An investigation into the combination of nifedipine with potassium channel openers as potential tocolytic therapy for preterm labour; and a novel potassium channel blocker as potential therapy for post-partum haemorrhage.

by

Elizabeth Helen Bailey

A thesis submitted in partial fulfilment of the requirements for the
degree of
Doctor of Philosophy in Medicine

University of Warwick, Warwick Medical School
July 2015

Contents:

1

Appendix 1 - ethical approval letters, participant information
sheet and consent form for obtaining myometrial biopsies

Appendix 2 - Publication relating to this thesis:

McCloskey, C; Rada, C; Bailey, E; McCavera, S; van den Berg, H;
Atia, J; Rand, DA; Shmygol, A; Chan, Y; Quenby, S; Brosens, JJ
Vatish, M; Zhang, J; Denton, JS; Taggart, MJ; Kettleborough, C;
Tickle, D; Jerman, J; Wright, P; Dale, T; Kanumilli, S; Trezise, DJ;
Thornton, S; Brown, P; Catalano, R; Lin, N; England, SK; Blanks,
AM (2014) The inwardly rectifying K+ channel KIR7.1 controls
uterine excitability throughout pregnancy *EMBO Molecular
Medicine* Jul 23;6(9): 1161-74

Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree at this, or any other University.

The work presented (including data generated and data analysis) was carried out by the author.

N.B: Myography experiments presented in section 3.3 were conducted by myself at the Institute of Cellular Medicine, Newcastle University in collaboration with and under the guidance of Dr Michele Sweeney and Professor Mike Taggart.

Parts of this thesis have been published by the author and collaborators:

McCloskey, C; Rada, C; Bailey, E; McCavera, S; van den Berg, H; Atia, J; Rand, DA; Shmygol, A; Chan, Y; Quenby, S; Brosens, JJ Vatish, M; Zhang, J; Denton, JS; Taggart, MJ; Kettleborough, C; Tickle, D; Jerman, J; Wright, P; Dale, T; Kanumilli, S; Trezise, DJ; Thornton, S; Brown, P; Catalano, R; Lin, N; England, SK; Blanks, AM (2014) The inwardly rectifying K+ channel KIR7.1 controls uterine excitability throughout pregnancy *EMBO Molecular Medicine* Jul 23;6(9): 1161-74 *(Appendix 2)*

Poster presentation: British Maternal and Fetal Medicine Society Conference 2011, Harrogate UK:

Bailey E, Quenby S, Thornton S, Blanks AM (2011) An investigation into the combination of nifedipine with potassium channel openers as potential tocolytic

therapy for preterm labour *Archives of Disease in Child Fetal Neonatal Ed* 2011;96:Fa76 (BMFMS Labour and Delivery Posters)

Oral presentation: 59th Annual meeting Society for Gynecologic Investigation 2012, San Diego USA

Bailey E, Quenby S, Thornton S, Blanks AM (2012) The Kir 7.1 Blocker VU590 Causes Profound Contractions in Human Myometrium *Reproductive Sciences* 19 (3) O-034

Abstract

Background
Preterm labour and post-partum haemorrhage are leading causes of pregnancy morbidity and mortality. Previous work identified potassium channels expressed in myometrium and hypothesized modulation of channels with greater expression in MSMC than VSMC will influence contractility and avoid cardiovascular effects. By combining calcium channel blockers with potassium channel openers an enhanced tocolytic effect is anticipated. VU590 inhibits K_{ir} 7.1 and it was hypothesised would elicit a contractile effect with therapeutic potential for post-partum haemorrhage.

Aim
To determine the effect of select potassium channel openers and a specific potassium channel blocker in myometrial contractility.

Methods
Human and murine myometrial strips were used in contractility organ bath experiments. Select combined doses were tested in myometrial small arteries using wire myography. Western blotting was carried out to determine the gestational and labour-state expression of potassium channels in human myometrium and myometrial small arteries.

Results
Pinacidil demonstrated a relaxatory effect on both myometrial and vascular smooth muscle. Riluzole reduced contractility alone and greater inhibition in combination with nifedipine than nifedipine alone. Riluzole appeared to have a mild effect on myometrial arteries.
K_{ir} 7.1 showed a trend of diminished expression by gestation and was down-regulated in term and preterm labour states.
VU590 elicited a significant increase contractility characterised by a prolonged contraction phase of up to 6.7 ± 1.9 hrs (VU590 10 µM). A gestational-dependent effect was seen on murine myometrium.

Conclusion
The combination of nifedipine with potassium channel openers has a more potent effect on reducing contractility than either compound alone. Riluzole combined with nifedipine warrants further investigation for potential tocolytic therapy.
VU590 augments spontaneous contractions profoundly in human myometrium in vitro and could have potential therapeutic benefits in the treatment of post-partum haemorrhage.

