)
Oxygen (O_2)
Oxytocin (OT)
Oxytocin receptor (OTR)
PGE synthase (PGES)
Phosphate buffered saline (PBS)
Phosphatidylinositol 3-kinase (PI3K)
Phosphatidylinositol 4,5-bisphosphate (PIP_2)
Phospholipase A_2 (PLA_2)
Phospholipase C (PLC)
Progesterone (P_4)
Progesterone receptor (PR)
Prostaglandin $F_{2\alpha}$ receptor (FP): refer to Page 11 for nomenclature of prostanoids.
Prostanoids (PGs)
Protein kinase A (PKA)
Relative luminescence units (RLU)
Reverse transcription polymerase chain reaction (RT-PCR)
Rho-associated coiled coil-forming protein kinase (ROCK)
Ribonucleases (RNases)
Standard error of the mean (S.E.)
Vascular endothelial growth factor (VEGF)
β-mercaptoethanol (β-ME)

Chapter 1: Introduction

1.1 The human uterus

The uterus is a dynamic organ. During the reproductive years, it responds to the hormonal milieu (Europe-Finner *et al*., 1994; Lopez Bernal, 2001; Blanks *et al*., 2007) and to physical stimuli, such as the arrival of a blastocyst (Lim *et al*., 2002; Critchley & Saunders, 2009). Maternal physiological adaptations to the menstrual cycle, pregnancy and parturition can be dramatic. Even so, some events may be accompanied by common gynaecological disorders, including excessive blood loss (menorrhagia), menstrual cramps (dysmenorrhoea) and premature labour.

The uterus, a hollow fibromuscular organ, is supported deep within the pelvic cavity by broad, round and uterosacral ligaments. At puberty the uterus is pyriform in shape with two anatomical regions, the body or corpus and the cervix. The isthmus, a slight constriction, marks the junction of the cervix and the body. In a non-pregnant adult, the uterine body narrows from the fundus caudally to the cervix, which projects through the anterior wall of the vagina (Figure 1.1).

The uterine wall is composed of three layers: the outer serosa, the thick muscular layer (myometrium) and the inner mucosal membrane (endometrium), tapering the uterine cavity. In the non-gravid state, myometrial tissue can be further structured into external (stratum supravasculare), mid (stratum vasculare) and inner (stratum subvasculare) layers. Even so, the boundaries between each stratum are not well defined.

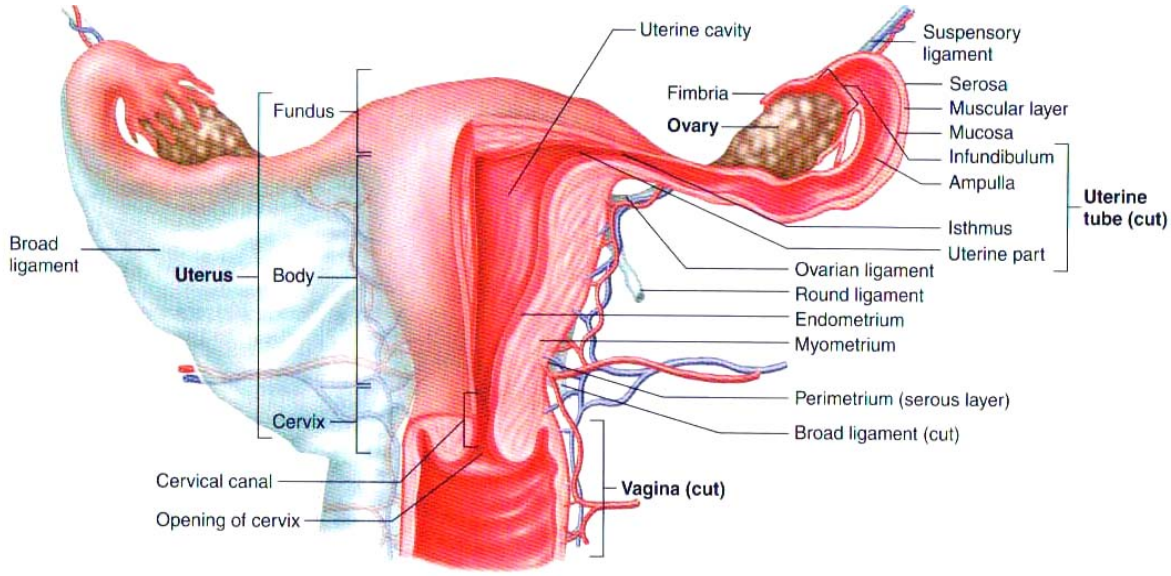


Figure 1.1: Anterior view of the uterus, uterine tubes and associated ligaments. Cut sections of the uterus and vagina illustrate their internal anatomy (Seeley *et al.*, 2006).

1.2 The menstrual cycle

The menstrual cycle is controlled by episodic changes in ovarian hormone secretion, which prepare the uterus to receive a fertilised ovum. In non-pregnant healthy women of reproductive age, the complete cycle is approximately 28 days in length. By convention, menses marks the first 4-5 days of the cycle, during which endometrial tissue is sloughed together with blood from ruptured arteries. After menstruation, circulatory oestrogen gradually rises due to its secretion by ovarian granulosa and theca cells within the developing dominant follicle. The binding of oestrogen to its abundant uterine receptors causes the endometrial cells and, to a lesser extent, the myometrial cells to proliferate (Yin *et al.*, 2007). Moreover, oestrogen induces the synthesis of specific receptors for progesterone (refer to Page 8). This primes the

uterus for progesterone binding after ovulation, signified by the transformation of the follicular cavity into the corpus luteum (Figure 1.2).

The subsequent luteal phase is dominated by progesterone secretion from the corpus luteum, concentrations of which peak at 7.48 ± 3.86 ng/ml between days 18 to 24 of the cycle (Erden *et al.*, 2005). During this phase, as well as antagonising oestrogen, progesterone suppresses myometrial activity and thickens the endometrial lining. Spiral arteries also fully develop in preparation for pregnancy. However, without successful fertilisation and implantation of the ovum, the corpus luteum regresses and progesterone and oestrogen synthesis declines. With the withdrawal of steroid support, uterine spiral arteries constrict causing ischaemia and oxidative damage to the endometrium. The shedding of the endometrial tissue then prepares the uterus for the next menstrual cycle.

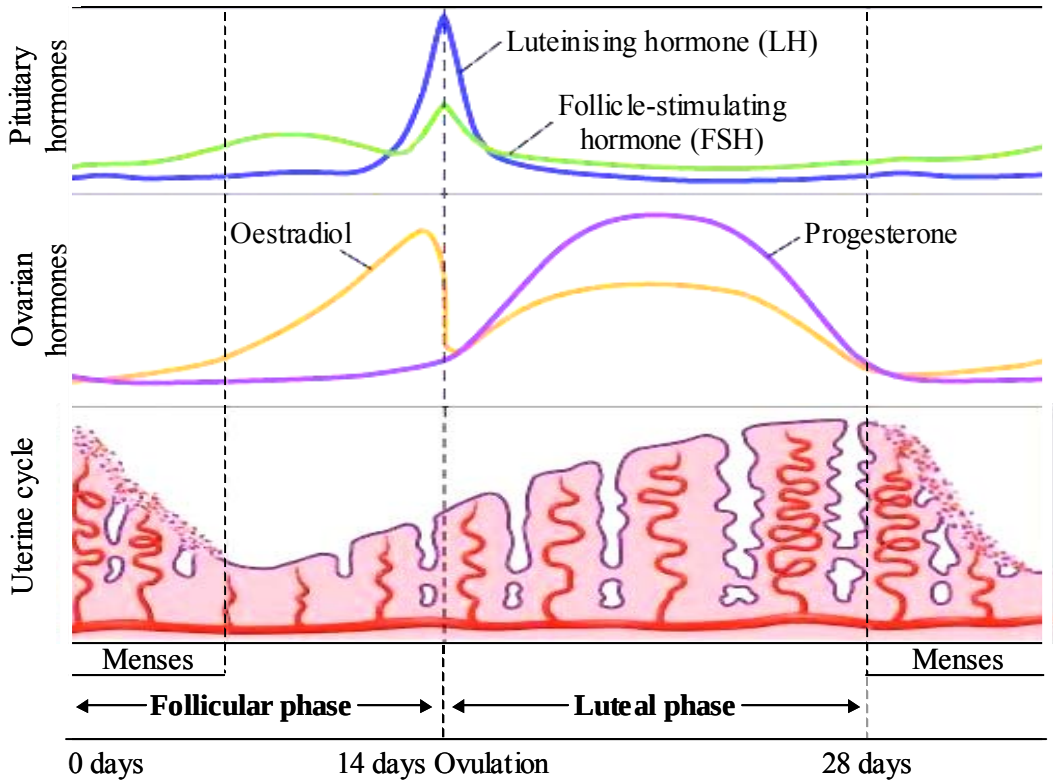


Figure 1.2: During a typical menstrual cycle, luteinising hormone (LH) stimulates the production of androgens by theca cells in the ovary, providing a substrate for oestrogen synthesis by ovarian granulosa cells. Follicle-stimulating hormone (FSH) facilitates follicle maturation and oestrogen-dependent proliferation of endometrial and myometrial cells. A mid-cycle surge in LH triggers ovulation, followed by a drop in FSH and LH release. At the site of the ruptured follicle a corpus luteum develops. This secretes progesterone and causes endometrial cells to differentiate and stabilise. If pregnancy is not established, menstruation results from endometrial shedding secondary to the rapid decline in oestrogen and progesterone synthesis from the demise of the corpus luteum (adapted from www.sciencedaily.com).

1.3 Myometrial structure

Although the myometrium is predominantly composed of smooth muscle cells, it also contains a heterogeneous arrangement of fibroblasts, blood vessels, lymphatic vessels and immune cells embedded in a matrix of connective tissue. In humans, the myometrial fibres tend to be oriented in a longitudinal direction close to the serosa and gradually form a circular muscle layer next to the endometrium (Weiss *et al*., 2006). However, the interconnection between bundles and numerous interspersed oblique fibres reduce any clear distinction between layers.

Human myometrial cells are long (300-600μm), narrow (5-10μm) and generally spindle-shaped (Finn & Porter, 1975). The complex cytoskeletal structure enables contractile forces to be generated within each cell and then transmitted through the myometrium via gap junctions, directly linking adjacent cells. Interaction with connective tissue collagen fibres facilitates the electrical coupling and transforms the uterus from its intrinsic relaxed state into an effective contractile syncytium. Although suppressed during pregnancy, the synchronous uterine contractions are phasic in nature and driven by action potentials propagated by pacemaker cells (Marshall, 1962). This myogenic activity imparts directional force essential for progressive cervical dilation and delivery of the foetus at parturition.

The structural basis of contractions involves actin and myosin filaments, which form cross-bridges to generate force. Myosin is a hexamer composed of two identical heavy chains and four light chains. As well as possessing actin binding-sites, myosin hydrolyses ATP to provide the energy required for contraction. As with other types of smooth muscle, the interaction between actin and myosin is regulated by the enzyme myosin light chain kinase (MLCK) (Word *et al.*, 1994). Action potentials depolarise the cell membrane causing calcium (Ca^{2+}) influx through voltage-operated channels

(Somlyo *et al.*, 1994). A crucial rise in intracellular-free Ca^{2+} promotes the binding of Ca^{2+} to calmodulin, which activates MLCK phosphorylating myosin light chain (MLC_{20}). As a result, myogenic activity is stimulated and amplitude, duration and frequency of contractions are enhanced. By contrast a rapid reduction in Ca^{2+} availability due to extrusion through the plasma membrane and Ca^{2+} uptake into the sarcoplasmic reticulum leads to muscle relaxation (Word *et al.*, 1994). This mechanism helps to maintain the uterus in an auto-inhibited, quiescent state during pregnancy.

1.4 Pregnancy

After conception, the uterus becomes conducive to blastocyst attachment and implantation to establish pregnancy. Embryonic trophoblast cells and maternal endometrial tissue develop to form the placenta, a specialised vascular region required for nutrient and waste product exchange between the mother and conceptus. Although blood supplies remain separate, the remodelling of arteries within these structures assists the progressive rise in uterine blood flow throughout gestation. Moreover, to protect the conceptus, the velocity of blood is slowed by the convoluted and dilated nature of terminal arteries supplying the endometrial decidua. This also acts to mediate steroid hormone transfer, important for embryo implantation and the timing and onset of parturition.

1.5 The role of ovarian steroids in the uterus

Changes in the hormonal milieu, especially ovarian steroid secretions, directly target the myometrium, endometrium and uterine vasculature. Whilst progesterone is crucial in preparing the endometrium during the luteal phase, a rise in oestrogen is required

for blastocyst metabolism and attachment (Lim *et al.*, 2002). As a result, the release of both ovarian hormones coordinates uterine events in a spatiotemporal manner.

With the onset of pregnancy, steroidogenesis is enhanced initially by conceptus signal cascades on the functional corpus luteum and then superseded by the foeto-placental unit. Interferon-τ is considered to be the recognition signal that has roles in embryogenesis and foetal development (Asselin *et al.*, 1997). Circulatory progesterone and oestrogen concentrations continue to increase throughout pregnancy (Table 1.1); this promotes uterine quiescence to accommodate the development and growth of the foetus. To reduce the capacity for myogenic activation, progesterone in the presence of oestrogen suppresses gap junction dynamics within the uterus (Garfield *et al.*, 1980). As gap junctions are transmembrane channels between myometrial cells, the absence of direct cytoplasmic linkage during pregnancy limits electric and metabolic communication (Garfield, 1984; Riemer *et al.*, 1998). This prevents synchronised contractions of the uterus. Nevertheless, in humans, labour is preceded by increased myometrial gap junction formation, identified by the elevated expression of connexin isoforms (Cx) 43, 26, 40 and 45, which constitute the channels between cells (Kilarski *et al.*, 2000; Di *et al.*, 2001). Although cellular mechanisms have not been fully elucidated, the rise in Cx43 transcripts, mRNA and protein expression correlate to oestrogen treatments (Kilarski *et al.*, 2000; Di *et al.*, 2001) and oestrogen-mediated gene regulation (Grummer *et al.*, 2004). Therefore, in contrast to progesterone, heightened oestrogen may be integral for priming the uterus for parturition.

Table 1.1 Plasma progesterone and oestrogen concentrations in women during the luteal phase of the cycle and late pregnancy (Johnson *et al.*, 1995).

Steroid	Progesterone	Oestriol	Oestrone	17β Oestradiol
Luteal phase (ng/ml)	11	-	0.2	0.2
Pregnancy (ng/ml)	125-200	113	53	15

Whilst plasma progesterone increases throughout gestation, oestriol synthesis is promoted at term by foetal cortisol secretion, which elevates the oestrogen: progesterone ratio. In contrast to other species, progesterone withdrawal is not detectable in humans at parturition. This implies that the hormonal control of parturition may involve a regulated change in myometrial receptors or their signalling pathways.

Two main isoforms of the human progesterone receptor (PR) exist encoded by a single gene. Despite being independently regulated, PR-A is a truncated form of PR-B, lacking the first 164 N-terminal amino acids. Due to receptor co-expression in myocytes, the relative proportions of each PR subtype have been proposed to regulate uterine activity during pregnancy. PR-A dominantly represses the transcriptional activity mediated by PR-B (Giangrande & McDonnell, 1999; Pieber *et al.*, 2001), and a substantial increase in the myometrial PR-A: PR-B ratio has been identified at term labour in humans (Smith *et al.*, 2002) and non-human primates (Haluska *et al*., 2002). In addition to reducing the active suppression of genes required for parturition, the increased expression of PR-A has been associated with enhanced myometrial responsiveness to oestrogen via the oestrogen receptor (ER)-α (Mesiano, 2001). This suggests that the interaction between PR and ER in human myometrium may contribute to coordinating the preparatory phase of labour.

1.6 Mechanisms of human parturition

The onset of parturition is under tight endocrine control. Although functional progesterone withdrawal is pivotal in the human parturition cascade (Astle *et al.*, 2003), other maternal and foetal mechanisms are required to prepare the uterus for labour. In women, this process is only partially understood.

Relative myometrial quiescence during pregnancy is maintained through the autocrine-paracrine actions of putative inhibitors, such as progesterone, prostacyclin, relaxin, parathyroid hormone-related peptide, nitric oxide and corticotrophin-releasing hormone. These compounds may both impede or stimulate uterine contractility via different signalling pathways (Challis *et al.*, 2000). In late pregnancy, a shift in genomic mechanisms is proposed to diminish the production of inhibitory agents and concomitantly upregulate contractile-associated proteins (CAPs), including gap-junctions, agonist receptors and proteins encoding ion channels (Norwitz *et al*., 1999; Challis *et al.*, 2000). Stimulation can then produce synchronised contractions of the uterus; this is predominantly orchestrated by prostanoids acting as uterotonins (Figure 1.3).

Concurrent to the establishment of regular uterine contractions, the effacement and dilation of the uterine cervix facilitates the passage of the foetus during labour. Cervical changes involve connective tissue remodelling, mediated by a decline in matrix metalloproteinase inhibitors (Becher *et al.*, 2004). Distension of the uterus and maturation of the foetal pituitary-adrenal axis appear to have only a supportive rather than essential role in labour (Lopez Bernal *et al.*, 1993; Alfaidy *et al.*, 2001;). However, alterations in both Ca^{2+} metabolism and prostaglandin synthesis are implicated as crucial parallel mechanisms, which coordinate the timing of parturition (Weiss, 2000).

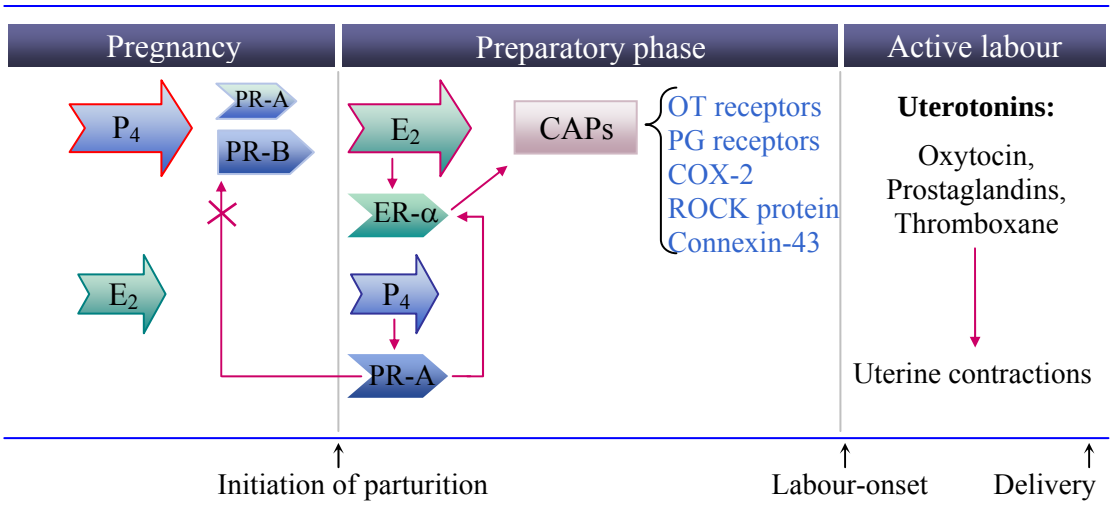


Figure 1.3: Hormones involved in the regulation of pregnancy and parturition. Labour-onset is preceded by an increase in the myometrial progesterone receptor (PR)-A: PR-B ratio, together with elevated oestrogen receptor (ER)-α expression. Oestrogen activation increases the genes encoding for contractile-associated proteins (CAPs), including oxytocin (OT) and prostaglandin (PG) receptors, cyclooxygenase (COX)-2, rho-associated coiled coil-forming protein kinase (ROCK) and connexin-43. These enhance myometrial responsiveness to uterotonins and promote labour contractions.

1.7 Prostanoids

Prostanoids are classified as prostaglandins (PGs) and thromboxane (TXA), consisting of a cyclopentane and cyclohexane ring respectively. Naturally existing PGs are further subdivided into prostaglandin (PG) D, E, F and I, according to slight modifications of the ring structure. Due to their chemical and metabolic instability, prostanoids act as local mediators and maintain homeostasis in a variety of tissues and cells (Tsuboi *et al.*, 2002). Although integral to inflammatory, ovulatory and luteolytic processes, changes in prostanoids are also essential in the initiation and maintenance of labour, reflected by changes in their biosynthesis (Giannoulias *et al.*, 2002; Jabbour & Sales, 2004).

1.7.1 Prostanoid biosynthesis

As part of the eicosanoid family, the PGs are regulated through a common biosynthetic pathway (Figure 1.4). In humans the primary precursor of PGs is arachidonic acid, a C20 esterified fatty acid stored in membrane phospholipids. To liberate free arachidonic acid for PG production, cytosolic phospholipase A_2 (PLA_2) translocates from the cytosol to the cell membrane upon activation by Ca^{2+}. Alternatively, phospholipase C (PLC) and diacylglycerol lipase indirectly catalyse the process. Free arachidonate is subsequently converted to form the unstable intermediate endoperoxide PGH_2. This requires oxidation by the cyclooxygenase enzyme (COX), of which three isoforms exist (Chandrasekharan *et al*., 2002). Despite catalytic and structural similarities, COX-1 is expressed in most cells whereas hormones, growth factors and cytokines are necessary to readily induce COX-2 and -3 (Morita, 2002). Following the biosynthesis of PGH_2, the endoperoxides are rapidly converted to the series-2 bioactive PGs by specific prostaglandin and thromboxane synthases. These terminal enzymes are named according to the prostanoid produced, such that PGD_2 PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 are synthesised by their respective synthases denoted PGDS, PGES, PGFS, PGIS and TXS (Narumiya *et al.*, 1999). Transporters mediate the rapid efflux of the newly synthesised PGs, facilitating local PG receptor binding.

1.7.2 Prostanoid receptors

PGs transmit their signals via rhodopsin-type seven transmembrane receptors that couple to different guanine nucleotide-binding (G) proteins and downstream effector systems. Each receptor type, classified as DP, EP, FP, IP and TP, is based on respective sensitivities to the five primary prostanoids PGD_2 PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 (Coleman *et al.*, 1994). Separate genes encode a further four EP subtypes (EP_{1-4}), differing in structure, signalling pathways and pharmacological action. A ninth subtype has recently been identified in mast cells of PGD_2 as a potent agonist at the chemoattractant receptor-homologous molecule expressed on T helper 2 cells ($CRTH_2$) (Hirai *et al.*, 2001). Through use of alternative splice variants, two isoforms of EP_1 have been cloned in the rat, whilst two TP and nine variants of the EP_3 receptor have been identified in humans (Negishi *et al.*, 1993; Pierce & Regan, 1998), in conjunction with two ovine FP isoforms (Pierce *et al.*, 1997). In each receptor type similar binding properties are maintained due to splice variant modifications 9-12 amino acids into the carboxyl-terminal domain. As a result, prostanoid receptor isoforms mainly differ in terms of localisation, G-protein coupling and agonist-induced desensitisation (Negishi *et al.*, 1993). Molecular cloning techniques have improved the analysis of prostanoid subtype ligand binding properties and signal transducing pathways (Table 1.2; Figure 1.4).

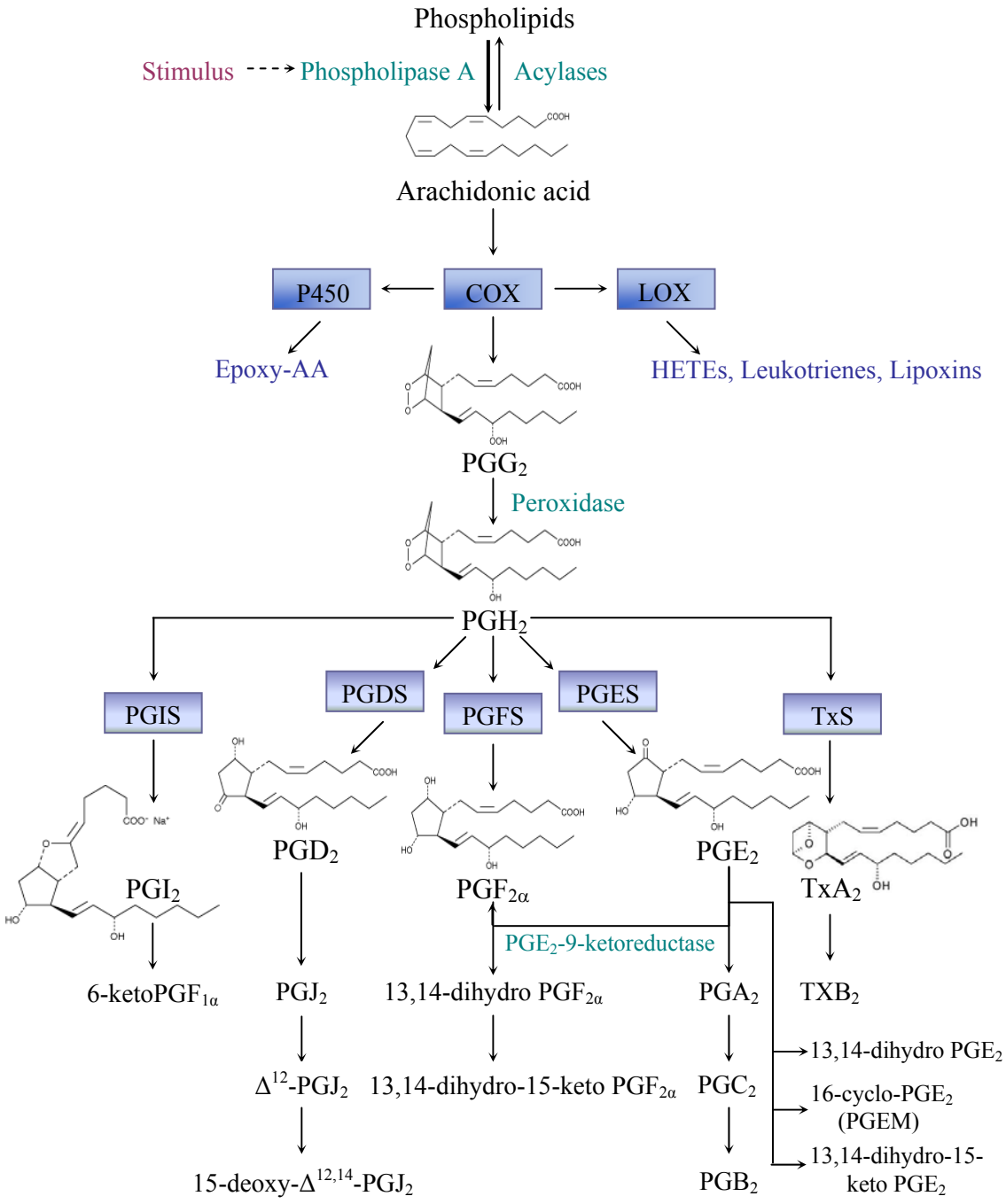


Figure 1.4: The biosynthetic pathway of the series-2 prostanoids (PGs) and their metabolites derived from arachidonic acid (adapted from Bos *et al.*, 2004). Arachidonic acid is metabolised by cyclooxygenase (COX)-1 or COX-2 to the unstable endoperoxide PGH_2. Thromboxane (TxA_2), PGD_2, PGE_2, PGI_2 and $PGF_{2\alpha}$ are generated from this common precursor by individual PG synthase enzymes (TxAS, PGDS, PGES, PGIS and PGFS) before eliciting biological effects at their cognate cell surface receptors.

Prostanoid receptors can be broadly divided into relaxant (DP, EP_2, EP_4, IP) and contractile (EP_1, EP_3, FP, TP) groups according to their actions on smooth muscle cells. Except for EP_1, specific G protein species have been identified for each prostanoid receptor (Kiriyama *et al.*, 1997), classified according to their α-subunit (Table 1.2).

Table 1.2: Signal transduction pathways of prostanoid and oxytocin receptors. The Gαq and Gαi proteins increase and potentiate uterine contractility, whilst Gαs proteins cause muscle relaxation. Corresponding secondary messengers mediate effects via inositol trisphosphate (IP_3) and changes in cyclic adenosine monophosphate (cAMP) (Narumiya *et al.*, 1999; Tsuboi *et al.*, 2002; Hata & Breyer, 2004).

Type	Subtype	Isoforms	G Protein	Second messenger
DP		DP	Gαs	↑ cAMP
		$CRTH_2$ (DP_2)	Gαi	↑ cAMP, ↑ Ca^{2+} ↑ IP_3
EP	EP_1	EP_1, EP_1-variant (rat)	Unknown	↑ Ca^{2+}, low↑ IP_3
	EP_2		Gαs	↑ cAMP
	EP_3	$EP_{3\text{-}1a, 1b, II, III, IV, V, VI, e, f}$	Gαi, Gαs, Gαq	↓↑ cAMP, IP_3
	EP_4		Gαs	↑ cAMP
FP		FP-A & FP-B (ovine)	Gαq	↑ IP_3
IP			Gαs, Gαq, Gαi	↓↑ IP_3, ↑ cAMP
OT			Gαi, Gαq	↑ Ca^{2+}, ↑ IP_3
TP		TPα	Gαs, Gαq, $G\alpha_{12,13}$, Gαh	↑ IP_3, ↑ cAMP
		TPβ	Gαi, Gαq, $G\alpha_{12,13}$	↑ IP_3, ↓ cAMP

Among the different receptors DP, EP_2, EP_4 and IP have a profound inhibitory effect on contractility by stimulating the Gαs protein coupled to adenylyl cyclase (AC). In contrast, TP, FP and EP_1 receptors enhance strong contractile effects through Gαq and Gαi proteins. Gαq activates PLC, catalysing the production of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), whilst Gαi inhibits cyclic adenosine monophosphate (cAMP). The resultant Ca^{2+} mobilisation is essential for the actions of uterotonins,

especially towards term pregnancy (Shlykov & Sanborn, 2004). Although associated with a general decline in cAMP, EP_3 may conversely increase AC activity depending on the splice variant and cell type (Narumiya *et al.*, 1999). Nevertheless, it is likely that cross-communication of downstream signalling pathways potentiate all prostanoid effects in the myometrium.

1.7.3 Prostanoids in the myometrium

PGs have long been implicated in regulating uterine contractility via myometrial receptors. In the non-pregnant and term pregnant state, functional studies on the human myometrium have characterised the expression of heterogeneous DP, EP, FP, IP and TP receptors (Senior *et al.*, 1992; Senior *et al.*, 1993). Differences in the response to PGs, both regional and hormone-related, indicate either altered receptor populations or the production of uterine contractile-associated proteins (Myatt & Lye, 2004). In addition, the expression of prostanoid receptors varies with tissue distribution. The TP receptor, for instance, is predominantly localised in platelets and blood vessels, EP_1 in fibroblasts, FP in renal cells and EP_2 and EP_4 in smooth muscle cells (Narumiya *et al.*, 1999; Bos *et al.*, 2004). In the myometrium these reflect uterine physiology and activity, especially towards the onset of parturition.

1.7.4 Prostaglandin receptors in pregnancy and parturition

PG synthesis and receptor expression are intimately involved in the parturition process. This involves the switch between relaxant to contractile receptor populations. Unlike DP, which is least abundant in human myometrium, the main arachidonic acid metabolite in uteri from non-pregnant and term pregnant donors is prostacyclin (PGI_2) (Christensen *et al.*, 1983). As well as an increase at menstruation, PGI_2 synthesis is

augmented during pregnancy to reduce myogenic activity and regulate uterine and placental blood flow. Pregnancy maintenance is also related to an increase in relaxatory EP receptor responses, primarily EP_2 (Senior *et al.*, 1993; Brodt-Eppley & Myatt, 1999), in conjunction with elevated coupling of Gαs-proteins to AC in response to PGE_2 (Europe-Finner *et al.*, 1994; Europe-Finner *et al.*, 1997). The concomitant rise in uterine cAMP formation activates cAMP-dependent protein kinase A (PKA) to phosphorylate MLCK. This promotes myometrial relaxation by reducing the affinity for the Ca^{2+}-calmodulin complex and inhibiting voltage-gated Ca^{2+} channels (Word *et al.*, 1994). The reduction in cAMP at labour indicates a loss of this inhibitory pathway (Europe-Finner *et al.*, 1994; Lopez Bernal *et al.*, 1995).

The onset of labour is associated with elevated PG synthesis within the uterus. This increase, particularly in PGE_2 and $PGF_{2\alpha}$ production by the foetal membranes and decidua, coincides with augmented COX-2 expression (Erkinheimo *et al.*, 2000). Others have reported no change in COX-2 mRNA at parturition but differences in contractile receptor responsiveness (Sparey *et al.*, 1999; Giannoulias *et al.*, 2002). Although downregulated during pregnancy (Matsumoto *et al.*, 1997), the expression of human myometrial EP_3 and FP receptors increase dramatically at parturition (Brodt-Eppley & Myatt, 1999). A concurrent loss of EP_2 receptors (Astle *et al.*, 2005) and withdrawal of PGIS in myometrium at term pregnancy may also contribute to the labour process (Giannoulias *et al.*, 2002).

PGE_2 is important in myometrial contractility as, clinically, PGE_1 and PGE_2 analogues are used for the induction of labour and for cervical ripening. Even so, other uterotonic agents such as $PGF_{2\alpha}$ and oxytocin contribute to successful parturition, their functional roles determined by gene knockout mice. As $PGF_{2\alpha}$ is a luteolytic agent, impaired parturition in FP-deficient mice was attributed to the

absence of a decline in progesterone and a lack of oxytocin receptor expression (Sugimoto *et al.*, 1999). Aberrant COX-1 genes are related to parturition failure, whereas COX-2 defects cause infertility with abnormalities in ovulation, fertilisation, implantation and decidualisation (Lim *et al*., 1997). In addition, gene ablation of EP_2 receptors inhibited ovulation in mouse models (Tilley *et al.*, 1999), whereas the failure to close the ductus arteriosus at birth in EP_4-deficient mice caused neonatal deaths (Nguyen *et al.*, 1997). As oxytocin also stimulates ovarian $PGF_{2\alpha}$ release in a positive feedback manner (Chibbar *et al*., 1993), it is likely that a balance of factors and signalling pathways ultimately regulate uterine activity. These complex interrelated receptor cascades are not well defined in humans and need further elucidation.

1.7.5 TP receptors in parturition

The TP receptor gene has been localised in the human myometrium, foetal membranes and placenta (Swanson *et al.*, 1992), corresponding to functional roles in uterine contractility and vascular tone. The TP splice variants have been identified in human myocytes from both non-pregnant and pregnant donors (Moore *et al.*, 2002; Moran *et al.*, 2002) although little is known about TP receptors in relation to labour-onset.

The two receptor isoforms, TPα and TPβ, differ exclusively in their carboxyl-terminal domains (Raychowdhury *et al.*, 1994). While both mediate identical ligand binding, each isoform exhibits critical differences in signalling and patterns of expression, indicative of distinct pathophysiological roles (Miggin & Kinsella, 1998; Moore *et al.*, 2002). The TPα receptor activates AC, increasing cAMP production through Gαs (Hirata *et al.*, 1996). By contrast, in addition to inhibiting AC through Gαi-protein

coupling, TPβ stimulates intracellular Ca^{2+} via the IP_3 pathway. Due to the synergism of signal cascades, this mechanism enhances myogenic contractility.

Both TP receptors activate RhoA, a small GTPase, stimulating the two target proteins, rho-associated coiled coil-forming protein kinase (ROCK)I and its isoform ROCKII (Amano *et al.*, 2000). By direct phosphorylation of MLC_{20} and inactivation of myosin phosphatase, the uterus becomes sensitised to Ca^{2+} (Figure 1.5).

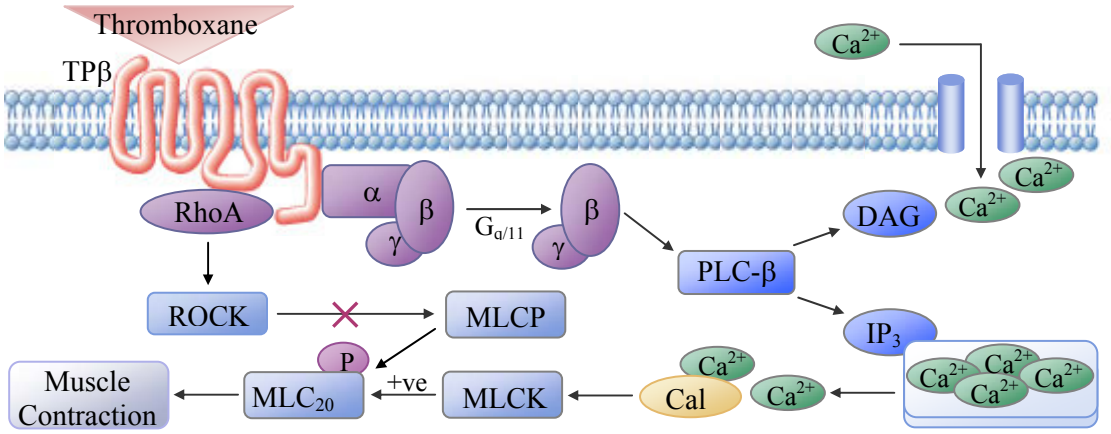


Figure 1.5: Thromboxane stimulates phospholipase C (PLCβ) production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{2+} from the sarcoplasmic reticulum intracellular stores. Upon binding with calmodulin (Cal), cytosolic-free Ca^{2+} activates myosin light chain kinase (MLCK), which catalyses MLC_{20} phosphorylation and induces uterine contractility. Activation of the rho-associated coiled coil-forming protein kinase (ROCK) pathway inhibits MLC_{20} phosphatase (MLCP), denoted by X, preventing MLC_{20} dephosphorylation; this sensitises the uterus to Ca^{2+} and potentiates contractility.

The increased myometrial contractility in non-pregnant donors was irrespective of the phase of the menstrual cycle (Senior *et al.*, 1992; Senchyna *et al.*, 1999), suggesting that small changes in steroid hormones or related transcription factors do not influence TP affinity and density. In contrast, ROCKI isoforms have been reported to both upregulate (Moore *et al.*, 2000; Moore & Lopez Bernal, 2003) and remain unaltered during human pregnancy (Friel *et al.*, 2005). However, an apparent increase

in RhoA mRNA at parturition implies a role for ROCKI in the preparatory phase and activation of pregnancy (Lartey *et al*., 2007). Moreover, aberrant ROCKI expression has been associated to uterine contractile dysfunctions, such as preterm labour or prolonged full term labour (Moore & Lopez Bernal, 2003; Lartey *et al*., 2007). Therefore greater understanding and control of these proteins may improve tocolytics for labour-associated disorders.

1.8 Oxytocin biosynthesis

Oxytocin is one of the most potent uterotonic agents used for augmenting uterine contractions during labour (Fuchs *et al*., 1985; Wathes *et al*., 1999; Nilsson *et al*., 2003). As a nonapeptide hormone, oxytocin is primarily synthesised in the paraventricular and supraoptic nuclei of the hypothalamus with storage and release from the posterior pituitary. Local production from the gravid uterus and chorio-decidua is also well documented (Chibbar *et al*., 1993), suggesting that oxytocin may act by both endocrine and paracrine mechanisms to promote parturition.

1.8.1 Oxytocin receptors in pregnancy and parturition

Although systemic oxytocin concentrations do not correlate with the progression of labour (Dawood *et al*., 1978) an increase in pulse frequency and the number of myometrial oxytocin receptors have been reported (Fuchs *et al*., 1984; Kimura *et al*., 1996; Riemer & Heymann, 1998). Oxytocin has been shown to bind to its cognate myometrial Gαq and Gαi receptors for enhanced phasic and tonic uterine activity (Phaneuf *et al*., 1993; Riemer & Heymann, 1998; Wathes *et al*., 1999). During pregnancy, myometrial oxytocin mRNA transcripts increase 100-fold at 32 weeks and 300-fold at parturition relative to the receptors expressed in the non-gravid uterus

(Kimura *et al*., 1996). This would likely sensitise the uterus to oxytocin immediately prior to labour-onset (Keelan *et al*., 1997). Even so, in the absence of maternal and foetal oxytocin, the delivery of a litter ends successfully at the same gestational age as wild-type mice (Young *et al*., 1996; Nishimori *et al*., 1996). Similarly in knockout mice deficient for both oxytocin and COX-1, labour contractions are timely but prolonged and neonatal deaths occur from maternal failure to establish lactation (Gross *et al*., 1998). This indicates possible compensatory or redundant mechanisms for oxytocin in the parturition process. Nevertheless, as only a few oxytocin receptors exist in the peripheral system (Kiss & Mikkelsen, 2005) and oxytocin stimulates preterm and term human uteri (Fuchs *et al*., 1984; Wathes *et al*., 1999; Nilsson *et al*., 2003), the oxytocin receptor is considered a suitable target in the management of preterm labour.

1.9 Preterm labour

Preterm birth, defined as parturition before 37 weeks (259 days) of gestation, is a major obstetric problem related to perinatal mortality and morbidity. In Western countries, although only 6-10 percent of deliveries are premature, the associated complications result in more than two-thirds of perinatal deaths (Lumley, 2003). This is most severe with both early gestational age and low birth-weight infants. Over the past 20-30 years, advances in neonatal medicine and perinatal care have reduced mortality rates (Tucker *et al*., 2004). However, this has been counterbalanced by increased short-term morbidity and long-term physical and mental disability in infant survivors of very preterm birth. The immature development of foetal organs in third trimester of pregnancy is linked to a high prevalence of respiratory distress syndrome. Moreover, preterm delivery contributes to 50% of childhood neurological disabilities,

including subnormal cognitive function, cerebral palsy, blindness and deafness (Hack *et al.*, 2000). As a result, the cost in terms of neonatal intensive care, long-term treatment and emotional trauma suffered by the parents are considerable. Therefore many risk factors have been identified in women to anticipate preterm delivery for improving neonatal outcome.

Despite the multiple aetiologies of preterm birth, poor socio-economic status is principally associated with an increased risk of spontaneous preterm delivery. Contributing factors include increased frequency of cigarette smoking, nutritional deprivation, greater use of recreational drugs such as cocaine, psychological stress and involvement in heavy physical work. Nevertheless, these lifestyle risk factors have not been shown to exert independent adverse effects on pregnancy outcome (Slattery *et al.*, 2002).

Table 1.3 The factors known to be associated with preterm delivery (Steer, 2005).

Factors associated with preterm delivery	
Spontaneous (70-80%)	**Iatrogenic (20-30%)**
Spontaneous rupture of the membranes	Hypertension/ pre-eclampsia
Infection	Diabetes
Multiple pregnancy	Intrauterine growth restriction
Cervical dysfunction/ uterine malformation	
Placental haemorrhage	
Malnutrition, stress	

Previous low neonatal birth weight or preterm delivery increases the prospect of subsequent premature births by 2.5-fold, implying the recurrence of active causal factors in subsequent pregnancies (Mercer *et al.*, 1999). A high incidence of preterm labours has also been associated with low maternal body mass index, height below 1.46m and very young or older maternal age groups (Steer, 2005). Large racial and ethnic disparities have been reported in the USA (Regan *et al.*, 2005) but are not as

notable in other developed countries (Steer, 2005). Moreover, regardless of race, approximately half of all twin pregnancies that reach 20 weeks of gestation end prematurely (Gardner *et al.*, 1995), linked to a high incidence of pre-eclampsia, placental abruption and uterine over-distension. Even so, some cases of preterm birth are iatrogenic because of maternal illness or developing foetal compromise (Table 1.3).

Systemic, intrauterine or genital tract-infections, arising at a preterm period of gestation, have been directly linked to the onset of labour (Keelan *et al.*, 2003). In response to bacterial endotoxins, leukocytes infiltrate the uterus and cytokines and other inflammatory mediators are produced. The resulting augmentation of PG synthesis and activation of metalloproteinases are postulated to be principal mechanisms of infection-driven preterm labour. Although antibiotic treatments can reduce maternal infection, a meta-analysis of neonatal outcome showed an associated increase in functional impairments and cerebral palsy (Kenyon *et al.*, 2008).

1.10 Current treatments for preterm labour

At present no effective diagnostic indicators exist for preterm labour. The only absolute proof is contractions of the uterus, accompanied by the progressive dilation of the cervix. However, by this time, tocolytic drugs and antibiotics are relatively ineffective, suppressing contractions temporarily in high-risk patients but rarely preventing preterm births (Caritis, 2005). Placebo-controlled studies suggest that current treatments only prolong pregnancy for 24-48 hours. This provides time to arrange transfer of the mother and the neonate to specialist care units and to administer glucocorticoids, which enhance foetal maturity and neonatal survival. Even

so, tocolytics are associated with a number of adverse maternal and foetal effects (Table 1.4).

Table 1.4 Tocolytic therapies used clinically for preterm labour and their associated adverse effects (Plested & Lopez Bernal, 2001; Goldenberg, 2002; Meis *et* al., 2005; Papatsonis *et al*., 2009).

Drug	Mechanism	Major side effects & comments
Nifedipine	Ca^{2+} blocker	Maternal hypotension, altered uteroplacental blood flow, foetal tachychardia. Recommended by Papatsonis *et al*., 2009.
Ritodrine, Terbutaline	β_2-adrenergic agonists	Cardiac arrhythmias, pulmonary oedema, myocardial ischaemia. Limited efficacy due to adverse effects and rapid desensitisation.
Atosiban	Oxytocin antagonist	Maternal nausea, hyperglycaemia and headaches; no reported foetal side effects.
Indometacin	COX inhibitor	Maternal gastrointestinal disturbance, ductus arteriosus constriction, lack of amniotic fluid. Not used >32 wks gestation.
Magnesium sulphate	Ca^{2+} antagonist	Respiratory arrest, cardiac arrest, maternal nausea and headaches. Often used in pre-eclampsia.
17-α-hydroxy-progesterone caproate	Progesterone agonist	Fatigue, depression and headaches. Reduced incidence of preterm labour only in high-risk patients with a history of previous spontaneous premature deliveries.

Current tocolytics include Ca^{2+} channel blockers, β_2-mimetics, magnesium sulphate, COX inhibitors, progesterone analogues and oxytocin receptor antagonists. Recent trends have favoured agents with lower maternal side-effect profiles. These include Ca^{2+} channel blockers and oxytocin receptor antagonists, which are better tolerated than β_2 agonists (Coomarasamy *et al*., 2002; Kashanian *et al*., 2005). To improve treatments all aspects of delayed labour, minimised side effects to mother and foetus and long-term health implications have to be evaluated. Agents with potential beneficial pharmacological properties include FP receptor antagonists, oxytocin antagonists and more novel inhibitory receptor agonists, such as those that target the EP_3 receptor.

1.11 Aims

The overall aim of this thesis is to investigate the functional expression of EP, FP, TP and oxytocin receptors in isolated human myometrium during the menstrual cycle, term pregnancy and parturition. At present, the relationship between PGs and oxytocin is not well elucidated. Whilst it is recognised that PG and oxytocin receptors modulate uterine contractions, cellular effects are dependent on the type of receptor engaged, coupled to divergent signalling pathways. Their responses have been difficult to define pharmacologically due to the moderate selectivity of some synthetic analogues (Coleman *et al*., 1994; Wilson *et al*., 2004). Section I of this thesis will address the effects of PGs and oxytocin using a range of standard and novel receptor agonists and antagonists. Assessment of their roles in smooth muscle contractility will be investigated using functional immersion and superfusion assays and tissues from different hormonal stages.

PG and oxytocin receptors are subject to hormonal and gestational-dependent regulation. Although precise endocrine changes remain elusive, progesterone is associated with uterine quiescence whilst oestrogen promotes PG and oxytocin-induced contractions (Thornton *et al*., 1999). This is regulated in a temporal and topographical manner (Giannopoulos *et al*., 1985; Adelantado *et al*., 1988; Brodt-Eppley *et al*., 1999; Smith *et al*., 2001). To study total uterine function in the non-pregnant state, spontaneous activity and PG and oxytocin receptor function will be investigated according to anatomical location and the stage of menstrual cycle of the donor. During pregnancy and parturition, in accordance with ethical constraints, myometrial specimens will only be recovered from the lower uterine segment. In this lower region, myogenicity and agonist-mediated contractions will be measured to identify the changes in PG and oxytocic effects at term and preterm pregnancy and

labour. As previous functional studies using human tissues obtained during labour are limited, the results of this thesis should particularly enhance knowledge of the parturient uterus.

In conjunction with *in vitro* studies, a primary cell culture model will be developed to examine the separate functions of uterine smooth muscle cells and fibroblasts at term pregnancy (Section II). By establishing a method for the high throughput screening of compounds, PG and oxytocin-induced activation of Ca^{2+} (Wray, 1993) and cAMP signals (Europe-Finner *et al*., 1997; Price *et al*., 2000) will be studied relative to receptor mRNA. In conjunction with the results from Section I, cellular and molecular studies should clarify the contribution of receptors or messenger targets that culminate in opposing actions on myometrial tone.

Using these methods, it may be possible to develop better therapeutic strategies to prevent or control myometrial disorders caused by uterine hypercontractility.

Section I:
Functional studies

Chapter 2: Materials and Methods

2.1 Surgical specimens

Human uterine smooth muscle was excised and donated for research from:

- Longitudinal, full-thickness sections of the anterior uterus from non-pregnant donors, taken at hysterectomy.
- Transverse, anterior, lower uterine segments from pregnant, non-labouring and labouring donors, taken at Caesarean section.

2.2 Approval and ethical consent

Ethical approval for all studies was obtained from the Local Regional Ethics Committees: Bradford Hospital NHS Trust and the University of Bradford Ethics Committee. All women who donated tissue gave informed written consent before surgical procedures were performed. Uterine specimens from non-pregnant donors were obtained from the Yorkshire Clinic and the Nucleus Theatres at the Bradford Royal Infirmary and samples from pregnant donors were collected from the Maternity unit at the Bradford Royal Infirmary. Patient consent forms and information sheets are included in the Appendix (Figures A1, A2, A3 and A4).

2.3 Uterine smooth muscle

Longitudinal uterine specimens, taken at hysterectomy, were pale with visible distinctions between dense muscle, serosa and cervical tissue (Figure 2.1). In contrast, transverse muscle from pregnant donors was highly vascular, especially in samples taken after labour-onset.

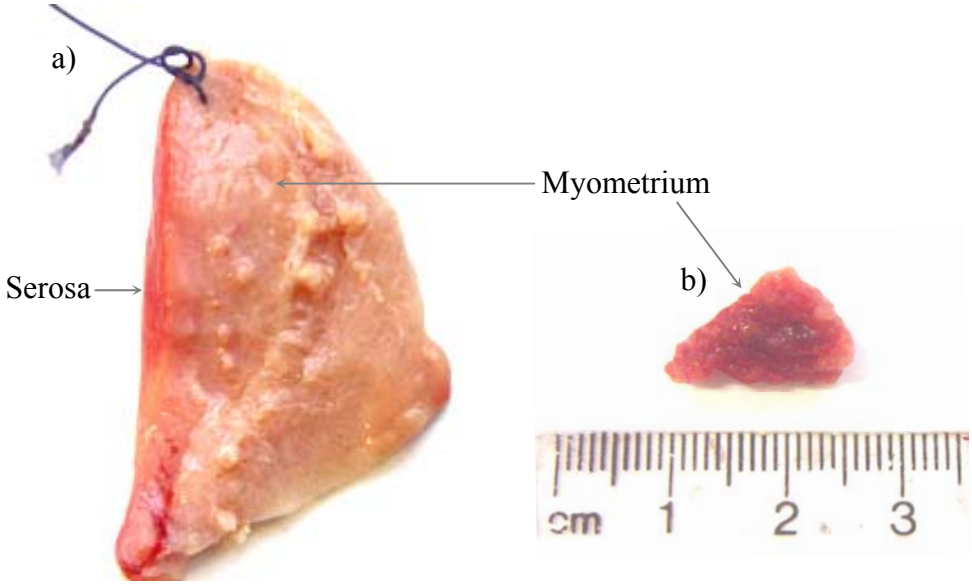


Figure 2.1: Uterine biopsies taken from a) non-pregnant and b) term pregnant, non-labouring donors.

2.3.1 Non-pregnant donors

Human myometrial samples were obtained from pre-menopausal women undergoing hysterectomy for benign disorders, such as menorrhagia and dysmenorrhoea (Figure 2.2). At the time of surgery, none of the donors were using oral contraceptives or had received any hormone therapy. The stage of menstrual cycle was recounted to the nearest week and recorded on patient information forms. Donor ages ranged from 29 to 52 years (median 42 years) and 80 percent of uteri were removed during the follicular stage of the menstrual cycle. Specimens were excised from the anterior wall of the corpus uteri and a ligature was tied at the fundus to indicate orientation.

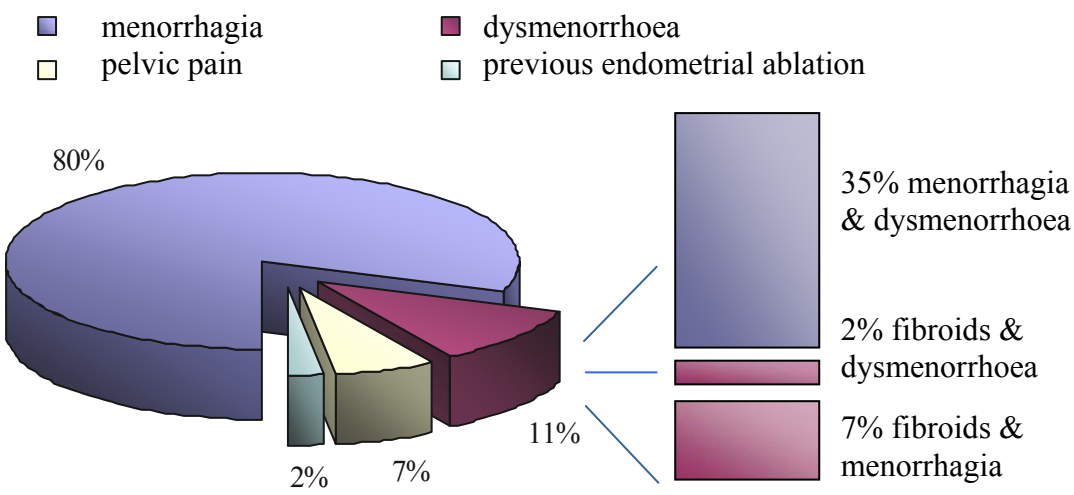


Figure 2.2: Reasons recorded on patient information forms for hysterectomy for the treatment of benign disorders.

2.3.2 Pregnant donors not in labour

Segments of the upper margin of lower uterine muscle were obtained from preterm (33-36^{+6} weeks) and term (37-41 weeks) pregnant women, aged between 19 to 44 years (median 30 years), undergoing elective, non-emergency Caesarean section. During this routine operation and due to ethical restrictions, myometrium was only removed from the superior edge of the transverse incision site, whilst fundal tissue was not available for biopsy. Subjects with maternal metabolic diseases, multiple foetuses or after labour-onset were grouped and analysed separately.

2.3.3 Pregnant donors in labour

At emergency Caesarean section, after delivery of the foetus and placenta, transverse myometrial biopsies were excised from the upper edge of lower uterine segments from labouring donors. Labour was defined as the presence of regular uterine contractions with early, mid and late labour in patients identified at 0-2.5cm, 3-8.5cm and ≥9cm cervical dilation respectively. Donors were between 18 and 37 years of age (median 29 years) at preterm (32-36^{+2} weeks) and term (37-42 weeks) gestations. The

indications for Caesarean delivery included foetal distress, failed progression of labour, breech presentation, previous Caesarean sections, placental abruption, maternal pregnancy-induced hypertension, epilepsy and asthma.

Table 2.1: Patient characteristics for term pregnant, not in labour (n=129), early-mid (n=35) and late (n=17) labouring donors.

	Term pregnancy		Early-mid labour		Late-labour	
Donor details	**n**	**%**	**n**	**%**	**n**	**%**
White British	89	69.0	17	48.6.7	6	35.3
Pakistani/ Indian	10	7.8	11	31.4	7	41.2
Other ethnic groups	12	9.3	2	5.7	1	5.9
Details not recorded	18	14.0	5	14.3	3	17.6
Previous abortions/ miscarriages	33	25.6	12	35.3	2	14.3
Previous labours	61	60.4	14	41.2	3	21.4
Previous Caesarean sections	88	68.8	13	61.9	4	28.6
Cigarette smokers[a]	32	24.8	8	23.5	2	11.8
Medical conditions[b]	18	14.0	11	32.4	7	41.2
Current medication	15	11.6	10	29.4	7	41.2

[a] Patients smoked 3 to 20 cigarettes per day throughout pregnancy
[b] Medical conditions included: gestational diabetes, pregnancy-induced hypertension, asthma, epilepsy, sickle cell anaemia, Streptococcus B infections and Crohn's disease.

2.4 Compounds

Agonists and antagonists used in functional studies were prepared as a 10mM stock solution according to the manufacturers' advice (Table 2.2). Serial dilutions were made with 0.9% w/v normal saline and kept on ice throughout experiments.

2.4.1 Solutions

Prior to immersion and superfusion assays, Krebs'-Heinseleit physiological salt (Krebs') solution (pH 7.4) was freshly prepared at the following composition (mM): NaCl 118.9; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 10.0 and oxygenated with 95% O_2 and 5% CO_2.

Table 2.2: Agonists and antagonists used to identify functional receptors in human myometrium. Time-matched vehicles, matched for solvent, caused no effect on myogenicity of tissue strips. The key indicates line colour on concentration-effect curves.

Compound	Key	Receptor target	Action	Chemical name	Stock Vehicle	Source
AGN201734 Elworthy *et al*. (2004)		EP_4	agonist	7-2-[(E)-3-hydroxy-4-(3-trifluoromethyl-phenyl)-phenyl)-but-1-enyl]-5-oxo-pyrrolidin-1-yl)-heptanoic acid)	ethanol	Allergan Inc., USA
AGN211329 Belley *et al*. (2005)		EP_3	antagonist	3-(-2-(3-[2-2, 6-dichloro-benzloxy)-3-methyl-phenyl]-allyl]-phenyl)-acrylic acid	ethanol	Allergan Inc., USA
AGN211330 Belley *et al*. (2005)		EP_2	agonist	1-(benzyloxy-2-vinylbenzene)-2-bromocinnamic acid	ethanol	Allergan Inc., USA
AH13205 Coleman *et al*. (1994)		EP_2	agonist	*trans*-2-(4-(1-Hydroxyhexyl)phenyl-5-oxocyclopentane-heptanoic acid	DMSO	Sigma-Aldrich, UK
AH-6809 Coleman *et al*. (1994)		EP_1, EP_2, EP_3 & DP_1	antagonist	6-Isopropoxy-9-xanthone-2-carboxylic acid	DMSO	Cayman chemicals, USA
Atosiban Nilsson *et al*., (2003)		OTR	antagonist	1-(3-mercaptopropanoic acid)-2-(O-ethyl-D-tyrosine)-4-L-threonine-8-L-ornithine-oxytocin	0.1% BSA in dH_2O	Sigma-Aldrich, UK
Butaprost Gardiner (1986)		EP_2	agonist	9-oxo-11α, 16S-dihydroxy-17-cyclobutyl-prost-13E-en-1-oic acid, methyl ester	ethanol	Cayman Chemicals, USA
Caspase-3 Inhibitor Moore *et al*., (2002)		Caspase-3 (6, 7, 8 & 10)	inhibitor	(3S)-3-[[(2S)-2-[(2S)-2-acetamido-3-methylbutanoyl]amino propanoyl]amino]-4-oxobutanoic acid	DMSO	Calbiochem, USA
CP533,536 Li *et al*., (2003); Paralkar *et al*. (2003)		EP_2	agonist	3-[(4-tert-butyl-benzyl)-pyridine-3-sulfonyl-amino)-methyl)-phenoxy]-acetic acid sodium salt	ethanol	Allergan Inc., USA a Pfizer compound
GR32191B Lumley *et al*. (1989)		TP	antagonist	7-[5-[[(1, 1'-biphenyl)-4-yl] methoxy]-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptanoic acid	ethanol	GlaxoSmith-Kline, UK
GW627368x Wilson *et al*. (2006)		EP_4	antagonist	(N-(2-[4[(4,9-diethoxy-1-oxo-1,3-dihydro-2H-benzo[f]isoindol-2-yl)phenyl]-acetyl]benzene-sulphonamide	DMSO	GlaxoSmith-Kline, UK
Indometacin Durn *et al*. (2010)		COX	inhibitor	1[p-chlorobenzoyl]5-methoxy-2-methlindole-3-acetic acid	ethanol	Sigma-Aldrich, UK

L-902688 Billot *et al*. (2003)		EP_4	agonist	5-(3-Hydroxy-4-phenyl-but-1-enyl)-1- [6-(1H-tetrazol-5-yl)-hexyl]-pyrrolidin-2-one.	ethanol	Allergan Inc., USA
Lanthanum chloride Fu *et al*. (2000)		Ca^{2+} channels	inhibitor	trichlorolanthanum	ethanol	Sigma-Aldrich, UK
Nifedipine Phillippe & Basa (1997)		L-type Ca^{2+} channels	inhibitor	dimethyl2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate	ethanol	Sigma-Aldrich, UK
ONO-D1-004 Oka *et al*. (2003)		EP_1	agonist	4-[2-[(1,2,3)-3-hydroxy-2-[3-hydroxy-5-methylnon-1-enyl]-5-oxocyclopentyl]acetyl]cyclohexane-1-carboxylic acid	ethanol	Allergan Inc., USA
Oxytocin Noe *et al*. (1999)		OTR	agonist	(1-(2-amino-2-oxoethylamino)-4-methyl-1-oxopentan-2-yl)-1-(19-amino-7-(2-amino-2-oxoethyl)-10-(3-amino-3-oxopropyl)-13-sec-butyl-16-(4-hydroxybenzyl)-pentaoxo-1,2-dithia-pentaazacycloicosane	dH_2O	Sigma-Aldrich, UK
Prostaglandin E_2 Coleman *et al*. (1994)		$EP_{1\text{-}4}$	agonist	9-oxo-11α, 15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid	ethanol	Cayman Chemicals, USA
Prostaglandin $F_{2\alpha}$ Coleman *et al*. (1994)		FP	agonist	9α, 11α, 15S-trihydroxy-prosta-5Z, 13E-dien-1-oic acid, tris (hydroxymethyl) aminomethane salt	ethanol	Cayman Chemicals, USA
Rho-kinase inhibitor Ikenoya *et al*. (2002)		ROCK	inhibitor	(S)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine, 2HCl	dH_2O	Calbiochem, USA
SQ29,548 Ogletree *et al*. (1985)		TP	antagonist	[1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid	ethanol	Cayman Chemicals, USA
Sulprostone Schaaf *et al*, 1981		$EP_{3/1}$	agonist	N-(methylsulfonyl)-9-oxo-11α,15R-dihydroxy-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide	ethanol	Cayman Chemicals, USA
Thapsigargin Fomin *et al*. (1999)		Ca^{2+}-ATPase	inhibitor	6-(acetyloxy)-4-(butyryloxy)-3-dihydroxy-3,6,9-trimethyl-8-([(2Z)-2-methylbut-2-enoyl]oxy)-2-oxo-2,3,4,5,6,7,8,9-decahydroazuleno[4,5]furan-7-yl octanoate	ethanol	Sigma-Aldrich, UK
U46619 Coleman *et al*. (1994)		TP	agonist	9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z, 13E-dien-1-oic acid	ethanol	Cayman Chemicals, USA

Alterative code names for the EP_3 antagonist AGN211329: L-826266 (Belley *et al*., 2005), the caspase-3 inhibitor: Z-D(OMe)E(Ome)VD(OMe)-FMK (Moore *et al*., 2002) and the rho-kinase inhibitor: H1152 (Shum *et al*., 2003).

2.5 Tissue collection and preparation

Immediately following surgical removal of uterine specimens, the biopsies were places in Krebs' solution for transport to the laboratory. The uterine samples were then trimmed of endometrial, serosal, fat and fibrous tissue and dissected to produce strips (10 x 2 x 3mm) of predominantly longitudinal muscle. Myometrial strips were then set-up for functional studies using immersion and superfusion techniques.

2.6 Immersion

Vilhelm Magnus (1871-1929) first introduced the idea of suspending an isolated portion of smooth muscle in a chamber containing a nutrient fluid and measuring changes in tissue tone. Traditional organ baths used by Sir Henry Dale (1875-1968) were modified for this study. The dissected strips of myometrium were mounted longitudinally in individual 8ml water-jacketed muscle baths (York Glassware Services, York, UK), containing aerated (95% O_2, 5% CO_2) Krebs' solution at 37°C (Figure 2.3). Immersed tissue strips were maintained at a constant temperature and pH (Table 2.3) with the volume of Krebs' solution adjusted via a tap and overflow system.

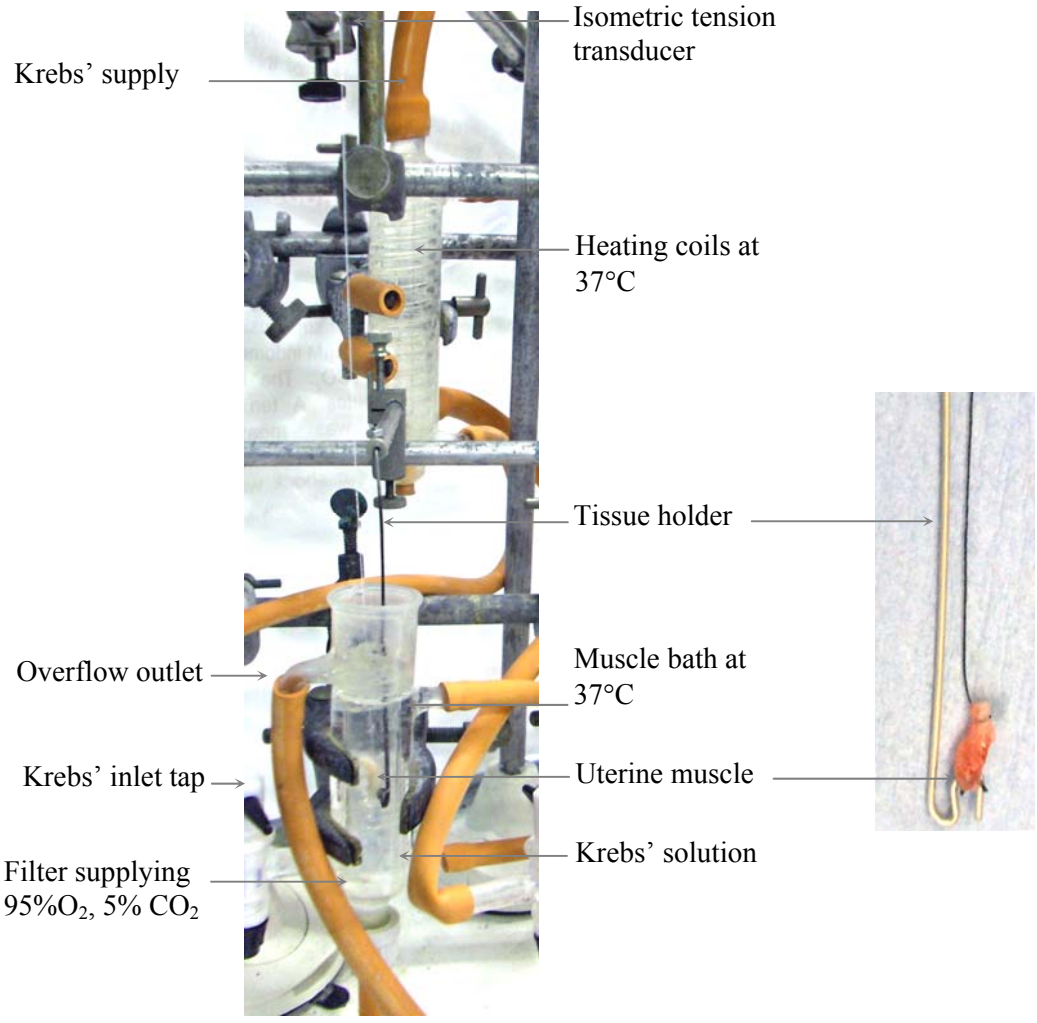


Figure 2.3: Immersion equipment used to examine functional receptors in the isolated human uterus and a myometrium sample anchored to a tissue holder.

Table 2.3: Changes in the pH of Krebs' solution over time whilst equilibrating human myometrial strips (n=3). Results are expressed as means ± S.E.

Equilibration Time:	**0hrs**	**3hrs**	**6hrs**
pH of Krebs' solution:	7.42 ± 0.03	7.41 ± 0.03	7.39 ± 0.07

Changes in tension were measured after attaching myometrial strips to isometric force transducers (Grass Instruments Inc., Rhode Island, USA), connected to a personal computer (Dell Inc) via bridge amplifiers (AD Instruments, Hastings, UK). Traces were recorded digitally on a PowerLab data acquisition system running Microsoft Chart v5.4 software (sampling frequency 2Hz; AD Instruments, Hastings, UK).

Muscle baths were refilled with Krebs' solution and an initial, optimum resting tension of 2g was applied to each strip (Morrison *et al*., 1993; Slattery *et al*., 2001). Isolated tissues from non-pregnant and term pregnant donors were equilibrated for a minimum of 60 and 90 minutes respectively or until regular phasic contractions had developed (Figure 2.4). This myogenic activity was recorded over a subsequent 30-minute period before addition of the study drug and remained stable for at least 5 hours (Popescu *et al*., 2006; Figure 2.4), demonstrating the viability of tissue strips within immersion baths. In myometrium from labouring donors, experiments were initiated after 2.5 hours of equilibration, regardless of the presence or absence of contractile activity.

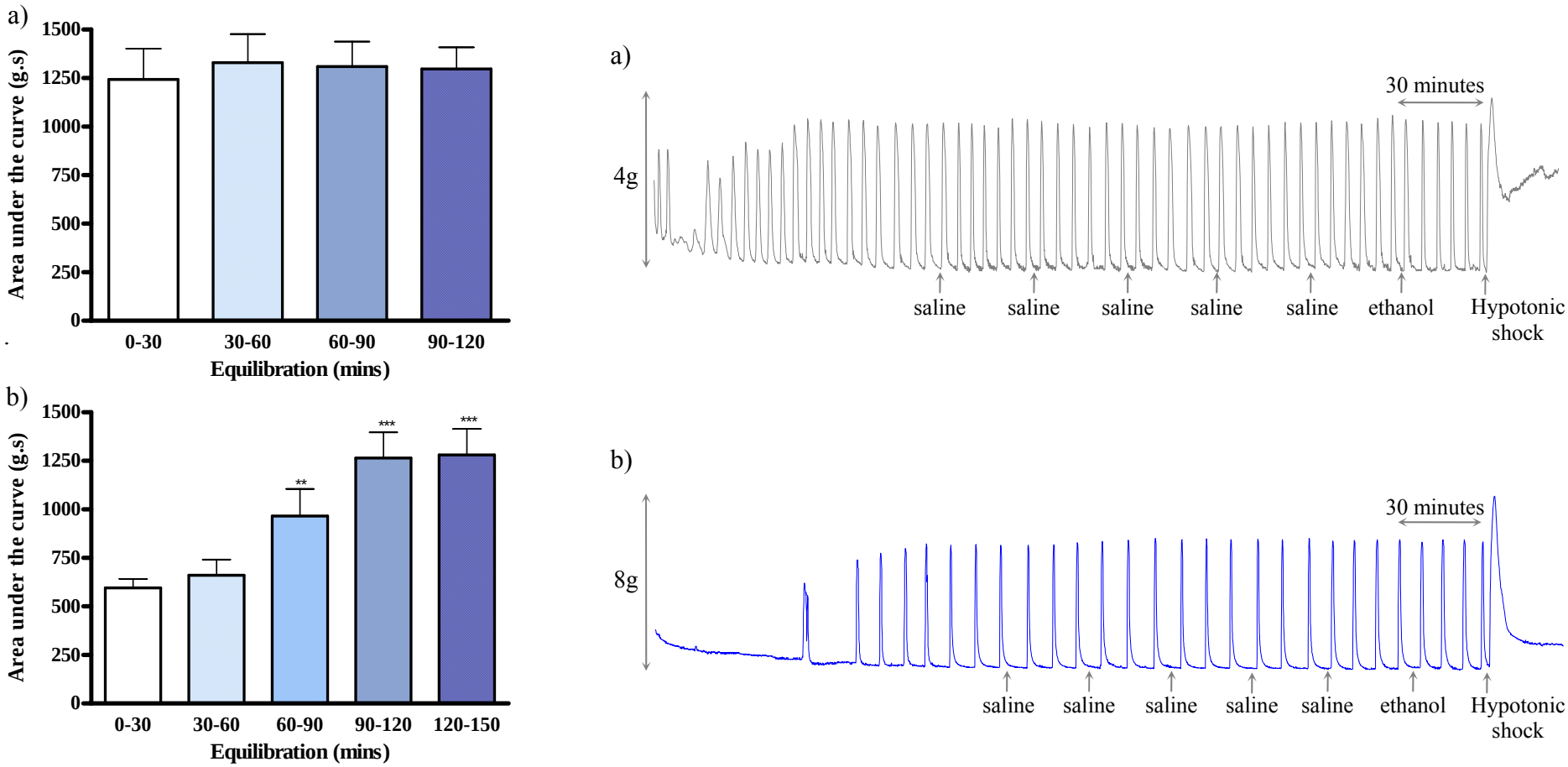


Figure 2.4: Equilibration for regular contractions to develop in myometrium taken at a) hysterectomy (fundus) (n=10) and b) elective Caesarean section from term pregnant, non-labouring women (n=10). Contractile activity was measured as the integrated area under the curve (g.s), expressed as means ± S.E. Univariate ANOVA with Bonferroni's *post-hoc* test showed an increase in myogenic activity compared to the activity established between 0-30 minutes of tissue set-up **p<0.01; ***p<0.001.
Traces are representative of stable contractions developed by immersed vehicle control strips after equilibrating for a) 60 and b) 90 minutes.

Although 89 percent of tissues were set-up within a 2-hour post-operative period, the samples that could not be processed immediately were stored in oxygenated Krebs' solution at room temperature for up to 18 hours. As well as no obvious signs of bacterial contamination, ambient temperatures avoided the reduction in spontaneous activity observed with tissues stored at 4ºC (personal communication, Hutchinson, 2005). Similar myogenic responses to prostanoid compounds between fresh and stored tissues have been previously demonstrated (Hillock & Crankshaw, 1999; Popat & Crankshaw, 2001) and maintenance of tissue viability was validated in this study (Figure 2.5). As a result, data from fresh and stored tissues were pooled and collectively analysed.

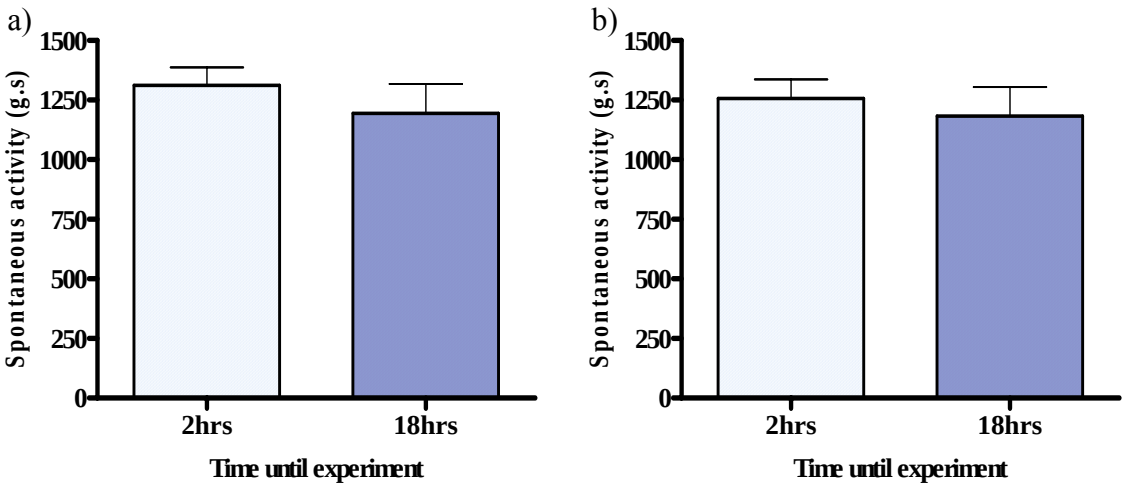


Figure 2.5: Spontaneous activity of isolated myometrium from a) non-pregnant (n=4) and b) pregnant (n=4) donors set-up for immersion within 2 or 18 hours of surgery. All hysterectomy samples were fundus-end and excised during the follicular stage of the menstrual cycle. Regular myogenicity was measured over 30 minutes as the integrated area under the curve (g.s) and expressed as means ± S.E.

2.6.1 Administration of drugs

Following tissue equilibration, vehicle and drugs were added to immersion baths at a maximum volume of 8µl to prevent fluctuations in temperature and pH. Tissue strips were randomly assigned treatments and cumulative concentration-effect curves were constructed at 30-minute intervals, using log unit concentration increases. The concentration range of agonists [10^{-12}M to 10^{-5}M] was adjusted to encompass full concentration-effect curves and spanned at least 5 log units. Responses were expressed as a percentage of hypotonic shock (details in Section 2.6.3).

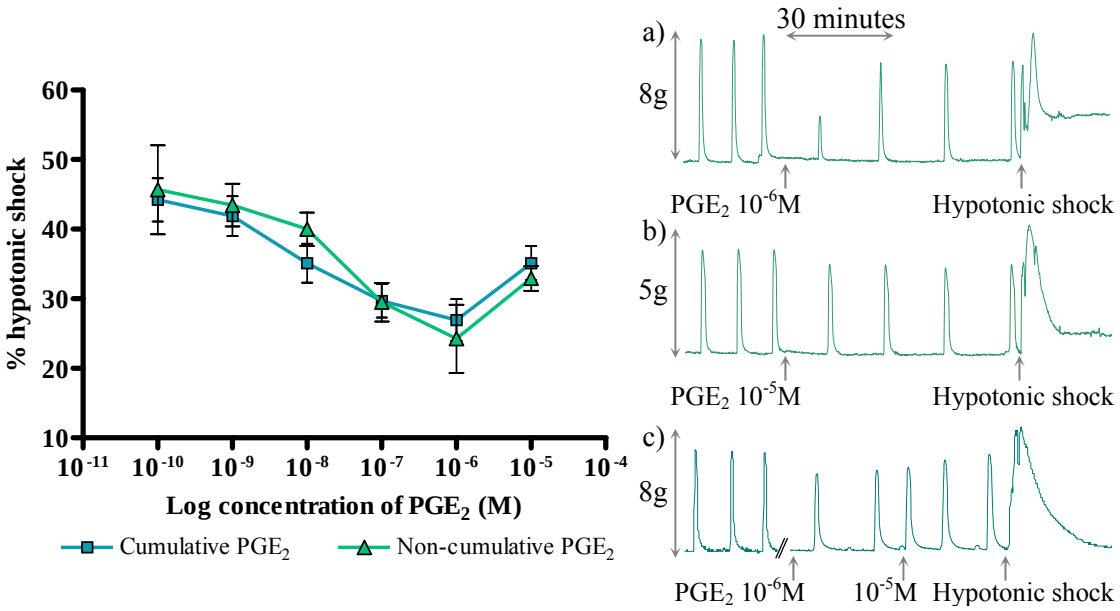


Figure 2.6: Concentration-response curves for PGE_2 in myometrium from term pregnant donors added in a cumulative (n=12) and a non-cumulative (n=5) manner to immersion baths. Traces show the changes in regular spontaneous activity with additions of PGE_2 a) at 10^{-6}M, b) at 10^{-5}M and c) added sequentially to myometrial strips.

By pooling successive agonists, a wide range of compounds could be assayed per donor tissue and individual drug effects were directly comparable to the same myometrial strip. Moreover, similar responses to PGE_2 in cumulative and non-

cumulative treatments indicated that myometrial receptors did not desensitise during experiments (Figure 2.6).

In some cases, at least one tissue strip from the same biopsy was challenged for 30 minutes with an antagonist before the addition of time-matched agonist concentrations. This served to validate the selectivity of agonists on receptors. Some agonist-treated tissue strips were washed with fresh Krebs' solution and equilibrated for 30 minutes to re-establish contractile activity (Figure 2.7). However, this was avoided when testing antagonists due to difficulties in complete removal of lipophillic compounds (Nilsson *et al*., 2003).

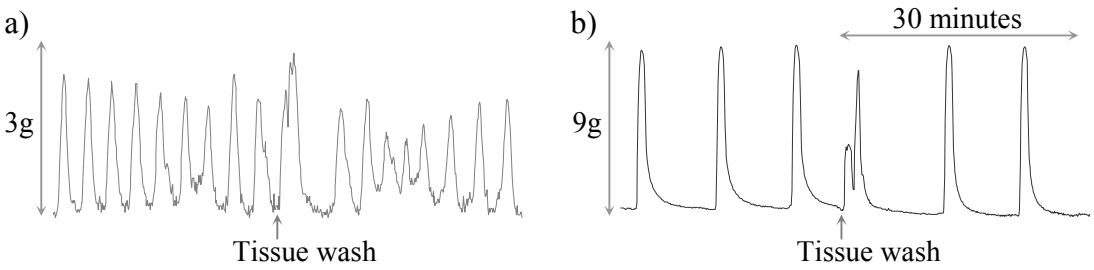


Figure 2.7: Representative traces of myometrial strips from a) non-pregnant and b) term pregnant, non-labouring donors after refilling immersion baths with fresh Krebs' solution. Washing tissue caused a brief period of quiescence before the return of myogenic activity.

2.6.2 Measurement of tissue activity

Myometrial activity was quantified as the integrated area under the contraction curve, which accounted for changes in contractile frequency, duration and amplitude. To set the lower base tension for area calculation, integral above mean settings were applied using Chart v5.4. Data were measured over 30-minute epochs (Thornton *et al*., 1999) as this time-period of activity most clearly distinguished drug-induced effects from spontaneous contractions (Figure 2.8).

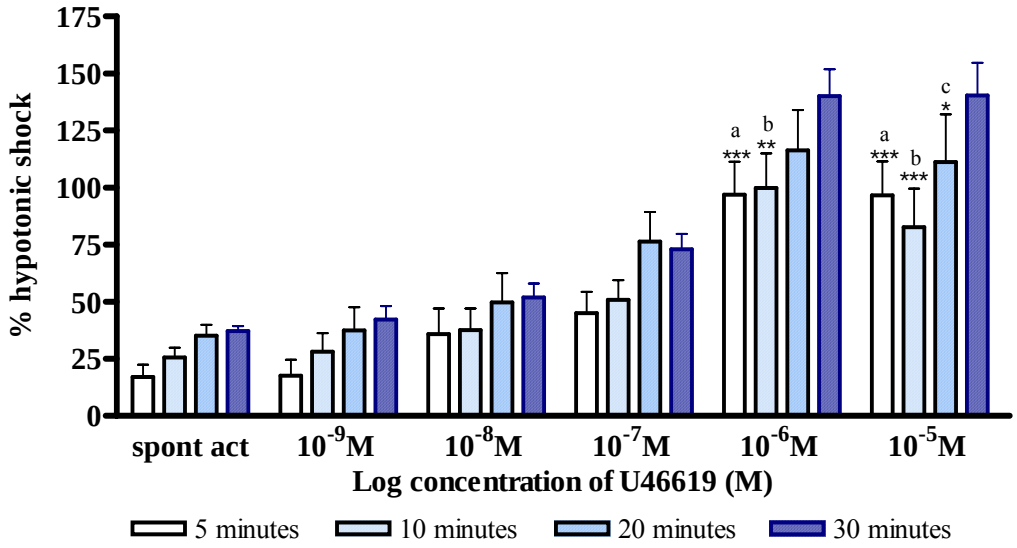


Figure 2.8: Spontaneous activity and the concentration-effect of U46619 in myometrial strips from term pregnant, non-labouring donors (n=6) measured at 5, 10, 20 and 30-minute intervals after agonist additions to muscle baths. Results are expressed as means ± S.E. and analysed using repeated measures ANOVA with Bonferroni's *post-hoc* test; *p<0.05; **p<0.01; ***p<0.001 for the reduction in measured activity over a) 5, b) 10 and c) 20 minutes compared to 30-minute responses to U46619.

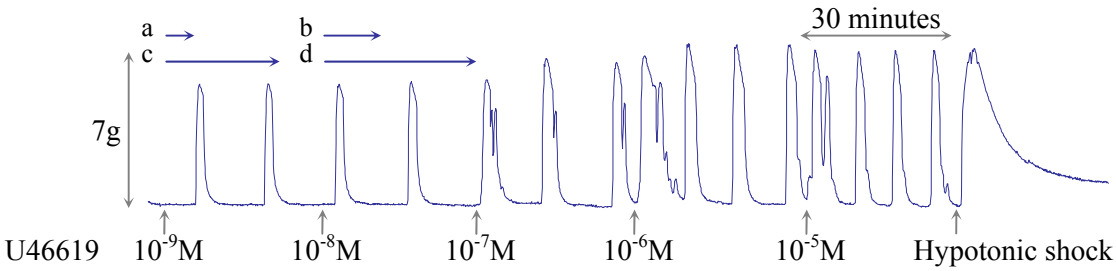


Figure 2.9: A representative trace showing the concentration-dependent increase in contractile activity to U46619 in an isolated myometrium taken at term pregnancy, not in labour. The trace was measured over a) 5, b) 10, c) 20 and d) 30 minutes after U46619 additions to the immersion bath.

2.6.3 Normalising responses

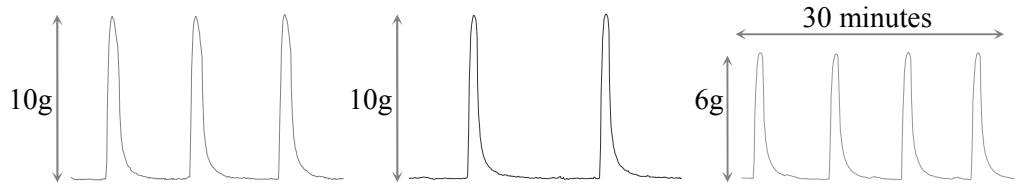


Figure 2.10: Traces showing unique 30-minute profiles of spontaneous activity from parallel myometrial strips taken from the same piece of isolated uterine tissue donated at term pregnancy.

Traces of spontaneous activity were unique for each muscle strip and functionally idiosyncratic (Figure 2.10). This was attributed to the intrinsic variability between donor hormonal states and the differences in receptor dominance, cell types and filament composition of myometrial strips (Crankshaw, 2001; Popat & Crankshaw, 2001). To reduce intra- and inter-assay variations, the data were therefore normalised.

Previous functional studies have quantified agonist-induced responses (T) as a ratio of the background activity (B) (Senior *et al*., 1991; Duckworth *et al*., 2002). However, this would not have represented isolated myometrium taken at late labour, which frequently lacked background contractility. Instead, measured responses were expressed as a percentage of a reference contraction, induced by displacing the Krebs' solution with distilled water (Popat & Crankshaw, 2001). This hypotonic shock was performed after the final drug incubation to avoid disrupting receptor and tissue activity. In addition, compounds interacting with G-protein coupled receptors would not alter the reference contraction, mediated via Ca^{2+}-activated potassium and chloride channels.

Preliminary experiments were conducted on isolated myometrium to ensure that the reference contraction was reliable and reproducible. Compared to sustained contractions by potassium chloride (KCl), maximal tissue responses to distilled water

were higher in amplitude and declined more steadily with time (Figures 2.11 & 2.12).

As a result, hypotonic shock was used to normalise response data in this study.

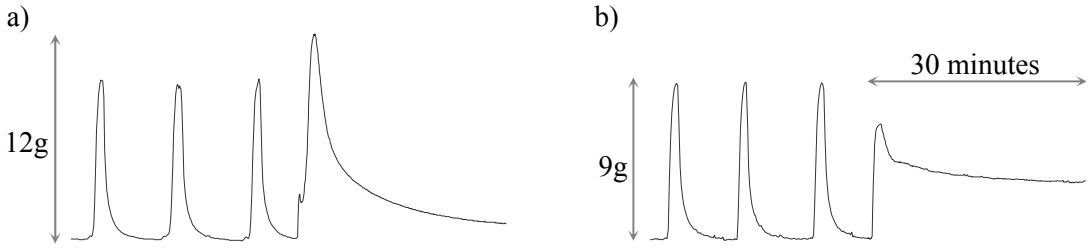


Figure 2.11: Reference contractions induced by the displacement of Krebs' solution in immersion baths with a) distilled water (hypotonic shock) and b) potassium chloride (KCl, 60mM) in myometrial strips from term pregnant, non-labouring donors displaying regular spontaneous activity. Traces show that the contraction by KCl was sustained but lower in amplitude than that produced by hypotonic shock.

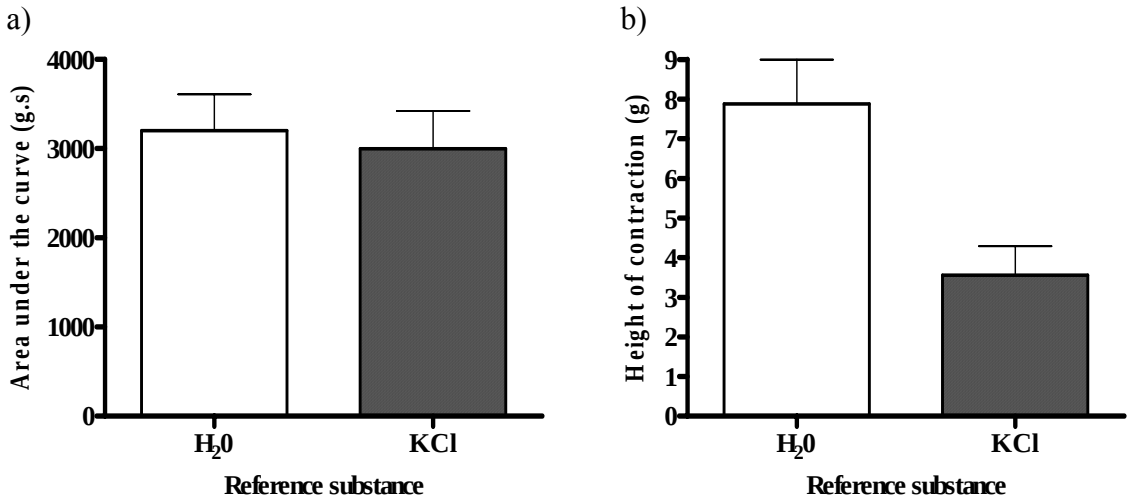


Figure 2.12: A comparison between the reference contractions induced by distilled water (hypotonic shock) and potassium chloride (KCl, 60mM) in myometrial strips from term pregnant donors (n=5). Activity was measured as a) the integrated area under the contraction curve over 30 minutes and b) the amplitude of contractions. Results are expressed as means ± S.E.

2.7 Superfusion

The superfusion technique was performed in parallel to the immersion procedure to measure myometrial responses to bolus doses of compounds. Although first described by Finkleman (1930), the method was modified to allow direct administration of test substances into the superfusate (Gaddum *et al*., 1939).