List of Abbreviations

AI	Activity Integral
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BNF	British National Formulary
CCB	Calcium channel blocker
CD	Contraction duration
CSC	Charged space competition
COX	Cyclooxygenase
DAG	Diacyl-glycerol
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EDHF	Endothelial dependent hyperpolarisation factor
ELLSCS	Elective lower section caesarean section
EMLSCS	Emergency lower section caesarean section
ER	Ednoplastic reticulum
hCG	Human chorionic gonadotropin
HCL	Hydrochloric acid
IUGR	Intra-uterine growth restriction
LHCGR	Lutenising hormone choriogonadotropin receptor
MF	Maximal force
MSMC	Myometrial smooth muscle cell
NO	Nitric oxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OT	Oxytocin
P_4	Progesterone
PCR	Polymerase chain reaction
PKA	Phosphokinase A
PKC	Phosphokinase C
PPH	Postpartum haemorrhage
PR	Progesterone receptor
PTL	Preterm labour
PTNL	Preterm non-labour
RCOG	Royal College of Obstetricians and Gynaecologists
RNA	Ribonucleic acid
RR	Risk ratio/relative risk
SE	Standard error
SEM	Standard error of the mean
SN	Substantia nigra
SR	Sarcoplasmic reticulum
SUR	Sulfonylurea receptor
TL	Term Labour
TNL	Term non-labour

UN	United Nations
VGCC	Voltage-gated calcium channel
VGLCC	Voltage gated L-type calcium channel
VSMC	Vascular smooth muscle cell
WHO	World health organisation
ZEB-1	Zinc finger E-box binding activated B cells

List of Tables

21

1. **Introduction**

1.1 The problem of preterm labour

Preterm birth continues to be a national and global problem (*Figure 1.1*). Complications of pre-term birth continue to be the largest direct cause of neonatal deaths and deaths of children under five, globally (Blencowe et al 2013). Recent data published by the World Health Organisation from 65 countries shows that in all but three countries, the rates of preterm birth are increasing (Blencowe et al 2012). The global average preterm birth rate for 2010 was 11.1% with a global increase of 14.7% from 1990 to 2010 (Blencowe et al 2012). There continues to be a disparity in preterm birth rates from high-income (9% average) to low-income countries (12% average) (WHO 2015) (*Figure 1.2*) . This disparity continues when looking at the trends over the past 25 years. There was a 19.4% increase in preterm birth rates in Developing regions from 1990 to 2010, an increase of 9.1% in Latin America, and an increase of 25.8% in the Caribbean for the same time period (Blencowe et al 2012). Although the increase in the Developed regions may seem disproportionately high, it is worth bearing in mind that in developed regions, deaths resulting from prematurity are reducing significantly (Blencowe et al 2012). Increased antenatal care and improved antenatal ultrasound surveillance leads to more iatrogenic preterm birth in high-income countries, largely to the benefit of both maternal and child health; supported by access to high quality and specialist neonatal care (Blencowe et al 2013). Aetiology of preterm birth differs from high income to

low-income countries, with assisted reproduction and higher multiple pregnancy rates contributing to the increase in preterm birth rates seen in high-income countries from 1990 to 2010 (Blencowe et al 2013). More than 90% preterm infants in low income countries die where as less than 10% die in high income settings (WHO 2015). In recognition of the significant medical, social, and economic impact that infant and pregnancy loss, or significant disability resulting from preterm birth has on worldwide communities; The United Nations focused on the reduction of childhood mortality as part of their Millennium Development Goal programme (UN 2015). From 1990 to 2015, *Goal 4*; to reduce child mortality recognised the significance of the number of worldwide neonatal deaths caused by preterm birth complications (35%) (UN 2015). This continues to be a priority in the new UN Sustainable Development Goals that are initiated in January 2016 with the aim of ending preventable deaths of newborns and children under 5 by 2030 (UN 2016). All countries are encouraged to aim to reduce neonatal mortality by < 12/1000 live births, and deaths of under fives to <25/1000 (UN 2016). Deaths by complications of immaturity represent 43% of all infant deaths in England and Wales (*Figure 1.3*). In both low-income and high-income settings, there are simple cost-effective and high-impact interventions that could improve survival and reduce morbidity (UN 2015). These include increased access to antenatal health care, better social support for pregnant women, and simple treatments and technique that could support the preterm newborn such as kangaroo care and oxygen support (UN 2015). As 84% of preterm births in low-income countries are moderate to late preterm births, a significant number could potentially survive, or have lessened disability with

minimal intervention and this remains a priority of intervention strategies (Blencowe et al 2012).

Although this data is insightful, and encourages national and global improvements in care to prevent preterm birth; issues with data quality must be considered. Access to antenatal ultrasound for pregnancy dating differs from country to country, and this affects accuracy of gestation-at-delivery data. Reporting systems and accuracy of birth outcome data from hospitals and medical centres vary country to country with a lack of consistency in classification, live birth and stillbirth reporting and birth and death registration requirements (Lawn et al 2010). In recognition of this, recommendations to improve data quality includes; using more household and survey data on birth outcome, using standardised definitions and reporting tools; and having more standardised death registration and certification systems (Lawn et al 2010). This will assist in monitoring whether the desired reduction in neonatal and infant mortality as a result of the complications of prematurity are being achieved.

World Health Organisation November 2015

Preterm Birth Factsheet No. 363

Key facts:

- 15 million babies born preterm (before 37 completed weeks)
- This number is rising
- Preterm birth complications are a leading cause of death among children under 5 years old
- Preterm birth is responsible for nearly 1 million deaths in 2013
- 3/4 of babies of babies lost could be saved with current cost-effective interventions
- Across 184 countries, preterm birth rates range from 5-18 % of babies

Figure 1.1 Key facts from World Health Organisation Factsheet on Preterm birth (2015)
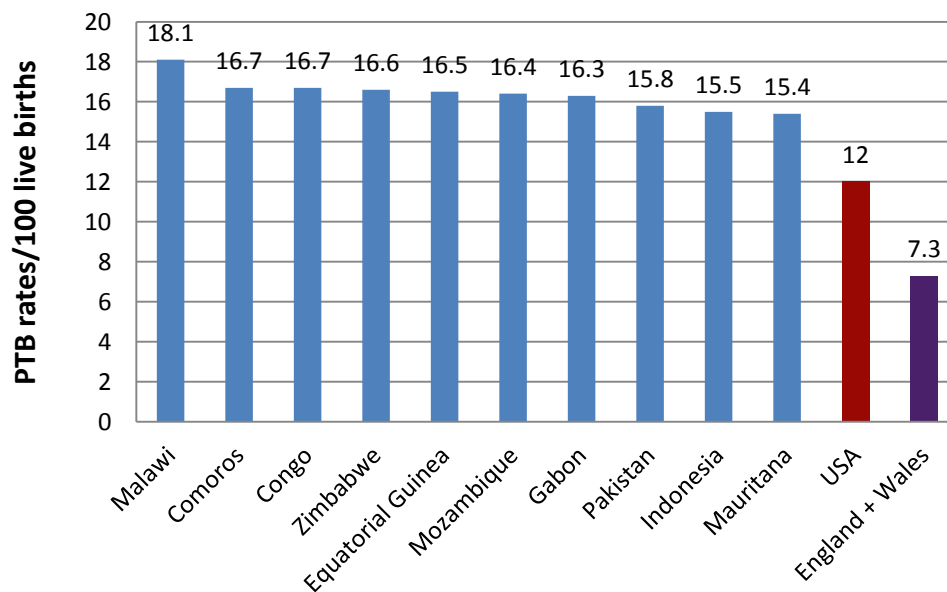
Figure 1.2: The 10 countries with the highest global preterm birth rates per 100 live births (blue), against the rate for the USA and England and Wales. All data from WHO (2012) other than data for England and Wales from ONS (2014).
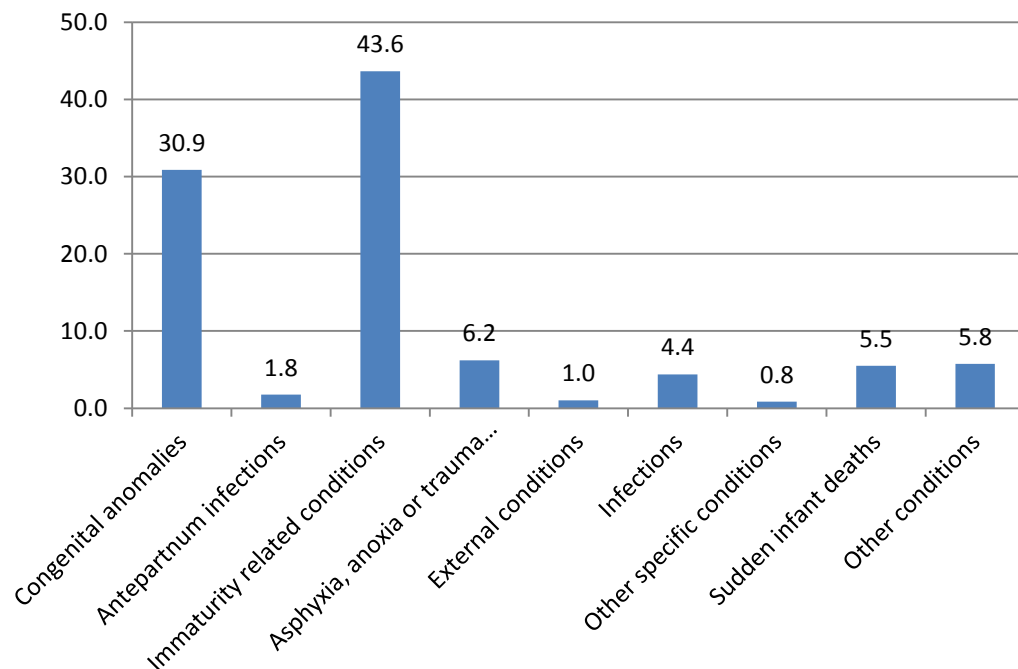
Figure 1.3: The percentage of total infant deaths, England and Wales (2012) by classification. (ONS 2014)

1.1.1 Risk and prevention

Numerous pre-pregnancy reproductive, medical, lifestyle and social factors have been associated with an increased risk of preterm birth (*Table 1.1*). In response to this a number of strategies have been implemented in order to reduce preterm birth rates. Improved access to healthcare, antenatal care, and lifestyle support for pregnant women may have an impact on reducing preterm birth rates both nationally and globally (WHO 2015). In addition to this, there are obstetric management strategies to reduce the likelihood of preterm birth in women who have been determined to be at risk (NICE 2015). As history of

preterm birth or mid-trimester loss is the biggest single indicator of risk of subsequent preterm birth, women reporting a history are offered screening in the antenatal period (Mercer et al 1996, Mazaki-Tovi et al 2007).

Clinical trials of both prophylactic progesterone and of cervical cerclage have shown that administration to women at risk of preterm birth may reduce the risk of subsequent preterm birth although further trial data is awaited (Meis et al 2003, Norman et al 2012, Deshpande et al 2013, Winer, 2015).