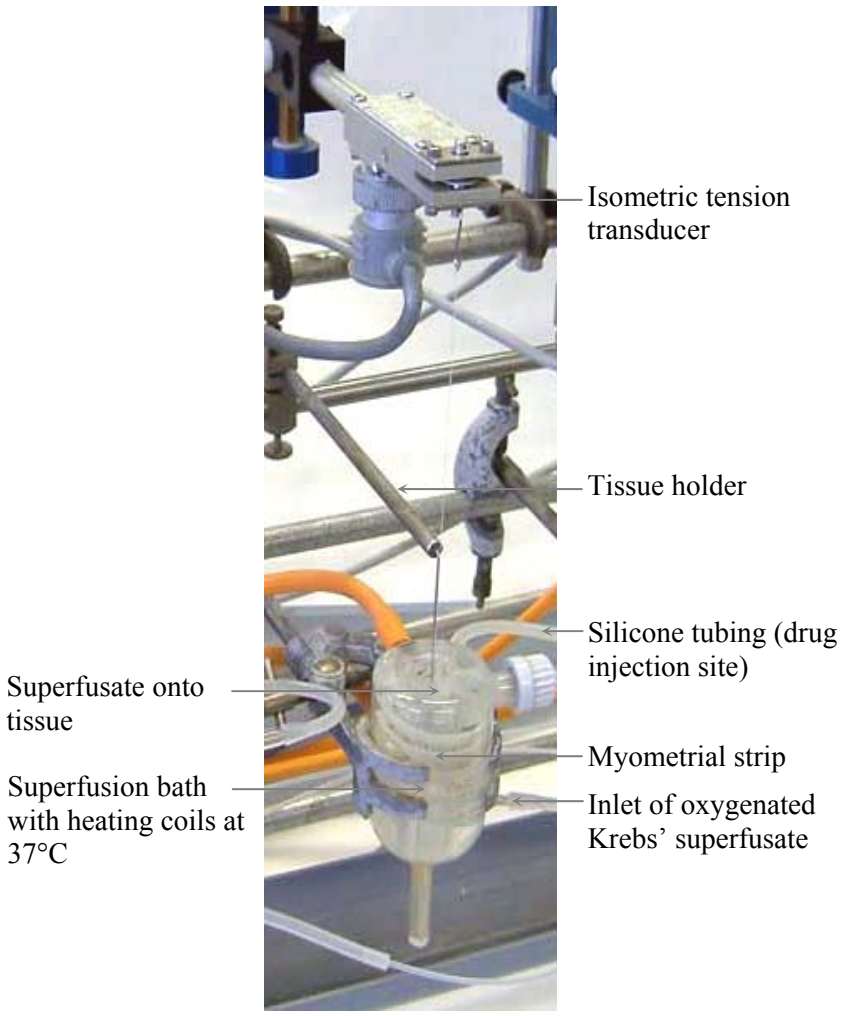


Figure 2.13: Superfusion apparatus for functional studies using isolated human myometrium.

Each uterine segment was secured to a metal tissue holder, and mounted longitudinally within a glass superfusion chamber (York Glassware Services, York, UK), heated to 37°C (Figure 2.13). After aerating with 95% O_2: 5% CO_2, the Krebs' solution was driven through heating coils within the superfusion chamber by a Watson & Marlow MHRE peristaltic pump at a rate of 2ml min^{-1} (Senior *et al*., 1991). A short section of re-sealable silicon tubing (Watson-Marlow Bredel Pumps Ltd., Cornwall, UK) was used to direct the constant flow of Krebs' solution out of the chamber and over the suspended myometrial strip.

Isometric tissue contractions were recorded using force transducers (Grass Instruments Inc., Rhode Island, USA) at a passive tension of 2g (Senior *et al*., 1991; Duckworth *et al*., 2002). The signals were amplified, digitally converted and stored in a personal computer (Dell Inc.) by PowerLab data acquisition software running Microsoft Chart v5.4 (sampling frequency 2Hz; AD Instruments, Hastings, UK).

Tissue strips from non-pregnant and pregnant donors were equilibrated for at least 60 and 90 minutes respectively or until regular phasic contractions were achieved. This mechanical activity was measured and analysed over the following 30 minutes. Compared to the immersion set-up, the development of tissue strip contractions were attenuated in amplitude rather than frequency (Figure 2.14), suggesting that fluid tension facilitated the contractile force of immersed myometrium. As the spontaneous activity of isolated tissues from labouring donors was more sporadic, experiments were initiated after 2.5 hours of equilibration.

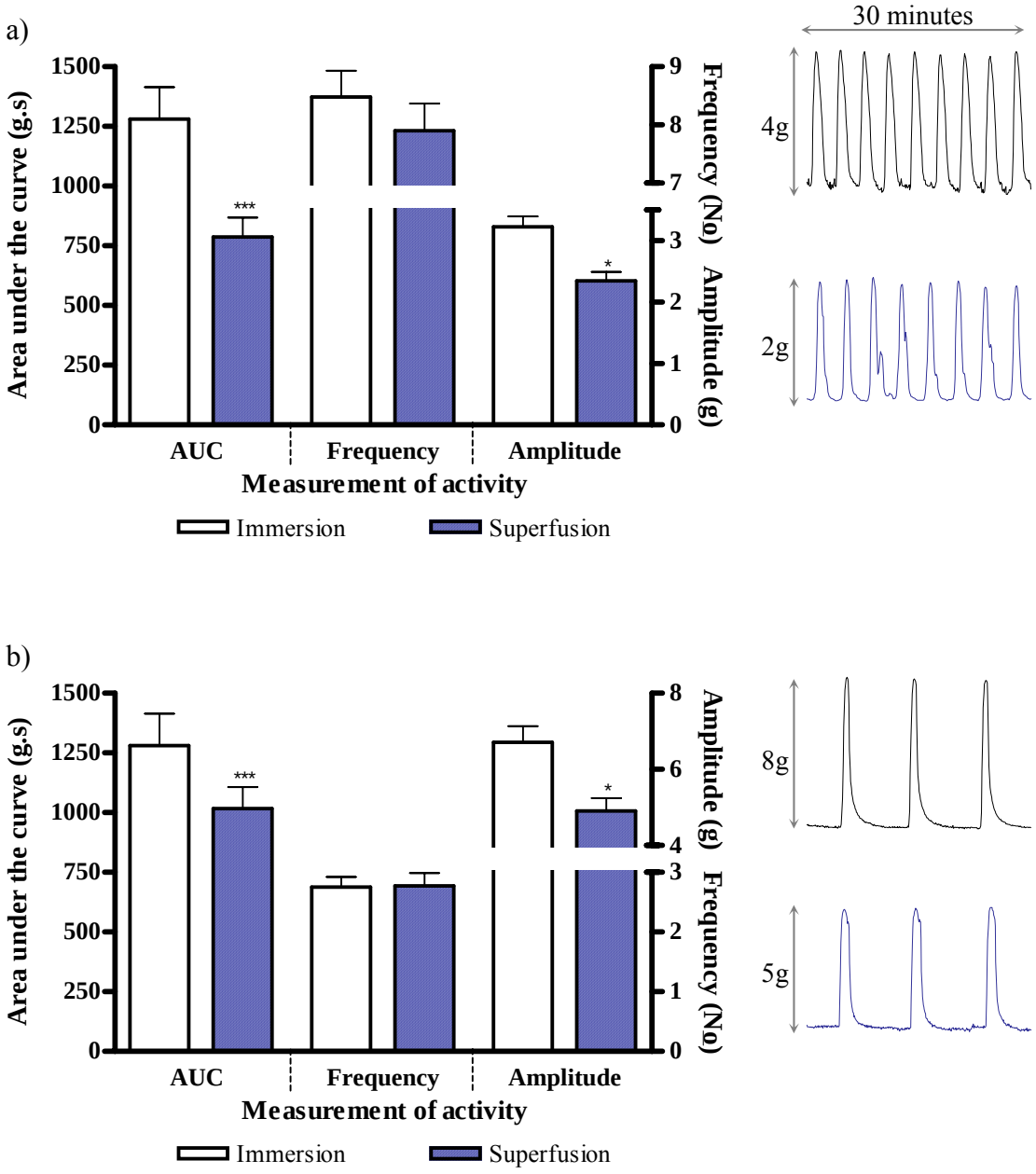


Figure 2.14: Spontaneous activity measured as area under the curve (AUC), frequency (number) and amplitude (g) of contractions over 30-minute intervals before the addition of test drugs in myometrium taken at a) hysterectomy and b) term elective Caesarean section using immersion and superfusion techniques. Results were expressed as means ± S.E and analysed using one-way ANOVA with Bonferroni's *post-hoc* test; *p<0.05; ***p<0.001 compared to contractions established in immersed tissue strips.
Traces show the intrinsic activity developed by isolated myometrium equilibrated in immersion and superfusion apparatus.

2.7.1 Administration of drugs

Agonists were injected directly into the Krebs' superfusate through re-sealable silicon tubing using a 100µl glass micropipette (Hamilton Co., Nevada, USA). For a consistent flow rate and accurate dosing, the maximum volume of agonist administered was 10µl. After tissue equilibration, vehicle and dose-response curves were constructed sequentially and doses, ranging from 10^{-14}mol to 10^{-7}mol, were adjusted according to drug potency. Tissues were treated at 30-minute intervals or until the resumption of baseline activity. This corresponded to immersion measurements and was sufficient to wash drugs from the tissues for uniform responses to repeated sub-maximal doses (Griffiths *et al*., 2006).

In parallel studies, 30 minutes prior to agonist dosing, antagonists were added to superfusate reservoirs for constant perfusion over the tissue. Muscle strips from the same biopsy were also assigned as time-matched vehicle controls with intrinsic activity sustained for the duration of each experiment (Figure 2.15). Only one dose-response curve was completed per tissue strip, after which the Krebs' solution in the reservoir was replaced with distilled water and perfused for 30 minutes. This induced a large hypotonic contraction unique to each myometrial strip (Popat & Crankshaw, 2001).

2.7.2 Measurement of tissue activity

Equivalent to immersion recordings, excitatory myometrial responses were measured immediately after dosing as the integrated area under the contraction curve over a 30-minute period. This was expressed as a percentage of the activity integral induced by 30 minutes of hypotonic shock.

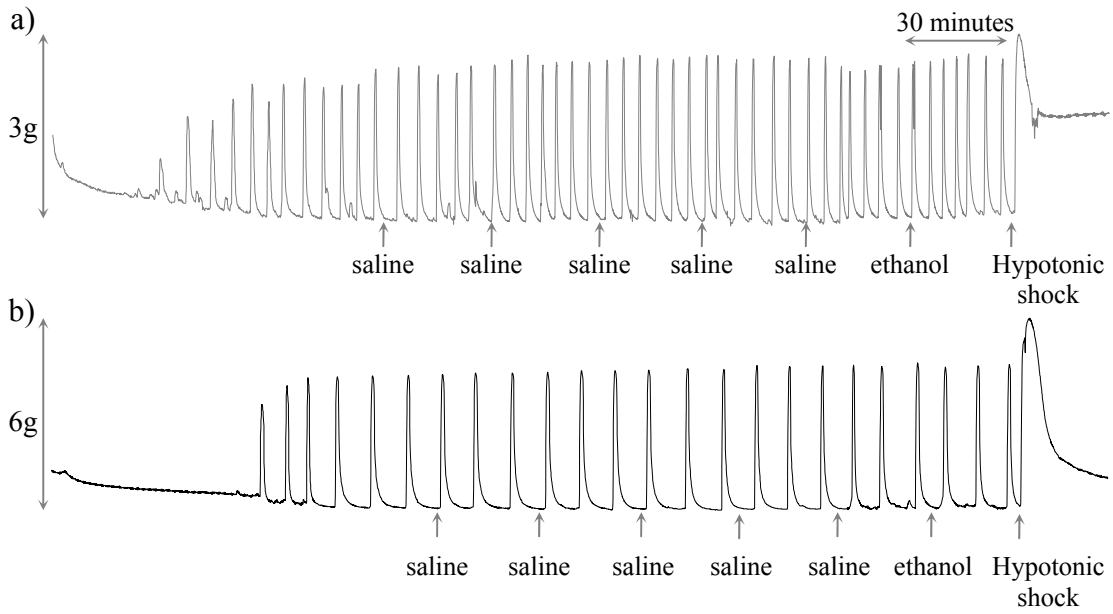


Figure 2.15: Traces showing the activity of myometrial strips from a) non-pregnant and b) term pregnant, non-labouring donors equilibrated in the superfusion apparatus. Vehicle controls demonstrated that there was no temporal effect on frequency and amplitude of contractions.

The hypotonic shock varied between non-pregnant and pregnant donor groups, with more transitory contractions reflecting smooth muscle remodelling and changes in proteins encoding ion channels during pregnancy (Challis *et al*., 2000). Even so, when measured as the area under the curve, responses to hypotonic shock were consistent regardless of the stage of menstrual cycle or gestational state (Table 2.4). This was applicable for both immersion and superfusion experiments validating further this method of data normalisation.

Table 2.4: The mean force of contractions induced by hypotonic shock (distilled water) in myometrial strips taken at menses (n=6), follicular (n=13) and luteal (n=5) stages of the menstrual cycle and at term pregnancy, not in labour (n=20), early-mid (n=13) and late (n=11) labour equilibrated in immersion and superfusion apparatus. Results were measured for 30 minutes as the integrated area under the curve (g.s) and expressed as arithmetic means ± S.E. The hypotonic shock was not significantly different, regardless of assay technique.

	Immersion	**Superfusion**
Stage of Menstrual Cycle	**Hypotonic shock (g.s)**	**Hypotonic shock (g.s)**
Menses	2093 ± 170	2282 ± 277
Follicular	2294 ± 123	2071 ± 125
Luteal	1996 ± 121	2234 ± 154
Stage of Gestation	**Hypotonic shock (g.s)**	**Hypotonic shock (g.s)**
Term pregnancy	2161 ± 388	2260 ± 133
Early-mid labour	2115 ± 367	2290 ± 467
Late labour	1777 ± 185	1809 ± 266

For inhibitory studies using superfusion, tissue myogenicity was not always re-established within the 30-minute intervals. As a result, the inhibitory response to agonists was expressed as the extended time between spontaneous contractions. Agonists were administered immediately following the repolarisation phase of a contraction to avoid superimposing responses on background activity. This time period was measured from the point of agonist injection to the recurrence of a spontaneous contraction, measuring at least 80 percent of the previous contraction peak height (Senior *et al*., 1991). Any initial excitatory responses were excluded from this measurement and background activity was corrected by subtracting the time interval between preceding regular contractions (Figure 2.16).

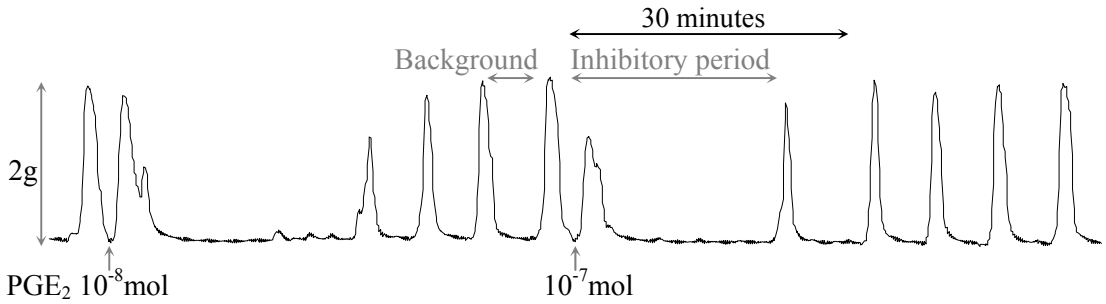


Figure 2.16: A sample trace showing measurement of the inhibitory period induced by PGE_2 added as bolus doses to myometrium from term pregnant, non-labouring donors. The response was measured as the time in minutes between agonist administration and the re-establishment of a defined spontaneous contraction minus the standard time between contractions before agonist dose administration.

2.8 Choice of technique

The contractile activity and receptor function of myometrial strips were comparable in immersion and superfusion studies, indicating the merits of using either technique. Due to the highly regulated tissue environments, each system provided data that were reliable and reproducible.

When added to immersion baths in a cumulative manner, the contact time of compounds could be determined and prolonged drug incubations mimicked *in vivo* conditions. Whilst limited compounds were conserved in muscle baths, the chemically unstable compounds were better identified using superfusion (Gaddum *et al*., 1939). As bolus doses were immediately washed, the constant perfusion of the Krebs' superfusate reduced any potential interactions of endogenous tissue metabolites. Therefore, both techniques were useful in characterising the receptors involved in myometrial activity of the isolated human uterus.

2.9 Statistical analysis

Data were first tested for normality using a Kolmogorov-Smirnov test. To examine the relationship between agonist concentrations and treatment, contractile activity of myometrial strips was compared using a student's t-test or one-way/ two-way analysis of variance (ANOVA) in a mixed model. *Post-hoc* comparisons were performed using Bonferroni's adjustment. Estimates of the maximal effect (E_m) and inhibitory (pIC_{50}) or excitatory (pEC_{50}) curve mid-points were calculated for agonists and antagonists at different stages of the menstrual cycle, term pregnancy and parturition using non-linear regression (GraphPad Prism 4.0, San Diego, CA, USA). When E_m was not reached, constraints for the lower asymptote were set at greater than zero to avoid curves that tended to infinity. For competitive antagonists, pA_2 values were also estimated as a measure of affinity using the Schild's equation. Results were expressed as arithmetic means ± S.E. and significance was attributed at $p<0.05$.

Chapter 3: Non-pregnant

Functional prostanoid receptors in isolated non-gravid myometrium

3.1 Introduction

The uterus undergoes dramatic changes during the menstrual cycle, facilitating sperm transport, ovum fertilisation and implantation of the developing blastocyst. In the absence of pregnancy, the corpus luteum involutes and the resultant withdrawal of progesterone causes cyclic degradation and shedding of the superficial endometrial layer (menstruation). This involves a process of tissue injury and spiral arteriole vasoconstriction predominantly mediated by elevated $PGF_{2\alpha}$, PGE_2, COX enzymes, oxytocin and endothelin-1 (Marsh *et al*., 1995; Smith *et al*., 2007). Further increases in circulatory prostanoids and oxytocin have been associated with common gynaecological disorders, including menorrhagia (excessive menstrual blood loss) and dysmenorrhoea (painful periods) (Adelantado *et al*., 1988; Noe *et al*., 1999; Dawood & Khan-Dawood, 2007; Smith *et al*., 2007). Despite the accompanied hypercontractility of the uterus (Leyendecker *et al*., 2004; Altunyurt *et al*., 2005; Kataoka *et al*., 2005; Dawood & Khan-Dawood 2007), myometrial responsiveness to PGs and oxytocin receptor agonists remain unclear.

To identify functional uterine PG and oxytocin receptor populations, *in vitro* studies were performed using myometrial biopsies taken at hysterectomy from pre-menopausal women (aged 29 to 52 years). Phasic myometrial activity and receptor-mediated effects were assessed according to the stage of menstrual cycle and excision site under physiological conditions. These results also provided a baseline comparison for gestational tissues taken before and after labour-onset.

Chapter 3.2: Results

3.3 Samples from non-pregnant donors

Although harvested at total hysterectomy, 83 percent of the provided uterine specimens were incomplete longitudinal sections taken from the fundus and adjoined upper corpus muscle. This corresponded to the fewer assays using lower segment tissues from non-pregnant donors. The orientation of uterine muscle was denoted by a ligature aligned with the upper serosal edge and experimental tissues were excised from the mid-uterine wall >5mm from either the endometrial or serosal surfaces. Regardless of regional location and stage of menstrual cycle, samples were equilibrated for 89 minutes (range: 77 to 153 minutes) to facilitate the development of regular phasic activity. In spite of intra- and inter-donor variations, myogenic contractile profiles were distinct in frequency and amplitude; this was particularly manifest during the different stages of the menstrual cycle.

3.4 Myogenic activity at different stages of the menstrual cycle

All myometrial strips obtained from non-pregnant donors at hysterectomy developed spontaneous activity. The undulating activity was most frequent, but sporadic during the menstrual phase of the cycle (Figure 3.1). However, the overall myogenicity was only at 37.2 ± 1.38 percent hypotonic shock due to the low amplitude of contractions (Table 3.1). In tissues taken mid-cycle from the fundus, the amplitude of contractions was 3.1 and 1.5 fold greater than myometrium from menstrual ($p<0.001$) and luteal phases, when contractions were slow and phasic. The rank order of myogenic activity was follicular > luteal > menstrual stages. Moreover, variation was exhibited

topographically during the follicular phase with activity 20 percent greater in lower compared with fundus-end tissues ($p<0.001$). This was due to the differences in amplitude rather than frequency of contractions.

3.5 Indications for hysterectomy

Hysterectomies were performed for benign gynaecological disorders, including menorrhagia, dysmenorrhoea and fibroids. All donors were premenopausal and use of oral contraceptives or hormone therapies had been discontinued for 6-8 weeks prior to surgery. Thereby, the only medications noted on patient forms for 3 individual donors were aspirin and Ventolin. Despite the different indications for hysterectomy, phasic contractile activity was not influenced by donor symptoms or underlying disease states (Table 3.2).

3.6 Longitudinal and transverse myometrial sections.

Although myometrial strips were dissected longitudinally for functional studies, the spontaneous contractions generated by transversely cut sections gained comparable intensity following equilibration in organ baths (Table 3.3). This was observed in both fundus and lower segment samples.

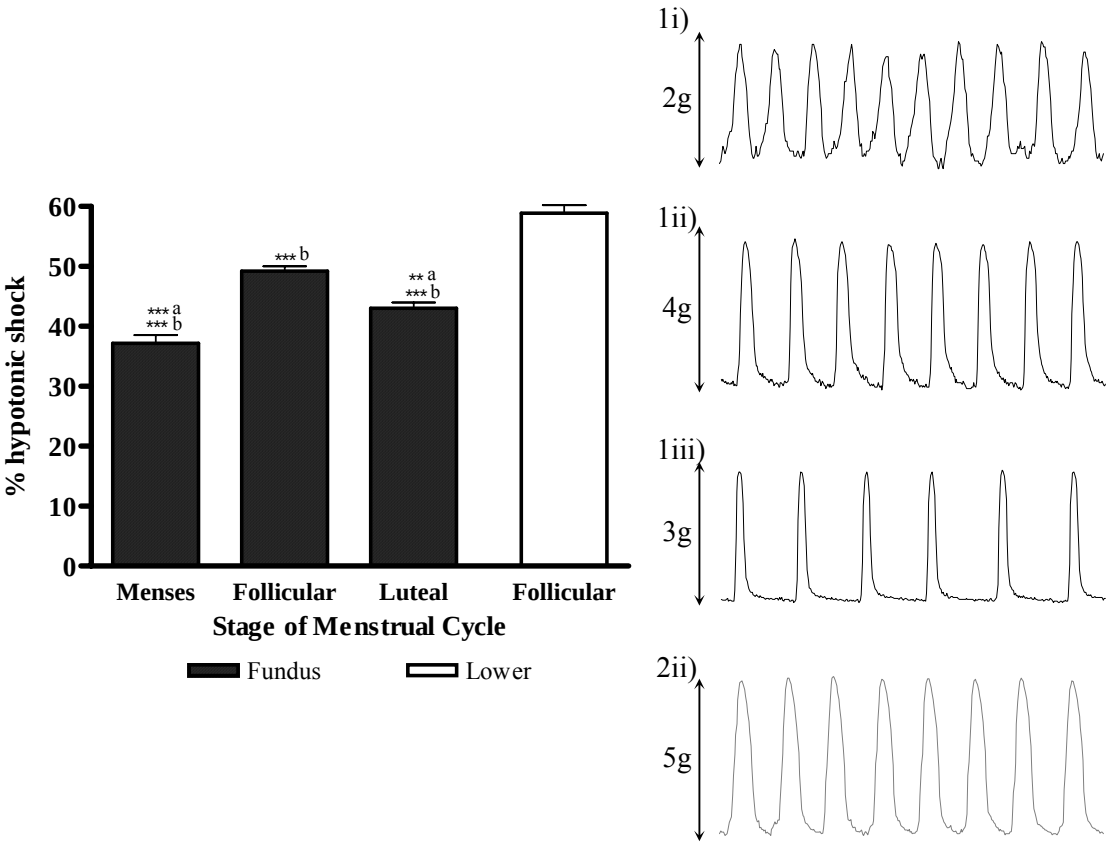


Figure 3.1: Spontaneous contractions of immersed myometrial strips taken at hysterectomy. Myometrium was excised from fundus (n=20) and lower (n=4) segment uterine muscle at the different stages of the menstrual cycle. After equilibration, myogenic activity was measured as 30 minutes area under the curve and expressed as a percentage of 30 minutes hypotonic shock. Data are arithmetic means ± S.E. and statistics were performed using univariate ANOVA with Bonferroni's *post-hoc* adjustment; **p<0.01; ***p<0.001 reduction in contractility compared with tissues taken during the follicular stage from [a]fundus and [b]lower segment tissues.

Traces display typical spontaneous activity of immersed 1) upper and 2) lower segment isolated myometrium obtained from non-pregnant donors during i) menses, ii) follicular and iii) luteal stages of the menstrual cycle. Each trace represents a 30-minute time period.

Table 3.1: Spontaneous activity measured as amplitude (g) and frequency of contractions over 30-minute intervals before the addition of study compounds in myometrium taken at hysterectomy (n=3-5). Myogenic profiles were evaluated according to menstrual cycle stage and uterine location using the immersion technique. Results are expressed as means ± S.E. and analysed using one-way ANOVA with Bonferroni's *post-hoc* adjustment; *p<0.05; **p<0.01; ***p<0.001 for myogenic contractions compared to fundus end tissues taken at [a]menses, [b]follicular and [c]luteal phases of the cycle.

Location	Fundus			Lower
Stage of cycle	Menses	Follicular	Luteal	Follicular
Amplitude (g)	1.3 ± 0.2	3.9 ± 2.6^{***a}	2.6 ± 0.4^{*a}	4.4 ± 0.6$^{*b;\ ***ac}$
Frequency (No)	9.9 ± 0.6	7.8 ± 1.3^{*a}	6.7 ± 0.5^{**a}	8.4 ± 1.7

Table 3.2: Spontaneous activity of myometrial strips equilibrated in immersion baths and categorised according to the main indications noted on patient information forms. Uterine samples were harvested from non-pregnant donors with each group in the follicular stage of the menstrual cycle (n=3-5). Data were measured as 30 minutes area under the curve, expressed as a percentage of 30 minutes hypotonic shock (HS) and results are arithmetic means ± S.E.

Indications	Menorrhagia	Dysmenorrhoea	Menorrhagia & dysmenorrhoea	Menorrhagia & fibroids
Activity (%HS)	47.0 ± 2.3	46.8 ± 1.9	51.5 ± 2.9	50.9 ± 2.2

Table 3.3: Spontaneous activity of immersed myometrial strips dissected longitudinally or transversely across fundus and lower segment uterine muscle (n=3). Myometrial samples were obtained at hysterectomy in the non-pregnant state. Following equilibration, regular myogenic activity was measured as 30 minutes integrated area under the curve and expressed as a percentage of 30 minutes hypotonic shock. Results are arithmetic means ± S.E.

Location	Fundus		Lower segment	
Dissected fibres	Longitudinal	Transverse	Longitudinal	Transverse
Activity (%HS)	36.0 ± 0.8	37.3 ± 1.8	53.7 ± 3.0	54.1 ± 1.8

3.7 Characterisation of EP receptors using PGE_2 and EP analogues

PGE_2 is well characterised in its targeting of $EP_{1\text{-}4}$ receptors (Coleman *et al*., 1994). To broadly identify myometrial responsiveness, PGE_2 was assayed in the absence and presence of indometacin and across the different phases of the menstrual cycle.

3.7.1 Indometacin on myometrial responses to PGE_2

Indometacin, a non-selective COX inhibitor, has been shown to block myometrial PG biosynthesis at 10^{-6}M (Durn *et al*., 2010). In immersion studies, PGE_2 alone diminished contractions in a concentration-dependent manner (10^{-10}M to10^{-6}M), with relative stimulation at 10^{-5}M (Figure 3.3). Pre-incubation with indometacin (10^{-6}M) did not significantly alter myogenic contractions or responses to PGE_2 ($F(1, 35) = 0.55$; ns). Therefore, to mimic physiological responses, indometacin was omitted from the Krebs' solution in all further immersion and superfusion experiments.

3.7.2 Myometrial EP receptors during the menstrual cycle

Responses to PGE_2 were similar in fundus-end myometrium taken at hysterectomy from non-pregnant donors at different stages of the menstrual cycle ($F(2, 77) = 1.56$; ns; Figure 3.4). PGE_2 attenuated myogenic activity (10^{-10}M to 10^{-6}M) to an analogous extent at each phase of the menstrual cycle. At the highest concentration of 10^{-5}M, 13 percent stimulation relative to contractions at 10^{-6}M was achieved. However, this varied between donors and appeared to be most pronounced, but not significantly so, in myometrium collected during the menstrual and luteal stages. With predominant collection of follicular stage tissues and comparable responses to PGE_2, myometrial tissues were grouped irrespective of phase in functional studies.

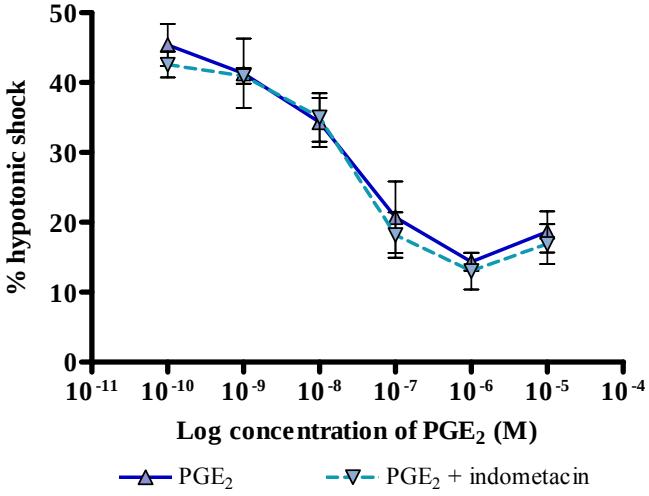


Figure 3.3: Concentration-effect curves for PGE_2 alone and in the presence of indometacin (10^{-6}M) on isolated fundus-end myometrium from non-pregnant donors (n=4). Contractility was measured as 30 minutes area under the curve and expressed as a percentage of 30 minutes hypotonic shock. Results are arithmetic means ± S.E. Indometacin, the non-selective COX inhibitor, did not influence tissue myogenicity or responsiveness to PGE_2.

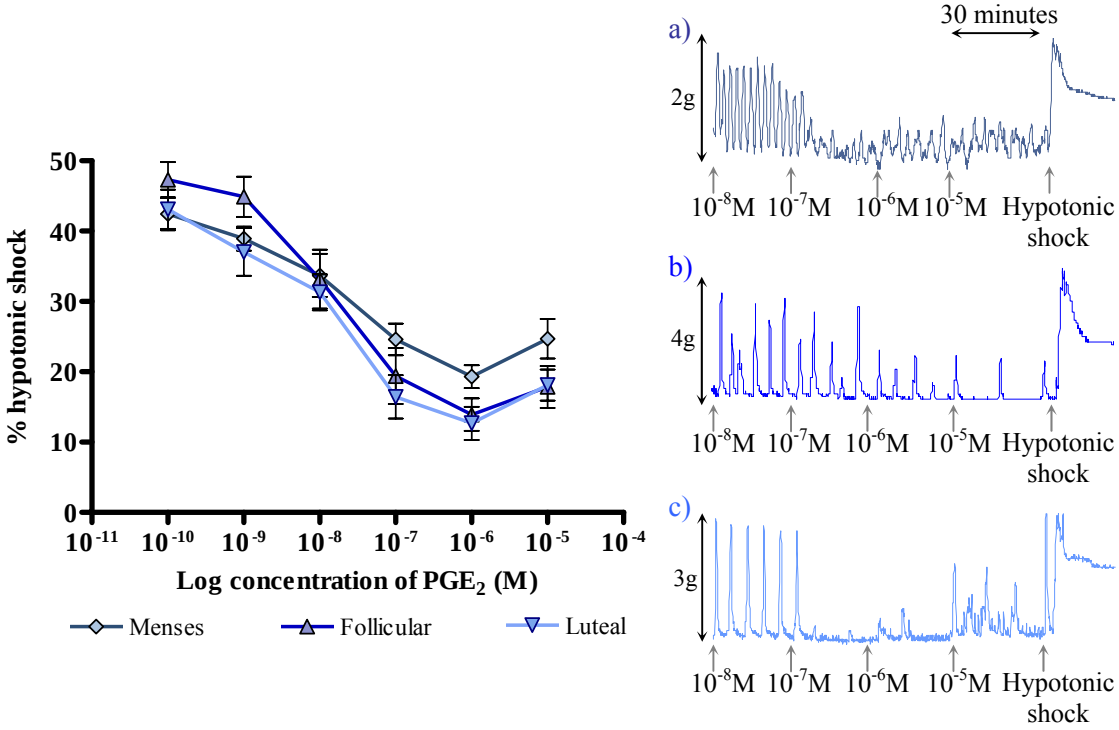


Figure 3.4: Concentration-effect curves and representative traces showing the response to PGE_2 in isolated fundus-end myometrium taken at a) menses (n=4), b) follicular (n=8) and c) luteal (n=4) phases of the menstrual cycle. PGE_2 was added in a cumulative manner to organ baths at 30-minute intervals and results are expressed as arithmetic means ± S.E.

3.7.3 Inhibitory effects of EP_2 agonists on myogenic activity

In immersed fundal myometrial strips from non-pregnant donors, the EP_2 mimetics butaprost and CP533,536 evoked concentration-dependent inhibition of myogenic activity (10^{-10}M to 10^{-5}M) via a reduction in the frequency and amplitude of contractions ($F(2, 90) = 35.54$; $p<0.001$; Figure 3.5). Similar potency values were shown by respective pEC_{50} values at 6.87 ± 0.47M and 6.99 ± 0.43M and a 3.2-fold and 4.0-fold decline in myogenicity (Table 3.4).

3.7.4 EP_4 agonist effects on myogenic activity

Compared to the sustained myogenicity with time-matched controls, the EP_4 agonist AGN201734 (10^{-10}M to 10^{-6}M) attenuated contractions in a concentration-dependent manner ($F(1, 82) = 37.15$; $p<0.001$; Figure 3.5). The gradual reduction in activity from 46.2 ± 2.78 to 21.7 ± 4.13 percent of hypotonic shock plateaued at 10^{-6}M with 9.2 percent relative excitation at 10^{-5}M. Despite similar biphasic curves and potency values, the efficacy of AGN201734 was lower than PGE_2 (Table 3.4).

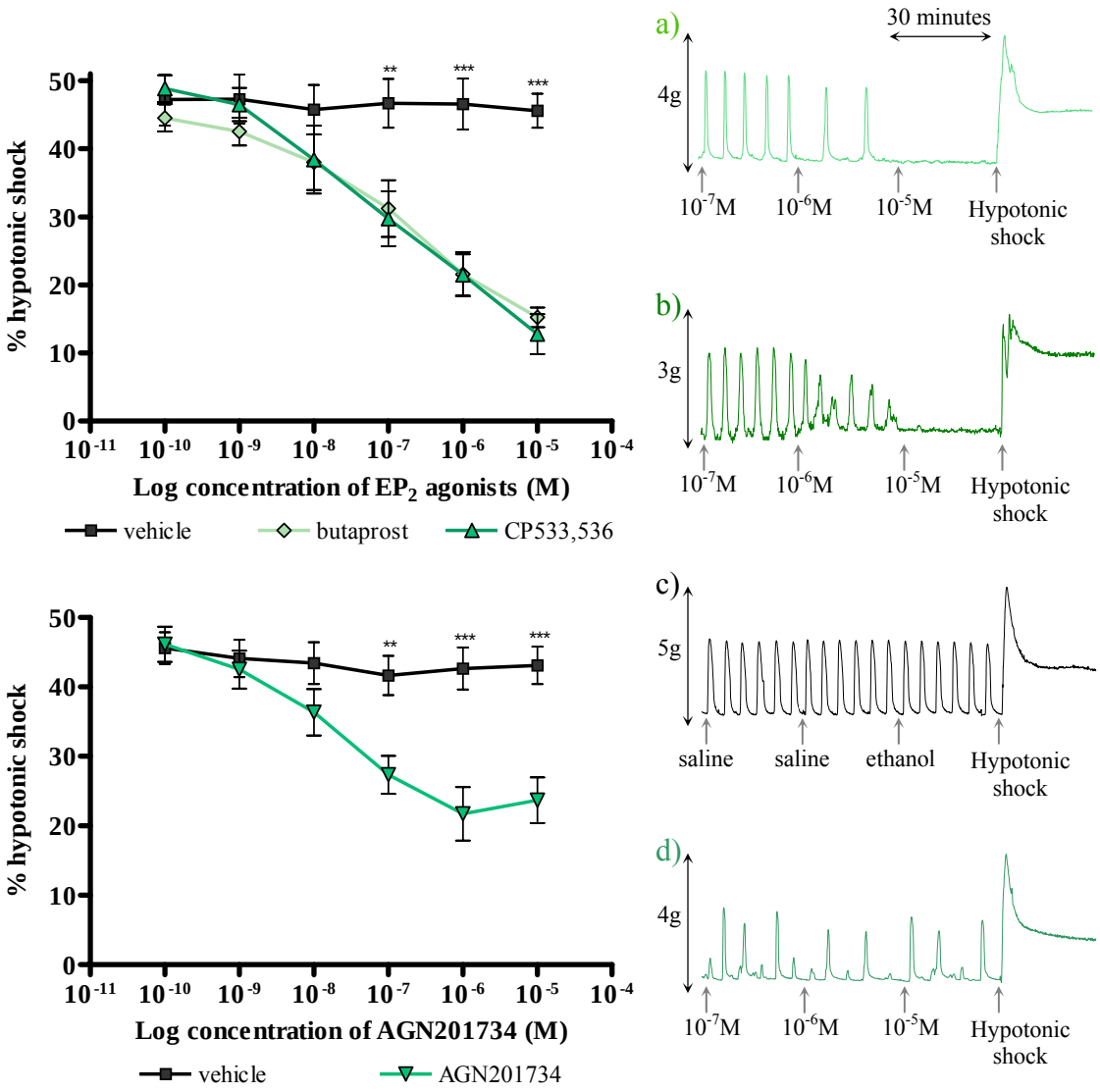


Figure 3.5: Concentration-effect curves and typical traces for the EP_2 agonists a) butaprost, b) CP533,536 and the EP_4 mimetic d) AGN201734 compared to c) vehicle in human fundal myometrium obtained from non-pregnant women (n=6-7). Agonists (10^{-10}M to 10^{-5}M) were added in a cumulative manner at 30-minute intervals to immersed myometrial strips. Results are expressed as arithmetic means ± S.E. and significant differences were determined using two-way ANOVA with Bonferroni's *post-hoc* test; **p<0.01; ***p<0.001 compared to vehicle controls.

Table 3.4: Mean pIC_{50} values (M) and percentage reduction in myogenicity for PGE_2, butaprost, CP533,536 and AGN201734 concentration-effect curves in immersed myometrium from non-pregnant, pre-menopausal donors (n=6-9).

Agonists:	**PGE_2**	**Butaprost**	**CP533,536**	**AGN201734**
pIC_{50}	7.7 ± 0.2	6.9 ± 0.5	7.0 ± 0.4	7.7 ± 0.3
% decrease	68.4	68.7	75.3	56.7

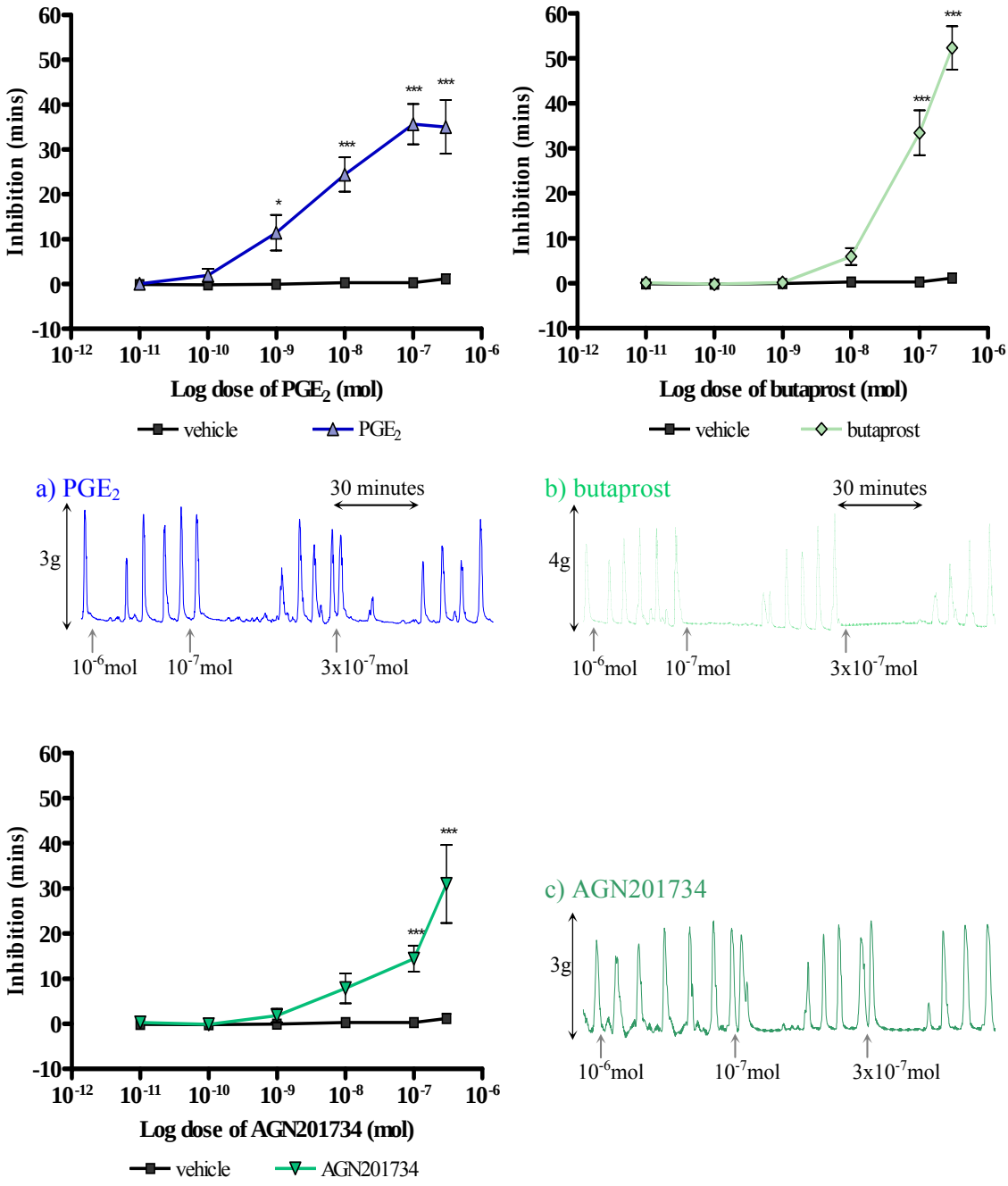


Figure 3.5: Vehicle, utero-relaxant effects and representative traces of a) PGE_2, b) butaprost and c) AGN201734 in human fundus-end myometrial tissue from non-pregnant women in the follicular stage of the cycle (n=3-5). Tissue strips were suspended in superfusion baths and bolus doses of agonists (10^{-11}mol to $3x10^{-7}$mol) and vehicle were administered directly into the superfusate after a contraction of similar amplitude to the myogenic activity. Results are expressed as means ± S.E. with statistical analysis performed using two-way ANOVA with Bonferroni's *post-hoc* adjustment; *p<0.05; ***p<0.001 for EP agonists compared with vehicle controls.

Using the superfusion technique, bolus doses of the EP mimetics evoked immediate responses in fundus-end myometrial strips from non-pregnant donors (Figure 3.5). Whilst myogenic contractions were sustained with time-matched vehicles, PGE_2 and AGN201734 exhibited biphasic responses (10^{-7}mol to $3x10^{-7}$mol), consisting of an initial contraction followed by a period of inhibition ($F(2, 84) = 54.37$; $p<0.001$). Despite the higher potency of PGE_2 (pEC_{50}: 8.41 ± 0.23M), delays in contractility were analogous for PGE_2 and AGN201734 at $3x10^{-7}$mol ($F(1, 36) = 2.30$; ns). This was observed as a significant reduction in the frequency of contractions before normal spontaneous activity was restored.

Unlike PGE_2 and AGN201734, exposure to butaprost produced a dose-related suppression of tissue activity without exerting tissue excitation ($F(1, 48) = 145.2$; $p<0.001$). The complete cessation of contractions reached 52.3 ± 5.38 minutes, which was 1.5 and 1.7-fold longer than challenge with PGE_2 and AGN201734 respectively. Due to the restricted bolus volume, maximal responses were not achieved for AGN201734 or butaprost.

3.7.5 EP agonists in the presence and absence of GW627368x

To more fully characterise functional receptors, responses to EP agonists were examined alone and in the presence of the EP_4 antagonist GW627368x (10^{-6}M; Wilson *et al*., 2006). As shown by time-matched vehicle controls, GW627368x had no effect on the myogenic activity of fundus-end uteri from non-pregnant donors ($F(1, 48) = 0.77$; ns; Figure 3.6). Likewise, the concentration-related inhibitory effects of butaprost ($F(1, 48 = 0.41$; ns) and PGE_2 ($F(1, 36 = 1.32$; ns) were unchanged, regardless of pre-incubation with GW627368x. Even so, GW627368x displaced the AGN201734 curve rightwards ($F(1, 48) = 4.13$; $p<0.05$). This was shown by a

change in pEC_{50} values ($p<0.001$; Table 3.5) and also by the estimate of pA_2 (see below), rather than the percentage reduction in spontaneous activity.

Table 3.5: Mean pEC_{50} values (M) and the percentage reduction in myogenicity for concentration-effect curves to EP agonists (10^{-10}M to 10^{-5}M) using immersed myometrial strips in the presence (treated) and absence (control) of the EP_4 antagonist GW627368x (10^{-6}M). Human myometrium was harvested from non-pregnant donors at hysterectomy (n=4-6). Data are shown as arithmetic means ± S.E. and statistical analysis was performed using Student's t-tests; ***$p<0.001$ for AGN201734 pEC_{50} values of control compared with treated groups.

EP agonists	**Control**		**Treated**	
	pEC_{50}	% decrease	pEC_{50}	% decrease
PGE_2	8.4 ± 0.2	69.7	8.6 ± 0.2	70.2
butaprost	7.5 ± 0.5	71.4	7.2 ± 0.4	68.5
AGN201734	7.5 ± 0.2	51.9	5.3 ± 0.3***	41.1

To estimate the pA_2 value for GW627368x, the Schild's equation was applied where KB was the dissociation constant of the antagonist:

$(EC_{50\ antagonist}/\ EC_{50\ agonist})\ -1 = [antagonist]/\ KB \qquad pA_2 = -\log (KB)$

$\therefore\ (4.6x10^{-6}M/\ 1.3x10^{-7}M)\ -1 = [10^{-6}M]/\ KB \qquad pA_2 = -\log (3x10^{-8}M) = 7.53$

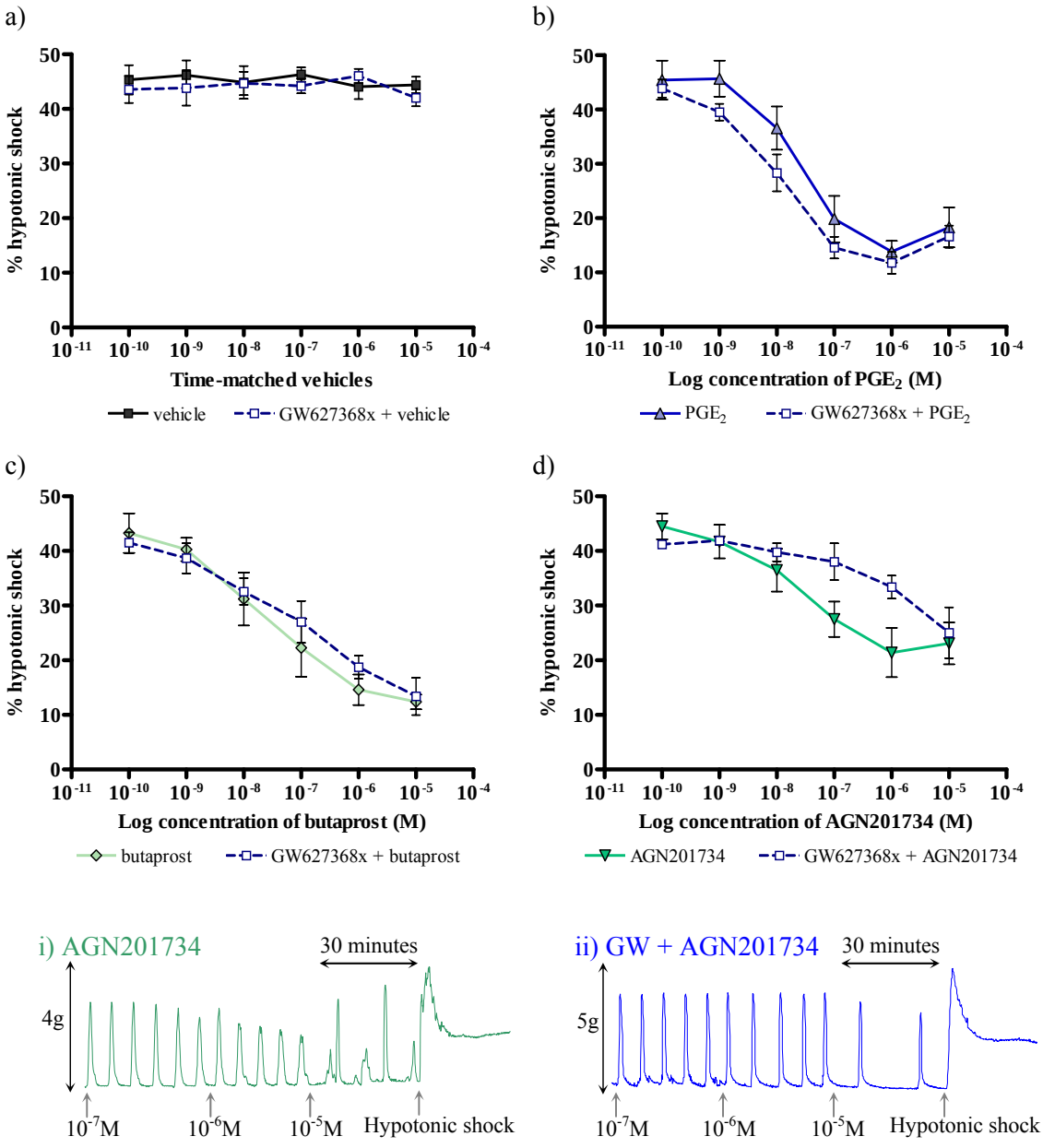


Figure 3.6: a) Vehicle and concentration-effect curves for b) PGE_2, c) butaprost and d) AGN201734 in isolated fundus-end myometrium donated by non-pregnant women at hysterectomy (n=4-6). Myometrial strips were incubated alone or in the presence of the EP_4 antagonist GW627368x (10^{-6}M), with agonists (10^{-10}M to 10^{-5}M) added in a cumulative manner at 30-minute intervals to immersion baths. Results are expressed as means ± S.E.

Traces show typical myometrial responses to i) AGN201734 alone and ii) pre-incubated with GW627368x (10^{-6}M) in immersed fundus-end samples harvested mid-cycle.

3.7.6 Excitatory EP agonists on myogenic activity

Using the immersion technique in fundus-end uterine samples, time-matched vehicle controls maintained myogenicity (Figure 3.7). Although the EP_1 agonist ONO-D1-004 had no effect on phasic contractions ($F(1, 48) = 1.32$; ns), excitation was evoked in response to the $EP_{3/1}$ agonist sulprostone ($F(1, 72) = 8.79$; $p<0.001$). As a result, myogenic activity was elevated from 48.3 ± 2.39 to 60.1 ± 2.17 of the hypotonic shock value; this was sustained by sulprostone (10^{-6}M to 10^{-5}M), without adjusting the muscle tonus.

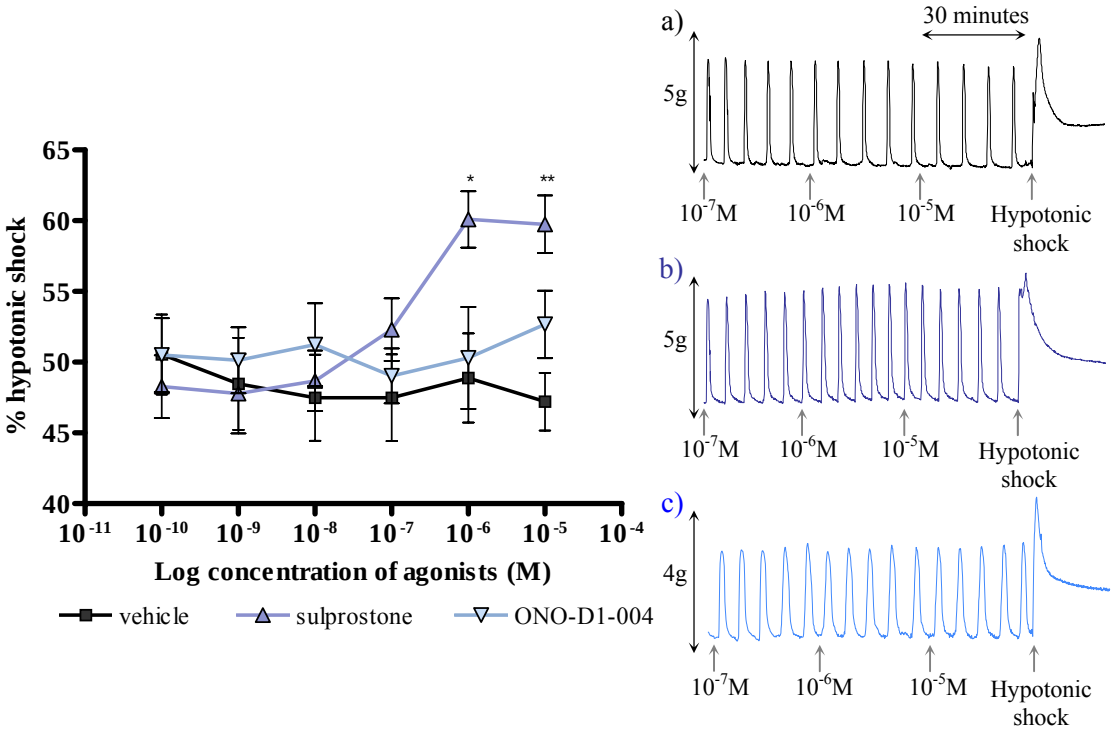


Figure 3.7: Concentration-effect curves and representative traces for a) vehicle, b) the $EP_{3/1}$ agonist sulprostone and c) the EP_1 mimetic ONO-D1-004 in fundus-end uterine samples obtained from non-pregnant donors (n=4-7). Time-matched vehicles and spasmogens were added in a cumulative manner (10^{-10}M to 10^{-5}M) to individual organ baths at 30-minute intervals. Results are arithmetic means ± S.E. with significance determined using multivariate ANOVA with Bonferroni's *post-hoc* test; *$p<0.05$; **$p<0.01$ for responses to sulprostone compared with vehicle controls.

3.7.7 Topographical effect of EP receptor agonists

After obtaining full-thickness longitudinal sections of the uterus at total hysterectomy, upper and lower segment myometrial strips were dissected from the cephalic and caudal extremities. To determine regional responsiveness to uterotonins, contractile parameters were established using immersed tissues from donors in the follicular stage of the menstrual cycle. Despite the 1.2-fold higher spontaneous activity exhibited by lower segment tissues compared with the fundus ($p<0.001$; Figure 3.1), the concentration-dependent trends of EP agonists were similar (Figures 3.8 & 3.9; Table 3.6). With PGE_2, butaprost and AGN201734, the monophasic *in vitro* utero-relaxant effects were more pronounced towards the cervix ($p<0.001$). Although differences were manifest between 10^{-10}M to 10^{-7}M ($p<0.001$), myogenic responses subsided to 15.7 ± 2.41 hypotonic shock at 10^{-5}M. This was equivalent to the abolition of activity at 10^{-5}M in fundus-end tissues.

By contrast, the $EP_{3/1}$ mimetic sulprostone evoked concentration-dependent excitation in myometrial strips, with peak contractility at 10^{-6}M ($F(1, 42) = 50.62$; $p<0.001$). Compared with time-matched vehicle controls, contractions were augmented by 23.7 and 25.1 percent in upper and lower tissue strips respectively. This demonstrated parallel responses to sulprostone, regardless of uterine topographical location (Table 3.6).

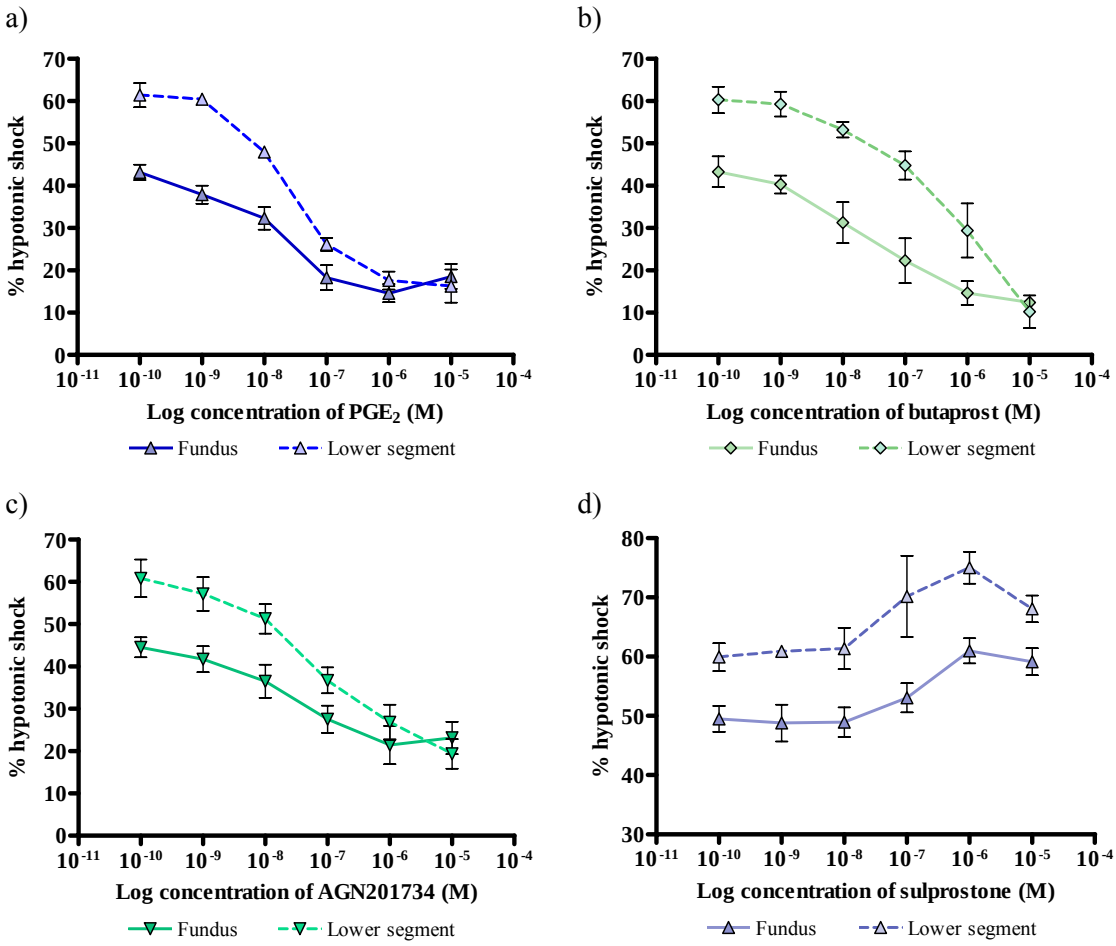


Figure 3.8: Concentration-effect curves for a) PGE_2, b) butaprost, c) AGN201734 and d) sulprostone in fundal and lower human myometrium taken at the follicular stage of the menstrual cycle (n=4-6). After equilibration in immersion baths, responses to EP mimetics were measured as 30 minutes integrated area under the curve and expressed as a percentage of 30 minutes hypotonic shock. Results are arithmetic means ± S.E.

Table 3.6: Mean pEC_{50} values (M) ± S.E. for PGE_2, butaprost, AGN201734 and sulprostone concentration-effect curves in immersed upper and lower segment myometrium from non-pregnant, pre-menopausal donors (n=4-6).

Agonists	Region	PGE_2	Butaprost	AGN201734	Sulprostone
pEC_{50}	Fundus	7.9 ± 0.1	7.3 ± 0.5	7.5 ± 0.2	6.9 ± 0.5
pEC_{50}	Lower	7.6 ± 0.2	6.6 ± 0.6	7.2 ± 0.1	7.2 ± 0.4

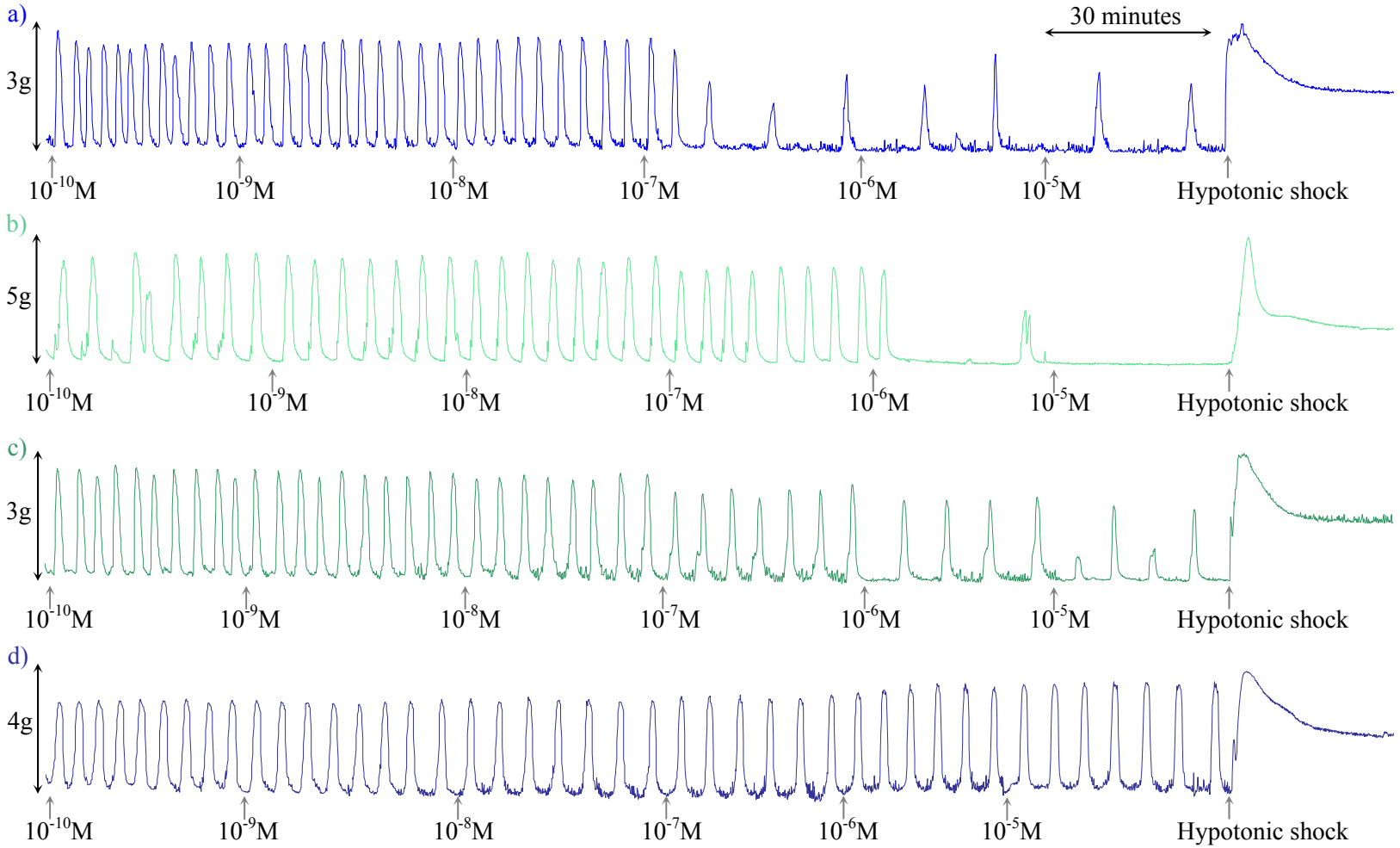


Figure 3.9: Typical traces showing the concentration-effect of a) PGE_2, b) butaprost, c) AGN201734 and d) sulprostone (10^{-10}M to 10^{-5}M) in immersed lower segment myometrium from non-pregnant donors. Agonists were added to organ baths at the time-points indicated by the arrows.

3.8 Topographical responsiveness to PGF_{2a} and U46619

The spasmogens $PGF_{2\alpha}$ and U46619 increased the amplitude and frequency of contractions in myometrial tissues from non-pregnant donors whilst maintaining a constant baseline tension (Figures 3.10 & 3.12). At the fundus-end, $PGF_{2\alpha}$ elicited a monophasic excitatory response (pEC_{50}: 6.9 ± 0.80M), parallelled by the effect of U46619 in immersed tissue strips (pEC_{50}: 6.8 ± 0.27M). Activity was increased to 59.3 ± 2.43 and 65.2 ± 5.86 percent hypotonic shock respectively (Figure 3.11). Likewise in superfused tissues, bolus doses of $PGF_{2\alpha}$ ($F(1, 30) = 16.73$; $p<0.001$) and U46619 ($F(1, 30) = 13.44$; $p<0.001$) augmented myometrial contractions by 2.6 and 2.4-fold compared with time-matched controls. This demonstrated their similar efficacy and potency values in upper myometrial strips.

In lower segment immersed tissues, $PGF_{2\alpha}$ stimulated activity to 83.1 ± 10.4 percent hypotonic shock at 10^{-6}M, after which the response was attenuated ($F(1, 30) = 71.27$; $p<0.001$; Figure 3.10). A similar concentration-related excitatory effect was observed with U46619; although contractions reached 104.5 ± 10.2 percent of the hypotonic shock value at 10^{-5}M ($F(1, 40) = 30.93$; $p<0.001$). Therefore, a topographical gradient to both $PGF_{2\alpha}$ and U46619 was observed, with responsiveness enhanced in lower segment uteri compared with fundus-end tissues.

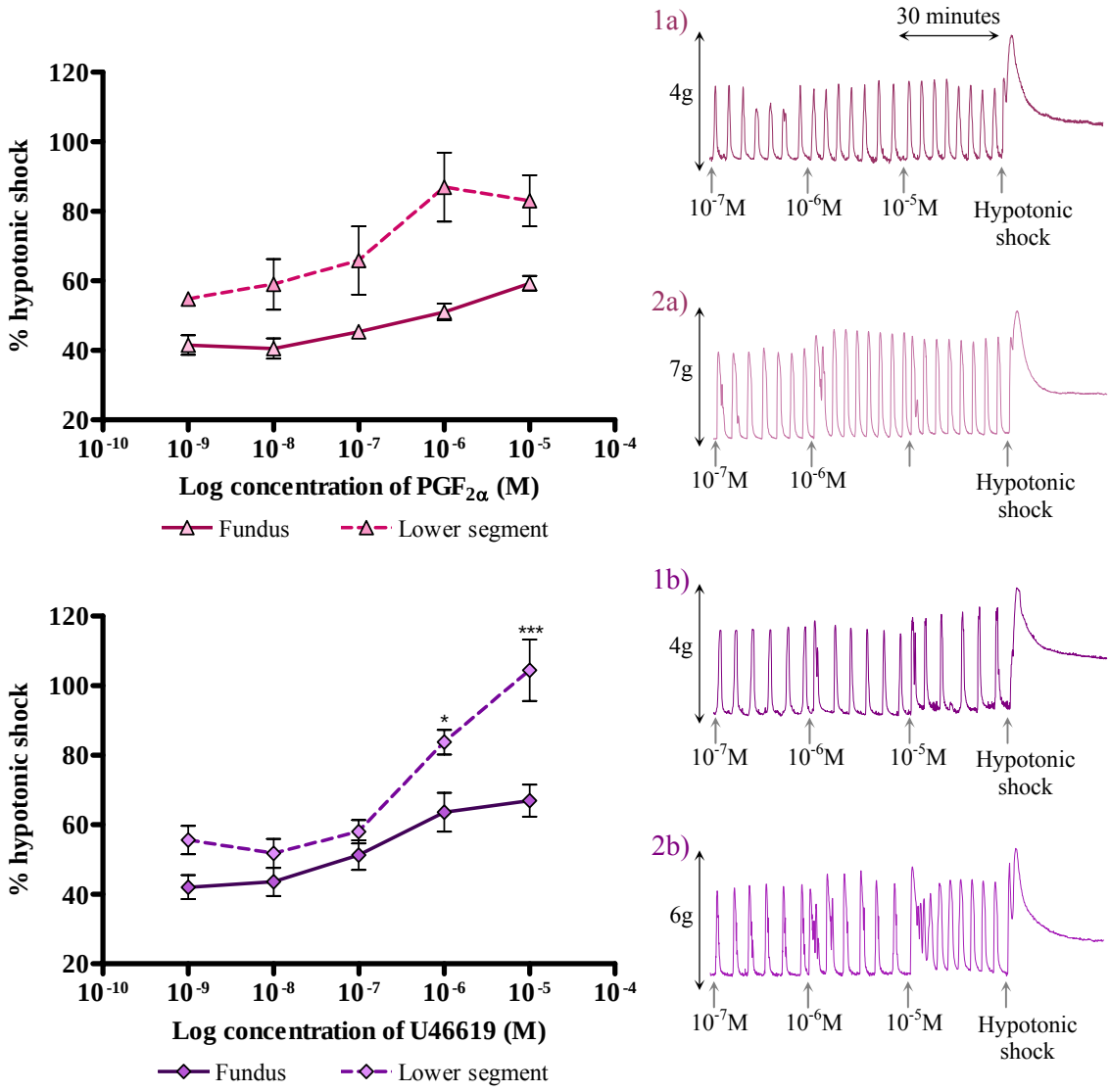


Figure 3.10: Concentration-effect curves and representative traces for a) $PGF_{2\alpha}$ and b) U46619 in 1) fundus and 2) lower segment human myometrium from non-pregnant donors in the follicular stage of the cycle (n=4-6). Myogenic activity was recorded per 30-minute agonist incubation in the immersion apparatus and expressed as a percentage of 30 minutes hypotonic shock. Results are arithmetic means ± S.E. with significance determined using a multivariate ANOVA and Bonferroni's *post-hoc* test; *p<0.05; ***p<0.001 for fundus compared with lower segment tissue responses.

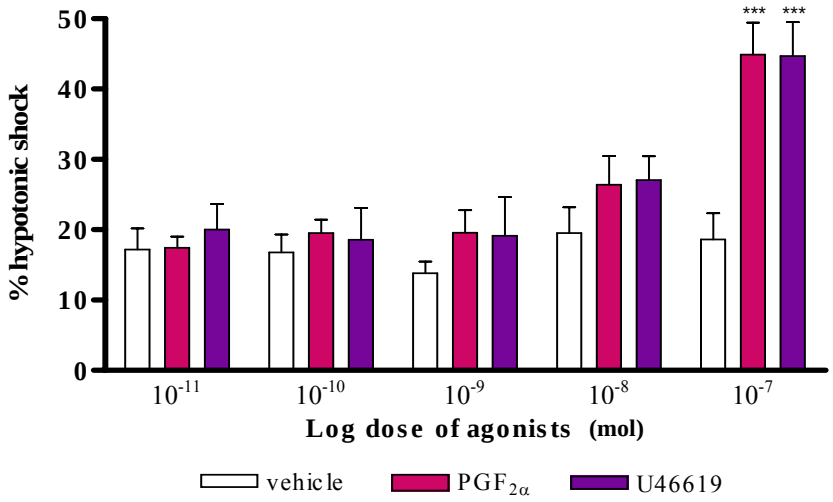


Figure 3.11: Vehicle and excitatory dose-responses for $PGF_{2\alpha}$ and U46619 in fundus-end myometrium harvested from non-pregnant donors in the follicular stage of the menstrual cycle (n=4). Using the superfusion technique, myometrial responsiveness to bolus doses of agonists were measured over a 30-minute period (area under the curve) and expressed as a percentage of 30 minutes hypotonic shock. Data are arithmetic means ± S.E. and statistical analysis was performed using two-way ANOVA with Bonferroni's *post-hoc* adjustment; ***$p<0.001$ for responsiveness to uterotonins at 10^{-7}mol compared with time-matched vehicle controls.

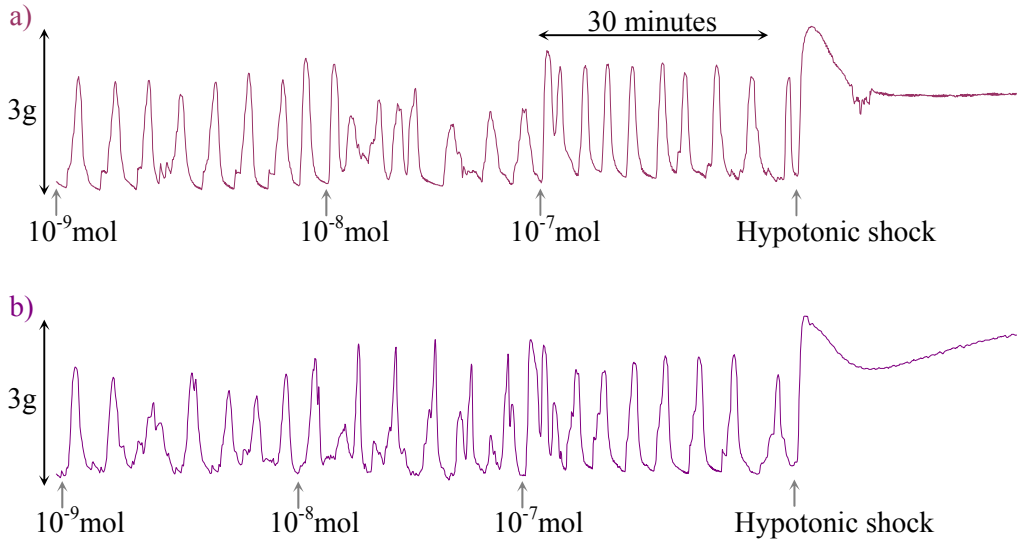


Figure 3.12: Representative traces showing the responses of upper segment uterine tissues taken mid-cycle to bolus doses of a) $PGF_{2\alpha}$ and b) U46619 (10^{-9}mol to 10^{-7}mol) in superfusion baths.

Chapter 3.9: Discussion

The results show that myometrial strips exhibited spontaneous activity relative to the stage of menstrual cycle and site of excision under *in vitro* conditions. Contraction amplitude was most pronounced during the follicular phase and was attenuated during menses. In contrast to the outer myometrial layers, sequential contractions are known to develop from the stratum subvasculare of the myometrium (Kunz *et al*., 1996; Noe *et al*., 1999; van Gestel *et al*., 2003). As the expression of oestrogen and its cognate receptors peak mid-cycle in this sub-endometrial layer (Noe *et al*., 1999), this suggests that the oestrogenic milieu is crucial in mediating uterine peristalsis.

The acute rise in oestrogen during the periovulatory period of the follicular phase coincides with an increased expression of ERα, implying positive feedback within the uterus (Lecce *et al*., 2001). In addition to increasing cellular division and growth, oestrogen exerts uterotrophic effects, which enhance myometrial contractility and excitability via the upregulated expression of gap junctions (Garfield *et al*., 1980; Kilarski *et al*., 2000). In accord with previous functional studies (Hutchinson, 2005), contractions in this study were highest in myometrium taken at the follicular stage. Myogenic activity declined and was more sporadic in tissues taken during menses corresponding to a reduction in circulatory oestrogens and decreased uterine expression of ERα and PR-A (Ijland *et al*., 1998). This infers that the myogenic activity established *in vitro* maintained the receptor profile and hormonal influences of the donor at the time of hysterectomy.

Whilst the underlying control of uterine activity is not well understood, it is recognised that paracrine ovarian steroids modulate myometrial transcriptional activity via specific growth factors, enzymes, hormones and receptors. The genomic

and non-genomic effects of oestrogen increase the expression of uterine progesterone receptor (PR) isoforms PR-A and PR-B (Lecce *et al*., 2001). PR-B mediates the proliferation of endometrial and, to a lesser extent, myometrial cells in the luteal phase, whilst the expression of PR-A suppresses progesterone effects in the follicular phase of the menstrual cycle (Ijland *et al*., 1998). The reported decrease in excitability of the uterine musculature in the luteal phase by magnetic resonance imaging (Bulletti *et al*., 1998) was reflected in the reduced spontaneous activity of isolated myometrial strips taken from non-pregnant donors at this stage. This quiescence was therefore likely to be facilitated by progesterone diminishing contractile associated proteins (CAPs) gene expression in the uterus (Garfield *et al*, 1980) as well as the paucity of oestrogen action.

Myogenic activity was 10 percent greater in isolated lower segment tissues compared to the fundus; this seemed to reflect the physiological state of the uterus. The apparent functional regionalisation of the uterus may be attributed to the highest local expression of progesterone receptors in the fundus (Lye *et al*., 1998; Challis *et al*., 2000, 2002). Moreover, the close apposition of the venous drainage of the ovary and ovarian bursa would enable sex steroids to reach the fundus prior to the lower segment of the uterus. This may facilitate spatial-hormonal effects, altering receptor-binding properties and myometrial contractions. As a result, autocrine and paracrine influences are likely to affect regional variation within the uterus.

In women of reproductive age, the structural and functional polarity of the non-gravid uterus has been discerned using magnetic resonance imaging, video-vaginosonography and immunohistochemistry. Whilst the internal layer or junctional zone consists of mainly circular running fibres, the myofilaments in the thick mid layer are oriented obliquely to the axis of the muscle fibre (Weiss et al., 2006). As

well as sustaining forceful and prolonged contractions (Wray et al., 2001), this fibre arrangement may explain the uniform myogenic contractions in this study regardless of longitudinal or transverse incisions. Uterine smooth muscle composition also decreases caudally from the fundus towards cervix, with connective tissue composition at 38 and 68 percent of the dry-defatted tissues for the uterine body and cervix respectively (Leppert & Yu, 1991). Moreover, myocyte density is greater at the myometrial-endometrial surface compared to the middle stratum vasculare (Weiss et al., 2006), perhaps accounting for the intra-donor variation in spontaneous contractility.

During the follicular phase, synchronised contractions of the uterus are primarily directed towards the fundus and fallopian tubes, facilitating the rapid transport of potential sperm (Kunz *et al*., 1996). Without conception, uterine activity decreases and is manifest as short and asymmetric bi-directional waves (Ijland *et al*., 1998; Bulletti *et al*., 2002). This may facilitate blastocyst implantation and the local supply of nutrients and oxygen during the luteal phase of the menstrual cycle. In addition, the caudal contractile direction at menses would assist uterine emptying. Even so, primary and secondary dysmenorrhoea (Leyendecker *et al*., 2004; Altunyurt *et al*., 2005; Kataoka *et al*., 2005; Dawood & Khan-Dawood 2007) and endometriosis (Bulletti *et al*., 2002) are associated with hyperactivity of the uterus, whilst chronic uterine infections attenuate contractile force (Hirsbrunner *et al*., 2006; Laird *et al*., 2003). Due to benign gynaecological conditions, it is recognised that the myometrial specimens taken for this study may have displayed compromised characteristic functions. Even so, over a third of the women at hysterectomy have normal uteri removed (Clarke, 1995) and myogenic activity could not be distinguished between samples, regardless of patient disorder. This demonstrates that menstrual cycle

dysfunctions, which contribute to major morbidity in reproductive health, require further elucidation at a cellular level.

The Spanish pathologist and Nobel laureate Santigo Ramon y Cajal discovered cells thought to have a pacemaker role in propagating smooth muscle contractility. These interstitial cells of Cajal have abundant thin cytoplasmic processes, numerous mitochondria, intermediate filaments and caveolae, which are localised between muscle bundles and layers to provide a pathway for the transmission of depolarisation (Ciontea *et al*., 2005; Popescu *et al*., 2006). Their regional distribution and inappropriate electric impulses has been implicated in reduced fecundity in adolescent females (Dixon *et al*., 2009). This appeared to be manifest as unstable baseline contractions in specimens inhibited by PG agonists or obtained at menses. As interstitial cells of Cajal express oestrogen and progesterone receptors (Cretoiu *et al*., 2006), their activation may vary temporally and spatially across the menstrual cycle. The over-expression of oestrogen and progesterone receptors in symptomatic uterine leiomyomata (fibroids), which are benign neoplasms of the smooth muscle cell, may also be responsible for abnormal contractile status (Severino *et al*., 1996; Kunz *et al*., 2000). Moreover, with endometriosis, the heightened retrograde activity facilitating the dissemination and implantation of endometrial tissue outside the uterine cavity is oestrogen-dependent and decreases the propensity for a typical menstrual cycle (Bulletti *et al*., 2002). Therefore, ovarian hormones contribute to the subtle and transient changes in uterine activity according to pathology (Kataoka *et al*., 2005). Even so, increases in PG synthesis that promote the inflammation, fibrosis and adhesion formation are symptoms of these conditions. Consequently, to elucidate uterine physiology and pathophysiology, contractile responses to exogenous PGs were examined.

PGE_2 and $PGF_{2\alpha}$ were the first abundant vasoactive substances to be detected in menstrual fluid and the endometrium (Pickles, 1967). These bioactive autacoids and their endoperoxide precursors diffuse through the walls of the effluent veins of the endometrium to reach the myometrium (Baird *et al*., 1996). For PGE_2 biosynthesis, membrane-derived arachidonic acid is liberated by phospholipases and metabolized to the labile intermediate PGH_2 by COX enzymes. Of the three co-existing isoforms in myometrial cells, COX-1 is constitutively expressed, whereas COX-2 and COX-3 are inducible by inflammatory, mitogenic and physical stimuli (Murakami *et al*., 1999). Downstream of the COX pathway, the conversion of PGH_2 to PGE_2 is catalysed by cytosolic and membrane-bound PGE synthase (cPGES and mPGES). Although the mechanisms are obscure, perturbed PGE_2 pathways have been particularly associated with reproductive disorders.

Whilst fibroids attenuate PG output (personal communication, Durn, 2009), the overproduction of PGE_2 and EP mRNA expression has been correlated to menorrhagia (Hofmann *et al*., 1983; Adelantado *et al*., 1988; Smith *et al*., 2007) and the hyperalgesic effects of dysmenorrhoea (Sales & Jabbour, 2003). In addition, COX-1 and COX-2 expression are elevated in women with menorrhagia (Smith *et al*., 2007). To prevent uterine PG generation, non-steroidal anti-inflammatory drugs (NSAIDs) have been employed as tocolytic agents that block COX signalling cascades. Indometacin, a well characterised NSAID, is widely reported to inhibit endogenous PG biosynthesis *in vitro* (Crankshaw, 2001; Jabbour & Sales, 2004) and to remove intrinsic tonus for a stable resting tension (Dong *et al*., 1986). To determine the role of endogenous prostanoids in uterine contractility, myogenic responsiveness to PGE_2 was assessed relative to the effect of indometacin.

Despite the presence of mPGES, COX-1 and COX-2 mRNA in human myometrium (Slater *et al*., 1999; Giannoulias *et al*., 2002; Sooranna *et al*., 2006), nonselective blockade of COX enzymes with indometacin [1μM] in this study did not influence spontaneous contractility or myometrial responses to PGE_2. However, at higher concentrations, the reported complete abolition of myogenic activity (Sawdy *et al*., 2003; Cao et al., 2004) implicates indometacin effects that are unrelated to PG production. This was substantiated by its direct attenuation of $PGF_{2\alpha}$-induced Ca^{2+} release (Landen *et al*., 2001; Smith & Langenbach, 2001; Sawdy *et al*., 2003) and inhibition of gap junction formation (Garfield *et al*., 1980). Due to its ambiguous mechanisms, the addition of indometacin was omitted from all other functional studies.

PGE_2 exhibits diverse effects on smooth muscle contractility via $EP_{1\text{-}4}$ receptor subtypes (Coleman *et al*., 1994), which are likely expressed on uterine myocytes as well as the interstitial cells of Cajal. By acting through divergent intracellular signalling pathways, a functional complement of heterogeneous uterine EP receptors has been identified. In isolated human non-gravid myometrium, time-matched vehicles sustained myogenicity throughout the duration of experiments. However, challenge with PGE_2 produced different types of response curves (Popat & Crankshaw, 2001), predominantly consisting of initial contractions before profound inhibition of myogenic activity (Senior *et al*., 1991; Hillock & Crankshaw, 1999; Popat & Crankshaw, 2001). In contrast, the observed biphasic response to PGE_2 in this study was characterised by concentration-dependent inhibition followed by partial restoration of contractility. This trend was uniform across the different phases of the menstrual cycle.

In premenopausal women, circulatory PGE_2 increases in conjunction with uterine activity at the follicular phase of the cycle (Maslow *et al*., 2004) and also at menstruation (Rees *et al*., 1984; Adelantado *et al*., 1998). This corresponds to the spatial and temporal expression of PGE synthase, EP_4 receptors and COX-2 in human epithelial cells (Milne *et al*., 2001). In ruminant endometrium and myometrium, whilst EP_4 receptors were undetectable, EP_2 mRNA and protein peaked mid-cycle (Arosh *et al*., 2003). EP_2 receptor expression correlated well with upregulated antiapoptotic genes and vascular endothelial growth factor (VEGF) (Jones *et al*., 1998). Due to the associated epithelial cell proliferation, angiogenesis and microvascular tube formation, roles of PGE_2 in decidualisation, menses, blastocyst implantation and embryonic development have been proposed (Lim & Dey, 1997; Arosh *et al*., 2003; Critchley & Saunders, 2009). Little is known of the cyclic expression pattern of EP subtypes in human non-gravid tissues. Even so, endothelial EP_2 and EP_4 receptors were expressed throughout the menstrual cycle without modulation (Milne *et al*., 2001) together with the ligand binding affinity of PGE (Giannopoulos *et al*., 1985). If transposed to the human myometrium, this would account for the observed consistent functional responsiveness to PGE_2. To elucidate further uterine EP receptor targets, the effect of PGE_2 was compared with selective EP analogues. However, due to limited myometrial specimens, tissues recovered from different stages of the menstrual cycle were combined.

Even though PGE_2 binds to its receptor subtypes with a rank order of affinity of EP_{3III} $>EP_4 >EP_2 >EP_1$ (Kiriyama *et al*., 1997; Abramovitz *et al*., 2000), its actions are also dependent on receptor density and signal transduction gain. With the abundant expression of EP_2 or EP_4 subtypes in uterine muscle (Katsuyama *et al*., 1995), the observed inhibitory effects on myogenic activity indicate that either of these EP

subtypes were engaged. In addition, treatment with PGE_2 evoked a concentration-dependent increase in cAMP (Arosh *et al*., 2003); this has been shown to directly impair mitogen-activated protein (MAP) kinase activity and Ca^{2+} signalling for utero-quiescence (Sanborn *et al*., 1998).

To identify the functional significance of EP_2 receptors, test compounds included butaprost (Gardiner, 1986) and CP533,536 (Li *et al*., 2003; Paralkar *et al*., 2003). As a methyl ester, butaprost was shown to have a 40-fold lower affinity for EP_2 compared to the non-commercially available free acid form of the molecule (Abramovitz *et al*., 2000). Even so, both butaprost and the pyridyl sulphonamide CP533,536 are well documented for high selectivity at the EP_2 receptor. Respective K_i values for EP_2 were 110nM and 50nM with negligible binding affinity at other EP or PG receptor subtypes (Kiriyama *et al*., 1997; Li *et al*., 2003; Paralkar *et al*., 2003). Accordingly, butaprost and CP533,536 were equipotent in this study at attenuating myogenic activity in a monophasic concentration-dependent manner. In immersion studies using human and guinea pig isolated myometrium, similar utero-relaxant effects were observed for butaprost (Hillock & Crankshaw, 1999; Popat & Crankshaw, 2001) and for CP533,536 (Lebel *et al*., 2004; Terry *et al*., 2007), re-enforcing the presence of operative EP_2 receptors in non-gravid uterine tissue.

Functional EP_4-mediated responses were distinguished using AGN201734 (Elworthy *et al*., 2004). As a lactam, this synthetic agonist is reported to confer greater than 1000-fold selectivity for EP_4 (Elworthy *et al*., 2004). Nevertheless, in immersion studies, AGN201734 produced biphasic curves with an initial reduction in myogenic activity followed by relative excitation. Although its efficacy was lower, the potency of AGN201734 was 6-fold higher than butaprost and CP533,536. Contractile responses, followed by a period of inhibition, were conversely elicited in superfused

myometrial strips. These profiles mimicked the *in vitro* effects of PGE_2 and indicated that EP_4 receptors contributed to utero-relaxation in fundus end myometrial segments with some off-target mechanisms displayed, perhaps at EP_1 and EP_3 receptors.

When compared to other EPs, the EP_4 receptor is a less compact structure with a long intracellular third loop and C-terminus (Regan *et al*., 1994). Of these receptors, the amino acid sequence of the relaxant EP_2 and EP_4 subtypes exhibit only 38 percent homology (Toh *et al*., 1995). As a result, their signalling pathways are not identical. The mechanisms of EP_2-induced cAMP accumulation mainly involve phosphorylation of glycogen kinase-3 (GSK-3) by protein kinase A (PKA), whereas EP_4 receptors also activate phosphatidylinositol kinase (Fujino *et al*., 2005; Fujino & Regan, 2006). This downstream activation of IP_3 and Ca^{2+} by EP_4 may account for the evoked contractile responses to AGN201734. Moreover, murine EP_4 receptors contain 38 serine and threonine residues. In response to PGE_2, these putative phosphorylation sites were shown to produce rapid desensitisation (Nishigaki *et al*., 1996) and internalisation (Desai *et al*., 2000) of EP_4 but not EP_2 receptors. Deactivation of the EP_4 subtype may also have contributed to restored myogenic activity with incubations and bolus doses of AGN201734. Even so, in order to clarify the identity of EP receptor subtypes, an EP_4 receptor antagonist was used.

Although EP_4 was purported to be devoid of action (Hillock & Crankshaw, 1999), the tested EP_4 antagonist AH23848 has since been shown to have moderate selectivity at EP_4 over $EP_{1\text{-}3}$ and IP receptors (Abramovitz *et al*., 2000; Jones & Chan, 2001). Instead, GW627368x was assayed due its more potent EP_4 selectivity in human (Wilson *et al*., 2006), piglet (Wilson *et al*., 2003) and rabbit (Jones & Chan, 2005) preparations. Despite additional TP receptor binding, GW627368x displayed at least 100-fold greater affinity for EP_4 over the other PG receptors (Wilson *et al*., 2006).

This was observed with GW627368x-mediated antagonism of the EP_4 receptor agonist AGN201734. The rightward displacement of the AGN201734 log concentration-effect curve with an estimated pA_2 value of 7.5 indicated competitive inhibition at EP_4 receptors. However, GW627368x did not alter responses to PGE_2, butaprost or spontaneous contractions in fundus segment tissues strips. Moreover, in the presence of the antagonist, PGE_2 produced maximal inhibition of the tissue and was the most potent agonist tested (pEC_{50}: 8.4 ± 0.2M). This implies that, despite the presence of EP_4 receptors, the EP_2 subtype is predominantly involved in PGE_2-induced utero-relaxation.