Prophylactic progesterone and prophylactic cervical cerclage is offered to women with a history of spontaneous preterm birth or mid-trimester loss between 16 and 34 weeks of pregnancy and in those in whom a cervical length of less than 25 mm has been identified (NICE 2015). Prophylactic vaginal progesterone is offered to women with no history of spontaneous preterm birth but cervical length of less than 25 mm (NICE 2015); and prophylactic cervical cerclage for women in whom cervical length of less than 25 mm has been identified and who have had preterm pre-rupture labour of the membranes in a previous pregnancy, or a history of cervical length (NICE 2015). Intra-uterine infection must always be suspected where threatened preterm birth presents. Antibiotic use has previously been used in obstetric practice in an attempt to prevent preterm birth. Findings of the ORACLE II study found an increased cerebral palsy rates in babies born in the arm of the trial where antibiotics were administered when fetal membranes were intact (Kenyon et al 2008). This highlights the importance of due vigilance when introducing new treatment strategies, and of long-term follow-up in trial outcome design. The OPPTIMUM study for prophylactic vaginal progesterone to prevent preterm birth in those

with a history of preterm birth, is currently ongoing and has a 3 to 5 year follow-up on infant development scores as part of the trial design (Norman et al 2012). This will inform practice as to whether prophylactic treatment such as this increases the primary outcome of birth at term, but will also inform any potential benefits or harms to ongoing child development. Previous trial findings in the success of cervical cerclage for prevention of subsequent preterm birth have varied in terms of timing, techniques used, and outcomes (Burghella 2011, Suhag 2015). As such, although guidelines instruct on when not to offer 'rescue' cervical cerclage; whether to undertake 'rescue' cerclage if symptoms of threatened preterm birth present in the absence of infection bleeding or active contractions, is currently left to physician judgement (NICE 2015). When a woman presents in active suspected preterm labour, then fetal fibronectin testing can be a useful diagnostic test to determine the likelihood of birth within 48 hours (Deshpande et al 2013, Abbott 2015). This can assist the obstetrician considering access to the level of neonatal care that may be required and is locally available, and in considering administrating feto-protective agents (*Table 1.2*). The decision on when or whether to administer tocolysis may depend on presenting symptoms and the time needed to achieve care provision and treatments to improve neonatal outcome.

Category	Risk Factor	Source
Reproductive/ Gynaecological history	History of Pre-term Labour	Mercer et al (1996), Mazaki-Tovi et al (2007)
	Multiple pregnancy	Bloom et al (2001), ONS (2014)
	Assisted reproduction	Dunietz et al (2015)
	Uterine abnormalities	Woefler et al (2001)
	Short cervix	Mercer et al (1996), Odibo et al (2003), Hughes et al (2015), Souka et al (2015)
	Short pregnancy interval	Smith et al (2003)
	Placenta praevia	Zlatnik et al (2007)
	Bacterial vaginosis	Leitch et al (2003)
Medical history	Diabetes (Gestational and exisiting)	Köck et al (2010)
Social and demographics	Ethnicity	Lu and Halfon (2003)
	Age (younger or older)	ONS (2014)
	Lifestyle	Copper et al (1996)
	No antenatal care	Hollowell et al (2009)
	Smoking	Kyrklund-Blomberg and Cnattingius (1998)
	Alcohol use (4-7 or over 7 drinks a week)	Albersten et al (2004)
	Domestic violence	Neggers et al (2004)
	Poor social support	Hollowell et al (2009)
	Stress	Cooper et al (1996)
	Long working hours	van Melick et al (2014)

Table 1.1: Reproductive, medical, lifestyle and social risk factors associated with spontaneous preterm birth.

1.1.2 Definition

Pre-term birth is defined as birth before 37 completed weeks of pregnancy with significantly higher morbidity and mortality in births occurring before 34 weeks. In the UK the age of viability was reduced from 28 completed weeks of gestation to 24 weeks in 1992 reflecting advances in neonatal support (Tucker & McGuire, 2004).

1.1.3 Cause

Williamson et al (2008) describe preterm birth as "a multi-factorial disease caused by genetic, social and environmental factors that most likely interact to increase risk". Iatrogenic causes of preterm birth account for approx 30% of all cases and this rate has recently increased due to assisted reproductive technologies with its associated rise in multiple births. Improvements in ultrasound technology have also led to recognition of intrauterine growth restriction and fetal compromise (Danielian & Hall, 2005). Other causes include maternal factors such as cervical incompetence, general medical conditions, history of previous preterm birth and infection. Exposure to stress may lead to immunosuppression and infection and many commentators have highlighted the association of poor socioeconomic status with preterm birth (Williamson et al, 2008). Lifestyle factors such as poor diet, obesity, smoking, economic and educational status have all been associated with preterm birth (Tucker & McGuire, 2004). The multifactorial causes and manifestations of preterm birth make it difficult to study. The label of preterm birth covers a variety of underlying physical conditions, some well understood and some not. Without further, in depth, understanding of the variance in the different causes of preterm birth, combined with appropriate diagnostic techniques to reliably differentiate between them, the treatment of preterm birth will remain problematic.

1.2. Tocolysis

The term tocolysis is derived from the Greek *toco for* contractions or birth and *lysis* to untie or destroy. The term was first used with regard to a therapeutic intervention in 1964. The primary aim of tocolysis is to delay delivery long enough to allow for the administration of antenatal steroids and/or in utero transfer. Antenatal steroids significantly reduce the occurrence of respiratory distress syndrome, neonatal death, intraventricular haemorrhage and cerebral palsy and in allowing time for their administration, tocolysis can improve neonatal outcomes (Eriksson et al, 2009). The secondary aim is to reduce perinatal morbidity and mortality associated with prematurity, however if the uterine environment is hostile with the presence of infection the additional time in utero can be harmful. In the absence of clear benefits it is therefore considered acceptable not to use tocolytics (Keirse, 2003). The difficult decision of when, or whether to use tocolysis, and which drug to use lies solely with the individual clinician. A number of compounds have been investigated as tocolytics including cyclooxygenase inhibitors, beta agonists, L-type calcium channel antagonists, magnesium sulphate and Oxytocin receptor antagonists. To date no tocolytic has been shown to reduce the incidence of perinatal morbidity and mortality and no study has been carried out with sufficient power to show such a benefit (Di Renzo & Roura, 2006). With a static or rising (dependent on the data source) preterm birth rate, increasing demand on neonatal services and the distress and long term social impact that preterm birth brings, there is an urgent need for new and effective treatments.