To evaluate the contribution of excitatory EP receptors, concentration-effect curves for sulprostone (Schaaf *et al*., 1981) and ONO-D1-004 (Oka *et al*., 2003) were constructed. Despite high affinities for EP_1 and FP receptors, sulprostone is most potent at EP_3 (Coleman *et al*., 1994; Kiriyama *et al*., 1997; Abramovitz *et al*., 2000). By contrast, ONO-D1-004 displays 70-fold higher selectivity at EP_1 than at the other EP receptors (Oka *et al*., 2003). In accord with previous functional studies, sulprostone evoked significant myometrial contractility (Senior *et al*., 1991; Popat & Crankshaw, 2001). However, ONO-D1-004 produced negligible uterotonic effects. This may be attributed to the lower binding affinity of ONO-D1-004 compared to sulprostone with respective K_i values of 150nM and 0.6nM at EP_1 (Oka *et al*., 2003) and EP_3 receptors (Kiriyama *et al*., 1997). Even so, activation of the EP_3 subtype is consistent with the greater EP_3 mRNA expression relative to EP_1 receptors (Astle *et al*., 2005; Sugimoto & Narumiya, 2007). On the basis of contraction, these results indicate that EP_3 receptors are predominant in non-gravid human myometrium and may contribute to the excitatory component of PGE_2 responses. Nevertheless, the

profound utero-relaxation exerted by PGE_2 implicates that inhibitory EP-coupled pathways exceed EP_1 and EP_3-mediated effects within this tissue.

The polarity of functional EP receptors was compared in upper and lower segment human myometrium harvested at the follicular phase of the menstrual cycle. Marked relaxatory responses to PGE_2, butaprost and AGN201734 were displayed in lower segment tissues relative to the fundus. The differences in spontaneous activity may have potentiated this regional effect, whereas excitatory responses to the highest concentration of these agonists were absence from lower segment uteri. Along the length of the uterus, however, functional EP_3 and EP_1 receptors were manifest with an identical contractile profile stimulated by sulprostone.

In longitudinal sections, uterine smooth muscle density depreciates from the fundus-corpus area to the lower segment in parallel with a decrease in PGE-binding affinity (Hofmann *et al*., 1983; Giannopoulos *et al*., 1985). This gradient conversely reflects blood flow into the lower uterus before distribution to the fundus. During the peri-ovulatory phase, the intrinsic intensity of lower uterine peristalsis and EP_1 and EP_3-mediated effects may aid retrograde contractions. As the concentration of PGE_2 in seminal plasma is considerable (Templeton *et al*., 1978; Muller *et al*., 2006), this could facilitate the transport of sperm towards the distal end of the fallopian tubes. Although the topographical distribution of EP receptors in the non-gravid uterus is not well defined, only genetic EP_2 knockout mice exhibit ovulation, fertilisation and peri-implantation defects (Tilley *et al*., 1999). Therefore, EP_2 receptors are a vital prerequisite for the successful establishment of pregnancy.

Anatomical responsiveness to $PGF_{2\alpha}$ and U46619, a stable thromboxane mimetic (Coleman *et al*., 1994), were also investigated in follicular stage non-gravid human myometrium. These spasmogens enhanced myogenic contractions in a concentration-

dependent manner. As in previous immersion (Word *et al*., 1992; Hutchinson, 2005; Janicek *et al*., 2007) and superfusion (Rees *et al*., 1984; Senior *et al*., 1992) studies, the contraction profiles displayed an initial tonic increase in force magnitude followed by a marked rise in frequency. Responses to both $PGF_{2\alpha}$ and U46619 were of similar potency, with greater FP and TP-mediated activity in lower compared to upper segment myometrium. For $PGF_{2\alpha}$, this was contrary to the reported functional uterine FP topography in the rat (Oropeza *et al*., 2002), mouse (Griffiths *et al*., 2006) and porcine myometrium (Cao *et al*., 2005). In human tissues, the gradient of uterine $PGF_{2\alpha}$ binding sites also decreased from the fundus to the lower uterine body (Hofmann *et al*., 1983; Giannopoulos *et al*., 1985). However, polarised regional FP-mediated effects appear to be reduced under oestrogen-primed conditions (Word *et al*., 1992; Griffiths *et al*., 2006). This might explain the observed $PGF_{2\alpha}$ sensitivity superimposed on the higher intrinsic contractions of cervical region tissues in this study.

Both PGE_2 and $PGF_{2\alpha}$ are principal metabolites of the human endometrium. As well as a peak in $PGF_{2\alpha}$ output (Downie *et al*., 1974), maximal FP receptor expression and signalling have been identified in endothelial and perivascular cells during the follicular phase of the menstrual cycle (Milne & Jabbour, 2003). This promotes endometrial thickening in case of ovum fertilisation (Milne & Jabbour, 2003) and luteal regression under non-pregnant conditions (Sugino *et al*., 2004). Moderate activity in the upper uterine region may also facilitate blastocyst implantation. However, $PGF_{2\alpha}$ consistently stimulates myometrial contractions with the greatest uterotonic contractions at menses (Rees *et al*., 1984; Myatt & Lye, 2004; Hutchinson, 2005). This is speculated to facilitate the expulsion of endometrial debris from the uterine lumen. Activation of FP receptors by $PGF_{2\alpha}$ results in tyrosine

phosphorylation, phosphatidylinositol hydrolysis and subsequent intracellular Ca^{2+} flux. Even so, at high concentrations, $PGF_{2\alpha}$ can also bind with EP_1, EP_3 and TP receptors (Coleman *et al*., 1994; Narumiya *et al*., 1999; Breyer *et al*., 2001). Whilst functional EP receptor interactions have not yet been confirmed, responses to $PGF_{2\alpha}$ remain unaltered in the presence of selective TP antagonists (Hutchinson, 2005; Griffiths *et al*., 2006). This indicates that the excitation evoked by $PGF_{2\alpha}$ is not due to off-target TP activity.

U46619 is a potent and stable thromboxane A_2 mimetic (Coleman *et al*., 1994) with full contractile activity in non-gravid uterine smooth muscle preparations (Senior *et al*., 1992; Senchyna & Crankshaw, 1999; Hutchinson, 2005). At putative TP receptors (Kiriyama *et al*., 1997; Abramovitz *et al*., 2000), TP receptor antagonists reduced U46619-induced excitation in isolated myometrial strips (Hutchinson, 2005; Griffiths *et al*., 2006). This confirms the operational expression of TP receptors in the human uterus.

Responsiveness to U46619 increased from the fundus towards the cervix in this study. In contrast, TP receptor activation was reported to be homogeneous in non-gravid human myometrium regardless of tissue excision site (Senchyna & Crankshaw, 1999). As differences in spontaneous contractility were not mentioned, anatomical variations may reflect the mechanical properties of the uterus. Even so, it is likely that the hormonal milieu could also account for these regional effects. Progesterone is associated with a decrease in TP receptor mRNA (Minshall *et al*., 2001) and TP binding affinity (Swanson *et al*., 1992). However, in porcine uterine muscle, the fundus-corpus area has more abundant TP receptors with lower binding affinity than cervical end tissues (Cao *et al*., 2004). Since two TP isoforms exist (Hirata *et al*., 1991; Raychowdhury *et al*., 1994), their expression ratio may account for regional

contractile effects. As a result, the roles of thromboxane in myometrial contraction, vascular tone and vascular haemostasis are likely to be intricately regulated. This could be important for reducing menstrual blood loss during menses (Dawood & Khan-Dawood, 2007). Nevertheless, FP and TP-deficient mice were fertile (Sugimoto *et al*., 1997; Kobayashi & Narumiya, 2002), indicating supportive rather than essential roles in non-pregnant reproductive processes.

In summary, this study confirms the heterogeneous array of functional EP, FP and TP receptors in the non-gravid human myometrium. Despite the gradient of spontaneous myometrial activity, responses to PGE_2 were consistent throughout the menstrual cycle and along the length of the uterus. This indicates that the complement of inhibitory to excitatory EP subtypes does not alter topographically. In accord with crucial effects on fertility (Tilley *et al*., 1999), PGE_2-induced utero-relaxation was predominantly mediated by the EP_2 receptor. Conversely, the spasmogens $PGF_{2\alpha}$ and thromboxane stimulated contractions. Enhanced TP and FP responsiveness related to the greater phasic motility at the lower segment of the uterus during the follicular phase of the cycle. This may facilitate retrograde contractions for successful oocyte fertilisation and implantation of the blastocyst. A balance in these PG receptor populations is likely to contribute to the total function of the uterus.

Chapter 4: Term pregnancy

Functional prostanoid receptors in isolated myometrium at term pregnancy

4.1 Introduction

Throughout the majority of pregnancy the uterus is maintained in a state of functional quiescence. As term approaches, integrated changes in hormonal, chemical and mechanical signals transform uterine contractures to contractions. This is mediated by the upregulated cascade of genes for CAPs, which include connexin-43, ion channel proteins and agonist-mediated receptors (Challis *et al*., 2000). As a result, the uterus is primed for parturition.

Myometrial prostanoid receptor proteins mediate responses via G-proteins coupled to distinct and complex signal transduction pathways (Coleman *et al*., 1994; Narumiya *et al*., 1999). Despite their similarities in structure, DP, EP_2, EP_4 and IP receptors inhibit smooth muscle activity whilst EP_1, EP_3, FP and TP receptors mediate contraction. Functional studies on the isolated human myometrium have characterised heterogeneous DP, EP_2, EP_3, FP, IP and TP receptor subtypes at term pregnancy (Senior *et al*., 1993; Hutchinson, 2005). However, responses have been difficult to distinguish pharmacologically due to a paucity of highly potent and selective PG ligands (Wilson *et al*., 2004). With the development of novel agonists and antagonists and the donation of tissue at elective Caesarean section, the aim of the present study was to characterise further functional PG receptors in isolated late gestational myometrium, taken prior to labour-onset (38-41 weeks).

Chapter 4.2: Results

4.3 Myometrial activity of isolated term gravid tissues

After equilibration, spontaneous regular contractions developed in lower myometrial segments from non-labouring, term pregnant donors (39.3 ± 0.4 weeks gestation). This phasic activity was maintained by vehicle controls (Figures 2.4b & 2.15b) with contractile episodes of 2-3 minutes duration, followed by relatively longer periods of quiescence. The mean amplitude and frequency of contractions were relatively uniform regardless of patient clinical profiles. This included factors such as ethnicity, smoking, hypertension and diabetes (Table 4.1). Whilst increased parity tended to reduce the contractile frequency, a history of abortions or miscarriages was associated with transitory contractions at lower amplitude ($F(5, 283) = 2.84$; $p<0.05$) (Figure 4.1). Nevertheless, variations in spontaneous activity were analogous between myometrial strips from the same and different biopsies with mean coefficents of variance at 17.1 and 16.8 percent respectively. Of the 757 myometrial strips investigated using the immersion technique, 5.4 percent were devoid of myogenic activity; these were excluded from the dataset.

Table 4.1: Contractility exhibited by immersed myometrial strips from term pregnant, non-laboring patients. Donor groups were distinguished by different ethnic, social and pathophysiological backgrounds with results measured over 30-minute periods of phasic activity after equilibration and expressed as arithmetic means ± S.E. Measurements of % hypotonic shock, amplitude and frequency of myogenic contractions were uniform, regardless of donor groups.

Patient info:	White	Indian subcontinents	Black	Non-smokers	smokers	diabetics	Hypertensives
number:	10	7	4	10	10	3	3
% hypotonic shock	48.0 ± 2.50	48.7 ± 2.97	54.5 ± 5.93	46.5 ± 2.29	47.3 ± 2.49	49.0 ± 0.67	46.5 ± 6.73
Amplitude (g)	5.4 ± 0.43	5.3 ± 0.40	5.0 ± 0.76	5.5 ± 0.72	5.2 ± 0.32	5.4 ± 0.34	5.4 ± 0.90
Frequency (/ 30 mins)	3.0 ± 0.19	3.4 ± 0.63	3.5 ± 0.58	3.3 ± 0.28	3.6 ± 0.40	3.3 ± 0.75	3.3 ± 0.54

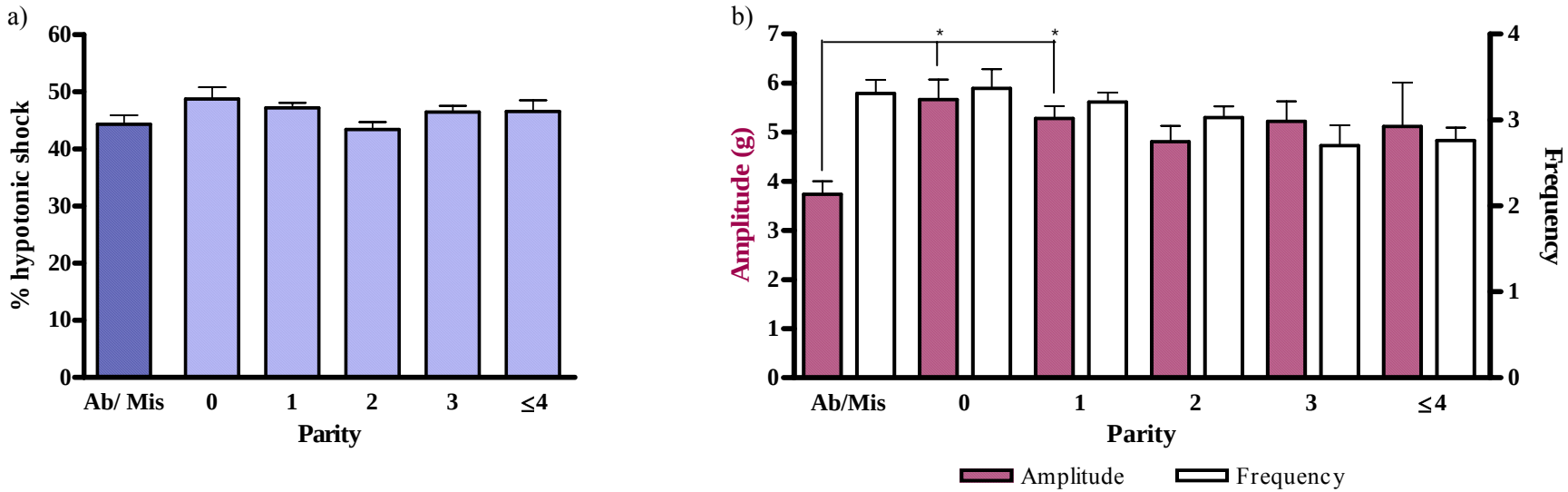


Figure 4.1: The a) % hypotonic shock and b) amplitude and frequency of spontaneous contractions exhibited by myometrial strips from term pregnant donors who had previously experienced abortions/ miscarriages (Ab/Mis), none or up to six previous live births (n=42). After equilibration in the immersion apparatus, regular phasic activity was measured for 30 minutes (area under the curve) with data expressed as arithmetic means ± S.E. Univariate analysis showed significance; *$p<0.05$ compared to myometrium from nulli and primiparous women.

4.4 Myometrial EP receptors at term pregnancy

In lower segment uteri obtained from term pregnant, non-labouring donors, PGE_2 (10^{-10}M to 10^{-5}M) evoked a predominant inhibitory effect on myogenicity via a reduction in the amplitude of myometrial contractions ($F(1, 92) = 23.86$; $p<0.01$; Figure 4.2). Myogenic activity was attenuated by 40 percent and some excitation was observed at 10^{-5}M.

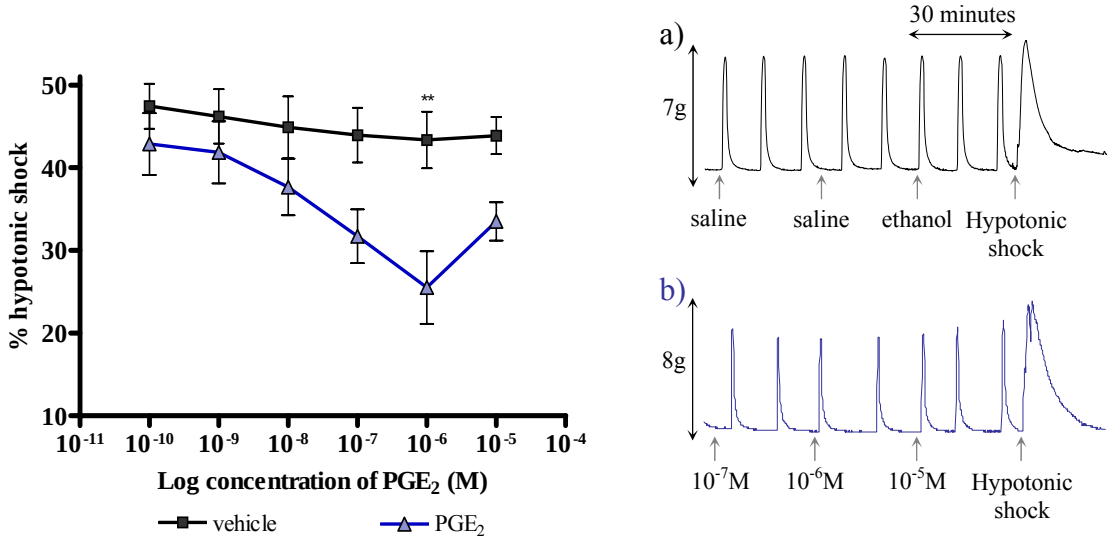


Figure 4.2: Concentration-effect curves and representative traces for a) vehicle and b) PGE_2 in isolated uterine muscle obtained from non-labouring term pregnant donors (n=9). Vehicle and PGE_2 (10^{-10}M to 10^{-5}M) were added to immersion baths in a cumulative manner at 30-minute intervals. Results are expressed as arithmetic means ± S.E. and data were analysed using a two-way ANOVA mixed model with Bonferroni's *post-hoc* test; **p<0.01 compared to the time-matched vehicle control.

4.5 Inhibitory effects of EP_2 agonists on myogenic activity

The EP_2 receptor agonists butaprost, CP533,536, AH13205 and AGN211330 attenuated myogenic activity in a monophasic concentration-dependent manner in immersed myometrial strips obtained at term pregnancy. Butaprost and CP533,536 produced a gradual decline in contractility with the complete cessation of activity at 10^{-5}M in some tissue strips ($F(2, 137) = 29.65$; $p<0.001$; Figure 4.3a).

Compared to the maintained activity of vehicle controls, AH13205 and AGN211330 predominantly suppressed contractions between 10^{-6}M and 10^{-5}M ($F(5, 60) = 9.64$; $p<0.001$; Figure 4.3b). This corresponded to their weaker potency values in relation to butaprost and CP533,536 (Table 4.2). Even so, none of the EP_2 mimetics evoked excitatory responses in isolated term gestational myometrium.

4.6 EP_4 agonist effects on myogenic activity

The concentration-effect curves for the EP_4 mimetics AGN201734 and L-902688 exhibited different responses in myometrial strips obtained at term pregnancy. AGN201734 induced a biphasic response, progressively inhibiting spontaneous activity ($F(1, 59) = 5.15$; $p<0.05$; Figure 4.4) until contractions were stimulated at 10^{-5}M. However, L-902688 produced no significant effect compared to vehicle, despite the decrease in contractile activity at 10^{-5}M from 45.8 ± 3.30 to 39.0 ± 3.57 percent hypotonic shock ($F(1, 41) = 0.43$; ns; Figure 4.4).

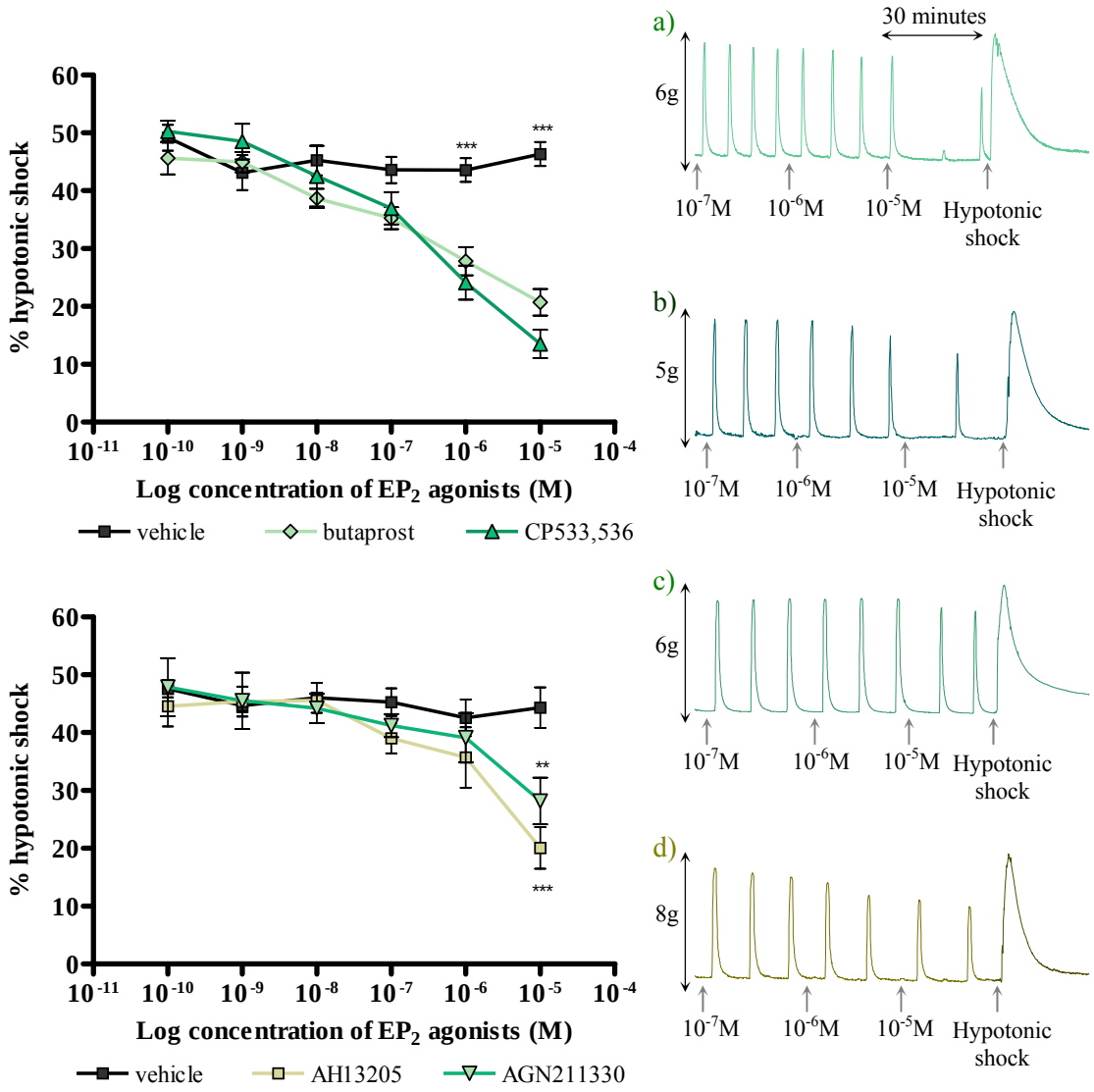


Figure 4.3: Vehicle, concentration-effect curves and typical traces for EP_2 agonists a) butaprost, b) C533,536, c) AGN211330 and d) AH13205 on immersed lower segment myometrium taken at term gestation (n=6-10). Responses were measured over a 30-minute period as area under the curve, expressed as percentage hypotonic shock and presented as arithmetic means ± S.E. Statistical significance was determined by two-way ANOVA with Bonferroni's *post-hoc* test; **p<0.01 for AGN211330 and ***p<0.001 for butaprost, CP533,536 and AH13205 compared with time-matched vehicle controls.

Table 4.2: Mean pIC_{50} values (M) and percentage reduction in myogenicity for EP_2 agonist concentration-effect curves in immersed myometrium from term pregnant, non-labouring donors (n=4-10).

Agonists:	**butaprost**	**CP533,536**	**AH-13205**	**AGN211330**
pIC_{50}	6.78 ± 0.26	6.62 ± 0.44	5.84 ± 0.56	5.73 ± 0.54
% decrease	54.9	72.7	59.4	39.5

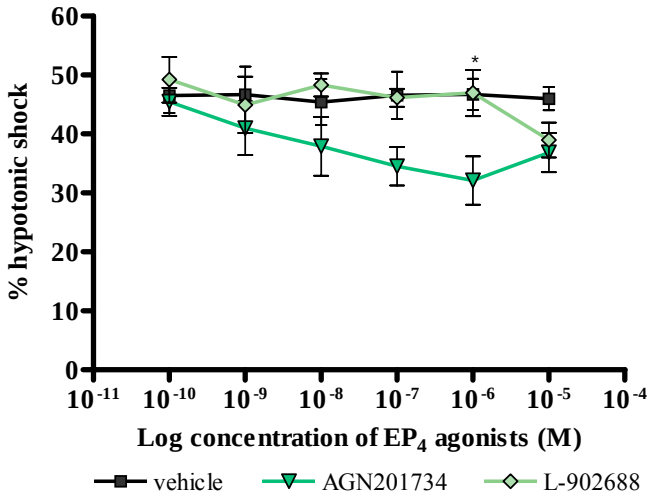


Figure 4.4: Vehicle and the concentration-effects of EP_4 agonists AGN201734 and L-902688 in isolated myometrium obtained at term pregnancy, not in labour (n=4-6). Agonists were added in a cumulative manner to immersion baths at 30-minute intervals with responses measured as area under the curve as a percentage of hypotonic shock. Data are expressed as arithmetic means ± S.E. and statistical analysis was performed using two-way ANOVA with Bonferroni's *post-hoc* test; *p<0.05 for AGN201734 compared with responses to vehicle.

The effects of PGE_2, butaprost and AGN201734 were also demonstrated in myometrial strips obtained from non-labouring donors using the superfusion technique (Figure 4.5). Unlike the immersion baths, responses were immediate and myogenic activity was restored before the addition of successive agonist concentrations. Bolus doses of PGE_2 and AGN201734 produced a biphasic effect, consisting of an initial contraction followed by a period of inhibition at all doses tested. Whilst AGN201734 delayed contractions for up to 37.4 ± 23.62 minutes ($F(1, 24) = 10.34$; $p<0.01$), the period of inhibition was extended by a third with doses of PGE_2 ($F(1, 24) = 54.50$; $p<0.001$). Butaprost did not evoke tissue excitation and, at the highest dose of $3x10^{-7}$mol, activity was suppressed for 75.5 ± 20.37 minutes ($F(1,30) = 22.85$; $p<0.001$). Maximal responses to EP agonists were not achieved due to the restricted volume of bolus doses that could be administered into the superfusate.

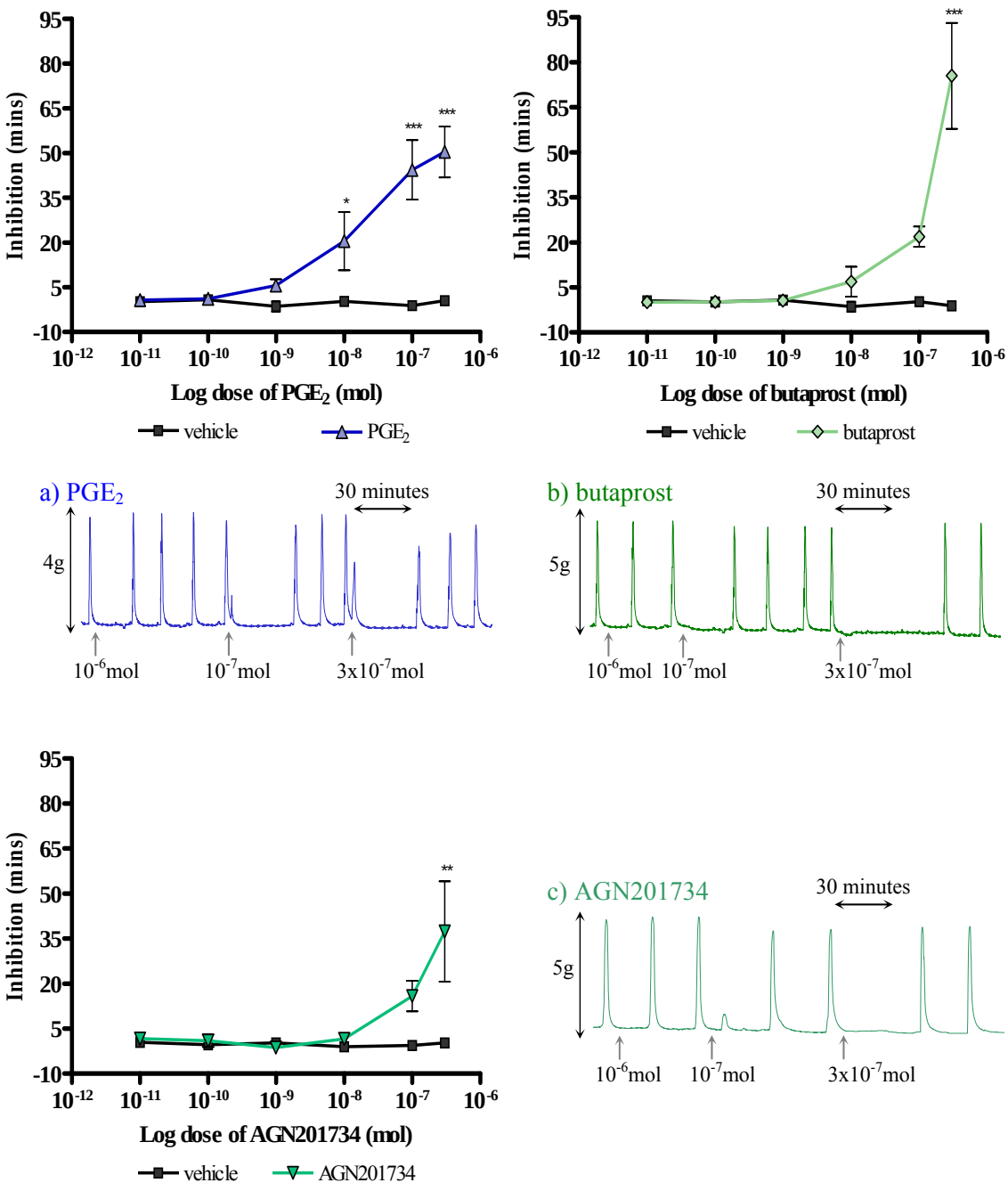


Figure 4.5: Vehicle and inhibitory dose-response curves with representative traces for a) PGE_2, b) butaprost and c) AGN201734 in isolated myometrium from term pregnant, non-labouring donors (n=4). Tissue strips were suspended in superfusion baths with bolus doses of agonist and vehicle administered into the superfusate after a contraction of similar magnitude to the myogenic activity. Results are expressed as arithmetic means ± S.E. and two-way ANOVA with Bonferroni's *post-hoc* test showed statistical significance; *p<0.05; **p<0.01; ***p<0.001 for EP agonists compared to vehicle.

4.7 Excitatory EP agonists on myogenic activity

In immersion baths, whilst myogenicity was not affected by the EP_1 agonist ONO-D1-004 ($F_{(5, 54)} = 0.245$; ns), the $EP_{3/1}$ agonist sulprostone evoked a 27.8 percent increase in the frequency and amplitude of contractions ($F_{(1, 72)} = 8.90$; $p<0.01$; Figure 4.6). This excitatory response reached 59.4 ± 2.59 percent hypotonic shock at 10^{-6}M.

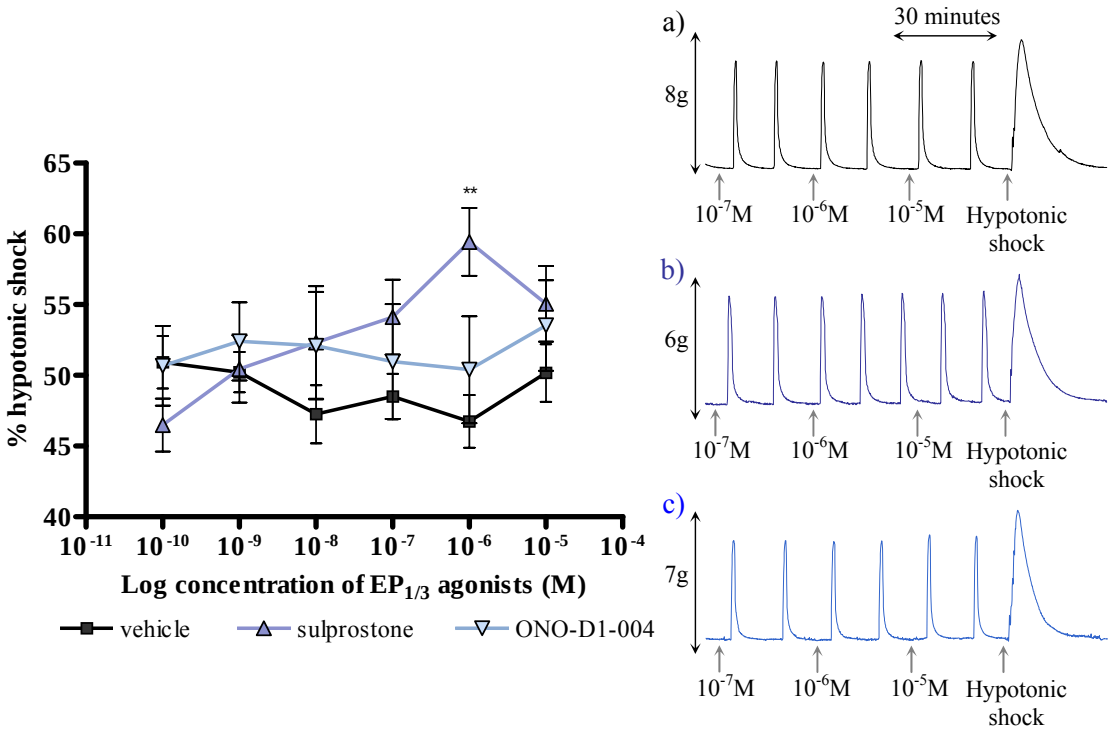


Figure 4.6: Vehicle and concentration-effect curves (10^{-10}M to 10^{-5}M) for the EP_1/EP_3 agonists sulprostone and ONO-D1-004 in isolated myometrium taken at Caesarean section from non-labouring donors (n=4-7). Treatments were added to individual organ baths and, every 30 minutes, agonist or vehicle applications were measured as area under the curve. Data are expressed as a percentage of the final contraction induced by hypotonic shock and analysed using two-way ANOVA with *post-hoc* Bonferroni's adjustment; **p<0.01 for sulprostone compared to vehicle.

Traces represent the typical myometrial responses to a) vehicle, b) sulprostone and c) ONO-D1-004 in tissue strips obtained at term pregnancy.

Using the superfusion technique, sulprostone displayed a monophasic contractile response (Figure 4.7). Following bolus doses of sulprostone, contractions increased by 54.0 percent to reach 61.2 ± 8.21 percent hypotonic shock at 10^{-7}mol ($p<0.01$). Although resultant contractions were quantitatively of a similar magnitude to immersion, the overall increase was more pronounced in superfusion experiments.

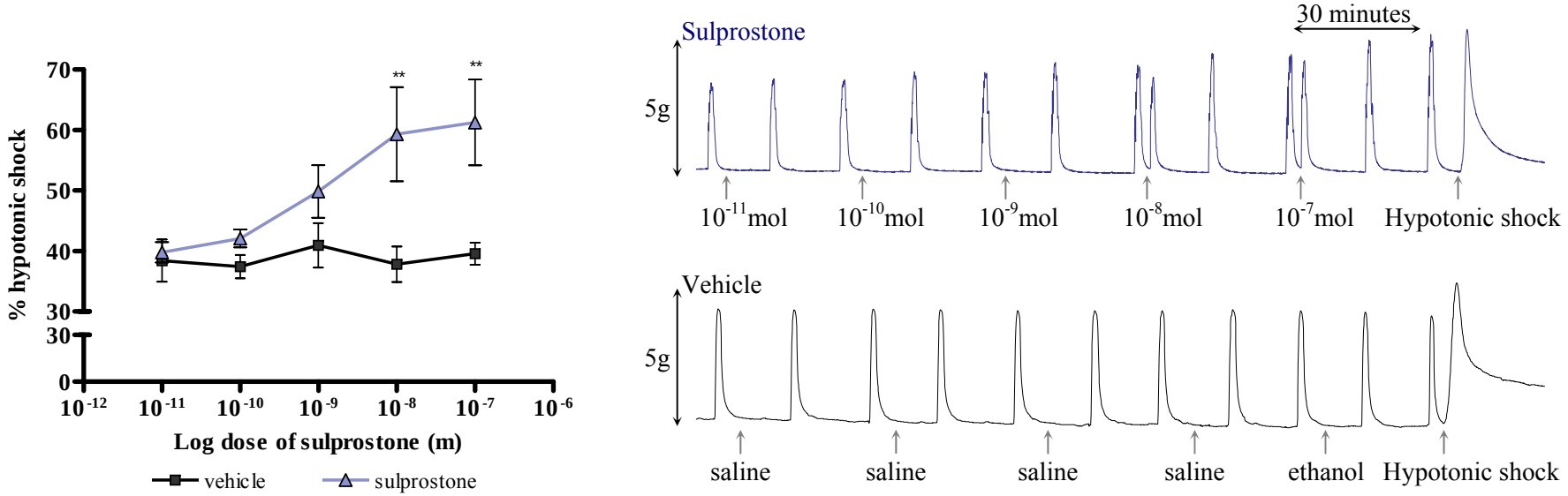


Figure 4.7: Vehicle and dose-response curves to bolus doses of the $EP_{3/1}$ agonist sulprostone in isolated myometrium obtained from term pregnant, non-labouring donors (n=4). Responses were measured over a 30-minute period (area under the curve) and expressed as a percentage of hypotonic shock. Data are arithmetic means ± S.E. and two-way ANOVA with Bonferroni's *post-hoc* test indicated statistical significance; **$p<0.01$ compared to vehicle.

Representative traces show vehicle and dose-related excitation evoked by sulprostone added at the time points indicated by the arrows.

Table 4.3: Mean pIC_{50} values (M) and percentage decrease in myogenicity for concentration-effect curves to EP agonists (10^{-10}M to 10^{-5}M) using immersed myometrial strips in the presence (treated) and absence (control) of the EP_4 antagonist GW627368x (10^{-6}M). Isolated human myometrium was obtained from donors at term pregnancy (n=3-6). Results are expressed as arithmetic means ± S.E. and significance was determined using paired t-tests; **p<0.01 for treated compared to control pEC_{50} values of AGN201734.

EP agonists	Control		Treated	
	pIC_{50}	% decrease	pIC_{50}	% decrease
PGE_2	8.4 ± 0.37	33.6	8.4 ± 0.36	36.8
Butaprost	7.1 ± 0.47	47.0	7.5 ± 0.51	46.5
CP533,536	6.6 ± 0.44	73.1	7.4 ± 0.92	60.6
AGN211330	7.2 ± 1.62	41.1	6.5 ± 0.20	34.0
AH13205	6.2 ± 0.53	54.9	6.9 ± 1.10	44.2
AGN201734	8.4 ± 0.50	29.7	6.2 ± 0.35**	15.9
L-902688	7.3 ± 0.64	20.8	6.6 ± 0.56	24.8

4.8 EP agonists in the presence and absence of GW627368x

In order to more fully characterise the EP receptors, EP agonists were tested in the presence of the EP_4 antagonist GW627368x. At 10^{-6}M, GW627368x did not significantly alter the spontaneous activity of immersed human myometrial strips obtained at term pregnancy (Figure 4.8). Likewise, GW627368x did not modify the profile of PGE_2-induced contractions nor the maximal inhibitory effects of the EP_2 agonists butaprost, CP533,536, AGN211330 and AH13205 ($F(10, 43) = 1.70$; ns; Table 4.3; Figure A5).

With EP_4 agonists, despite the lack of effect of L-902688, GW627368x produced a rightward displacement of the AGN201734 concentration-effect curve, sustaining activity between 10^{-8}M to 10^{-6}M. The pA_2 value was estimated at 8.1 and the pIC_{50} value for AGN201734 was significantly attenuated in the presence of the EP_4 antagonist ($F(5, 5) = 2.51$; $p<0.01$; Table 4.3).

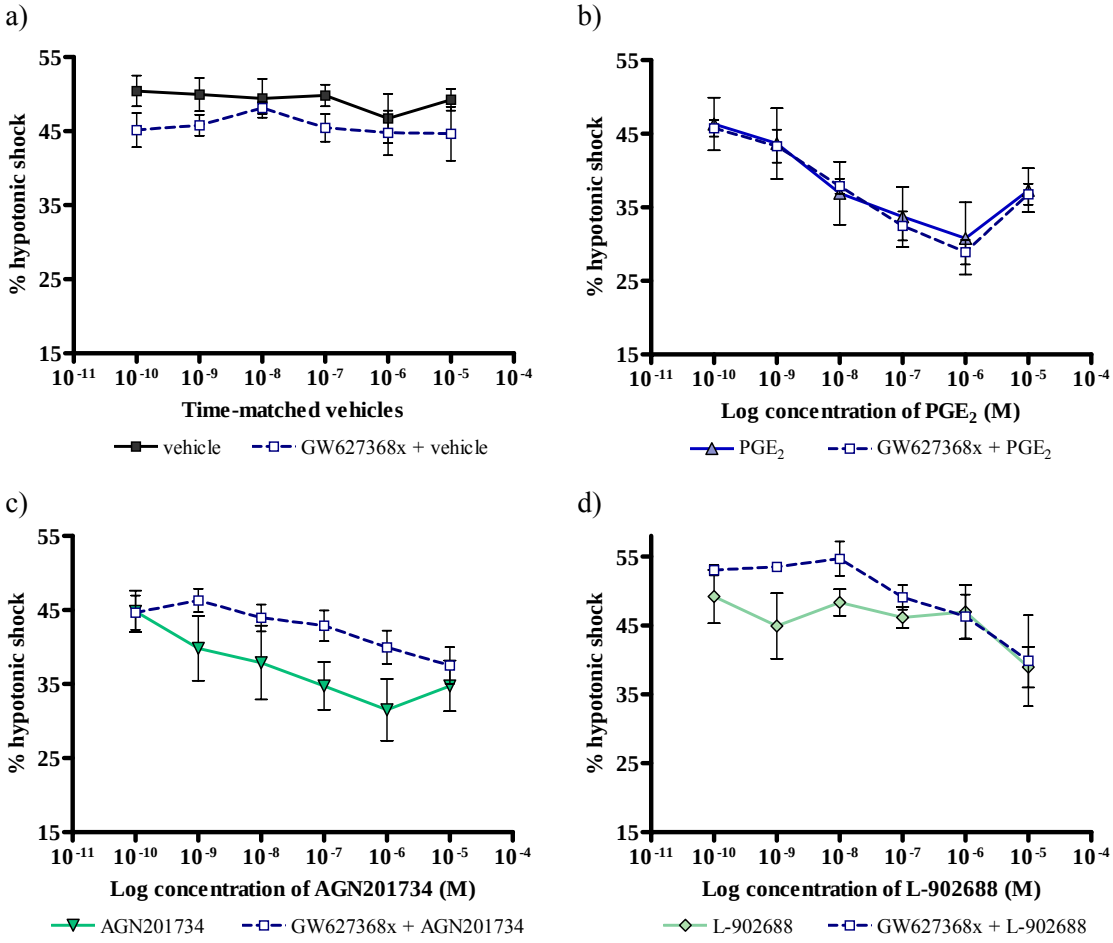


Figure 4.8: Vehicle and responses to PGE_2, AGN201734 and L-902688 in the presence and absence (control) of the selective EP_4 antagonist GW627368x (10^{-6}M) in isolated human myometrium taken at term pregnancy (n=3-6). Agonist concentration-effect curves were performed in immersion baths at 30-minute intervals and expressed as percentage of the final contraction induced by hypotonic shock. Results are arithmetic means ± S.E.

4.9 EP agonists in the presence and absence of AH6809

Due to its lack of specificity, the EP_1, EP_2, EP_3 and DP antagonist AH6809 was used at a concentration of 10^{-5}M. The presence of AH6809 alone did not affect tissue myogenicity (F (1, 83) = 0.10; ns; Figure 4.9). However, AH6809 significantly antagonised PGE_2, producing a rightward displacement of the biphasic curve with an estimated pA_2 value of 7.2 (Figure 4.9; Table 4.4). Unlike PGE_2 alone, AH6809 fully inhibited the contractile response characteristically produced at 10^{-5}M (F (1, 59) = 5.01; $p<0.001$).

Each EP_2 agonist elicited concentration-dependent inhibitory effects in the rank order of potency: butaprost > CP533,536 > AH13205 > AGN211330 (Table 4.4). In the presence of AH6809, activities were shifted rightwards and similar monophasic inhibitory curves were produced for butaprost, CP533,536 and AH13205 (Figure 4.10) with pA_2 values of about 5.5. This displacement was significant for CP533,536 at 10^{-6}M (F (1, 60) = 13.16; $p<0.05$). Even so, the effect of AH6809 was more pronounced with AGN211330, preventing the typical decline in activity at 10^{-5}M (F (1, 36) = 8.06; $p<0.01$; Figure 4.10).

AH6809 did not antagonise either of the EP_4 agonists AGN201734 or L-902688 and had no effect on the EP_1 agonist ONO-D1-004. Although the excitation evoked by sulprostone was potentiated in the presence AH6809, this response was variable (F (1, 60) = 1.99; ns; Figure A6; Table 4.4).

Table 4.4: Relative pEC_{50} values (M) and the percentage change in activity for EP agonists determined in isolated lower segment myometrium obtained at Caesarean section from non-labouring donors (n=4-8). Agonists were added to parallel myometrial strips in the absence (control) or presence (treated) of the EP_1, EP_2, EP_3 and DP receptor antagonist AH6809 (10^{-5}M). Data are shown as arithmetic means ± S.E. and statistical analysis was performed using paired t-tests; *p<0.05 for % change in AGN211330; ***p<0.001 for PGE_2 pEC_{50} values of control compared with treated groups.

EP agonists	**Control**		**Treated**	
	pEC_{50}^{+}	% change	pEC_{50}	% change
PGE_2	8.4 ± 0.37	33.6	6.0 ± 0.25***	61.6
butaprost	7.1 ± 0.47	47.0	6.2 ± 0.33	57.7
CP533,536	6.6 ± 0.44	73.1	5.5 ± 0.21	66.4
AGN211330	5.8 ± 0.56	41.1	5.1 ± 1.20	1.5*
AH13205	6.4 ± 1.23	54.9	6.2 ± 0.71	52.8
AGN201734	8.1 ± 0.34	29.7	7.7 ± 0.06	28.5
L-902688	7.3 ± 0.64	20.8	7.7 ± 0.39	19.5
Sulprostone	7.4 ± 0.64	20.6	8.2 ± 0.39	44.7
ONO-D1-004	7.3 ± 0.35	5.6	7.8 ± 0.82	7.5

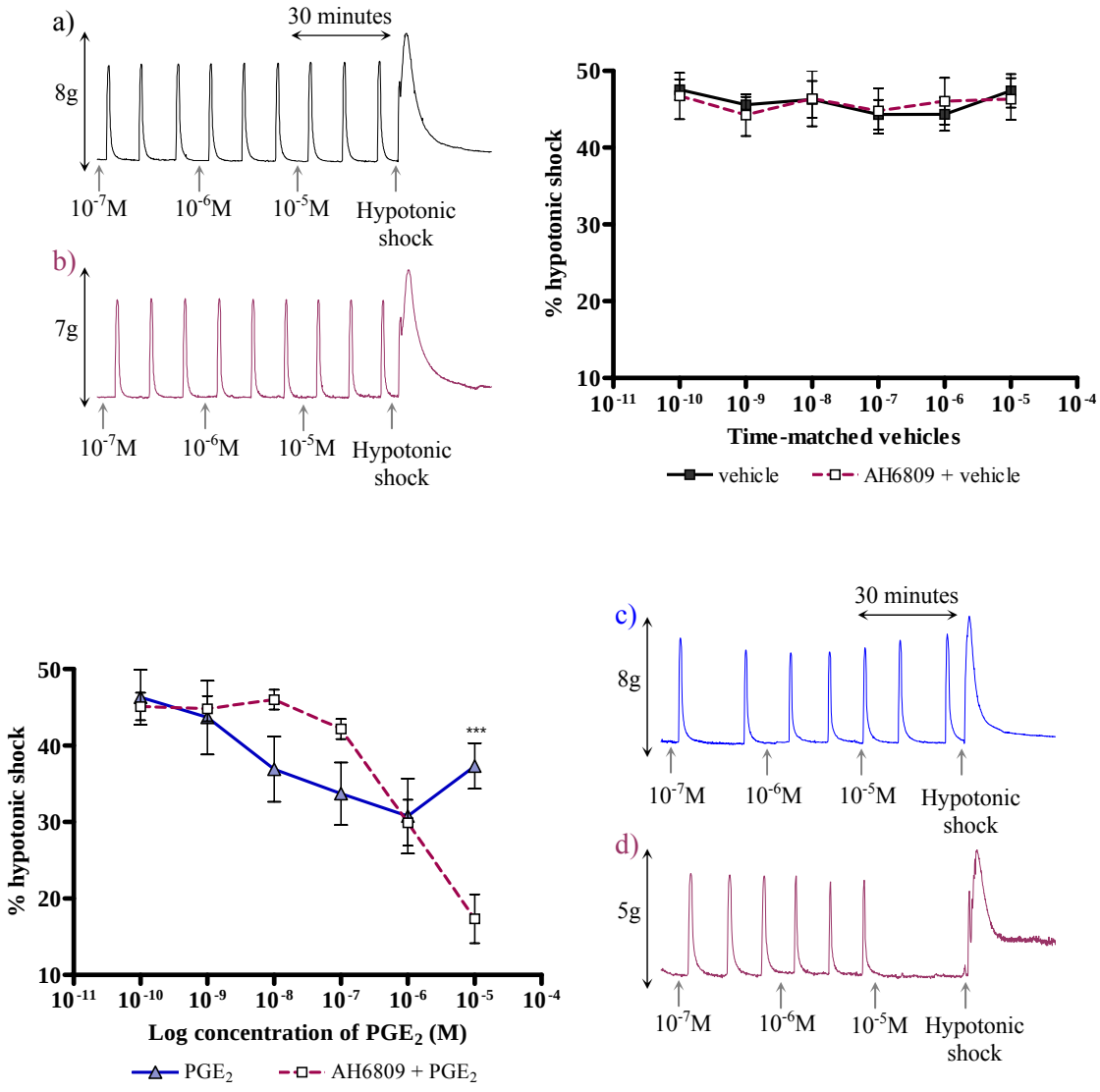


Figure 4.9: Vehicle and concentration-effect curves for PGE_2 in the absence (control) and presence of the EP_1, EP_2, EP_3 and DP receptor antagonist AH6809 (10^{-5}M) in myometrial strips from term pregnant, non-labouring donors (n=6-8). Vehicle and PGE_2 (10^{-10}M to 10^{-5}M) were added to immersion baths at 30-minute intervals with myometrial activity expressed as a percentage of the final contraction induced by hypotonic shock. Results are arithmetic means ± S.E. and statistical analysis was performed using two-way ANOVA with Bonferroni's adjustment; ***$p<0.001$ for PGE_2 compared to PGE_2 incubated with AH6809.

Representative traces showing the effects of vehicle and PGE_2 in the absence (a, c) and presence (b, d) of AH6809 (10^{-5}M) on isolated late gestational myometrium.

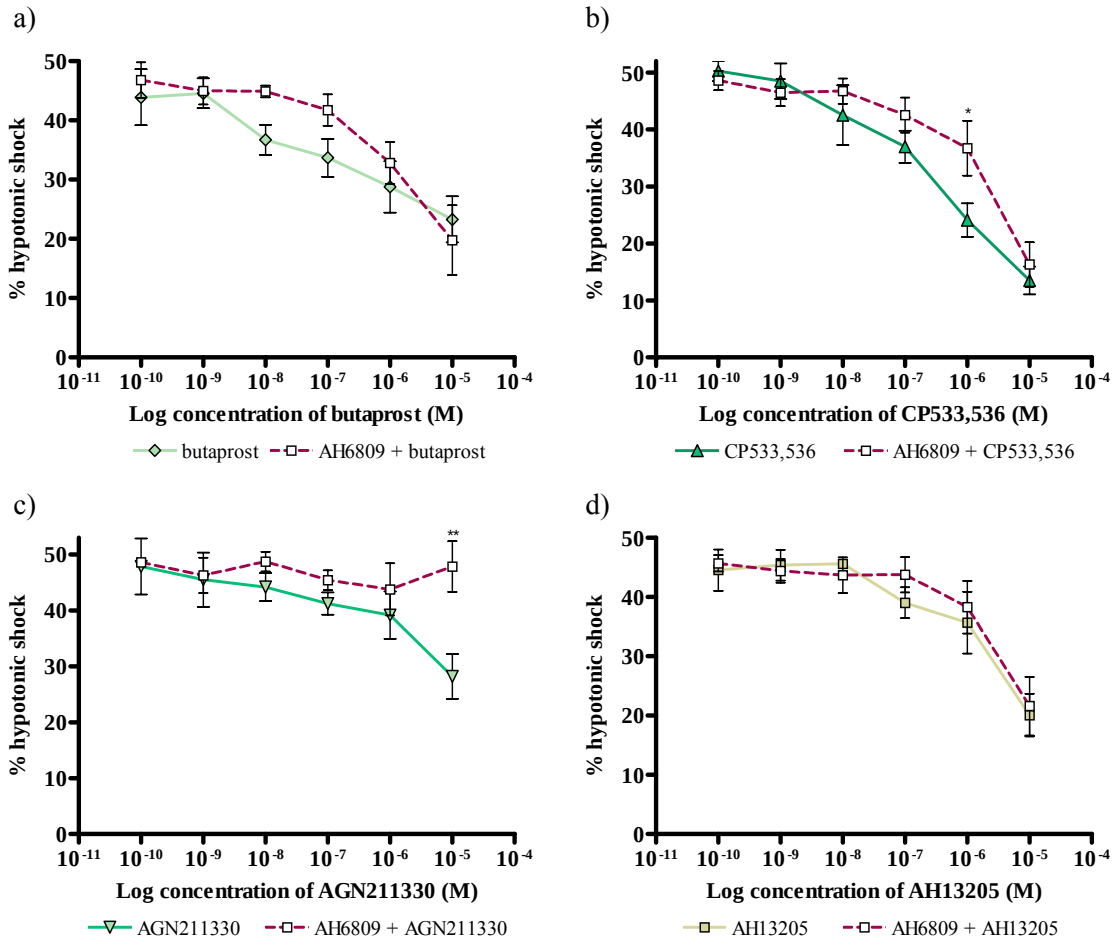


Figure 4.10: *In vitro* utero-relaxant effects of the EP_2 agonists a) butaprost, b) CP533,536, c) AGN211330 and d) AH13205 in human lower segment myometrium obtained from term pregnant, non-labouring women (n=4-8). EP agonists (10^{-10}M to 10^{-5}M) were added in a cumulative manner at 30-minute intervals to isolated myometrial biopsies either alone (control) or in the presence of AH6809 (10^{-5}M). Results are expressed as arithmetic means ± S.E. and statistical analysis was performed using two-way ANOVA with Bonferroni's *post-hoc* test; *p<0.05; **p<0.01 compared to control EP myogenic activity.

4.10 Contractile EP agonists in the presence and absence of AGN211329

At 10^{-6}M, the EP_3 antagonist AGN211329 maintained myogenic activity at 47.5 ± 6.89 percent hypotonic shock in vehicle-treated myometrial strips from term pregnant, non-labouring donors (Figure 4.11). Although the inhibitory phase of PGE_2-induced activity was extended by 13.4 percent in the presence of AGN211329, the excitation exhibited at 10^{-5}M was equivalent ($F(1, 47) = 0.94$; ns). Likewise, responses to sulprostone and ONO-D1-04 were not altered by AGN211329.

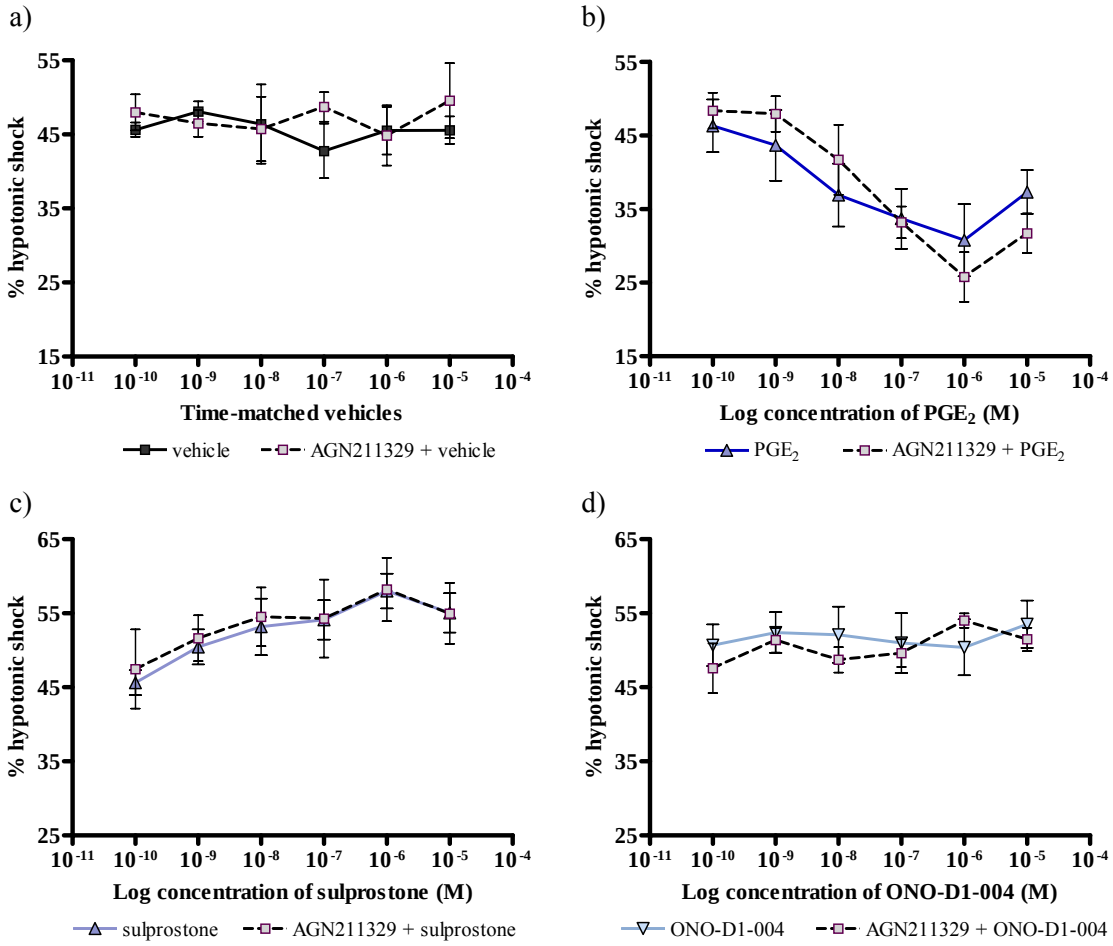


Figure 4.11: a) Vehicle and concentration-effect curves (10^{-10}M to 10^{-5}M) for b) PGE_2 and the $EP_{1/3}$ agonists c) sulprostone and d) ONO-D1-004 in isolated late gestational myometrium from donors not in labour (n=3-6). Increasing concentrations of EP agonists were added to parallel immersed myometrial strips either alone or in the presence of the EP_3 antagonist AGN211329 (10^{-6}M). Activity was determined over 30-minute periods (area under the curve) and expressed as percentage hypotonic shock. Data are shown as arithmetic means ± S.E.

4.11 Relative uterotonic effects of $PGF_{2\alpha}$ and U46619

The spasmogens $PGF_{2\alpha}$ and U46619 increased both the amplitude and frequency of phasic contractions without elevating the muscle tonus in myometrium from term pregnant, non-labouring donors. Using immersed myometrial strips, $PGF_{2\alpha}$ elicited an excitatory monophasic response (Figure 4.12). This reached 77.1 ± 6.3 percent hypotonic shock, which was significant compared to sustained myogenicity with time-matched controls ($F(1, 55) = 26.55$; $p<0.001$). Using the superfusion technique, contractions were evoked immediately after bolus doses of $PGF_{2\alpha}$. In contrast to immersion, enhanced activity was sigmoidal in shape, reaching 64.4 ± 2.64 percent hypotonic shock at 10^{-7}mol ($F(1, 60) = 49.66$; $p<0.001$).

U46619 augmented myogenic activity to 137.6 ± 19.2 percent hypotonic shock, which was nearly 2-fold higher than the concentration-effect curves generated by $PGF_{2\alpha}$. When added to the same organ bath, U46619 and $PGF_{2\alpha}$ did not produce synergistic effects (Figure 4.14). In addition to the increase in amplitude, U46619 enhanced the duration of contractions and between 10^{-6}M and 10^{-5}M responses were potentiated ($F(1, 55) = 83.56$; $p<0.001$).

4.11.1 Antagonism of U46619 with SQ29,548 and GR32191B

Whilst the TP antagonists SQ29,548 (10^{-6}M) and GR32191B (10^{-6}M) had no effect on myogenicity, each suppressed U46619 concentration-effect curves in immersed and superfused isolated term gestational myometrium (Figures 4.15 & 4.16) with estimated pA_2 values of 6.5 and 7.0 respectively. The reduction in U46619-induced contractility was notable with the addition of TP antagonists between repeated doses of U46619.

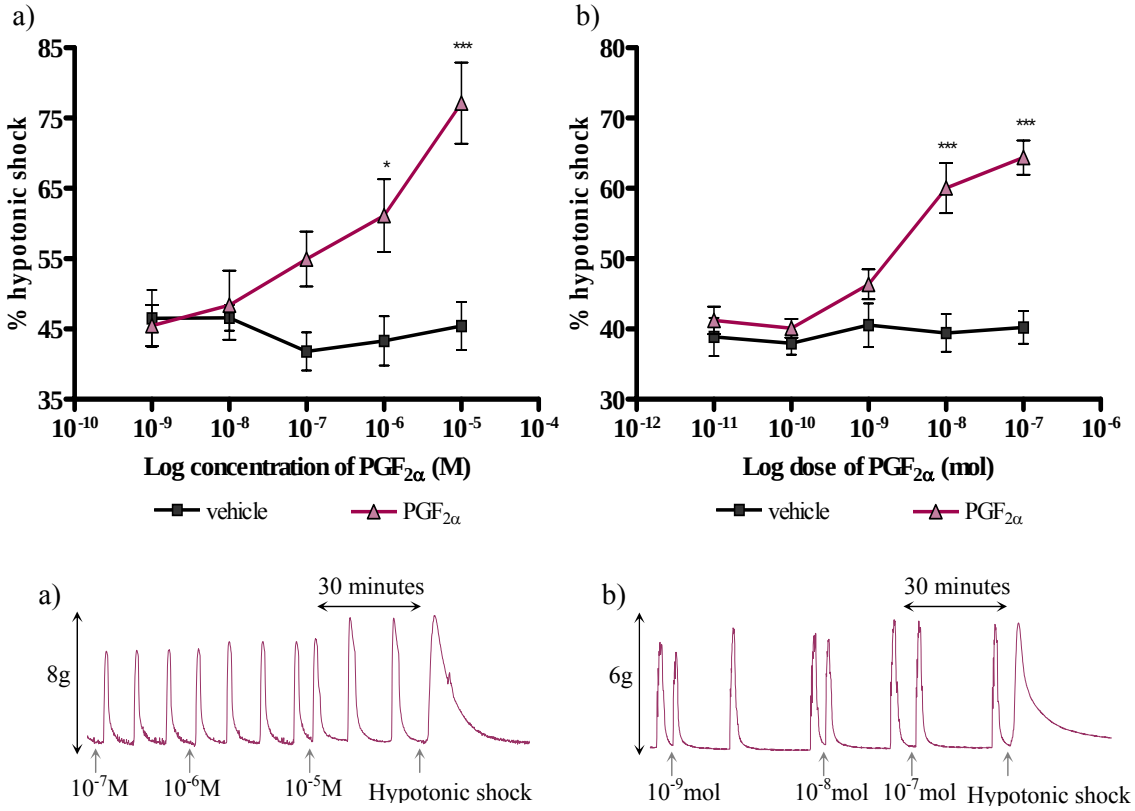


Figure 4.12: Vehicle, concentration-effect curves and typical traces for $PGF_{2\alpha}$ in myometrium from term pregnant, non-labouring donors set-up using a) immersion (n=7) and b) superfusion techniques (n=7). $PGF_{2\alpha}$ was respectively added in a cumulative manner to immersion baths and administered as bolus doses to the superfusate. Results are expressed as arithmetic means ± S.E. and statistical significance was determined using two-way ANOVA with Bonferroni's *post-hoc* test; *p<0.05; ***p<0.001 for $PGF_{2\alpha}$ compared with vehicle.

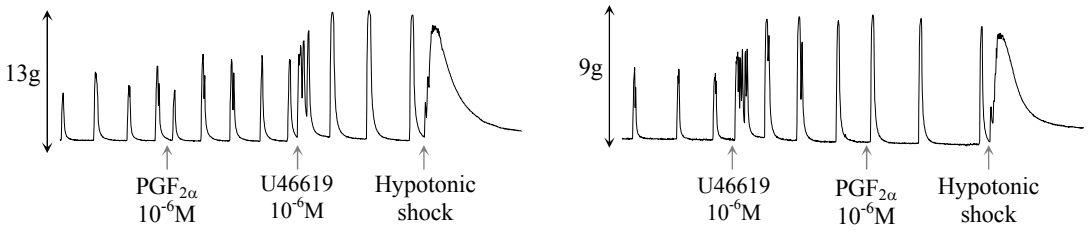


Figure 4.13 Representative traces showing the excitatory effects evoked by $PGF_{2\alpha}$ compared to the thromboxane mimetic U46619 in myometrium from term pregnant donors, before labour-onset. Agonists were added at 30-minute intervals to the immersion apparatus.

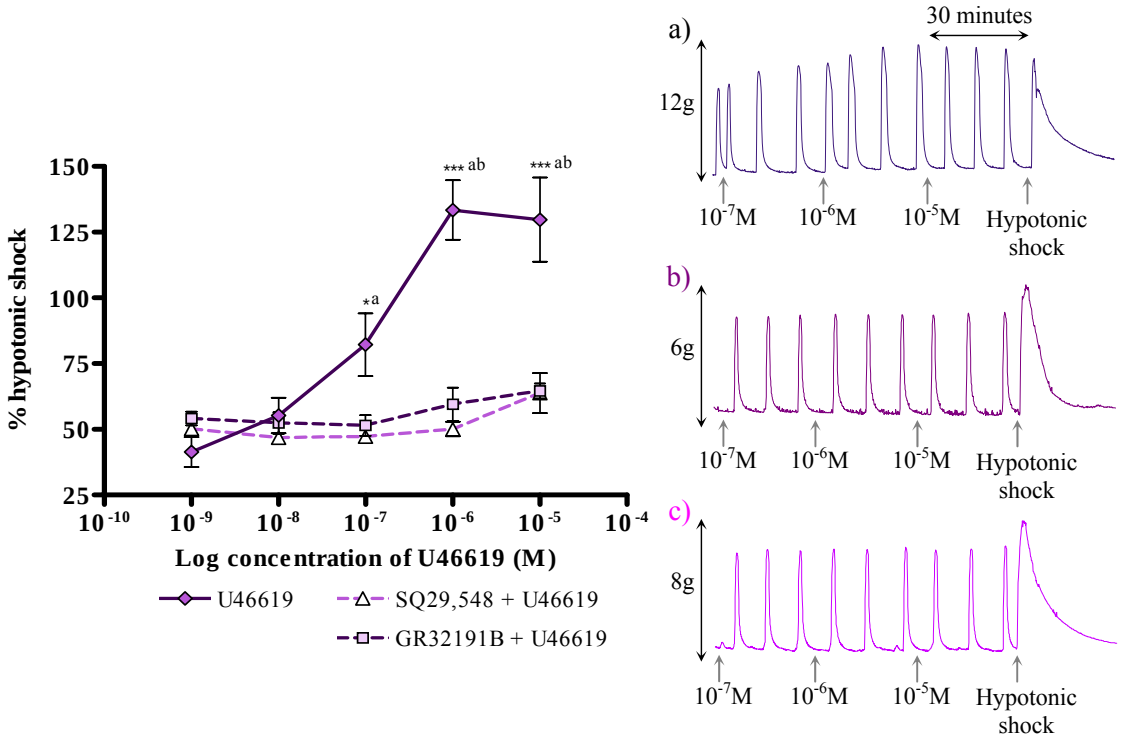


Figure 4.14: Concentration-effect curves for the uterotonin U46619 a) alone or in the presence of either TP antagonist b) SQ29,548 (10^{-6}M) or c) GR32191B (10^{-6}M) in myometrium from term pregnant, non-labouring donors (n=6-7). U46619 (10^{-9}M to 10^{-5}M) was added to immersion baths in a cumulative manner and maximal responses for excitation are expressed as percentage hypotonic shock. Data were analysed using two-way ANOVA mixed model with Bonferroni's *post-hoc* adjustment and expressed as arithmetic means ± S.E.; *p<0.05; ***p<0.001 significant attenuation by [a]SQ29,548 and [b]GR32191B compared with U46619 alone.

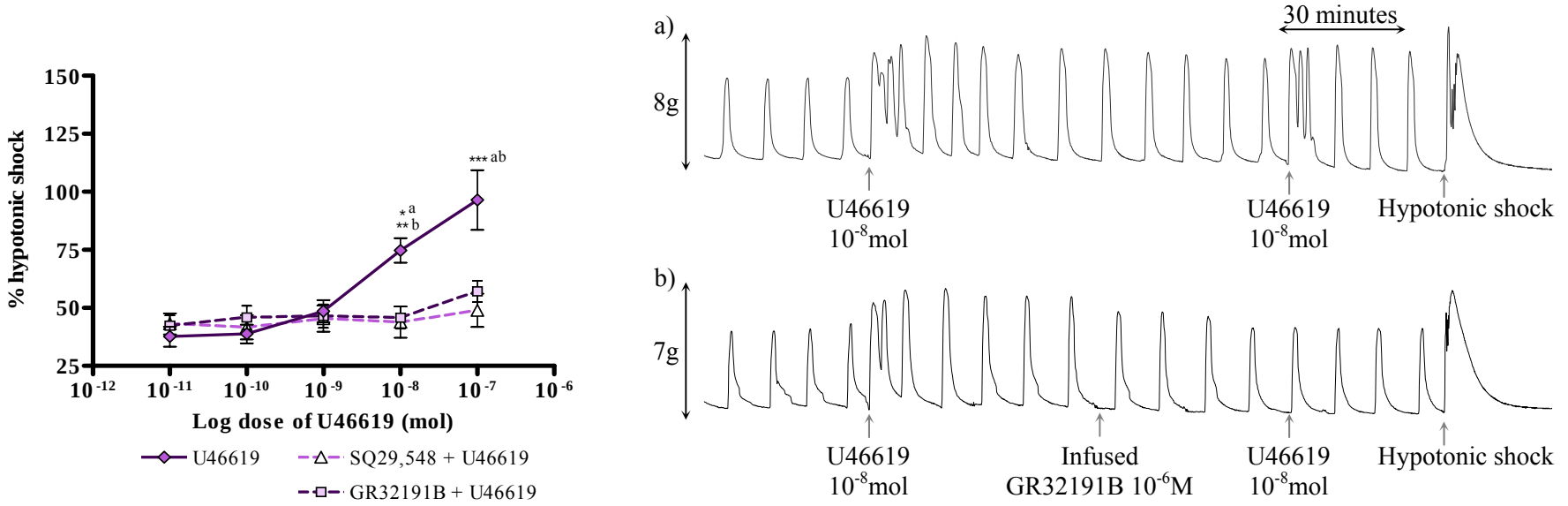


Figure 4.15: Concentration-effect curves for U46619 in the absence (control) and presence of the TP receptor antagonists SQ29,548 (10^{-8}mol) or GR32191B (10^{-8}mol) in myometrial strips from term pregnant, non-labouring donors (n=4-8). Bolus doses of U46619 (10^{-11}mol to 10^{-7}mol) were added to the superfusate immediately after myogenic contractions with activity expressed as a percentage of the final contraction induced by hypotonic shock. Results are arithmetic means ± S.E. and statistical analysis was performed using two-way ANOVA with Bonferroni's adjustment; *p<0.05; **p<0.01; ***p<0.001 for U46619 alone compared to tissues superfused with [a]SQ29,548 and [b]GR32191B.

Representative traces show the excitatory responses evoked in isolated gestational myometrium by a) repeated doses of U46619 (10^{-8}mol) and b) antagonised by the addition of GR32191B (10^{-6}M) into the superfusate.

Chapter 4.12: Discussion

The results show that late gestational human myometrium exhibited spontaneous and agonist-induced contractions in functional *in vitro* studies. Compared to the non-gravid state (Chapter 3), myometrial tissue had been subjected to hypertrophy, hyperplasia and stretching due to the 400-fold increase in uterine volume during the course of pregnancy (Shynlova *et al*., 2010). The consequential changes in cell signalling and mechanotransduction pathways were manifested as well-defined intrinsic contractions in immersion and superfusion systems. This may reflect the lower segment uterine function to prevent premature delivery of the foetus. Even so, due to a lack of definitive markers, the imminent timing of parturition and related preparatory stage of tissues were unknown in this study.

In spite of differences in donor clinical profiles, variations in myogenic activity did not relate to groups at high risk of preterm labour, including heavy smoking status and those with pathophysiological conditions. This indicates that compensatory changes in uterine physiology are responsible for pregnancy maintenance. Compared to uncomplicated normal pregnancies, isolated myometrium from diabetics have previously been shown to achieve similar isometric forces (Kaya *et al*., 1999). Likewise, the increased vascular resistance in preeclamptic pregnant women was related to peripheral rather than myometrial blood vessels (Wimalsundera *et al*., 2005). This suggests that the haemodynamic changes to the uterus during pregnancy may minimise inter-donor variations.

Spontaneous contractions of myometrial strips were also not affected by donor parity. The distension of the parturient uterus at term to fill almost the entire abdominal cavity may have standardised differences between individuals. Similarly, no

correlation was found between patient parity and the peak frequency of *in vivo* uterine electromyograph recordings (Maner *et al*., 2003). Even so, the contractile amplitude of isolated myometrium was relatively attenuated in women with a history of previous abortions or miscarriages. The timing and manner of pregnancy termination was not disclosed on patient information forms but accounted for 26 percent of donors.

Distinct contractile profiles between and within donor tissues reflected differences in myometrial tensile strength (Buhimschi *et al*., 2006). This included variations in muscle content, the distribution and number of PG receptors, gap junctions, ion channel proteins, pacemaker cells, signal transduction pathways and endogenous PG production. In particular, the upregulated decidual synthesis of PGE_2 and $PGF_{2\alpha}$ has been reported for miscarriage and for preterm labour (Calder, 1990). This suggests that an aberrant release of endogenous PGs may be responsible for changes in uterine motility leading to pregnancy-related disorders.

Increasing intrauterine PGE_2 synthesis during the third trimester of pregnancy implicates its pivotal role for regulating myometrial tonus (Soloff, 1989). This was demonstrated in the present study by use of PGE_2 and selective analogues for $EP_{1\text{-}4}$ receptors. In immersed myometrium taken from term pregnant, non-labouring donors, PGE_2 attenuated contractions in a concentration-dependent manner followed by relative tissue excitation. Bell-shaped responses to PGE_2 were also observed using superfusion techniques and corroborated previous functional studies (Word *et al*., 1992; Senior *et al*., 1993).

In lower segment myometrium, PGE_2 was the most potent agonist tested with predominant utero-relaxatory effects (pEC_{50}: 8.4 ± 0.37M). To mediate inhibition, PGE_2 has been shown to augment intracellular cAMP in human myometrial cells (Oger *et al*., 2002; Asboth *et al*., 1997), suggesting the involvement of Gαs coupled

EP_2 and EP_4 receptors. Due to their common pathways and co-localisation, the physiological significance of these receptors remains obscure. Nevertheless, higher proportions of serine and threonine residues in the carboxyl-terminal tail of EP_4 distinguish it from EP_2 (Regan *et al*., 1994). Accordingly a variety of selective agonists have been developed, which were used to determine the relative effects of EP_2 and EP_4 in isolated myometrial tissue.

For EP_2, the selective agonists assayed included butaprost (Gardiner, 1986), CP533,536 (Li *et al*., 2003; Paralkar *et al*., 2003), AH13205 (Coleman *et al*., 1994) and AGN211330 (Belley *et al*., 2005). The EP_2 analogue butaprost was synthesised by replacing the α-carboxylic moiety with a methyl ester. In contrast, CP533,536 was a non-prostanoid pyridyl sulfonamide, AH13205 a heptanoic acid and AGN211330 an ortho-substituted cinnamic acid. Using myometrial strips in this study, the rank order of potency for EP_2-mediated inhibition was butaprost = CP533,536 > AH13205 > AGN211330. In accordance with superfusion, butaprost attenuated activity in a monophasic concentration-related manner (Senior *et al*., 1993; Duckworth *et al*., 2002). Although these studies employed the commercially available methyl ester, cellular de-esterification of butaprost to its free acid form would have enabled butaprost to attain full agonist potency (Abramovitz *et al*., 2000; Wilson *et al*., 2004; Alexander *et al*., 2007). The EP_2 agonist, AH13205 only possessed modest affinity at EP_2 receptors, but was essentially inactive at human EP_1 and EP_3 receptors (Coleman *et al*., 1994). The decline in activity with AH13205 indicated functional EP_2 receptors in the isolated myometrium. In addition, AGN211330 was identified as a full EP_2 agonist, with some TP receptor activity (Belley *et al*., 2005). However, the low potency in this study (pEC_{50}: 5.73 ± 0.54M) corresponded to its recent classification as an EP_2 agonist with only partial EP_2 affinity (personal communication, Woodward,

2007). Myocyte stimulation with PGE_2 and EP_2 agonists were also shown to produce similar rises in cAMP (Asboth *et al*., 1997), which indicates further predominant EP_2 receptor binding.

To determine EP_4 receptor action, concentration-effect curves for AGN201734 (Elworthy *et al*., 2004) and L-902688 (Billot *et al*., 2003) were performed on myometrial strips. Each ligand was composed of a lactam template and maintained natural stereochemistry at C-12 and C-15 for optimal EP_4 activity. Even so, myometrial responses to AGN201734 mimicked the biphasic response of PGE_2, whilst L-902688 only attenuated myogenic activity at 10^{-5}M. This was surprising as each agonist had respectively demonstrated a 1000-fold greater affinity to EP_4 compared with the other EP receptors (Elworthy *et al*., 2004) and PG receptors (Billot *et al*., 2003). Even so, as the heptanoic acid moiety of AGN201734 is similar in structure to PGE_1 analogues, it is plausible that AGN201734 either activated EP_4 or exhibited off-target agonist potency at IP receptors. A common structural motif of some IP and EP_4 receptor agonists has been demonstrated in human (Abramovitz *et al*., 2000; Wilson *et al*., 2004), porcine (Jones & Chan, 2001), guinea pig and rabbit preparations (Jones & Chan, 2005). As a result, EP_4 activation was further investigated by use of the selective EP_4 antagonist, GW627368x (Jones & Chan, 2005; Wilson & Giles, 2005).

Despite its affinity for human TP receptors, GW627368x is a highly potent and competitive EP_4 antagonist (Wilson *et al*., 2006; Alexander *et al*., 2007). The selectivity of GW627368x was 100-fold greater for EP_4 receptors over the EP_2 subtype (Jones & Chan, 2005; Wilson & Giles, 2005; Wilson *et al*., 2006). This was demonstrated in the present study, since GW627368x produced no effects in vehicle and EP_2-treated myometrial strips. However, with EP_4 agonists, the addition of

GW627368x caused a rightward displacement of AGN201734 curves, without affecting responses to L-902688. These contradictory results increased the ambiguity of EP_4-mediated events. However, as GW627368x failed to shift PGE_2-induced responses, it seems most likely that EP_2 is the dominant functional isoform in the lower uterus at term pregnancy.

Both EP_2 and EP_4 transcripts are highly expressed in isolated human lower uterine segments at term gestation (Leonhardt *et al*., 2003; Astle *et al*., 2005; Sooranna *et al*., 2005; Grigsby *et al*., 2006), which confounds further the diversity of their actions. However, with only 38 percent homology between transmembrane domains, it has been reported that EP_2 and EP_4 receptors differ in their sensitivity and additional signalling pathways. The agonist-induced desensitisation (Nishigaki *et al*., 1996) and PGE_2-mediated internalisation (Desai *et al*., 2000) of EP_4 receptors may attribute to the lower PGE_2-stimulated cAMP formation in EP_4 compared to EP_2-transfected cells (Fujino *et al*., 2002; Fujino *et al*., 2005). In addition, although both stimulate adenylyl cyclase, the EP_2 receptor is mediated through a cAMP/ PKA-dependent mechanism, whereas the EP_4 receptor predominantly activates phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK)-dependent systems (Fujino *et al*., 2002) via a cAMP-inhibitory G-protein ($G\alpha i$; Fujino & Regan, 2006). Moreover, the induction of early growth response factor-1 (ERG-1) by PI3K and ERK regulates the growth and motility of cells (Sheng *et al*., 2001). This implicates the involvement of EP_4 receptors in uterine remodelling during pregnancy. Gene transcription by ERG-1 further enhances PGE synthase, inflammatory responses and hyperalgesia (Naraba *et al*., 2002; Slater *et al*., 2006). As a result, myometrial EP_4 receptors may primarily mediate PG synthesis, inflammation and nociception whilst EP_2 receptors appear to be more functional in maintaining uterine quiescence.

To identify responses to EP_1 and EP_3 analogues in gravid human myometrium, concentration-effect curves for sulprostone (Schaaf *et al*., 1981) and ONO-D1-004 (Oka *et al*., 2003) were constructed. As a stable acyl sulphonamide, sulprostone showed affinity for both EP_3 and EP_1 receptors (Coleman *et al*., 1994; Alexander *et al*., 2007) with K_i values of 0.60 and 14nM respectively (Kiriyama *et al*., 1997). The excitatory responses to sulprostone were monophasic using immersion and superfusion techniques, indicating the absence of EP_2 (Coleman *et al*., 1994) and EP_4 receptor activation (Wilson *et al*., 2004). A decline in the majority of EP_3 receptors, including $EP_{3\text{-II}}$ mRNA and increased $EP_{3\text{-VI}}$ transcripts by term gestation (Matsumoto *et al*., 1997; Wing *et al*., 2003; Astle *et al*., 2005) may have reflected the 10-fold reduction in the potency of sulprostone compared with the non-pregnant state (Senior *et al*., 1993). However, the signalling cascades and transcriptional mechanisms for each of the EP_3 splice variants have yet to be elucidated.

In contrast, the novel EP_1 receptor agonist, ONO-D1-004, produced negligible myogenic effects on tissues relative to time-matched vehicle controls. Although highly selective for the mouse EP_1 receptor, the binding affinity of ONO-D1-004 was relatively weak with a K_i value of 150nM (Kiriyama *et al*., 1997). Moreover, its potency was 8-fold lower than PGE_2 (Oka *et al*., 2003). Therefore, rather than discounting the influence of EP_1 receptors, the population of myometrial EP receptors was further examined by use of selective antagonists.

AH6809 has been used as a putative EP_1 antagonist for receptor classification purposes (Coleman *et al*., 1994). However, in human recombinant receptors, the affinity of AH6809 was equipotent for EP_1 and EP_2 receptors (Woodward *et al*., 1995) with further weak, but specific antagonism displayed at human EP_3 and DP receptors (Keery & Lumley, 1988; Kiriyama *et al*., 1997; Abramovitz *et al*., 2000). As the pA_2

for AH6809 has been reported to be between 5.3 and 7.0 (Keery & Lumley, 1988; Meja *et al*., 1997; Abramovitz *et al*., 2000), it was added at 10^{-5}M to competitively block functional receptors.

Accordingly, AH6809 had no effect on spontaneous myometrial activity or responses to the EP_4 receptor agonists AGN201734 and L-902688 in this study. However, a notable change in the concentration-effect curves for PGE_2 was elicited in the presence of AH6809 with an apparent pA_2 value of 7.2. Between 10^{-10}M and 10^{-7}M, the relative enhancement of contractions indicated the antagonism of EP_2 receptors. However, the subsequent 2.6 ± 0.21 fold attenuation in activity may have represented blocking of EP_1 and EP_3 receptors, enabling PGE_2 to target relaxatory EP receptors. Despite the lower affinity of the EP_2 mimetics (pA_2 values: 5.3 to 5.7), the inhibitory effects of PGE_2 with AH6809 were similar to butaprost, CP533,536 and AH13205. This implies that high concentrations of PGE_2 couple to functional EP_2 receptors due to their prevalence in lower myometrial tissue at term pregnancy. The full antagonism of AGN211330 also demonstrated its weak affinity for EP_2 receptor sites.

In accord with previous functional studies (Senior *et al*., 1993), AH6809 did not alter contractile responses to the $EP_{3/1}$ agonist sulprostone. With AH6809 having 50-fold greater affinity for EP_1 over EP_3 receptors (Abramovitz *et al*., 2000), this suggests a paucity of functional EP_1 receptors in isolated human myometrium. It would also substantiate the lack of excitation observed with the EP_1 agonist ONO-D1-004. Even so, AH6809 decreased the height of the PGE_2-induced Ca^{2+} peak in myometrial cells (Asboth *et al*., 1997), which was displayed as the loss of contractility by PGE_2 at 10^{-5}M. This suggests that AH6809 can effectively block $EP_{1/3}$ receptors when inhibitory receptors supersede contractile EP receptor function in late gestational lower myometrium.

Unfortunately, the EP_3 receptor antagonist, AGN211329, did not modify excitatory responses to PGE_2 and sulprostone in this study. This was in contrast to its reported high affinity for EP_3 receptors in transfected human embryonic kidney cells (Belley *et al*., 2005) and guinea pig vas deferens and tracheal tissues (Clarke *et al*., 2004). The lack of inhibitory effect, however, was also determined using isolated mouse uterus (Griffiths *et al*., 2006) and additional antagonism for AGN211329 was shown at DP, EP_4 and TP receptors using recombinant human receptors (personal communication, Woodward, 2007). Functional data on EP_3 receptor activation has been particularly limited due to the multiple splice variants and the cross-reactivity of ligands. Whilst EP_3 antagonists are just emerging, potent selective antagonists for EP_2 receptors are yet to be developed (Woodward *et al*., 1995; Alexander *et al*., 2007). As a result, a more comprehensive pharmacological classification of EP receptor function still remains to be established.

Unlike PGE_2, $PGF_{2\alpha}$ elicited monophasic excitation in isolated lower tissues taken at term pregnancy. Uterotonic responses were quantitatively similar to previous superfusion and immersion studies (Wikland *et al*., 1984; Word *et al*., 1992; Senior *et al*., 1993; Crankshaw & Dyal, 1994; Friel *et al*., 2005; Hutchinson, 2005), indicating the presence of myometrial FP receptors. This was verified by the 16-phenoxy FP analogues fluprostenol and 17-phenyl $PGF_{2\alpha}$, which produced equivalent *in vitro* myogenic contractions (Senior *et al*., 1993; Hutchinson, 2005). High affinity for FP receptors was calculated in the rank order of $PGF_{2\alpha}$ = fluprostenol > PGD_2 > PGE_2 > U46619 > iloprost with K_i values of 2.1, 2.7, 5.4, 65, 112 and 920nM respectively (Abramovitz *et al*., 1994). Although $PGF_{2\alpha}$ was reported to also bind to EP_1 and EP_3 receptors with significant affinity (Kiriyama *et al*., 1997; Breyer *et al*., 2001), $PGF_{2\alpha}$-induced contractility was 23 percent more pronounced in this study compared to

sulprostone. This indicates the predominant involvement of FP-mediated effects on uterine motility.

Human FP receptors consist of 359 amino acid residues with a predicted molecular mass of 40,060Da (Abramovitz *et al*., 1994). Despite the clone of a second FP carboxyl-terminal splice variant from ovine (Pierce *et al*., 1997) and bovine corpus luteal cells (Ishii & Sakamoto, 2001), only one isoform has so far been identified in human tissues. To support pregnancy, uterine FP mRNA expression was shown to decline with gestational age in humans (Matsumoto *et al*., 1997; Brodt-Eppley & Myatt, 1999; Sooranna *et al*., 2005) and in rats (Brodt-Eppley & Myatt, 1998). At term pregnancy, the human gene for myometrial FP receptors was downregulated by 45 percent compared with the non-pregnant state (Matsumoto *et al*., 1997). This correlated with a decrease in the potency of $PGF_{2\alpha}$-induced contractions (Senior *et al*., 1992; Senior *et al*., 1993) also observed in the present study.

Although FP receptors are coupled to Gαq, the postreceptor actions of $PGF_{2\alpha}$ are still uncertain. Signalling pathways are mediated via IP_3 to enhance intracellular Ca^{2+} entry (Carrasco *et al*., 1996). However, functional studies have also suggested involvement of the Ca^{2+}-independent RhoA pathway (Woodcock *et al*., 2006) and required adenosine-5'-trisphosphate (ATP) treatment to potentiate activity (Ziganshin *et al*., 2005). Challenge using the stable thromboxane mimetic, U46619, in this study augmented myometrial contractility by 1.8-fold above $PGF_{2\alpha}$, without enhancing responsiveness to $PGF_{2\alpha}$. This indicates differences in FP and TP receptor densities and specific signalling cascades to generate uterine force.

The best characterised of the synthetic thromboxane analogues is U46619 (Coleman *et al*., 1994). As a potent and full contractile agonist, U46619 produced dynamic contractions in isolated human myometrium taken at term pregnancy. Significant

antagonism of U46619 was shown with selective TP antagonists SQ29,548 (Ogletree *et al*., 1985) and GR32191B (Lumley *et al*., 1989). These compounds both have K_i values at 13nM (Tsuboi *et al*., 2002) and respective pA_2 values of 6.5 and 7.0, which confirmed TP-mediated responses in this study. In addition, SQ29,548 did not alter $PGF_{2\alpha}$-induced activity in myometrium obtained near term (Hutchinson, 2005; Griffiths *et al*., 2006); this demonstrates the paucity of off-target $PGF_{2\alpha}$ action at TP receptors.

The expression of thromboxane A_2 synthase and splice variants TPα and TPβ has been identified in human myocytes and vasculature at term pregnancy (Swanson *et al*., 1992; Hirata *et* al., 1996; Sooranna *et al*., 2005). Although both TP isoforms exhibit identical ligand binding, each oppositely regulates adenylyl cyclase activity (Hirata *et al*., 1996). TPα receptors increase cytoplasmic cAMP, whilst TPβ receptors inhibit cAMP and stimulate PLC-IP_3 and RhoA-mediated pathways (Moore *et al*., 2002). U46619 activates PLC at lower concentrations than adenylyl cyclase (Hirata *et al*., 1991; Moore *et al*., 2002), which may account for the contractile effect of U46619 in intact myometrial strips. Even so, the relative distribution of each TP subtype has yet to be discriminated in late gestational myometrium.

In uterine myocytes, U46619 produced a transient rise in IP_3 and intracellular Ca^{2+}, consistent with TPβ receptor activation (Moore *et al*., 2002). Chronic challenge with U46619 generated two target mediators in the TP signalling cascade, rho-associated coiled coil-forming protein kinase (ROCKI; p160 ROCKI) and its isoform ROCKII (Kureishi *et al*., 1997; Moore *et al*., 2002; Moran *et al*., 2002; Moore & Lopez Bernal, 2003). These activate the small G-protein RhoA, which sensitises the uterus to Ca^{2+}. For further Ca^{2+} sensitisation, U46619 induced the cleavage of the p160 ROCKI protein to yield p130 ROCKI by caspase-3 (Moore & Lopez Bernal, 2003), associated

with enhanced uterine contractility and apoptosis (Coleman *et al*., 2001). Although the caspase-3 inhibitor Z-DEVD-FMK blocked this pathway (Moore *et al*., 2002; Moore & Lopez Bernal, 2003), it caused no appreciable effect on U46619-induced activity in the present study. This suggests that the expression of each ROCK protein type has similar efficacy on myometrial contractions.