1.3 Clinical challenges in tocolytic use

The clinical guidelines produced by the Royal College of Obstetricians and Gynaecologists (RCOG 2011) suggest that there is no clear evidence that tocolytics improve pregnancy outcome and as such it is reasonable not to use them (RCOG 2011). However, other interventions that have been demonstrated to improve neonatal outcomes include moving the expectant mother to a clinical area with availability of high level neonatal critical care facilities and expertise (in-utero transfer) (Marlow et al 2014), and the administration of corticosteroids to promote lung maturation (Roberts and Dalziel 2006). The antenatal administration of corticosteroids has been shown to significantly reduce the risk of neonatal death, respiratory distress syndrome, cerebroventricular haemorrhage, necrotising enterocolitis, need for respiratory support, intensive care admissions and systemic infections within the first 48 hours after birth (Roberts and Dalziel 2006). Current RCOG guidelines recommend either two intramuscular doses of betamethasone 12mg, 24 hours apart or four intramuscular doses of dexamethasone 6mg, 12 hours apart (RCOG 2010). With such advantages to neonatal wellbeing, often the aim of tocolysis is to delay birth sufficiently to allow for full 48 hour administration of corticosteroids.

A number of pharmacological agents have been, or still are, used routinely as tocolytics. In the UK a commonly used tocolytic was the β-adrenergic receptor antagonist, ritodrine. Although being effective in increasing time from administration to birth, ritodrine was also associated with maternal side-effects such as palpations, nausea and vomiting, tremor and cardiovascular effects, often resulting in discontinuation of treatment (Gyetvia et al 1999, de Heus

2009). Indomethacin, a COX-1 and COX-2 inhibitor has similarly been shown to increase time to birth but carries significant fetal side effects including reduced fetal urine output (oligohydrmanious), and if used after 32 weeks gestation can bring about the premature closing of the ductus arteriosus which has a detrimental effect on fetal circulation (Groom et al 2005). In view of these detrimental side effects ritodrine and indomethacin are not recommended as tocolytics of choice by the RCOG (RCOG 2011).

Magnesium sulphate ($MgSO_4$) is also used as a tocolytic agent, however it has been shown in trials to be ineffective in delaying birth (Crowther et al 2002). There have been recent suggestions that $MgSO_4$ is useful as a neuroprotective agent to benefit neonatal outcomes and as such is used by some clinicians, particularly favoured in the USA, but evidence for this is not yet conclusive (Doyle et al 2009). Atosiban, an Oxytocin receptor antagonist is licensed for use as a tocolytic in the UK. In terms of increasing time to birth from administration, it is comparable to Nifedipine with Nifedipine demonstrating a significant reduction in respiratory distress syndrome (OR 0.55 CI 0.32-0.97) over atosiban (Coomarasamy 2003). There is currently a Dutch clinical trial (Assessment of Perinatal Outcome after Specific Tocolysis in Early Labour: APOSTEL III-Trial) in progress directly comparing Nifedipine to atosiban (van Vliet et al 2014). The primary outcome is neonatal morbidity, with maternal side effects leading to discontinuation as a secondary outcome. Long-term developmental follow-up is desirable but dependent on future funding (van Vliet et al 2014). Due to the methods of administration of both agents, Nifedipine oral and atosiban IV, the study design is random treatment allocation but is not blinded to administration

(van Vliet et al 2014). The results of this trial will hopefully add information as to the clinical advantage, if any, of one agent over the other. Without a demonstrated advantage the clinical decision on which tocolytic to use within the context of the NHS is often economic and policy driven. The cost of a full 48 hour course of atosiban is approximately £500 with each 5 ml vial costing £52.82, whereas Nifedipine is considerably cheaper at less than £1 for a 48 hour course (max 60 mg) with an 84-blister pack of 10mg Nifedipine costing £6.53 (BNF 2015). Anecdotally many NHS local policies reserve the use of atosiban for situations where Nifedipine does not appear to be effective and the prescriptions often require consultant level agreement.

Nifedipine is not licensed as a tocolytic but is a commonly used calcium channel blocker (CCB) for the purpose of delaying birth with a similar agent, nicardipine also used. In a large systematic review including 38 trials where CCB's were compared to other tocolytics, 35 used Nifedipine and the remaining 3; nicardipine (Flenady et al 2014). CCB's were compared to β-mimetics (23 trials), $MgSO_4$ (7 trials), atosiban (2 trials), terbutaline (4 trials), indomethacin (2 trials) with 1 trial comparing high dose verses low dose CCB (Flenady et al 2014). Overall, there was a reduction in birth within 48 hours (RR 0.30, CI 0.21 to 0.43) after administration with CCB's compared with other tocolytics; but an increase in maternal side effects RR 49.89 CI 3.13 to 795.02). When Nifedipine was compared with β-mimetic specifically, there was a significant reduction in very preterm birth (before 34 completed weeks) (RR 0.78 CI 0.66 to 0.93) and an increase in interval between trial entry and birth (Average 4.38 days) (Flenady et al 2014). Again, a significant reduction in respiratory distress syndrome was seen