Specific inhibitors of the RhoA cascade have been investigated as smooth muscle relaxant agents. SQ29,548 has been shown to promote increased proteolysis of pre-existing p160 ROCKI to p130 ROCKI (Moore *et al*., 2002). Even so, in myometrial strips the caspase-3 inhibitor did not reverse SQ29,548 actions, indicating that attenuated U46619 responses were blocked at cognate TP receptor sites. Use of the specific ROCKI and ROCKII inhibitor, Y-267632, also reduced smooth muscle contractility (Moran *et al*., 2002). This demonstrates ROCK protein involvement in spontaneous myometrial activity. Upregulated expression of the ROCK isoforms was shown in rat and human myometrial tissues either during the third trimester of pregnancy (Niiro *et al*., 1997; Moore *et al*., 2000) or after labour-onset (Friel *et al*., 2005). Therefore, ROCK has the potential to regulate uterine contractions in a Ca^{2+}-independent manner during late gestation. In addition, U46619 stimulates mitogenesis and hypertrophic growth of uterine smooth muscle cells via mitogen-activated protein kinase (MAPK) cascades (Miggin & Kinsella, 2001). Accordingly, thromboxane may also influence muscle tone and uterine remodelling during pregnancy.

In summary, this study demonstrates the heterogeneous population of functional EP, FP and TP receptors in lower segment myometrium from term pregnant, non-labouring donors. To maintain quiescence, EP agonists induced predominant utero-relaxant effects; this was primarily mediated by EP_2 rather than EP_4 receptors. Despite the multitude of isoforms, some excitation was elicited via EP_3 receptors, whilst

responsiveness to the EP_1 agonist ONO-D1-004 was negligible. Even so, functional EP_3 and EP_1 receptors appeared to represent a minor component of the overall receptor complement in this tissue. In contrast, activated FP receptors augmented myometrial activity with contractions further potentiated by the TP mimetic U46619. As EP, FP and TP receptors also contribute to hyperplasia and hypertrophy, it is likely that these receptors facilitate uterine distension and maintain myometrial tone throughout the majority of pregnancy.

Chapter 5: Labour

Functional prostanoid receptors in isolated myometrium during parturition

5.1 Introduction

Successful parturition requires powerful and coordinated uterine contractions, in conjunction with cervical ripening and dilation. This is synchronised by changes in local maternal, foetal and mechanical factors (Keelan *et al*., 2003; Hertelendy & Zakar, 2004). Even so, due to its complexity, the physiological and molecular mechanisms underlying the transition from uterine quiescence to activation are not fully understood.

Compelling evidence has shown that PGs, particularly those produced within the intrauterine tissues, are central in the initiation and progression of labour. In late pregnancy, enhanced PGE_2 and $PGF_{2\alpha}$ biosynthesis by intrauterine tissues precedes labour-onset (Gibb, 1998), whilst clinical applications of PGE analogues are widely used for labour induction, cervical effacement and to maintain patency of the ductus arteriosus. Moreover, in terms of tocolysis, many PG synthesis inhibitors can prolong gestation via temporary suppression of myometrial contractility (Vermillion & Landen, 2001). Even so, the functional dynamics of myometrial PG receptors during parturition have yet to be elucidated.

To better understand parturient uterine function, myometrial responsiveness to selective EP analogues, $PGF_{2\alpha}$ and U46619 were investigated in this study in lower segment myometrium obtained from labouring donors. After the onset of regular, painful *in vivo* uterine contractions, labour was categorised at cervical dilations of 0-2cm, 3-8.5cm and 9-10cm for early, mid and late stages respectively. Myometrial

responses to PGs were examined to improve the efficacy of available tocolytics for labour-associated disorders.

Chapter 5.2: Results

5.3 Myogenic activity at term pregnancy and labour

Spontaneous contractions varied markedly between donor tissues taken at different stages of pregnancy and labour (Figure 5.1). In immersed isolated myometrium, the greatest activity was exhibited by samples taken at term (39.3 ± 0.4 weeks gestation) from pregnant non-labouring donors. By the early stages of parturition, myogenicity was variable and decreased by 14.9 percent ($F(1, 18) = 1.34$; ns). This group included patients labouring for 1.5 to 8 hours, with cervical dilation at 0-2cm; two donors presented placental praevia. Myometrial contractions substantially declined by 2.6 and 3.3-fold in tissues collected during the mid ($F(1, 18) = 47.33$; $p<0.001$) and late ($F(1, 18) = 96.41$; $p<0.001$) stages of labour. These groups were determined by cervical dilation at 3-8.5cm and 9-10cm respectively, correlating with the reduction in both the frequency and amplitude of *in vitro* contractions. Of the myometrial strips, 59 percent were devoid of rhythmic undulating myogenic activity late after labour-onset. This may have related to the relatively prolonged duration of labour in fully dilated donors. Even so, labour-associated pharmacological compounds, such as entonox and syntocinon, did not influence *in vitro* spontaneous myometrial contractions (Table 5.1).

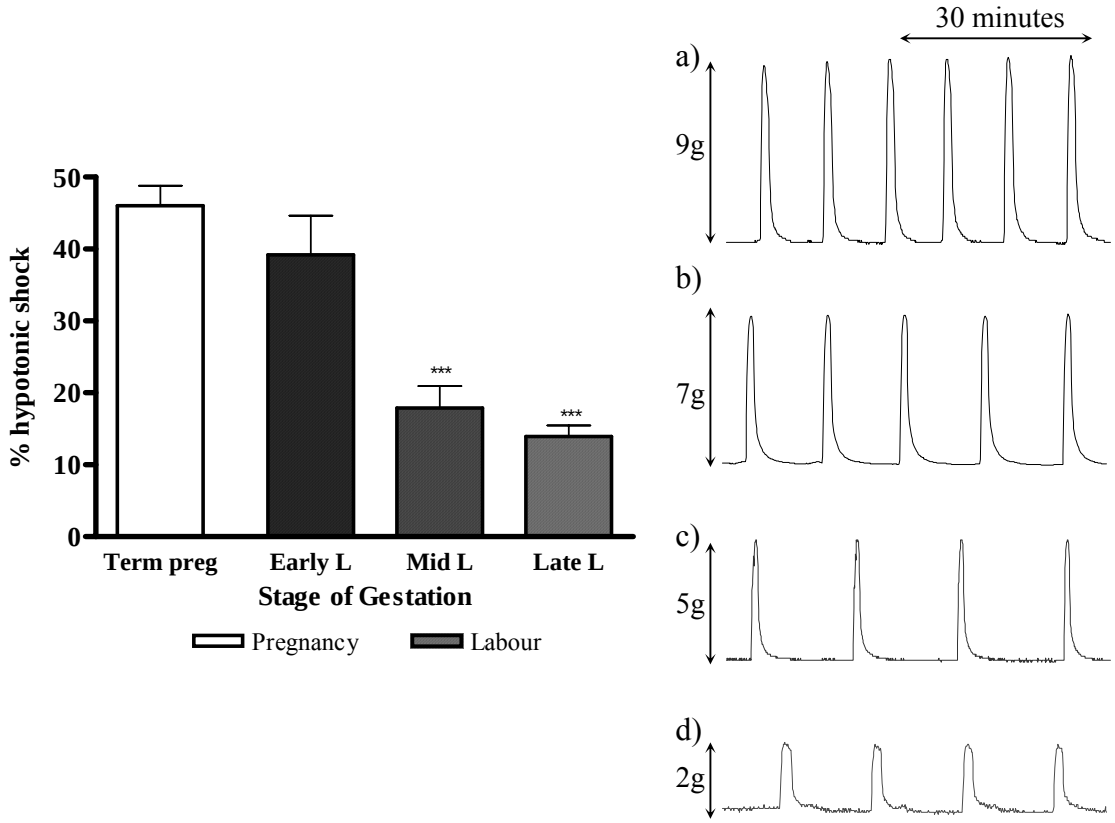


Figure 5.1: Spontaneous activity of lower segment myometrial strips obtained from term pregnant donors, not in labour (n=10) and in early (n=9), mid (n=9) and late (n=9) stages of labour. Labour was defined as regular uterine contractions with early, mid and late stages categorised at 0-2cm, 3-8.5cm and ≥9cm cervical dilation respectively. Myometrium was immersed, equilibrated for up to 2.5 hours and myogenic activity was measured as 30 minutes area under the curve and expressed as a percentage of 30 minutes hypotonic shock, regardless of the presence of phasic activity. Results were displayed as arithmetic means ± S.E. and significant differences were identified using univariate ANOVA with Bonferroni's *post-hoc* adjustment: ***$p<0.001$ reduction in spontaneous activity relative to tissues taken at term pregnancy and early labour.

Representative traces show typical activity of isolated myometrium equilibrated in immersion baths from donors at term pregnancy a) not in labour and after labour-onset at cervical dilations of b) 2cm, c) 5-6cm and d) 9cm.

Table 5.1: Spontaneous contractions of donated lower segment myometrium obtained during labour. Early, mid and late stages of labour were defined as regular *in vivo* contractions at cervical dilations of 0-2cm, 3-8.5cm and 9-10cm respectively. In addition to epidural anaesthetics, labouring women had been given entonox or pethidine for analgesic relief, Phenergan or Maxolon to prevent nausea and vomiting and/ or syntocinon to induce or accelerate labour before donation of tissues. These pharmaceutical agents did not influence myogenic activity established using the immersion technique, measured for 30 minutes after the equilibration period as area under the contraction curve.

	Entonox/ Pethidine	**n**	**Phenergan/ Maxolon**	**n**	**Syntocinon**	**n**
Early labour	1143.4 ± 172.4	**4**	1170.1 ± 207.7^{+}	**1**	n/a	**0**
Mid labour	569.4 ± 92.9	**5**	417.2 ± 80.3	**6**	614.5 ± 107.2	**4**
Late labour	174.0 ± 20.7	**5**	157.7 ±17.3	**2**	128.5 ± 12.5^{+}	**1**

$^{+}$Mean activity of 6-8 myometrial strips from the same biopsy

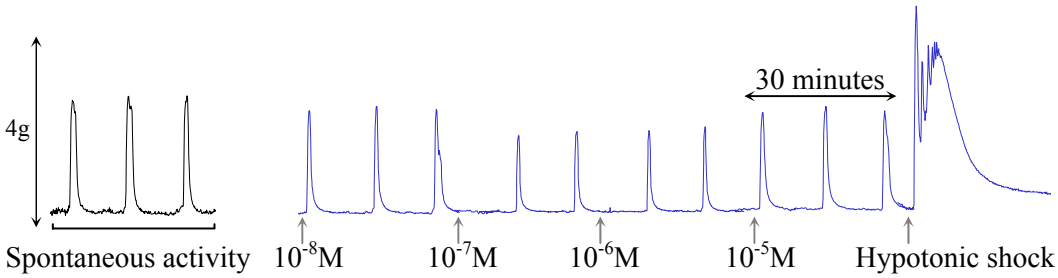


Figure 5.2: Trace showing the concentration-effect of PGE_2 relative to spontaneous activity of myometrium obtained at 32 weeks of gestation from a fully dilated donor.

5.4 Myometrial EP receptors after labour-onset

In lower segment myometrium taken at term early labour, PGE_2 caused a biphasic response, similar to that observed before parturition. PGE_2 (10^{-8}M to 10^{-6}M) initially attenuated the amplitude and frequency of myogenic contractions by 2.1-fold ($F(1, 60) = 15.98$; $p<0.001$), with tissue excitation subsequently evoked at 10^{-5}M (Figure 5.3a). This corresponded to the profile of myometrial strips taken preterm, at 32 weeks of gestation from a fully dilated donor (Figure 5.2). However, by mid and late stage term labour, PGE_2 (10^{-8}M to 10^{-5}M) fully inhibited myogenic activity in a monophasic concentration-related manner (Figures 5.3b & 5.3c). This was achieved by PGE_2 suppressing contractile amplitude until the threshold was no longer reached. Compared to spontaneous activity, the main effects of PGE_2 were significant for both mid ($F(1, 72) = 7.03$; $p<0.01$) and late ($F(1, 60) = 4.31$; $p<0.05$) stages of term parturition.

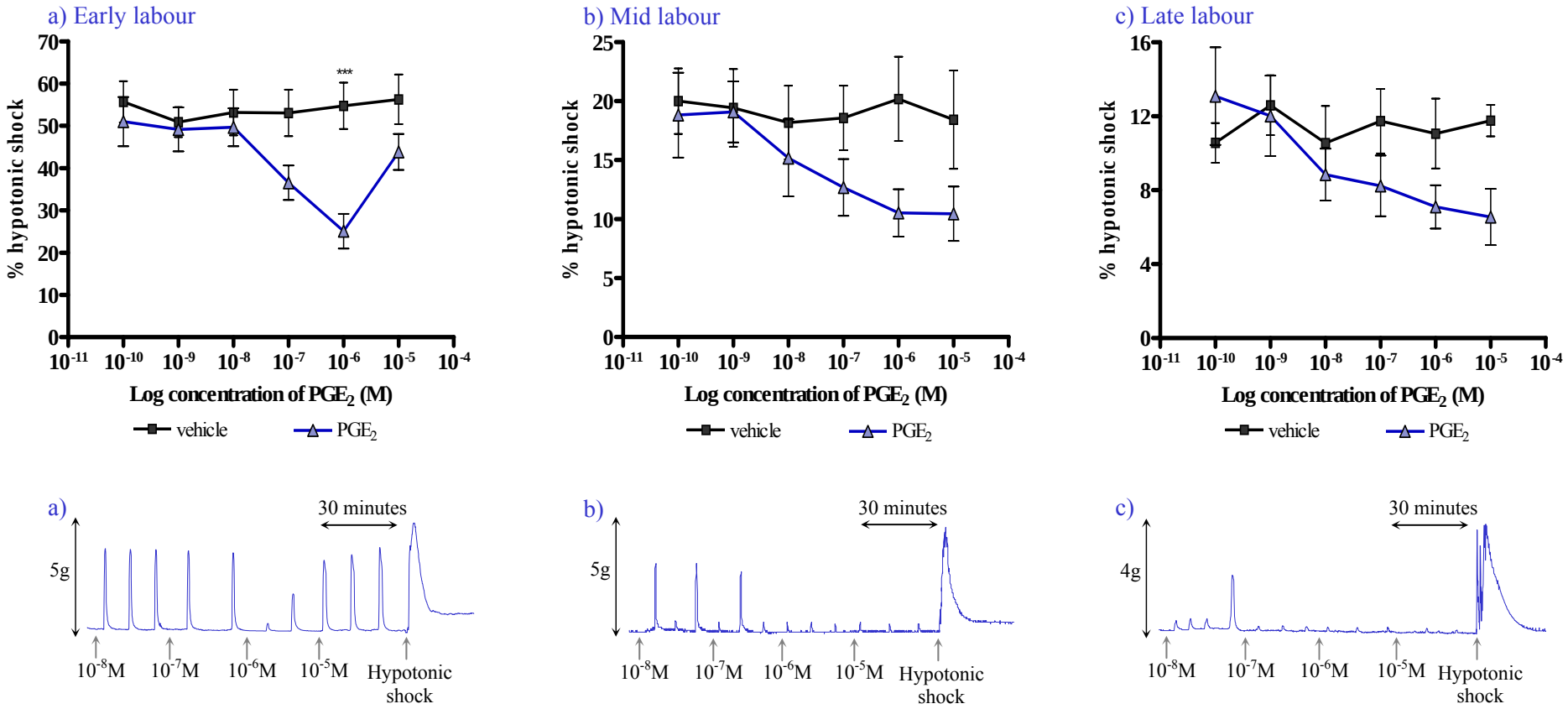


Figure 5.3: Vehicle and concentration-effect curves for PGE_2 in lower myometrium obtained from term pregnant donors in a) early (n=6), b) mid (n=7) and c) late (n=6) stages of labour. Labour was defined as *in vivo* contractions accompanied by cervical dilation at 0-2cm, 3-8.5cm and 9-10cm respectively. Vehicle and PGE_2 were added to individual organ baths at 30-minute intervals and excitatory responses to PGE_2 were only evoked at 10^{-5}M during early labour. Results are expressed as arithmetic means ± S.E. and univariate analysis using Bonferroni's *post-hoc* test showed significant differences ***$p<0.001$ for PGE_2 compared to vehicle.

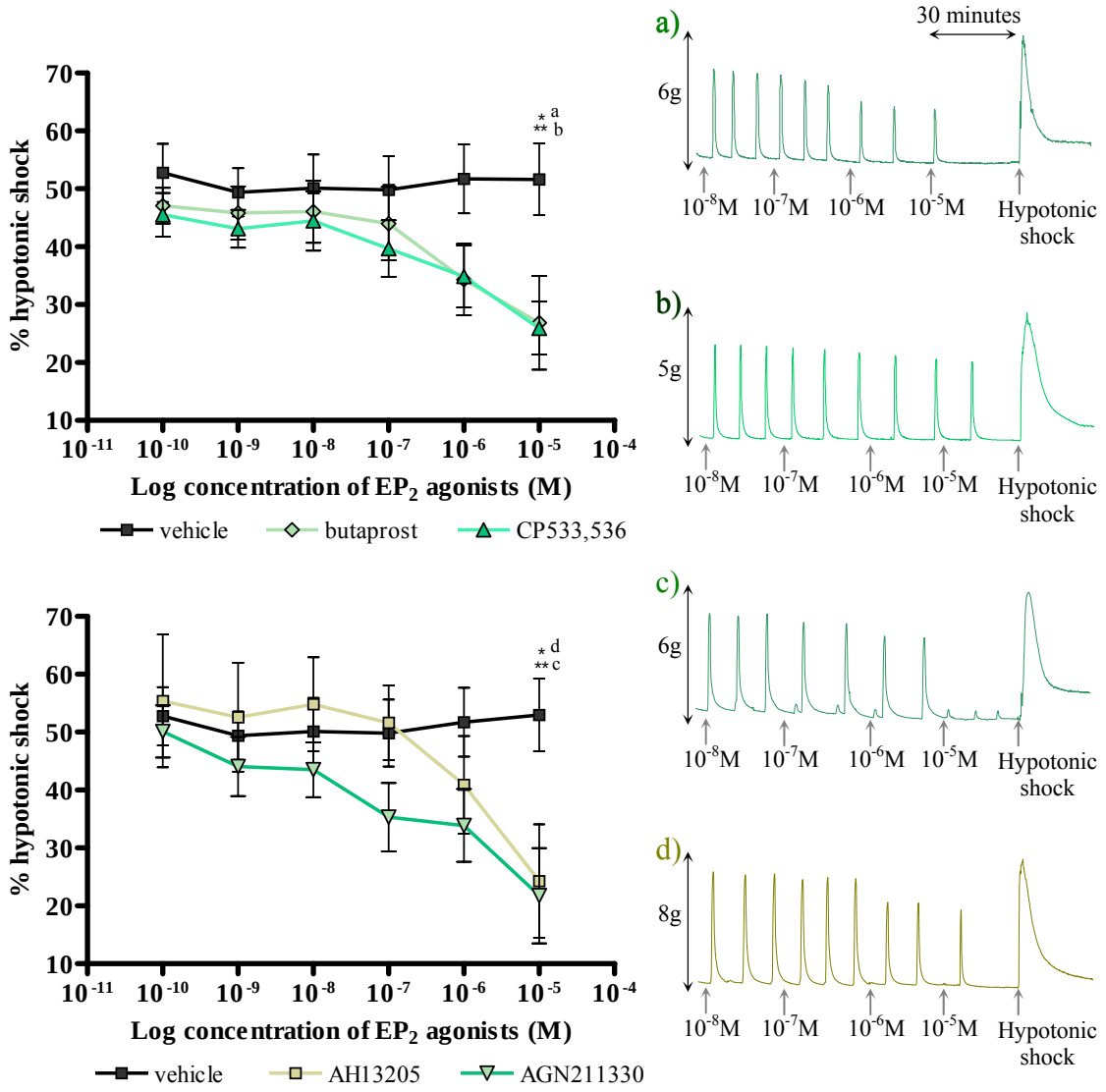


Figure 5.4: Vehicle, concentration-effect curves and representative traces for the EP_2 agonists a) butaprost, b) CP533,536, c) AGN211330 and d) AH13205 in immersed myometrial strips taken at term, early labour (n=4-6). Early labour was defined as painful contractions accompanied by cervical dilation between 0-2cm. Myometrial responses to EP_2 agonists were measured over 30-minute intervals as area under the curve, expressed as percentage hypotonic shock and presented as arithmetic means ± S.E. Statistical significance was determined using multivariate ANOVA with Bonferroni's *post-hoc* adjustment; *p<0.05 for butaprost and AH13205; **p<0.01 for CP533,536 and AGN211330 compared with vehicle treatments.

Table 5.2: Mean pIC_{50} values (M) for EP_2 agonist concentration-effect curves (10^{-10}M to 10^{-5}M) in immersed myometrium from term pregnant donors after labour-onset (0-2cm cervical dilation; n=4-6). Results are expressed as arithmetic means ± S.E.

EP_2 agonists	**butaprost**	**CP533,536**	**AGN211330**	**AH13205**
pIC_{50}	6.20 ± 0.30	6.02 ± 0.55	5.81 ± 0.29	6.03 ± 0.19

5.5 Inhibitory effects of EP_2 agonists on myogenic activity

The steady and active nature of phasic myometrial contractions in tissues obtained early after labour-onset enabled inhibitory agonists to be assayed at this stage of parturition. Using immersed myometrial strips butaprost, CP533,536, AH13205 and AGN211330 attenuated myogenic activity in a linear concentration-dependent manner (Figure 5.4). This was achieved primarily between 10^{-7}M and 10^{-5}M due a reduction in both the amplitude and frequency of contractions. The rank order of potency was similar between EP_2 agonists with AGN211330 having least efficacy (Table 5.2). Inhibitory responses were due to the main effects of butaprost ($F(1, 48) = 9.35$; $p<0.01$), CP533,536 ($F(1, 54) = 16.75$; $p<0.001$), AH13205 ($F(1, 36) = 6.23$ $p<0.05$) and AGN211330 ($F(1, 36) = 15.52$; $p<0.001$).

5.6 EP_4 agonist effects on myogenic activity

In isolated myometrium obtained during early labour, the steady decline in myogenic activity for the EP_4 agonist AGN201734 (10^{-8}M to 10^{-5}M) did not reach statistical significance ($F(1, 48) = 2.85$; ns; Figure 5.5). In comparison, the effects of L-902688 were more variable. Contractions were predominantly maintained until 10^{-5}M, whereby L-902688 attenuated activity from 55.5 ± 4.0 to 34.6 ± 18.6 hypotonic shock ($F(1, 36) = 0.37$; ns); this corresponded to its low potency value (Table 5.3). Even so, neither of the EP_4 agonists altered myometrial tonus or induced excitatory responses.

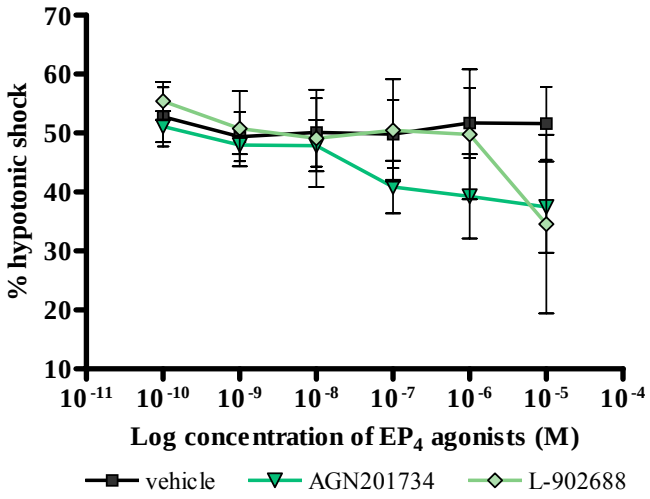


Figure 5.5: Vehicle and concentration-effect curves for EP_4 agonists AGN201734 and L-902688 in isolated myometrium obtained from term pregnant, early labouring donors (n=4-5). The contractions associated with labour were accompanied by cervical dilation between 0-2cm. Vehicle and EP_4 agonists (10^{-10}M to 10^{-5}M) were added to immersed myometrial strips in a cumulative manner at 30-minute intervals. Results are expressed as arithmetic means ± S.E.

Table 5.3: Mean pIC_{50} values (M) determined for the EP_4 agonists AGN201734 and L-902688 (10^{-10}M to 10^{-5}M) using myometrium obtained from donors during early labour at emergency Caesarean section (n=4-5). Concentration-effect curves were performed using the immersion technique and results are expressed as arithmetic means ± S.E.

EP_4 agonists	**AGN201734**	**L-902688**
pIC_{50}	7.53 ± 1.04	5.69 ± 0.86

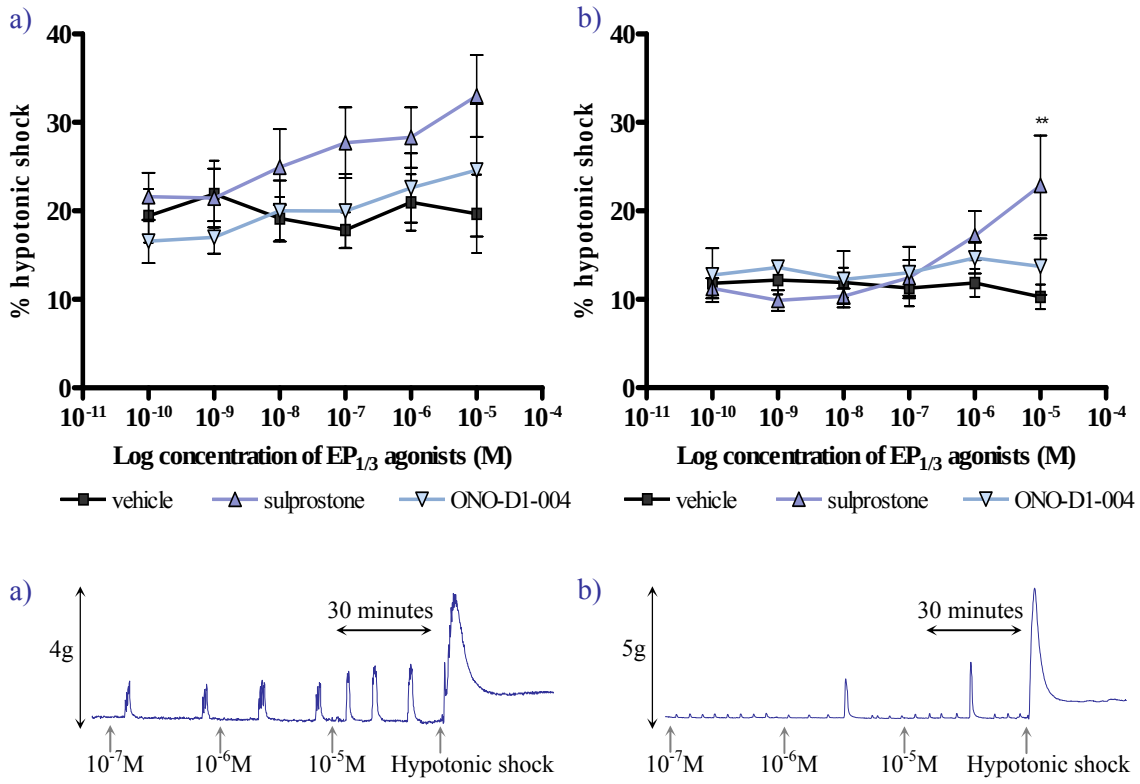


Figure 5.6: Vehicle and concentration-effect curves for the $EP_{1/3}$ agonists ONO-D1-004 and sulprostone in myometrium obtained from donors in a) mid and b) late stages of term parturition (n=4-7). *In vivo* contractions accompanied by cervical dilation at 3-8.5cm and 9-10cm respectively were used to distinguish the stages of labour. Immersed myometrial responses to $EP_{1/3}$ agonists were measured over 30-minute periods as area under the curve and expressed as a percentage hypotonic shock. Results are arithmetic means ± S.E. and statistical significance was determined using two-way ANOVA with Bonferroni's *post-hoc* test; **p<0.01 for sulprostone compared with vehicle.

Typical traces show myometrial tissue in organ baths, contracting in response to sulprostone (10^{-7}M to 10^{-5}M). Myometrium was obtained from donors after the onset of a) mid and b) late stages of labour.

5.7 Excitatory EP agonists on myogenic activity

To ensure that responses were apparent, contractile agonists were primarily added to tissues with low myogenic activity. Although the EP_1 agonist ONO-D1-004 did not evoke excitation either at mid ($F(1, 48) = 0.18$; ns) or late ($F(1, 42) = 2.15$; ns) stages of term labour, the $EP_{3/1}$ agonist sulprostone produced a monophasic increase in activity (Figure 5.6). Despite a lack of significance compared with vehicle controls ($F(5, 66) = 0.70$; ns), sulprostone exhibited a main treatment effect at mid labour ($F(1, 66) = 9.36$; $p<0.01$). This was better displayed during late labour with a 2-fold increase in activity at 10^{-5}M relative to intrinsic contractions ($F(5, 66) = 8.70$; $p<0.01$). Using superfused tissues, the excitatory responses to bolus doses of sulprostone were observed throughout parturition; this included tissues taken early after labour-onset (Figure 5.9.1).

5.8 Contractile effects of $PGF_{2\alpha}$

$PGF_{2\alpha}$ produced concentration-dependent excitation in uterine muscle obtained during early, mid and late stages of parturition (Figures 5.7 & 5.9.2). In immersed myometrial strips, the increase in activity was nearly 60 percent higher in tissues taken during mid-term labour rather than late labour at 10^{-5}M ($p<0.001$). Whilst during mid labour $PGF_{2\alpha}$ augmented myometrial contractions in a monophasic manner, by late labour, responsiveness was attenuated and was sigmoidal shaped ($F(1, 50) = 22.18$; $p<0.001$). The excitation evoked by $PGF_{2\alpha}$ was more pronounced in superfused tissues with the greatest phasic activity reaching 48.7 ± 4.4 percent hypotonic shock early after labour-onset. In a late labour preterm myometrial sample, the $PGF_{2\alpha}$-induced contractile response increased to a similar extent from 25.8 to 46.1 percent hypotonic shock between 10^{-7}M and 10^{-5}M (Figure 5.8).

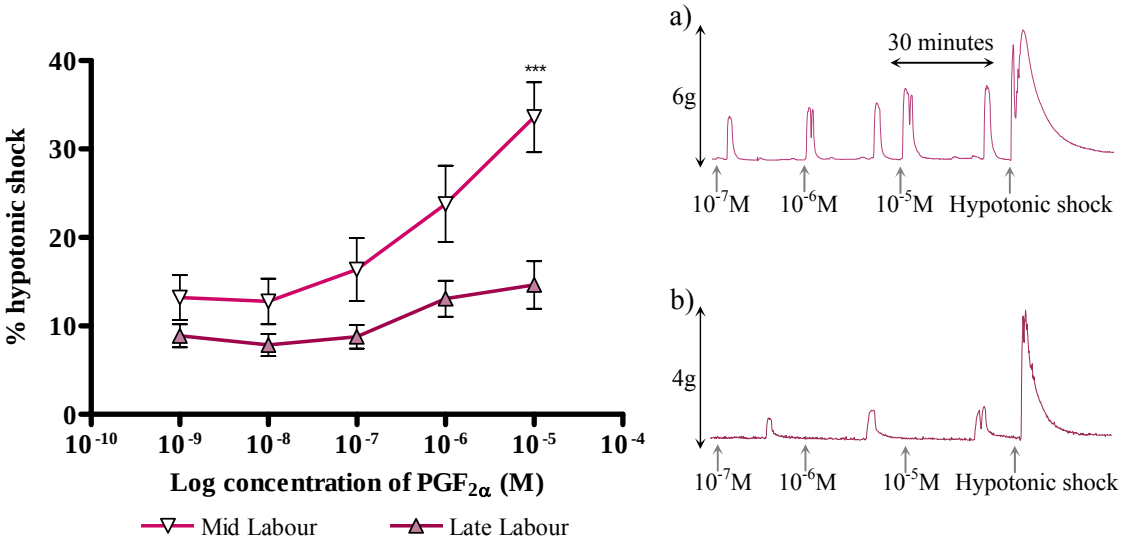


Figure 5.7: Concentration-effect curves and representative traces for $PGF_{2\alpha}$ in myometrium obtained from term pregnant donors at a) mid and b) late stages of labour (n=6-7). Labour was defined as *in vivo* contractions with respective stages determined at 3-8.5cm and 9-10cm cervical dilation. $PGF_{2\alpha}$ was added to organ baths in a cumulative manner at 30-minute intervals with responses were measured as area under the curve relative to the contraction achieved by hypotonic shock. Results are expressed as means ± S.E. and statistical analysis was performed using multivariate ANOVA with Bonferroni's *post-hoc* test; $p<0.001$ contractions in response to $PGF_{2\alpha}$ at 10^{-5}M for mid compared to late labour.

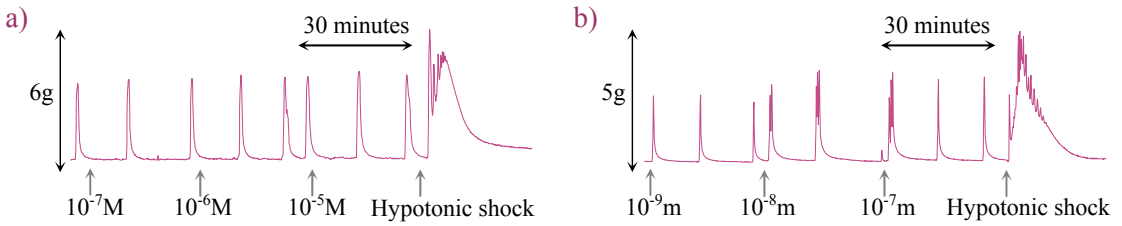


Figure 5.8: Representative traces for $PGF_{2\alpha}$ in myometrium obtained at 32 weeks gestation from a fully dilated donor. Responses to $PGF_{2\alpha}$ were similar regardless of agonist addition to a) immersed and b) superfused myometrial strips.

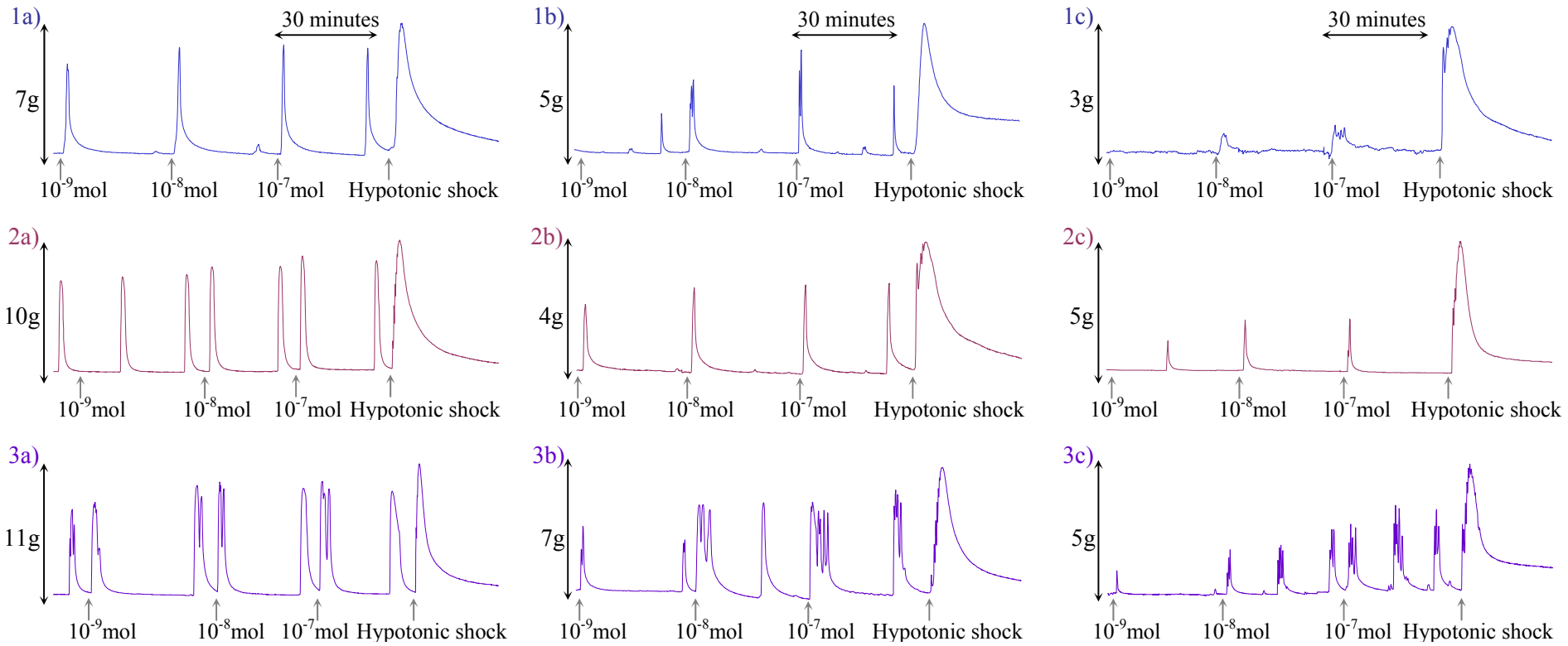


Figure 5.9: Representative traces showing the contractile effect of increasing bolus doses of 1) sulprostone, 2) $PGF_{2\alpha}$ and 3) U46619 superfused on lower segment myometrium obtained during parturition at emergency Caesarean section. Labour was defined as *in utero* contractions with a) early, b) mid and c) late stages determined by cervical dilations at 0-2cm, 3-8.5cm and 9-10cm respectively.

5.9 Maintained uterotonic effects of U46619

The thromboxane mimetic U46619 reliably produced a contractile phenotype in myometrial strips obtained during parturition. U46619 induced substantial tissue excitation and particularly enhanced contractile frequency. In immersed tissue strips during mid stage labour, activity was increased from 14.7 ± 2.6 to 89.7 ± 13.0 percent hypotonic shock. These contractile responses were substantially higher than in tissues taken during late term labour ($p<0.001$), despite the 13.6 percent attenuation of activity at 10^{-5}M ($p<0.05$; Figure 5.10). Concentration-effects of U46619 reached 42.7 ± 6.6 percent hypotonic shock in myometrium obtained during late labour. Even so, responses to U46619 were greatly augmented in relation to the low amplitude and frequency of spontaneous contractions ($F(1, 55) = 23.36$; $p<0.001$). U46619-induced excitation was also maintained throughout parturition in superfused tissues (Figure 5.9.3) as well as in the late labour sample obtained at 32 weeks of pregnancy (Figure 5.11). In addition, the caspase-3 inhibitor had no effect on the augmented contractile frequency and amplitude of U46619 concentration-effect curves at mid and late stages of term parturition (Figures 5.12 & A7).

5.10 TP antagonism by SQ29,548 and GR32191B

Neither TP antagonists SQ29,548 (10^{-6}M) or GR32191B (10^{-6}M) affected myogenic activity. However, both antagonists suppressed U46619-induced contractions in myometrium taken at mid and late stages of term labour (Table 5.4).

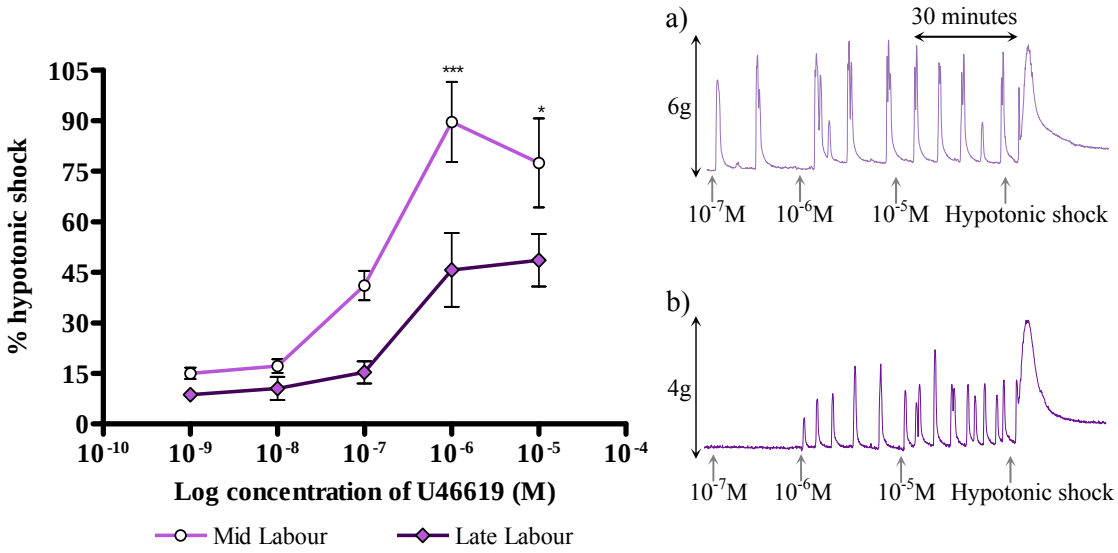


Figure 5.10: Concentration-effect curves and representative traces for U46619 in myometrial strips from donors at a) mid and b) late stages of labour (n=6-7). Mid and late labour was categorised at 3-8.5cm and 9-10cm cervical dilation respectively. In all immersed myometrial strips, addition of the stable thromboxane mimetic U46619 provoked contractile responses relative to spontaneous activity. This was measured as area under the curve relative to the contraction induced by hypotonic shock. Results are expressed as means ± S.E. and statistics were performed using two-way ANOVA with Bonferroni's adjustment; *p<0.05; ***p<0.001 for U46619-induced contractions in samples taken at mid compared with late labour.

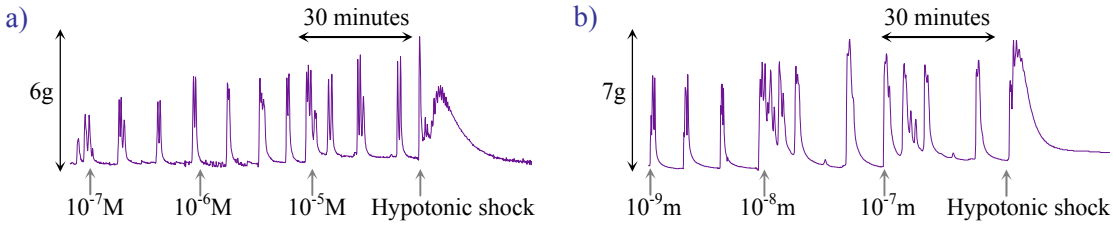


Figure 5.11: Representative traces showing the concentration-effect and dose-response of U46619 in myometrial strips set-up using a) immersion and b) superfusion techniques. The muscle biopsy was obtained from a fully dilated donor at 32 weeks of gestation.

Table 5.4: Mean pEC_{50} values (M) and maximal excitatory responses (E_m) for U46619 (10^{-9}M to 10^{-5}M) in the absence and presence of either SQ29,548 (10^{-6}M) or GR32191B (10^{-6}M) in myometrium from term pregnant donors in mid (n=6-8) and late stages of labour (n=5-8). Maximal responses for excitation are expressed as percentage hypotonic shock. Data were analysed using univariate ANOVA mixed model with Bonferroni's *post-hoc* test and results were expressed as arithmetic means ± S.E. Excitatory responses to U46619 were significantly different in myometrium taken at mid labour compared with [a]late gestation ($p<0.001$). Responses to U46619 were also attenuated by action of SQ29,548 in [c]mid labour ($p<0.001$) and [d]late labour ($p<0.001$) with similar antagonism by GR32191B at [e]mid and [f]late labour

	U46619 alone		+ SQ29,548		+ GR32191B	
	pEC_{50}	**E_m**	**pEC_{50}**	**E_m**	**pEC_{50}**	**E_m**
Mid labour	7.1 ± 0.2	106.2 ± 9.6	6.2 ± 0.5	28.7 ± 9.9[c]	6.7 ± 0.3	39.3 ± 14.1[e]
Late labour	6.8 ± 0.1	56.9 ± 9.7[a]	6.1 ± 0.3	21.0 ± 7.6[d]	6.2 ± 0.6	20.1 ± 0.7[f]

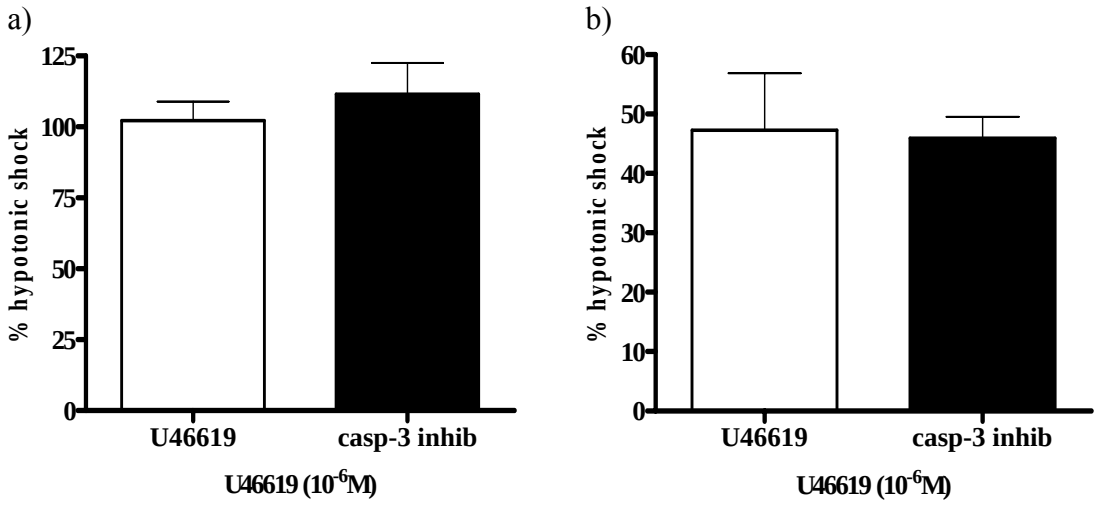


Figure 5.12: Mean U46619-induced excitation in the presence or absence of the caspase-3 inhibitor (10^{-6}M) on myometrial strips obtained at a) mid (n=6-8) and b) late (n=5-8) stages of labour. The caspase-3 inhibitor had no effect on contractions potentiated by U46619. Mid and late labour were defined at 3-8.5cm and 9-10cm cervical dilation respectively. Results are expressed as means ± S.E.

Chapter 5.11: Discussion

During parturition, regular and forceful uterine muscle contractions develop in a caudal direction from the fundus towards the cervix. These were exhibited as well-defined spontaneous contractions in immersed (Hutchinson, 2005) and superfused myometrial tissues obtained early after labour-onset. However, as labour progressed into mid and late stages, the frequency and amplitude of phasic contractions in lower segment myometrial strips declined by 2.6 and 3.3-fold respectively. This would likely correspond to extensive *in utero* collagen tissue remodelling, which facilitates cervical effacement and dilation for delivery of the foetus (Leppert, 1995).

To soften and retract the cervix prior to labour-onset, metalloproteinase inhibitors decrease the stability of collagen, whilst the water, glycosaminoglycan and noncollagenic protein content of tissues increase (Osmers *et al*., 1993). Cervical dilation and myometrial remodelling are advanced by the concurrent apoptosis of smooth muscle cells (Leong *et al*., 2008). Since muscle bundles transmit action potentials within the uterus, the changes in cellular composition would attenuate myometrial excitability (Wray *et al*., 2001; Blanks *et al*., 2007). Moreover, the presenting foetus exerts a constant tension against the cervix and begins stretching and passively dilating the lumen. This may further contribute to the reduction in myogenic activity observed with labour progression in this study.

The attenuated myogenic force in lower segment tissues during labour was consistent with the topographical changes in contractile-associated protein expression and Ca^{2+} transients (Astle *et al*., 2005; Riley *et al*., 2005). For the uterus to act in synchrony, specialised pacemaker cells transduce electrical signals via gap junctions between myocytes (Kilarski *et al*., 2000; Duquette *et al*., 2005). These gap junction transcripts

become more pronounced in upper rather than lower segment myometrium during labour (Sparey *et al*., 1999), facilitating the vigorous spontaneous contractions of isolated fundus myometrium towards the cervix (Griffiths *et al*., 2006). At parturition, less sensitive isoforms of Ca^{2+}-activated potassium channels are expressed in isolated lower segment tissues (Curley *et al*., 2004) whilst Ca^{2+}-ATPase activity is reduced with uterine dystocia (Zyrianov *et al*., 2003). As a result, contractile constituents may have been depleted in prolonged labours, contributing to the low intrinsic myometrial activity.

In this study, emergency Caesarean sections were performed due to labour-associated disorders. Indications included foetal distress, breech presentation, previous Caesarean sections, labour dystocia, placental abruption, maternal pregnancy-induced hypertension, epilepsy and asthma. Due to the limited sample numbers, no exclusion criteria were applied. Pharmacological medications provided for analgesia, nausea-relief and to augment labour did not influence *in vitro* myogenic activity. In addition, similar tensile strengths of uterine muscle were demonstrated with intact and previously scarred tissues (Buhimschi *et al*., 2006). Even so, it was recognised that the tissue properties in this study may not have resembled uterine muscle obtained from normal, uncomplicated labours.

Despite a high incidence of premature deliveries (Lumley, 2003), only one late labour myometrial biopsy was donated at 32 weeks of gestation. The spontaneous contractions by immersed preterm myometrial strips were twice as active as biopsies harvested from fully dilated donors at term. This suggests differences in preterm and term parturition cascades.

Although functional progesterone withdrawal is a prerequisite for labour, its mechanisms are unclear. As systemic progesterone does not decline before labour-

onset in women (Boroditsky *et al*., 1978), events appear to be mediated by biochemical changes within the uterus. These include the upregulation of cognate progesterone receptor (PR)-A, which repress PR-B function (Pieber *et al*., 2001; Mesiano *et al*., 2002), association of PR with nuclear factor kappa B (NF-κB) and a reduction in steroid receptor co-activators (Allport *et al*., 2001). Moreover, the progesterone block is positively correlated to rising oestrogen receptor-α (ERα) mRNA (Winkler *et al*., 2002). In the presence of ERα, oestrogen treatments have been shown to increase connexin-43 transcripts (Garfield *et al*., 1980; Kilarski *et al*., 2000; Di *et al*., 2001; Grummer *et al*., 2004), COX-2 and oxytocin receptors (Mesiano, 2001; Mesiano *et al*., 2002), which are required for transient myometrial contractions. Thereby, the close apposition of placental oestrogen to the uterine fundus may attribute to forceful contractions, which dissipate towards the cervix for parturition to succeed. In addition, the heightened spontaneous activity observed in this study implicates sustained oestrogenic effects during preterm relative to term deliveries. Nevertheless, rather than exogenous steroids, uterotonins are crucial mediators in orchestrating and driving labour contractions (Challis *et al*., 2000).

Profound increases in PG synthesis and metabolism by foetal membranes and myometrium precede the initiation and progression of labour (Gibb, 1998; Durn *et al*., 2010). The biosynthetic capacity for PGs, especially of amnion-derived PGE_2, is elevated prior to term and preterm labour in women (Gibb, 1998; Olson *et* al., 2003). This corresponds to the increased activities of PLA_2 type-IIA and COX-2 in amniotic and lower myometrial tissues that catalyse PG output (Slater *et al*., 1999; Slater *et al*., 2004; Sooranna *et al*., 2006). Concurrently, the conversion of active PGs into inactive 15-keto metabolites by chorionic prostaglandin dehydrogenase (PGDH) diminishes (Cheung *et al*., 1992). This would facilitate passage of PGE_2 from the amnion to the

underlying maternal decidua and myometrium for activation of spontaneous term and preterm labour (Giannoulias *et al*., 2005). Moreover, at labour-onset, microsomal synthase mPGES-1 expression is upregulated within lower rather than upper segment myometrium and vasculature (Sooranna *et al*., 2006). The PGE_2-induced vasodilatation and tissue remodelling (Kimura *et al*., 1995) perhaps reflected the dark red pigmentation observed in tissues obtained during late labour.

PGE_2 is associated with both inhibitory and excitatory mechanisms (Senior *et al*., 1993). This was demonstrated using immersion and superfusion techniques in this study. Using myometrial strips obtained from labouring donors, PGE_2 attenuated contractions in a concentration and dose-dependent manner. Relative tissue excitation was displayed at 10^{-5}M in early term and late preterm labour samples. Despite limited functional data, this biphasic response corresponded to heterogeneous $EP_{1\text{-}4}$ receptor subtypes within plasma and nuclear membranes of myometrial cells (Coleman *et al*., 1994; Bhattacharya *et al*., 1999; Leonhardt *et al*., 2003). However, in the latter stages of term parturition, full cessation of myogenic activity was exhibited. These results substantiate a previous *in vitro* study using myometrium obtained during active labour (Wikland *et al*., 1984). Excitatory responses to PGE_2 were solely at the fundus whilst lower myometrial activity was suppressed; this indicates a regional change in the complement of functional EP receptor subtypes.

Myometrial responses to specific EP agonists during human labour were not previously examined. In this study, inhibitory compounds were tested in tissues harvested early after labour-onset, corresponding to the stability of active myogenic contractions. To determine EP_2 receptor function, concentration-effect curves for butaprost (Gardiner, 1986), CP533,536 (Li *et al*., 2003; Paralkar *et al*., 2003), AH13205 (Coleman *et al*., 1994) and AGN211330 (Belley *et al*., 2005) were

constructed. In accord with myometrial biopsies from non-labouring donors, each EP_2 agonist attenuated spontaneous activity in a monophasic concentration-dependent manner (10^{-7}M to 10^{-5}M). Despite marginal differences between EP_2-mediated inhibitions, the rank order of potency was butaprost > CP533,536 = AH-13205 > AGN211330.

EP_2 receptors have particularly been implicated in labour-associated events due to altered temporal and regional myometrial expression. EP_2 receptors decline towards term gestation (Brodt-Eppley & Myatt, 1999; Leonhardt *et al*., 2003), although remain unaltered (Brodt-Eppley & Myatt, 1999; Astle *et al*., 2005; Sooranna *et al*., 2005) or increase during parturition (Grigsby *et al*., 2006). With advancing gestation with or without labour, the expression of total EP_2 mRNA and nuclear EP receptors were more intense in lower compared with upper myometrial segments (Astle *et al*., 2005; Grigsby *et al*., 2006). This corresponds to consistent EP_2 responses in myometrial strips obtained before and after labour-onset.

Although EP_4 mRNA and protein expression are abundant in lower segment myometrial tissue (Leonhardt *et al*., 2003; Astle *et al*., 2005; Grigsby *et al*., 2006), EP_4 agonist AGN201734 (Elworthy *et al*., 2004) and L-902688 (Billot *et al*., 2003) effects were moderate. Whilst AGN201734 produced a gradual decline in spontaneous activity (10^{-8}M to 10^{-5}M), L-902688 solely attenuated contractions at 10^{-5}M. As these ligands have high affinity for EP_4 receptors (Elworthy *et al*., 2004; Billot *et al*., 2003), the low responses may relate to poor receptor-effector coupling in isolated human myometrium. Nevertheless, myometrial EP_4 mRNA and protein expression did not change regardless of gestational age, labour and regional location (Leonhardt *et al*., 2003; Astle *et al*., 2005; Grigsby *et al*., 2006). This suggests that EP_4 may mediate PGE_2-induced inflammatory responses (Slater *et al*., 2006) or

cervical ripening (Schmitz *et al*., 2001) rather than the functional suppression of myometrial activity.

Using myometrial strips from mid and late stages of labour, the novel EP_1 agonist ONO-D1-004 (Oka *et al*., 2003) did not influence myogenicity compared with time-matched controls. This was surprising as the expression of EP_1 subtypes was detected in upper and lower segments at term pregnancy (Astle *et al*., 2005; Grigsby *et al*., 2006) and increased further in lower (Astle *et al*., 2005) or both lower and upper myometrial segments during labour (Grigsby *et al*., 2006). However, as ONO-D1-004 has poor binding affinity and low potency values (Kiriyama *et al*., 1997; Oka *et al*., 2003), alternative agonists such as 17-phenyl PGE_2 are required to elucidate EP_1 function.

By contrast, the $EP_{3/1}$ agonist sulprostone (Schaaf *et al*., 1981) evoked myogenic contractions in both immersed (10^{-6}M to 10^{-5}M) and superfused (10^{-8}mol to 10^{-7}mol) tissue strips. Contractile responses were displayed at each stage of parturition. This was associated with the maintained expression of EP_3 mRNA before and after labour-onset. For uterine emptying, EP_3 receptors were predominantly expressed in the fundus compared with lower segment myometrial biopsies (Astle *et al*., 2005; Grigsby *et al*., 2006). However, as PGE_2 did not produce a contractile phenotype during late labour, the predominant inhibitory EP_2 receptors may supersede $EP_{1/3}$ mediated effects. Thereby, a shift in EP receptor dynamics and secondary signal cascades are likely to regulate labour-onset and postpartum involution.

$PGF_{2\alpha}$ elicited monophasic excitatory responses in isolated lower myometrium taken after labour-onset. The spasmogenic activity was quantitatively similar to previous superfusion and immersion studies (Wikland *et al*., 1984; Hutchinson, 2005). This was related to a transient rise in intracellular Ca^{2+} release in both intact myometrium

and myocytes (Carrasco *et al*., 1996; Shlykov & Sanborn, 2004). During pregnancy, myometrial FP mRNA was shown to decrease relative to the non-pregnant state (Matsumoto *et al*., 1997; Sooranna *et al*., 2005); this corresponded to the decline in potent $PGF_{2\alpha}$-induced contractions (Senior *et al*., 1992; Senior *et al*., 1993). At term parturition, human FP receptor expression significantly increased indicating hormonal and physiological influences on PG receptors (Brodt-Eppley & Myatt, 1999). Even so, this study showed that tissue responsiveness to $PGF_{2\alpha}$ was attenuated through early, mid and late stages of labour in lower segment myometrial strips. Weak responses were also observed in superfusion, with marked stimulation by $PGF_{2\alpha}$ evoked only in paired fundus end specimens taken during active labour (Wikland *et al*., 1984). This topographical difference in FP receptor activity parallels the decline in smooth muscle content of cervical tissue compared to the fundus (Adelantado *et al*., 1988). Moreover, instead of modulating activity, it is plausible that FP receptor populations mediate $PGF_{2\alpha}$-stimulated glycosaminoglycan activity for uterine compliance in the lower segment during labour (Weiss, 2000). This may account for the high $PGF_{2\alpha}$ synthesis in lower myometrial segments at this time (Durn *et al*., 2010). Combined doses of PGE_2 and $PGF_{2\alpha}$ suppressed contractile activity in lower isolated myometrium after labour-onset (Wikland *et al*., 1984). Whilst reflecting a high PGE_2 binding affinity, this also indicates the predisposition of the lower uterus to relax in order to subserve the birth process.

The thromboxane mimetic U46619 potentiated contractions in isolated human myometrium taken after labour-onset. Significant attenuation with TP antagonists SQ29,548 (Ogletree *et al*., 1985) and GR32191B (Lumley *et al*., 1989) confirmed constitutive TP-mediated responses in this study. As SQ29,548 failed to shift $PGF_{2\alpha}$ concentration-effect curves in myometrium obtained during labour (Hutchinson,

2005; Griffiths *et al*., 2006), off-target $PGF_{2\alpha}$ activation was not displayed. TPα and TPβ splice variants have been identified in human myocytes and vasculature from both non-pregnant and term pregnant donors (Moore *et al*., 2002; Moran *et al*., 2002). However, little is known about labour-associated changes in human TP receptor function and expression.

Despite a 2-fold reduction in U46619-induced contractile activity between early and late stages of labour, tissue excitation was significantly augmented compared with initial low myogenic contractions. At late gestation, urinary thromboxane excretion has been shown to increase and heighten during labour (Noort & Keirse, 1990), alongside thromboxane synthase (Swanson *et al*., 1992), augmenting uterine activity. This implicates a maintained function for TP receptors during the parturition-process. Two target mediators in the TP signalling cascade, ROCKI and its isoform ROCKII sensitise the uterus to Ca^{2+} (Kureishi *et al*., 1997) and may account for the potentiated contractile responses in this study. Aberrant ROCKI expression has been associated with uterine contractile dysfunctions such as preterm and prolonged labour at term (Moore & Lopez Bernal, 2003). Moreover, an increase in RhoA mRNA at parturition implies thromboxane involvement in the preparatory and stimulatory phases of labour (Noort & Keirse, 1990). This corresponded to the enhanced U46619 response at preterm late labour in this study. As a result, cognate TP receptors may control uterine tone required for foetal descent during labour and possibly uterine involution postpartum.

For further Ca^{2+} sensitisation, U46619 induced the cleavage of the p160 ROCKI protein to yield p130 ROCKI by caspase-3 (Moore & Lopez Bernal, 2003), associated with enhanced uterine contractility and apoptosis (Coleman *et al*., 2001; Leong *et al*., 2008). Although the caspase-3 inhibitor Z-DEVD-FMK blocked this pathway (Moore

et al., 2002; Moore & Lopez Bernal, 2003), no significant effect on U46619-induced activity was observed in the present study. This may be attributed to already high caspase-3 in myometrial cells resulting from shrinkage, oedema and apoptosis with tissue remodelling (Leong *et al*., 2008). It also suggests that p160 and p130 ROCKI proteins have similar efficacy for enhanced Ca^{2+} sensitisation in the uterus.

In conclusion, this study showed that myometrial EP, FP and TP receptors are dynamic in nature at term pregnancy and during parturition. It seems likely that a change in the balance of these receptors and signal transduction pathways would mediate the transition from uterine quiescence to activation. Despite ethical constraints limiting research to the lower uterus in the present study, TP receptor function seemed to predominate. Therefore, targeting TP receptors or their downstream regulatory pathways in the parturient uterus may help to improve tocolytic therapy for labour-associated disorders.

Section II:
Myometrial cell assays

Chapter 6: Methods for myometrial cell assays

Cell culture models to assess intracellular signalling pathways

6.1 Introduction

Cell culture methodology was developed to separate and purify smooth muscle cells and fibroblasts from uterine biopsies taken at term pregnancy. The primary cell culture model enabled aspects of the human myometrium to be studied without the influence of endogenous steroid hormones. Despite the reported change in myometrial cell morphology from the *in vivo* state (Hongpaisan, 2000), cell cultures retain many characteristics of the intact tissue (Pressman *et al*., 1988; Carrasco *et al*., 1996). This includes sensitivity to PG and oxytocin agonists, measured by intracellular Ca^{2+} mobilisation (Thornton *et al*., 1999) and signal transduction pathways (Phaneuf *et al*., 1993).

The aim of this study was to isolate pure cultures of smooth muscle cells and fibroblasts from term gravid human myometrium. Subsequent optimisation and development of FLIPR and RT-PCR techniques were performed. By characterising myometrial cell populations, the contribution of PG and oxytocin-mediated intracellular Ca^{2+} and cAMP release could be assessed relative to receptor mRNA expression (Chapter 7). Study of these intracellular and molecular events would facilitate the high throughput screening of drugs to improve tocolysis for preterm labour.

Chapter 6: Cell Materials & Methods

6.2 Materials

Table 6.1: Materials used in this study were purchased from the following sources:

Company	Town	County/ State	Country
Alpha Innotech Corporation	San Leandro	California	USA
Ambion	Austin	Texas	USA
Amersham Biosciences	Björkgatan	Uppsala	Sweden
BDH Laboratory Supplies	Poole	Dorset	UK
Becton-Dickinson	Franklin Lakes	New Jersey	USA
Bio-Rad Laboratories	Hercules	California	USA
Calbiochem	Nottingham	Nottinghamshire	UK
Cayman Chemicals	Ann Arbor	Michigan	USA
Cellgro Inc.	Pensacola	Florida	USA
Costar	Lowell	Massachusetts	USA
Decon Laboratories Ltd.	Hove	East Sussex	UK
EMD Chemicals Inc.	Gibbstown	New Jersey	USA
Fisher Scientific Ltd.	Loughborough	Leicestershire	UK
Gibco BRL	Inchinnan	Renfrewshire	UK
Invitrogen Ltd.	Paisley	Greater Glasgow	UK
Invitrogen Ltd.	Carlsbad	California	USA
Molecular Devices	Sunnyvale	California	USA
Perkin Elmer	Waltham	Massachusetts	USA
PromoCell GmbH	Sickingenstraße	Heidelberg	Germany
Qiagen	Valencia	California	USA
Riedel-de-haen	Seelze	Hanover	Germany
Roche Ltd.	Grenzacherstrasse	Basel	Switzerland
Serologicals Corporation	Norcross	Georgia	USA
Sigma Chemicals Co.	Poole	Dorset	UK
Vector Laboratories	Burlingame	California	USA

6.3 Compounds and solutions for cell culture

Table 6.2.1: Enzyme solution A diluted in Hanks' balanced salt solution (HBSS) for initial collagen dissociation of myometrial tissues.

Enzyme Solution A	Final Conc.	Source	Catalogue N^o
HBSS w/o phenol red, $CaCl_2$ or $MgSO_4$		Promocell	C-40390
Dispase enzyme solution (grade 2)	10mg/ml	Roche (BD)	354235
Calcium chloride	2.5mM	Riedel-de-haen	12074
Magnesium chloride	0.9mM	Sigma	M8266

Table 6.2.2: Enzyme solution B for cell isolation from myometrial biopsies; in the final working solution, elastase was omitted.

Enzyme Solution B	Final Conc.	Source	Catalogue N^o
HBSS w/o phenol red, $CaCl_2$ or $MgSO_4$		Promocell	C-40390
Collagenase type II	300U/ml	Sigma	C6885
DNAse I type IV	30U/ml	Sigma	D5025
Elastase type I	2U/ml	Sigma	E1250
Fatty acid-free bovine serum albumin	1mg/ml	Sigma	A8806

Table 6.3.1: Fully defined Dulbecco's modified eagles medium (DMEM) A for culture of myocytes and fibroblasts.

Basic defined medium	Concentration	Source	Catalogue N^o
DMEM with high glucose & sodium bicarbonate		Sigma	D6171
Foetal calf serum	10%	Sigma	F6178
L-glutamine	2mM	Promocell	C-42210
HEPES solution	25mM	Sigma	H0887
Penicillin (10,000U/ml) Streptomycin (10mg/ml)		Sigma	P0781
Amphotericin B	2.5ng/ml	Sigma	A2942

Basic defined medium was also used with Waymouth's Medium MB 752/1, Invitrogen 31220-023 or PromoCell C-73440, instead of DMEM A.

Table 6.3.2: Fully defined DMEM B with reduced oestrogen content for seeding cells.

Basic defined medium	Concentration	Source	Catalogue N^o
DMEM with high glucose and without phenol red		Sigma	D1145
Charcoal stripped foetal calf serum	1%	Sigma	F6765
L-glutamine	2mM	Promocell	C-42210
HEPES solution	25mM	Sigma	H0887
Penicillin (10,000U/ml) Streptomycin (10mg/ml)		Sigma	P0781
Amphotericin B	2.5ng/ml	Sigma	A2942

Table 6.3.3: Fully defined DMEM C to culture cells for FLIPR assays.

Basic defined medium	Concentration	Source	Catalogue N^o
DMEM with high glucose, L-glutamine & pyruvate		Invitrogen	11995-065
Foetal calf serum	10%	Invitrogen	14040-133
Geneticin	200μg/ml	Invitrogen	10131-027
Hygromycin B	200μg/ml	Invitrogen	10687-010

Table 6.3.4: Fully defined DMEM D with reduced oestrogen content for seeding cells in FLIPR assays.

Basic defined medium	Concentration	Source	Catalogue N^o
DMEM with high glucose and without phenol red		Sigma	D1145
Charcoal stripped foetal calf serum	1%	Sigma	F6765
L-glutamine	2mM	Promocell	C-42210
Penicillin-Streptomycin (10,000U) Amphotericin B (25μg)		Gibco	15240-062

Table 6.4: Solutions for cell trypsinisation.

Compounds for cell passage	Concentration	Source	Catalogue N^o
Trypsin-EDTA solution	0.25%	Sigma	T4049
Foetal calf serum	20%	Sigma	F6178

Table 6.5: Preparation of coverslips (22mm^2) and slides for cell or tissue adhesion.

Compounds for coverslips	Concentration	Source	Catalogue N^o
Urea	1mM	Sigma	U2709
Decon 90	5%	Decon Lab Ltd.	1310-58-3
Bovine fibronectin	50μg/ml	Sigma	F1141
Ethanol	70%	Fisher Scientific	E/0400/17
Poly-L-lysine solution	0.01% w/v	Sigma	P8920

6.4 Compounds and solutions for cell assays

Table 6.6: Buffers and solutions for immunocytochemistry & immunohistochemistry.

Buffers for immunofluorescence	Concentration	Source	Catalogue N^o
HBSS without phenol red		Promocell	C-40390
Phosphate buffered saline (PBS)	0.01M	Sigma	P3813
Tween 20	1.1g/ml	Sigma	T2700
Bovine serum albumen (BSA)	10%	Sigma	A3059
Paraformaldehyde solution	4%	Sigma	P6148
Sodium hydroxide (NaOH)	1N	Sigma	S8045
Mounting medium with DAPI	1.5μg/ml	Vector Laboratories	H1200

Table 6.7: Solutions to quantify intracellular cyclic AMP (cAMP).

HitHunter cAMP XS+ assay (Cat N°: 90-0075L)	Volume	Catalogue N°
cAMP XS+ Lysis Buffer	76ml	30-213
cAMP XA+ EA Reagent	200ml	30-352
cAMP XS+ ED Reagent	100ml	30-353
cAMP XS+ Antibody Reagent	50ml	30-354
cAMP XS+ Standard (250μM)	13ml	30-355
Galacton-Star®	4.0ml	10-069
Emerald-II™	20ml	10-068

Table 6.8: Agonists and antagonists used to identify functional receptors in human myocytes and fibroblasts.

Compound	Receptor target	Stock vehicle	Source	Catalogue N°
AH13205	EP_2	DMSO	Sigma	A9102
AH6809	EP_1, EP_2, EP_3, DP_1	DMSO	Sigma	A1221
Atosiban	Oxytocin receptors	0.1% BSA in dH_2O	Sigma	A3480
β-oestradiol	Oestrogen α & β	ethanol	Sigma	E2257
Butaprost	EP_2	ethanol	Cayman Chemicals	13740
Caspase-3 inhibitor II		DMSO	Calbiochem	264155
EDTA	Ca^{2+} chelator	DMEM D	Sigma	E6758
Indometacin	COX inhibitor	ethanol	Sigma	I7378
$LaCl_3$	Ca^{2+} channel blocker	ethanol	Sigma	L4131
Nifedipine	L-type Ca^{2+} channels	ethanol	Sigma	N7634
Oxytocin	Oxytocin receptors	DMEM D	Sigma	O3251
Progesterone	Progesterone A & B	ethanol	Sigma	P6149
PGE_2	EP_{1-4}	ethanol	Cayman Chemicals	14010
$PGF_{2\alpha}$	FP	ethanol	Cayman Chemicals	16010
Rho-kinase inhibitor		DMEM D	Cayman Chemicals	555550
SQ29,548	TP	ethanol	Cayman Chemicals	19025
Sulprostone	EP_1, EP_3	ethanol	Cayman Chemicals	14765
Thapsigargin	Ca^{2+}-ATPase inhibitor	ethanol	Sigma	T9033
U46619	TP	ethanol	Cayman Chemicals	16450

Table 6.9. Solutions for SuperScript III one-step quantitative RT-PCR system.

Solutions for SuperScript III one-step RT-PCR	Source	Catalogue N°
DNase/ RNase free water	Gibco	10977-015
PCR supermix	Invitrogen	10572
Platinum PCR supermix	Invitrogen	11306-016
Run mix 2X	Invitrogen	52202
SuperScript III RT/ Platinum Taq mix	Invitrogen	52118
SuperScript III	Invitrogen	12574

6.5 Isolated myometrium for cellular and molecular studies

Uterine tissue was obtained from term pregnant women (aged 22-38) undergoing elective Caesarean sections before the onset of labour (n=17). To standardise myocyte cultures, women associated with any major complication of pregnancy, such as hypertension, pre-eclampsia and diabetes were excluded from cellular and molecular studies. Other exclusion criteria included donors taking prescription medicines and those who smoked. All donors signed informed written consent forms prior to surgery; this was in accordance with ethical approval from the Bradford Hospital NHS Trust and the University of Bradford Ethics Committee (Figure A2).

At Caesarean delivery, full-thickness uterine biopsies were taken from the upper margin of lower transverse incisions. These were transported to the laboratory in sterile Krebs' solution and processed for cell dispersion within 2 hours of excision. Adherent decidua, serosa, fat and connective tissues were trimmed using a sterile scalpel and the remaining myometrium was washed several times in sterile Hanks' balanced salt solution (HBSS) to clean and remove residual mesentery and blood. Isolated myometrium was further processed for primary cell culture using explant or enzymatic digestion techniques.

6.6 Primary cell culture

Myometrial biopsies (~0.5g) were minced using a McIlwain Tissue chopper (Mickle Laboratory Engineering, Guilford, UK) into pieces <1mm^3 and washed in HBSS.

6.6.1 Myometrial explant cultures

For crude explant cultures, 30-50mg of minced myometrial tissues were added to 0.5ml basic defined Dulbecco's modified eagles medium (DMEM A), containing 10% v/v foetal calf serum (FCS) and 1% v/v antibiotic and antimycotic solutions. The explant suspensions were aliquoted into 25cm^2 culture flasks or onto sterile treated coverslips in 35mm culture dishes (Section 6.10). These containers were carefully inverted to improve explant adhesion and DMEM A was added beneath to create a humidified atmosphere (Figure 6.1). After incubating at 37°C (5% CO_2) overnight, the culture flasks and dishes were returned to their upright position and explants were submerged with fresh basic defined medium. The medium was changed every other day thereafter with cells maintained under the same conditions until sub-confluence.

a) b)

Figure 6.1: Myometrial explants adhering to treated coverslips in culture dishes a) inverted overnight and b) subsequently returned to an upright position. Addition of basic defined medium and incubation at 37°C facilitated myometrial cell growth.

6.6.2 Enzymatic dispersion of myometrium

The technique for enzymatic myometrial cell isolation was based on a protocol previously described by Phaneuf *et al*. (1993) with some adaptations. Between 0.5-1.0g of the minced tissue was placed in 10ml HBSS (solution A) containing calcium chloride ($CaCl_2$; 2.5mM), magnesium chloride ($MgCl_2$; 0.9mM) and dispase (10mg/ml), which was incubated at 37°C for 1-2 hour with continuous stirring. After washing in Ca^{2+}- and magnesium- (Mg^{2+}) free HBSS, the mesh of softened tissue was triturated. Cell dispersion was achieved in the same solution, supplemented with collagenase (300U/ml), elastase (2U/ml), DNAse I (30U/ml) and fatty acid free-bovine serum albumen (BSA; 1mg/ml) (solution B) at 37°C with gentle shaking. After 2 hours, the cell suspension was transferred to a centrifuge tube and washed three times with HBSS by centrifugation at 450g (~1200rpm). Basic defined medium composed of DMEM A, 10% v/v FBS, 100IU/ml penicillin, 10mg/ml streptomycin and 2mmol/l L-glutamine was used to resuspend the final cell pellet.

6.7 Cell number and viability

Before plating, a trypan blue exclusion assay was performed to determine cell number and viability. The cell suspension was diluted 1:1 with 0.4% v/v trypan blue solution and 10µl of this mixed solution was added to each chamber of a Neubauer haemocytometric slide with a coverslip in place. Using the 10x focus on the Olympus CK40 microscope, live (unstained) and dead (trypan blue positive) cells were counted in five $1mm^2$ grids per chamber and an average was calculated per grid from the two chambers. Since the depth was 0.1mm, each grid represented a total volume of $0.1mm^3$ or $10^{-4}cm^3$.

The total number of cells and cell viability was therefore determined using the following calculations:

Cells/ ml = average cell count per grid x 10^4 x dilution factor

Total cells = Cells/ ml x volume of original cell suspension (ml)

% cell viability = total viable cells (unstained)/ total cells x 100

If cells were too concentrated to count, the cell suspension was diluted further and the dilution factor was adjusted accordingly. In all cases cell viability was greater than 82 percent (Table 6.10).

6.8 Optimising isolation parameters

Although cell viability with the trypan blue exclusion assay was at 90 percent using the method outlined by Phaneuf *et al*., (1993) (Section 6.6.2; Table 6.10), enzyme combinations were altered to optimise tissue digestion. Dissociation with trypsin and collagenase for 1 hour (Karasinski *et al*., 2000) increased cell damage by 8.9 percent. However, by omitting elastase from the digestion step, the yield and viability of cells was improved. As a result, elastase was subsequently excluded from solution B in the digestion process.

Table 6.10: Enzymatic digestion of minced isolated myometrium following exposure to solution A. The composition of enzyme solutions was based upon protocols described by a) Phaneuf *et al*. (1993), b) Fomin *et al*., (1999) and c) Karasinski *et al*., (2000). Cell number and viability were quantified using a haemocytometer and trypan blue exclusion assays; approximately 100,000 to 1,000,000 cells were isolated per gram of myometrial tissue.

	Collagenase (U/ml)	**Elastase (U/ml)**	**DNase I (U/ml)**	**FAF BSA (mg/ml)**	**Total cell number (range; n=6)**	**Average cell viability (%)**
a	300	2	30	1	$1.2x10^5 – 1.1x10^6$	90 ± 10.8
b	300	0	30	1	$1.1x10^5 – 1.3x10^6$	94 ± 6.5
	Collagenase (U/ml)	**Trypsin (%)**	**DNase I (U/ml)**	**FAF BSA (mg/ml)**	**Total cell number (n=1)**	**Cell viability (%)**
c	300	0.1	0	1	$5.2x10^5$	82

FAF BSA: Fatty acid-free bovine serum albumen.

6.9 Purification of myometrial cells

Different techniques were applied to purify smooth muscle cells from fibroblasts. The cell suspension was initially separated using Percoll two-phase discontinuous density gradient (Amersham Biosciences, Uppsala, Sweden). This consisted of the following solutions:

Table 6.11: 10X Ads buffer composition in 100ml milli-Q water.

10X Ads buffer	Volume added	Source	Catalogue N^{o}
Sodium chloride (NaCl)	6.8g	Sigma	S5629
HEPES	4.76g	Sigma	H3784
Sodium dihydrogen phosphate (NaH_2PO_4)	0.12g	Sigma	S5011
Glucose	1.0g	Sigma	G7021
Potassium chloride (KCl)	0.4g	Sigma	P5405
Magnesium sulphate ($MgSO_4$)	0.1g	Analar	203726

These substances were dissolved in milli-Q water to a final volume of 100ml. After adjusting the pH to 7.35 with 1N NaOH, the 10X Ads buffer was filter sterilised (Nalgene, 0.20 μm) and stored at 2-8°C.

For 1X Ads solution preparation, the 10X Ads buffer was diluted with sterile dH_2O. Percoll (density = 1.130g/ml) was diluted 9:1 with 10X Ads buffer to formulate the Percoll stock (density: 1.110g/ml). Gradients of Percoll for the top (density: 1.059g/ml) and lower (density: 1.082g/ml) layers were prepared by diluting Percoll stock with 1X Ads buffer at ratios of 9:11 and 13:7 respectively.

To establish the Percoll interface, 5ml top layer Percoll was transferred into a 15ml centrifuge tube. For this layer to rise, 5ml lower layer Percoll was added a drop at a time to the bottom of the tube. This two-phase Percoll gradient was overlaid with the myocyte/ fibroblast cell suspension ($1x10^{-6}$ cells). By centrifuging for 20 minutes at 2500g (~3000rpm), room temperature in a Mistral 3000 centrifuge, myocytes

sedimented at the Percoll interface, whilst fibroblasts and non-myocyte cells aggregated on the upper surface.

Individual bands of cells were carefully removed, resuspended in DMEM A and centrifuged at 450g for 5 minutes. Despite the observed cell pellets, once seeded in flasks, recovery of viable Percoll-enriched myocytes and fibroblasts was poor. Therefore, alternative methods to this gradient fractioning were explored.

Following enzymatic dissociation, myometrial cells were plated into 25cm^2 vent-cap culture flasks (Costar, Lowell, Massachusetts, USA) at a density of 0.5-2x10^4 cells/ ml and stored at 37°C in a water-saturated atmosphere containing 5% CO_2. To purify cultures based on differential adhesion, after 18 hours the supernatant containing late adherent cells was centrifuged at 450g for 5 minutes, resuspended and transferred to a separate 25cm^2 flask with fresh basic defined medium added to both cultures. This preplating technique has been shown to isolate rapidly adherent fibroblast cells from myocytes (Hongpaisan, 2000; Rouger *et al*., 2007). The media was changed every 48 hours thereafter to enhance cell proliferation. Culture growth and cell morphology were repeatedly examined using a phase contrast microscope (Olympus CK40) with pictures imaged using a JVC digital colour video camera (Figure 6.4). Near confluence, the cells were harvested using trypsin (Section 6.11).

6.10 Coating coverslips for cell adhesion

To enable myometrial cells to anchor, move and proliferate, the sterile flasks, plates and dishes were composed of treated polystyrene plastic (Costar, Lowell, Massachusetts, USA). However, when plating cells on glass coverslips (Ultima, 22mm^2), fibronectin was added as an extracellular matrix coating to facilitate the

attachment of cell surface cytoplasmic proteins, such as integrins and α-actinin (Sinanan *et al*., 2008).

The coverslips were cleaned using 5% v/v Decon 90 detergent followed by urea (1mM). After rinsing with sterile 1x phosphate buffered saline (PBS) and ethanol in a laminar flow hood, glass coverslips were incubated with 50μg/ml bovine fibronectin for 30-45 minutes at room temperature. The coverslips were then rinsed with PBS and secured in 6-well plates or 35mm Petri dishes with tissue explant or cell suspensions ($5x10^4$ cells/ ml) added immediately. Following a 30-minute incubation in a water-saturated atmosphere at 37°C, Petri dishes were flooded with media and incubated overnight.

A time-lapse video was performed using Adobe Premiere software with images taken at 0 and 18 hours showing cell adhesion to a fibronectin-coated glass coverslip (Figure 6.2). To maintain cell pH and temperature, HEPES buffer (25mM) was added to the culture medium and a heating stage (RS) set to 38°C was used.

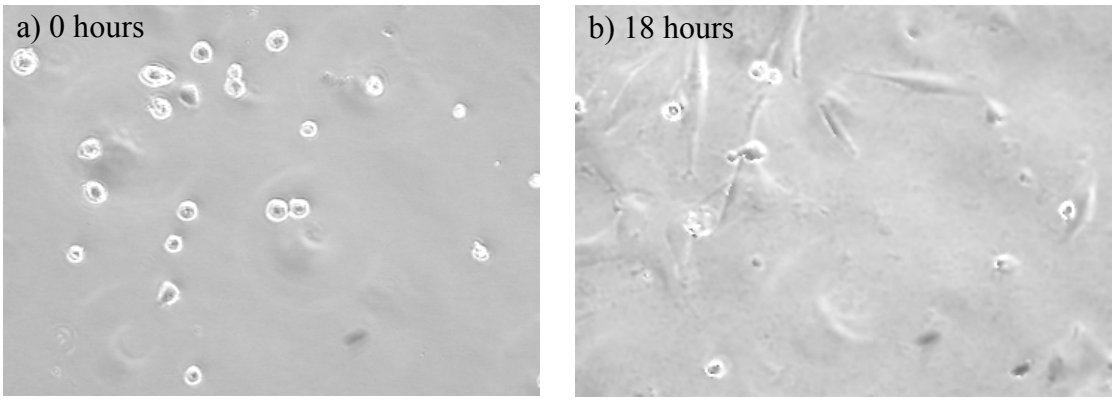


Figure 6.2: Isolated primary myometrial cells incubated on a fibronectin-coated slide at 37°C for a) 0 hours and b) 18 hours under a phase contrast microscope (x10) equipped with a JVC digital camera. The rapid adhesion properties have been associated with cultures enriched in fibroblast cells (Rouger *et al*., 2007).

6.11 Harvesting cells

To passage cells for subculture, myocytes were harvested upon reaching a semi-confluent state. This was achieved after removing the conditioned medium by adding 0.25% trypsin containing 2mM ethylendiamine tetra-acetic acid (EDTA) to the cell flasks. As a Ca^{2+} chelator, EDTA worked synergistically with the protease enzyme trypsin to cleave cell-matrix adhesion proteins. Thereby, as monitored under the phase contrast microscope (Figure 6.3), after 5-15 minutes at 37°C, the cells were rounded and detached from the flask surface. Due to its inhibitor proteins and divalent cations, FCS was immediately added to neutralise trypsin action. The cells were then centrifuged at 450g for 5 minutes and the cell pellet was diluted with fresh medium for culture or for freezing.

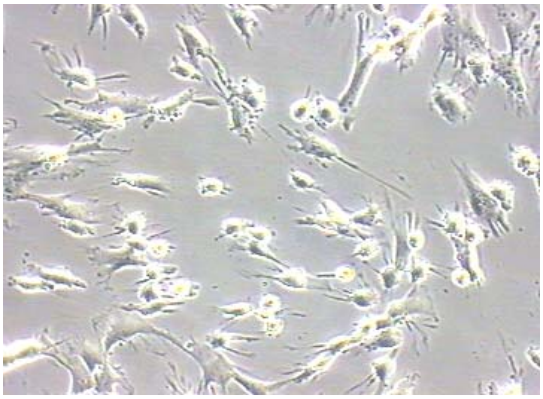


Figure 6.3: Trypsin-EDTA incubated at 37°C/ 5% CO_2 for 3 minutes in a flask of cultured myocytes. The rounding of the cells visualised under a phase contrast microscope (x10) indicates near detachment from the flask surface.

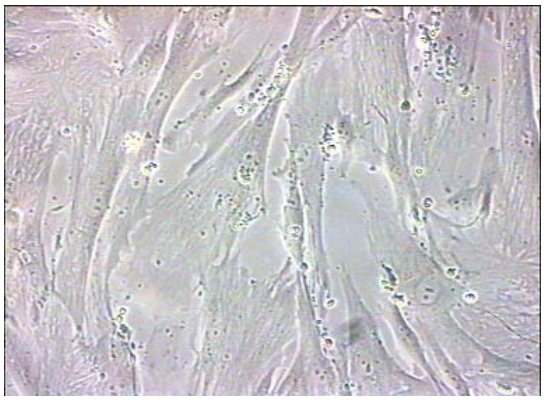
6.12 Preservation, storage and recovery of cultured cells

For freezing and storage of cells near confluence, the cells were trypsinised, harvested and counted (Sections 6.7 & 6.11). Following centrifugation at 450g for 5 minutes, the supernatant was discarded and the remaining cell pellet was resuspended at a final density of $1x10^6$ cells/ ml in freshly prepared DMEM A, containing 10% v/v dimethyl sulfoxide (DMSO) and 10% v/v FCS. This freezing medium both prevented crystallisation of water, which would lyse cells during cryopreservation, and also

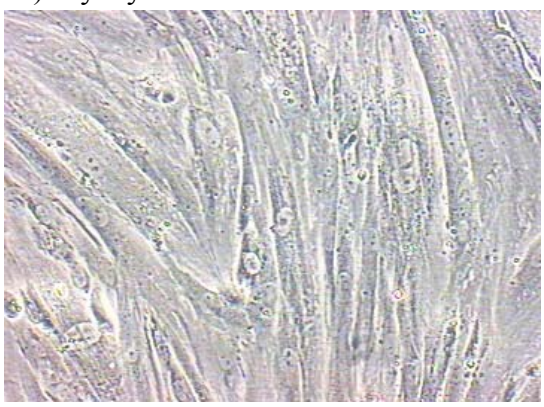
provided nutrients during the thawing process. The cells were stored in a cryovial within a freezing container (Nalgene Ltd., Ridderstraat, Neerijse, Belgium) at -80°C, which was transferred to liquid nitrogen the following day.

Frozen cells were recovered within a year of storage. The cells were quickly thawed in a 37°C water bath, diluted in pre-warmed basic defined medium and plated for culture.

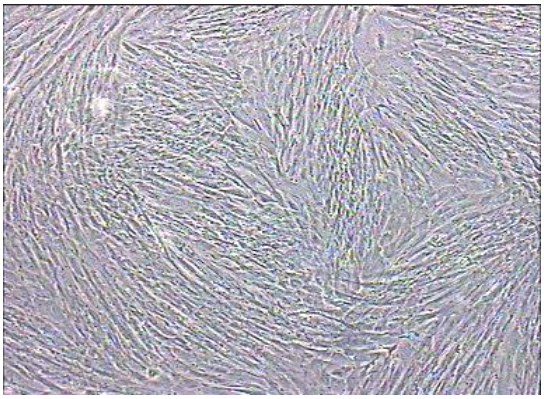
a) Fibroblasts x25



b) Myocytes x25



c) Myocytes x4



d) Myocytes x40

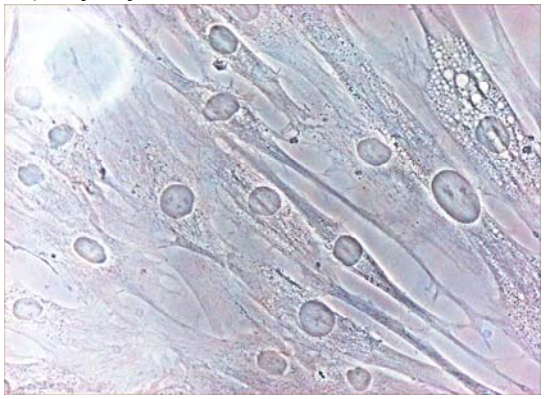


Figure 6.4: Images of primary human a) fibroblasts and b, c, d) smooth muscle cells dissociated from myometrial biopsies and visualised under magnifications of a-b) x25, c) x4 and d) x40. Cell populations were separated using differential adhesion and each culture formed a typical whorl pattern near confluence. As myocytes and fibroblasts were difficult to distinguish morphologically, both immunohistochemistry and immunocytochemistry staining techniques were employed.

6.13 Solutions for immunofluorescent staining

The purity of cell cultures was assessed using immunohistochemistry and immunocytochemistry techniques. The protein markers assayed included α-actinin, actin, desmin, vimentin and anti-human fibroblast surface protein to distinguish between myocytes and fibroblasts.

6.13.1 Phosphate buffered saline (PBS)

PBS was prepared by dissolving a sachet of 10x PBS in 1 litre of autoclaved milli-Q water for a working solution of 1x PBS (0.01M, pH 7.4).

6.13.2 Wash buffer (PBS-Tween 0.5%)

The mild detergent Tween 20 was added to PBS (0.01M, pH 7.4) at 0.5% v/v and stored at 2-8°C.

6.13.3 Blocking buffer

After addition of 1g BSA (Fraction V) to 100ml PBS-Tween (0.05%), the blocking buffer was heat inactivated at 65°C for 15 minutes and cooled. This was diluted to 3% v/v and 1% v/v working solutions using PBS-Tween (0.5%).

6.13.4 Paraformaldehyde fixing buffer

To prepare 4% paraformaldehyde-fixing buffer, PBS (0.01M) was pre-heated to 65°C and 4g of paraformaldehyde powder was added in a fume hood. This was maintained at 65°C for 15 minutes and then stirred at a low heat until the paraformaldehyde solution became clear. After cooling, the pH was adjusted to 7.35 ± 0.5 with 1N sodium hydroxide (NaOH) and this fixing solution was stored in the dark.

6.13.5 Permeabilising buffer

The permeabilising buffer was composed of the following substances (Table 6.12) dissolved in milli-Q water to a final volume of 100ml. The pH was adjusted to 7.2 using 1N NaOH and this buffer was then filter sterilised (Nalgene, 0.20 μm) and stored at 2-8°C.

Table 6.12: Permeabilising buffer composition in 100ml milli-Q water.