with CCB's but no significant difference in perinatal mortality. Fewer women required discontinuation of therapy for adverse effects (RR 0.21 CI 0.11 to 0.40). High dose Nifedipine resulted in a statistically significant higher gestational age at birth but other than that there were no significant outcomes with low-dose compared to high-dose. The varied dose regimes and durations studied in this review make interpretation problematic. Long-term outcomes of death and neurosensory impairment was only available for one study (children aged 9-12) showed no difference between CCB's and Ritodrine in terms of parental reported quality of life, behaviour, emotion, educational and motor quality (Flenady et al 2014). In a review of side effects from tocolytic use, the most commonly occurring serious (4/5) and mild (4/6) adverse event associated with Nifedipine was hypotension. In 6/7 cases, hypotension developed within 2 to 4 hours after the start of tocolysis where the dose used was 2 to 4 doses of 10 mg sublingual Nifedipine every 15 minutes followed by 20 mg slow release every four hours (de Heus et al 2009). It is not clear if in these cases the treatment was continued beyond a total amount of 60 mg, which is used as a cautionary cut-off threshold for increased likelihood of side-effects within the RCOG guidelines (RCOG 2011). Despite these incidents there was an absence of associated fetal compromise (de Heus et al 2009). de Heus et al (2009) also demonstrated an increased likelihood of adverse effects if more than one type of tocolytic was used in combination and their caution against this practice is echoed in the RCOG guidelines (RCOG 2011). When single doses were administered, the rate of serious adverse drug reactions associated with β-mimetics was 1.7% whereas with Nifedipine the rate was 0.9% (de Heus et al 2009).

Overall Nifedipine, used as a short-term tocolytic, has an effect in delaying time from administration to birth, is economically favourable over other tocolytics, and has a more favourable safety profile than alternative agents. However, the cardiovascular side-effects remain of concern, and in order to avoid increasing this risk the dose is limited within currently recommended dose regimes; which may not always be sufficient to delay birth for administration of antenatal corticosteroids or allow for safe in-utero transfer. As such a new tocolytic regime that can delay birth for up to 48 hours without adding to existing side effects would be desirable. Drug regimes currently clinically recommended for tocolysis and feto-protection are summarised in *Table 1.2*.

Drug	Initial dose	Route	Maintenance	Source
Tocolysis				
Atosiban	6.75 mg	Intravenous	18 mg/hour for 3 hours, the 6 mg/hour for up to 45 hours (max of 330 mg/48 hrs)	RCOG Green top guideline No 1B (2011)
Nifedipine	20 mg	Oral	10-20 mg three to four times daily up to 48 hrs (total dose over 60 mg associated with three to four-fold increase in side effects	RCOG Green top guideline No 1B (2011)
Fetal protection				
Magnesium Sulphate (neuroprotection)	4-6 mg	Intravenous	1-2 mg/hour (up to 24 hours)	ACOG Committee Opinion No. 455 (2010 (revised 2015)) RCOG Scientific Impact Paper No. 29 (2011)
Betamethasone (Fetal lung maturation)	12 mg (2 doses 24 hours apart)	Intramuscular	Total dose (24 mg) over 48 hours - most effective from 24 hours - 7 days from last dose	RCOG Green–top Guideline No. 7 (2010)
Dexamethasone (Fetal lung maturation)	6 mg (4 doses 12 hours apart)	Intramuscular	Total dose (24 mg) over 48 hours - most effective from 24 hours - 7 days from last dose	RCOG Green–top Guideline No. 7 (2010)

Table 1.2: Main tocolytic drugs and drugs for feto-protection currently recommended for use in the UK

1.4 The problem of Post-Partum Haemorrhage

Post partum hemorrhage (PPH) remains a significant clinical issue in women's health. Primary PPH is a significant blood loss that occurs within 24hrs of delivery and secondary PPH when occurring from 24hrs to 6 weeks post-partum. PPH is defined as a blood loss of 500 mls or more; but further categorized as a minor PPH when the loss is from 500ml-1000 mls, major PPH if 1000mls-2000mls, and severe PPH when over 2000mls (RCOG, 2009). Despite these classifications any blood loss can be significant if it leads to hemodynamic instability. This is of particular significance in women that are anemic prior to delivery. This is more common in developing countries where PPH poses a significant risk of death to childbearing women. In the UK it is estimated that PPH occurs in 18% of all births and with 3% of all vaginal deliveries resulting in a severe PPH. WHO estimates the rate worldwide to be 6% rising to 10.5% in Africa. PPH accounts for 30% of all maternal deaths in Africa and Asia (WHO, 2007). Interventions to prevent PPH are a priority in the UN Millennium development Goals global effort to reduce the maternal mortality rates by three-quarters by 2015 (UN, 2010) *(Figure 1.4).*

The causes of PPH are either traumatic bleeding or atonic bleeding. Traumatic bleeding is as a result of mechanical injury to the uterus, cervix or genito-urinary tract during delivery. Atonic bleeding is due to the inability of the uterus to contract preventing compression of blood vessels and control of bleeding. Conditions that can impair contraction are retained placenta, retained placental tissue or membrane fragments, or the accumulation of blood clots. Loss of

uterine tone is the most common cause of primary PPH. Risk factors for uterine atony include prolonged labour, high parity, multiple pregnancies, large babies and polyhydramnios (WHO, 2008).

PPH is an obstetric emergency and remains a leading cause of morbidity and mortality due to difficulty in management. Despite active management of the 3rd stage being routinely practiced in the UK, deaths from post-partum hemorrhage continue to occur. The most recent report on confidential enquiries into maternal deaths show the number of deaths has halved to 5 from the previous report. Although this is a welcome reduction the trend throughout triennial reports from 1985 remains stable *(Figure 1.5)*. These enquiries point to substandard care as contributing factors to maternal deaths from PPH, which are considered preventable. With a potential blood loss of 500mls per minute, death from exsanguination can occur within 10 mins with an untreated severe PPH (WHO, 2008). Renal damage is possible sequelae in survivors due to renal tissue damage from hypovolemia.
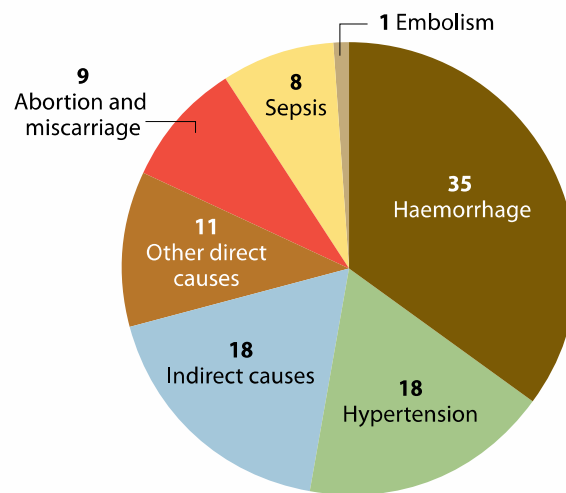
Figure 1.4: Causes of maternal deaths from developing countries (percentage)

from (UN, 2010)
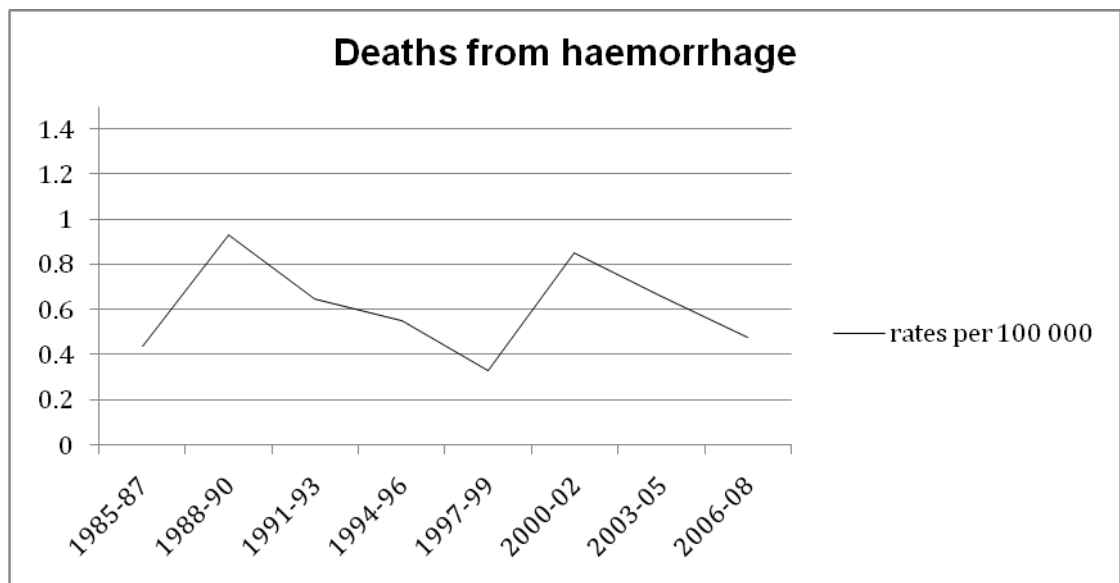
Figure 1.5: Graph showing deaths per 100 000 from haemorrhage from triennial

Confidential Enquiry reports into maternal deaths from 1985. Year to year

variance is due to low overall numbers but the trend remains stable.

1.5 Current treatments for the prevention and management of Post-partum haemorrhage

1.5.1 Prevention of PPH

Antenatal observation for risk factors is essential for the prevention and expectant management of PPH, however PPH cannot always be predicted. Active management of the third stage of pregnancy is a strategy that combines prophylactic administration of Oxytocin by intramuscular injection combined with delivery of the placenta by controlled cord traction. Active management of the 3rd stage reduces the risk of PPH by 60% and reduces the risk for additional therapeutic oxytocics by 50% (RCOG, 2009). There continues to be consistent evidence of the benefits of active management strategies from trials comparing Oxytocin prophylaxis with placebo or no treatment (Westhoff et al 2013). Active management with the administration of Oxytocin over any other currently available pharmaceutical agents is recommended globally (WHO, 2007). However, in a review of international guidelines, little agreement was found in timing and type of uterotonic use and dosage (Bohlmann and Rath 2014). There was agreement on Oxytocin as the first line choice for both prevention and management but with varied dosing protocols and methods of administration and much disagreement on the order and administration of second line agents (Bohlmann and Rath 2014).

In a comparison of misoprostol versus Oxytocin for the prevention of PPH, 12% of the study group had PPH and 10 % of the control. There were no significant difference in other outcomes or in side effect reporting suggesting that

misoprostol is equally effective as Oxytocin for prevention of PPH (Firouzbakht et al 2013), however this finding was not held up in systematic review which found Oxytocin to be most effective (Westhoff et al 2013) . One benefit of misoprostol over Oxytocin is it stability at various temperatures and ease of administration which makes it an ideal choice in areas where access to other drugs is difficult or in the hands of unskilled attendants (Nasreen et al 2011). There is currently a planned study for the combination of the antifibrinolytic agent tranexamic acid with prophylactic Oxytocin administration after singleton term planned vaginal births in order to prevent PPH (Sentilhes et al 2015), which indicates the desire to seek more effective methods of PPH prevention.