Permeabilising buffer	Volume added	Source	Catalogue N^{o}
Sucrose	10.3g	EMD Chemicals	102745C
Sodium chloride (NaCl)	0.292g	Sigma	S5629
Magnesium chloride ($MgCl_2$)	0.06g	Sigma	M8266
HEPES	0.476g	Sigma	H3784
Triton X-100	0.5ml	Sigma	9284

6.13.6 Primary & secondary antibodies

Table 6.13: Characteristics of primary antibodies diluted in 1% blocking solution for immunofluorescence.

Primary antibody	Donor species	Working dilution	Source	Cat No
α-actinin	Mouse	1:200	Sigma	A5044
Fibroblast surface protein	Mouse	1:100	Sigma	F4771
Vimentin	Goat	1:100	Sigma	V4630
Desmin	Rabbit	1:100	Sigma	D8281

Table 6.14: Secondary conjugated antibodies diluted in 1% blocking solution for immunofluorescence, stored in dark to prevent photobleaching.

Secondary antibody	Conjugate	Working Dilution	Protein target	Source	Cat No
Alexafluor 488, rabbit anti-mouse IgG	FITC (green)	1:200	α-actinin, Fibroblast protein	Invitrogen	A11059
Alexafluor 568, rabbit anti-goat IgG	TRITC (red)	1:200	Desmin	Invitrogen	A11079
Goat anti-rabbit IgG	FITC (green)	1:50	Vimentin	Sigma	F0382

6.14 Immunohistochemistry of myometrial sections

To identify cell subsets in isolated human myometrium, tissue biopsies obtained at term pregnancy were cut to approximately 1 cm^3 using a surgical blade (size 23). The specimens were submerged in optimal cutting temperature (OCT; a cryoprotective compound) in cuvettes, which were frozen and stored at -20°C.

6.14.1 Preparation of poly-L-lysine coated slides

Glass slides (BDH Laboratory Supplies, Poole, Dorset, UK) used for immunostaining were cleaned thoroughly to eliminate contamination. The slides were individually spaced on a slide carrier, soaked in 5% Decon 90 detergent for 5 minutes and washed twice with distilled water. After transferring to 100% ethanol, the slides were placed in a heated drying cabinet until the ethanol had evaporated. To optimise tissue section adherence, the slides were soaked in 0.01% w/v poly-L-lysine solution for 5 minutes and left to dry overnight.

6.14.2 Preparation of frozen sections using the cryostat

Myometrial biopsies frozen in the cryo-embedding substance OCT were left to thaw at room temperature. The chamber of the cryostat (Leica CM 1800) was adjusted to -25°C and a small disc of OCT was frozen onto the metal chuck. Once thawed, the myometrial tissue was placed in fresh OCT using forceps, which was then mounted on the metal chuck to freeze. Further layers of OCT were added and frozen to fully embed the tissue. Tissue sections were cut at 10μm using the cryostat and placed onto poly-L-lysine coated slides for storage at -20°C.

6.15 Immunofluorescent staining of myometrial sections and cells

Before immunostaining, frozen sections were immersed in cold PBS for 5 minutes within a Coplin Jar and a small region above and below each tissue section was blotted with Whatman paper. To retain solutions during incubation steps, a liquid-repellent slide marker pen (PAP pen, Sigma Chemicals, Poole, Dorset, UK) was used to encircle the tissue sections. For myometrial cells, coverslips with subconfluent cultures (Section 6.10) were washed three times with HBSS to remove medium. All preparations were fixed in 4% paraformaldehyde for 5 minutes at 37°C.

Staining for α-actinin, desmin and vimentin was performed by rinsing slides and coverslips with PBS-Tween (0.5%) before treating with a permeabilising buffer containing 0.5% solution of the detergent Triton X-100 (Table 6.12). After washing twice, the cells and myometrial sections were blocked with a 3% BSA solution to minimise non-specific background staining. Fibre-free Whatman filter paper was used to decant the buffer. The tissue sections and cells were then incubated with primary monoclonal antibodies at their optimal concentrations (Table 6.13). To visualise proteins after a further three washes, secondary antibodies conjugated to a fluorochrome were incubated for an hour at 37°C; this corresponded to the donor species of the primary antibody (Table 6.14). The slides and coverslips were subsequently kept in the dark to prevent photobleaching. After three washes with PBS, cells stained with the α-actinin antibody were incubated for a further hour at room temperature with phalloidin-tetramethylrhodamine B isothiocyanate (1μg/ml), a toxin that binds to polymeric filamentous actin. Three final washes with PBS were performed and coverslips were mounted on glass slides (BDH Laboratory Supplies, 76mm x 26mm) using 1.5μg/ml DAPI (Vector Laboratories, Burlingame, California, USA). This mounting medium was used to counterstain cell nuclei and the edges of each

coverslip were sealed using clear nail varnish. Negative controls were performed in parallel, except that tissues and cells were treated with a solution of 1% v/v BSA in PBS without primary antibody. The slides were stored in the dark at 4°C for up to two days before imaging.

Table 6.15: Overview of the procedure used to fix, permeabilise and stain intracellular protein markers.

Step	Procedure	Temperature	Time
1	Aspirated medium and washed 3 times with HBSS	37°C	5 minutes x 3
2	Fixed with 4% paraformaldehyde solution	37°C	5 minutes
3	Washed 3 times with PBS-Tween (0.5%)	37°C	5 minutes x 3
4	Incubated with permeabilising solution	4°C	5 minutes
5	Washed 2 times with PBS-Tween (0.5%)	37°C	5 minutes x 2
6	Incubated with 3% blocking solution	37°C	1 hour
7	Incubated with primary antibody solution	37°C	1 hour
8	Washed 3 times with PBS-Tween (0.5%)	37°C	5 minutes x 3
9	Incubated with secondary antibody in dark	37°C	1 hour
10	Washed 3 times with PBS on shaker in dark	RT	5 minutes x 3
11	Rinsed with milli-Q purified water and DAPI used to mount the coverslips on to slides. Once dry, slides were stored in dark at 4°C until examined under microscope.		

RT: room temperature

6.15.1 Immunostaining for fibroblast surface proteins

Unlike the above protocol (Section 6.15; Table 6.15), to detect fibroblast surface proteins the permeabilising step and addition of Tween 20 to wash and blocking buffers were omitted. This ensured that the integrity of the cell membrane remained intact and also prevented the monoclonal antibody from binding to cytosolic proteins.

6.15.2 Fluorescence microscopy

After immunostaining, tissue sections and cell cultures were viewed and photographed under a Nikon 32 phase contrast microscope equipped with a camera.

ACT-2U software was used to capture both fluorescent and bright field pictures; these images were superimposed using Adobe Photoshop version 6.

6.16 Intracellular Ca^{2+} recordings

To determine receptor-mediated intracellular Ca^{2+} signalling, a fluorescence-based assay was adopted. This was performed using a fluorometric imaging plate reader (FLIPR-Tetra; Molecular Devices).

To prepare plates for the FLIPR system, myometrial smooth muscle cells and fibroblasts (passages 1-5) were harvested at 80-90% confluence using trypsin (Section 6.11). The cells were diluted 100-fold into 10ml saline and total cell number was determined using a Coulter Counter (Beckman-Coulter Z_2 Cell Counter). Following centrifugation at 450g for 5 minutes, myocytes and fibroblasts were resuspended in fresh basic defined medium C (Table 6.3.3) and plated at optimal concentrations (Chapter 7). The cells were then dispensed at 100µl or 50µl/ well into poly-D-lysine-coated, black-wall and clear-bottom 96-well or 384-well plates (Becton-Dickinson, New Jersey, USA), covered and incubated overnight at 37°C under 5% CO_2.

6.16.1 Preparation of buffers for FLIPR

Table 6.16: HBSS-HEPES buffer

Stock solutions	Volume added	Source	Catalogue N^o
10x HBSS	600ml	Gibco	14065-056
1M HEPES buffer	120ml	Gibco	15630-080

Distilled water was added to prepare 6 litres of HBSS-HEPES buffer. The pH was adjusted to pH 7.4 using 1N NaOH and the buffer was then filter sterilised and stored at ambient room temperature for up to 2 weeks.

Table 6.17: Dye-loading buffer (2μM fluo-4)

Solutions	Final Concentration	Source	Catalogue Nº
Fluo-4 AM dye	2μM	Invitrogen	F-23917
Pluronic acid	0.02%	Invitrogen	P-3000MP

The stock fluo-4 dye (2mM) was diluted in DMSO and pluronic acid was added at 20%. To prepare the dye loading buffer, the fluo-4 mixture was diluted 1000-fold with HBSS-HEPES buffer. This was stored in the dark at room temperature and used on the same day.

6.16.2 Standard agonist plates

Test drugs were prepared at 10^{-2}M in the appropriate diluent and stored at -20ºC (Table 6.8).

To prepare standard agonist plates, all wells in a 96-well V-bottom Greiner plate were pre-filled with HBSS-HEPES buffer. Stock agonists (10^{-2}M) were diluted to $4x10^{-4}$M with buffer and further diluted 10-fold in Row H for a working concentration of $4x10^{-5}$M. The agonists were serially diluted 1:10 up the plate, leaving Row A with buffer alone as a negative control. Similarly for 384-well V-bottom Greiner plates, two serial dilutions were prepared from Rows H and P, with Rows A and I saved as blanks. The plates were stored at 4ºC and used on the same day after equilibrating to room temperature.

6.16.3 Standard antagonist plates

Antagonists (10^{-2}M) were diluted in HBSS-HEPES buffer to 4 times the required final concentration. For negative controls, buffer was pipetted into columns 1-3 of 96-well V-bottom Greiner plates. Antagonists were then added to each of 24 wells in the three adjacent columns. Blank wells (columns 1-3; 13-15) and antagonists were plated in an

equivalent manner for 384-well plates, with a total of 6 antagonists assayed. These plates were stored at room temperature in the dark before use on the same day.

In incubation experiments, compounds prepared in DMEM B were added to adherent plated cells, substituting the original seeding medium. Plates were incubated for 24 hours before agonist additions using the FLIPR.

6.16.4 FLIPR assay

To determine intracellular Ca^{2+} responses, the cells were washed twice with HBSS-HEPES buffer using a Microplate Cell Washer (BioTek Instruments, Winooski, Vermont, USA) leaving a resting volume of 50µl and 25µl in 96- and 384-well plates respectively. The cells were then incubated with the cytoplasmic Ca^{2+} indicator fluo-4 dye in the acetylmethyl form (fluo-4 AM, 2µM) at 37°C for 45 minutes in the dark. During this time, the FLIPR was started, equilibrated to 39°C and an optics signal test was performed at set excitation/ emission wavelengths of 470-495nm/ 515-575nm using ScreenWorks software.

To remove extracellular fluo-4 AM dye, myocytes and fibroblasts were washed three times with HBSS-HEPES solution leaving 100µl or 50µl buffer in each well of 96- or 384-well plates. The FLIPR system was then loaded with disposable black tips alongside agonist and antagonist plates. After equilibrating at 37°C for 3 minutes, cell plates were placed in the FLIPR instrument and treated with antagonists for 4 minutes before an additional 4 minutes of agonist stimulation. A fluorescent signal emitted from the binding of cytosolic free Ca^{2+} with fluo-4 AM dye was detected using the FLIPR camera at an exposure time of 0.4 seconds. Fluorescence was also captured at excitation and emission wavelengths of 400nm and 506nm for Ca^{2+}-bound and free

dye respectively. Average changes in these fluorescent light units (FLU) were calculated for triplicate wells.

Responses were normalised relative to the maximum signal from control agonist wells and a 4-parametric sigmoidal curve was used to fit each data set. The background signal (negative control) of buffer alone was also taken into account using the following equation:

$$\% \text{ Change in FLU} = \frac{(\text{average FLU} - \text{background})}{(\text{average FLU (control wells)} - \text{background})} \times 100$$

6.17 Metabolic response assay: MTT

To identify changes in cell proliferation, cytotoxicity was quantified using the yellow tetrazolium dye, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). For this assay, stock MTT (5mg/ml) was diluted 1:10 in phenol red-free medium (DMEM B), which was added to adherent cells in 96-well plates at 37°C for 4 hours. During this time, the mitochondrial succinate-tetrazolium reductase system and cytoplasmic pyridine nucleotide cofactors within viable cells reduced the MTT to a blue/ purple, water-insoluble formazan salt. Following exposure to MTT, the conditioned media was aspirated and the formazan formed was solubilised by addition of 100µl DMSO per well. Colour development was read in a Bio-Rad plate reader at 630nm.

6.18 cAMP assay for smooth muscle cells

The cAMP assay was performed according to the HitHunter cAMP XS+ kit protocol (GE Healthcare Ltd., Buckinghamshire, UK). This was used for the quantitative determination of cAMP from cells and is based upon the complementation of the large enzyme acceptor (EA) and small enzyme donor (ED) fragments of *Escherichia coli* β-galactosidase (β-gal). For this assay, the cAMP from cell lysates and ED-labelled cAMP (ED-cAMP) compete for antibody binding sites. In the bound state, the activated β-gal enzyme produces a luminescent signal, which indicates the presence of intracellular cAMP.

6.18.1 Reagent preparation

Each reagent was equilibrated to ambient room temperature before use and protected from light during all incubations.

Lysis buffer/ antibody (Ab) working solution was gently mixed at a ratio of 3:1. cAMP XS+ ED/ lysis/ chemiluminescent (CL) substrate working solution was pipetted as a mixture. Galacton-star, Emerald-II and the Lysis buffer were gently mixed by inversion at a ratio of 1: 5: 19 before mixing with equal parts of ED at a ration of 1:1.

To activate adenylyl cyclase and increase intracellular cAMP, forskolin was added to cells. Stock forskolin (9mM) was diluted 10-fold in PBS to 900μM and ten 3-fold serial dilutions were prepared. These dilutions were 3-fold higher than the final plate concentration.

6.18.2 Standard curve

A cAMP standard curve was run to test the performance of cAMP XS+ kit reagents. The stock cAMP standard ($2.5x10^{-4}$M) was diluted 1:8 with PBS and nine 3-fold serial dilutions of cAMP were then prepared using PBS as the diluent for a final range of $2.35x10^{-10}$M to $4.63x10^{-6}$M. PBS alone was used for zero standards. Zero and cAMP standard dilutions were dispensed at 10µl/ well, followed by 5µl/ well PBS, 5µl/ well antibody reagent and 20µl/ well of ED/ CL/ Lysis mix. These compounds were incubated for 60 minutes at room temperate. After the addition of 20µl/ well EA, the plate was incubated for a further 4 hours at room temperature before the luminescent signal was read at 1 second/ well using a Victor Light precisely 1420 Counter (Perkin Elmer, Waltham, Massachusetts, USA).

6.18.3 Cell-based assay

Myocytes were harvested (Section 6.11), counted and resuspended in PBS to the appropriate cell density. Isobutylmethylxanthine (IBMX, 0.5mM), an inhibitor of phosphodiesterase, was also added to the cell suspension to prevent the degradation of cAMP. The assay was performed in triplicate per condition within a white OptiPlate-384 (Perkin-Elmer, Waltham, Massachusetts, USA). Controls included cAMP measurements in untreated cells and substitution of PBS for ED reagent to obtain background enzyme fragment complementation (EFC) signal.

6.18.4 Optimal cell conditions

Myocytes were trypsinised, washed, resuspended and diluted in IBMX-PBS solution for seeding densities of 2.5k, 5k, 10k, 20k and 40k cells/ well. Cell suspensions were added at 10µl/ well into the white OptiPlate-384 and treated with 5µl forskolin or PBS

as a zero control. To determine optimal conditions for cAMP induction, 3-fold forskolin for working concentrations of $5.1x10^{-9}$M to $3x10^{-4}$M were incubated with cells for 30 minutes at 37°C. Antibody reagent and ED/ Lysis/ CL Working Solution were added at 5µl and 20µl/ well respectively for 60 minutes incubation at room temperature. All cells were then treated with 20µl/ well EA solution for 4 hours at room temperature and the luminescence signal was read on the Victor Light plate reader (Table 6.18).

6.18.5 Agonist-induced cAMP production

The titre of myocytes in the preliminary assay established optimal cell density at 20k cells/ well (Figure 6.5). Based on this cell number, the EC_{80} concentration of Forskolin was calculated to be 10µM. To account for the working 3-fold dilution-factor, concentration-effect curves for PG and oxytocin agonists ($3x10^{-11}$M to $3x10^{-5}$M) were constructed using 30µM forskolin. The 10µl/ well seeded myocytes were then incubated with 5µl of each agonist at 37°C for 30 minutes. After addition of the antibody reagent and ED/ CL/ Lysis mix at 5µl and 20µl/ well respectively, the plate was incubated for 60 minutes at room temperature. EA solution was then dispensed at 20µl/ well before a 4-hour incubation step at room temperature and the chemiluminescent signal was read on the Victor Light plate reader.

Table 6.18: Overview of the 3-reagent addition protocol for the HitHunter cAMP XS+ assay to perform a standard curve and cell-based assay in a 384-well plate format.

Steps	Standard curve	Steps	Cell-based assay
1	10µl diluted cAMP standard	**1**	10µl cells (20µl/ well)
2	5µl PBS	**2**	5µl agonists in 30µM forskolin
3	5µl antibody reagent	**3**	30 minutes incubation at 37°C
4	20µl ED/Lysis/CL solution	**4**	5µl antibody reagent
5	1 hour incubation at RT	**5**	20µl ED/Lysis/CL solution
6	20µl EA reagent	**6**	1 hour incubation at RT
7	4 hours incubation at RT	**7**	20µl EA reagent
8	Read luminescence signal	**8**	4 hours incubation at RT
		9	Read luminescence signal

RT = room temperature

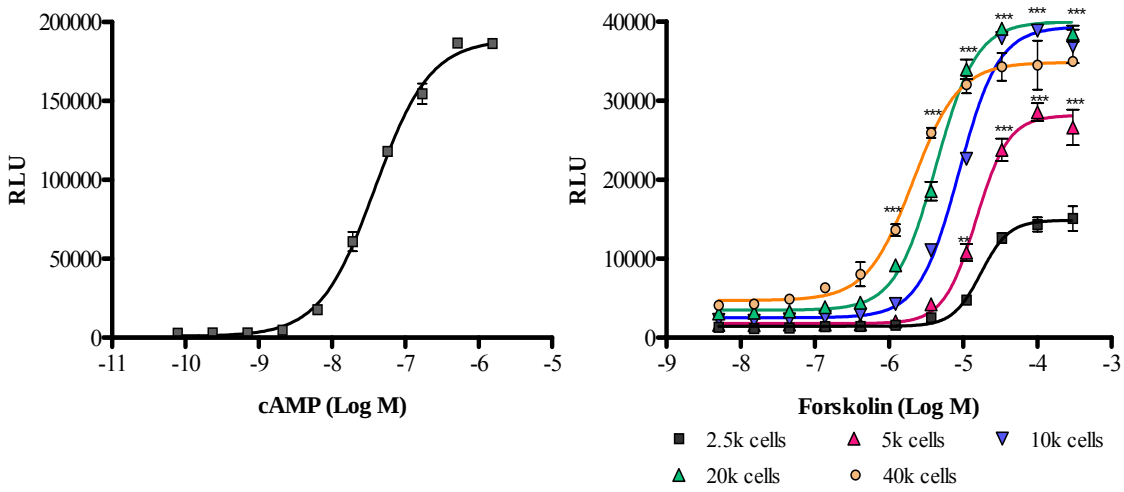


Figure 6.5: Standard curve for cAMP and forskolin-induced cell response data after subtraction of background relative luminescence units (RLU). Myocytes were seeded at different densities with optimal responsiveness determined in this preliminary assay at 20,000 cells/ well. Results are arithmetic means ± S.E. and two-way ANOVA with Bonferroni's *post-hoc* test showed statistical significance; **p<0.01; ***p<0.001 for RLU compared to 2,500 cells/ well.

6.19 Protocol for mRNA isolation

The reverse transcription polymerase chain reaction (RT-PCR) technique was applied to myocytes to analyse changes in gene transcripts. To avoid degradation by exogenous ribonucleases (RNases) and obtain high-quality RNA, the work area was cleaned thoroughly with 70% ethanol and RNaseZap solution (Ambion, Austin, Texas, USA). Gloves were changed on a regular basis and all plastics were supplied as sterile, DNase and RNase-free. In addition, glassware was either sprayed with RNaseZap or rinsed with 0.1% (w/v) diethyl pyrocarbonate (DEPC)-treated water (Sigma Chemicals Co, Poole, Dorset, UK) followed by autoclaving.

6.19.1 Effects of PGs and oxytocin on myocyte transcription

Myocytes were grown in a monolayer in 7x15cm^2 Petri dishes (passages 1-3; n=6). At about 80 percent confluence, myocytes were either exposed to vehicle (DMEM B), or to combinations of agonists and antagonists (Table 6.19). Although all cells were harvested at 24 hours incubation, oxytocin was added at 21 hours for a 3-hour exposure time.

Table 6.19: Myocytes were incubated with vehicle (DMEM B), oxytocin, the oxytocin antagonist atosiban, U46619 or the TP antagonist SQ29,548 at the following concentrations and incubation times before harvesting for RNA isolation.

Compounds	Concentration(s)	Incubation Time
Atosiban	10^{-5}M	24 hours
Oxytocin	10^{-6}M	3 hours
Atosiban + Oxytocin	10^{-5}M + 10^{-6}M	24 hours + 3 hours
U46619	10^{-6}M	24 hours
SQ29,548	10^{-6}M	24 hours
SQ29,548 + U46619	10^{-6}M + 10^{-6}M	24 hours + 24 hours

6.20 Purification of mRNA

mRNA was extracted using RNeasy Mini Kits (Qiagen, Valencia, California, USA) following the manufacturer's instructions. Before the start of experiments, RNA gels were prepared alongside the buffers.

6.20.1 RNA agarose gels

Electrophoresis tanks were cleaned with detergent solution, thoroughly rinsed with RNase-free water and then sprayed with ethanol and allowed to dry before use. Each RNA gel was prepared by mixing 0.5g agarose with 45ml of distilled water in a glass bottle. This solution was microwaved for 1-2 minutes to dissolve and sterilise the agarose mix. After cooling to about 65°C, 10x denaturing gel buffer at 1:10 dilution and 3µl ethidium bromide were added to the agarose gel mixture in a fume cupboard. This solution was immediately poured into an electrophoresis Sub-Cell (Bio-Rad, Hercules, California, USA) with 8 or 15 prong combs in place according to sample numbers. The final 1% agarose gel was covered and left to set without the addition of buffer.

6.20.2 Preparation of buffers

The running buffer 10X MOPS (Serological Corporation, Norcross Georgia, USA) and 10X TAE buffer (Cellgro Inc., Pensacola, Florida, USA) were diluted 1:10 with distilled water and stored for up to 1 week at 4°C before use. Buffer RPE concentrate was diluted 4-fold in 100% ethanol to obtain a working solution and stored at room temperature for use in all subsequent experiments. Buffer RW1 was provided as a working solution and stored at room temperature. To inhibit RNase function,

β-mercaptoethanol (β-ME) was diluted in buffer RLT at 10µl/ml in a fume hood. This was stored at room temperature for up to 1 month before use.

6.20.3 Cell lysis

After 24 hours incubation with vehicle or compounds (Table 6.19), the conditioned medium was removed from Petri dishes and myocytes were washed with PBS. The cells were then harvested with trypsin-EDTA (Section 6.11), transferred to individual centrifuge tubes and total cell number was determined using a Coulter Counter (Beckman-Coulter Z_2 Cell Counter). Trypsinised cell mixtures were subsequently centrifuged for 5 minutes at 300g (~12,000rpm) and the supernatant was completely aspirated. As total cell count was below $5x10^6$ cells (range: $1.0x10^{-6}$ to $4.4x10^6$ cells), the cell pellets were resuspended in 350µl buffer RLT containing β-ME. This lysed the cells before RNA isolation. The high guanine-thiocyanate content of this buffer also inactivated endogenous RNases to ensure purification of intact RNA.

6.20.4 Lysate homogenisation

The cell lysates were directly added into individual QIAshredder spin columns (Ambion, Austin, Texas, USA), placed in 2ml collection tubes and centrifuged for 2 minutes at 8,000g (~15,000rpm), 25°C. By shearing the high molecular weight genomic DNA and cellular components, the QIAshredder reduced the viscosity of lysates and improved RNA yields. Cleared lysates were then mixed in collecting tubes with 350µl ethanol to create appropriate conditions for RNA binding to the RNeasy membrane.

6.20.5 RNA elution

Samples were transferred to individual RNeasy spin columns, placed in 2ml collection tubes and centrifuged for 15 seconds at 8,000g, 25°C. After discarding the flow-through, 700µl buffer RW1 was added to the RNeasy spin columns and centrifuged for 15 seconds at 8,000g, 25°C to wash the spin column membrane. Two further washes were performed for each sample by the addition of 500µl buffer RPE to the RNeasy spin columns with centrifugation for 15 seconds and then for 2 minutes at 8,000g. This was followed by a further 2-minute centrifuge without buffer to dry the spin column membranes and remove any residual ethanol. After placing in a new 1.5ml collection tube, 50µl RNase-free water was directly added to each spin column membrane. To elute the RNA, tubes were centrifuged for 1 minute at 8,000g, 25°C. These samples were kept on ice or aliquoted and stored at -70°C to avoid RNA degradation.

6.21 Quantification of mRNA

After extracting RNA from smooth muscle cells, 1µl mRNA was diluted 100-fold with TE buffer (Qiagen, Valencia, California, USA) into a cuvette. Optical densities (OD) were read for each RNA sample on a Beckman DU640 spectrophotometer at 260nm (A_{260}) and 280nm (A_{280}). The cuvette was washed with RNase-free water and TE buffer was used as a blank between absorbance readings. To calculate 1) RNA content and 2) estimate RNA purity, the following equations were used:

1) RNA concentration (µg/ml) = [OD (A_{260}) x40 (constant) x100 (dilution factor)]

2) A_{260}/ A_{280} ratio

A ratio value of 1.5 and above was indicative of low RNA contamination. Denaturing agarose gel electrophoresis was also used to confirm the purity of RNA samples.

6.21.1 Examining RNA integrity

To eliminate DNA for RNA purification, deoxyribonuclease I (DNase I) was added to 10µg RNA samples on ice (Equation 1) together with DNase buffer and DEPC-treated water (Table 6.20). The tubes were incubated for 15 minutes at 25°C. The addition of 10µl of EDTA solution (25mM) to the reaction mixture inactivated the DNase I. This was heated for 10 minutes at 65°C on a heat block and transferred back to the ice. After removing the volume required for gel electrophoresis, samples were aliquoted for storage at -70°C for use in reverse transcription, prior to amplification.

Table 6.20: Solutions added in the following order to an RNase-free eppendorf on ice.

DEPC-treated water (µl)	10x DNase I buffer (µl)	10µg RNA sample (µl)	DNase I (µl)
*	10	= 1µg/ RNA (µg/ml) x 10	10

*DEPC-treated water (µl) = 100µl - 20µl - RNA sample (µl)

To examine RNA integrity, 10µl of DNase-treated RNA (1µg) was added to 10µl isopropanol and 1µl glycoblue (Ambion, Austin, Texas, USA). Following centrifugation for 10 minutes, 8,000g at 4°C, the supernatant was aspirated and the sample was air dried for 5 minutes. Blue RNA precipitates were resuspended with 3µl DEPC-treated water and 15µl NorthernMax Formaldehyde Load dye (Ambion, Austin, Texas, USA) on ice. To denature the RNA, these samples were incubated at 70°C, placed on ice for 2-3 minutes and briefly spun.

Gel electrophoresis was run in 1X MOPS buffer at 80V/ cm after loading the DNase-treated samples into each well. RNA was visualised by ethidium bromide staining on a UV light box. The larger ribosomal RNA bands 28S and 18S at an intensity ratio of 2:1 indicated that cellular mRNA was intact (Figure 6.6). The RNA was then reverse-transcribed into complementary DNA (cDNA); this was used as a template for PCR.

6.21.2 Reverse transcription and PCR

To perform RT-PCR, sense and antisense primers were designed using the computer software D-LUX programme (Invitrogen, Carlsbad, California, USA). PCR primer pairs were 23-25 nucleotides in length with similar A/T and G/C content for optimal annealing conditions (Table 6.22).

DNase-treated RNA was converted into cDNA using the Platinum Taq Maloney murine leukaemia virus (M-MLV) reverse transcription system (Invitrogen, Carlsbad, California, USA). Each reaction mix, prepared on ice in a 0.5ml eppendorf tube in a reaction volume of 30µl contained the following: SuperScript III reverse transcription buffer (deoxyribonucleotide trisphosphates (dNTP; 0.4mM), magnesium sulphate ($MgSO_4$; 6mM)), RNase-free water, sense and antisense primer pairs (10µM each), total RNA (100ng), 2x reaction mix and 200IU M-MLV Superscript III RT Platinum Taq (Table 6.21). Control RNA samples with Supermix of Platinum Taq M-MLV without the reverse transcription enzyme were included to confirm that no genomic DNA contamination was present.

The tubes were placed in a PCR Sprint thermal cycler (Thermo Scientific, New York, USA) and set to run the reverse transcription programme: incubation for 30 minutes at 50°C to allow cDNA synthesis from mRNA by reverse transcription. This was followed by denaturing samples at 94°C for 2 minutes to inhibit reverse transcription

activity. cDNA amplification was subsequently carried out by 35 cycles of denaturing at 94°C for 15 seconds, annealing at 57°C for 30 seconds and elongation at 68°C for 30 seconds. PCR products were finally heated at 68°C for 7 minutes and either stored at 4°C or placed on ice for immediate analysis by agarose gel electrophoresis.

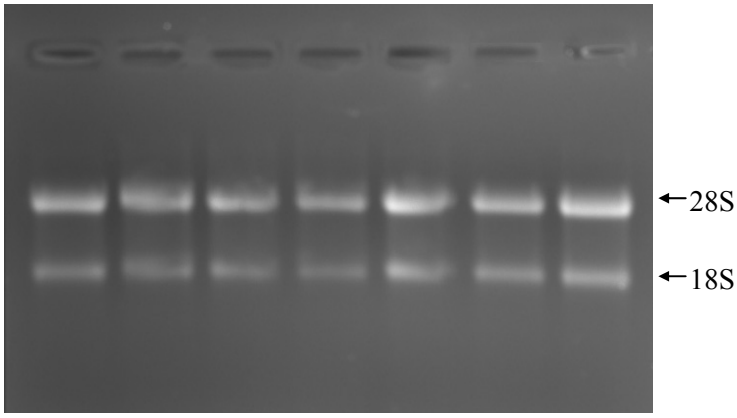


Figure 6.6: Intact RNA (1μg) samples from treated myocytes were run on a 1% denaturing agarose gel. The 28S and 18S ribosomal RNA bands were visualised on a light box with an even background smear of cellular mRNA for each sample.

Table 6.21: The following solutions were added to 0.2ml thin-wall PCR tube on ice:

Sample	**Control (μl)**	**β-actin (μl)**	**PGs (μl)**
Super mix of Platinum Taq	25	0	0
2x Reaction mix	0	15	15
RNase-free water	3	10	10
10μM Human β-actin (sense)	1	1	0
10μM Human β-actin (antisense)	1	1	0
10μM Human PG primers (sense)	0	0	1
10μM Human PG primers (antisense)	0	0	1
Human mRNA 100ng/μl	0	1	1
SuperScript III RT Platinum Taq	0	2	2

Table 6.22: PCR primer sequences. The accession number of each sequence for the EMBL/ Genbank/ DDBJ data library is provided.

PCR primers		Sequence	nt	Size	Accession N^o
β-actin	s	5'-GTA CCA CTG GCA TCG TGA TGG AC-3'	513	403	NM_001101
	as	5'-GAG TTG AAG GTA GTT TCG TGG ATG-3'	915		
EP_1	s	5'-GGT ATC ATG GTG GTG TCG TGC ATC-3'	875	238	L22647
	as	5'-CAG GAT GTG GTT CCA GGA GGC AAG-3'	1112		
EP_2	s	5'-TGG CAT CTG ACT GTG TAG AAC AGG-3'	876	336	NM_000956
	as	5'-CTC CGC TCC TGA TAA TGA TGT TGA-3'	1211		
EP_3	s	5'-CTC CGC TCC TGA TAA TGA TGT TGA-3'	998	197	NM_198716
	as	5'-CTT CTC CGT GTG TGT CTT GCA GTG-3'	1194		
EP_4	s	5'-GAT GGT CAT CTT ACT CAT TGC CAC-3'	1309	186	NM_000958
	as	5'-CTT GGC TGA TAT AAC TGG TTG ACG-3'	1494		
DP	s	5'-GTG CTT TAT CCA GAT GGT CCA CG-3'	595	377	U31332
	as	5'-CCA TGA GGC TGG AGT AGA GCA CAG-3'	971		
$CRTH_2$	s	5'-CAA CAC CAG CAT CCG CTA CAT CGA-3'	84	197	AB008535
	as	5'-CAC GAA GAG GAT GAC TCC ATT CTC-3'	280		
FP	s	5'-CAG CAC AGA CAA GGC AGA TCT CAT C-3'	772	384	NM_000959
	as	5'-GAT TCC ATG TTG CCA TTC GGA GAG-3'	1155		
IP	s	5'-CCT CTG CTC CTG TGG GAA AGG AG-3'	1063	130	L29016
	as	5'-GCT TCT GCT TTG GAC GAC GTT CC-3'	1192		
TP	s	5'-GTG GAG ATG ATG GCT CAG CTC CTG-3'	735	290	NM_201636
	as	5'-CAG CAC TGT CTG GGC GAT GAA GAC-3'	1024		
Oxytocin receptor	s	5'-TGG CCT CCT ACA CGT ACT TCT ACC-3'	1891	235	NM_000916
	as	5'-TGG TCA CCA ATC CTA TAT TTA CCG-3'	2125		
Rho A	s	5'-GGT TGG GAA TAA GAA GGA TCT TCG-3'	233	339	L25080
	as	5'-GCC ATA TCT CTG CCT TCT TCA GGT-3'	571		
ROCK I	s	5'-TTA AGA ATC TAA CCC TGC AAC TGG A-3'	2917	372	NM_005406
	as	5'-CAA TTC ATT TTG TAA CAA CAG CCG-3'	3288		
ROCK II	s	5'-GTG TGT GGC AGT ATT TTA GTA CCG-3'	5481	380	NM_004850
	as	5'-TTT GAG GCC ACT TCT TCC CAG TTG-3'	5860		
MLCK	s	5'-CTC TAT GAC GCC TTC GAG AGC AAG-3'	1609	306	NM_182493
	as	5'-GTG GTA CTT CTC ATC TGT GAT CCG-3'	1914		
IP_3 receptor	s	5'-TTT CGA GCT AGA TCC CAC CAC TCT-3'	972	405	D26070
	as	5'-ATT AGT ACA TAG GTG TCT GAG CCG-3'	1376		
Telokin	s	5'-GAA CTT TAC AAG GGT GTG TTG GCG-3'	772	320	U40712
	as	5'-TGG CCT CCT ACA CGT ACT TCT ACC-3'	1091		

s: sense; as: antisense; nt: nucleotides

6.22 DNA electrophoresis

RT-PCR products were analysed using a 3% pre-cast ready agarose gel (Bio-Rad, Hercules, California, USA), which was placed in an electrophoresis tank containing 1X TAE buffer. A total of 10µl of each PCR product was combined with 2µl of DNA loading dye (Ambion, Austin, Texas, USA) to aid loading and to monitor the progression of the product through the gel during electrophoresis. Once the PCR products and negative controls were carefully loaded into the wells, 10µl of standard DNA fragments (1Kb plus DNA ladder; Invitrogen, Carlsbad, California, USA) was loaded on the left side of the gel for sizing. The tank was set to run at a constant 100V/ cm for approximately 25 minutes when the DNA fragments were sufficiently resolved. Images of PGs, oxytocin and protein DNA products were then captured using a UV transilluminator UVP at a wavelength of 312nm and analysed with Fluor Chem H_2 software (Alpha Innotech, Leandro, California, USA).

6.23 Summary diagram of cell-based assays

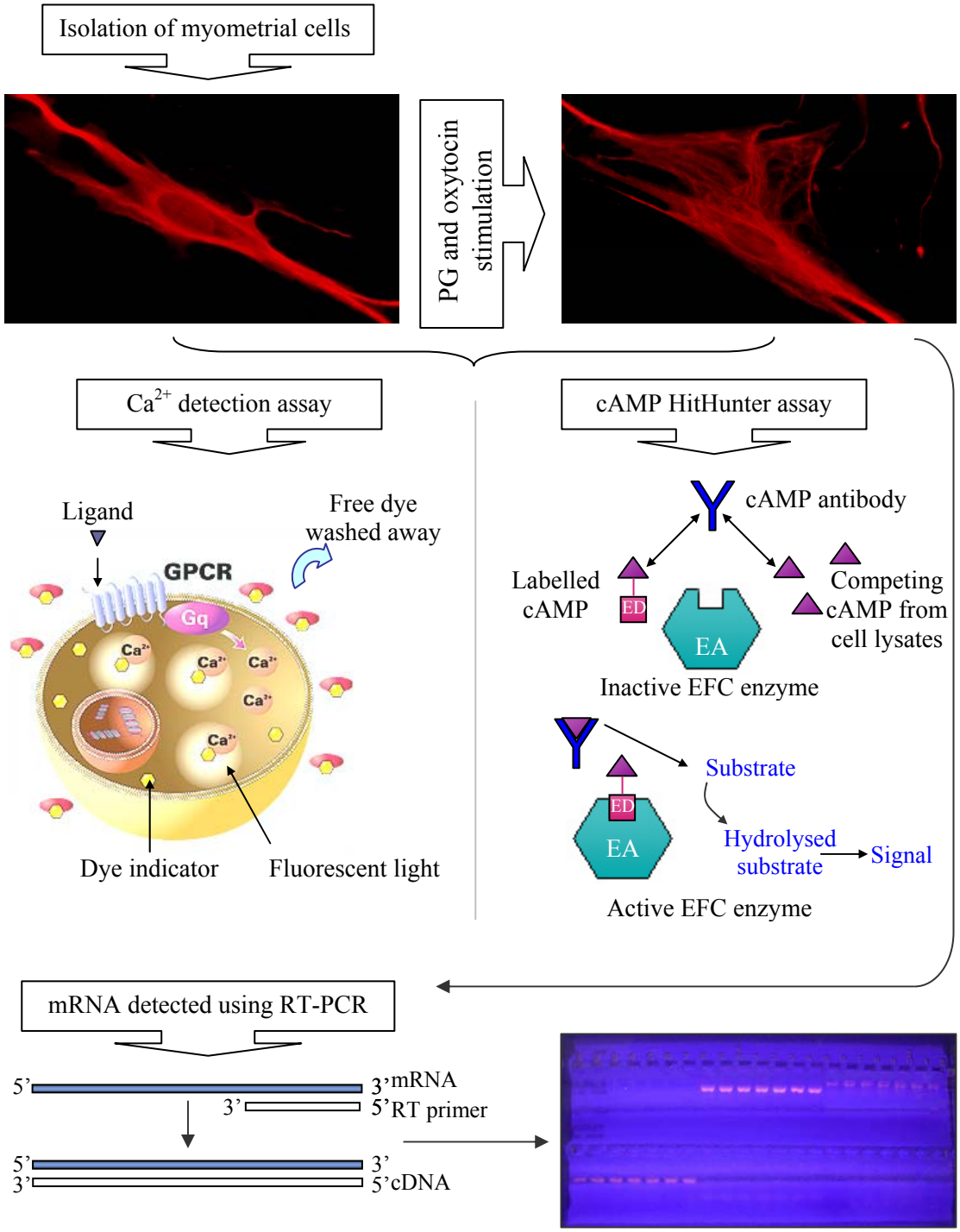


Figure 6.7: An overview schematic principle of the assays performed to develop an *in vitro* cell culture model for myocytes and fibroblasts. To isolate and characterise cell populations, enzymatic digestion, differential adhesion and immunocytochemistry techniques were performed on myometrial tissue taken at term pregnancy. Changes in Ca^{2+}, cAMP and gene transcripts were assessed using the FLIPR-Tetra, a competitive cAMP assay based upon the complementation (EFC) of enzyme fragments (EA and ED) and RT-PCR respectively. Measurements were used to screen the effects of uterotonic agents on normal and pathological downstream signalling cascades.

Chapter 7: Uterine myocytes & fibroblasts

Signal transduction in myometrial cells at term pregnancy

7.1 Introduction

The uterus adapts to the physiological process of pregnancy by achieving enormous expansion whilst sustaining muscle tone (Yu & Lopez Bernal, 1998). Such dynamic tissue remodelling depends on cell hyperplasia and hypertrophy, alongside smooth muscle cell (myocyte) and fibroblast interactions (Varayoud *et al*., 2001; Ono *et al*., 2007; MacCannell *et al*., 2007; Wu *et al*., 2008; Shynlova *et al*., 2010). In response to mechanical stretch and biochemical stimuli, myometrial smooth muscle exhibits considerable plasticity, mediated in part by actin polymerisation (Taggart & Morgan, 2007) and the recruitment of additional signalling proteins (Berridge, 2008). Gestational-related changes in the temporal and tissue-specific expression of PG and oxytocin receptors then contribute to the transition from uterine quiescence to contractility (Charpigny *et al*., 2003; Grigsby *et al*., 2006). This activates secondary signalling cascades, principally targeting cAMP, IP_3 and intracellular Ca^{2+} pathways. The cAMP system suppresses myometrial activity, whereas IP_3 generation and Ca^{2+} release augment contractions. Although PGs and oxytocin participate in cellular events, the signal transduction systems that orchestrate these striking changes at term gestation are not fully understood.

This chapter focuses on the structural, functional and molecular aspects of myometrial cells at term pregnancy. As well as characterising primary cell cultures, PG and oxytocin-induced activation of signalling pathways involving Ca^{2+} and cAMP were studied relative to receptor mRNA. By using this cell culture model, high throughput screening of potential tocolytics could be performed. In particular, the nature of EP_2-

based interactions that antagonise oxytocic contractile effects (Duckworth *et al*., 2002) would be investigated to develop a PG based therapy for preterm labour. Alongside responses in functional studies, these results would help to elucidate the contribution of receptors or messenger targets in regulating myometrial contractility.

Chapter 7.2: Results

7.3 Immunostaining of uterine smooth muscle cells and fibroblasts

Morphologic features of uterine cell populations were assessed using immunoassay and bright field images. The ultrastructure was identified with markers for smooth muscle actin, α-actinin, vimentin and desmin, generally used to characterise myometrial cells (Hongpaisan, 2000; Lopez Bernal & TambyRaja, 2000; Montes *et al*., 2002). In myometrial sections, positive expression and uniform distribution of these cytoskeletal elements were demonstrated (Figure 7.1). However, as clarity was compromised by tissue thickness, the structural components of monolayer primary cultures of myometrial cells were visualised.

Images of myocytes and fibroblasts cultured by differential adhesion techniques were captured under a phase contrast microscope (Figure 7.2). Smooth muscle cells and fibroblasts each displayed a central oval nucleus containing several nucleoli. In their cytoplasm, bright field pictures revealed prominent dense bodies located throughout the bundles of thin filaments. These were interspersed with less discernable large areas of ribosomes, rough endoplasmic reticulum, mitochondria and Golgi complexes. Despite their characteristic flat, elongated and spindle-shaped morphologies, myocytes tended to be about 5-10μm narrower than fibroblasts. Likewise, the protruding flat sheets of membrane-enclosed cytoplasm, *lamellipodia*, were observed at polar ends in myocytes but were more spread in fibroblasts. Due to these subtle differences, immunocytochemistry was performed to distinguish cell types.

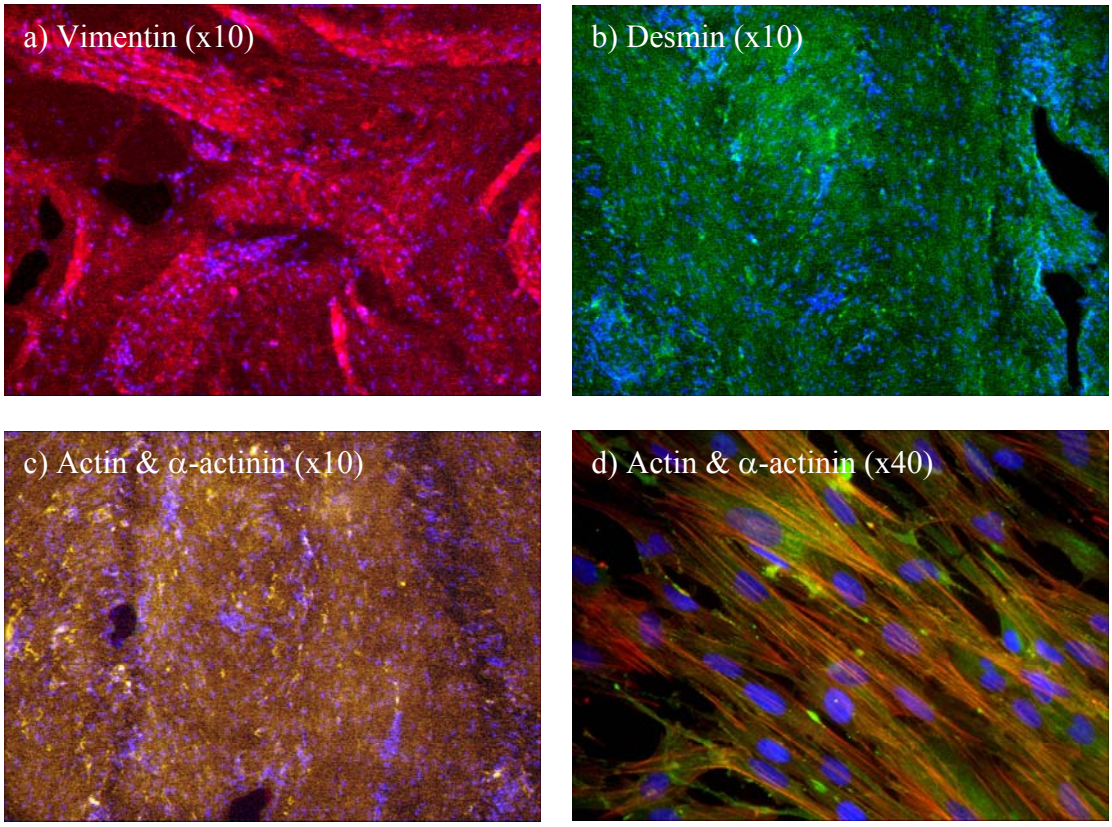


Figure 7.1: Typical micrographs showing sections of human myometrium taken at term pregnancy. Using immunohistochemistry techniques, sections were stained with a) vimentin (red) and b) desmin (green) at x10 magnification. Actin (red) and α-actinin (green) were also imaged at c) x10 and d) x40 magnifications. Staining by the fluorescent DNA-binding probe DAPI (blue) indicated the uniform positive staining of cell filaments and proteins throughout myometrial tissue sections.

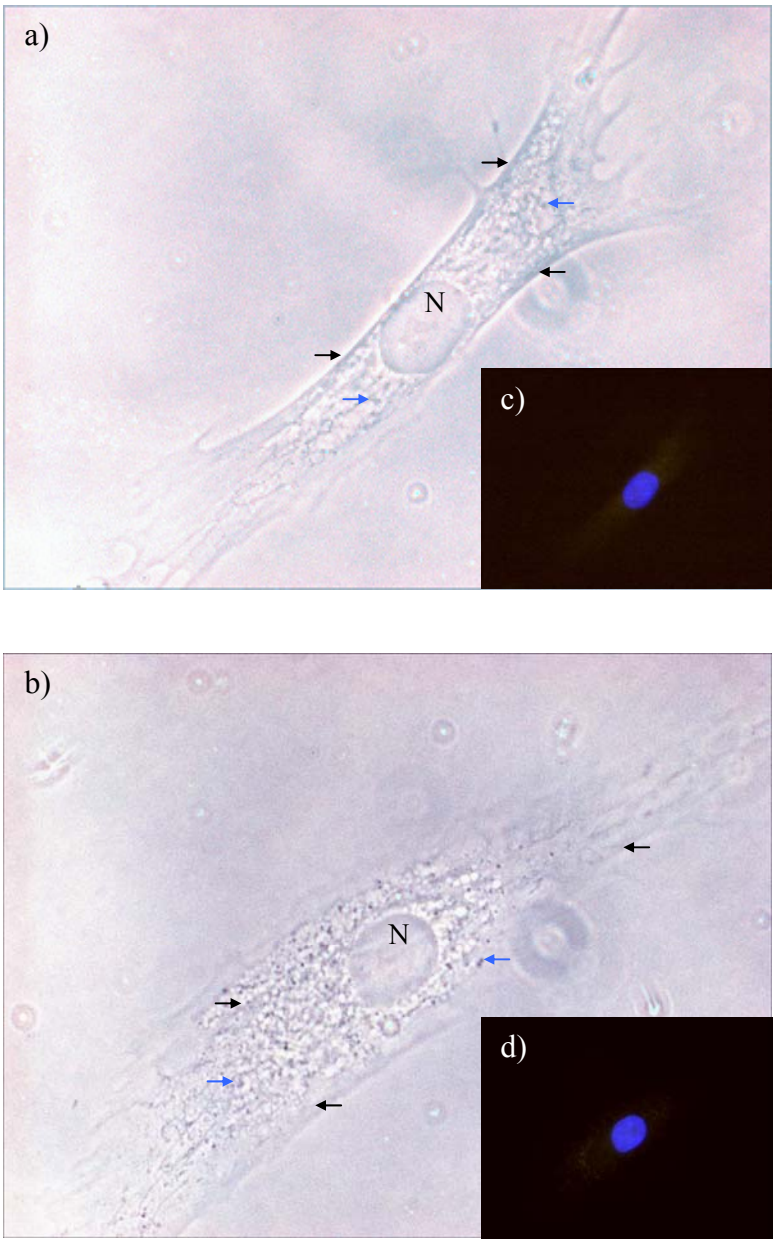


Figure 7.2: Phase contrast micrographs of primary a) smooth muscle and b) fibroblast cells cultured from human myometrium at term pregnancy (passages 1-2). Cells were separated by differential adhesion techniques and photographed at x100 magnification. Bright field images of cells show nuclei (N), black arrows indicate bundles of contractile filaments, and blue arrows more prominent dense bodies dispersed throughout the cytoplasm. In control fluorescent images of the same cells (c & d), nuclei were labelled with the fluorescent DNA-binding probe DAPI (blue). The green background staining was barely visible due to the addition of secondary but not primary antibodies.

7.4 Positive staining for α-actinin and actin

All nuclei were labelled with the fluorescent DNA-binding probe DAPI (blue). In the absence of primary antibodies, negative controls displayed very low background fluorescence (Figure 7.2). Even so, myocytes and fibroblasts showed positive double immunostaining for smooth muscle actin and α-actinin (Figure 7.3). The peripheral region largely consisted of a dense meshwork of thin actin filaments. These microfilaments were arranged into parallel bundles with fewer actin filaments in the central region close to the nucleus. As a cross-linking protein, α-actinin was highly dispersed throughout the cytoplasm and staining was punctate. Towards the nucleus, the co-localisation of α-actinin with actin was evident in merged images for myocytes and fibroblasts. For each cell phenotype, staining intensity and alignment of cytoskeletal structures were similar. Although colour intensity related to the plane of focus, mixed cell populations and myometrial tissue in explant cultures were also positive for α-actinin and actin (Figure 7.5).

7.5 Positive staining for intermediate filaments

Immunostaining of the two intermediate filaments vimentin and desmin were analogous in both primary myocyte and fibroblast cultures (Figures 7.4 & 7.5). The cells expressed abundant interconnected networks of vimentin with branches extending from the nuclear envelope to junctions in the lipid membrane. Distinguished in this longitudinal arrangement, all isolated myometrial cells and explant tissue stained positive for vimentin.

Desmin was also detected in the cytoplasm of myometrial cells, even in their proliferating state. With attachment to the dense plaques, desmin filaments were closely packed in a more centrally located position when cells were elongated. Fewer

specks of desmin were also present at the cell periphery in both smooth muscle cells and fibroblasts. In myometrial explants, due to the difficulties in focusing on raised and flat surfaces together, the tissue was brighter than desmin-labelled cells.

7.6 Positive staining for fibroblast surface proteins

To better distinguish between smooth muscle cells and fibroblasts, immunostaining was performed using a fibroblast antigen. This monoclonal antibody was reported to recognise fibroblast surface proteins on human fibroblasts as well as macrophages and blood monocytes (Singer *et al*., 1989; Esterre *et* al., 1992; Ronnov-Jessen *et al*., 1992). Even so, this study showed identical staining patterns in myocytes, fibroblasts and explant tissues (Figures 7.4 & 7.6). The punctate staining of scaffold proteins extended over the surface to the periphery of each cell. Searches through the literature indicated that specific conjugation of this fibroblast-associated marker and alternative smooth muscle specific antibodies, such as smoothelin, had not previously been tested to distinguish any cultures of both myocytes and fibroblasts in the uterus. As a result, differences in proliferation rate, morphology and intracellular events were used to characterise myometrial cell types.

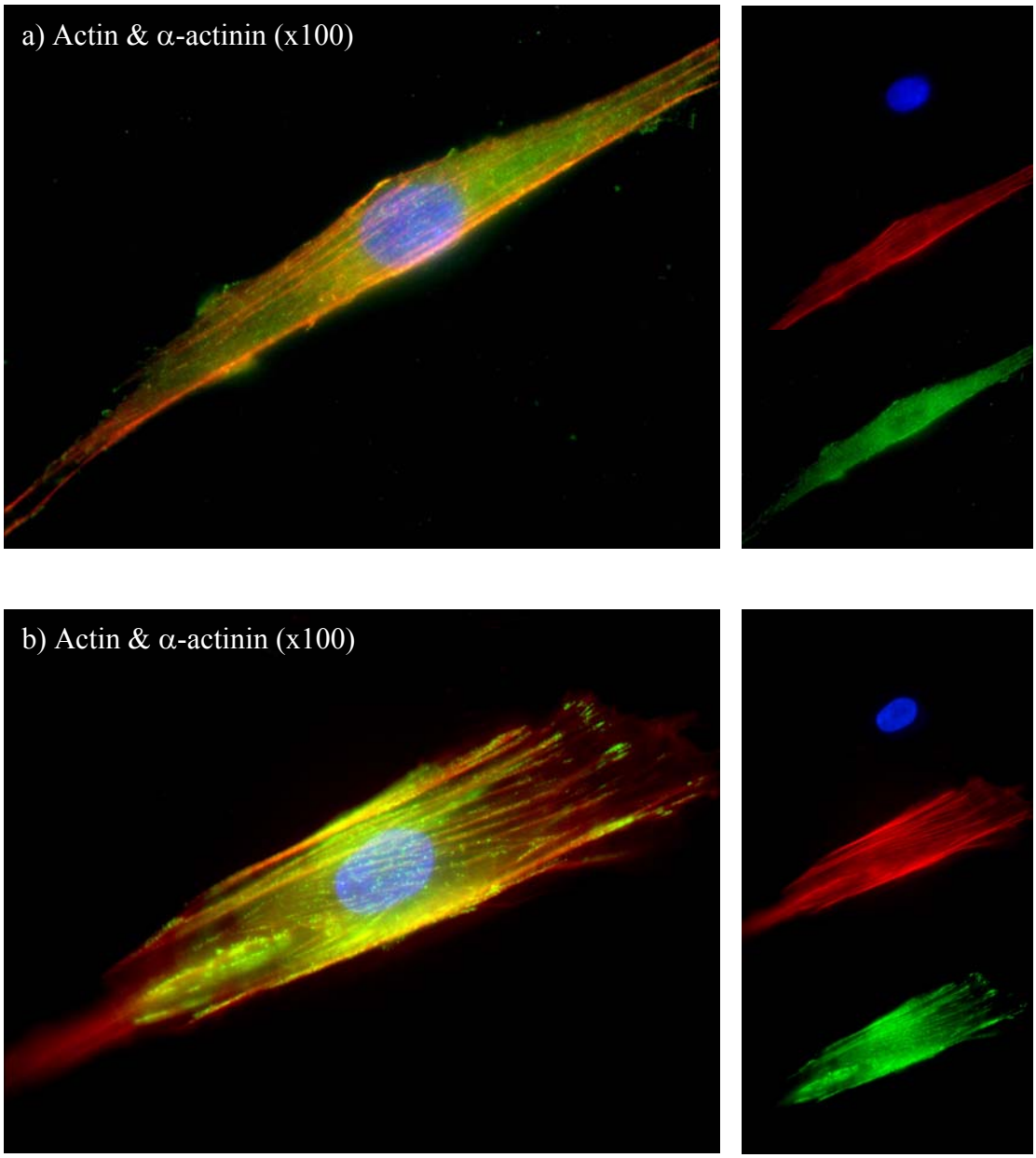


Figure 7.3: Phase contrast micrographs showing the co-expression of longitudinal actin filaments and α-actinin in primary cultures of a) myocytes and b) fibroblasts. Cells were cultured from myometrium taken at term pregnancy and separated by differential adhesion techniques (passages 1-2). Actin filaments (red), α-actinin (green) and cell nuclei, labelled with the fluorescent DNA-binding probe DAPI, (blue) were individually photographed at x100 magnification before images were merged.

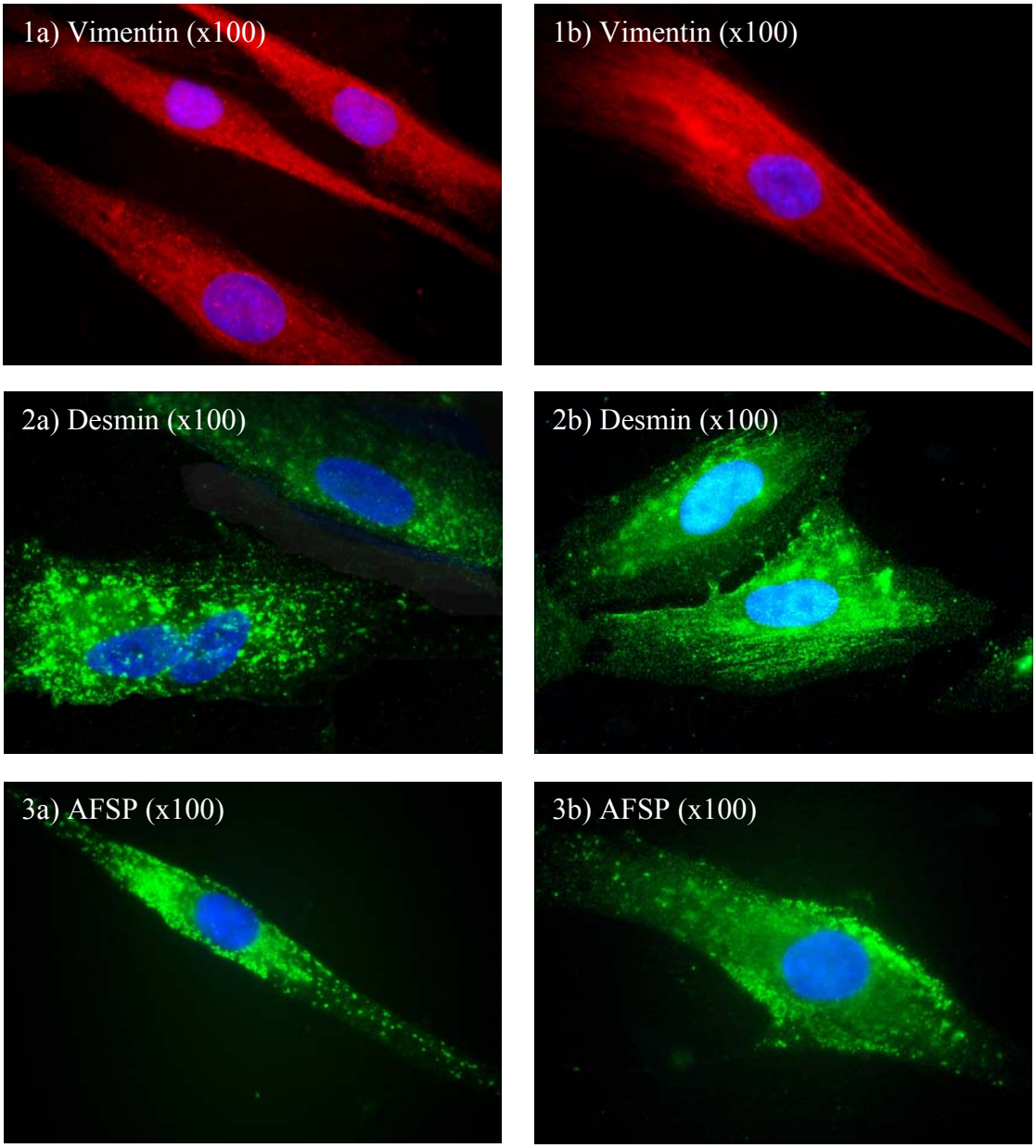


Figure 7.4: Phenotypic assessment of a) smooth muscle cells and b) fibroblasts cultured from myometrium taken at term pregnancy and separated by differential adhesion techniques (passages 1-2). Positive expression of 1) filamentous vimentin, 2) desmin and 3) anti-human fibroblast surface protein (AFSP) were detected in both cell types using immunocytochemistry. All nuclei were labelled with the fluorescent DNA-binding probe DAPI (blue). Photographs of these cytoskeletal proteins and nuclei were taken at x100 magnification and superimposed.

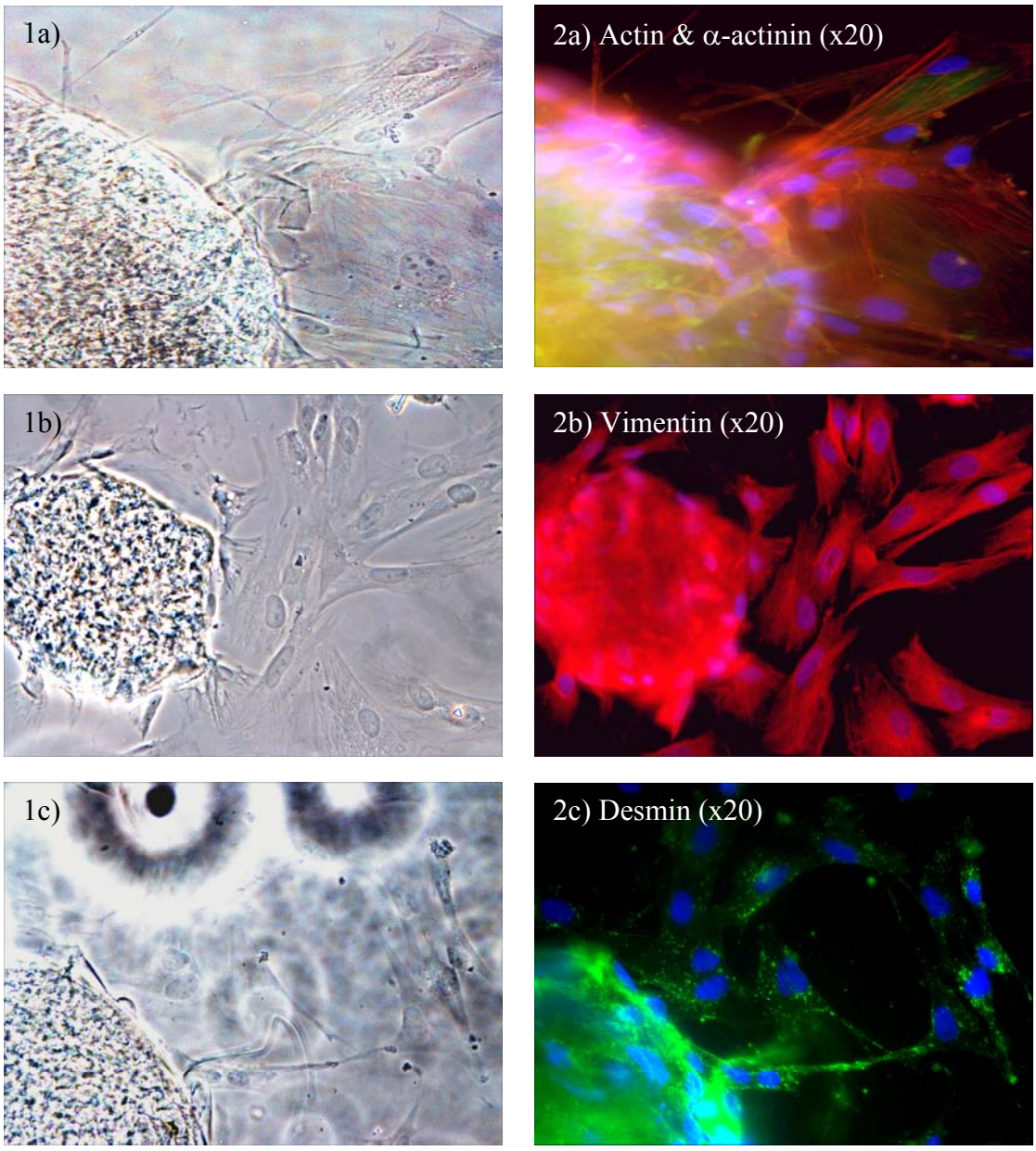


Figure 7.5: Phase contrast micrographs showing 1) bright field and 2) fluorescent images of primary cells cultured for 7-10 days from myometrial explant tissue taken at term pregnancy. Positive expression of a) actin (red) and α-actinin (green), b) vimentin and c) desmin filaments were detected in cells and explant tissues using immunocytochemistry. All nuclei were labelled with the fluorescent DNA-binding probe DAPI (blue). Photographs of these cytoskeletal proteins and nuclei were taken at x20 magnification and superimposed.

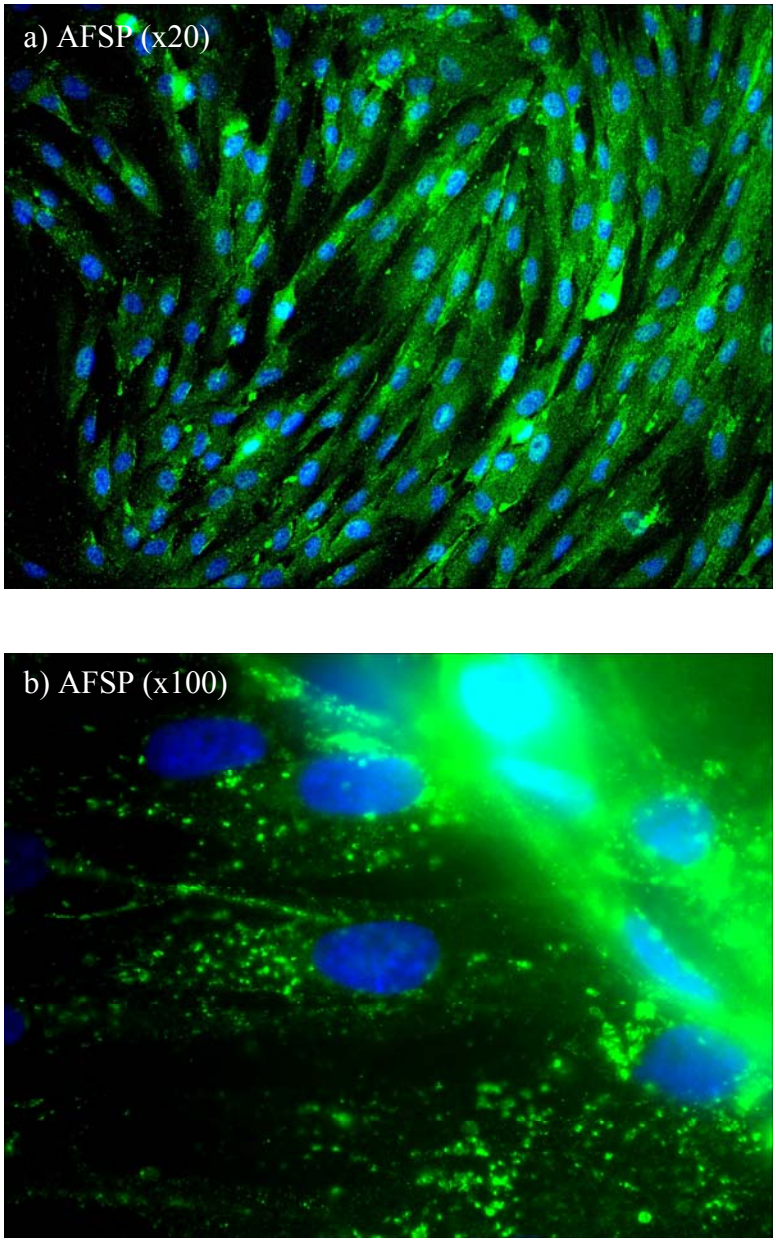


Figure 7.6: Micrographs showing myometrial cells formed from explant tissue after 14 days in primary culture. Uterine tissue was excised at term pregnancy. A consistent pattern of reactivity for anti-human fibroblast surface protein (AFSP) was expressed in all cells and explant tissue using immunocytochemistry. Nuclei were labelled with the fluorescent DNA-binding probe DAPI (blue) and photographs of the cells taken at a) x20 and b) x100 magnifications were superimposed.

Chapter 7.7: Results - FLIPR

7.8 Optimising agonist-induced Ca^{2+} readings

Receptor-mediated mobilisation of Ca^{2+} was assessed using the FLIPR-Tetra (Molecular Devices, Sunnyvale, California, USA). In preliminary studies to optimise cell parameters, myocytes were seeded in 96-well plates overnight at 15k, 30k and 60k cells/ well (Figures 7.7 and 7.8). Stimulation with vehicle (DMEM D), PGE_2 and U46619 for 4 minutes evoked little flux in intracellular-free Ca^{2+}. Even so, $PGF_{2\alpha}$ increased the Ca^{2+} signal by nearly 2-fold ($p<0.05$), whilst exposure to oxytocin significantly augmented Ca^{2+} transients at all seeding densities ($P<0.001$). Since maximum responses were observed with 30k cells/ well, this plating density was continued for all FLIPR assays performed in 96-well plates.

7.9 Responsiveness to PGs and oxytocin

When oxytocin was assayed separately, the concentration-dependent increases in Ca^{2+} flux to PGE_2, $PGF_{2\alpha}$ and U46619 were observed above 10^{-7}M (Figure 7.9). Whilst vehicle evoked negligible Ca^{2+} release, exposure to $PGF_{2\alpha}$ (10^{-5}M) peaked at 316 ± 26.75 FLU counts; this signal was attenuated by 17 and 93 percent in response to U46619 and PGE_2 respectively. As oxytocin-induced Ca^{2+} transients were more robust and reached better limits of detection at 1435 ± 53.20 FLU counts, oxytocin was used as the predominant agonist to study intracellular Ca^{2+} oscillations.

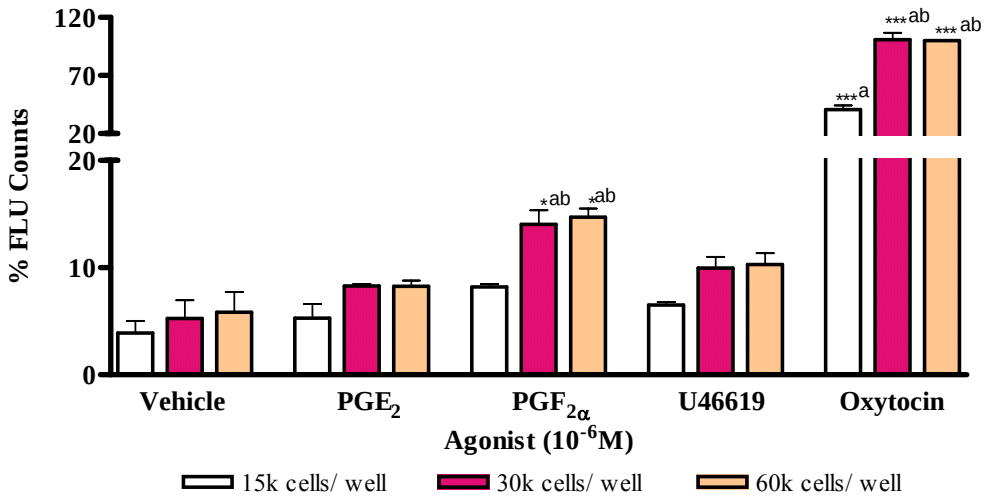


Figure 7.7: Primary myocytes cultured from isolated myometrium obtained at term pregnancy were seeded overnight at densities of 15k, 30k and 60k cells/ well in 96-well plates (n=4). After loading with fluo-4 AM dye (2μM), cells were exposed to vehicle (DMEM B), PGE_2, $PGF_{2\alpha}$, U46619 or oxytocin at 10^{-6}M for 4 minutes. Ca^{2+} transients, indicated by emitted fluorescence, were recorded using the FLIPR. Data are arithmetic means ± S.E. and statistics were performed using univariate ANOVA with Bonferroni's *post-hoc* adjustment; *p<0.05; ***p<0.001 elevation in Ca^{2+} flux compared with [a]vehicle and [b]15k cells/ well for the same agonist.

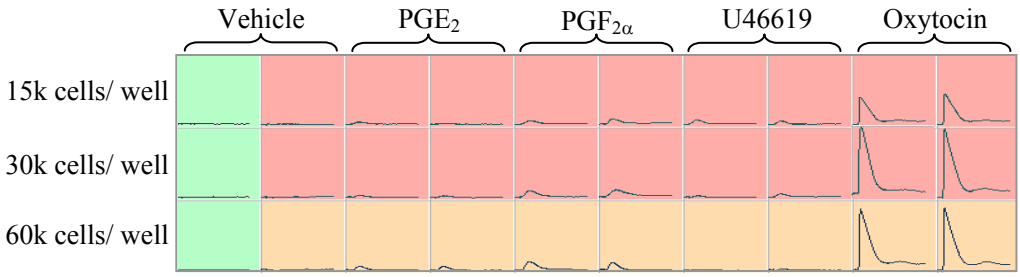


Figure 7.8: Typical Ca^{2+} mobilisation in primary human myocytes seeded at 15k, 30k and 60k cells/ well and incubated with the Ca^{2+} sensitive fluo-4 AM dye. The screenshot traces recorded on the FLIPR represent myocyte responses to PG and oxytocin agonists (10^{-6}M) with oxytocin challenge inducing the largest Ca^{2+} release.

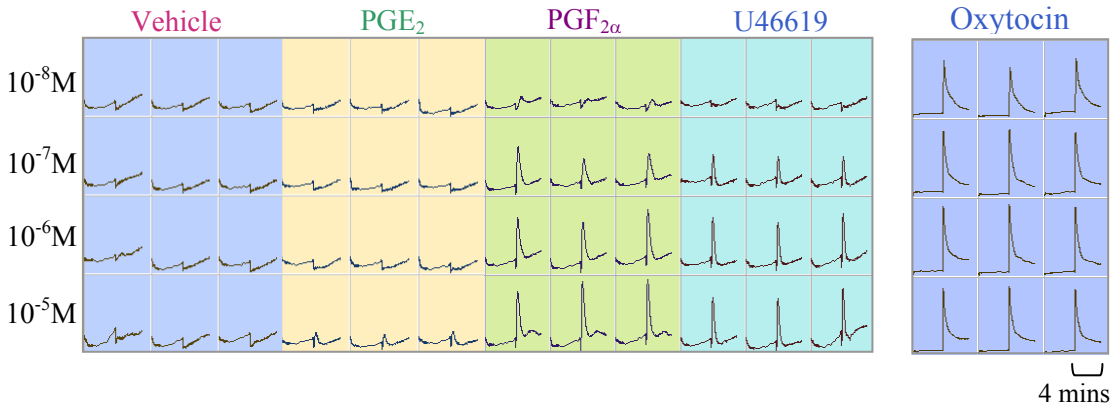


Figure 7.9: Vehicle (DMEM D) and concentration-dependent changes in intracellular-free Ca^{2+} for PGE_2, $PGF_{2\alpha}$, U46619 and oxytocin using FLIPR. Primary myocytes from term pregnant donors were seeded at 30,000 cells/ well and Ca^{2+} was detected using the fluo-4 AM indicator dye. Signals peaked at 316 ± 26.75 and 1435 ± 53.20 FLU counts for plates run in the absence and presence of oxytocin respectively.

7.10 384-well plate validation

In later experiments, the cell seeding density was also validated for 384-well plates. To determine cell titre, several dilutions of myocytes and fibroblast populations were serially prepared (Figures 7.10 & 7.11). Responses to oxytocin (10^{-6}M) peaked at 5k cells/ well before the signal plateaued ($p<0.001$). Accordingly for optimal Ca^{2+} recordings, the cell seeding density in 384-well plates was adjusted to 5k cells/ well.

7.11 Intracellular-free Ca^{2+} in myocytes and fibroblasts

When directly compared between cell types, oxytocin-induced Ca^{2+} mobilisation was nearly 2-fold higher in myocytes than fibroblasts ($p<0.001$). Therefore, to better distinguish Ca^{2+} release in fibroblasts, subsequent assays for each cell population were performed separately.

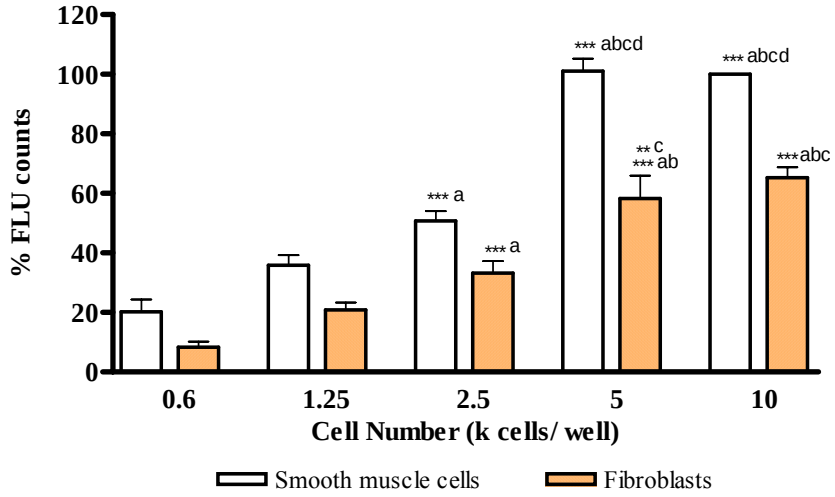


Figure 7.10: Intracellular Ca^{2+} flux induced by oxytocin (10^{-6}M) in primary myocytes and fibroblasts cultured from myometrial biopsies (n=8). Cells were seeded overnight at titrations of 600 to 10k cells/ well in 384-well plates. After loading with fluo-4 AM dye, responses to oxytocin were read using the FLIPR and calculated as % change in fluorescence units over baseline (% FLU). Statistics were performed using univariate analysis with Bonferroni's *post-hoc* test; **p<0.01; ***p<0.001 compared to cell seeding densities of [a]0.6k, [b]1.25k and [c]2.5k cells/ well and between [d]myocyte and fibroblast responses.

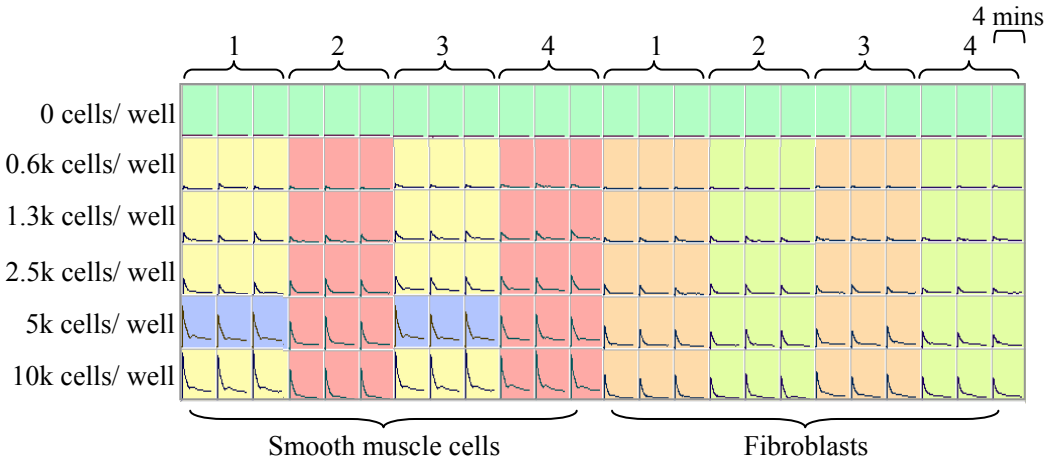
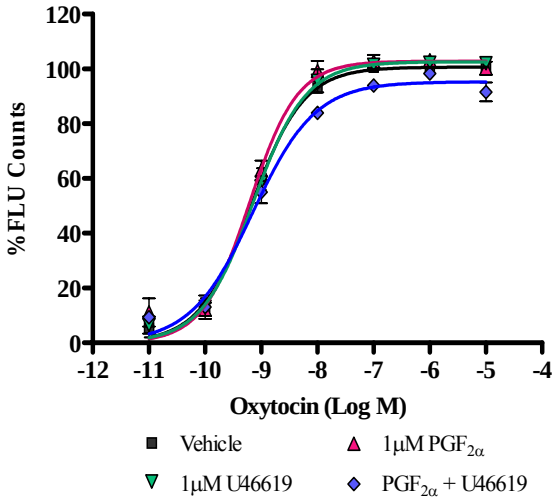


Figure 7.11: Changes in intracellular free Ca^{2+} after addition of oxytocin at 10^{-6}M in duplicate to fluo-4 AM loaded primary myocytes (n=4) and fibroblasts (n=4) from term pregnant donors recorded on the FLIPR. Typical Ca^{2+} profiles for smooth muscle cells were higher than fibroblasts over 4 minutes of oxytocin stimulation.

7.12 Pre-incubation with FP and TP agonists

Although $PGF_{2\alpha}$ (10^{-6}M; $p<0.001$) and U46619 (10^{-6}M) challenge each mobilised Ca^{2+} (Figures 7.7, 7.8 & 7.9), pre-incubation with these uterotonic agents did not affect the concentration-dependent signals evoked by oxytocin in myocytes (pEC_{50}: 9.17 ± 0.02M) and fibroblasts (pEC_{50}: 8.98 ± 0.03M; Figure 7.12). In smooth muscle cells, even the 8.6 percent attenuation in maximal oxytocin-induced Ca^{2+} signals after exposure to both $PGF_{2\alpha}$ and U46619 was not significant (F (1, 48) = 2.87; ns).

a) smooth muscle cells



b) fibroblasts

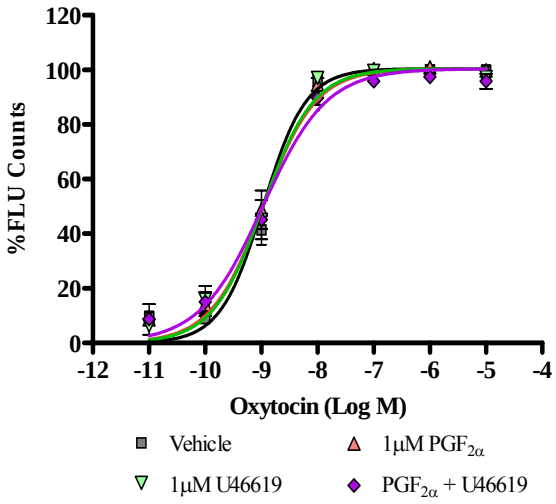


Figure 7.12: Concentration-effect curves for oxytocin (10^{-11}M to 10^{-5}M) in combination with vehicle, $PGF_{2\alpha}$ (10^{-6}M) and U46619 (10^{-6}M) for a) myocytes and b) fibroblasts. Primary cells cultures from myometrial tissue taken at term pregnancy (n=4) were incubated with the Ca^{2+} indicator fluo-4 AM dye (2μM) and washed. Changes in fluorescence counts from baseline (% FLU counts) were detected using the FLIPR-Tetra. Pre-incubation with the FP and TP agonists did not evoke a synergistic effect on oxytocin-induced Ca^{2+} transients.

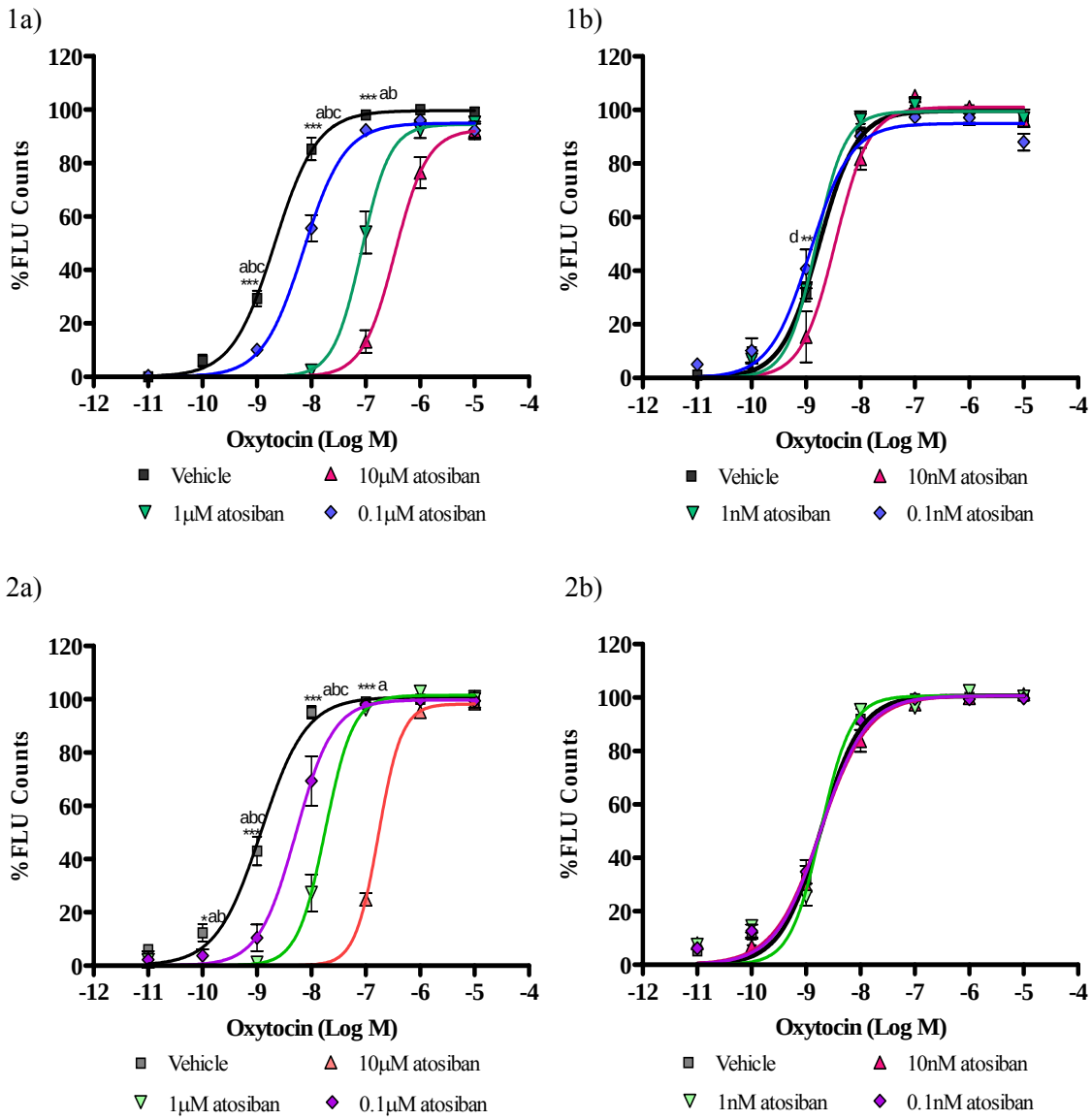


Figure 7.13: Changes in intracellular free Ca^{2+} by atosiban on oxytocin-induced Ca^{2+} curves in human myometrial 1) smooth muscle cells and 2) fibroblasts. Cells were loaded with the Ca^{2+} indicator fluo-4 AM dye (2μM), washed and exposed to vehicle (DMEM D) or atosiban at concentrations of a) 10^{-7}M to 10^{-5}M or b) 10^{-10}M to 10^{-8}M for 4 minutes before stimulation with oxytocin (10^{-11}M to 10^{-5}M) for 4 minutes. Data are expressed as arithmetic means ± S.E. and multivariate analysis showed statistical significance; *p<0.05; **p<0.01; ***p<0.001 rightward shift for atosiban at $^{a}10^{-5}$M, $^{b}10^{-6}$M, $^{c}10^{-7}$M and $^{d}10^{-8}$M compared to vehicle.

Table 7.1: Mean pEC_{50} values (M) for concentration-effect curves to oxytocin (10^{-11}M to 10^{-5}M) in myocytes and fibroblasts (n=8) pre-incubated with vehicle (DMEM D) or atosiban (10^{-9}M to 10^{-5}M) for 4 minutes. Primary cells were cultured from myometrium harvested at term pregnancy. Results are expressed as arithmetic means ± S.E. and significance was determined using one-way ANOVA with Bonferroni's *post-hoc* test; *p<0.05; ***p<0.001 for atosiban shifted curves compared to vehicle.

	Vehicle	Atosiban (10μM)	Atosiban (1μM)	Atosiban (0.1μM)	Atosiban (10nM)	Atosiban (1nM)
Smooth muscle cell s						
pEC_{50}	8.7 ± 0.1	6.5 ± 0.1***	7.1 ± 0.1***	8.2 ± 0.1*	8.5 ± 0.2	8.9 ± 0.03
Fibroblasts						
pEC_{50}	8.9 ± 0.1	6.9 ± 0.2***	7.7 ± 0.1***	8.3 ± 0.2*	8.7 ± 0.1	8.7 ± 0.04

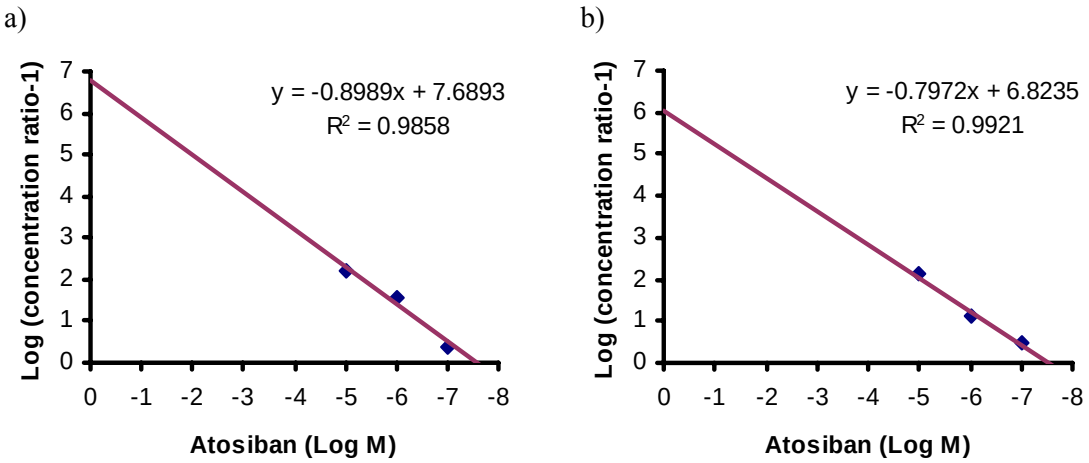


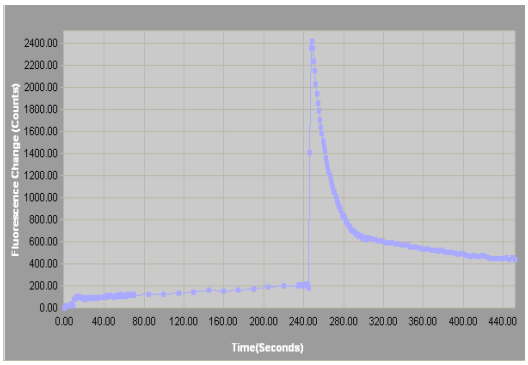
Figure 7.14: Representative Schild plots for human myometrial a) myocytes and b) fibroblasts obtained at term pregnancy. Cells were pre-incubated with atosiban (10^{-7}M to 10^{-5}M) before concentration-effect curves for oxytocin (10^{-11}M to 10^{-5}M) were performed using the FLIPR-Tetra.

7.13 Pre-incubation with atosiban

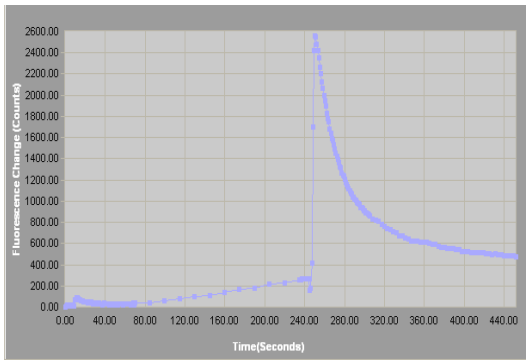
In myocytes and fibroblasts, challenge with oxytocin induced a concentration-dependent increase in intracellular-free Ca^{2+}. The resultant sigmoidal curve plateaued at 98.8 ± 0.79 percent fluorescent counts with a pEC_{50} of 8.8 ± 0.1M (Figure 7.13; Table 7.1). Although no change was evoked in the upper asymptote, the oxytocin antagonist atosiban (10^{-7}M to 10^{-5}M) produced parallel rightward shifts of the

oxytocin curve. Using Schild plot analysis, pA_2 values of 7.55 and 7.56 were calculated for myocytes and fibroblasts (Figure 7.14). Atosiban at 10^{-7}M displaced oxytocin-induced Ca^{2+} transients by a pEC_{50} value of 0.57 ± 0.15M ($F(1, 32) = 2.85$; $p<0.05$). Higher concentrations of atosiban further shifted the oxytocin-effect curve to the right ($F(2, 64) = 263$; $p<0.001$). The observed change in peak Ca^{2+} signals was displayed in screenshots from the FLIPR (Figure 7.15). Whilst responses to oxytocin were attenuated by atosiban at 10^{-8}M in myocytes ($F(1, 64) = 5.64$; $p<0.01$), lower concentrations of atosiban did not competitively antagonise or modify the profile of oxytocin in either cell type.

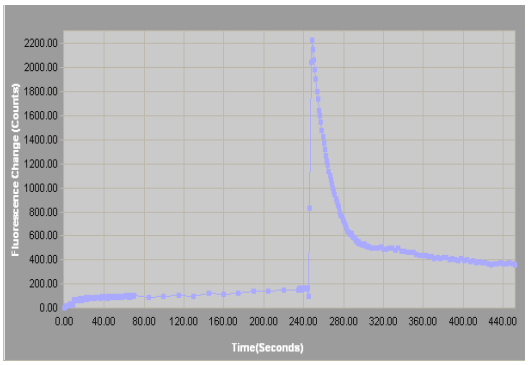
1a) Vehicle + oxytocin (10^{-6}M)



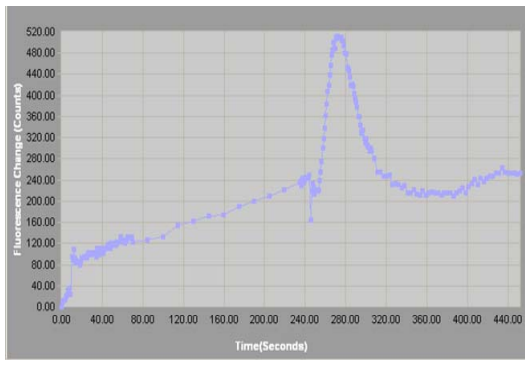
1b) Atosiban (10^{-5}M) + oxytocin (10^{-6}M)



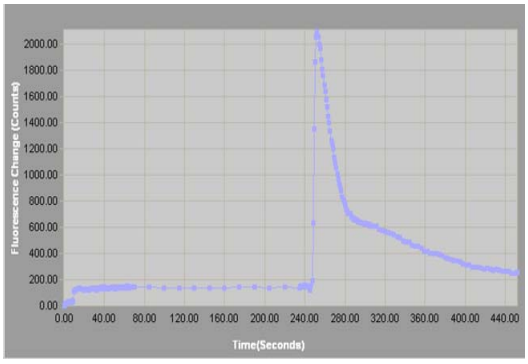
2a) Vehicle + oxytocin (10^{-7}M)



2b) Atosiban (10^{-5}M) + oxytocin (10^{-7}M)



3a) Vehicle + oxytocin (10^{-8}M)



3b) Atosiban (10^{-5}M) + oxytocin (10^{-8}M)

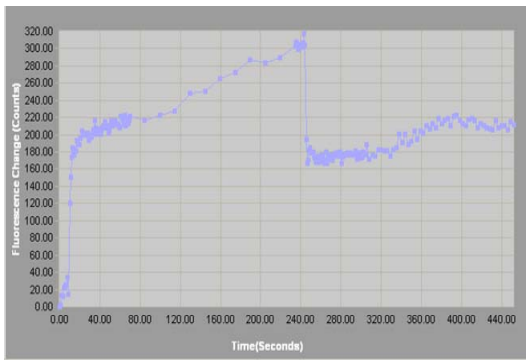


Figure 7.15: Screenshot images of Ca^{2+} transients in primary myocytes in response to oxytocin at 1) 10^{-6}M, 2) 10^{-7}M and 3) 10^{-8}M pre-incubated with either a) vehicle (DMEM D) or b) atosiban for 4 minutes. Fluorescent change counts were detected using the FLIPR camera from the binding of intracellular-free Ca^{2+} with fluo-4 AM dye at excitation and emission wavelengths of 400nm and 506nm respectively. Oxytocin-induced Ca^{2+} release was attenuated in the presence of atosiban at 10^{-5}M.

Table 7.2: Mean maximum percentage fluorescent count (E_m) and effective half maximal concentrations (pEC_{50}) (M) for smooth muscle cells (n=4) pre-exposed to vehicle (DMEM D), EP receptor agonists (10^{-6}M) or an EP_3 antagonist (10^{-5}M) for 4 minutes before stimulation with oxytocin (10^{-11}M to 10^{-5}M). Data are expressed as arithmetic means ± S.E. EP receptor compounds evoked no effect on oxytocin-induced Ca^{2+} release curves.

	Smooth muscle cells					
	Vehicle	**PGE_2**	**Butaprost**	**CP533,536**	**AGN211330**	**AGN211329**
Receptor target		**EP_{1-4}**	**EP_2**	**EP_2**	**EP_2**	**EP_3 antag**
E_m	100.5	100.5	104.0	104.3	98.2	99.0
S.E.	0.3	2.9	2.7	1.0	4.8	1.3
pEC_{50}	8.91	8.78	9.04	8.98	8.69	8.97
S.E.	0.05	0.2	0.04	0.04	0.1	0.03

Table 7.3: Mean maximum percentage fluorescent count (E_m) and effective half maximal concentrations (pEC_{50}) (M) for fibroblasts (n=4) pre-exposed to vehicle (DMEM D), PGE_2 (10^{-6}M), butaprost (10^{-6}M) or the EP_3 receptor antagonist AGN211329 (10^{-5}M) for 4 minutes before stimulation with oxytocin (10^{-11}M to 10^{-5}M). Data are expressed as arithmetic means ± S.E. EP receptor compounds evoked no effect on oxytocin-induced Ca^{2+} release curves.

	Fibroblasts			
	Vehicle	**PGE_2**	**Butaprost**	**AGN211329**
Receptor target	-	**EP_{1-4}**	**EP_2**	**EP_3 antag**
E_m	100.2	102.5	100.4	97.5
S.E.	1.12	2.1	2.2	1.8
pEC_{50}	8.82	8.88	8.80	8.87
S.E.	0.1	0.2	0.1	0.1

7.14 Pre-incubation with EP receptor compounds

Treatments with oxytocin (10^{-11}M to 10^{-5}M) depolarised the membrane of smooth muscle cells. This was characterised by Ca^{2+} influx, which plateaued at 100.5 ± 0.3 percent fluorescence counts (pEC_{50}: 8.91 ± 0.05M; Table 7.2). Pre-incubation with PGE_2 or the EP_2 receptor agonists butaprost, CP533,536 and AGN211330 did not alter the sigmoidal oxytocin-effect curve ($F(4, 120) = 1.44$; ns). Likewise, PGE_2 and butaprost had no affect on oxytocin-induced Ca^{2+} release in fibroblasts ($F(2, 72) = 1.53$; ns). Although the EP_3 receptor agonist AGN211329 induced a transient rise of 41.2 ± 5.15 percent fluorescent counts, the effect of oxytocin on cytosolic-free Ca^{2+} remained unaltered in both cell populations.

a) smooth muscle cells b) fibroblasts

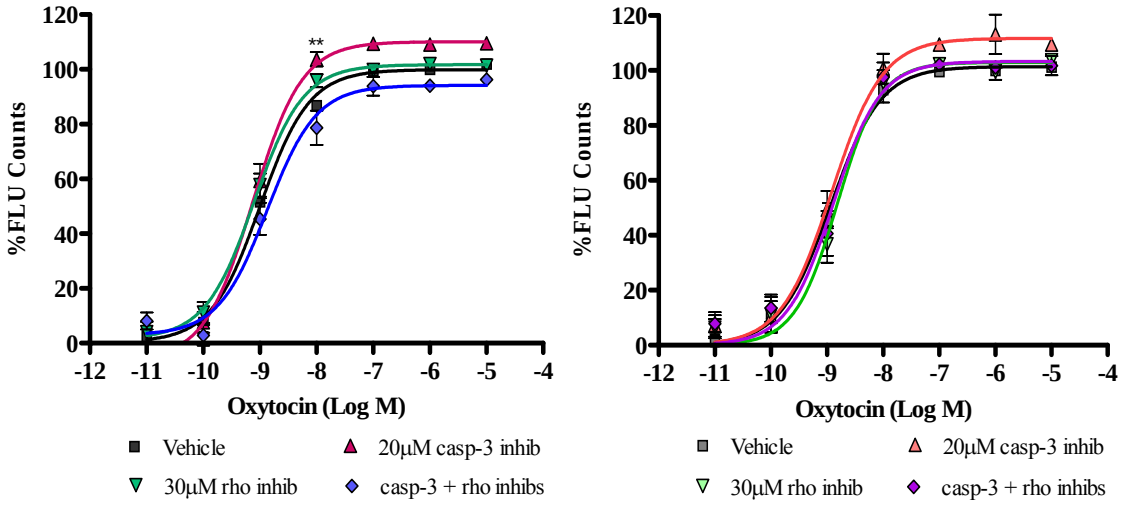


Figure 7.16: Concentration-effect curves for oxytocin alone (10^{-11}M to 10^{-5}M) and in combination with a caspase-3 inhibitor ($2x10^{-5}$M) and rho-kinase inhibitor ($3x10^{-5}$M) for a) myocytes and b) fibroblasts. Primary cells cultures from myometrial tissue taken at term pregnancy (n=4) were incubated with the Ca^{2+} indicator fluo-4 AM dye (2μM) and washed. Cells were exposed to vehicle (DMEM D) or inhibitors and oxytocin each for 4 minutes with changes in fluorescence detected using the FLIPR-Tetra. Data are expressed as arithmetic means ± S.E. and statistical significance was determined using two-way ANOVA with Bonferroni's *post-hoc* test; **p<0.01 for caspase-3 inhibitor compared to vehicle.

7.15 Effects of rho-kinase compounds on oxytocin signalling

In primary myometrial cells, oxytocin mobilised intracellular-free Ca^{2+} in a concentration-dependent manner reaching 99.6 ± 2.25 percent fluorescence counts between 10^{-7}M and 10^{-5}M (Figure 7.16). To elucidate their contribution on oxytocin-induced transduction pathways, rho kinase compounds were investigated as described by Moore & Lopez Bernal (2003). Challenge with the caspase-3 inhibitor potentiated the oxytocin-effect curve by 10.2 and 8.1 percent fluorescence counts in myocytes ($F(1, 48) = 8.77$; $p<0.01$) and fibroblasts respectively ($F(1, 48) = 1.45$; ns). Short exposure to caspase-3 and rho-kinase inhibitors alone did not influence oxytocin-induced Ca^{2+} transients. Despite the 5.7 percent reduction in fluorescence counts in myocytes, pre-incubation with both inhibitors for 4 minutes had no large effect on oxytocin signalling in either cell type.

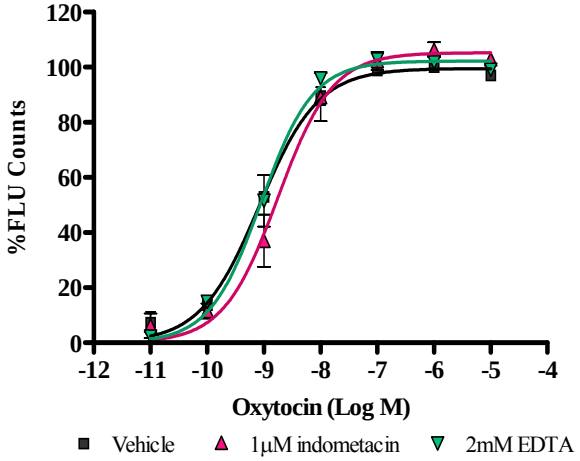


Figure 7.17: Concentration-effect curves for oxytocin (10^{-11}M to 10^{-5}M) pre-incubated with vehicle (DMEM D), indometacin (10^{-6}M) or EDTA ($2x10^{-3}$M) for 4 minutes. Primary cells were cultured from myometrial tissue taken at term pregnancy (n=4). After loading with fluo-4 AM dye (2μM), Ca^{2+} flux was read using the FLIPR and calculated as percentage change in fluorescence units over baseline (% FLU). Exposure to indometacin and EDTA did not alter oxytocin signalling.

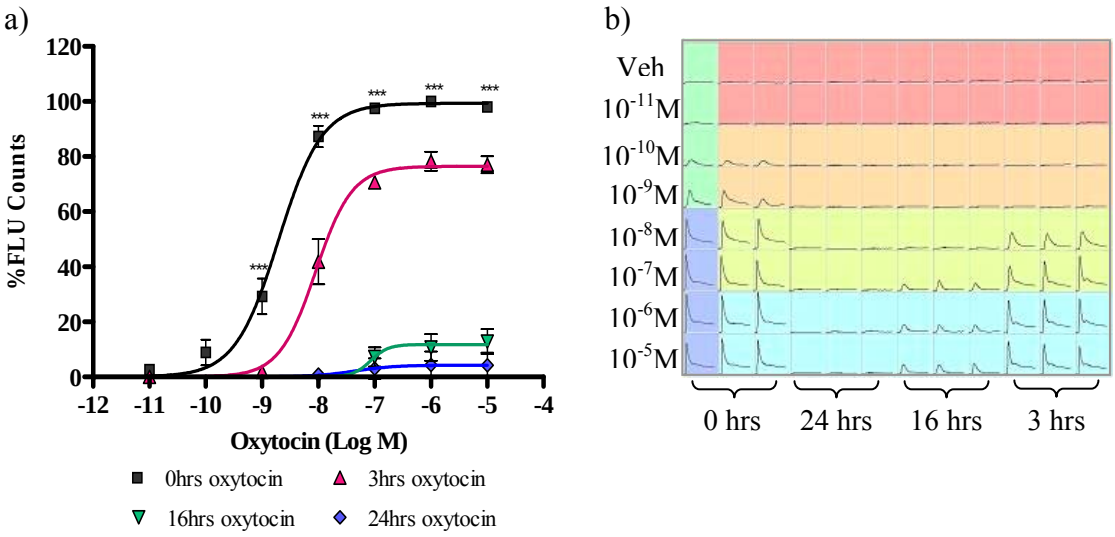


Figure 7.18: a) Concentration-effect curves for oxytocin (10^{-11}M to 10^{-5}M) pre-incubated with oxytocin (10^{-6}M) for 0, 3, 16 and 24 hours. Primary smooth muscle cells were cultured from myometrial tissue excised at term pregnancy (n=4). After loading with fluo-4 AM dye (2μM), responses to oxytocin were measured as percentage change in fluorescence units over baseline (% FLU) using the FLIPR. Results are expressed as arithmetic means ± S.E. and statistical significance was determined using multivariate ANOVA with Bonferroni's *post-hoc* test; ***$p<0.001$ for oxytocin incubated over 3, 16 and 24 hours compared to 0 hours.

b) FLIPR traces showing the decline in Ca^{2+} transients by oxytocin (10^{-11}M to 10^{-5}M) when myocytes were pre-incubated with oxytocin (10^{-6}M) for 3, 16 and 24 hours.

7.16 Pre-incubation with indometacin and EDTA

Pre-incubation with indometacin, a non-selective COX inhibitor (10^{-6}M) and ethylenediaminetetraacetic acid (EDTA), a Ca^{2+} chelating agent ($2x10^{-3}$M), for 4 minutes had no effect on Ca^{2+} influx within smooth muscle cells (Figure 7.17). Challenge with indometacin (pEC_{50}: 8.81 ± 0.25M) and EDTA (pEC_{50}: 9.12 ± 0.15M) similarly had no effect on oxytocin, which mobilised Ca^{2+} in a concentration-dependent manner (pEC_{50}: 9.07 ± 0.04M; F (2, 72) = 0.49; ns).

7.17 Pre-incubation with oxytocin.

Oxytocin at 0 hours (vehicle) liberated intracellular-free Ca^{2+} in a concentration-dependent manner, reaching 99.3 ± 0.82 percent fluorescence counts above 10^{-7}M (pEC_{50} of 8.83 ± 0.19M; Figure 7.18a). When smooth muscle cells were pre-incubated with oxytocin (10^{-6}M), oxytocic responses were attenuated in a time-related manner ($p<0.001$). Exposure to oxytocin for 3 hours produced an oxytocin-induced sigmoidal curve, which was 23 percent lower and 0.8 log M right shifted compared to oxytocin at 0 hours. Whilst challenge with oxytocin for 16 hours diminished oxytocin-induced Ca^{2+} transients by nearly 90 percent (pEC_{50}: 6.81 ± 0.37M; F (1, 48) = 1163; $p<0.001$), pre-incubation with oxytocin for 24 hours suppressed Ca^{2+} influx by a further 2.5 fold (pEC_{50}: 3.37 ± 2.81M; F (1, 48) = 1276; $p<0.001$). This marked decline in Ca^{2+} mobilisation was manifest in the 4-minute traces recorded on the FLIPR (Figure 7.18b).

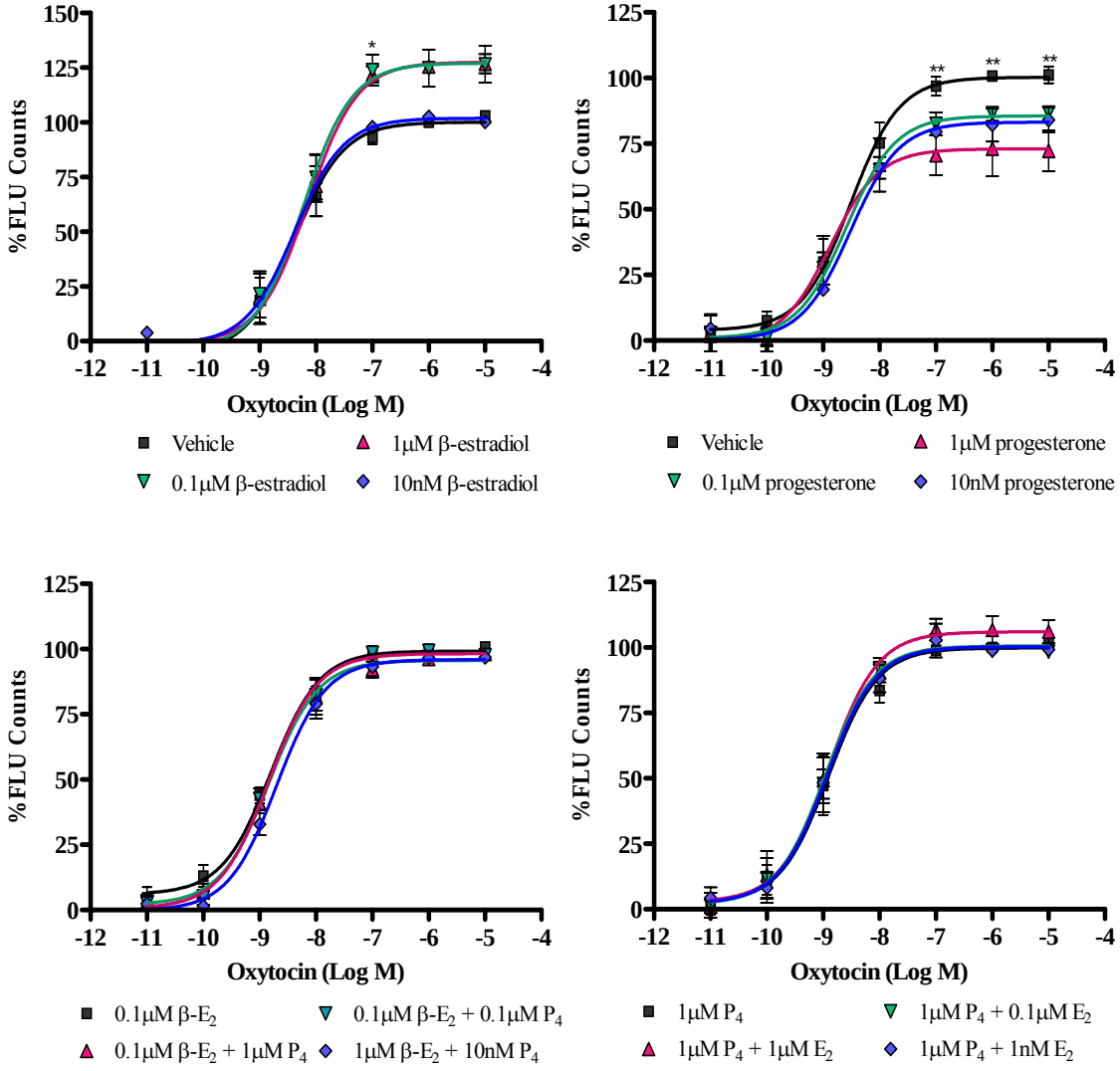


Figure 7.19: Concentration-effect curves for oxytocin alone (10^{-11}M to 10^{-5}M) and in combination with 17-β oestradiol (E_2; 10^{-6}M to 10^{-8}M) and progesterone (P_4; 10^{-6}M to 10^{-8}M) in primary myometrial smooth muscle cells obtained at term pregnancy (n=4). Once exposed to vehicle (DMEM D) or steroid hormones for 24 hours, the cells were loaded with fluo-4 AM dye (2μM) before intracellular Ca^{2+} responses to oxytocin were measured using the FLIPR. Data are expressed as arithmetic means ± S.E. and statistical significance was determined using two-way ANOVA with Bonferroni's *post-hoc* adjustment; *p<0.05; **p<0.01 respectively for E_2 (10^{-6}M & 10^{-7}M) and P_4 (10^{-6}M) compared to vehicle.

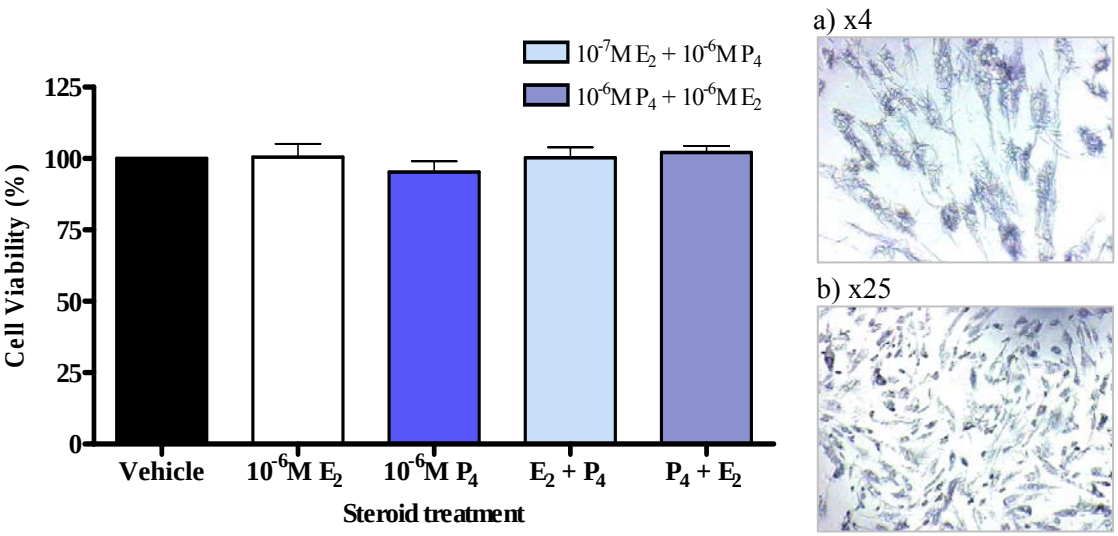


Figure 7.20: Cell viability for vehicle (DMEM B) and treatments with 17-β oestradiol (E_2; 10^{-6}M) and progesterone (P_4; 10^{-6}M) for 24 hours. Myocytes were cultured from myometrial tissue taken at term pregnancy (n=4) and seeded at a density of 30k cells/ well. Cytotoxicity was quantified using MTT dye (0.5mg/ml), which was reduced to a purple formazan salt by viable cells; this was visualised at a) x4 and b) x25 under a phase contrast microscope. Compared to vehicle controls, incubation with the steroids did not significantly alter cell proliferation or apoptosis.

7.18 Steroidogenic effects on oxytocin signalling

To elucidate the effect of steroid hormones on oxytocin-mediated Ca^{2+} signalling pathways, myocytes were exposed to vehicle or combinations of progesterone and 17β-oestradiol for 24 hours as described by Backlin *et al*. (2003). In vehicle-treated cells, oxytocin evoked a concentration-dependent Ca^{2+} flux, which plateaued at 100.7 ± 1.33 percent fluorescence counts with a pEC_{50} value of 8.61 ± 0.23M (Figure 7.19). 17-β oestradiol at 10^{-6}M and 10^{-7}M augmented oxytocin-induced Ca^{2+} transients by 24 percent ($p<0.05$), without shifting the curve (pEC_{50}: 8.28 ± 0.29M and pEC_{50}: 8.36 ± 0.24M respectively). Exposure to progesterone (10^{-6}M) attenuated oxytocic responses by 28 percent fluorescence counts ($p<0.01$), but also had no effect on the position of the curve (pEC_{50}: 8.86 ± 0.07M). The performed MTT assay indicated that this was not associated with cell proliferation, which was uniform irrespective of

steroid treatment (Figure 7.20). The effects of oxytocin on Ca^{2+} oscillations were restored in myocytes pre-incubated with a combination of oestrogen and progesterone.

7.19 The effects of Ca^{2+}-channel blockers on oxytocin

The mechanisms of oxytocin-induced Ca^{2+} release were initially assessed using the L-type Ca^{2+} channel blocker, nifedipine. To inhibit these cell membrane channels, myocytes were pre-treated with vehicle and nifedipine (10^{-5}M to 10^{-7}M) for 4 minutes before stimulation with oxytocin (10^{-11}M to 10^{-5}M; Table 7.4). For each concentration-effect curve, oxytocin mobilised Ca^{2+} until a plateau phase was sustained between 10^{-7}M and 10^{-5}M. Nifedipine at 10^{-5}M increased oxytocic effects from 104.3 ± 2.01 to 122.0 ± 9.02 percent fluorescent counts ($p<0.01$), without shifting the curve. Although exposure to nifedipine at 10^{-6}M and 10^{-7}M also enhanced Ca^{2+} influx, the <9 percent change was not significant compared to vehicle ($F(2, 72) = 2.60$; ns).

The combined effects of thapsigargin, lanthanum chloride ($LaCl_3$) and nifedipine on oxytocin-induced responses in myometrial cells were subsequently investigated to elucidate localised Ca^{2+} dynamics. $LaCl_3$ (25μM) and thapsigargin (2μM) were chosen due to their respective inhibition of store-operated Ca^{2+} channels (Fu *et al*., 2000) and endoplasmic reticulum Ca^{2+} pumps (Fomin *et al*., 1999; Kupittayanant *et al*., 2002; Wray & Shmygol, 2007). As before, uterine myocytes responded to oxytocin with a concentration-dependent increase in Ca^{2+} transients, followed by a plateau at 100.5 ± 1.54 percent fluorescent counts (pEC_{50}: 8.66 ± 0.23M; Figures 7.22 and 7.23). Potent responses to oxytocin were maintained in the presence of $LaCl_3$ alone and together with nifedipine (pEC_{50}: 8.53 ± 0.28M and 8.81 ± 0.20M

respectively). Whilst exposure to nifedipine alone augmented oxytocin-induced Ca^{2+} signalling by 16.5 percent ($F(1, 48) = 9.36$; $p<0.01$), thapsigargin attenuated Ca^{2+} transduction mechanisms. The 20 percent decrease in oxytocic effects ($F(1, 48) = 10.39$; $p<0.01$) may relate to the direct mobilisation of intracellular-free Ca^{2+} by thapsigargin (Figure 7.21). Even so, this reduction was potentiated in the presence of both nifedipine and $LaCl_3$ ($p<0.001$).

Table 7.4 Maximal responses (E_m) and effective half maximal concentrations (pEC_{50}) (M) for myocytes challenged with nifedipine at 10^{-5}M, 10^{-6}M or 10^{-7}M for 4 minutes before stimulation with oxytocin (10^{-11}M to 10^{-5}M) (n=4). Data are expressed as arithmetic means ± S.E. and analysed using multivariate ANOVA with Bonferroni's *post-hoc* test; **p<0.01 for nifedipine (10^{-5}M) compared to vehicle.

	Vehicle	**Nifedipine (10μM)**	**Nifedipine (1μM)**	**Nifedipine (0.1μM)**
E_m	104.3 ± 2.01	122.0 ± 9.02**	113.3 ± 5.11	107.3 ± 5.48
pEC_{50}	9.3 ± 0.11	9.1 ± 0.14	9.4 ± 0.07	9.3 ± 0.17

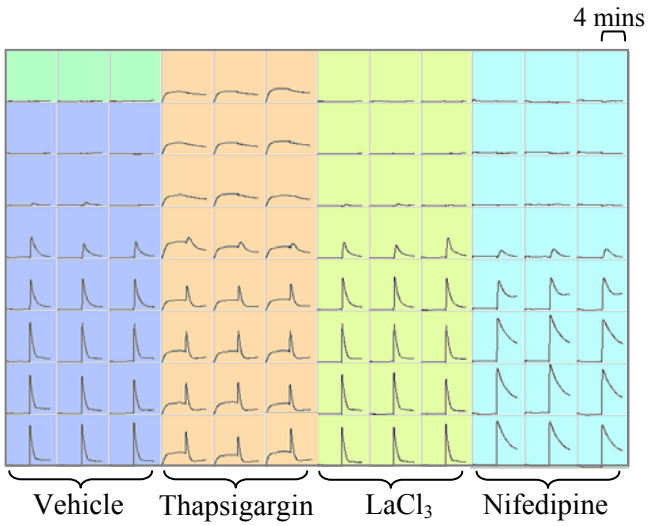
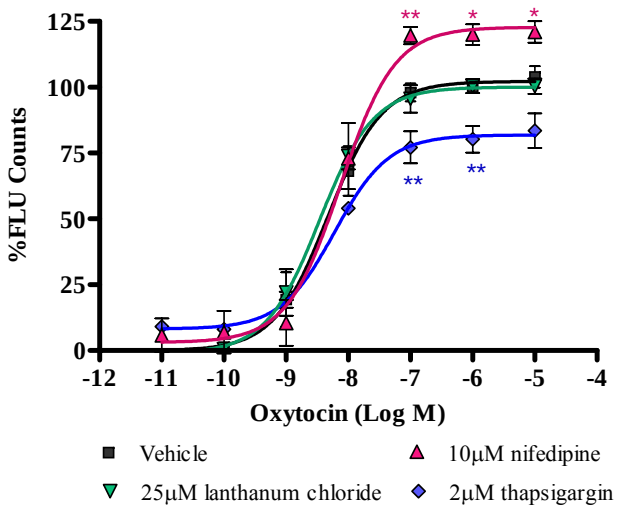
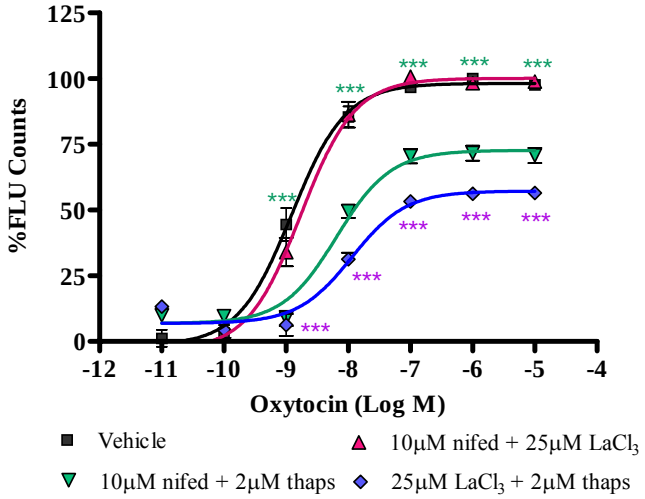


Figure 7.21: Concentration-dependent changes in intracellular-free Ca^{2+} for myocytes challenged with oxytocin (10^{-11}M to 10^{-5}M) for 4 minutes after pre-incubation with vehicle (DMEM D), thapsigargin ($2x10^{-6}$M), lanthanum chloride ($LaCl_3$; 25μM) or nifedipine (10μM) for 4 minutes. To detect Ca^{2+}, primary smooth muscle cells from term pregnant donors were loaded with the Ca^{2+} indicator fluo-4 AM dye (2μM) for 60 minutes and washed. Traces were recorded using the FLIPR-Tetra.

Similar trends were observed in fibroblasts (Figures 7.24 and 7.25). The characteristic concentration-effect curve evoked by oxytocin reached a plateau at 101.6 ± 0.86 percent fluorescence counts (pEC_{50}: 8.93 ± 0.12M). Again $LaCl_3$ alone and with nifedipine did not alter temporal changes in Ca^{2+}, whilst challenge with nifedipine (10^{-5}M) increased oxytocic effects by 18 percent ($F(1, 48) = 10.81$; $p<0.01$). Responses to thapsigargin and oxytocin were marginally more pronounced in fibroblasts compared to myocytes. Thapsigargin alone and in the presence of nifedipine decreased oxytocic effects by 13 percent ($F(2, 72) = 6.02$; $p<0.05$). Conversely, thapsigargin with oxytocin at 10^{-11}M stimulated relatively high Ca^{2+} release, reaching 27.0 ± 6.20 percent fluorescence counts; this created a more linear sigmoidal curve. Thapsigargin combined with $LaCl_3$ further suppressed oxytocin-mediated Ca^{2+} influx to 70.9 ± 7.72 percent fluorescence counts ($p<0.001$). In both myocytes and fibroblasts, pEC_{50} values were consistent throughout.





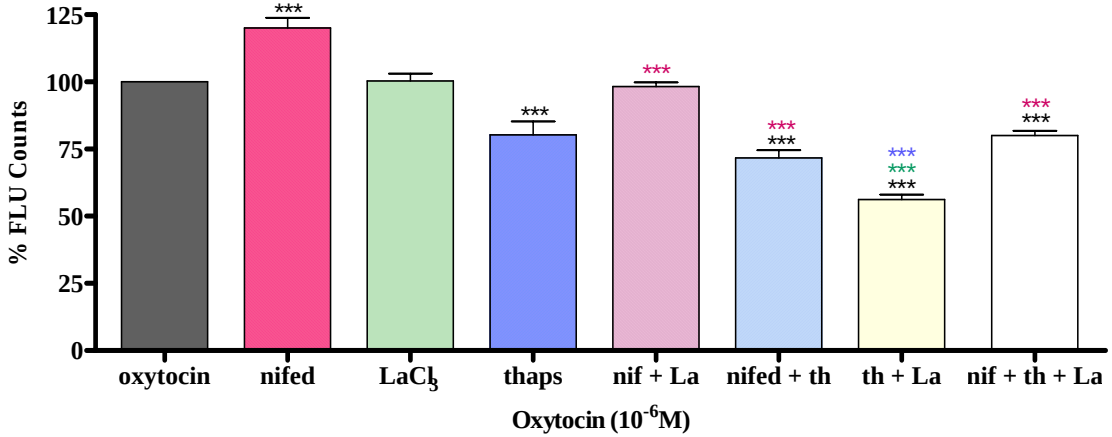
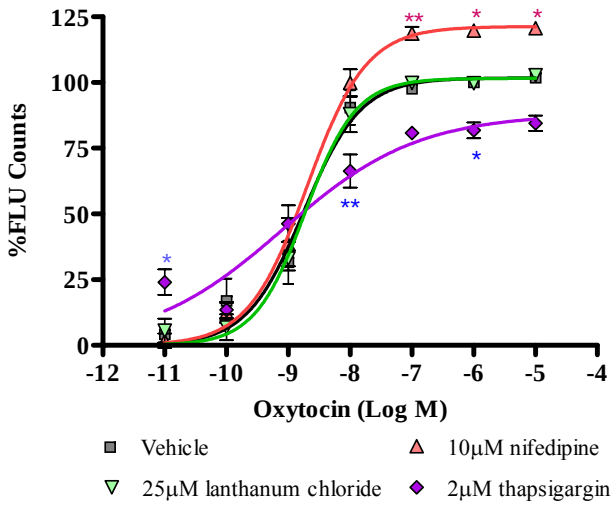
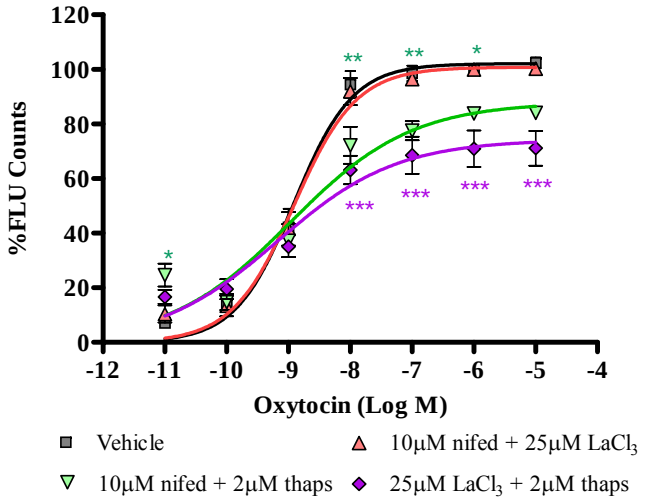


Figure 7.22: Concentration-effect curves (left) for oxytocin (10^{-11}M to 10^{-5}M) pre-incubated with combinations of vehicle (DMEM D), nifedipine (nifed; 10^{-6}M), lanthanum chloride ($LaCl_3$; $2.5x10^{-5}$M) and thapsigargin (thaps; $2x10^{-6}$M). To detect Ca^{2+} flux, **primary smooth muscle cells** obtained at term pregnancy were loaded with Ca^{2+} fluo-4 AM indicator dye (2μM; n=4). Ca^{2+} channel blockers and oxytocin were each added to cell plates for 4 minutes using the FLIPR-Tetra. Data are expressed as arithmetic means ± S.E. and analysed using two-way ANOVA with Bonferroni's *post-hoc* test; *p<0.05; **p<0.01; ***p<0.001 for nifedipine (red), thapsigargin (blue), nifed + thaps (green) and $LaCl_3$ + thaps (purple) compared to vehicle.

Figure 7.23: Bar chart (above) showing the effects of nifedipine (nifed; 10^{-6}M), lanthanum chloride ($LaCl_3$; $2.5x10^{-5}$M) and thapsigargin (thaps; $2x10^{-6}$M) on oxytocin at 10^{-6}M in **human myocytes** (n=4). Responses to oxytocin were read using the FLIPR and calculated as % change in fluorescence units over baseline (% FLU). Univariate ANOVA showed statistical differences; ***p<0.001 compared to oxytocin (black), nifed (red), $LaCl_3$ (green) and thaps (blue) alone.





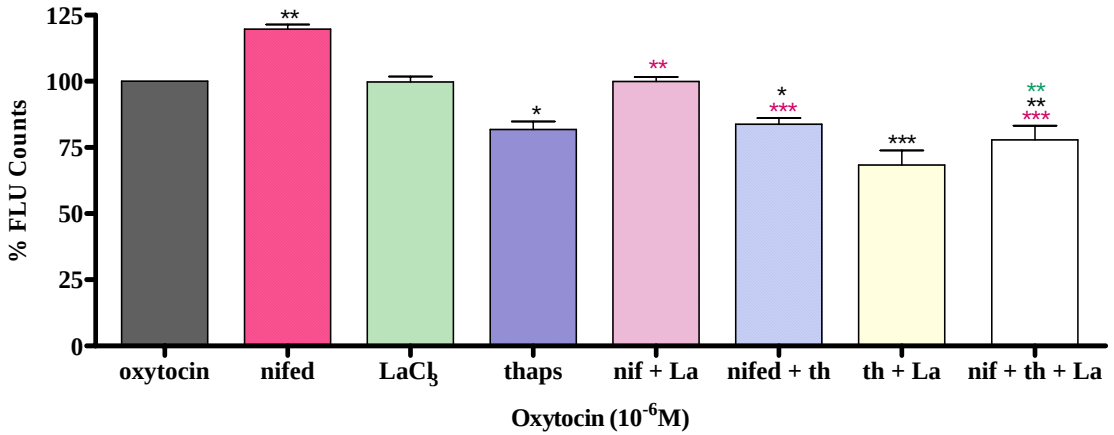


Figure 7.24: Concentration-effect curves (left) for oxytocin (10^{-11}M to 10^{-5}M) pre-incubated with combinations of vehicle (DMEM D), nifedipine (nifed; 10^{-6}M), lanthanum chloride ($LaCl_3$; $2.5x10^{-5}$M) and thapsigargin (thaps; $2x10^{-6}$M). To detect Ca^{2+} flux, **primary myometrial fibroblasts** obtained at term pregnancy were loaded with Ca^{2+} fluo-4 AM indicator dye (2μM; n=4). Ca^{2+} channel blockers and oxytocin were each added to cell plates for 4 minutes using the FLIPR-Tetra. Data are expressed as arithmetic means ± S.E. and analysed using two-way ANOVA with Bonferroni's *post-hoc* test; *p<0.05; **p<0.01 for nifedipine (red), thapsigargin (blue), nifed + thaps (green) and $LaCl_3$ + thaps (purple) compared to vehicle.

Figure 7.25: Bar chart showing the effects of nifedipine (nifed; 10^{-6}M), lanthanum chloride ($LaCl_3$; $2.5x10^{-5}$M) and thapsigargin (thaps; $2x10^{-6}$M) on oxytocin at 10^{-6}M in **human fibroblasts** (n=4). Responses to oxytocin were read using the FLIPR and calculated as % change in fluorescence units over baseline (% FLU). Univariate ANOVA showed statistical differences; ***p<0.001 compared to oxytocin (black), nifed (red) and $LaCl_3$ (green) alone.

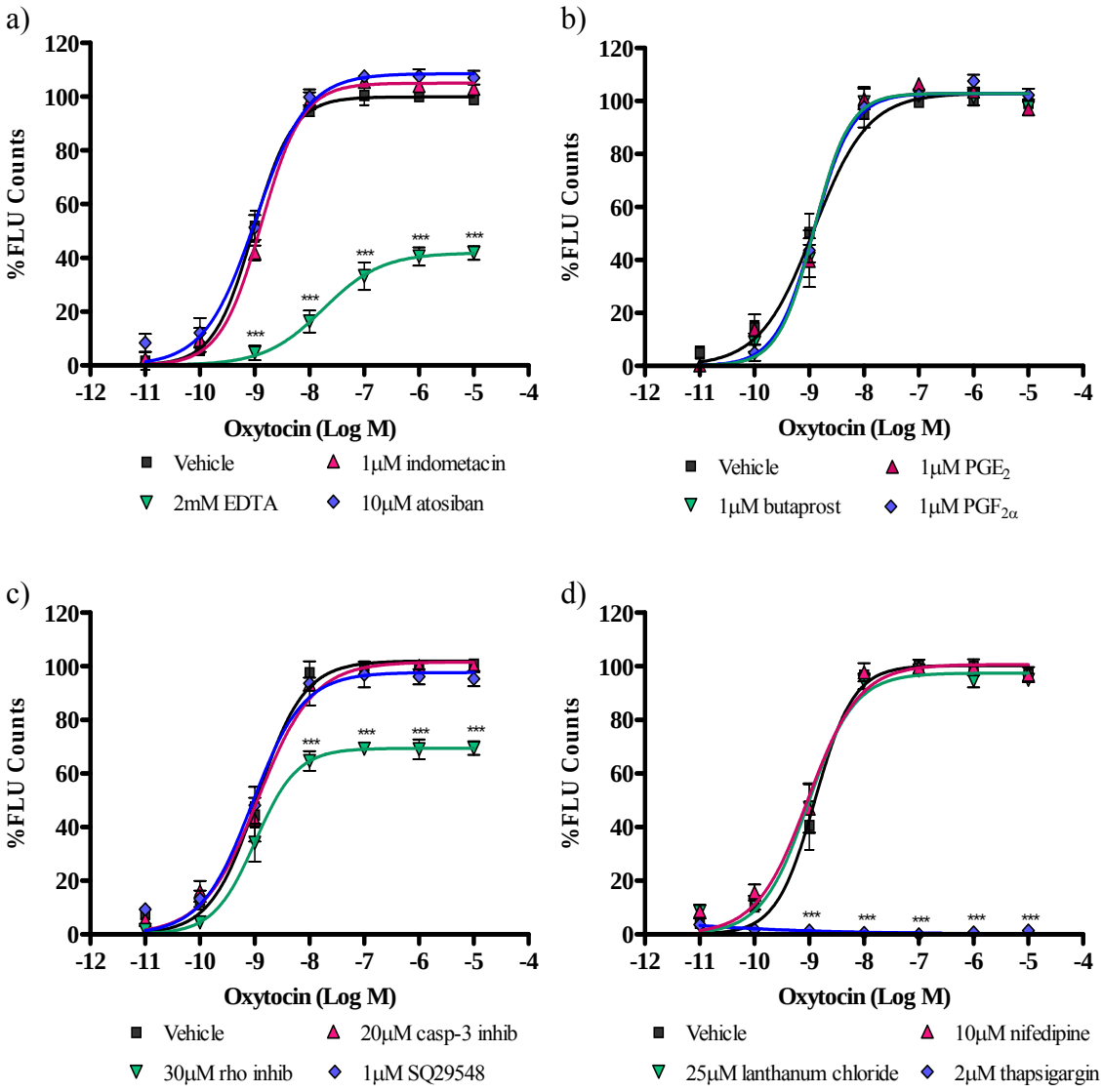


Figure 7.26: Concentration-effect curves for oxytocin (10^{-11}M to 10^{-5}M) pre-incubated with a) vehicle (DMEM D), indometacin (10^{-6}M), EDTA ($2x10^{-3}$M) and atosiban (10^{-7}M); b) vehicle, PGE_2 (10^{-6}M), butaprost (10^{-6}M) and $PGF_{2\alpha}$ (10^{-6}M); c) vehicle, caspase-3 inhibitor ($2x10^{-5}$M), rho-kinase inhibitor ($3x10^{-5}$M) and SQ29,548 (10^{-6}M); d) vehicle, nifedipine (10^{-5}M), lanthanum chloride ($2.5x10^{-5}$M) and thapsigargin ($2x10^{-6}$M) for 24 hours. Primary smooth muscle cells were cultured from myometrial tissue excised at term pregnancy (n=4). After loading with fluo-4 AM dye (2μM), responses to oxytocin were measured as percentage change in fluorescence units over baseline (% FLU) using the FLIPR-Tetra. Results are expressed as arithmetic means ± S.E. and statistical significance was determined using multivariate ANOVA with Bonferroni's *post-hoc* test; ***p<0.001 compared to vehicle.

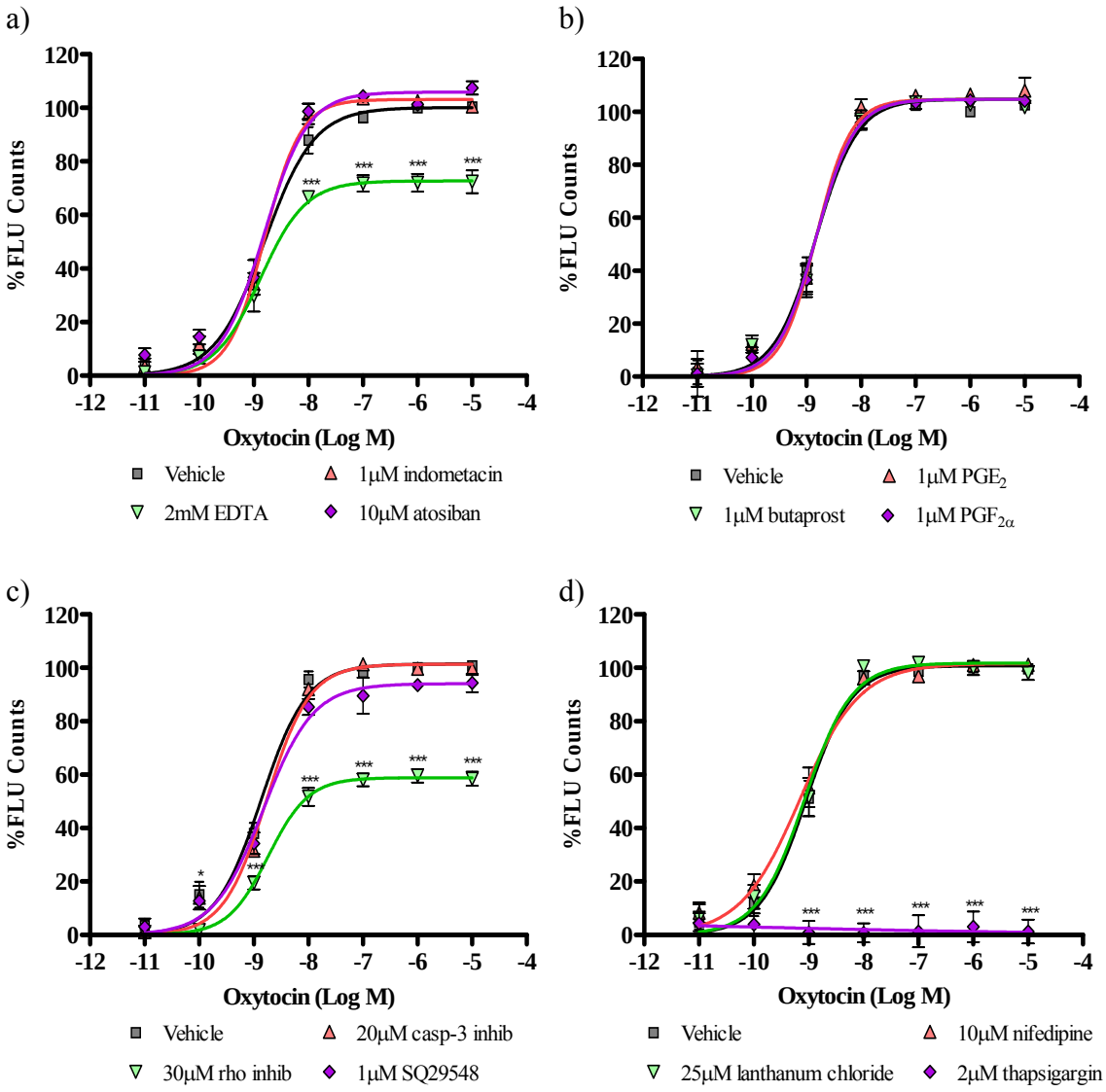


Figure 7.27: Concentration-effect curves for oxytocin (10^{-11}M to 10^{-5}M) pre-incubated with a) vehicle (DMEM D), indometacin (10^{-6}M), EDTA ($2x10^{-3}$M) and atosiban (10^{-7}M); b) vehicle, PGE_2 (10^{-6}M), butaprost (10^{-6}M) and $PGF_{2\alpha}$ (10^{-6}M); c) vehicle, caspase-3 inhibitor ($2x10^{-5}$M), rho-kinase inhibitor ($3x10^{-5}$M) and SQ29,548 (10^{-6}M); d) vehicle, nifedipine (10^{-5}M), lanthanum chloride ($2.5x10^{-5}$M) and thapsigargin ($2x10^{-6}$M) for 24 hours. Uterine fibroblasts were cultured from myometrial tissue excised at term pregnancy (n=4). After loading with fluo-4 AM dye (2μM), responses to oxytocin were measured as percentage change in fluorescence units over baseline (% FLU) using the FLIPR-Tetra. Results are expressed as arithmetic means ± S.E. and statistical significance was determined using multivariate ANOVA with Bonferroni's *post-hoc* test; *p<0.05; ***p<0.001 compared to vehicle.

7.20 The effects of overnight incubations on oxytocin

To identify their prolonged effects on the oxytocin cascade PGs, rho-kinase compounds and Ca^{2+} channel blockers were exposed to myometrial cells for 24 hours before oxytocin challenge (10^{-11}M to 10^{-5}M). In myocytes, pre-incubation with vehicle (DMEM D) did not influence typical oxytocin-stimulated Ca^{2+} entry, which plateaued at 100.6 ± 1.13 above 10^{-7}M (pEC_{50}: 9.0 ± 0.12M; Figure 7.26). The transient rise in Ca^{2+} was consistent in the presence of the COX inhibitor indometacin ($F(1, 48) = 1.27$; ns), the TP antagonist SQ29,548 ($F(1, 48) = 0.19$; ns) and PG agonists PGE_2, butaprost and $PGF_{2\alpha}$ ($F(3, 96) = 0.51$; ns).

Overnight pre-incubation with the oxytocin antagonist atosiban (10^{-5}M) also did not alter Ca^{2+} signals ($F(1, 48) = 3.88$; ns); this was in contrast to the results for short-term atosiban treatments (Figures 7.13 & 7.15; Table 7.1). In the presence of the Ca^{2+} chelator EDTA ($2x10^{-3}$M), the concentration-response curve for oxytocin was displaced 1.33 log M rightward, accompanied by a 57 percent reduction in peak Ca^{2+} release ($F(1, 48) = 758.7$; $p<0.001$). Likewise, 24-hour incubation with rho-kinase but not caspase-3 inhibitors attenuated oxytocin-induced Ca^{2+} transients. Although Ca^{2+} mobilisation plateaued at 69.2 ± 2.94 fluorescence counts ($F(1, 48) = 169.6$; $p<0.001$), the oxytocin-effect curve was not shifted to the right (pEC_{50}: 8.98 ± 0.15M). Myocytes challenged overnight with nifedipine (10^{-5}M; L-type) and lanthanum chloride (2.5×10^{-5}M; store-operated) Ca^{2+} channel blockers did not modify the profile of oxytocin. Even so, 24-hour exposure to thapsigargin ($2x10^{-6}$M), an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, completely prevented oxytocin-induced cytosolic Ca^{2+} influx ($p<0.001$).

In fibroblasts, oxytocin similarly mobilised intracellular-free Ca^{2+} in a concentration-dependent manner, reaching a sustained plateau at 101.4 ± 0.32 fluorescence counts (pEC_{50}: 8.9 ± 0.08M; Figure 7.27). No changes in integrated Ca^{2+} elevation were evoked by 24-hour pre-incubation with indometacin ($F(1, 48) = 3.31$; ns), SQ29,548 ($F(1, 48) = 3.89$; ns), PGE_2, butaprost or $PGF_{2\alpha}$ ($F(3, 96) = 1.78$; ns). The sigmoidal uterotonic action of oxytocin was also unchanged after overnight exposure to atosiban (10^{-5}M; $F(1, 48) = 1.69$; ns), the caspase-3 inhibitor ($F(1, 48) = 0.047$; ns) and Ca^{2+} channel blockers nifedipine and lanthanum chloride ($F(2, 72) = 0.69$; ns). Unlike in myocytes, external Ca^{2+} sequestered by EDTA ($2x10^{-3}$M) suppressed oxytocin-induced Ca^{2+} transients by 28 percent ($F(1, 48) = 80.27$; $p<0.001$), without shifting the curve (pEC_{50}: 8.92 ± 0.17). Even so, pre-treatment with the rho-kinase inhibitor had a more pronounced effect, decreasing peak fluorescence counts to 59.1 ± 3.14 percent ($F(1, 48) = 404.0$; $p<0.001$). Again challenge with thapsigargin for 24 hours totally blocked the stimulatory effect of oxytocin on Ca^{2+} channel activity.

Chapter 7.21: Results - cyclic AMP

7.22 Agonist-induced cAMP formation in myocytes

Agonist activation of cAMP was quantified to elucidate alternative intracellular signalling pathways (Figure 7.28). In smooth muscle cells, 30-minute oxytocin treatments produced a moderate monophasic increase in relative luminescence units from 3099 ± 858 to 6877 ± 909, which was not significant compared to vehicle ($F(1, 48) = 3.26$; ns). Butaprost conversely augmented cAMP formation by 18-fold in a concentration-dependent manner ($F(1, 48) = 451$; $p<0.001$). When pre-incubated at 10^{-6}M, butaprost also enhanced oxytocin-mediated cAMP mobilisation by nearly 100 percent ($F(1, 48) = 23.11$; $p<0.001$; pEC_{50}: 6.66 ± 0.11M). This concentration-related increase in liberated cAMP was analogous to the curve evoked by U46619 ($F(1, 48) = 25.46$; $p<0.001$; pEC_{50}: 6.54 ± 0.26M). Even so, in myocytes treated with PGE_2, cAMP signalling substantially increased 22-fold ($F(1, 48) = 1148$; $p<0.001$) and 20-fold ($F(1, 48) = 692.3$; $p<0.001$) above basal cAMP respectively.

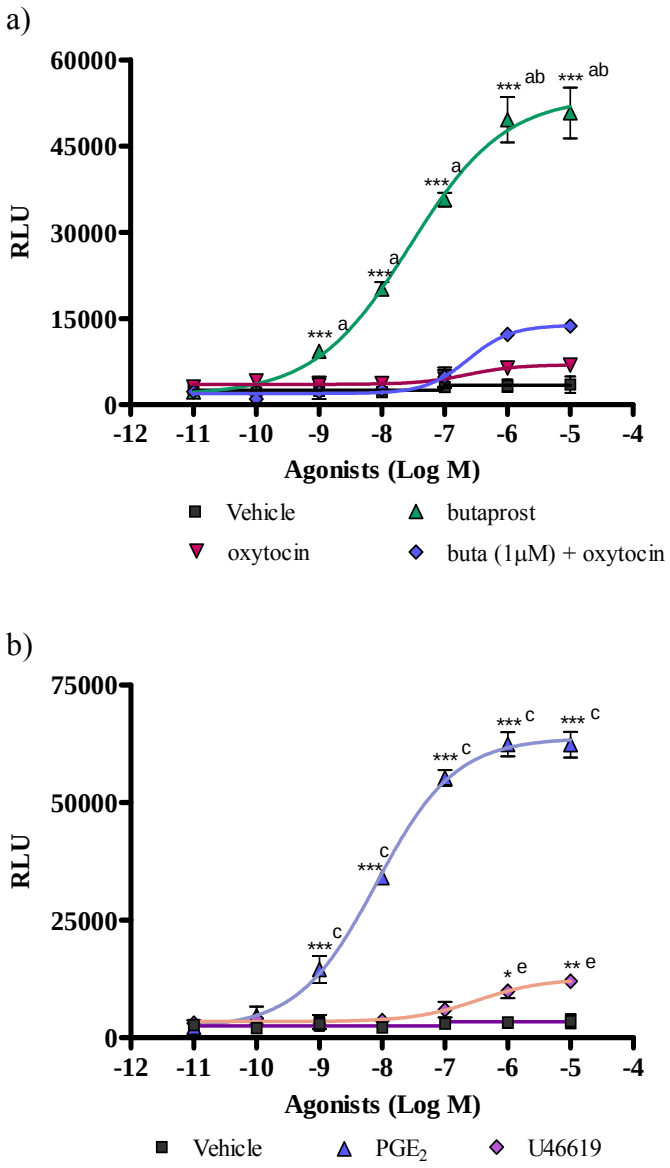


Figure 7.28: Myocyte cAMP flux for vehicle (DMEM D) and concentration-effect curves (10^{-11}M to 10^{-5}M) for a) butaprost, oxytocin and oxytocin together with butaprost (buta; 10^{-6}M) and b) PGE_2 and U46619. Primary smooth muscle cells were cultured from myometrium harvested at term pregnancy (n=4) and agonist treatments were pre-incubated for 30 minutes. Free cAMP was measured using a competitive-based immunoassay with cell-response signals, relative luminescence units (RLU), directly proportional to the presence of intracellular cAMP. Results are expressed as arithmetic means ± S.E. and analysed using two-way ANOVA with Bonferroni's *post-hoc* test; *p<0.05; **p<0.01, ***p<0.001 for [a] butaprost, [b] buta + oxytocin, [c] PGE_2 and [e] U46619 compared to vehicle.

Chapter 7.23: Results - RT-PCR

7.24 PG, oxytocin and rho-related mRNA expression in uterine myocytes

Qualitative mRNA expression for PGs, oxytocin receptors and target proteins in Ca^{2+} sensitisation was determined using RT-PCR in uterine myocytes harvested at term pregnancy. As well as their relative contribution in signal transduction pathways, the effects of vehicle (DMEM D), PG and oxytocin treatments were assessed. Only high quality, intact RNA was assayed, as shown by the clear 28S and 18S ribosomal RNA bands imaged before RT-PCR (Figure 6.6). In each PCR reaction, the absence of product in control RNA samples confirmed that no genomic DNA contamination had occurred (Figures 7.30 & 7.32).

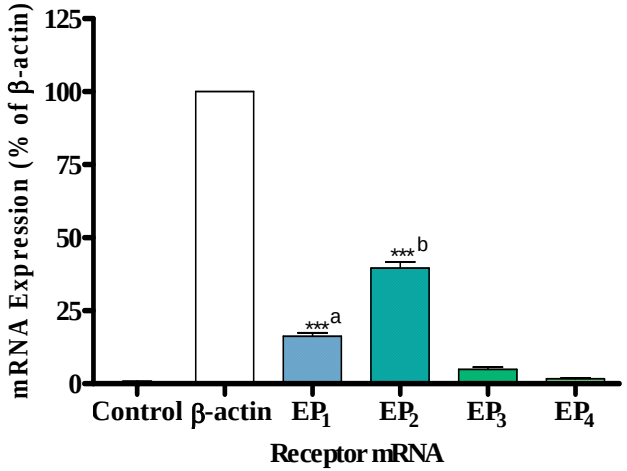
Myocyte mRNA was normalised as a percentage of the housekeeping gene β-actin, which was highly expressed at 377bp. All primer sets yielded RT-PCR products of the expected sizes (Table 6.21; Figures 7.30 & 7.32). DP and MLCK were the only amplified transcripts showing marked multiple bands; this indicated poor primer design. Of the EP isoforms, EP_2 receptor expression was most abundant at 40 ± 2.12 percent of β-actin ($p<0.001$; Figure 7.29). Although the density of EP_1 mRNA was 2.4-fold lower ($p<0.001$), only negligible compliments of EP_3 and EP_4 receptors could be detected. At 107.9 ± 2.58 percent β-actin, the expression of FP mRNA was considerably higher than the EP subset and other PGs ($p<0.001$). Despite greater effects on Ca^{2+} transients, oxytocin receptors were half as abundant as FP receptor transcripts. Even lower was the expression of DP and TP mRNA, followed by IP

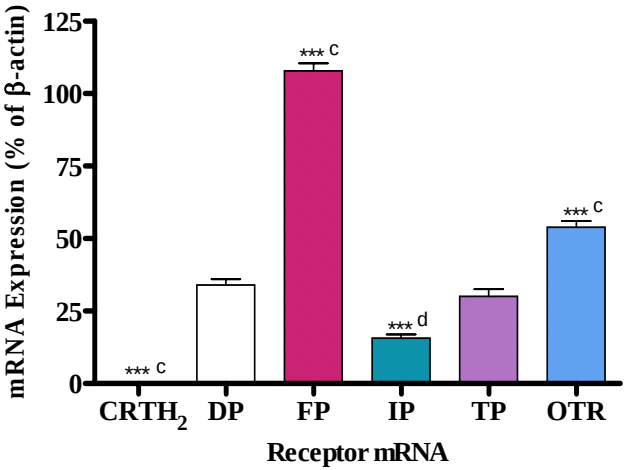
receptors at 15.6 ± 1.31 percent of β-actin ($p<0.001$). The chemoattractant receptor homologous molecule ($CRTH_2$) was used as a negative control due to its expression only on T helper cells (Hirai *et al*., 2001). Therefore, $CRTH_2$ was absent from the purified PCR products.

To address rho-related pathways that regulate uterine sensitisation, the relative expression of smooth muscle specific proteins were also investigated (Figures 7.31 & 7.32). Although the structure of telokin is identical to the C-terminus of MLCK, its expression was most abundant at 92.2 ± 1.42 percent β-actin. The density of RhoA and ROCKI bands were only 6.8 percent ($p<0.05$) and 11.5 percent ($p<0.001$) lower respectively. Inositol trisphosphate receptors (IP_3R), which mediate Ca^{2+} release into the cytosol, were still abundant at 66.7 ± 1.69 percent β-actin and ROCKII isoforms were two-thirds lower at 22.6 ± 1.85 percent β-actin ($p<0.001$). Surprisingly, the detection of MLCK mRNA was only very faint.

7.25 Effects of PG and oxytocin compounds on mRNA transcription

As well as the above vehicle (DMEM D) treatments, myocytes were exposed to U46619 (10^{-6}M), SQ29,548 (10^{-6}M) and the oxytocin antagonist atosiban (10^{-6}M) for 24 hours. Due to the rapid desensitisation of receptors (Phaneuf *et al*., 1998; Plested & Lopez Bernal, 2001), oxytocin (10^{-6}M) was only incubated with cells for 3 hours. The expression of PG, oxytocin and rho-related mRNA was consistent regardless of uterotonin or antagonist challenge (Tables 7.5 & 7.6). As a result, post-receptor transcriptional events appeared to be unchanged.





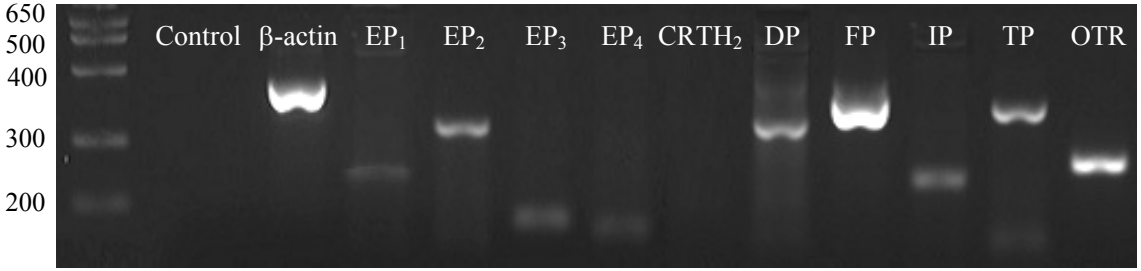


Figure 7.29: Relative expression of β-actin, EP_{1-4}, $CRTH_2$, DP, FP, IP, TP and oxytocin receptor (OTR) mRNA detected in human myocytes taken at term pregnancy (n=5). The density of each mRNA band was quantified using Fluor-Chem IS-8000 programme and normalised as a percentage of β-actin. Results are expressed as arithmetic means ± S.E. and analysed using univariate ANOVA with Bonferroni's *post-hoc* test: ***p<0.001 for [a] EP_1 and [b] EP_2 compared to the other EP receptors and for PG mRNA compared to [c] the other PG receptors and to [d] DP alone.

Figure 7.30: Representative agarose gel stained with ethidium bromide and photographed showing relative β-actin, PGs and oxytocin receptor (OTR) mRNA expression in human uterine myocytes taken at term pregnancy. PCR products were absent from the reverse transcriptase negative control lane and β-actin was used as a loading control. A 1Kb plus DNA ladder was run alongside for molecular weight sizing.

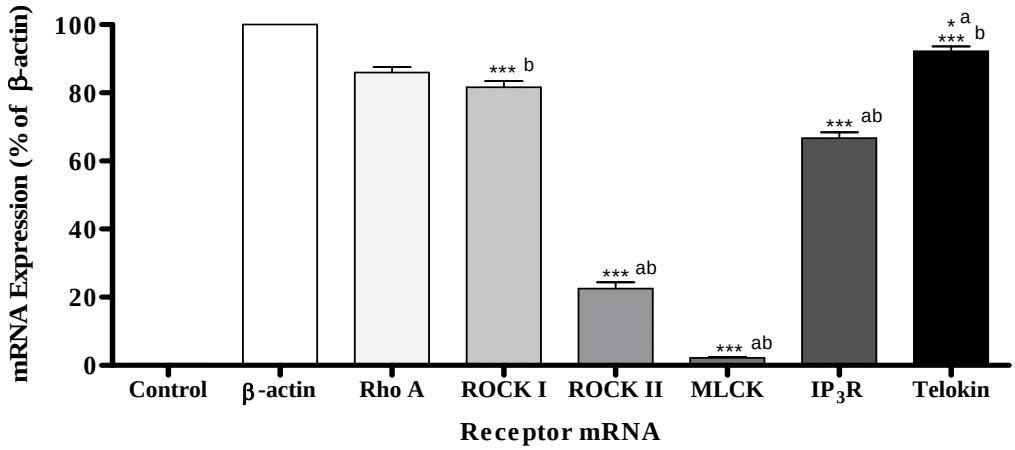


Figure 7.31: Relative expression of β-actin, RhoA protein, ROCKI, ROCKII, MLCK, IP_3 receptors and telokin mRNA detected in human myocytes taken at term pregnancy (n=5). The density of each mRNA band was quantified using Fluor-Chem IS-8000 programme and normalised as a percentage of β-actin. Results are expressed as arithmetic means ± S.E. and analysed using univariate ANOVA with Bonferroni's *post-hoc* test: *p<0.05; ***p<0.001 compared to [a] RhoA and [b] all other mRNA transcripts, excluding β-actin.

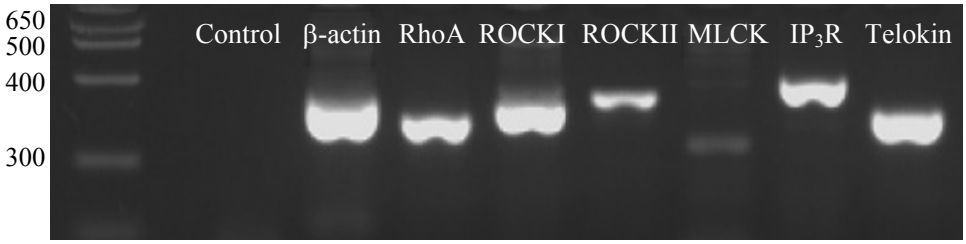


Figure 7.32: Representative agarose gel stained with ethidium bromide and photographed showing relative β-actin, RhoA, ROCKI, ROCKII, MLCK, IP_3 receptors and telokin mRNA expression in human uterine myocytes taken at term pregnancy. PCR products were absent from the reverse transcriptase negative control lane and β-actin was used as a loading control. A 1Kb plus DNA ladder was run alongside for molecular weight sizing.

Table 7.5: The effects of vehicle (DMEM D), oxytocin (10^{-6}M), atosiban (10^{-6}M), U46619 (10^{-6}M) and SQ29,548 (10^{-6}M) on PG and oxytocin mRNA receptor expression in smooth muscle cells cultured from myometrium at term pregnancy (n=5). Except for 3-hour oxytocin treatments, cells were incubated with agonists and antagonists for 24 hours. The density of mRNA transcripts were analysed with Fluor Chem IS-8000 and expressed as a percentage of β-actin ± S.E. Compared to vehicle, mRNA expression did not significantly change.

mRNA	Vehicle	Oxytocin	Atosiban	Atosiban + OT	U46619	SQ29,548	SQ + U19
EP_1	14.7 ± 2.84	14.2 ± 2.80	14.8 ± 3.02	13.2 ± 3.12	19.6 ± 4.12	18.0 ± 4.81	19.3 ± 2.58
EP_2	36.2 ± 6.88	42.3 ± 4.43	37.0 ± 5.30	41.5 ± 3.79	33.5 ±11.04	43.3 ± 5.41	44.0 ± 5.77
EP_3	4.0 ± 2.05	4.73 ± 2.42	4.7 ± 2.06	5.0 ± 2.41	5.3 ± 2.42	5.3 ± 2.03	5.4 ± 2.43
EP_4	1.6 ± 0.54	1.7 ± 0.74	2.1 ± 0.38	1.8 ±0.31	1.8 ± 0.81	1.5 ± 0.57	1.4 ± 0.67
DP	31.7 ± 4.8	32.3 ± 4.11	32.4 ± 4.69	29.9 ± 6.80	32.2 ± 7.91	31.9 ± 3.47	38.1 ± 6.35
FP	107.2 ± 9.26	106.0 ± 5.35	115.0 ± 11.87	104.6 ± 8.99	107.7 ± 7.17	106.6 ± 5.64	108.3 ± 6.43
IP	11.5 ± 2.28	10.6 ± 1.84	13.8 ± 3.49	15.34 ± 3.59	15.9 ± 5.27	16.8 ± 3.09	18.9 ± 3.54
TP	23.6 ± 8.24	25.6 ± 6.21	28.9 ± 6.10	31.2 ± 10.01	30.5 ± 10.20	35.2 ± 5.77	35.3 ± 7.17
OT	53.2 ± 4.77	53.2 ± 5.56	49.7 ± 8.52	52.1 ± 4.66	50.5 ± 11.40	58.8 ± 2.96	60.1 ± 3.22

Table 7.6: The effects of vehicle (DMEM D), oxytocin (10^{-6}M), atosiban (10^{-6}M), U46619 (10^{-6}M) and SQ29,548 (10^{-6}M) on rho-related mRNA expression in smooth muscle cells cultured from myometrium at term pregnancy (n=5). Except for 3-hour oxytocin treatments, cells were incubated with agonists and antagonists for 24 hours. The density of mRNA transcripts were analysed with Fluor Chem IS-8000 and expressed as a percentage of β-actin ± S.E. Compared to vehicle, mRNA expression did not significantly change.

mRNA	Vehicle	Oxytocin	Atosiban	Atosiban + OT	U46619	SQ29,548	SQ + U19
RhoA	85.2 ± 6.04	81.9 ± 7.18	85.9 ± 4.99	87.8 ± 3.44	83.9 ± 3.61	84.4 ± 4.52	89.9 ± 4.64
ROCK I	78.5 ± 5.61	74.0 ± 4.53	81.6 ± 6.49	84.6 ± 1.97	79.8 ±3.71	78.0 ± 4.87	89.6 ± 2.98
ROCK II	21.6 ± 3.19	27.4 ± 2.79	17.9 ± 4.17	18.1 ± 4.22	20.7 ± 4.82	19.6 ± 3.57	20.3 ± 5.60
MLCK	2.1 ± 0.67	1.8 ± 0.43	2.0 ± 0.41	1.7 ± 0.19	2.11 ± 0.46	2.9 ± 0.56	2.9 ± 0.60
IP_3	61.2 ± 4.38	69.8 ± 3.66	62.3 ± 4.77	65.7 ± 2.84	65.4 ± 5.44	67.6 ± 5.12	72.75 ± 3.21
Telokin	93.3 ± 3.90	90.3 ± 4.58	89.5 ± 4.99	92.0 ± 3.85	91.6 ± 2.97	89.8 ± 5.17	95.3 ± 2.69

Chapter 7.26: Discussion

In this study, smooth muscle cells and fibroblasts were isolated from human myometrial biopsies taken at term pregnancy. Although myocytes occupy most of the tissue volume, functional coupling with fibroblastic cells propagate electrotonic signals, contributing to the mechanical syncytium required for labour contractions (Lopez Bernal *et al*., 1993; Koumas *et al*., 2003; MacCannell *et al*., 2007). Myogenic activity is mediated through the ATP-dependent binding of myosin to actin, which is augmented by PG and oxytocin in the absence of autonomic innervation (Wray, 1993). The associated liberation of intracellular-free Ca^{2+} was induced by uterotonins in this study; however, oxytocic effects were most pronounced. In spite of the noted similarities between myometrial cell populations, subtle distinctions in their morphologies and signalling mechanisms may be of physiological importance in preparing the uterus for pregnancy and labour.

Myometrial cells are composed of intracellular filamentous systems that govern structural integrity and contraction. In the uterus, myometrial smooth muscle cells are arranged in bundles embedded in a matrix of connective tissue that enhance the transmission of contractile forces (Yu & Lopez Bernal, 1998) without directly altering Ca^{2+} flux (Shaw *et al*., 2006). In this study, uterine cryotome sections and cultured myometrial cells were similarly arranged in parallel arrays. This myofilament lattice, interspersed with focal adhesion dense plaques, was identified to envelop a centrally located nucleus by phase contrast microscopy. In isolated cells, immunocytochemistry was subsequently performed to characterise the distribution and intrinsic activity of myocyte and fibroblast populations in term gravid human myometrium.

Superficially, monolayer cultures of myometrial smooth muscle cells and fibroblasts exhibited similar morphologies. Although each cell type was fusiform in shape, myometrial fibroblasts were larger flattened cells with many processes that extended from the periphery. Due to their abundance in motile cells, these *lamellipodia* protrusions have been associated with cell survival, migration and aggregation to mediate tissue remodelling of the myometrium and pubic symphysis during pregnancy and delivery (Moraes *et al*., 2004). These ultrastructural features may also account for the faster attachment of fibroblast cells compared to myocytes, which constituted the basis of the adhesion separation technique. As the actin polymer constituents also have an integral role in traction and force generation, staining for smooth muscle specific α-actin was employed.

Of the six different isoforms, α-actin and γ-actin are the major constituents of myometrial smooth muscle cells (Shynlova *et al*., 2005). In the uterus, these thin filaments have directly been shown to activate myosin-ATPase activity in a Ca^{2+}-dependent manner for the development of tension (Borovikov *et al*., 1996). By binding with cross-linking proteins, such as α-actinin, filamentous actin forms complex architectures that enhance the mechanical response (Tseng *et al*., 2005). In both myometrial cell types, double immunofluorescent images showed strong co-localisation of α-actin and α-actinin markers. Although expression of these proteins appeared consistent in this study, a greater abundance of actin filaments has previously been indicated in myocytes compared to myometrial fibroblasts taken from non-pregnant women (Casey *et al*., 1984). In contrast to the increase in γ-actin polymers, the expression of α-actin has been reported to decline (Skalli *et al*., 1987) or remain high (Shynlova *et al*., 2005) with the progression of pregnancy. Mechanical stretch is thought to contribute to this switch from α-actin to the γ isoform in smooth

muscle cells at late gestation due to enrichment in stress fibres and focal adhesion sites (Katoh *et al*., 2001; Wang *et al*., 2006). This may account for the similarities in myocyte and fibroblast structural phenotypes in the term gravid uterus.

To identify further the cytoskeletal assembly, specific antibodies against desmin and vimentin were employed. These intermediate filaments displayed prominent networks, associated with providing mechanical integrity for myometrial cells (Leoni *et al*., 1990; Hongpaisan, 2000; Tang, 2008). In this study, filamentous vimentin was distributed throughout the cytoplasm, whereas desmin was located at the dense bodies of each cell. Spatial reorganisation and co-localisation of these filaments with the type IV protein synemin have been shown to regulate cell shape and alignment of myofibrils by linking intercellular apparatus (Gimona, 2008). This appears to facilitate mechanical transduction in smooth muscle tissues, contributing to mitosis and force development (Wang *et al*., 2006). In myometrial cells at term pregnancy, the upregulation of desmin content (Leoni *et al*., 1990) and genes encoding for intermediate filament-associated proteins have been related to the hypertrophic shape change and preparation of the cell architecture for labouring contractions (Salomonis *et al*., 2005; Shynlova *et al*., 2010). The positive immunostaining for both intermediate filaments in this study was therefore indicative of a smooth muscle contractile phenotype for both myocytes and fibroblasts.

Distinguishing features of myometrial cell types were evaluated further using a monoclonal antibody against fibroblast surface protein. Immunocytochemistry showed dispersed fine staining of the cell surface, with slightly stronger granular staining over the perinuclear region. This was in accord with the ultrastructural pattern of antigen immunoreactivity detected on the cell surface of human fibroblasts (Ronnov-Jessen *et al*., 1992), tissue macrophages and peripheral monocytes (Singer *et*

al., 1989). Despite its reported lack of expression in vascular smooth muscle cells (Ronnov-Jessen *et al*., 1992), all myometrial cells stained positive for the fibroblast-associated antigen in this study. With fibroblasts and endothelial cells comprising an estimated 5 to 10 percent of myometrial cell types in a digestion (Hongpaisan, 2000; Duquette *et al*., 2005), it is likely that the surface protein was present on smooth muscle cells as well as fibroblasts. This suggests that an alternative cytoskeletal marker would be required for the absolute identification of cell subsets. However, lineage-specific monoclonal antibodies were limited and even several smooth muscle-associated antigens, including smoothelin, calponin, h-caldesmon and vinculin were positively expressed in some fibroblastic cells (Esterre *et al*., 1992; Lazard *et al*., 1993; Hasegawa *et al*., 2003; D'Addario *et al*., 2002). Despite the concerted smooth muscle phenotype, cultured flasks appeared to contain enriched cell populations. This was based on differences in their morphology and the faster proliferation rate of fibroblasts compared with myocytes (Casey *et al*., 1984; Phaneuf *et al*., 1993; Hongpaisan, 2000; Rouger *et al*., 2007). To characterise separate contractile functions of these cells, PG and oxytocin-induced post-receptor signalling cascades were next assessed.

PG and oxytocin agonists are known to modulate spontaneous uterine activity via functional excitation-contraction coupling between myometrial cells. The transmission of action potentials is reported to originate from myometrial Cajal-like interstitial cells, which are responsible for the endogenous pacemaker system (Ciontea *et al*., 2005; Young, 2007; Allix *et al*., 2008). Although undetected in primary cell cultures in this study, the Cajal-like interstitial cells are dependent on Ca^{2+} signalling cascades for generating the coordinated activity of ion channel pumps and exchanges throughout uterine muscle (Berridge, 2008). To induce phasic myometrial

contractions, Ca^{2+} in combination with calmodulin activates myosin light chain kinase (MLCK). This enzyme phosphorylates 20kDa myosin light chains (MLC_{20}), enhancing both adenosine trisphosphatase (ATPase) activity and cross-bridge formation between the myosin head and actin filament (Word *et al*., 1994; Sanborn, 2001). This activation only occurs when cytosolic Ca^{2+} reaches the threshold above resting potential for regenerative membrane depolarisation. Perturbations of this pathway are likely to cause untimely parturition (Word *et al*., 1994; Riley *et al*., 2005) and so the effects of PGs and oxytocin on Ca^{2+} release were tested using the FLIPR-Tetra system.

Cell loading with the Ca^{2+}-sensitive fluo-4 AM dye indicated concentration-dependent stimulation of Ca^{2+} transients by PG and oxytocin agonists. Even so, low amplitude PG-induced responses were at the limits of detection, whilst oxytocic effects were marked in this study. Oxytocin, EP_1, EP_3, FP and TP receptors activate phospholipase C (PLC) β1, β2 or β3 through large heterotrimeric G-proteins (Phaneuf *et al*., 1996; Sanborn *et al*., 1998). Due to the induction of β1 and β2 isoforms during pregnancy, PLC efficiently hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to release IP_3 and diacylglycerol (DAG) (Somlyo *et al*., 1999; Hurd *et al*., 2000). In myometrial tissue, IP_3 binding to its cognate receptors on the endoplasmic reticulum liberates stored Ca^{2+} into the cytosol; this is vital for sustained uterotonic contractile activity at term (Yamada *et al*., 1994; Sanborn, 2001). By contrast, DAG activates protein kinase C (PKC) leading to the phosphorylation of cellular proteins. A direct correlation between intracellular Ca^{2+} transients, MLC_{20} phosphorylation and temporal force development has been shown in mouse (Matthew *et al*., 2004) and human myometrial tissues (MacKenzie *et al*., 1990; Word *et al*., 1990; Woodcock *et al*., 2004; Jie *et al*., 2007). Therefore, based on the ability of PGs to modulate contractions (Chapter 4),

induce abortion during pregnancy as well as parturition and to treat postpartum haemorrhage, the minor Ca^{2+} flux detected using the FLIPR-Tetra was surprising.

In primary cultures of human myometrial cells, PGE_2 was shown to elevate cytosolic Ca^{2+} with peak depolarisation only 1.5-fold (Thornton *et al*., 1992) and 4-fold (Asboth *et al*., 1996) lower than oxytocin. The respective addition of PGE_2 at 140μM and 20μM relative to the 10μM stimulation of cells in this study may account for some of the discrepancies between results. In particular, chronic exposure to PGE_2 is more likely to evoke biochemical changes rather than act through EP receptor sites. The effect of PGE_2 may also be attributed to cross-reaction with the FP receptor (Kiriyama *et al*., 1997; Breyer *et al*., 2001), especially with $PGF_{2\alpha}$ evoking large Ca^{2+} oscillations in primary human myocytes (Phaneuf *et al*., 1993; Fu *et al*., 2000). The expression of FP receptors was particularly abundant in myometrial cells compared to TP receptors in this study. Moreover, $PGF_{2\alpha}$ was reported to be equipotent with oxytocin using a myometrial cell line (Molnar & Hertelendy, 1990), whilst U46619 produced either a marginal Ca^{2+} release (Himpens & Somlyo, 1988) or analogous Ca^{2+} transients to PGE_2 (Asboth *et al*., 1996; Moore *et al*., 2002). Whilst the trend in results was similar, it appeared that use of fura-2 tetraoxymethylester loaded cells with an epifluorescence microscope was more sensitive than the FLIPR-Tetra system for Ca^{2+} flux detection. However, due to its potent uterotonic effects, Ca^{2+} signalling pathways for oxytocin were further investigated.

Despite the clinical significance of oxytocin as a pharmaceutical agent for augmenting uterine contractions during dysfunctional labour, its role in parturition is controversial. For myometrial depolarisation to occur, challenge with oxytocin has been shown to elevate inward Ca^{2+} and sodium (Na^+) flux, in exchange for the outward flow of potassium (K^+) and chloride (Cl^-) ions (Thornton *et al*., 1992;

Rezapour *et al*., 1996; Shmigol *et al*., 2001). This change in ionic gradient produces an initial transient followed by slower repolarisation essential for effective excitation-contraction coupling (Thornton *et al*., 1992). In human myometrium, whilst the resting membrane potential is generally between -45 and -50mV (Inoue *et al*., 1990), the threshold for activation becomes lower at term pregnancy (Sanborn, 2001). These action potentials are driven through large conductance Ca^{2+} channels and are potentiated by reduced sensitisation of prevalent voltage-dependent K^+ channels (Szal *et al*., 1994; Korovkina *et al*., 2006).

In this study, oxytocin mobilised intracellular Ca^{2+} in a concentration-dependent manner. This was almost 2-fold higher in uterine myocytes compared to fibroblasts, suggesting that cultures of enriched cell populations were isolated in this study. The results were substantiated by the higher incidence of typical oxytocin-generated Ca^{2+} transients (Hongpaisan, 2000) and electrotonic coupling (MacCannell *et al*., 2007) in myocyte cultures, compared to cultures contaminated with fibroblasts. As fibroblasts are the main effector cells in the cervix (Montes *et al*., 2002), such changes in action potential waveform could promote uterine contractions without modulating the cervix. The larger Ca^{2+} flux within smooth muscle cells may also relate to their extensively developed sarcoplasmic reticulum for enhanced Ca^{2+} storage (Sanborn, 2001) and high-density functional oxytocin receptors (Fuchs *et al*., 1984).

For uterine contractions to proceed efficiently at term it is likely that Ca^{2+} signals are regulated by oxytocin in conjunction with other autacoids and hormones. Through DAG stimulation of PKC, oxytocin has been shown to activate the mitogen-activated protein kinase (MAPK) cascade promoting PG biosynthesis in human myometrial (Hoare *et al*., 1999; Molnar *et al*., 1999) and decidual cells (Fuchs *et al*., 1984; Asselin *et al*., 1997). The uterotonic action of these PGs may increase the

concentration of oxytocin receptors (Melin, 1993) and initiate crosstalk between pathways for positive or negative feedback loops, which could impact Ca^{2+} homeostasis (Sanborn, 2007). In particular, it is thought that heightened myometrial responsiveness to circulatory oxytocin and locally produced PGs may promote parturition (Molnar *et al*., 1999; Lopez Bernal & TambyRaja, 2000). As a result, possible cumulative effects of PGs on oxytocin-induced Ca^{2+} signalling were assessed in this study.

Upon ligand binding, oxytocin interacts with two G-proteins of the Gαi and $G\alpha q_{/11}$ subfamily, determined according to pertussis toxin (PT) sensitivity (Phaneuf *et al*., 1993; Phaneuf *et al*., 1996; Sanborn *et al*., 1998). Although $PGF_{2\alpha}$ also operates through a PT-resistant pathway (Gαq), unlike oxytocin, a non-selective protein kinase inhibitor has been shown to partly inhibit $PGF_{2\alpha}$ induced IP_3 formation (Phaneuf *et al*., 1996). This suggests that $PGF_{2\alpha}$ may also activate an alternative G-protein pathway via $G\alpha_{13}$ (Cao *et al*., 2005), phospholipase A_2 (Hertelendy & Molnar, 1995) or stimulate PLC-γ through the phosphorylation of tyrosine kinase receptors (Carrasco *et al*., 1996). Similarly, TPα and TPβ coupling proteins include $G\alpha q/_{11}$, $G\alpha_{12/13}$ and Gαi subunits and so signal via alternative transduction mechanisms (Vezza *et al*., 1999; Moore *et al*., 2002). Despite these different routes of activating Ca^{2+} entry, short or 24-hour incubation of cells with combinations of $PGF_{2\alpha}$ and U46619 did not amplify oxytocic Ca^{2+} signals. This lack of synergism may be attributed to the paucity of large PG-induced Ca^{2+} oscillations detected by the FLIPR-Tetra in this study.

Likewise, oxytocin-evoked Ca^{2+} fluxes were not altered by the presence of PGE_2 or the COX-inhibitor indometacin. In myometrial cell cultures, PGE_2 was shown to elevate Ca^{2+} entry via voltage-sensitive membrane channels, without release from intracellular Ca^{2+} stores (Molnar & Hertelendy, 1990; Thornton *et al*., 1992). This

suggests that PGE_2 binds to high affinity EP_1 receptors coupled to Gαi without PLC activation (Thornton *et al*., 1992). Even so, the use of pharmacological analogues has since shown predominant Ca^{2+} signalling via the low affinity EP_3 receptors coupled to Gαq and the PLC inositol messenger system (Asboth *et al*., 1996). Surprisingly in this study, addition of the EP_3 antagonist AGN211329 immediately evoked Ca^{2+} transients in smooth muscle cells and fibroblasts. This indicates partial agonist activity, perhaps at FP receptor sites which stimulate the IP_3-Ca^{2+} cascade. In contrast, PGE_2 and EP_2 receptor agonists activate the cyclase adenylyl cascade, inhibiting Ca^{2+} transport through intracellular and extracellular membrane channels (Sanborn, 2001; Sanborn *et al*., 2005; Yuan & Lopez Bernal, 2007). Nevertheless, as the complex downstream events of cAMP also involve phosphorylation of MLCK (Word, 1995) and PLC target proteins (Sanborn *et al*., 2005), PGE_2 and EP compounds did not appear to modulate oxytocic effects on Ca^{2+} influx in this study.

Although the local paracrine effects of oxytocin are unclear, the uterus is sensitised to oxytocin mid-cycle (Janicek *et al*., 2007) and just before labour-onset (Keelan *et al*., 1997). This correlates with the density of myometrial oxytocin receptors and their relative contractile effects (Richter *et al*., 2006; Janicek *et al*., 2007). In this study (Figures A8 & A9), responsiveness to oxytocin was potentiated in lower, but not fundal regions of non-gravid myometrium obtained during the follicular phase. This corresponds to the enhanced topographical distribution of oxytocin receptors towards the cervix around ovulation (Richter *et al*., 2004; Steinwall *et al*., 2004), perhaps facilitating retrograde sperm transport at this time of the menstrual cycle. Despite the reported low or negligible expression of myometrial oxytocin mRNA (Kimura *et al*., 1996; Fuchs *et al*., 1998; Helmer *et al*., 1998; Steinwall *et al*., 2004; Wing *et al*., 2006), uterotonic activation was notable in this study. Such responsiveness indicates

the potential involvement of oxytocin in the pathogenesis of primary dysmenorrhoea (Liedman *et al*., 2008) or preterm labour (Phaneuf *et al*., 2000).

Compared to the non-gravid condition, uterine sensitivity to oxytocin is enhanced at term pregnancy by an increase in oxytocin binding to its cognate cell surface membrane receptors (Fuchs *et al*., 1984). Similarly high expression of oxytocin receptor mRNA was detected in this study. The potent spasmogenic responses were observed *in vitro* as concentration-dependent phasic activity with superimposed tonic contractions. Whilst the mRNA expression is reported to be 10 to 100-fold higher at late gestation (Fuchs *et al*., 1984; Kimura *et al*., 1996; Riemer & Heymann, 1998; Terzidou *et al*., 2005), oxytocin receptors were 300-fold higher in the parturient uterus compared to myometrium in the non-gravid state (Kimura *et al*., 1996). Even so, other studies have shown a lack of change in mRNA expression with labour-onset (Charpigny *et al*., 2003; Havelock *et al*., 2005; Tattersall *et al*., 2008). This suggests that the increase in oxytocin receptor sensitivity may be of greater importance in activating parturition alongside its role in preventing postpartum haemorrhage and establishing lactation (Kimura *et al*., 1996; Zeeman *et al*., 1997; Tattersall *et al*., 2008; Turton *et al*., 2009).

To block specific receptor binding activity, the oxytocin antagonist atosiban was incubated with myocytes and fibroblasts. Atosiban is a synthetic derivative of oxytocin and possesses equal affinity for oxytocin and vasopressin V_{1a} receptors (Nilsson *et al*., 2003). In contrast to oxytocin, circulatory arginine vasopressin and the expression of myometrial V_{1a} receptors remain constant during late pregnancy and labour (Maggi *et al*., 1990; Helmer *et al*., 1998). Thereby, oxytocin rather than V_{1a} receptors more likely represents a therapeutic target for parturition.

In smooth muscle cells and fibroblasts, atosiban caused parallel rightward shifts of the oxytocin effect curves in a competitive and concentration-dependent manner. This corresponds to reports that atosiban attenuates oxytocin-stimulated IP_3 production and intracellular-free Ca^{2+} release with pEC_{50} values of 4.7 and 6.8 (Fuchs *et al*., 1984; Phaneuf *et al*., 1994). In this study, the pA_2 for atosiban was 7.6 in both myocytes and fibroblasts. Likewise, atosiban reduces the amplitude and frequency of myometrial contractions *in vitro* with pA_2 values of 7.6, 7.8 and 5.9 for immersed (Wilson *et al*., 2001; Pierzynski *et al*., 2004) and superfused myometrial strips (Duckworth *et al*., 2002). The enhanced inhibitory effect of atosiban may reflect its relative equilibration in the immersion rather than superfusion apparatus. Atosiban alone was also shown to reduce basal myogenic contractions, without partial agonism, indicating high functional activity of constitutive oxytocin receptors (Buscher *et al*., 2001; Wilson *et al*., 2001; Duckworth *et al*., 2002). Nevertheless, exposure to atosiban or vehicle for 24 hours, followed by drug removal from the cell plates, did not influence cell responsiveness to oxytocin in this study. This confirmed a lack of intrinsic activity on Ca^{2+} channels and oxytocin transduction pathways as well as the competitive antagonistic properties of atosiban.

With the promising reduction of uterine contractility in clinical trials (Akerlund *et al*., 1987), atosiban was registered in 2002 on the indication of delaying imminent preterm birth. Even so, the efficacy and beneficial use of atosiban as a tocolytic agent is controversial. Early reports showed that intravenous infusions of atosiban were at least as effective as β-agonists in prolonging pregnancy with few maternal side effects (Goodwin *et al*., 1996; Coomarasamy *et al*., 2002). However, systematic literature reviews have since determined that atosiban and the more selective oxytocin antagonist barusiban were no better than placebo in delaying parturition or improving

neonatal outcome (Papatsonis *et al*., 2009; Thornton *et al*., 2009). This paucity of *in vivo* effect may be attributed to oxytocin receptor changes in the physiology or pathophysiology of the parturient uterus.

It is acknowledged that myometrial oxytocin receptors lose hormonal responsiveness with repeated or prolonged oxytocin treatments (Phaneuf *et al*. 1994; Brenninkmeijer *et al*., 1999; Plested & Lopez Bernal, 2001). This homologous desensitisation was confirmed by a decline in oxytocin-induced Ca^{2+} transients in myocytes following continuous exposure to oxytocin. Although the mechanisms are unclear, the three intracellular loops and C-terminal tail of the oxytocin receptor are rich in putative phosphorylation sites for G-protein coupled receptor kinases or other kinases, such as PKA, PKC and calmodulin-dependent protein kinase II (Carrasco *et al*., 1996; Plested & Lopez Bernal, 2001; Willets *et al*., 2009). These kinases are thought to activate β-arrestin for steric hindrance and uncoupling of G-proteins from receptors (Brenninkmeijer *et al*., 1999; Plested & Lopez Bernal, 2001). In primary myometrial cell cultures, the progressive decrease in oxytocin-stimulated PLC pathways correlates with a substantial loss of oxytocin binding sites (Phaneuf *et al*., 1994; Phaneuf *et al*., 1997). This process maintained the expression of total oxytocin receptor protein. To reverse the desensitisation of these oxytocin receptors, oxytocin was pre-incubated with atosiban (Phaneuf *et al*., 1994). However, on the basis of clinical practice outcomes, very early therapeutic intervention with atosiban would be necessary to successfully delay preterm labour.

Desensitisation of oxytocin receptors is likely to occur *in vivo* during the course of spontaneous and induced labour at term (Bossmar *et al.,* 1994; Phaneuf *et al*., 1998; Liedman *et al*., 2009). The decline in tissue responsiveness to oxytocin was observed in this study (Figure A10). Although the most potent contractions were evoked at term

and in tissues harvested shortly after labour-onset, oxytocin caused little effect in isolated myometrium from fully dilated donors. This may be attributed to the transient decrease in myometrial oxytocin binding sites (Fuchs *et al*., 1984; Bossmar *et al*., 1994) and the steep decline in oxytocin mRNA with labour progression (Phaneuf *et al*., 1998; Liedman *et al*., 2009). During this time, plasma oxytocin concentrations remain stable (Dawood *et al*., 1978; Thornton *et al*., 1992), whilst the local production of oxytocin in intrauterine tissues and the frequency of pulsatile oxytocin release are elevated (Fuchs *et al*., 1991; Chibbar *et al*., 1993). Due to the slow process of oxytocin receptor desensitisation, the myometrium maintains its oxytocin-mediated effects for several hours during active labour. This may be an important feature of regulating early labour-contractions rather than the advanced stages of the birth process.

Mechanical stretch and cervical effacement may also modulate myometrial sensitivity to oxytocin. The distension of the uterus in clinical conditions, such as multiple pregnancies and polyhydramnios, is associated with a marked increase in myometrial and decidual oxytocin mRNA and the increased risk of preterm labour (Fuchs *et al*., 1991; Turton *et al*., 2009). Moreover, oxytocin receptor mRNA and oxytocin gene promoter activity are elevated in primary human myocytes exposed to acute stretch (Terzidou *et al*., 2005). In contrast, the lowest numbers of oxytocin receptor sites are located near the cervical end of the uterus at term pregnancy (Fuchs *et al*., 1984; Havelock *et al*., 2005). As the cervix effaces into lower segment tissue following long episodes of labour, this perhaps would also account for the observed attenuated responsiveness to oxytocin.

In this study, 17β oestradiol enhanced oxytocin-induced Ca^{2+} transients, whilst exposure to progesterone attenuated responses. The changes in Ca^{2+} flux were not

associated with cell proliferation, which was uniform irrespective of steroid treatment, and were restored in myocytes pre-incubated with a combination of oestrogen and progesterone. These effects concur with changes in the ovarian hormonal milieu that influence further myometrial responsiveness to oxytocin (Word *et al*., 1992). Throughout most of pregnancy, progesterone promotes utero-quiescence by genomic and non-genomic mechanisms. Its predominant actions, mediated via the progesterone receptor (PR)-B isoform, are associated with the repression of many genes, including gap junctions (Garfield *et al*., 1980) and oxytocin receptor transcription (Soloff *et al*., 1983). As a result, electrical coupling between cells, Ca^{2+} oscillations and *in vitro* oxytocin-induced myometrial contractions are attenuated (Thornton *et al*., 1999; Chanrachakul *et al*., 2005). Acute progesterone treatments have also been shown to inhibit transmembrane Ca^{2+} entry (Fomin *et al*., 1999) and deplete Ca^{2+} stores (Gehrig-Burger *et al*., 2010) by direct interaction with oxytocin receptors (Grazzini *et al*., 1998). Therefore, progesterone most likely reduced oxytocin receptor binding or intrinsic receptor activity in this study, decreasing cytosolic-free Ca^{2+}.

Although in most mammals parturition is associated with a marked decline in maternal progesterone, in humans, circulatory progesterone (Darne *et al*., 1987) and total PR in myometrium and decidua remain elevated (Haluska *et al*., 2002). Instead the myometrial PR-A: PR-B mRNA expression ratio increases from late pregnancy to spontaneous labour at term. This shift to the PR-A isoform represses PR-B mediated transactivation and may contribute to the reputed functional progesterone withdrawal at labour-onset (Pieber *et al*., 2001; Mesiano *et al*., 2002). Other proposed mechanisms include increased transcription nuclear factor kappa B (NF-κB) activity, inactivating PR-sensitive promoters (Allport *et al*., 2001), elevated progesterone metabolism (Astle *et al*., 2003) and a reduction in steroid receptor co-activators at

term (Condon *et al*., 2006). Oestrogen priming is also a necessary for PR function (Backlin *et al*. 2003; Thijssen, 2005). In accord, oestrogenic phenol red and serum-containing growth medium appeared to maintain functional PR dynamics even after removal from seeded primary cells in this study. Also, unlike other studies (Sadovsky *et al*., 1992; Severino *et al*., 1996), the presence of functional oestrogen receptors in cultured uterine cells was confirmed.

It is well accepted that oestrogens assist the development, growth and differentiation of the myometrium via oestrogen receptor (ER)-α as a transcription regulator. Despite the constant rise in systemic oestrogen before labour (Walsh *et al*., 1984; Darne *et al*., 1987), oestrogens, particularly 17β-oestradiol, increase the formation of uterine gap junctions (Garfield *et al*., 1980; Dong & Yallampalli, 2000; Kilarski *et al*., 2000) and oxytocin receptors (Fuchs & Fuchs, 1984; Richter *et al*., 2004). Although analysis of the human oxytocin promoter does not reveal classical palindromic oestrogen response elements, eleven putative transcription motifs have been identified (Kimura *et al*., 1999; Terzidou *et al*., 2005). In addition, oestrogen-dependent transcription of Ca^{2+} channels in the parturient uterus has been proposed (Garfield *et al*., 1998; Dalrymple *et al*., 2004). These factors may account for the heightened oxytocin-induced Ca^{2+} mobilisation in both myocytes and fibroblasts. Therefore, under oestrogenic conditions at term pregnancy, the enhanced sensitivity to oxytocin and resultant Ca^{2+} influx may facilitate uterine contractions; this could be an important prerequisite for the parturition process in women.

Although oestrogen enhances myometrial excitation-contraction coupling, the relative contributions of localised Ca^{2+} dynamics have yet to be established. Extracellular Ca^{2+} entry through membrane voltage-gated Ca^{2+} channels principally mediates action potentials and sustained force development in the human gravid myometrium

(Somlyo & Somlyo, 1994; Sanborn *et al*., 2005; Wray *et al*., 2005). These L-type dihydropyridine-sensitive channels are upregulated during the parturition process (Longo *et al*., 2003). Consequently, nifedipine, an L-type Ca^{2+} channel blocker, is used as a tocolytic agent to delay preterm labour (Papatsonis *et al*., 2009). *In vitro* studies show that nifedipine attenuates both myogenic and oxytocin-induced contractions (Phillippe & Basa, 1997; Parkington *et al*., 1999) and either reduces (Young *et al*., 1993; Fu *et al*., 2000) or does not affect Ca^{2+} oscillations (Burghardt *et al*., 1999). However, in both primary myocytes and fibroblasts, exposure to nifedipine alone augmented oxytocin-induced Ca^{2+} signalling. Although the mechanisms for this are unknown, nifedipine appears to prevent the release of cytosolic-free Ca^{2+} from the cells.

As well as nifedipine, the inhibitor lanthanum chloride has been shown to block store-operated Ca^{2+} channels (Fu *et al*., 2000; Young, 2002; Kim *et al*., 2005; Moynihan *et al*., 2008). Activation of these plasma membrane channels, termed either Ca^{2+} release activated currents or capacitative Ca^{2+} entry, is triggered via the emptying of Ca^{2+} from intracellular stores. In human myometrial cells, the Ca^{2+} release channels in the sarcoplasmic reticulum are gated by ryanodine and IP_3 receptors. Whilst ryanodine receptors are expressed, functional units do not appear to be formed in human uterine smooth muscle cells (Kupittayanant *et al*., 2002). In contrast, mRNA encoding for the three IP_3 isoforms are present in myometrial tissue (Shmygol & Wray, 2004) and the predominant type I isoform was displayed in myocytes. Oxytocin-generated IP_3 induces a global Ca^{2+} rise in myocytes contributing to potent oxytocic contractile effects (Luckas *et al*., 1999; Shmygol &Wray, 2004). In this study, responses to oxytocin were maintained in the presence of lanthanum chloride alone and together with nifedipine. Whilst lanthanum chloride was previously shown not to affect

Ca^{2+} waves (Young, 2002), it tempered additional Ca^{2+} entry. This may correspond with its role of mediating a low and sustained influx of Ca^{2+} to replenish the sarcoplasmic reticulum (Wray *et al*., 2005; Berridge, 2008). Therefore, this store-operated system may facilitate Ca^{2+} homeostasis following cell depolarisation.

Another mechanism for refilling intracellular Ca^{2+} stores is via the sarcoplasmic reticulum Ca^{2+}-ATPase. The expression of this ATPase is upregulated from term pregnancy to labour, suggesting a functional role in potentiating myometrial contractions (Tribe *et al*., 2000). As a specific inhibitor of sarcoplasmic reticulum Ca^{2+} pumps, thapsigargin depletes intracellular Ca^{2+} stores without a concomitant rise in IP_3 (Fomin *et al*., 1999). Accordingly, thapsigargin directly mobilised intracellular-free Ca^{2+} in uterine myocytes and fibroblasts. Although oxytocin signalling was reduced, functional studies show that Ca^{2+}-ATPase inhibitors decrease contractile frequency but enhance spontaneous uterine force (Kupittayanant *et al*., 2002). This suggests that Ca^{2+} sequestered into these stores regulates myometrial contractions.

Combined incubations of thapsigargin with the L-type channel blocker nifedipine attenuated further oxytocin transduction pathways, associated with Ca^{2+} mobilised from both intracellular and extracellular pools. However, Ca^{2+} influx was even lower in the presence of lanthanum chloride, indicating the synergistic uptake of Ca^{2+} via ATPase and store-operated channels. To identify the contribution of each system, 24-hour treatments with the strong Ca^{2+} chelating agent ethylenediaminetetraacetic acid (EDTA) and with thapsigargin were performed. Similar to previous studies (Thornton *et al*., 1992; Kupittayanant *et al*., 2002), the removal of extracellular Ca^{2+} using EDTA did not completely suppress Ca^{2+} signals. However, thapsigargin abolished Ca^{2+} release within myometrial cells. Taken together, these results suggest that in spite of the Ca^{2+} boost from the extracellular milieu, the sarcoplasmic reticulum is

particularly important for oxytocin-mediated uterine contractions. Even so, these effects would also depend on other membrane channels, secondary messenger cascades and the sensitivity of the uterus to Ca^{2+} (Taggart *et al*., 1999; Somlyo & Somlyo, 2000).

As well as liberating cytosolic Ca^{2+}, oxytocin stimulates the GTPase RhoA and ROCK cascade (Woodcock *et al*., 2004). This signalling process is Ca^{2+}-independent and involves direct phosphorylation of MLC_{20} and inactivation of the myosin binding subunit of MLC_{20} phosphatase (Kimura *et al*., 1996; Amano *et al*., 2000). As this maintains MLC_{20} in a phosphorylated state, the uterus becomes sensitised to Ca^{2+}. *In vitro* studies show that myometrial tissues are more responsive to Ca^{2+} and oxytocin in the term pregnant compared to the non-gravid state (Word *et al*., 1993; Riley *et al*., 2005). This corresponds to the dramatic increase in RhoA mRNA, but not protein (Friel *et al*., 2005; Lartey *et al*., 2007), and elevated ROCKI and ROCKII mRNA and protein expression in rat (Niiro *et al*, 1997) and human myometrium at term pregnancy (Moore *et al*., 2000; Moran *et al*., 2002; Moore & Lopez Bernal, 2003). Although conflicting reports have been published (Friel *et al*., 2005; Lartey *et al*., 2006), it is likely that the ROCK pathway is involved in augmenting uterine contractions during labour (Niiro *et al*., 1997; Moore *et al*., 2000; Moran *et al*., 2002; Lartey & Lopez Bernal, 2009).

RT-PCR using total RNA revealed relatively high expression of RhoA and ROCKI in primary uterine myocytes compared to ROCKII at term pregnancy. The detection of ROCK proteins was also similar in human myometrial tissue (Moran *et al*., 2002; Friel *et al*., 2005). As the two ROCK isoforms have only 64 percent homology in sequence structure (Ishizaki *et al*., 1996) each isoform may have distinct functions. With abundant expression in the uterus, ROCKI may be the primary effector of RhoA

for actin myofilament assembly and indirect regulation of MLCK phosphorylation. However, despite the wide distribution of MLCK in smooth muscle tissues (Word *et al*., 1993; Moore & Lopez Bernal, 2001), MLCK transcripts showed faint and multiple banding after gel electrophoresis in this study. Therefore, it is likely that the complementary sequences of primers for MLCK were poorly designed and the experiments need repeating. In contrast the protein telokin, whose sequence is identical to the C-terminus of smooth muscle MLCK (Ito *et al*., 1989), was richly expressed in myocytes. *In vitro*, telokin blocks the unphosphorylated S2 region of the myosin head to competitively inhibit MLCK binding (Katayama *et al*., 1995). Smooth muscle telokin also acts a negative modulator of Ca^{2+} sensitisation by enhancing MLC_{20} phosphatase activity (Khromov *et al*., 2006). Such events resulting in utero-relaxation and may be important before labour-onset.

The contribution of rho on oxytocin-mediated Ca^{2+} transduction signals was investigated in this study. Agonist activation of RhoA has been shown to increase the stability of the p160 ROCKI isoform (Moore & Lopez Bernal, 2003) for enhanced tonic phase myogenic activity (Kupittayanant *et al*., 2001). As a result, several inhibitors of serine/ threonine protein kinase have been manufactured to block RhoA effects for smooth muscle relaxation (Uehata *et al*., 1997; Kupittayanant *et al*., 2001; Oh *et al*., 2003; Woodcock *et al*., 2004; Lartey & Lopez Bernal, 2009).

Although the cyclohexane carboxamide Y-27632 is the most widely used ATP-competitive inhibitor with equal potency against ROCKI and ROCKII (Fu *et al*., 1998; Moran *et al*., 2002; Uehata *et al*., 1997; Ikenoya *et al*., 2002), the isoquinolinesulfonamide rho-kinase inhibitor was chosen for pre-incubation with myometrial cells. This was reported to be more potent, selective and membrane-permeable than Y-26732 (Ikenoya *et al*., 2002; Sasaki *et al*., 2002). Unlike short

exposure to the rho-kinase inhibitor, challenge for 24 hours attenuated oxytocin-induced Ca^{2+} transients by up to 50 percent. This reduction was more pronounced in uterine fibroblasts than myocytes, indicating a larger involvement of rho transduction signals in these fibroblastic cells. Pharmacological inhibition of ROCK by Y-27632 (Somlyo & Somlyo, 1998) or by ADP ribosylation (Otto *et al*., 1996) has also been shown to decrease oxytocin-induced Ca^{2+} sensitisation of uterine smooth muscle cell force. The mechanism of inhibiting this agonist-mediated Ca^{2+} pathway is not well understood. Even so, RhoA is known to promote cell migration and actin myofilament assembly, regulating the formation of stress fibres and *lamellipodia* protrusions (Matsumoto *et al*., 1997; Kawabata *et al*., 2004; Katoh *et al*., 2001; Lartey *et al*., 2007). Therefore, it is likely that RhoA in fibroblasts improves the capacity and mechanotransduction properties of the gravid uterus by contributing to uterine hypertrophy and hyperplasia (Varayoud *et al*., 2001). In fibroblasts, the enhanced RhoA may also reflect their morphological features and faster proliferation rate compared to myocytes. Moreover, tissue remodelling is associated with the caspase-3 effector, which is anti-contractile and activates cell hypertrophy at mid-gestation and apoptosis at term (Shynlova *et al*., 2010) by cleaving ROCKI (Sebbagh *et al*., 2005). It is possible that caspase-3 reduces the cell viability of fibroblasts over myocytes during postpartum involution, enabling the uterus to return to its non-gravid smooth muscle phenotype.

To conversely assess the effects of enhanced ROCK activity, the caspase-3 inhibitor Z-DEVD-FMK was used in this study. Inhibition of caspase-3, a cysteine-dependent protease, prevents the cleavage of four putative functional domains within ROCKI (Ishizaki *et al*., 1996; Moore *et al*., 2002; Moore & Lopez Bernal, 2003), without affecting ROCKII (Sebbagh *et al*., 2005). This blocks the irreversible proteolysis of

pre-existing active p160 ROCKI to yield relatively inactive p130 ROCKI (Moore *et al*., 2002; Moore & Lopez Bernal, 2003). Despite the enhanced p160 stability, treatment with the caspase-3 inhibitor caused negligible effects on oxytocin-induced Ca^{2+} transients in both uterine myocytes and fibroblasts. This may reflect the mechanism of ROCK generating actomyosin cross-bridging and force generation at a constant concentration of intracellular-free Ca^{2+}.

The lack of effect on oxytocin-induced Ca^{2+} mobilisation was substantiated by the TP receptor antagonist SQ29,548. Unlike caspase-3 inhibition, chronic stimulation with SQ29,548 mediates cleavage of the C-terminal autoinhibitory domain of p160 ROCKI to produce p130 ROCKI (Moore & Lopez Bernal, 2003). In spite of promoting apoptosis, p130 ROCKI has been shown to irreversibly stimulate Ca^{2+} independent pathways (Moore & Lopez Bernal, 2003). Due to the contrast in results, this suggests that the rho-kinase inhibitor may have targeted an alternative pathway. As well as the six possible rho effectors identified in human myometrium (Lartey *et al*., 2007), the rho-kinase inhibitor has been reported to have weak affinity binding with other serine/threonine kinases, including protein kinase A (PKA) and PKC (Ikenoya *et al*., 2002; Sasaki *et al*., 2002). Due to their reported interaction with Ca^{2+} transport systems (Sanborn *et al*., 1998), these kinases may have altered the Ca^{2+} dynamics observed in this study. In this regard, the secondary messenger cAMP cascade was also investigated.

Activation of the effector enzyme adenylyl cyclase through Gαs is responsible for generating cAMP from ATP for muscle relaxation (Price & Lopez Bernal, 2001). Whilst the exact mechanisms remain obscure, cAMP-dependent PKA is reported to phosphorylate MLCK, inhibit PLC activation and block Ca^{2+} entry via K^+ channels and Ca^{2+} pumps (Sanborn *et al*, 1998). As a result, cAMP attenuates the oxytocin-

stimulated PLC pathway in rat myometrium (Anwer *et al*., 1990), but not in human myometrial cells (Phaneuf *et al*., 1993). In both species, the expression of Gαs increases throughout gestation and declines during labour (Europe-Finner *et al*., 1994; Sanborn *et al*., 1998) by a mechanism that is reported to involve alternative splice variants of Gαs (Europe-Finner *et al*., 1997). This may contribute to the loss of quiescence at labour-onset.

As well as the diterpene forskolin, PGE_2 and butaprost were shown to substantially increase cAMP in primary cultures of human myocytes. The formation of cAMP by the specific EP_2 receptor agonist butaprost was nearly as potent as PGE_2, which activates EP_2, $EP_{3\text{-II}}$, $EP_{3\text{-IV}}$ and EP_4 receptors coupled to Gαs (Coleman *et al*., 1994; Regan *et al*., 1994; Kotani *et al*., 1995). Accordingly, previous studies have shown weaker Gαs binding by EP_4 receptors than EP_2 (Fujino *et al*., 2002) and a paucity of elevated cAMP in EP_4-mediated PGE_2 signalling (Pozzi *et al*., 2004). This indicates that EP_4 receptors have a minor role in generating cAMP compared to the EP_2 subtype. As PGE_2 also acts through EP_1 and EP_3 pathways coupled to Ca^{2+} influx and inhibition of adenylyl cyclase respectively (Regan *et al*., 1994; Asboth *et al*., 1997), the results also suggest that EP_2-induced cAMP signalling predominates at term pregnancy.

Of the EP receptor transcripts in cultured human myocytes, EP_2 mRNA was more abundant than EP_1, whilst the complement of EP_3 and EP_4 subtypes was low. A similar pattern of dominant EP_2 expression over EP_4 receptors was reported in late gestational myometrial cells (Erkinheimo *et al*., 2000) and lower segment biopsies (Brodt-Eppley & Myatt, 1999; Leonhardt *et al*., 2003; Astle *et al*., 2005; Grigsby *et al*., 2006). Given that EP_2 receptors are more prevalent in the lower uterus, this further substantiates EP_2-mediated PGE_2 signalling for relaxation of the uterus. In both upper

and lower segments, EP_1 transcripts are upregulated with labour (Astle *et al*., 2005), converse to the decreases in EP_3 at late gestation (Matsumoto *et al*., 1997; Wing *et al*., 2003; Astle *et al*., 2005). Unlike EP_1 receptors, staining for EP_3 proteins is weak and diffuse in myometrial smooth muscle cells (Astle *et al*., 2005) or only expressed by stromal and epithelial cells (Leonhardt *et al*., 2003). This was consistent with the low detection of EP_3 in this study. Therefore, direct changes in the relative expression of EP_2 and EP_1 receptors are more likely to mediate functional myometrial effects.

To elucidate the underlying mechanisms of EP_2 on oxytocin receptors, uterine smooth muscle cells were pre-incubated with butaprost. As butaprost is shown to antagonise oxytocin-induced contractions, this EP_2 receptor agonist has suggested tocolytic effects (Duckworth *et al*., 2002). In this study, the attenuated responses to oxytocin were inversely related to an increase in cAMP formation, without affecting Ca^{2+} release. As each receptor-binding site is distinct (Coleman *et al*., 1994; Kimura *et al*., 1996), butaprost is likely to attenuate oxytocin-induced myometrial excitation through cAMP transduction mechanisms. In addition to implicating cAMP as a major component for maintaining utero-quiescence at term, the results suggest that a balance in these receptors and their downstream pathways influence the timing of labour-onset.

Gene transcripts for DP, $EP_{1\text{-}4}$, IP, FP, TP, oxytocin receptors and contractile elements were detected in uterine myocytes in this study. Although the receptors are temporally and spatially regulated in the uterus, the underlying genomic changes are not well understood (Havelock *et al*., 2005). To regulate gene transcription, myometrial EP and FP receptors are reported to co-localise in the nuclear membrane (Bhattacharya *et al*., 1998; Leonhardt *et al*., 2003; Astle *et al*., 2005) together with COX enzymes for PG biosynthesis (Spencer *et al*., 1998). PGs have also been hypothesised to directly

interact and increase the expression of oxytocin receptors (Wing *et al*., 2006). Even so, pre-incubation of myocytes with combinations of U46619 and SQ29,548 or oxytocin and atosiban had no genomic effect on mRNA expression. It is possible that higher concentrations of these compounds, a longer duration of exposure or the measurement of translated proteins would have shown a more marked response in this study. Other endocrine factors, including oestrogen, progesterone or inflammatory cytokines may have also been required for transcription (Mohan *et al*., 2004). Even so, further studies would be needed to clarify their complex regulation.

Taken together, these studies show that myometrial cells can be used as a model of the gravid uterus for high throughput screening of drugs. Whilst primary cell cultures showed similar ultrastructures, uterine fibroblasts had a lower capacity for Ca^{2+} release but higher sensitivity to the ROCK pathway than myocytes. This may have important implications in uterine tissue remodelling and postpartum involution. Compared with PGs, oxytocin-induced contractility and Ca^{2+} oscillations were of greater amplitude. However, complex Ca^{2+} and cAMP signalling pathways are part of a multiplicity of downstream events at term gestation. As it is probable that a loss of uterine quiescence is pivotal to successful parturition (Price & Lopez Bernal, 2001), maintaining EP_2 receptors or intrinsic adenylyl cyclase activity may represent a novel tocolytic approach for hypercontractile uterine disorders, such as preterm labour.

Chapter 8: Final discussion

The main aim of this thesis was to determine the functional expression of EP, FP, TP and oxytocin receptors in isolated human myometrial tissues. PGs and oxytocin have a critical role in orchestrating the changes between uterine quiescence and contractility. It is suggested that uterine receptor function is regulated by the hormonal milieu (Lopez Bernal, 2001), as well as the structural composition of the uterus (Luckas & Wray, 2000). This was demonstrated in the present study by changes in the *in vitro* responsiveness to PGs and oxytocin during the menstrual cycle, pregnancy and labour.

Under physiological conditions, myometrial tissue strips exhibited sustained spontaneous contractions, which were most pronounced during the follicular phase of the cycle. The decline in fundus tissue activity through the luteal phase to sporadic contractions at menses corresponded to progesterone action at cognate PR-B receptors (Bulletti *et al*., 1998; Ijland *et al*., 1998) and the decrease in oestrogen and ERα expression at this time (Noe *et al*., 1999). Topographical differences were also shown mid-cycle with the most active contractile waves generated by lower segment non-gravid myometrium. Although not anatomically defined (Wray *et al*., 2001), these *in vitro* intrinsic action potentials indicated the presence of pacemaker Cajal-like interstitial cells (Ciontea *et al*., 2005; Young, 2007). During the periovulatory period, this is reported to direct the retrograde transport of potential sperm (Kunz *et al*., 1996; Bulletti *et al*., 1998).

With pregnancy, the uterus undergoes dramatic expansion whilst remaining relatively quiescent. The changes in tissue remodelling involve two growth phases of cell hyperplasia followed by cell hypertrophy, primarily within the distensible lower

segment tissue (Luckas & Wray, 2000; Shynlova *et al*., 2010). At Caesarean section, only lower segment gestational myometrium was available to study. This tissue was used in functional studies (Section I) and myometrial cell cultures (Section II) to elucidate better the underlying mechanisms of uterine contractility.

At term, *in vitro* contractions were defined and up to 5-fold greater in amplitude, although less frequent, than tissues obtained from non-gravid donors. As well as mechanical stretch and foetal signalling, the elevated plasma oestrogen to progesterone ratio in late gestation may account for these changes in activity (Johnson *et al*., 1995). Progesterone represses the transcription of contractile-associated proteins (Garfield *et al*., 1980; Soloff *et al*., 1983) and also directly interacts with oxytocin receptors to maintain utero-quiescence (Grazzini *et al*., 1998). Conversely oestrogens activate the oestrogen response element on target genes for enhanced myogenic activity. In particular, 17β-oestradiol is shown to enhance the formation of gap junctions in the late gestational uterus for coordinated excitation-electrical coupling between cells (Garfield *et al*., 1990; Dong & Yallampalli, 2000; Kilarski *et al*., 2000). In this study, 17β-oestradiol also elevated oxytocin-induced Ca^{2+} release, whilst progesterone counteracted this effect. This confirms the importance of the sex steroids in sensitising the myometrium to stimulatory agonists.

Mechanical responses to PGs and oxytocin reflected tissue spontaneous activity and appeared to be spatially and temporally regulated during the menstrual cycle. At the fundus, bell-shaped responses to PGE_2 consisted of concentration-dependent utero-relaxation, followed by partial restoration of activity. This demonstrated PGE_2 coupling at EP receptor subtypes (Coleman *et al*., 1994) in addition to the possible activation of FP receptors (Kiriyama *et al*., 1997). Corresponding to other studies (Senior *et al*., 1991; Popat & Crankshaw, 2001) use of sulprostone and ONO-D1-004

showed excitatory effects through the EP_3 subtype and only a small compliment of EP_1 receptors (Senior *et al*., 1991). In contrast, the selective agonists butaprost and AGN201734 attenuated myogenic activity via EP_2 and EP_4 subtypes respectively. Despite their presence, EP_4 receptors are reported to be devoid of function (Hillock & Crankshaw, 1999), and did not contribute to PGE_2-mediated utero-relaxation in this study. This suggests a prominent role for EP_2 receptors in the non-gravid uterus.

As little is known about the topography of functional PG receptors, responsiveness to PGs and oxytocin was investigated according to anatomical location in follicular phase myometrium. The inhibitory effects of PGE_2, butaprost and AGN201734 were more pronounced towards the cervix, whereas active $EP_{3/1}$ receptors were uniform. This may relate to the more abundant EP_2 receptors in the lower segment tissues (Astle *et al*., 2005) or presence of EP_4 receptors within uterine arteries that branch into the lower uterus before reaching the fundus and ovary (Baxter *et al*., 1995). The apparent functional regionalisation may also reflect total uterine physiology. Epithelial and vascular cells of the endometrium synthesise PGE_2 and co-express EP_2 and EP_4 receptors (Milne *et al*., 2001; Arosh *et al*., 2003). Through cellular proliferation and angiogenesis, the associated endometrial thickening at the fundus may reduce uterine activity for blastocyst attachment and implantation. On the contrary, PGE_2 within the seminal plasma is likely to intensify intrinsic retrograde contractions for sperm delivery to the upper uterus (Templeton *et al*., 1978).

Similar to PGE_2, the uterotonic effects of $PGF_{2\alpha}$, U46619 and oxytocin were greater in lower segment myometrium compared to the fundus. This corresponds to the fundus to cervix gradient of oxytocin receptors around ovulation (Richter *et al*., 2004; Steinwall *et al*., 2004) but not the reported decrease in PGF-binding affinity (Hofmann *et al*., 1983; Adelantado *et al*., 1988) and homogeneous distribution of TP

receptors (Senchyna & Crankshaw, 1999). As a result, it is likely that the hormonal milieu and mechanical properties of the uterus account for these regional differences. This is further implied in mice knockout studies with only the EP_2 subtype vital for successful establishment of pregnancy (Tilley *et al*., 1999). Even so, interactions between the receptor subtypes and their downstream effector signals are proposed to compensate for overall reproductive outcomes (Lopez Bernal *et al*., 2001; Duckworth *et al*., 2002). This was investigated further at term pregnancy.

PGE_2 is particularly important in the induction of labour by mediating both cervical compliance and myometrial activity (Gibb, 1998). Functional $EP_{1\text{-}3}$ receptors have been characterised at term (Senior *et al*., 1993) with the temporal and tissue-specific expression of EP receptors shown over the course of pregnancy and labour (Astle *et al*., 2005; Grigsby *et al*., 2006; Sooranna *et al*., 2006). In this study, PGE_2 and AGN201734 caused predominant utero-relaxatory effects with excitation re-established at 10^{-5}M. The EP_2 agonists were consistent in mediating uterine quiescence, whilst the other EP_4 mimetic L-902688 produced no response. The reason for this discrepancy in EP_4 action was unclear. However, in cultured myocytes, similar EP_2 and PGE_2-induced elevations in cAMP were observed and the EP_4 antagonist GW627368x did not modify the profile of PGE_2-induced contractions. These data suggest that PGE_2 supports utero-quiescence via activation of the EP_2 subtype.

PGE_2 also serves as an uterotonin via the EP_1 and EP_3 receptor complement during pregnancy. To mediate excitation, EP_1 receptors couple to Ca^{2+} influx whilst EP3 inhibits adenylyl cyclase (Coleman *et al*., 1994; Narumiya *et al*., 1999). In functional studies, sulprostone evoked moderate stimulation at $EP_{3/1}$ receptors, whilst ONO-D1-004 and the EP_3 antagonist AGN211329 produced negligible effects. Only minimal

Ca^{2+} mobilisation was induced by PGE_2 in cultured human myocytes and fibroblasts; however, $EP_{3/1}$ and EP_2 receptor stimulation of myometrial strips were greater at term gestation than in follicular phase tissues (Table 8.1). This corresponds to previous functional studies (Senior *et al*., 1991; Senior *et al*., 1993) and the reported upregulated (Grigsby *et al*, 2006) or maintained expression of EP_1 transcripts with advancing gestation and labour (Astle *et al*., 2005). Given the abundance of EP_2 mRNA over EP_1, EP_3 and EP_4 receptors in primary uterine myocytes at term, the results further indicate predominant effects via EP_2 receptors, whilst $EP_{1/3}$ receptors regulate muscle tonus. This suggests that these receptors may be crucial tocolytic targets for maintaining utero-quiescence before parturition. Nevertheless, further studies using more selective compounds including ONO-AE-248 and ONO-AE1-329 for EP_3 and EP_4 receptors respectively are still required to comprehensively elucidate $EP_{1\text{-}4}$ receptor function.

In late pregnancy, $PGF_{2\alpha}$-induced myogenic contractions were two-fold higher than in the non-gravid state (Table 8.2). This was inconsistent with the reported decrease in myometrial FP gene expression towards term (Matsumoto *et al*., 1997; Sooranna *et al*., 2005), perhaps reflecting differences in gestational age. In this study, however, U46619 was more potent than $PGF_{2\alpha}$ and neither showed additive effects. This substantiates previous studies showing a lack of off-target $PGF_{2\alpha}$ action at myometrial TP receptors (Hutchinson, 2005; Griffiths *et al*., 2006). Within uterine myocytes, exposure to $PGF_{2\alpha}$ produced greater Ca^{2+} transients than U46619 via the abundant expression of FP mRNA over TP receptors. Even so, uterine sensitivity to TP activation indicates that alternative signalling cascades are involved.

Table 8.1: pEC_{50} values (M) and the percentage change in myogenicity for concentration-effect curves to EP agonists (10^{-10}M to 10^{-5}M) in immersed lower segment myometrial tissues obtained during the follicular phase (n=4), at term pregnancy (n=4-10) and during early labour (n=4-6). Early labour was defined as regular *in vivo* contractions at a cervical dilation of 0-2cm with % change calculated from baseline activity as a change in % hypotonic shock. Results are expressed as arithmetic means ± S.E. and univariate analysis was performed using Bonferroni's *post-hoc* test; *p<0.05 for % difference in response to AGN201734 at early labour compared to the follicular phase of the menstrual cycle. ND = not determined.

		Follicular phase		**Term pregnancy**		**Early labour**	
EP agonist	**Receptors**	pEC_{50}	% change	pEC_{50}	% change	pEC_{50}	% change
PGE_2	$EP_{1\text{-}4}$	7.6 ± 0.1	73.5	8.4 ± 0.4	33.6	8.1 ± 0.4	52.9
Butaprost	EP_2	6.2 ± 0.4	83.1	7.1 ± 0.5	47.0	6.2 ± 0.3	45.2
AGN201734	EP_4	8.0 ± 0.1	68.2	8.1 ± 0.3	29.7	7.5 ± 1.0	31.5*
Sulprostone	$EP_{3/1}$	7.0 ± 0.1	25.1	7.4 ± 0.6	20.6	ND	ND

Table 8.2: pEC_{50} values (M) and the percentage increase in myogenicity for concentration-effect curves to FP, TP and oxytocin agonists in immersed lower segment myometrial tissues obtained during the follicular phase (n=4), at term pregnancy (n=5-7) and during early labour (n=5-6). Late labour was defined as donors at full dilatation (9-10cm) with % change calculated from baseline activity as a change in % hypotonic shock. Results are expressed as arithmetic means ± S.E. and univariate analysis was performed using Bonferroni's *post-hoc* test; *p<0.05 for % increase in response to U46619 for term pregnancy and late labour compared to the follicular phase of the menstrual cycle. N = no response.

		Follicular phase		**Term pregnancy**		**Late labour**	
Agonists	**Receptors**	pEC_{50}	% change	pEC_{50}	% change	pEC_{50}	% change
$PGF_{2\alpha}$	FP	7.0 ± 0.2	51.4	6.8 ± 1.8	100.3	6.1 ± 0.7	36.7
U46619	TP	6.2 ± 0.2	81.6	7.3 ± 0.5	267.5*	6.8 ± 0.1	322.0*
Oxytocin	oxytocin	7.5 ± 0.7	132.1	7.4 ± 0.4	271.0	N	N

Signal transduction via RhoA and ROCK are important in sensitising the uterus to Ca^{2+}. Challenge with U46619 is shown to generate p160 ROCKI and ROCKII, which maintains MLC_{20} phosphorylation (Moore *et al*., 20002; Moran *et al*., 2002; Moore & Lopez Bernal, 2003) and may account for the enhanced contractile responses at term in this study. Further Ca^{2+} sensitisation is mediated by irreversible caspase-3 cleavage

of p160 ROCKI to p130 ROCKI (Moore & Lopez Bernal, 2003). Despite the lack of change in caspase-3 inhibited tissues, the rho-kinase inhibitor attenuated Ca^{2+} signalling. This was more pronounced in fibroblasts over myocytes, suggesting a role for fibroblast-directed cell migration and apoptosis in tissue remodelling during pregnancy as well as postpartum involution.

In functional studies, oxytocin-induced myogenic contractions were twice as high at term than in follicular stage tissues, relating to the upregulated expression of oxytocin receptors before labour-onset (Fuchs *et al*., 1984; Kimura *et al*., 1996; Terzidou *et al*., 2005). Within primary uterine myocytes, Ca^{2+} influx by oxytocin was over 13-fold greater than that stimulated by $PGF_{2\alpha}$ or U46619, perhaps due to principal Ca^{2+} release from the intracellular stores. Compared to oxytocin, PG responses were at the limits of detection. Whilst this implied the significance of oxytocin-induced Ca^{2+} signals, a more sensitive method such as the fura-2 loaded cell system (Asboth *et al*., 1998) should replace use of the Flipr-tetra in further studies.

Limitations of the Flipr-tetra may also account for the paucity of effect by butaprost on oxytocin-induced Ca^{2+} transients. However, the reported EP_2-mediated attenuation of oxytocic effects *in vitro* (Duckworth *et al*., 2002) appeared to be mediated through cAMP rather than the Ca^{2+} signalling pathway in this study. This confirms cAMP as a major pro-relaxant mediator at term pregnancy and indicates that a decrease in uterine Gαs coupled receptors may lead to labour-onset.

Successful parturition requires coordinated contractions of the uterus in a caudal direction for delivery of the foetus. This involves the differential expression of uterine PG, oxytocin receptors and gap junction proteins to transmit action potentials in syncytium (Sparey *et al*., 1999; Astle *et al*., 2005; Grigsby *et al*., 2006). Functional progesterone withdrawal is a considered a pivotal event for labour-onset, but its

mechanisms are unclear. In humans, plasma progesterone remains high until postpartum delivery of the placenta (Broditsky *et al*., 1978; Darne *et al*., 1987). Instead a rise in nuclear PR-A receptors, which act as a transrepressor of the PR-B isoform, may mediate the timing of labour (Pieber *et al*., 2001; Mesiano *et al*., 2002). Since PG receptors are also important mediators of parturition, novel functional effects were demonstrated in this study.

Spontaneous contractions were attenuated in lower segment myometrial strips with advancing labour. During this time the extracellular collagen matrix in the cervix remodels and effaces upwards against the presenting foetus. The associated changes in tissue physiology include an increase in cervical fibroblasts within the lower uterus (Montes *et al*., 2002) as well as reduced ATPase activity (Zyrianov *et al*., 2003). Within cell culture systems, Ca^{2+} mobilisation was two-times lower in fibroblasts compared to myocytes. This may be a mechanism to moderate uterine resistance at the cervical end for controlled foetal passage.

In parallel with PGE_2 effects at term, predominant utero-relaxation was followed by relative contractility in tissues obtained early after labour-onset. In the latter stages of labour, however, the contractile component of the PGE_2 curve was suppressed. *In vitro* studies also confirmed the loss of PGE_2-mediated contractions in the lower, but not upper segment parturient uterus (Wikland *et al*., 1984); this corresponds to a regional change in the EP receptor complement.

Due to its temporal and topographical distribution, EP_2 receptors are particularly implicated in the parturition process. In the lower segment uterus, EP_2 mRNA and protein expression decreases towards term (Brodt-Eppley & Myatt, 1999; Leonhardt *et al*., 2003), but either shows no change (Brodt-Eppley& Myatt, 1999; Astle *et al*., 2005; Sooranna *et al*., 2006) or increases with advancing labour (Grigsby *et al*.,

2006). The abundance of EP_2 receptors in the lower compared with the upper uterus in pregnancy and labour relates to the continued relaxatory effects in myometrial strips at this time. For EP_4 receptors, a reduction in mRNA expression from the non-gravid to term gravid state (Sooranna *et al*., 2006) also corresponds to the attenuated myometrial responses to AGN201734 in this study. However, no change in EP_4 receptors is reported by gestational age or anatomical location (Astle *et al*., 2005; Grigsby *et al*., 2006). As a result, the EP_4 subtype may be responsible for cervical ripening (Schmitz *et al*., 2003) or vasodilatation (Baxter *et al*., 1995), rather than for modulating contractions during labour.

In the lower uterus obtained during labour, ONO-D1-004 had no effect on myogenic activity. This was in contrast to the reported presence of myometrial EP_1 receptors at this time (Astle *et al*., 2005; Grigsby *et al*., 2006). Although a more potent EP_1 agonist is required to clarify function (Kiriyama *et al*., 1997; Oka *et al*., 2003), it is suggested that EP_1 receptors mediate intracellular signalling pathways rather than promote contractility pre and postpartum (Grigsby *et al*., 2006). Conversely with advancing labour, sulprostone and $PGF_{2\alpha}$ stimulated weak *in vitro* myogenic contractions of low amplitude and frequency. This is consistent with the reported contractility induced by PGE_2 and $PGF_{2\alpha}$ only in paired fundus end specimens obtained during active labour (Wikland *et al*., 1984). The results also reflect the relative abundance of EP_3 (Astle *et al*., 2005; Grigsby *et al*., 2006) and FP mRNA in upper rather than lower segment tissues (Grigsby *et al*., 2006). As well as to promote uterine emptying, this may contribute to the relaxant phenotype of the lower uterus during parturition (Figure 8.1).

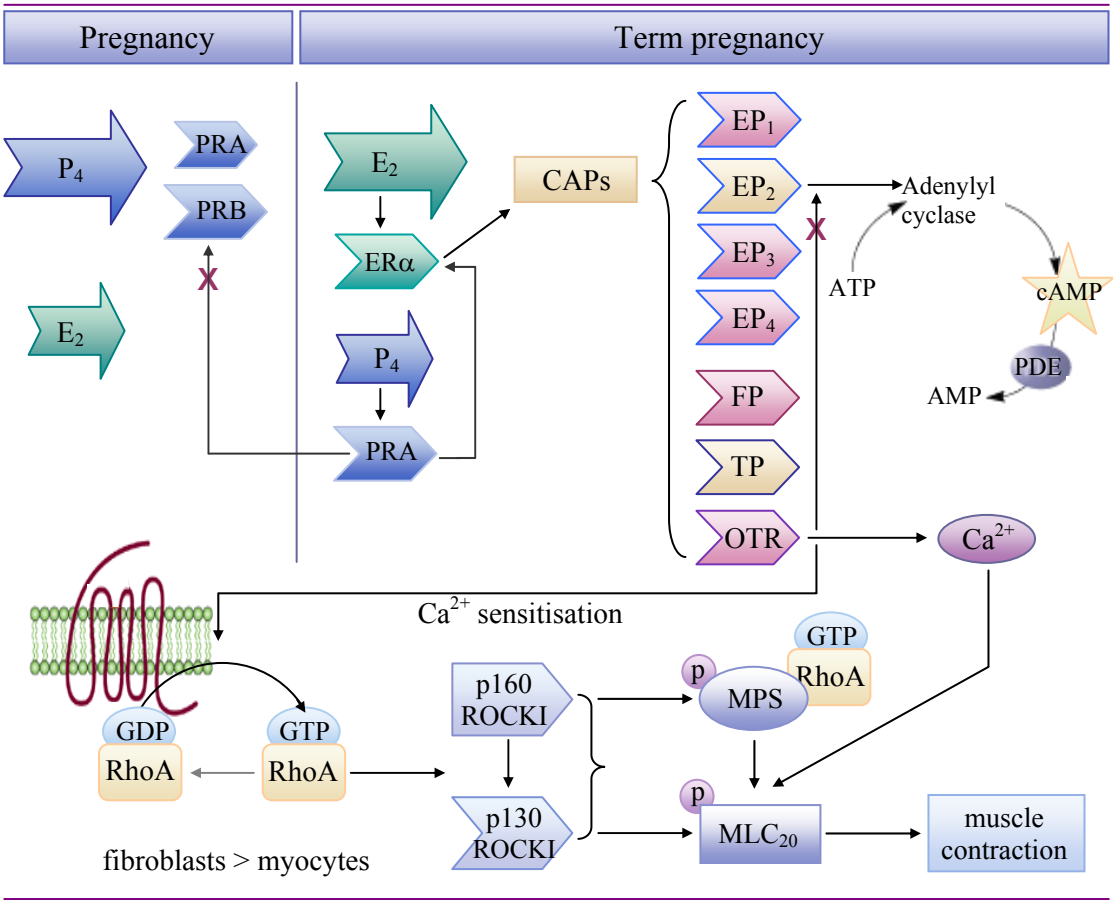


Figure 8.1: A summary diagram showing a proposed model for labour-onset in the lower segment human uterus. For the majority of pregnancy, progesterone (P_4) dominantly represses uterine activity. At term, increases in oestrogen (E_2), myometrial progesterone receptor (PR)-A and oestrogen receptor (ER)-α correspond with a rise in contractile associated proteins (CAPs). Of the PG receptors, uterorelaxation is predominantly mediated via the EP_2 receptor. This is coupled to the adenylyl cyclase pathway, which also attenuates oxytocic effects. Oxytocin stimulates potent intracellular-free Ca^{2+} flux to drive muscle contractions. The small GTPase RhoA and rho-kinase (ROCKI) pathway further sensitises the uterus to Ca^{2+} by phosphorylating (p) myosin light chain (MLC_{20}) directly and indirectly through inactivation of myosin phosphatase (MPS). With the onset and progression of labour, the expression of functional PG and oxytocin receptors are decreased (red), maintained or upregulated (yellow). To facilitate the delivery of the foetus, the overall decline in myogenic activity relates to the relaxation and stretch of the lower segment uterus.

Responsiveness to U46619 was enhanced in lower segment myometrial strips taken after labour-onset relative to low spontaneous contractions. Increased thromboxane synthesis at parturition (Noort *et al*., 1990; Swanson *et al*., 1992) alongside ROCK protein expression (Noort & Keirse, 1990; Friel *et al*., 2005) likely potentiates its contractile effects during active labour. Accordingly, thromboxane may maintain muscle tonus in the lower uterine segment as well as contribute to postpartum involution of the uterus. However, functional oxytocin desensitisation was observed with advancing labour and chronic oxytocin pretreatments. As the untimely loss of oxytocin receptor function is associated with labour-associated disorders (Rezapour *et al*., 1996), accurate diagnostic indicators would be crucial to allow for early therapeutic intervention. This would particularly be useful for improving the efficacy of atosiban clinical treatments.

Although myometrial responses to PG and oxytocin agonists were comparable, immersed tissue strips developed more stable phasic contractions of higher amplitude than tissues superfused with Krebs' solution. This approach was particularly suitable for myometrium from labouring donors with intrinsically low myogenic activity. In spite of the larger volumes of antagonists required for superfusion, the findings complemented immersion studies and clarified direct agonist effects on receptor activation. Given the limited number of myometrial tissues taken in menstrual, luteal and preterm states, a wider range of these samples would assist future research. Subject to ethical approval, paired fundus and lower segment tissues would also enable functional regionalisation to be defined during pregnancy and labour.

Based on the primary cell culture experiments in this study, cell isolation could be applied to tissues taken from differing hormonal and gestational states. This would aid direct comparisons of intracellular signalling events relating to the original tissue

dynamics. In screening for suitable tocolytics, receptor-binding studies would indicate the efficacy of receptor-effector coupling; this would be of particular interest for myometrial EP_1 and EP_4 receptors. By reconstituting cells into a collagen matrix (Dallot *et al*., 2003) or onto a polyglactin mesh (Young *et al*., 2002), the measurement of cellular contractile responses would also support immersion and superfusion studies. This use of cells in a regulated microenvironment may enhance understanding of the mechanisms that transform the uterus from a quiescent to an active state.

It would also be beneficial to clarify uterine properties throughout pregnancy and during the involution process postpartum. Due to ethical constraints in women, the use of an animal model would be of great importance. Previous studies using mice indicate that the uterus expresses similar PG receptors to the human, but it more resembles expected fundus rather than lower segment characteristics (Griffiths, 2007). This is complicated further by the species-specific differences in the parturition process. As a result, the animal models alone would not be sufficient to translate into human uterine function.

Taken together, the findings of this thesis have clarified further EP, FP, TP and oxytocin receptor functions in isolated myometrium taken during the menstrual cycle, pregnancy and at different stage of labour. Primary myocytes and fibroblasts models of the gravid uterus indicate that EP_2 receptors are crucial in attenuating oxytocic effects via cAMP signal cascades. Oxytocin action corresponds to myogenic profiles and was desensitised in the lower uterus with advancing labour. However, TP-mediated effects were maintained. These receptors and their secondary messenger systems therefore represent effective myometrial targets for uterine hypercontractile disorders. The identification of therapies with minimal side effects for the management of women's reproductive health remains an important challenge.

References

ABRAMOVITZ, M., ADAM, M., BOIE, Y., CARRIERE, M., DENIS, D., GODBOUT, C., LAMONTAGNE, S., ROCHETTE, C., SAWYER, N., TREMBLAY, N. M., BELLEY, M., GALLANT, M., DUFRESNE, C., GAREAU, Y., RUEL, R., JUTEAU, H., LABELLE, M., OUIMET, N. & METTERS, K. M. (2000). The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. *Biochim Biophys Acta,* 1483**,** 285-293.

ABRAMOVITZ, M., BOIE, Y., NGUYEN, T., RUSHMORE, T. H., BAYNE, M. A., METTERS, K. M., SLIPETZ, D. M. & GRYGORCZYK, R. (1994). Cloning and expression of a cDNA for the human prostanoid FP receptor. *J Biol Chem,* 269**,** 2632-2636.

ADELANTADO, J. M., LOPEZ BERNAL, A. & TURNBULL, A. C. (1988). Topographical distribution of prostaglandin E receptors in human myometrium. *Brit J Obstet Gynaec,* 95**,** 348-353.

AKERLUND, M., STROMBERG, P., HAUKSSON, A., ANDERSEN, L. F., LYNDRUP, J., TROJNAR, J. & MELIN, P. (1987). Inhibition of uterine contractions of premature labour with an oxytocin analogue. Results from a pilot study. *Brit J Obstet Gynaec,* 94**,** 1040-1044.

ALEXANDER, S. P., MATHIE, A. & PETERS, J. A. (2007). Guide to Receptors and Channels (GRAC), 2nd edition (2007 Revision). *Brit J Pharmacol,* 150 Suppl 1**,** S1-168.

ALFAIDY, N., XIONG, Z. G., MYATT, L., LYE, S. J., MACDONALD, J. F. & CHALLIS, J. R. (2001). Prostaglandin $F_{2\alpha}$ potentiates cortisol production by stimulating 11β-hydroxysteroid dehydrogenase 1: a novel feedback loop that may contribute to human labor. *J Clin Endocr Metab,* 86**,** 5585-5592.

ALLIX, S., REYES-GOMEZ, E., AUBIN-HOUZELSTEIN, G., NOEL, D., TIRET, L., PANTHIER, J. J. & BERNEX, F. (2008). Uterine contractions depend on KIT-positive interstitial cells in the mouse: genetic and pharmacological evidence. *Biol Reprod,* 79**,** 510-517.

ALLPORT, V. C., PIEBER, D., SLATER, D. M., NEWTON, R., WHITE, J. O. & BENNETT, P. R. (2001). Human labour is associated with nuclear factor-kappa β activity which mediates cyclooxygenase-2 expression and is involved with the 'functional progesterone withdrawal'. *Mol Hum Reprod,* 7**,** 581-586.

ALTUNYURT, S., GOL, M., SEZER, O. & DEMIR, N. (2005). Primary dysmenorrhea and uterine blood flow: a color Doppler study. *J Reprod Med,* 50**,** 251-255.

AMANO, M., FUKATA, Y. & KAIBUCHI, K. (2000). Regulation and functions of Rho-associated kinase. *Exp Cell Res,* 261**,** 44-51.

AROSH, J. A., BANU, S. K., CHAPDELAINE, P., EMOND, V., KIM, J. J., MACLAREN, L. A. & FORTIER, M. A. (2003). Molecular cloning and characterization of bovine prostaglandin E_2 receptors EP_2 and EP_4: expression and regulation in endometrium and myometrium during the estrous cycle and early pregnancy. *Endocrinology,* 144**,** 3076-3091.

ASBOTH, G., PHANEUF, S., EUROPE-FINNER, G. N., TOTH, M. & BERNAL, A. L. (1996). Prostaglandin E_2 activates phospholipase C and elevates intracellular calcium in cultured myometrial cells: involvement of EP_1 and EP_3 receptor subtypes. *Endocrinology,* 137**,** 2572-2579.

ASBOTH, G., PHANEUF, S. & LPEZ BERNAL, A. L. (1997). Prostaglandin E receptors in myometrial cells. *Acta Physiol Hung,* 85**,** 39-50.

ASSELIN, E., BAZER, F. W. & FORTIER, M. A. (1997). Recombinant ovine and bovine interferons tau regulate prostaglandin production and oxytocin response in cultured bovine endometrial cells. *Biol Reprod,* 56**,** 402-408.

ASTLE, S., KHAN, R. N. & THORNTON, S. (2003). The effects of a progesterone metabolite, 5β-dihydroprogesterone, on oxytocin receptor binding in human myometrial membranes. *Brit J Obstet Gynaec,* 110**,** 589-592.

ASTLE, S., SLATER, D. M. & THORNTON, S. (2003). The involvement of progesterone in the onset of human labour. *Eur J Obstet Gyn R B,* 108**,** 177-181.

ASTLE, S., THORTON, S. & SLATER, D. M. (2005). Identification and localization of prostaglandin E_2 receptors in upper and lower segment human myometrium during pregnancy. *Mol Hum Reprod,* 11**,** 279-287.

BACKLIN, B. M., BREDHULT, C. & OLOVSSON, M. (2003). Proliferative effects of estradiol, progesterone, and two CB congeners and their metabolites on gray seal (Halichoerus grypus) uterine myocytes in vitro. *Toxicol Sci,* 75**,** 154-160.

BAIRD, D. T., CAMERON, S. T., CRITCHLEY, H. O., DRUDY, T. A., HOWE, A., JONES, R. L., LEA, R. G. & KELLY, R. W. (1996). Prostaglandins and menstruation. *Eur J Obstet Gyn R B,* 70**,** 15-17.

BAXTER, G. S., CLAYTON, J. K., COLEMAN, R. A., MARSHALL, K., SANGHA, R. & SENIOR, J. (1995). Characterization of the prostanoid receptors mediating constriction and relaxation of human isolated uterine artery. *Brit J Pharmacol,* 116**,** 1692-1696.

BECHER, N., HEIN, M., DANIELSEN, C. C. & ULDBJERG, N. (2004). Matrix metalloproteinases and their inhibitors in the cervical mucus plug at term of pregnancy. *Am J Obstet Gynecol,* 191**,** 1232-1239.

BELLEY, M., CHAN, C. C., GAREAU, Y., GALLANT, M., JUTEAU, H., HOUDE, K., LACHANCE, N., LABELLE, M., SAWYER, N., TREMBLAY, N., LAMONTAGNE, S., CARRIERE, M. C., DENIS, D., GREIG, G. M., SLIPETZ, D., GORDON, R., CHAURET, N., LI, C., ZAMBONI, R. J. & METTERS, K. M. (2006). Comparison between two classes of selective EP_3 antagonists and their biological activities. *Bioorg Med Chem Lett,* 16**,** 5639-5642.

BERRIDGE, M. J. (2008). Smooth muscle cell calcium activation mechanisms. *J Physiol,* 586**,** 5047-5061.

BHATTACHARYA, M., PERI, K., RIBEIRO-DA-SILVA, A., ALMAZAN, G., SHICHI, H., HOU, X., VARMA, D. R. & CHEMTOB, S. (1999). Localization of functional prostaglandin E_2 receptors EP_3 and EP_4 in the nuclear envelope. *J Biol Chem,* 274**,** 15719-15724.

BHATTACHARYA, M., PERI, K. G., ALMAZAN, G., RIBEIRO-DA-SILVA, A., SHICHI, H., DUROCHER, Y., ABRAMOVITZ, M., HOU, X., VARMA, D. R. & CHEMTOB, S. (1998). Nuclear localization of prostaglandin E_2 receptors. *P Natl Acad Sci USA,* 95**,** 15792-15797.

BILLOT, X., CHATEAUNEUF, A., CHAURET, N., DENIS, D., GREIG, G., MATHIEU, M. C., METTERS, K. M., SLIPETZ, D. M. & YOUNG, R. N. (2003). Discovery of a potent and selective agonist of the prostaglandin EP_4 receptor. *Bioorg Med Chem Lett,* 13**,** 1129-1132.

BLANKS, A. M., SHMYGOL, A. & THORNTON, S. (2007). Regulation of oxytocin receptors and oxytocin receptor signalling. *Semin Reprod Med,* 25**,** 52-59.

BORODITSKY, R. S., REYES, F. I., WINTER, J. S. & FAIMAN, C. (1978). Maternal serum estrogen and progesterone concentrations preceding normal labor. *Obstet Gynecol,* 51**,** 686-691.

BOROVIKOV YU, S., HORIUCHI, K. Y., AVROVA, S. V. & CHACKO, S. (1996). Modulation of actin conformation and inhibition of actin filament velocity by calponin. *Biochemistry,* 35**,** 13849-13857.

BOS, C. L., RICHEL, D. J., RITSEMA, T., PEPPELENBOSCH, M. P. & VERSTEEG, H. H. (2004). Prostanoids and prostanoid receptors in signal transduction. *Int J Biochem Cell B,* 36**,** 1187-1205.

BOSSMAR, T., AKERLUND, M., FANTONI, G., SZAMATOWICZ, J., MELIN, P. & MAGGI, M. (1994). Receptors for and myometrial responses to oxytocin and vasopressin in preterm and term human pregnancy: effects of the oxytocin antagonist atosiban. *Am J Obstet Gynecol,* 171**,** 1634-1642.

BRENNINKMEIJER, C. B., PRICE, S. A., LOPEZ BERNAL, A. & PHANEUF, S. (1999). Expression of G-protein-coupled receptor kinases in pregnant term and non-pregnant human myometrium. *J Endocrinol,* 162**,** 401-408.

BREYER, R. M., BAGDASSARIAN, C. K., MYERS, S. A. & BREYER, M. D. (2001). Prostanoid receptors: subtypes and signalling. *Annu Rev Pharmacol,* 41**,** 661-690.

BRODT-EPPLEY, J. & MYATT, L. (1998). Changes in expression of contractile FP and relaxatory EP_2 receptor in pregnant rat myometrium during late gestation, at labor and postpartum. *Biol Reprod,* 59**,** 878-883.

BRODT-EPPLEY, J. & MYATT, L. (1999). Prostaglandin receptors in lower segment myometrium during gestation and labor. *Obstet Gynecol,* 93**,** 89-93.

BUHIMSCHI, C. S., BUHIMSCHI, I. A., YU, C., WANG, H., SHARER, D. J., DIAMOND, M. P., PETKOVA, A. P., GARFIELD, R. E., SAADE, G. R. & WEINER, C. P. (2006). The effect of dystocia and previous Caesarean uterine scar on the tensile properties of the lower uterine segment. *Am J Obstet Gynecol,* 194**,** 873-883.

BULLETTI, C., DE ZIEGLER, D., ALBONETTI, A. & FLAMIGNI, C. (1998). Paracrine regulation of menstruation. *J Reprod Immunol,* 39**,** 89-104.

BULLETTI, C., DE ZIEGLER, D., POLLI, V., DEL FERRO, E., PALINI, S. & FLAMIGNI, C. (2002). Characteristics of uterine contractility during menses in women with mild to moderate endometriosis. *Fertil Steril,* 77**,** 1156-1161.

BURGHARDT, R. C., BARHOUMI, R., SANBORN, B. M. & ANDERSEN, J. 1999. Oxytocin-induced Ca^{2+} responses in human myometrial cells. *Biol Reprod,* 60**,** 777-782.

BUSCHER, U., CHEN, F. C., RIESENKAMPFF, E., VON DEHN, D., DAVID, M. & DUDENHAUSEN, J. W. (2001). Effects of oxytocin receptor antagonist atosiban on pregnant myometrium in vitro. *Obstet Gynecol,* 98**,** 117-121.

CALDER, A. A. (1990). Prostaglandins as therapy for labour induction or therapeutic abortion. *Reprod Fert Develop,* 2**,** 553-556.

CAO, J., WAKATSUKI, A., YOSHIDA, M., KITAZAWA, T. & TANEIKE, T. (2004). Thromboxane A_2 (TP) receptor in the non-pregnant porcine myometrium and its role in regulation of spontaneous contractile activity. *Eur J Pharmacol,* 485**,** 317-327.

CAO, J., YOSIDA, M., KITAZAWA, T. & TANEIKE, T. (2005). Uterine region-dependent differences in responsiveness to prostaglandins in the non-pregnant porcine myometrium. *Prostag other Lipid M,* 75**,** 105-122.

CARITIS, S. (2005). Adverse effects of tocolytic therapy. *Brit J Obstet Gynaec,* 112 Suppl 1**,** 74-78.

CARRASCO, M. P., PHANEUF, S., ASBOTH, G. & LOPEZ BERNAL, A. (1996). Fluprostenol activates phospholipase C and Ca^{2+} mobilization in human myometrial cells. *J Clin Endocr Metab,* 81**,** 2104-2110.

CASEY, M. L., MACDONALD, P. C., MITCHELL, M. D. & SNYDER, J. M. (1984). Maintenance and characterization of human myometrial smooth muscle cells in monolayer culture. *In Vitro,* 20**,** 396-403.

CHALLIS, J. R., MATTHEWS, S. G., GIBB, W. & LYE, S. J. (2000). Endocrine and paracrine regulation of birth at term and preterm. *Endocr Rev,* 21**,** 514-550.

CHALLIS, J. R., SLOBODA, D. M., ALFAIDY, N., LYE, S. J., GIBB, W., PATEL, F. A., WHITTLE, W. L. & NEWNHAM, J. P. (2002). Prostaglandins and mechanisms of preterm birth. *Reproduction,* 124**,** 1-17.

CHANDRASEKHARAN, N. V., DAI, H., ROOS, K. L., EVANSON, N. K., TOMSIK, J., ELTON, T. S. & SIMMONS, D. L. (2002). COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *P Natl Acad Sci USA,* 99**,** 13926-13931.

CHANRACHAKUL, B., PIPKIN, F. B., WARREN, A. Y., ARULKUMARAN, S. & KHAN, R. N. (2005). Progesterone enhances the tocolytic effect of ritodrine in isolated pregnant human myometrium. *Am J Obstet Gynecol,* 192**,** 458-463.

CHARPIGNY, G., LEROY, M. J., BREUILLER-FOUCHE, M., TANFIN, Z., MHAOUTY-KODJA, S., ROBIN, P., LEIBER, D., COHEN-TANNOUDJI, J., CABROL, D., BARBERIS, C. & GERMAIN, G. (2003). A functional genomic study to identify differential gene expression in the preterm and term human myometrium. *Biol Reprod,* 68**,** 2289-2296.

CHEUNG, P. Y., WALTON, J. C., TAI, H. H., RILEY, S. C. & CHALLIS, J. R. (1992). Localization of 15-hydroxy prostaglandin dehydrogenase in human fetal membranes, decidua and placenta during pregnancy. *Gynecol Obstet Invest,* 33**,** 142-146.

CHIBBAR, R., MILLER, F. D. & MITCHELL, B. F. (1993). Synthesis of oxytocin in amnion, chorion, and decidua may influence the timing of human parturition. *J Clin Invest,* 91**,** 185-192.

CHRISTENSEN, N. J. & GREEN, K. (1983). Bioconversion of arachidonic acid in human pregnant reproductive tissues. *Biochem Med,* 30**,** 162-180.

CIONTEA, S. M., RADU, E., REGALIA, T., CEAFALAN, L., CRETOIU, D., GHERGHICEANU, M., BRAGA, R. I., MALINCENCO, M., ZAGREAN, L., HINESCU, M. E. & POPESCU, L. M. (2005). C-kit immunopositive interstitial cells (Cajal-type) in human myometrium. *J Cell Mol Med,* 9**,** 407-420.

CLARKE, A., BLACK, N., ROWE, P., MOTT, S. & HOWLE, K. (1995). Indications for and outcome of total abdominal hysterectomy for benign disease: a prospective cohort study. *Brit J Obstet Gynaec,* 102**,** 611-620.

CLARKE, D. L., GIEMBYCZ, M. A., PATEL, H. J. & BELVISI, M. G. (2004). E-ring 8-isoprostanes inhibit ACh release from parasympathetic nerves innervating guinea-pig trachea through agonism of prostanoid receptors of the EP_3-subtype. *Brit J Pharmacol,* 141**,** 600-609.

COLEMAN, M. L., SAHAI, E. A., YEO, M., BOSCH, M., DEWAR, A. & OLSON, M. F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCKI. *Nat Cell Biol,* 3**,** 339-345.

COLEMAN, R. A., SMITH, W. L. & NARUMIYA, S. (1994). International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev,* 46**,** 205-229.

CONDON, J. C., HARDY, D. B., KOVARIC, K. & MENDELSON, C. R. (2006). Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function. *Mol Endocrinol,* 20**,** 764-775.

COOMARASAMY, A., KNOX, E. M., GEE, H. & KHAN, K. S. (2002). Oxytocin antagonists for tocolysis in preterm labour - a systematic review. *Med Sci Monitor,* 8**,** RA268-273.

CRANKSHAW, D. J. (2001). Pharmacological techniques for the in vitro study of the uterus. *J Pharmacol Toxicol,* 45**,** 123-140.

CRANKSHAW, D. J. & DYAL, R. (1994). Effects of some naturally occurring prostanoids and some cyclooxygenase inhibitors on the contractility of the human lower uterine segment in vitro. *Can J Physiol Pharm,* 72**,** 870-874.

CRETOIU, D., CIONTEA, S. M., POPESCU, L. M., CEAFALAN, L. & ARDELEANU, C. (2006). Interstitial Cajal-like cells (ICLC) as steroid hormone sensors in human myometrium: immunocytochemical approach. *J Cell Mol Med,* 10**,** 789-795.

CRITCHLEY, H. O. & SAUNDERS, P. T. (2009). Hormone receptor dynamics in a receptive human endometrium. *Reprod Sci,* 16**,** 191-199.

CURLEY, M., MORRISON, J. J. & SMITH, T. J. (2004). Analysis of Maxi-K alpha subunit splice variants in human myometrium. *Reprod Biol Endocrinol,* 2**,** 67-75.

D'ADDARIO, S. F., MORGAN, M., TALLEY, L. & SMOLLER, B. R. (2002). h-Caldesmon as a specific marker of smooth muscle cell differentiation in some soft tissue tumors of the skin. *J Cutan Pathol,* 29**,** 426-429.

DALLOT, E., POUCHELET, M., GOUHIER, N., CABROL, D., FERRE, F. & BREUILLER-FOUCHE, M. (2003). Contraction of cultured human uterine smooth muscle cells after stimulation with endothelin-1. *Biol Reprod,* 68**,** 937-942.

DALRYMPLE, A., SLATER, D. M., POSTON, L. & TRIBE, R. M. (2004). Physiological induction of transient receptor potential canonical proteins, calcium entry channels, in human myometrium: influence of pregnancy, labor, and interleukin-1 beta. *J Clin Endocr Metab,* 89**,** 1291-1300.

DARNE, J., MCGARRIGLE, H. H. & LACHELIN, G. C. (1987). Saliva oestriol, oestradiol, oestrone and progesterone levels in pregnancy: spontaneous labour at term is preceded by a rise in the saliva oestriol: progesterone ratio. *Brit J Obstet Gynaec,* 94**,** 227-235.

DAWOOD, M. Y. & KHAN-DAWOOD, F. S. (2007). Differential suppression of menstrual fluid prostaglandin $F_{2\alpha}$, prostaglandin E_2, 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2 by suprofen in women with primary dysmenorrhea. *Prostag other Lipid M,* 83**,** 146-153.

DAWOOD, M. Y., RAGHAVAN, K. S. & POCIASK, C. (1978). Radioimmunoassay of oxytocin. *J Endocrinol,* 76**,** 261-270.

DESAI, S., APRIL, H., NWANESHIUDU, C. & ASHBY, B. (2000). Comparison of agonist-induced internalization of the human EP_2 and EP_4 prostaglandin receptors: role of the carboxyl terminus in EP_4 receptor sequestration. *Mol Pharmacol,* 58**,** 1279-1286.

DI, W. L., LACHELIN, G. C., MCGARRIGLE, H. H., THOMAS, N. S. & BECKER, D. L. (2001). Oestriol and oestradiol increase cell to cell communication and connexin-43 protein expression in human myometrium. *Mol Hum Reprod,* 7**,** 671-679.

DIXON, R. E., HWANG, S. J., HENNIG, G. W., RAMSEY, K. H., SCHRIPSEMA, J. H., SANDERS, K. M. & WARD, S. M. (2009). Chlamydia infection causes loss of pacemaker cells and inhibits oocyte transport in the mouse oviduct. *Biol Reprod,* 80**,** 665-673.

DONG, Y. J., JONES, R. L. & WILSON, N. H. (1986). Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *Brit J Pharmacol,* 87**,** 97-107.

DONG, Y. L. & YALLAMPALLI, C. (2000). Pregnancy and exogenous steroid treatments modulate the expression of relaxant EP_2 and contractile FP receptors in the rat uterus. *Biol Reprod,* 62**,** 533-539.

DOWNIE, J., POYSER, N. L. & WUNDERLICH, M. (1974). Levels of prostaglandins in human endometrium during the normal menstrual cycle. *J Physiol,* 236**,** 465-472.

DUCKWORTH, N., MARSHALL, K. & CLAYTON, J. K. (2002). An investigation of the effect of the prostaglandin EP_2 receptor agonist, butaprost, on the human isolated myometrium from pregnant and non-pregnant women. *J Endocrinol,* 172**,** 263-269.

DUQUETTE, R. A., SHMYGOL, A., VAILLANT, C., MOBASHERI, A., POPE, M., BURDYGA, T. & WRAY, S. 2005. Vimentin-positive, c-kit-negative interstitial cells in human and rat uterus: a role in pacemaking? *Biol Reprod,* 72**,** 276-283.

DURN, J. H., MARSHALL, K. M., FARRAR, D., O'DONOVAN, P., SCALLY, A. J., WOODWARD, D. F. & NICOLAOU, A. (2010). Lipidomic analysis reveals prostanoid profiles in human term pregnant myometrium. *Prostaglandins Leukot Essent Fatty Acids,* 82**,** 21-26.

ELWORTHY, T. R., BRILL, E. R., CHIOU, S. S., CHU, F., HARRIS, J. R., HENDRICKS, R. T., HUANG, J., KIM, W., LACH, L. K., MIRZADEGAN, T., YEE, C. & WALKER, K. A. (2004). Lactams as EP_4 prostanoid receptor agonists. 3. Discovery of N-ethylbenzoic acid 2-pyrrolidinones as subtype selective agents. *J Med Chem,* 47**,** 6124-6127.

ERDEN, V., YANGIN, Z., ERKALP, K., DELATIOGLU, H., BAHCECI, F. & SEYHAN, A. (2005). Increased progesterone production during the luteal phase of menstruation may decrease anesthetic requirement. *Anesth Analg,* 101**,** 1007-1011.

ERKINHEIMO, T. L., JALKANEN, J., YLIKORKALA, O., SAUKKONEN, K., NARKO, K. & RISTIMKI, A. (2000). Expression of cyclooxygenase-2 and prostanoid receptors by human myometrium. *J Clin Endocr Metab,* 85**,** 3468-3475.

ESTERRE, P., MELIN, M., SERRAR, M. & GRIMAUD, J. A. (1992). New specific markers of human and mouse fibroblasts. *Cell Mol Biol,* 38**,** 297-301.

EUROPE-FINNER, G. N., PHANEUF, S., CARTWRIGHT, E., MARDON, H. J. & LOPEZ BERNAL, A. (1997). Expression of human myometrial Gαs messenger ribonucleic acid transcript during pregnancy and labour: involvement of alternative splicing pathways. *J Mol Endocrinol,* 18**,** 15-25.

EUROPE-FINNER, G. N., PHANEUF, S., TOLKOVSKY, A. M., WATSON, S. P. & LOPEZ BERNAL, A. (1994). Down-regulation of G alpha s in human myometrium in term and preterm labor: a mechanism for parturition. *J Clin Endocr Metab,* 79**,** 1835-1839.

FINN, C. A. & PORTER, D. G. (1975). The myometrial cell. *In:* FINN, C. A. & PORTER, D. G. (eds.) *The Uterus.* London: Elek Science.

FOMIN, V. P., COX, B. E. & WORD, R. A. (1999). Effect of progesterone on intracellular Ca^{2+} homeostasis in human myometrial smooth muscle cells. *Am J Physiol,* 276**,** C379-385.

FRIEL, A. M., CURLEY, M., RAVIKUMAR, N., SMITH, T. J. & MORRISON, J. J. (2005). Rho A/ Rho kinase mRNA and protein levels in human myometrium during pregnancy and labor. *J Soc Gynecol Investig,* 12**,** 20-27.

FRIEL, A. M., O'REILLY, M. W., SEXTON, D. J. & MORRISON, J. J. (2005). Specific $PGF_{2\alpha}$ receptor (FP) antagonism and human uterine contractility in vitro. *Brit J Obstet Gynaec,* 112**,** 1034-1042.

FU, X., GONG, M. C., JIA, T., SOMLYO, A. V. & SOMLYO, A. P. (1998). The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, GTPγS-, and phorbol ester-induced Ca^{2+} sensitization of smooth muscle. *FEBS Lett,* 440**,** 183-187.

FU, X., LIU, Y. J., CIRAY, N., OLOVSSON, M., ULMSTEN, U. & GYLFE, E. (2000). Oxytocin-induced oscillations of cytoplasmic Ca^{2+} in human myometrial cells. *Acta Obstet Gynecol Scand,* 79**,** 174-179.

FUCHS, A. R., BEHRENS, O., MASCHEK, H., KUPSCH, E. & EINSPANIER, A. (1998). Oxytocin and vasopressin receptors in human and uterine myomas during menstrual cycle and early pregnancy. *Hum Reprod Update,* 4**,** 594-604.

FUCHS, A. R., FUCHS, F., HUSSLEIN, P. & SOLOFF, M. S. (1984). Oxytocin receptors in the human uterus during pregnancy and parturition. *Am J Obstet Gynecol,* 150**,** 734-741.

FUCHS, A. R., FUCHS, F. & SOLOFF, M. S. (1985). Oxytocin receptors in non-pregnant human uterus. *J Clin Endocr Metab,* 60**,** 37-41.

FUCHS, A. R., ROMERO, R., KEEFE, D., PARRA, M., OYARZUN, E. & BEHNKE, E. (1991). Oxytocin secretion and human parturition: pulse frequency and duration increase during spontaneous labor in women. *Am J Obstet Gynecol,* 165**,** 1515-1523.

FUJINO, H. & REGAN, J. W. (2006). EP_4 prostanoid receptor coupling to a pertussis toxin-sensitive inhibitory G protein. *Mol Pharmacol,* 69**,** 5-10.

FUJINO, H., SALVI, S. & REGAN, J. W. (2005). Differential regulation of phosphorylation of the cAMP response element-binding protein after activation of EP_2 and EP_4 prostanoid receptors by PGE_2. *Mol Pharmacol,* 68**,** 251-259.

FUJINO, H., WEST, K. A. & REGAN, J. W. (2002). Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signalling following activation of EP_2 and EP_4 prostanoid receptors by prostaglandin E_2. *J Biol Chem,* 277**,** 2614-2619.

GADDUM, J. H., JANG, C. S. & KWIATKOWSKI, H. (1939). The effect on the intestine of the substance liberated by adrenergic nerves in a rabbit's ear. *J Physiol,* 96**,** 104-108.

GARDINER, P. J. 1986. Characterization of prostanoid relaxant/ inhibitory receptors (psi) using a highly selective agonist, TR4979. *Brit J Pharmacol,* 87**,** 45-56.

GARDNER, M. O., GOLDENBERG, R. L., CLIVER, S. P., TUCKER, J. M., NELSON, K. G. & COPPER, R. L. (1995). The origin and outcome of preterm twin pregnancies. *Obstet Gynecol,* 85**,** 553-557.

GARFIELD, R. E. (1984). Control of myometrial function in preterm versus term labor. *Clin Obstet Gynecol,* 27**,** 572-591.

GARFIELD, R. E., MERRETT, D. & GROVER, A. K. (1980). Gap junction formation and regulation in myometrium. *Am J Physiol,* 239**,** C217-228.

GARFIELD, R. E., SAADE, G., BUHIMSCHI, C., BUHIMSCHI, I., SHI, L., SHI, S. Q. & CHWALISZ, K. (1998). Control and assessment of the uterus and cervix during pregnancy and labour. *Hum Reprod Update,* 4**,** 673-695.

GEHRIG-BURGER, K., SLANINOVA, J. & GIMPL, G. (2010). Depletion of calcium stores contributes to progesterone-induced attenuation of calcium signalling of G protein-coupled receptors. *Cell Mol Life Sci*. 140.

GIANGRANDE, P. H. & MCDONNELL, D. P. (1999). The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. *Recent Prog Horm Res,* 54**,** 291-314.

GIANNOPOULOS, G., JACKSON, K., KREDENTSER, J. & TULCHINSKY, D. (1985). Prostaglandin E and $F_{2\alpha}$ receptors in human myometrium during the menstrual cycle and in pregnancy and labor. *Am J Obstet Gynecol,* 153**,** 904-910.

GIANNOULIAS, D., ALFAIDY, N., HOLLOWAY, A. C., GIBB, W., SUN, M., LYE, S. J. & CHALLIS, J. R. (2002). Expression of prostaglandin I_2 synthase, but not prostaglandin E synthase, changes in myometrium of women at term pregnancy. *J Clin Endocr Metab,* 87**,** 5274-5282.

GIANNOULIAS, D., HALUSKA, G. J., GRAVETT, M. G., SADOWSKY, D. W., CHALLIS, J. R. & NOVY, M. J. (2005). Localization of prostaglandin H synthase, prostaglandin dehydrogenase, corticotropin releasing hormone and glucocorticoid receptor in rhesus monkey fetal membranes with labor and in the presence of infection. *Placenta,* 26**,** 289-297.

GIBB, W. (1998). The role of prostaglandins in human parturition. *Ann Med,* 30**,** 235-241.

GOLDENBERG, R. L. (2002). The management of preterm labor. *Obstet Gynecol,* 100**,** 1020-1037.

GOODWIN, T. M., VALENZUELA, G., SILVER, H., HAYASHI, R., CREASY, G. W. & LANE, R. (1996). Treatment of preterm labor with the oxytocin antagonist atosiban. *Am J Perinatol,* 13**,** 143-146.

GRAZZINI, E., GUILLON, G., MOUILLAC, B. & ZINGG, H. H. (1998). Inhibition of oxytocin receptor function by direct binding of progesterone. *Nature,* 392**,** 509-512.

GRIFFITHS, A. L., MARSHALL, K. M., SENIOR, J., FLEMING, C. & WOODWARD, D. F. (2006). Effect of the oestrous cycle, pregnancy and uterine region on the responsiveness of the isolated mouse uterus to prostaglandin $F_{2\alpha}$ and the thromboxane mimetic U46619. *J Endocrinol,* 188**,** 569-577.

GRIGSBY, P. L., SOORANNA, S. R., ADU-AMANKWA, B., PITZER, B., BROCKMAN, D. E., JOHNSON, M. R. & MYATT, L. (2006). Regional expression of prostaglandin E_2 and $F_{2\alpha}$ receptors in human myometrium, amnion, and choriodecidua with advancing gestation and labor. *Biol Reprod,* 75**,** 297-305.

GROSS, G. A., OLSON, L. M., VOGT, S. K., SADOVSKY, Y., IMAMURA, T., LUEDKE, C., NELSON, D. M. & MUGLIA, L. J. (1998). Opposing actions of prostaglandins and oxytocin determine the onset of murine labor. *P Natl Acad Sci USA,* 95**,** 11875-11879.

GRUMMER, R., HEWITT, S. W., TRAUB, O., KORACH, K. S. & WINTERHAGER, E. (2004). Different regulatory pathways of endometrial connexin expression: pre-implantation hormonal-mediated pathway versus embryo implantation-initiated pathway. *Biol Reprod,* 71**,** 273-281.

HACK, M. & FANAROFF, A. A. (2000). Outcomes of children of extremely low birthweight and gestational age in the 1990s. *Semin Neonatol,* 5**,** 89-106.

HALUSKA, G. J., WELLS, T. R., HIRST, J. J., BRENNER, R. M., SADOWSKY, D. W. & NOVY, M. J. (2002). Progesterone receptor localization and isoforms in myometrium, decidua, and fetal membranes from rhesus macaques: evidence for functional progesterone withdrawal at parturition. *J Soc Gynecol Investig,* 9**,** 125-136.

HASEGAWA, T., HASEGAWA, F., HIROSE, T., SANO, T. & MATSUNO, Y. (2003). Expression of smooth muscle markers in so called malignant fibrous histiocytomas. *J Clin Pathol,* 56**,** 666-671.

HATA, A. N. & BREYER, R. M. (2004). Pharmacology and signalling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacol Therapeut,* 103**,** 147-166.

HAVELOCK, J. C., KELLER, P., MULEBA, N., MAYHEW, B. A., CASEY, B. M., RAINEY, W. E. & WORD, R. A. (2005). Human myometrial gene expression before and during parturition. *Biol Reprod,* 72**,** 707-719.

HELMER, H., HACKL, T., SCHNEEBERGER, C., KNOFLER, M., BEHRENS, O., KAIDER, A. & HUSSLEIN, P. (1998). Oxytocin and vasopressin 1a receptor gene expression in the cycling or pregnant human uterus. *Am J Obstet Gynecol,* 179**,** 1572-1578.

HERTELENDY, F., MOLNAR, M. & RIGO, J., JR. (1995). Proposed signalling role of arachidonic acid in human myometrium. *Mol Cell Endocrinol,* 110**,** 113-8.

HERTELENDY, F. & ZAKAR, T. (2004). Regulation of myometrial smooth muscle functions. *Curr Pharm Design,* 10**,** 2499-2517.

HILLOCK, C. J. & CRANKSHAW, D. J. (1999). Inhibitory prostanoid EP receptors in human non-pregnant myometrium. *Eur J Pharmacol,* 378**,** 99-108.

HIMPENS, B. & SOMLYO, A. P. (1988). Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J Physiol,* 395**,** 507-530.

HIRAI, H., TANAKA, K., YOSHIE, O., OGAWA, K., KENMOTSU, K., TAKAMORI, Y., ICHIMASA, M., SUGAMURA, K., NAKAMURA, M., TAKANO, S. & NAGATA, K. (2001). Prostaglandin D_2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor $CRTH_2$. *J Exp Med,* 193**,** 255-261.

HIRATA, M., HAYASHI, Y., USHIKUBI, F., YOKOTA, Y., KAGEYAMA, R., NAKANISHI, S. & NARUMIYA, S. (1991). Cloning and expression of cDNA for a human thromboxane A_2 receptor. *Nature,* 349**,** 617-620.

HIRATA, T., USHIKUBI, F., KAKIZUKA, A., OKUMA, M. & NARUMIYA, S. (1996). Two thromboxane A_2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J Clin Invest,* 97**,** 949-956.

HIRSBRUNNER, G., REIST, M., COUTO, S. S., STEINER, A., SNYDER, J., VANLEEUWEN, E. & LIU, I. (2006). An in vitro study on spontaneous myometrial contractility in the mare during estrus and diestrus. *Theriogenology,* 65**,** 517-527.

HOARE, S., COPLAND, J. A., STRAKOVA, Z., IVES, K., JENG, Y. J., HELLMICH, M. R. & SOLOFF, M. S. (1999). The proximal portion of the COOH terminus of the oxytocin receptor is required for coupling to G_q, but not G_i. Independent mechanisms for elevating intracellular calcium concentrations from intracellular stores. *J Biol Chem,* 274**,** 28682-28689.

HOBEL, C. & CULHANE, J. (2003). Role of psychosocial and nutritional stress on poor pregnancy outcome. *J Nutr,* 133**,** 1709S-1717S.

HOFMANN, G. E., SANFILIPPO, J. S., RAO, C. V. & BARROWS, G. H. (1983). Topography of human uterine prostaglandin E and $F_{2\alpha}$ receptors and their profiles during pathological states. *J Clin Endocr Metab,* 57**,** 360-366.

HONGPAISAN, J. (2000). Inhibition of proliferation of contaminating fibroblasts by D-valine in cultures of smooth muscle cells from human myometrium. *Cell Biol Int,* 24**,** 1-7.

HURD, W. W., FOMIN, V. P., NATARAJAN, V., BROWN, H. L., BIGSBY, R. M. & SINGH, D. M. (2000). Expression of protein kinase C isozymes in non-pregnant and pregnant human myometrium. *Am J Obstet Gynecol,* 183**,** 1525-1531.

HUTCHINSON, J. A. (2005). *A study into the roles of FP, TP and EP receptors in the uterus.* University of Bradford.

IJLAND, M. M., EVERS, J. L., DUNSELMAN, G. A. & HOOGLAND, H. J. (1998). Endometrial wavelike activity, endometrial thickness, and ultrasound texture in controlled ovarian hyperstimulation cycles. *Fertil Steril,* 70**,** 279-283.

IKENOYA, M., HIDAKA, H., HOSOYA, T., SUZUKI, M., YAMAMOTO, N. & SASAKI, Y. (2002). Inhibition of rho-kinase induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho-kinase inhibitor. *J Neurochem,* 81**,** 9-16.

INOUE, Y., NAKAO, K., OKABE, K., IZUMI, H., KANDA, S., KITAMURA, K. & KURIYAMA, H. (1990). Some electrical properties of human pregnant myometrium. *Am J Obstet Gynecol,* 162**,** 1090-1098.

ISHII, Y. & SAKAMOTO, K. (2001). Suppression of protein kinase C signalling by the novel isoform for bovine $PGF_{2\alpha}$ receptor. *Biochem Biophys Res Co,* 285**,** 1-8.

ISHIZAKI, T., MAEKAWA, M., FUJISAWA, K., OKAWA, K., IWAMATSU, A., FUJITA, A., WATANABE, N., SAITO, Y., KAKIZUKA, A., MORII, N. & NARUMIYA, S. (1996). The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/ Thr protein kinase homologous to myotonic dystrophy kinase. *Embo J,* 15**,** 1885-1893.

ITO, M., TANABE, F., SATO, A., ISHIDA, E., TAKAMI, Y. & SHIGETA, S. (1989). Inhibition of natural killer cell-mediated cytotoxicity by ML-9, a selective inhibitor of myosin light chain kinase. *Int J Immunopharmacol,* 11**,** 185-190.

JABBOUR, H. N. & SALES, K. J. (2004). Prostaglandin receptor signalling and function in human endometrial pathology. *Trends Endocrinol Metab,* 15**,** 398-404.

JANICEK, F., FRANOVA, S., NOSALOVA, G. & VISNOVSKY, J. (2007). In vitro contractile response of human myometrium to oxytocin, $PGF_{2\alpha}$, bradykinin and ET-1. *Bratisl Lek Listy,* 108**,** 174-178.

JIE, Z., KENDRICK, A., QUENBY, S. & WRAY, S. (2007). Contractility and calcium signalling of human myometrium are profoundly affected by cholesterol manipulation: implications for labor? *Reprod Sci,* 14**,** 456-466.

JOHNSON, M. R., BROOKS, A. A., ABDALLA, H. I. & TOOZS-HOBSON, P. (1995). Serum concentrations of luteinizing hormone and progesterone in ovum recipients: their relationship with pregnancy and miscarriage. *Hum Reprod,* 10**,** 1228-1231.

JONES, R. L. & CHAN, K. (2001). Distinction between relaxations induced via prostanoid EP_4 and IP_1 receptors in pig and rabbit blood vessels. *Brit J Pharmacol,* 134**,** 313-324.

JONES, R. L. & CHAN, K. M. (2005). Investigation of the agonist activity of prostacyclin analogues on prostanoid EP_4 receptors using GW627368 and taprostene: evidence for species differences. *Prostaglandins Leukot Essent Fatty Acids,* 72**,** 289-299.

JONES, R. L., CRITCHLEY, H. O., BROOKS, J., JABBOUR, H. N. & MCNEILLY, A. S. (1998). Localization and temporal expression of prolactin receptor in human endometrium. *J Clin Endocr Metab,* 83**,** 258-262.

KARASINSKI, J., SEMIK, D. & KILARSKI, W. (2000). Connexin-43 in porcine myocardium and non-pregnant myometrium. *Tissue Cell,* 32**,** 133-140.

KASHANIAN, M., AKBARIAN, A. R. & SOLTANZADEH, M. (2005). Atosiban and nifedipine for the treatment of preterm labor. *Int J Gynaecol Obstet,* 91**,** 10-14.

KATAOKA, M., TOGASHI, K., KIDO, A., NAKAI, A., FUJIWARA, T., KOYAMA, T. & FUJII, S. (2005). Dysmenorrhea: evaluation with cine-mode-display MR imaging - initial experience. *Radiology,* 235**,** 124-131.

KATAYAMA, E., SCOTT-WOO, G. & IKEBE, M. (1995). Effect of caldesmon on the assembly of smooth muscle myosin. *J Biol Chem,* 270**,** 3919-3925.

KATOH, K., KANO, Y., AMANO, M., KAIBUCHI, K. & FUJIWARA, K. (2001). Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. *Am J Physiol Cell Physiol,* 280**,** C1669-1679.

KATSUYAMA, M., MORIMOTO, K., ICHIKAWA, A., NEGISHI, M., NISHIGAKI, N., SUGIMOTO, Y. & NARUMIYA, S. (1995). The mouse prostaglandin E receptor EP_2 subtype: cloning, expression, and northern blot analysis. *FEBS Lett,* 372**,** 151-156.

KAWABATA, S., USUKURA, J., MORONE, N., ITO, M., IWAMATSU, A., KAIBUCHI, K. & AMANO, M. (2004). Interaction of Rho-kinase with myosin II at stress fibres. *Genes Cells,* 9**,** 653-660.

KAYA, T., CETIN, A., CETIN, M. & SARIOGLU, Y. (1999). Effects of endothelin-1 and calcium channel blockers on contractions in human myometrium. A study on myometrial strips from normal and diabetic pregnant women. *J Reprod Med,* 44**,** 115-121.

KEELAN, J. A., BLUMENSTEIN, M., HELLIWELL, R. J., SATO, T. A., MARVIN, K. W. & MITCHELL, M. D. (2003). Cytokines, prostaglandins and parturition - a review. *Placenta,* 24 Suppl A**,** S33-46.

KEELAN, J. A., COLEMAN, M. & MITCHELL, M. D. (1997). The molecular mechanisms of term and preterm labor: recent progress and clinical implications. *Clin Obstet Gynecol,* 40**,** 460-478.

KENYON, S., BROCKLEHURST, P., JONES, D., MARLOW, N., SALT, A. & TAYLOR, D. (2008). MRC ORACLE Children Study. Long term outcomes following prescription of antibiotics to pregnant women with either spontaneous preterm labour or preterm rupture of the membranes. *BMC Pregnancy Childbirth,* 8**,** 14-22.

KEERY, R. J. & LUMLEY, P. (1988). AH6809, a prostaglandin DP-receptor blocking drug on human platelets. *Brit J Pharmacol*, 94, 745-754.

KHROMOV, A. S., WANG, H., CHOUDHURY, N., MCDUFFIE, M., HERRING, B. P., NAKAMOTO, R., OWENS, G. K., SOMLYO, A. P. & SOMLYO, A. V. (2006). Smooth muscle of telokin-deficient mice exhibits increased sensitivity to Ca^{2+} and decreased cGMP-induced relaxation. *P Natl Acad Sci USA,* 103**,** 2440-2445.

KILARSKI, W. M., ROOMANS, G. M., HONGPAISAN, J. & SEMIK, D. (2000). Effect of progesterone and oestradiol on expression of connexin-43 in cultured human myometrium cells. *Folia Histochem Cytobiol,* 38**,** 3-9.

KIM, B. Y., CHO, C. H., SONG, D. K., MUN, K. C., SUH, S. I., KIM, S. P., SHIN, D. H., JANG, B. C., KWON, T. K., CHA, S. D., BAE, I. & BAE, J. H. (2005). Ciglitizone inhibits cell proliferation in human uterine leiomyoma via activation of store-operated Ca^{2+} channels. *Am J Physiol Cell Physiol,* 288**,** C389-395.

KIMATRAI, M., OLIVER, C., ABADIA-MOLINA, A. C., GARCIA-PACHECO, J. M. & OLIVARES, E. G. (2003). Contractile activity of human decidual stromal cells. *J Clin Endocr Metab,* 88**,** 844-9.

KIMURA, K., ITO, M., AMANO, M., CHIHARA, K., FUKATA, Y., NAKAFUKU, M., YAMAMORI, B., FENG, J., NAKANO, T., OKAWA, K., IWAMATSU, A. & KAIBUCHI, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science,* 273**,** 245-248.

KIMURA, T., OKAMURA, T., YOSHIDA, Y. & TODA, N. (1995). Relaxant responses to prostaglandin $F_{2\alpha}$ and E_2 of isolated human uterine arteries. *J Cardiovasc Pharmacol,* 26**,** 333-338.

KIRIYAMA, M., USHIKUBI, F., KOBAYASHI, T., HIRATA, M., SUGIMOTO, Y. & NARUMIYA, S. (1997). Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. *Brit J Pharmacol,* 122**,** 217-224.

KISS, A. & MIKKELSEN, J. D. (2005). Oxytocin - anatomy and functional assignments: a minireview. *Endocr Regul,* 39**,** 97-105.

KOBAYASHI, T. & NARUMIYA, S. (2002). Function of prostanoid receptors: studies on knockout mice. *Prostag other Lipid M,* 68-69**,** 557-573.

KOROVKINA, V. P., BRAINARD, A. M. & ENGLAND, S. K. (2006). Translocation of an endoproteolytically cleaved maxi-K channel isoform: mechanisms to induce human myometrial cell repolarization. *J Physiol,* 573**,** 329-341.

KOTANI, M., TANAKA, I., OGAWA, Y., USUI, T., MORI, K., ICHIKAWA, A., NARUMIYA, S., YOSHIMI, T. & NAKAO, K. (1995). Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP_3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. *Mol Pharmacol,* 48**,** 869-879.

KOUMAS, L., SMITH, T. J., FELDON, S., BLUMBERG, N. & PHIPPS, R. P. (2003). Thy-1 expression in human fibroblast subsets defines myofibroblastic or lipofibroblastic phenotypes. *Am J Pathol,* 163**,** 1291-1300.

KUNZ, G., BEIL, D., DEININGER, H., WILDT, L. & LEYENDECKER, G. (1996). The dynamics of rapid sperm transport through the female genital tract: evidence from vaginal sonography of uterine peristalsis and hysterosalpingoscintigraphy. *Hum Reprod,* 11**,** 627-632.

KUNZ, G., BEIL, D., HUPPERT, P. & LEYENDECKER, G. (2000). Structural abnormalities of the uterine wall in women with endometriosis and infertility visualized by vaginal sonography and MRI. *Hum Reprod,* 15**,** 76-82.

KUPITTAYANANT, S., BURDYGA, T. & WRAY, S. (2001). The effects of inhibiting Rho-associated kinase with Y-27632 on force and intracellular calcium in human myometrium. *Pflugers Arch,* 443**,** 112-114.

KUPITTAYANANT, S., LUCKAS, M. J. & WRAY, S. (2002). Effect of inhibiting the sarcoplasmic reticulum on spontaneous and oxytocin-induced contractions of human myometrium. *Brit J Obstet Gynaec,* 109**,** 289-296.

KUREISHI, Y., KOBAYASHI, S., AMANO, M., KIMURA, K., KANAIDE, H., NAKANO, T., KAIBUCHI, K. & ITO, M. (1997). Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem,* 272**,** 12257-12260.

LAIRD, S. M., TUCKERMAN, E. M., CORK, B. A., LINJAWI, S., BLAKEMORE, A. I. & LI, T. C. (2003). A review of immune cells and molecules in women with recurrent miscarriage. *Hum Reprod Update,* 9**,** 163-174.

LANDEN, C. N., JR., ZHANG, P. & YOUNG, R. C. (2001). Differing mechanisms of inhibition of calcium increases in human uterine myocytes by indomethacin and nimesulide. *Am J Obstet Gynecol,* 184**,** 1100-1103.

LARTEY, J., GAMPEL, A., PAWADE, J., MELLOR, H. & BERNAL, A. L. (2006). Expression of RND proteins in human myometrium. *Biol Reprod,* 75**,** 452-461.

LARTEY, J. & LOPEZ BERNAL, A. (2009). RHO protein regulation of contraction in the human uterus. *Reproduction,* 138**,** 407-424.

LARTEY, J., SMITH, M., PAWADE, J., STRACHAN, B., MELLOR, H. & LOPEZ BERNAL, A. (2007). Up-regulation of myometrial RHO effector proteins (PKN1 and DIAPH1) and CPI-17 (PPP1R14A) phosphorylation in human pregnancy is associated with increased GTP-RHOA in spontaneous preterm labor. *Biol Reprod,* 76**,** 971-982.

LAZARD, D., SASTRE, X., FRID, M. G., GLUKHOVA, M. A., THIERY, J. P. & KOTELIANSKY, V. E. (1993). Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. *P Natl Acad Sci USA,* 90**,** 999-1003.

LEBEL, W., RICCARDI, K., GRASSER, W. A., TERRY, K., THOMPSON, D. & PARALKAR, V. M. (2004). Prostaglandin E_2 receptor subtype EP_2 is not involved in the induction of non-pregnant guinea pig uterine contractions associated with terminal pregnancy. *Prostaglandins Leukot Essent Fatty Acids,* 71**,** 399-404.

LECCE, G., MEDURI, G., ANCELIN, M., BERGERON, C. & PERROT-APPLANAT, M. (2001). Presence of estrogen receptor beta in the human endometrium through the cycle: expression in glandular, stromal, and vascular cells. *J Clin Endocr Metab,* 86**,** 1379-1386.

LEONG, A. S., NORMAN, J. E. & SMITH, R. (2008). Vascular and myometrial changes in the human uterus at term. *Reprod Sci,* 15**,** 59-65.

LEONHARDT, A., HACKENBERG, R., NSING, R. M., GLASER, A. & WEGMANN, M. (2003). Expression of prostanoid receptors in human lower segment pregnant myometrium. *Prostaglandins Leukot Essent Fatty Acids,* 69**,** 307-313.

LEONI, P., CARLI, F. & HALLIDAY, D. (1990). Intermediate filaments in smooth muscle from pregnant and non-pregnant human uterus. *Biochem J,* 269**,** 31-34.

LEPPERT, P. C. (1995). Anatomy and physiology of cervical ripening. *Clin Obstet Gynecol,* 38**,** 267-279.

LEPPERT, P. C. & YU, S. Y. (1991). Three-dimensional structures of uterine elastic fibers: scanning electron microscopic studies. *Connect Tissue Res,* 27**,** 15-31.

LEYENDECKER, G., KUNZ, G., HERBERTZ, M., BEIL, D., HUPPERT, P., MALL, G., KISSLER, S., NOE, M. & WILDT, L. (2004). Uterine peristaltic activity and the development of endometriosis. *Ann N Y Acad Sci,* 1034**,** 338-355.

LI, M., KE, H. Z., QI, H., HEALY, D. R., LI, Y., CRAWFORD, D. T., PARALKAR, V. M., OWEN, T. A., CAMERON, K. O., LEFKER, B. A., BROWN, T. A. & THOMPSON, D. D. (2003). A novel, non-prostanoid EP_2 receptor-selective prostaglandin E_2 agonist stimulates local bone formation and enhances fracture healing. *J Bone Miner Res,* 18**,** 2033-2042.

LIEDMAN, R., HANSSON, S. R., HOWE, D., IGIDBASHIAN, S., MCLEOD, A., RUSSELL, R. J. & AKERLUND, M. (2008). Reproductive hormones in plasma over the menstrual cycle in primary dysmenorrhea compared with healthy subjects. *Gynecol Endocrinol,* 24**,** 508-513.

LIEDMAN, R., HANSSON, S. R., IGIDBASHIAN, S. & AKERLUND, M. (2009). Myometrial oxytocin receptor mRNA concentrations at preterm and term delivery - the influence of external oxytocin. *Gynecol Endocrinol,* 25**,** 188-193.

LIM, H. & DEY, S. K. (1997). Prostaglandin E_2 receptor subtype EP_2 gene expression in the mouse uterus coincides with differentiation of the luminal epithelium for implantation. *Endocrinology,* 138**,** 4599-4606.

LIM, H., LANGENBACH, R., DINCHUK, J. E., DEY, S. K., PARIA, B. C., DAS, S. K. & TRZASKOS, J. M. (1997). Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell,* 91**,** 197-208.

LIM, H., SONG, H., PARIA, B. C., REESE, J., DAS, S. K. & DEY, S. K. (2002). Molecules in blastocyst implantation: uterine and embryonic perspectives. *Vitam Horm,* 64**,** 43-76.

LONGO, M., JAIN, V., VEDERNIKOV, Y. P., HANKINS, G. D., GARFIELD, R. E. & SAADE, G. R. (2003). Effects of L-type Ca^{2+}-channel blockade, K^+ (ATP)-channel opening and nitric oxide on human uterine contractility in relation to gestational age and labour. *Mol Hum Reprod,* 9**,** 159-164.

LOPEZ BERNAL, A. (2001). Overview of current research in parturition. *Experimental Physiology,* 86**,** 213-222.

LOPEZ BERNAL, A., EUROPE-FINNER, G. N., PHANEUF, S. & WATSON, S. P. (1995). Preterm labour: a pharmacological challenge. *Trends Pharmacol Sci,* 16**,** 129-133.

LOPEZ BERNAL, A. & TAMBYRAJA, R. L. (2000). Preterm labour. *Baillieres Best Pract Res Clin Obstet Gynaecol,* 14**,** 133-153.

LOPEZ BERNAL, A., WATSON, S. P., PHANEUF, S. & EUROPE-FINNER, G. N. (1993). Biochemistry and physiology of preterm labour and delivery. *Baillieres Clin Obstet Gynaecol,* 7**,** 523-552.

LUCKAS, M. J., TAGGART, M. J. & WRAY, S. (1999). Intracellular calcium stores and agonist-induced contractions in isolated human myometrium. *Am J Obstet Gynecol,* 181**,** 468-476.

LUCKAS, M. J. & WRAY, S. (2000). A comparison of the contractile properties of human myometrium obtained from the upper and lower uterine segments. *Brit J Obstet Gynaec,* 107**,** 1309-1311.

LUMLEY, J. (2003). Defining the problem: the epidemiology of preterm birth. *Brit J Obstet Gynaec,* 110 Suppl 20**,** 3-7.

LUMLEY, P., WHITE, B. P. & HUMPHREY, P. P. (1989). GR32191, a highly potent and specific thromboxane A_2 receptor blocking drug on platelets and vascular and airways smooth muscle in vitro. *Brit J Pharmacol,* 97**,** 783-794.

LYE, S. J., OU, C.-W., TEOH, T.-G., ERB, G., STEVENS, Y., CASPER, R., PATEL, F. A. & CHALLIS, J. R. G. (1998). The molecular basis of labour and tocolysis. *Fetal and Maternal Medicine Review,* 10**,** 121-136.

MACCANNELL, K. A., BAZZAZI, H., CHILTON, L., SHIBUKAWA, Y., CLARK, R. B. & GILES, W. R. (2007). A mathematical model of electrotonic interactions between ventricular myocytes and fibroblasts. *Biophys J,* 92**,** 4121-4132.

MACKENZIE, L. W., WORD, R. A., CASEY, M. L. & STULL, J. T. (1990). Myosin light chain phosphorylation in human myometrial smooth muscle cells. *Am J Physiol,* 258**,** C92-98.

MAGGI, M., DEL CARLO, P., FANTONI, G., GIANNINI, S., TORRISI, C., CASPARIS, D., MASSI, G. & SERIO, M. (1990). Human myometrium during pregnancy contains and responds to V_1 vasopressin receptors as well as oxytocin receptors. *J Clin Endocr Metab,* 70**,** 1142-1152.

MANER, W. L., GARFIELD, R. E., MAUL, H., OLSON, G. & SAADE, G. (2003). Predicting term and preterm delivery with transabdominal uterine electromyography. *Obstet Gynecol,* 101**,** 1254-1260.

MARSH, M. M., BUTT, A. R., RILEY, S. C., ROGERS, P. A., SUSIL, B., AFFANDI, B., FINDLAY, J. K. & SALAMONSEN, L. A. (1995). Immunolocalization of endothelin and neutral endopeptidase in the endometrium of users of subdermally implanted levonorgestrel (Norplant). *Hum Reprod,* 10**,** 2584-2589.

MARSHALL, J. M. (1962). Regulation of activity in uterine smooth muscle. *Physiol Rev* Suppl 213-227.

MASLOW, K. D. & LYONS, E. A. (2004). Effect of prostaglandin and antiprostaglandin on midcycle myometrial contractions. *Fertil Steril,* 82**,** 511-513.

MATSUMOTO, T., MORI, T., KOTANI, M., TANAKA, I., SAGAWA, N., YOSHIDA, M., MUKOYAMA, M. & NAKAO, K. (1997). The prostaglandin E_2 and $F_{2\alpha}$ receptor genes are expressed in human myometrium and are down-regulated during pregnancy. *Biochem Biophys Res Co,* 238**,** 838-841.

MATTHEW, A., KUPITTAYANANT, S., BURDYGA, T. & WRAY, S. (2004). Characterization of contractile activity and intracellular Ca^{2+} signalling in mouse myometrium. *J Soc Gynecol Investig,* 11**,** 207-212.

MEJA, K. K., BARNES, P. J. & GIEMBYCZ, M. A. (1997). Characterization of the prostanoid receptors on human blood monocytes at which prostaglandin E_2 inhibits lipopolysaccharide-induced tumour necrosis factor-alpha generation. *Brit J Pharmacol,* 122**,** 149-157.

MELIN, P. (1993). Oxytocin antagonists in preterm labour and delivery. *Baillieres Clin Obstet Gynaecol,* 7**,** 577-600.

MERCER, B. M., MEIS, P. J., CARITIS, S. N., IAMS, J. D., GOLDENBERG, R. L., MOAWAD, A. H., MENARD, M. K., DAS, A. F. & MIODOVNIK, M. (1999). The preterm prediction study: effect of gestational age and cause of preterm birth on subsequent obstetric outcome. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. *Am J Obstet Gynecol,* 181**,** 1216-1221.

MESIANO, S. (2001). Roles of estrogen and progesterone in human parturition. *Front Horm Res,* 27**,** 86-104.

MESIANO, S., CHAN, E. C., FITTER, J. T., KWEK, K., YEO, G. & SMITH, R. (2002). Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. *J Clin Endocr Metab,* 87**,** 2924-2930.

MIGGIN, S. M. & KINSELLA, B. T. (1998). Expression and tissue distribution of the mRNAs encoding the human thromboxane A_2 receptor (TP) α and β isoforms. *Biochim Biophys Acta,* 1425**,** 543-559.

MIGGIN, S. M. & KINSELLA, B. T. (2002). Regulation of extracellular signal-regulated kinase cascades by alpha- and beta-isoforms of the human thromboxane A_2 receptor. *Mol Pharmacol,* 61**,** 817-831.

MILNE, S. A. & JABBOUR, H. N. (2003). Prostaglandin (PG) $F_{2\alpha}$ receptor expression and signalling in human endometrium: role of $PGF_{2\alpha}$ in epithelial cell proliferation. *J Clin Endocr Metab,* 88**,** 1825-1832.

MILNE, S. A., PERCHICK, G. B., BODDY, S. C. & JABBOUR, H. N. (2001). Expression, localization, and signalling of PGE_2 and EP_2/ EP_4 receptors in human non-pregnant endometrium across the menstrual cycle. *J Clin Endocr Metab,* 86**,** 4453-4459.

MINSHALL, R. D., PAVCNIK, D., HALUSHKA, P. V. & HERMSMEYER, K. (2001). Progesterone regulation of vascular thromboxane A_2 receptors in rhesus monkeys. *Am J Physiol Heart Circ Physiol,* 281**,** H1498-1507.

MOHAN, A. R., LOUDON, J. A. & BENNETT, P. R. (2004). Molecular and biochemical mechanisms of preterm labour. *Semin Fetal Neonatal Med,* 9**,** 437-444.

MOLNAR, M. & HERTELENDY, F. (1990). Regulation of intracellular free calcium in human myometrial cells by prostaglandin $F_{2\alpha}$: comparison with oxytocin. *J Clin Endocr Metab,* 71**,** 1243-1250.

MOLNAR, M., RIGO, J., JR., ROMERO, R. & HERTELENDY, F. (1999). Oxytocin activates mitogen-activated protein kinase and up-regulates cyclooxygenase-2 and prostaglandin production in human myometrial cells. *Am J Obstet Gynecol,* 181**,** 42-49.

MONTES, G. S., ZUGAIB, M., JOAZEIRO, P. P., VARAYOUD, J., RAMOS, J. G., MUNOZ-DE-TORO, M. & LUQUE, E. H. (2002). Phenotypic modulation of fibroblastic cells in the mucous layer of the human uterine cervix at term. *Reproduction,* 124**,** 783-790.

MOORE, F., ASBOTH, G. & LOPEZ, B. A. (2002). Thromboxane receptor signalling in human myometrial cells. *Prostag other Lipid M,* 67**,** 31-47.

MOORE, F. & BERNAL, A. L. (2001). Myosin light chain kinase and the onset of labour in humans. *Exp Physiol,* 86**,** 313-318.

MOORE, F., DA SILVA, C., WILDE, J. I., SMARASON, A., WATSON, S. P. & LOPEZ BERNAL, A. (2000). Up-regulation of p21- and RhoA-activated protein kinases in human pregnant myometrium. *Biochem Biophys Res Co,* 269**,** 322-326.

MOORE, F. & LOPEZ BERNAL, A. (2003). Chronic exposure to TXA_2 increases expression of ROCKI in human myometrial cells. *Prostag other Lipid M,* 71**,** 23-32.

MORAES, S. G., CAMPOS PINHEIRO, M., TOLEDO, O. M. & JOAZEIRO, P. P. (2004). Phenotypic modulation of fibroblastic cells in mice pubic symphysis during pregnancy, partum and postpartum. *Cell Tissue Res,* 315**,** 223-231.

MORAN, C. J., FRIEL, A. M., SMITH, T. J., CAIRNS, M. & MORRISON, J. J. (2002). Expression and modulation of Rho kinase in human pregnant myometrium. *Mol Hum Reprod,* 8**,** 196-200.

MORITA, I. (2002). Distinct functions of COX-1 and COX-2. *Prostag other Lipid M,* 68-69**,** 165-175.

MORRISON, J. J., ASHFORD, M. L., KHAN, R. N. & SMITH, S. K. (1993). The effects of potassium channel openers on isolated pregnant human myometrium before and after the onset of labor: potential for tocolysis. *Am J Obstet Gynecol,* 169**,** 1277-1285.

MOYNIHAN, A. T., SMITH, T. J. & MORRISON, J. J. (2008). The relaxant effect of nifedipine in human uterine smooth muscle and the BK(Ca) channel. *Am J Obstet Gynecol,* 198**,** 237 e1-8.

MULLER, M., SALES, K. J., KATZ, A. A. & JABBOUR, H. N. (2006). Seminal plasma promotes the expression of tumorigenic and angiogenic genes in cervical adenocarcinoma cells via the E-series prostanoid 4 receptor. *Endocrinology,* 147**,** 3356-3365.

MURAKAMI, M., KAMBE, T., SHIMBARA, S. & KUDO, I. (1999). Functional coupling between various phospholipase A_2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. *J Biol Chem,* 274**,** 3103-3115.

MYATT, L. & LYE, S. J. (2004). Expression, localization and function of prostaglandin receptors in myometrium. *Prostaglandins Leukot Essent Fatty Acids,* 70**,** 137-148.

NARABA, H., YOKOYAMA, C., TAGO, N., MURAKAMI, M., KUDO, I., FUEKI, M., OH-ISHI, S. & TANABE, T. (2002). Transcriptional regulation of the membrane-associated prostaglandin E_2 synthase gene. Essential role of the transcription factor Egr-1. *J Biol Chem,* 277**,** 28601-28608.

NARUMIYA, S., SUGIMOTO, Y. & USHIKUBI, F. (1999). Prostanoid receptors: structures, properties, and functions. *Physiol Rev,* 79**,** 1193-1226.

NEGISHI, M., NARUMIYA, S., ICHIKAWA, A., SUGIMOTO, Y. & IRIE, A. (1993). Two isoforms of prostaglandin E receptor EP_3 subtype. Different COOH-terminal domains determine sensitivity to agonist-induced desensitization. *J Biol Chem,* 268**,** 9517-9521.

NGUYEN, M., CAMENISCH, T., SNOUWAERT, J. N., HICKS, E., COFFMAN, T. M., ANDERSON, P. A., MALOUF, N. N. & KOLLER, B. H. (1997). The prostaglandin receptor EP_4 triggers remodelling of the cardiovascular system at birth. *Nature,* 390**,** 78-81.

NIIRO, N., NISHIMURA, J., SAKIHARA, C., NAKANO, H. & KANAIDE, H. (1997). Up-regulation of rho A and rho-kinase mRNAs in the rat myometrium during pregnancy. *Biochem Biophys Res Co,* 230**,** 356-359.

NILSSON, L., REINHEIMER, T., STEINWALL, M. & AKERLUND, M. (2003). FE 200 440: a selective oxytocin antagonist on the term-pregnant human uterus. *Brit J Obstet Gynaec,* 110**,** 1025-1028.

NISHIGAKI, N., NEGISHI, M. & ICHIKAWA, A. (1996). Two Gs-coupled prostaglandin E receptor subtypes, EP_2 and EP_4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist. *Mol Pharmacol,* 50**,** 1031-1037.

NISHIMORI, K., YOUNG, L. J., GUO, Q., WANG, Z., INSEL, T. R. & MATZUK, M. M. (1996). Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. *P Natl Acad Sci USA,* 93**,** 11699-11704.

NOE, M., KUNZ, G., HERBERTZ, M., MALL, G. & LEYENDECKER, G. (1999). The cyclic pattern of the immunocytochemical expression of oestrogen and progesterone receptors in human myometrial and endometrial layers: characterization of the endometrial-subendometrial unit. *Hum Reprod,* 14**,** 190-197.

NOORT, W. A. & KEIRSE, M. J. (1990). Prostacyclin versus thromboxane metabolite excretion: changes in pregnancy and labor. *Eur J Obstet Gyn R B,* 35**,** 15-21.

NORWITZ, E. R., ROBINSON, J. N. & CHALLIS, J. R. (1999). The control of labor. *N Engl J Med,* 341**,** 660-666.

OGER, S., MEHATS, C., DALLOT, E., FERRE, F. & LEROY, M. J. (2002). Interleukin-1β induces phosphodiesterase $4B_2$ expression in human myometrial cells through a prostaglandin E_2- and cyclic adenosine 3',5'-monophosphate-dependent pathway. *J Clin Endocr Metab,* 87**,** 5524-5531.

OGLETREE, M. L., HARRIS, D. N., GREENBERG, R., HASLANGER, M. F. & NAKANE, M. (1985). Pharmacological actions of SQ29,548, a novel selective thromboxane antagonist. *J Pharmacol Exp Ther,* 234**,** 435-441.

OH, J. H., YOU, S. K., HWANG, M. K., AHN, D. S., KWON, S. C., TAGGART, M. J. & LEE, Y. H. (2003). Inhibition of rho-associated kinase reduces MLC_{20} phosphorylation and contractility of intact myometrium and attenuates agonist-induced Ca^{2+} sensitization of force of permeabilized rat myometrium. *J Vet Med Sci,* 65**,** 43-50.

OKA, T., OKA, K. & SAPER, C. B. (2003). Contrasting effects of E type prostaglandin (EP) receptor agonists on core body temperature in rats. *Brain Res,* 968**,** 256-262.

OLSON, D. M. (2003). The role of prostaglandins in the initiation of parturition. *Best Pract Res Clin Obstet Gynaecol,* 17**,** 717-730.

ONO, M., MARUYAMA, T., MASUDA, H., KAJITANI, T., NAGASHIMA, T., ARASE, T., ITO, M., OHTA, K., UCHIDA, H., ASADA, H., YOSHIMURA, Y., OKANO, H. & MATSUZAKI, Y. (2007). Side population in human uterine myometrium displays phenotypic and functional characteristics of myometrial stem cells. *P Natl Acad Sci USA,* 104**,** 18700-18705.

OROPEZA, M. V., PONCE-MONTER, H., VILLANUEVA-TELLO, T., PALMA-AGUIRRE, J. A. & CAMPOS, M. G. (2002). Anatomical differences in uterine sensitivity to prostaglandin $F_{2\alpha}$ and serotonin in non-pregnant rats. *Eur J Pharmacol,* 446**,** 161-166.

OSMERS, R., RATH, W., PFLANZ, M. A., KUHN, W., STUHLSATZ, H. W. & SZEVERENYI, M. (1993). Glycosaminoglycans in cervical connective tissue during pregnancy and parturition. *Obstet Gynecol,* 81**,** 88-92.

OTTO, B., STEUSLOFF, A., JUST, I., AKTORIES, K. & PFITZER, G. (1996). Role of Rho proteins in carbachol-induced contractions in intact and permeabilized guinea-pig intestinal smooth muscle. *J Physiol,* 496(2)**,** 317-329.

PAPATSONIS, D., FLENADY, V. & LILEY, H. (2009). Maintenance therapy with oxytocin antagonists for inhibiting preterm birth after threatened preterm labour. *Cochrane Database Syst Rev***,** C5938-5959.

PARALKAR, V. M., BOROVECKI, F., KE, H. Z., CAMERON, K. O., LEFKER, B., GRASSER, W. A., OWEN, T. A., LI, M., DASILVA-JARDINE, P., ZHOU, M., DUNN, R. L., DUMONT, F., KORSMEYER, R., KRASNEY, P., BROWN, T. A., PLOWCHALK, D., VUKICEVIC, S. & THOMPSON, D. D. (2003). An EP_2 receptor-selective prostaglandin E_2 agonist induces bone healing. *P Natl Acad Sci USA,* 100**,** 6736-6740.

PARKINGTON, H. C., TONTA, M. A., BRENNECKE, S. P. & COLEMAN, H. A. (1999). Contractile activity, membrane potential and cytoplasmic calcium in human uterine smooth muscle in the third trimester of pregnancy and during labor. *Am J Obstet Gynecol,* 181**,** 1445-1451.

PHANEUF, S., ASBOTH, G., CARRASCO, M. P., LINARES, B. R., KIMURA, T., HARRIS, A. & BERNAL, A. L. (1998). Desensitization of oxytocin receptors in human myometrium. *Hum Reprod Update,* 4**,** 625-633.

PHANEUF, S., ASBOTH, G., EUROPE-FINNER, G. N., WATSON, S. P. & LOPEZ BERNAL, A. (1995). Second messenger pathways for oxytocin and prostaglandins in human myometrium. *Biochem Soc Trans,* 23**,** 21S.

PHANEUF, S., ASBOTH, G., MACKENZIE, I. Z., MELIN, P. & LOPEZ BERNAL, A. (1994). Effect of oxytocin antagonists on the activation of human myometrium in vitro: atosiban prevents oxytocin-induced desensitization. *Am J Obstet Gynecol,* 171**,** 1627-1634.

PHANEUF, S., EUROPE-FINNER, G. N., VARNEY, M., MACKENZIE, I. Z., WATSON, S. P. & LOPEZ BERNAL, A. (1993). Oxytocin-stimulated phosphoinositide hydrolysis in human myometrial cells: involvement of pertussis toxin-sensitive and -insensitive G-proteins. *J Endocrinol,* 136**,** 497-509.

PHANEUF, S., RODRIGUEZ LINARES, B., TAMBYRAJA, R. L., MACKENZIE, I. Z. & LOPEZ BERNAL, A. (2000). Loss of myometrial oxytocin receptors during oxytocin-induced and oxytocin-augmented labour. *J Reprod Fertil,* 120**,** 91-97.

PHILLIPPE, M. & BASA, A. (1997). Effects of sodium and calcium channel blockade on cytosolic calcium oscillations and phasic contractions of myometrial tissue. *J Soc Gynecol Investig,* 4**,** 72-77.

PICKLES, V. R. (1967). The myometrial actions of six prostaglandins: consideration of a receptor hypothesis. *In:* BERGSTROM, S. & SAMUELSSON, B. (eds.) *Prostaglandins*. New York: Interscience.

PIEBER, D., ALLPORT, V. C., HILLS, F., JOHNSON, M. & BENNETT, P. R. (2001). Interactions between progesterone receptor isoforms in myometrial cells in human labour. *Mol Hum Reprod,* 7**,** 875-879.

PIERCE, K. L., GIL, D. W., WOODWARD, D. F., BAILEY, T. J., HOYER, P. B. & REGAN, J. W. (1997). Cloning of a carboxyl-terminal isoform of the prostanoid FP receptor. *J Biol Chem,* 272**,** 883-887.

PIERCE, K. L. & REGAN, J. W. (1998). Prostanoid receptor heterogeneity through alternative mRNA splicing. *Life Sci,* 62**,** 1479-1483.

PIERZYNSKI, P., LEMANCEWICZ, A., REINHEIMER, T., AKERLUND, M. & LAUDANSKI, T. (2004). Inhibitory effect of barusiban and atosiban on oxytocin - induced contractions of myometrium from preterm and term pregnant women. *J Soc Gynecol Investig,* 11**,** 384-387.

PLESTED, C. P. & BERNAL, A. L. (2001). Desensitisation of the oxytocin receptor and other G-protein coupled receptors in the human myometrium. *Exp Physiol,* 86**,** 303-312.

POPAT, A. & CRANKSHAW, D. J. (2001). Variable responses to prostaglandin E_2 in human non-pregnant myometrium. *Eur J Pharmacol,* 416**,** 145-152.

POPESCU, L. M., VIDULESCU, C., CURICI, A., CARAVIA, L., SIMIONESCU, A. A., CIONTEA, S. M. & SIMION, S. (2006). Imatinib inhibits spontaneous rhythmic contractions of human uterus and intestine. *Eur J Pharmacol,* 546**,** 177-181.

POZZI, A., YAN, X., MACIAS-PEREZ, I., WEI, S., HATA, A. N., BREYER, R. M., MORROW, J. D. & CAPDEVILA, J. H. (2004). Colon carcinoma cell growth is associated with prostaglandin E_2/ EP_4 receptor-evoked ERK activation. *J Biol Chem,* 279**,** 29797-29804.

PRESSMAN, E. K., TUCKER, J. A., JR., ANDERSON, N. C., JR. & YOUNG, R. C. (1988). Morphologic and electrophysiologic characterization of isolated pregnant human myometrial cells. *Am J Obstet Gynecol,* 159**,** 1273-1279.

PRICE, S. A. & LOPEZ BERNAL, A. (2001). Uterine quiescence: the role of cyclic AMP. *Exp Physiol,* 86**,** 265-272.

PRICE, S. A., POCHUN, I., PHANEUF, S. & LOPEZ BERNAL, A. (2000). Adenylyl cyclase isoforms in pregnant and non-pregnant human myometrium. *J Endocrinol,* 164**,** 21-30.

RAYCHOWDHURY, M. K., KENT, K. C., MCGRAIL, S. H., YUKAWA, M., COLLINS, L. J. & WARE, J. A. (1994). Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A_2 receptor. *J Biol Chem,* 269**,** 19256-19261.

REAGAN, P. B. & SALSBERRY, P. J. (2005). Race and ethnic differences in determinants of preterm birth in the USA: broadening the social context. *Soc Sci Med,* 60**,** 2217-2228.

REES, M. C., ANDERSON, A. B., DEMERS, L. M. & TURNBULL, A. C. (1984). Endometrial and myometrial prostaglandin release during the menstrual cycle in relation to menstrual blood loss. *J Clin Endocr Metab,* 58**,** 813-818.

REGAN, J. W., BAILEY, T. J., PEPPERL, D. J., PIERCE, K. L., BOGARDUS, A. M., DONELLO, J. E., FAIRBAIRN, C., KEDZIE, K. M., WOODWARD, D. F. & GIL, D. W. (1994). Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP_2 subtype. *Mol Pharmacol,* 46**,** 213-220.

REZAPOUR, M., HONGPAISAN, J., FU, X., BACKSTROM, T., ROOMANS, G. M. & ULMSTEN, U. (1996). Effects of progesterone and oxytocin on intracellular elemental composition of term human myometrium in vitro. *Eur J Obstet Gyn R B,* 68**,** 191-197.

RICHTER, O. N., BARTZ, C., DOWAJI, J., KUPKA, M., REINSBERG, J., ULRICH, U. & RATH, W. (2006). Contractile reactivity of human myometrium in isolated non-pregnant uteri. *Hum Reprod,* 21**,** 36-45.

RICHTER, O. N., KUBLER, K., SCHMOLLING, J., KUPKA, M., REINSBERG, J., ULRICH, U., VAN DER VEN, H., WARDELMANN, E. & VAN DER VEN, K. (2004). Oxytocin receptor gene expression of estrogen-stimulated human myometrium in extracorporeally perfused non-pregnant uteri. *Mol Hum Reprod,* 10**,** 339-346.

RIEMER, R. K. & HEYMANN, M. A. (1998). Regulation of uterine smooth muscle function during gestation. *Pediatr Res,* 44**,** 615-627.

RILEY, M., BAKER, P. N., TRIBE, R. M. & TAGGART, M. J. (2005). Expression of scaffolding, signalling and contractile-filament proteins in human myometria: effects of pregnancy and labour. *J Cell Mol Med,* 9**,** 122-134.

RONNOV-JESSEN, L., CELIS, J. E., VAN DEURS, B. & PETERSEN, O. W. (1992). A fibroblast-associated antigen: characterization in fibroblasts and immunoreactivity in smooth muscle differentiated stromal cells. *J Histochem Cytochem,* 40**,** 475-486.

ROUGER, K., FORNASARI, B., ARMENGOL, V., JOUVION, G., LEROUX, I., DUBREIL, L., FERON, M., GUEVEL, L. & CHEREL, Y. (2007). Progenitor cell isolation from muscle-derived cells based on adhesion properties. *J Histochem Cytochem,* 55**,** 607-618.

SADOVSKY, Y., RIEMER, R. K. & ROBERTS, J. M. (1992). The concentration of estrogen receptors in rabbit uterine myocytes decreases in culture. *Am J Obstet Gynecol,* 167**,** 1631-1635.

SALES, K. J. & JABBOUR, H. N. (2003). Cyclooxygenase enzymes and prostaglandins in reproductive tract physiology and pathology. *Prostag other Lipid M,* 71, 97-117.

SALOMONIS, N., COTTE, N., ZAMBON, A. C., POLLARD, K. S., VRANIZAN, K., DONIGER, S. W., DOLGANOV, G. & CONKLIN, B. R. (2005). Identifying genetic networks underlying myometrial transition to labor. *Genome Biol,* 6**,** R12-28.

SANBORN, B. M. (2001). Hormones and calcium: mechanisms controlling uterine smooth muscle contractile activity. The Litchfield Lecture. *Exp Physiol,* 86**,** 223-237.

SANBORN, B. M. (2007). Hormonal signalling and signal pathway crosstalk in the control of myometrial calcium dynamics. *Semin Cell Dev Biol,* 18**,** 305-314.

SANBORN, B. M., DODGE, K., MONGA, M., QIAN, A., WANG, W. & YUE, C. (1998). Molecular mechanisms regulating the effects of oxytocin on myometrial intracellular calcium. *Adv Exp Med Biol,* 449**,** 277-286.

SANBORN, B. M., KU, C. Y., SHLYKOV, S. & BABICH, L. (2005). Molecular signalling through G-protein-coupled receptors and the control of intracellular calcium in myometrium. *J Soc Gynecol Investig,* 12**,** 479-487.

SASAKI, Y., SUZUKI, M. & HIDAKA, H. (2002). The novel and specific Rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for Rho-kinase-involved pathway. *Pharmacol Therapeut,* 93**,** 225-232.

SAWDY, R. J., SULLIVAN, M. H. & BENNETT, P. R. (2003). The effects of non-steroidal anti-inflammatory compounds on human myometrial contractility. *Eur J Obstet Gyn R B,* 109**,** 33-40.

SCHAAF, T. K., BINDRA, J. S., EGGLER, J. F., PLATTNER, J. J., NELSON, A. J., JOHNSON, M. R., CONSTANTINE, J. W., HESS, H. J. & ELGER, W. (1981). N - (Methanesulfonyl) - 16 - phenoxyprostaglandincarboxamides: tissue-selective, uterine stimulants. *J Med Chem,* 24**,** 1353-1359.

SCHMITZ, T., DALLOT, E., LEROY, M. J., BREUILLER-FOUCHE, M., FERRE, F. & CABROL, D. (2001). EP_4 receptors mediate prostaglandin E_2-stimulated glycosaminoglycan synthesis in human cervical fibroblasts in culture. *Mol Hum Reprod,* 7**,** 397-402.

SEBBAGH, M., HAMELIN, J., BERTOGLIO, J., SOLARY, E. & BREARD, J. (2005). Direct cleavage of ROCKII by granzyme B induces target cell membrane blebbing in a caspase-independent manner. *J Exp Med,* 201**,** 465-471.

SEELEY, R. R., STEPHENS, T. D. & TATE, P. (2006). Reproduction and Development. *In:* CALLANAN, R. J. & MCKINLEY, L. (eds.) *Anatomy & Physiology.* 7th ed. St Louis: McGraw-Hill Publishers.

SENCHYNA, M. & CRANKSHAW, D. J. (1999). Operational correlates of prostanoid TP receptor expression in human non-pregnant myometrium are unaffected by excision site or menstrual cycle status of the donor. *Brit J Pharmacol,* 128**,** 1524-1528.

SENIOR, J., BAXTER, G. S., CLAYTON, J. K., MARSHALL, K. & SANGHA, R. (1991). In vitro characterization of prostanoid EP-receptors in the non-pregnant human myometrium. *Brit J Pharmacol,* 102**,** 747-753.

SENIOR, J., CLAYTON, J. K., MARSHALL, K. & SANGHA, R. (1993). In vitro characterization of prostanoid receptors on human myometrium at term pregnancy. *Brit J Pharmacol,* 108**,** 501-506.

SENIOR, J., MARSHALL, K., CLAYTON, J. K., SANGHA, R. & BAXTER, G. S. (1992). In vitro characterization of prostanoid FP-, DP-, IP- and TP-receptors on the non-pregnant human myometrium. *Brit J Pharmacol,* 107**,** 215-221.

SEVERINO, M. F., MURRAY, M. J., BRANDON, D. D., CLINTON, G. M., BURRY, K. A. & NOVY, M. J. (1996). Rapid loss of oestrogen and progesterone receptors in human leiomyoma and myometrial explant cultures. *Mol Hum Reprod,* 2**,** 823-828.

SHAW, L., SWEENEY, M. A., O'NEILL, S. C., JONES, C. J., AUSTIN, C. & TAGGART, M. J. (2006). Caveolae and sarcoplasmic reticular coupling in smooth muscle cells of pressurised arteries: the relevance for Ca^{2+} oscillations and tone. *Cardiovasc Res,* 69**,** 825-835.

SHENG, H., SHAO, J., WASHINGTON, M. K. & DUBOIS, R. N. (2001). Prostaglandin E_2 increases growth and motility of colorectal carcinoma cells. *J Biol Chem,* 276**,** 18075-18081.

SHLYKOV, S. G. & SANBORN, B. M. (2004). Stimulation of intracellular Ca^{2+} oscillations by diacylglycerol in human myometrial cells. *Cell Calcium,* 36**,** 157-164.

SHMIGOL, A. V., EISNER, D. A. & WRAY, S. (2001). Simultaneous measurements of changes in sarcoplasmic reticulum and cytosolic. *J Physiol,* 531, 707-713.

SHMYGOL, A. & WRAY, S. (2004). Functional architecture of the SR calcium store in uterine smooth muscle. *Cell Calcium,* 35**,** 501-508.

SHYNLOVA, O., DOROGIN, A. & LYE, S. J. (2010). Stretch-induced uterine myocyte differentiation during rat pregnancy: involvement of caspase activation. *Biol Reprod*, 82: 1248-1255.

SHYNLOVA, O., TSUI, P., DOROGIN, A., CHOW, M. & LYE, S. J. (2005). Expression and localization of alpha-smooth muscle and gamma-actins in the pregnant rat myometrium. *Biol Reprod,* 73**,** 773-780.

SHUM, W. W., LE, G. Y., JONES, R. L., GURNEY, A. M. & SASAKI, Y. (2003). Involvement of Rho-kinase in contraction of guinea-pig aorta induced by prostanoid EP_3 receptor agonists. *Brit J Pharmacol,* 139**,** 1449-1461.

SINANAN, A. C., MACHELL, J. R., WYNNE-HUGHES, G. T., HUNT, N. P. & LEWIS, M. P. (2008). Alpha v beta 3 and alpha v beta 5 integrins and their role in muscle precursor cell adhesion. *Biol Cell,* 100**,** 465-477.

SINGER, K. H., SCEARCE, R. M., TUCK, D. T., WHICHARD, L. P., DENNING, S. M. & HAYNES, B. F. (1989). Removal of fibroblasts from human epithelial cell cultures with use of a complement fixing monoclonal antibody reactive with human fibroblasts and monocytes/ macrophages. *J Invest Dermatol,* 92**,** 166-170.

SKALLI, O., ROPRAZ, P., TRZECIAK, A., BENZONANA, G., GILLESSEN, D. & GABBIANI, G. (1986). A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol,* 103**,** 2787-2796.

SLATER, D. M., ASTLE, S., WOODCOCK, N., CHIVERS, J. E., DE WIT, N. C., THORNTON, S., VATISH, M. & NEWTON, R. (2006). Anti-inflammatory and relaxatory effects of prostaglandin E_2 in myometrial smooth muscle. *Mol Hum Reprod,* 12**,** 89-97.

SLATER, D. M., DENNES, W. J., CAMPA, J. S., POSTON, L. & BENNETT, P. R. (1999). Expression of cyclooxygenase types-1 and -2 in human myometrium throughout pregnancy. *Mol Hum Reprod,* 5**,** 880-884.

SLATTERY, M. M., FRIEL, A. M., HEALY, D. G. & MORRISON, J. J. (2001). Uterine relaxant effects of cyclooxygenase-2 inhibitors in vitro. *Obstet Gynecol,* 98**,** 563-569.

SLATTERY, M. M. & MORRISON, J. J. (2002). Preterm delivery. *Lancet,* 360**,** 1489-1497.

SMITH, O. P., JABBOUR, H. N. & CRITCHLEY, H. O. (2007). Cyclooxygenase enzyme expression and E series prostaglandin receptor signalling are enhanced in heavy menstruation. *Hum Reprod,* 22**,** 1450-1456.

SMITH, R., MESIANO, S. & MCGRATH, S. (2002). Hormone trajectories leading to human birth. *Regulatory Peptides,* 108**,** 159-164.

SMITH, W. L. & LANGENBACH, R. (2001). Why there are two cyclooxygenase isozymes. *J Clin Invest,* 107**,** 1491-1495.

SOLOFF, M. S., FERNSTROM, M. A., PERIYASAMY, S., SOLOFF, S., BALDWIN, S. & WIEDER, M. (1983). Regulation of oxytocin receptor concentration in rat uterine explants by estrogen and progesterone. *Can J Biochem Cell Biol,* 61**,** 625-630.

SOMLYO, A. P. & SOMLYO, A. V. (1994). Signal transduction and regulation in smooth muscle. *Nature,* 372**,** 231-236.

SOMLYO, A. P. & SOMLYO, A. V. (2000). Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol,* 522 Pt 2**,** 177-185.

SOMLYO, A. P., WU, X., WALKER, L. A. & SOMLYO, A. V. (1999). Pharmacomechanical coupling: the role of calcium, G-proteins, kinases and phosphatases. *Rev Physiol Biochem Pharmacol,* 134**,** 201-234.

SOORANNA, S. R., GRIGSBY, P., MYATT, L., BENNETT, P. R. & JOHNSON, M. R. (2005). Prostanoid receptors in human uterine myocytes: the effect of reproductive state and stretch. *Mol Hum Reprod,* 11**,** 859-864.

SOORANNA, S. R., GRIGSBY, P. L., ENGINEER, N., LIANG, Z., SUN, K., MYATT, L. & JOHNSON, M. R. (2006). Myometrial prostaglandin E_2 synthetic enzyme mRNA expression: spatial and temporal variations with pregnancy and labour. *Mol Hum Reprod,* 12**,** 625-631.

SPAREY, C., ROBSON, S. C., BAILEY, J., LYALL, F. & EUROPE-FINNER, G. N. (1999). The differential expression of myometrial connexin-43, cyclooxygenase-1 and -2, and Gs alpha proteins in the upper and lower segments of the human uterus during pregnancy and labor. *J Clin Endocr Metab,* 84**,** 1705-1710.

SPENCER, A. G., WOODS, J. W., ARAKAWA, T., SINGER, II & SMITH, W. L. (1998). Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. *J Biol Chem,* 273**,** 9886-9893.

STEER, P. (2005). The epidemiology of preterm labour. *Brit J Obstet Gynaec,* 112 Suppl 1**,** 1-3.

STEINWALL, M., HANSSON, S., BOSSMAR, T., LARSSON, I., PILKA, R. & AKERLUND, M. (2004). Oxytocin mRNA content in the endometrium of non-pregnant women. *Brit J Obstet Gynaec,* 111**,** 266-270.

SUGIMOTO, Y. & NARUMIYA, S. (2007). Prostaglandin E receptors. *J Biol Chem,* 282**,** 11613-11617.

SUGIMOTO, Y., SEGI, E., TSUBOI, K., ICHIKAWA, A. & NARUMIYA, S. (1998). Female reproduction in mice lacking the prostaglandin F receptor. Roles of prostaglandin and oxytocin receptors in parturition. *Adv Exp Med Biol,* 449**,** 317-321.

SUGINO, N., KARUBE-HARADA, A., TAKETANI, T., SAKATA, A. & NAKAMURA, Y. (2004). Withdrawal of ovarian steroids stimulates prostaglandin $F_{2\alpha}$ production through nuclear factor-kappaβ activation via oxygen radicals in human endometrial stromal cells: potential relevance to menstruation. *J Reprod Dev,* 50**,** 215-225.

SWANSON, M. L., LEI, Z. M., SWANSON, P. H., RAO, C. V., NARUMIYA, S. & HIRATA, M. (1992). The expression of thromboxane A_2 synthase and thromboxane A_2 receptor gene in human uterus. *Biol Reprod,* 47**,** 105-117.

SZAL, S. E., REPKE, J. T., SEELY, E. W., GRAVES, S. W., PARKER, C. A. & MORGAN, K. G. (1994). Ca^{2+} signalling in pregnant human myometrium. *Am J Physiol,* 267**,** E77-87.

TAGGART, M. J., LEE, Y. H. & MORGAN, K. G. (1999). Cellular redistribution of PKCα, rhoA and ROKα following smooth muscle agonist stimulation. *Exp Cell Res,* 251**,** 92-101.

TAGGART, M. J. & MORGAN, K. G. (2007). Regulation of the uterine contractile apparatus and cytoskeleton. *Semin Cell Dev Biol,* 18**,** 296-304.

TANG, D. D. (2008). Intermediate filaments in smooth muscle. *Am J Physiol Cell Physiol,* 294**,** C869-878.

TATTERSALL, M., ENGINEER, N., KHANJANI, S., SOORANNA, S. R., ROBERTS, V. H., GRIGSBY, P. L., LIANG, Z., MYATT, L. & JOHNSON, M. R. (2008). Pro-labour myometrial gene expression: are preterm labour and term labour the same? *Reproduction*, 135**,** 569-579.

TEMPLETON, A. A., COOPER, I. & KELLY, R. W. (1978). Prostaglandin concentrations in the semen of fertile men. *J Reprod Fertil,* 52**,** 147-150.

TERRY, K. K., LEBEL, W. S., RICCARDI, K. A., GRASSER, W. A., THOMPSON, D. D. & PARALKAR, V. M. (2008). Effects of gestational age on prostaglandin EP receptor expression and functional involvement during in vitro contraction of the guinea pig uterus. *Prostaglandins Leukot Essent Fatty Acids,* 78**,** 3-10.

TERZIDOU, V., SOORANNA, S. R., KIM, L. U., THORNTON, S., BENNETT, P. R. & JOHNSON, M. R. (2005). Mechanical stretch up-regulates the human oxytocin receptor in primary human uterine myocytes. *J Clin Endocr Metab,* 90**,** 237-246.

THIJSSEN, J. H. H. (2005). Progesterone receptors in the human uterus and their possible role in parturition. *J Steroid Biochem,* 97**,** 397-400.

THORNTON, S., GILLESPIE, J. I., GREENWELL, J. R. & DUNLOP, W. (1992). Mobilization of calcium by the brief application of oxytocin and prostaglandin E_2 in single cultured human myometrial cells. *Exp Physiol,* 77**,** 293-305.

THORNTON, S., GOODWIN, T. M., GREISEN, G., HEDEGAARD, M. & ARCE, J. C. (2009). The effect of barusiban, a selective oxytocin antagonist, in threatened preterm labor at late gestational age: a randomized, double-blind, placebo-controlled trial. *Am J Obstet Gynecol,* 200**,** 627 e1-10.

THORNTON, S., TERZIDOU, V., CLARK, A. & BLANKS, A. (1999). Progesterone metabolite and spontaneous myometrial contractions in vitro. *Lancet,* 353**,** 1327-1329.

TILLEY, S. L., KIM, H. S., KOLLER, B. H., FLANNERY, P. J., AUDOLY, L. P., HICKS, E. H. & COFFMAN, T. M. (1999). Reproductive failure and reduced blood pressure in mice lacking the EP_2 prostaglandin E_2 receptor. *J Clin Invest,* 103**,** 1539-1545.

TOH, H., ICHIKAWA, A. & NARUMIYA, S. (1995). Molecular evolution of receptors for eicosanoids. *FEBS Lett,* 361**,** 17-21.

TSENG, Y., KOLE, T. P., LEE, J. S., FEDOROV, E., ALMO, S. C., SCHAFER, B. W. & WIRTZ, D. (2005). How actin crosslinking and bundling proteins cooperate to generate an enhanced cell mechanical response. *Biochem Biophys Res Co,* 334**,** 183-192.

TSUBOI, K., SUGIMOTO, Y. & ICHIKAWA, A. (2002). Prostanoid receptor subtypes. *Prostag other Lipid M,* 68-69**,** 535-556.

TUCKER, J. & MCGUIRE, W. (2004). Epidemiology of preterm birth. *Brit Med J,* 329**,** 675-678.

TURTON, P., NEILSON, J. P., QUENBY, S., BURDYGA, T. & WRAY, S. (2009). A short review of twin pregnancy and how oxytocin receptor expression may differ in multiple pregnancy. *Eur J Obstet Gyn R B,* 144 Suppl 1**,** S40-44.

UEHATA, M., ISHIZAKI, T., SATOH, H., ONO, T., KAWAHARA, T., MORISHITA, T., TAMAKAWA, H., YAMAGAMI, K., INUI, J., MAEKAWA, M. & NARUMIYA, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature,* 389**,** 990-994.

VAN GESTEL, I., MM, I. J., HOOGLAND, H. J. & EVERS, J. L. (2003). Endometrial wave-like activity in the non-pregnant uterus. *Hum Reprod Update,* 9**,** 131-138.

VARAYOUD, J., RAMOS, J. G., JOAZEIRO, P. P., MONTES, G. S., MUNOZ DE TORO, M. M. & LUQUE, E. H. (2001). Characterization of fibroblastic cell plasticity in the lamina propria of the rat uterine cervix at term. *Biol Reprod,* 65**,** 375-383.

VERMILLION, S. T. & LANDEN, C. N. (2001). Prostaglandin inhibitors as tocolytic agents. *Semin Perinatol,* 25**,** 256-262.

VEZZA, R., HABIB, A. & FITZGERALD, G. A. (1999). Differential signalling by the thromboxane receptor isoforms via the novel GTP-binding protein, G_h. *J Biol Chem,* 274**,** 12774-12779.

WANG, J., ZOHAR, R. & MCCULLOCH, C. A. (2006). Multiple roles of alpha-smooth muscle actin in mechanotransduction. *Exp Cell Res,* 312**,** 205-214.

WATHES, D. C., BORWICK, S. C., TIMMONS, P. M., LEUNG, S. T. & THORNTON, S. (1999). Oxytocin receptor expression in human term and preterm gestational tissues prior to and following the onset of labour. *J Endocrinol,* 161**,** 143-151.

WEISS, G. (2000). Endocrinology of parturition. *J Clin Endocr Metab,* 85**,** 4421-4425.

WEISS, S., JAERMANN, T., SCHMID, P., STAEMPFLI, P., BOESIGER, P., NIEDERER, P., CADUFF, R. & BAJKA, M. (2006). Three-dimensional fiber architecture of the non-pregnant human uterus determined ex vivo using magnetic resonance diffusion tensor imaging. *Anat Rec Part A,* 288**,** 84-90.

WIKLAND, M., LINDBLOM, B. & WIQVIST, N. (1984). Myometrial response to prostaglandins during labor. *Gynecol Obstet Invest,* 17**,** 131-138.

WILLETS, J. M., BRIGHTON, P. J., MISTRY, R., MORRIS, G. E., KONJE, J. C. & CHALLISS, R. A. (2009). Regulation of oxytocin receptor responsiveness by G protein-coupled receptor kinase 6 in human myometrial smooth muscle. *Mol Endocrinol,* 23**,** 1272-1280.

WILSON, R. J., ALLEN, M. J., NANDI, M., GILES, H. & THORNTON, S. (2001). Spontaneous contractions of myometrium from humans, non-human primate and rodents are sensitive to selective oxytocin receptor antagonism in vitro. *Brit J Obstet Gynaec,* 108**,** 960-966.

WILSON, R. J., GIBLIN, G. M., ROOMANS, S., RHODES, S. A., CARTWRIGHT, K. A., SHIELD, V. J., BROWN, J., WISE, A., CHOWDHURY, J., PRITCHARD, S., COOTE, J., NOEL, L. S., KENAKIN, T., BURNS-KURTIS, C. L., MORRISON, V., GRAY, D. W. & GILES, H. (2006). GW627368X ((N-(2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2H-benzo[f]isoindol-2-yl)phenyl] acetyl) benzene sulphonamide) a novel, potent and selective prostanoid EP_4 receptor antagonist. *Brit J Pharmacol,* 148**,** 326-339.

WILSON, R. J. & GILES, H. (2005). Piglet saphenous vein contains multiple relaxatory prostanoid receptors: evidence for EP_4, EP_2, DP and IP receptor subtypes. *Brit J Pharmacol,* 144**,** 405-415.

WILSON, R. J., RHODES, S. A., WOOD, R. L., SHIELD, V. J., NOEL, L. S., GRAY, D. W. & GILES, H. (2004). Functional pharmacology of human prostanoid EP_2 and EP_4 receptors. *Eur J Pharmacol,* 501**,** 49-58.

WIMALASUNDERA, R. C., THOM, S. A., REGAN, L. & HUGHES, A. D. (2005). Effects of vasoactive agents on intracellular calcium and force in myometrial and subcutaneous resistance arteries isolated from preeclamptic, pregnant, and non-pregnant woman. *Am J Obstet Gynecol,* 192**,** 625-632.

WING, D. A., GOHARKHAY, N., FELIX, J. C., ROSTAMKHANI, M., NAIDU, Y. M. & KOVACS, B. W. (2006). Expression of the oxytocin and V_{1a} vasopressin receptors in human myometrium in differing physiologic states and following misoprostol administration. *Gynecol Obstet Invest,* 62**,** 181-185.

WING, D. A., GOHARKHAY, N., HANNA, M., NAIDU, Y. M., KOVACS, B. W. & FELIX, J. C. (2003). $EP_{3\text{-}2}$ receptor mRNA expression is reduced and $EP_{3\text{-}6}$ receptor mRNA expression is increased in gravid human myometrium. *J Soc Gynecol Investig,* 10**,** 124-129.

WINKLER, M., KEMP, B., CLASSEN-LINKE, I., FISCHER, D. C., ZLATINSI, S., NEULEN, J., BEIER, H. M. & RATH, W. (2002). Estrogen receptor alpha and progesterone receptor A and B concentration and localization in the lower uterine segment in term parturition. *J Soc Gynecol Investig,* 9**,** 226-232.

WOODCOCK, N. A., TAYLOR, C. W. & THORNTON, S. (2004). Effect of an oxytocin receptor antagonist and rho-kinase inhibitor on the Ca^{2+} sensitivity of human myometrium. *Am J Obstet Gynecol,* 190**,** 222-228.

WOODCOCK, N. A., TAYLOR, C. W. & THORNTON, S. (2006). Prostaglandin $F_{2\alpha}$ increases the sensitivity of the contractile proteins to Ca^{2+} in human myometrium. *Am J Obstet Gynecol*, 195, 1404-1406.

WOODWARD, D. F., PEPPERL, D. J., BURKEY, T. H. & REGAN, J. W. (1995). 6-Isopropoxy-9-oxoxanthene-2-carboxylic acid (AH6809), a human EP_2 receptor antagonist. *Biochem Pharmacol,* 50**,** 1731-1733.

WORD, R. A. (1995). Myosin phosphorylation and the control of myometrial contraction/ relaxation. *Semin Perinatol,* 19**,** 3-14.

WORD, R. A., KAMM, K. E. & CASEY, M. L. (1992). Contractile effects of prostaglandins, oxytocin, and endothelin-1 in human myometrium in vitro: refractoriness of myometrial tissue of pregnant women to prostaglandins E_2 and $F_{2\alpha}$. *J Clin Endocr Metab,* 75**,** 1027-1032.

WORD, R. A., KAMM, K. E., STULL, J. T. & CASEY, M. L. (1993). Contractile elements and myosin light chain phosphorylation in myometrial tissue from non-pregnant and pregnant women. *J Clin Invest,* 92**,** 29-37.

WORD, R. A., TANG, D. C. & KAMM, K. E. (1994). Activation properties of myosin light chain kinase during contraction/relaxation cycles of tonic and phasic smooth muscles. *J Biol Chem,* 269**,** 21596-21602.

WRAY, S. (1993). Uterine contraction and physiological mechanisms of modulation. *Am J Physiol,* 264**,** C1-18.

WRAY, S., BURDYGA, T. & NOBLE, K. (2005). Calcium signalling in smooth muscle. *Cell Calcium,* 38**,** 397-407.

WRAY, S., KUPITTAYANANT, S., SHMYGOL, A., SMITH, R. D. & BURDYGA, T. (2001). The physiological basis of uterine contractility: a short review. *Exp Physiol,* 86**,** 239-246.

WU, X., MORGAN, K. G., JONES, C. J., TRIBE, R. M. & TAGGART, M. J. (2008). Myometrial mechanoadaptation during pregnancy: implications for smooth muscle plasticity and remodelling. *J Cell Mol Med,* 12**,** 1360-1373.

YAMADA, N., MAKINO, Y., CLARK, R. A., PEARSON, D. W., MATTEI, M. G., GUENET, J. L., OHAMA, E., FUJINO, I., MIYAWAKI, A., FURUICHI, T. & ET AL. 1994. Human inositol 1,4,5-trisphosphate type-1 receptor, $InsP_3R$-1: structure, function, regulation of expression and chromosomal localization. *Biochem J,* 302**,** 781-790.

YIN, P., LIN, Z., CHENG, Y. H., MARSH, E. E., UTSUNOMIYA, H., ISHIKAWA, H., XUE, Q., REIERSTAD, S., INNES, J., THUNG, S., KIM, J. J., XU, E. & BULUN, S. E. (2007). Progesterone receptor regulates Bcl-2 gene expression through direct binding to its promoter region in uterine leiomyoma cells. *J Clin Endocr Metab,* 92**,** 4459-4466.

YOUNG, R. C. (2002). Sarcoplasmic reticulum, calcium waves and myometrial signalling. *Novartis Found Symp,* 246**,** 174-188.

YOUNG, R. C. (2007). Myocytes, myometrium, and uterine contractions. *Ann N Y Acad Sci,* 1101**,** 72-84.

YOUNG, R. C., SMITH, L. H. & MCLAREN, M. D. (1993). T-type and L-type calcium currents in freshly dispersed human uterine smooth muscle cells. *Am J Obstet Gynecol,* 169**,** 785-792.

YOUNG, W. S., HENNIGHAUSEN, L., MCKINNEY, C., WAGNER, K. U., SHEPARD, E., AMICO, J., LAMARCA, M. E. & GINNS, E. I. (1996). Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition. *J Neuroendocrinol,* 8**,** 847-853.

YU, J. T. & LOPEZ BERNAL, A. (1998). The cytoskeleton of human myometrial cells. *J Reprod Fertil,* 112**,** 185-198.

YUAN, W. & LOPEZ BERNAL, A. (2007). Cyclic AMP signalling pathways in the regulation of uterine relaxation. *BMC Pregnancy Childbirth,* 7 Suppl 1**,** S10-16.

ZIGANSHIN, A. U., ZEFIROVA, J. T., ZEFIROVA, T. P., ZIGANSHINA, L. E., HOYLE, C. H. & BURNSTOCK, G. (2005). Potentiation of uterine effects of prostaglandin $F_{2\alpha}$ by adenosine 5'-triphosphate. *Obstet Gynecol,* 105**,** 1429-1436.

ZYRIANOV, V. V., SUMOVSKAYA, A. Y. & SHOSTAK, A. A. (2003). Application of electron spin resonance for evaluation of the level of free radicals in the myometrium in full-term pregnancy with normal labour and uterine inertia. *J Biosci,* 28**,** 19-21.

Appendix

Bradford Hospitals NHS
NHS Trust

Name:

Address:

Bradford Royal Infirmary
Duckworth Lane
Bradford
BD9 6RJ

Date of Birth:

Minicom: 01274 382555
www.bradfordhospitals.nhs.uk

DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY

THE EFFECT OF DRUGS ON UTERINE MUSCLE

We are at present working with the Reproductive Pharmacology Group at the University of Bradford in performing medical research into the causes and treatment of problems associated with irregular periods and heavy bleeding. The purpose of the research is in the hope that in the future we may provide a new treatment for these conditions, therefore reducing the problems and pains often associated with periods.

We need samples of womb tissue to carry out our experiments. We would very much appreciate it if you would allow the surgeon to remove a small piece of tissue from your womb after it has been removed at your hysterectomy.

The tissue taken from your womb will be about the size of a £1 coin. The tissue donated will be treated with respect at all times and will be disposed of wholly and carefully when the experiment is completed, within a short time of the tissue being donated.

None of the samples are retained for further use and be assured that this will in no way affect your surgery or recovery.

Thank you very much for your help and assistance.

Mr J K Clayton
Director of Obstetrics and Gynaecology

I understand the nature of the research being performed and agree to a small piece of muscle being removed from my womb after it has been removed at the time of my hysterectomy.

The nature of the procedure was explained to me by: .....

Signed: .....
(Patient)

Signature of Doctor: .....

Appendix v

Figure A1: Copy of consent form used to obtain smooth muscle samples from non-pregnant donors.

Bradford Hospitals NHS
NHS Trust

Name:

Address:

Bradford Royal Infirmary
Duckworth Lane
Bradford
BD9 6RJ

Minicom: 01274 382555
www.bradfordhospitals.nhs.uk

Date of Birth:

DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY

THE EFFECT OF DRUGS ON PREGNANT UTERINE MUSCLE

We are at present working with the University of Bradford in looking at the effect of certain drugs on the muscle of the uterus. We hope at some stage that this will be useful in either the treatment of women who go into premature labour or who have severe period pains.

We would very much appreciate it if you would allow us at the time of your Caesarean section to remove a small fragment of muscle from the cut edge of the opening in the uterus. We can assure you that this does not affect the operation, neither does it affect your recovery or future child-bearing. We can also reassure you that that small piece of muscle is treated with respect, the investigations on the muscle are performed shortly after its transport to the University of Bradford and the muscle is disposed of the same day. No tissues are kept for further research.

It may also be necessary for a member of the medical team to look back at your case notes to tie up the results of the laboratory procedure with your own clinical history, though when the final report is produced, no patients will be identifiable.

Thank you very much for your help and assistance.

Mr J K Clayton
Director of Obstetrics and Gynaecology

I understand the nature of the research being performed and agree to a small piece of muscle being removed from the cut edge of my uterus at the time of Caesarean section.

The nature of the procedure was explained to me by:

.....

Signed: .....
(Patient)

Signature of Doctor: .....

Figure A2: Copy of consent form used to obtain smooth muscle samples from pregnant donors.

Uterine Smooth Muscle Research

Postgraduate Studies
School of Pharmacy
Richmond Road
University of Bradford
BRADFORD
Telephone: (01274)234669 or 232323 ext. 4732/ 4675

Debbie Fischer, Anna Griffiths & Dr. K.M. Marshall

Patient Number:	
Age:	
Blood pressure (mmHg):	/
Last Menstrual Period:	
Reason for Hysterectomy:	
Ethnicity:	
Consultant:	
Any current medication:	

Figure A3: Patient information form for uterine muscle taken at hysterectomy from non-pregnant donors.

Uterine Smooth Muscle Research

Miss Debbie Fischer, Miss Anna Griffiths, Dr. Kay Marshall

Reproduction Research Group, School of Pharmacy, University of Bradford
Tel: 234669, 232323 ext. 4732 or 4675

Date:
Time of delivery:

Patient Number	
Duration of Pregnancy	
Blood Pressure (mmHg)	
Number of previous pregnancies	
Number of previous labours	
Number of previous caesareans	
Age	
Ethnicity	
Consultant	
Does the mother smoke? If so, how many per day?	
Does the mother suffer from any of the following: - hypertension - pre-eclampsia/ PH - diabetes-IDDM NIDDM Gestational - Rh factor - Other (please state)	
Any current medication	
Anaesthetic: General/ Spinal	
If labouring: duration (hrs) stage of labour + dilation (cm)	
Drugs used in labour	

Figure A4: Patient information form for myometrial tissue taken at Caesarean section from pregnant donors.

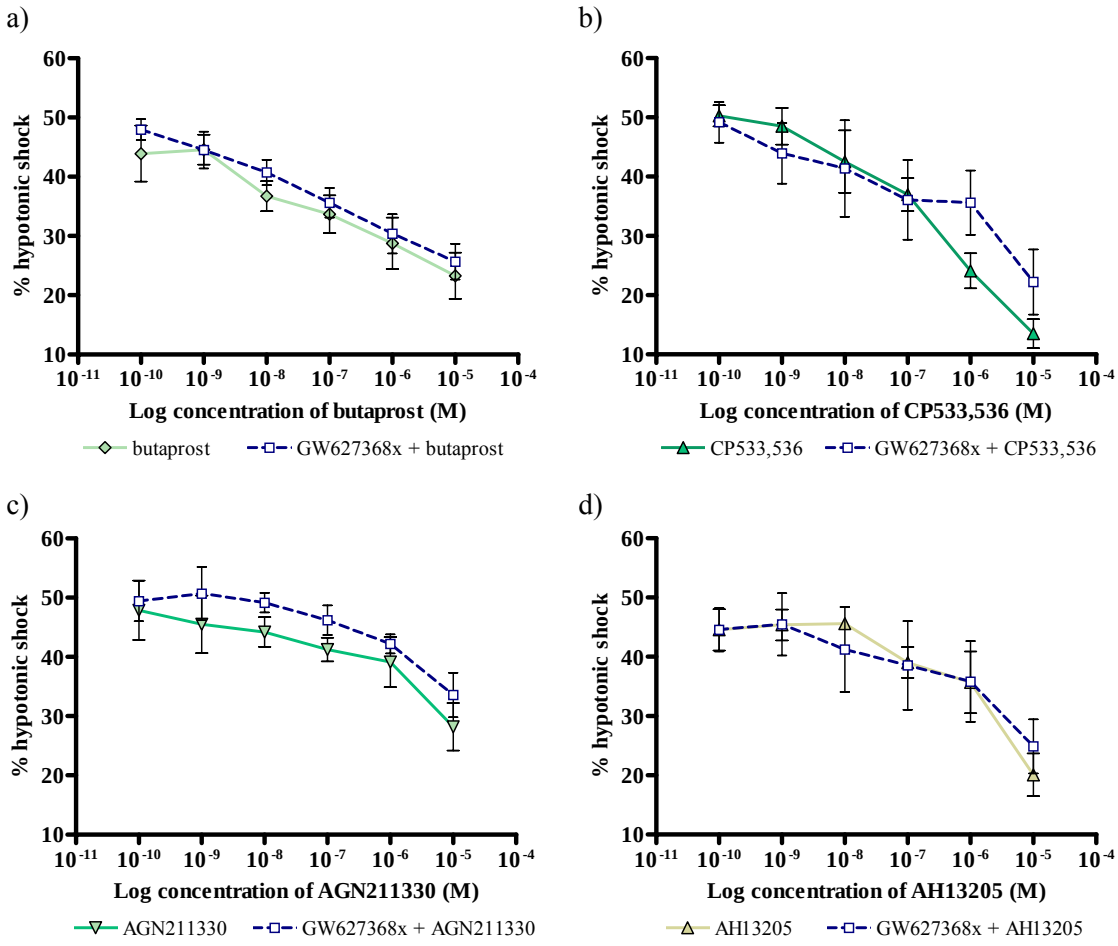


Figure A5: *In vitro* uterorelaxant effects of the EP_2 agonists butaprost, CP533,536, AGN211330 and AH13205 on myometrial strips from term pregnant donors (n=3-6). Agonists (10^{-10}M to 10^{-5}M) were added to organ baths in a cumulative manner at 30-minute intervals in the presence and absence of the EP_4 antagonist GW627368x (10^{-6}M). Responses were measured over a 30-minute period as area under the curve, expressed as percentage hypotonic shock and presented as arithmetic means ± S.E.

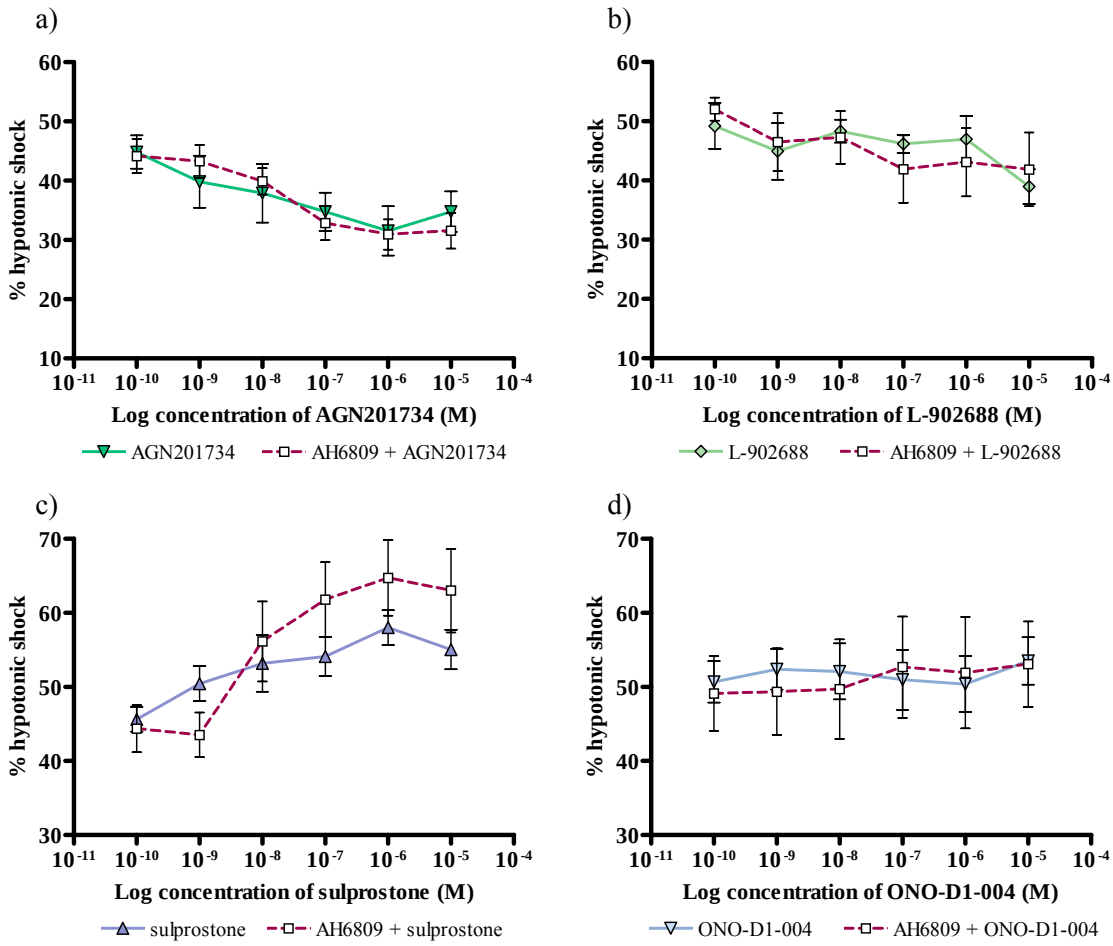


Figure A6: The lack of effect of the EP_1, EP_2, EP_3 and DP antagonist AH6809 on concentration-activity curves for a) AGN201734, b) L-902688, c) sulprostone and d) ONO-D1-004 in myometrial strips from term pregnant, non-labouring donors (n=4-6). Agonists were added to parallel organ baths at 30-minute intervals either alone or after preincubation of tissues with AH6809 (10^{-5}M). Responses were measured over a 30-minute period as area under the curve, expressed as percentage hypotonic shock and presented as arithmetic means ± S.E.

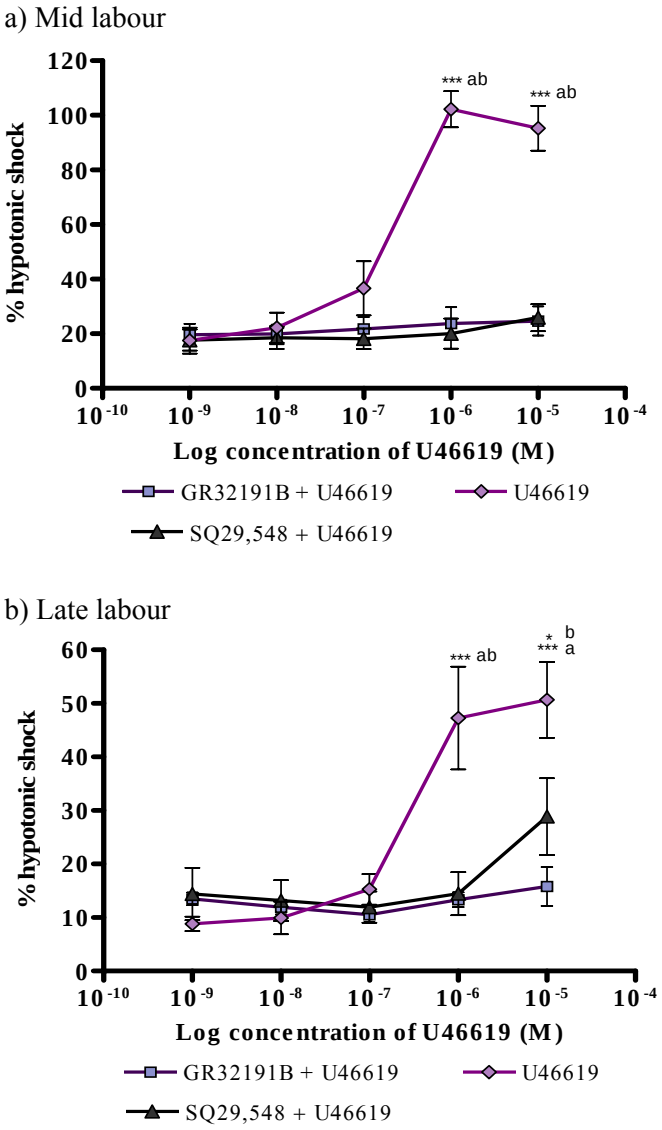


Figure A7: Concentration-effect curves for U46619 alone or in the presence of either TP antagonist SQ29,548 (10^{-6}M) or GR32191B (10^{-6}M) in lower myometrium obtained from term pregnant donors in a) mid (n=6-8) and c) late (n=5-8) stages of labour. Labour was defined as *in vivo* contractions with respective stages determined at 3-8.5cm and 9-10cm cervical dilation. U46619 was added to organ baths in a cumulative manner at 30-minute intervals with responses measured as area under the curve relative to the contraction achieved by hypotonic shock. Results are expressed as means ± S.E. and statistical analysis was performed using multivariate ANOVA with Bonferroni's *post-hoc* test; *p<0.05; ***p<0.001 for [a]GR32191B and [b]SQ29,548 compared to U46619 alone.

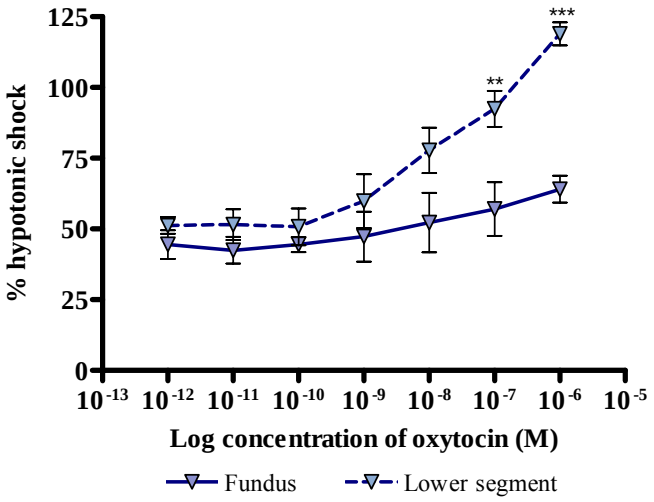


Figure A8: Concentration-effect curves for oxytocin in fundal (n=4) and lower (n=4) segment human myometrium taken at the follicular stage of the menstrual cycle. After equilibration in immersion baths, responses to oxytocin were measured as 30 minutes integrated area under the curve and expressed as a percentage of 30 minutes hypotonic shock. Results are arithmetic means ± S.E. with analysis performed using two-way ANOVA with Bonferroni's *post-hoc* adjustment; **p<0.01; ***p<0.001 for responsiveness to oxytocin in fundus compared with lower segment tissue strips.

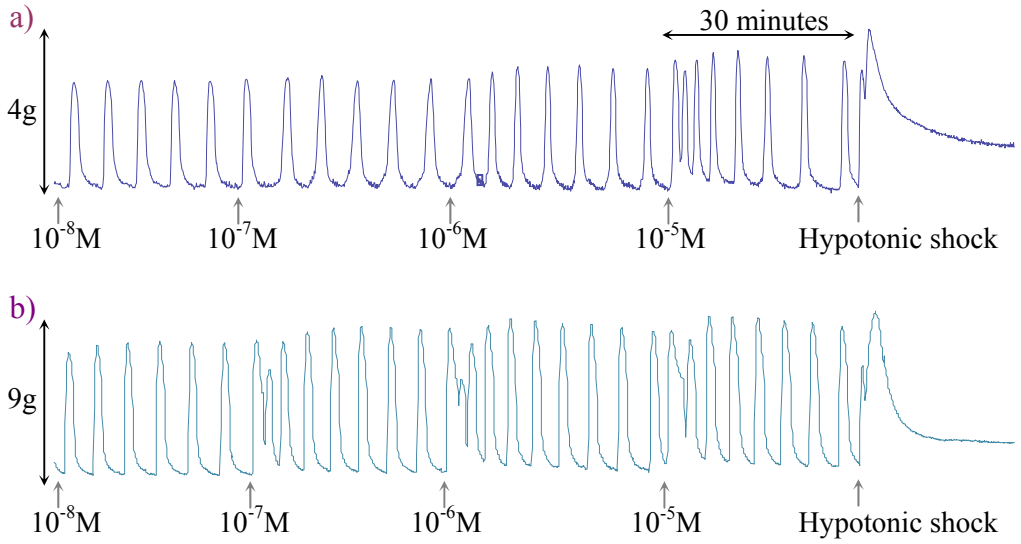


Figure A9: Representative traces showing the excitatory effects evoked by oxytocin in a) fundal and b) lower segment myometrium harvested from non-pregnant donors during the follicular phase of the cycle. Agonists were added to the immersion apparatus at 30-minute intervals.

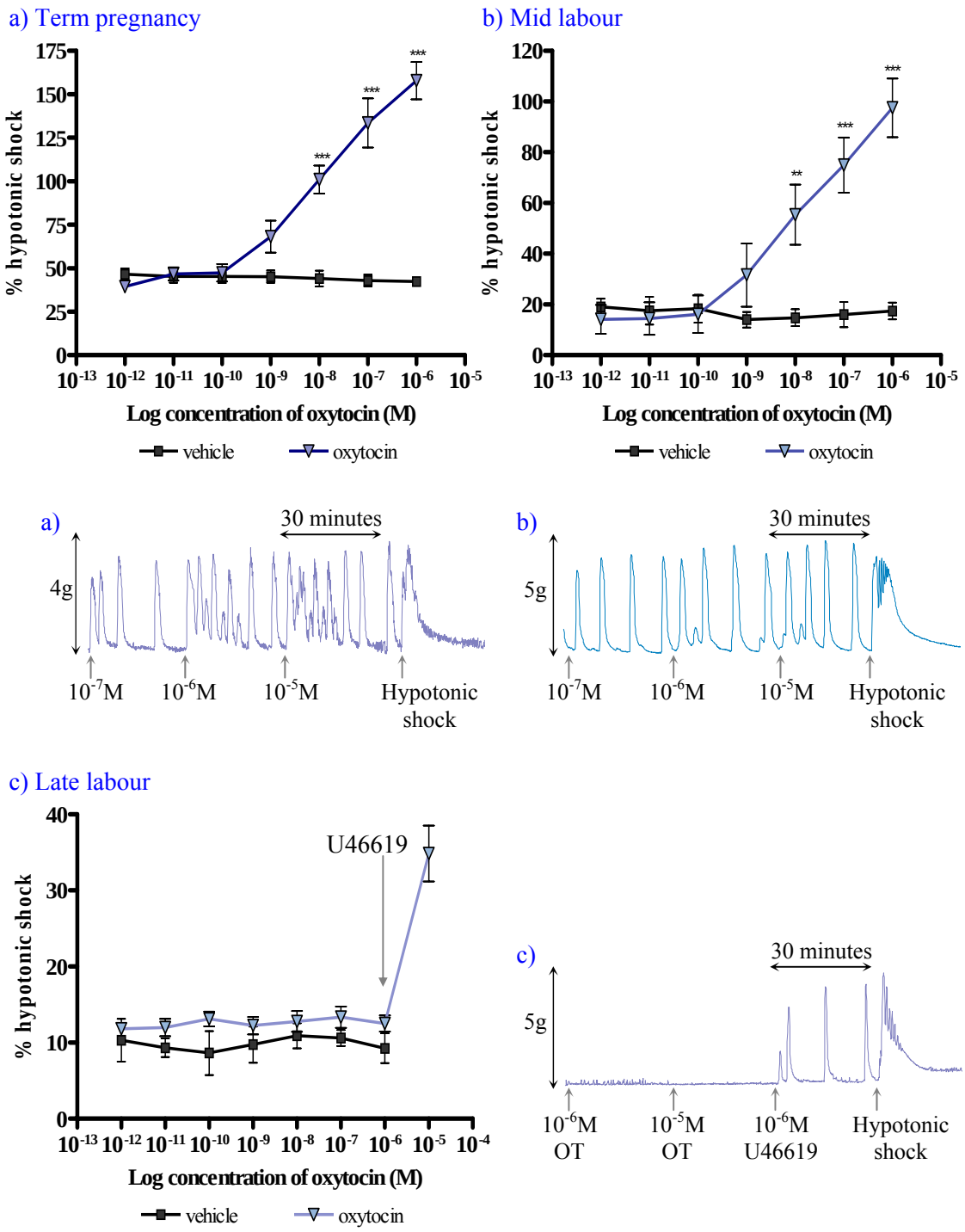


Figure A10: Vehicle, concentration-effect curves and representative traces for oxytocin in myometrial strips obtained from donors at a) term pregnancy, not in labour (n=5), b) mid (n=5) and c) late stages of labour (n=6). Mid and late labour was categorised at 3-8.5cm and 9-10cm cervical dilation respectively. In myometrial strips from fully dilated donors, the addition of the stable thromboxane mimetic U46619 (10^{-6}M) provoked contractile responses relative to spontaneous activity, indicating tissue viability. Responses were measured as area under the curve relative to the contraction induced by hypotonic shock. Results are expressed as means ± S.E. and statistics were performed using two-way ANOVA with Bonferroni's adjustment; **p<0.01; ***p<0.001 for oxytocin-induced contractions compared to vehicle.