1.5.2 Management of PPH

Management strategies include clinical interventions such as catheterization and manual compression of the uterus as well as pharmacological management with the administration of uterotonics, (Oxytocin by intravenous infusion, prostaglandins - carboprost ($PGF_2\alpha$) by infusion or direct intramyometrial injection, or misoprostal (prostaglandin E_1) by rectal administration). If pharmacological and clinical interventions fail then surgical interventions may be indicated (balloon tapenade, haemostatic suturing, internal iliac artery ligation) with ultimately obstetric hysterectomy being the only effective solution. The RCOG guidelines on the management of PPH (*Figure 1.6*) re-iterate the gravity of such an obstetric emergency. Management includes effective and timely communication, escalation to clinical seniors and access to appropriate facilities as essential aspects of care and crucial to a successful outcome as any individual

treatment. Individual cases may require specific approaches to management and escalation of recue measures. Any additional therapeutic options that can prevent morbidity would improve outcomes for women at risk of PPH.

Resuscitation, monitoring, investigation and treatment should occur simultaneously

Major obstetric haemorrhage
Blood loss > 1000 ml
Continuing major obstetric
haemorrhage or clinical shock

Call for help
Senior midwife/obstetrician and anaesthetist
Alert haematologist
Alert blood transfusion laboratory
Alert consultant obstetrician on-call

Resuscitation
Airway
Breathing
Circulation
Oxygen mask (15 litres)
Fluid balance (2 litres Hartmann's, 1.5 litres colloid)
Blood transfusion (O RhD negative or group-specific blood)
Blood products (FFP, PLT, cryoprecipitate, factor VIIa)
Keep patient warm

Monitoring and investigations
14-g cannulae x 2
FBC, coagulation, U&Es, LFTs
Crossmatch (4 units, FFP, PLT, cryoprecipitate)
ECG, oximeter
Foley catheter
Hb bedside testing
Blood products
Consider central and arterial lines
Commence record chart
Weigh all swabs and estimate blood loss

Medical treatment
Bimanual uterine compression
Empty bladder
Oxytocin 5 iu x 2
Ergometrine 500 micrograms
Oxytocin infusion (40 u in 500 ml)
Carboprost 250 micrograms IM every 15 minutes up to 8 times
Carboprost (intramyometrial) 0.5 mg
Misoprostol 1000 micrograms rectally

Theatre
Is the uterus contracted?
Examination under anaesthesia
Has any clotting abnormality been corrected?

Intrauterine balloon tamponade
Brace suture
Consider interventional radiology

Surgery
Bilateral uterine artery ligation
Bilateral internal iliac ligation
Hysterectomy (second consultant)
Uterine artery embolisation

Consider high-dependency unit
or intensive care unit

Figure 1.6: Algorithm from RCOG guidelines (green top guideline no.52, 2009) on the management of major PPH illustrating the complex aspect of management from communication, escalation and cascade of mechanical and medical and surgical therapies.

45

1.6. The physiology of myometrial contraction.

1.6.1 Uterine quiescence

1.6.1.1 Progesterone/Progesterone receptor

The progestogen, progesterone (P_4) plays an important role in the maintenance of quiescence by suppressing expression of genes that increase myometrial contractility and by inhibiting inflammatory pathways.

In many mammalian species circulating progesterone withdrawal is critical for increased uterine contractility leading to labour. In humans, circulating P_4 levels remain elevated throughout the third trimester of pregnancy and into labour; increasing uterine contractility and the onset of labour maybe due to events that impair progesterone receptor (PR) function rather than a reduction of circulating P_4.

P_4 is secreted from the corpus luteum following ovulation. In absence of pregnancy, falling circulating progesterone in the late secretory phase of the menstrual cycle leads to sloughing of the decidual layer and menstruation. If pregnancy occurs, human chorionic gonadotropin (hCG) secreted by trophoblast cells maintain the corpus luteum stimulating steroid hormone secretion. Interaction with the lutenising hormone choriogonadotropin receptor (LHCGR) promotes the secretion of P_4 (Järvelä 2008). Increased angiogeneis and increased blood supply supplies the cholesterol needed for P_4 synthesis (Takasaki 2009). At around 7-8 weeks there is a shift from extra-uterine P_4 production to intra-uterine and the regression of the corpus luteum can be seen from 10 weeks

onwards after which hCG levels start to decline. (Rowan et al 2008, Järvelä 2008). The corpus luteum is declining at around week 5-7 with the secretion of P_4 reaching its lowest ebb before increasing once supported by the placenta (Järvelä 2008).

P_4 exerts its action through binding to progesterone receptor (PR). PR receptor is a ligand-activated transcription factor with sequence specific binding regions. In *in vitro* experiments, P_4 exerts a dose-dependent inhibitory effect on myometrial contractility (Anderson et al 2009, Chen at al 2014, Arrowsmith et al 2016) and maintains uterine quiescence by promoting relaxatory factors. P4 suppresses the expression of connexin-43 (CX-43); a protein which is essential for the formation of gap junctions in the myometrium (Petrocelli and Lye 1993, Sheldon et al 2014). Gap junctions are vital for the propagation of the action potential across myometrial smooth muscle cells in order to elicit global excitability and contraction (Sheldon et al 2014). CX-43 ribonucleic acid demonstrates a gestation-dependent functional increase towards term with a further increase at the onset of labour (Chow and Lye 1994). P_4 also promotes cAMP to inhibit the immune regulating protein nuclear factor kappa-light-chain-enhancer of activated B cells, (NF-κB) activation downstream of protein kinase A (PKA); and in turn enhances P_4 activity to drive gene expression (Chen at al 2014). PKA reverses progesterone-induced suppression of inflammation leading to the onset of labour (Chen at al 2014).

Two estrogen-regulated promoters generate transcripts encoding two functionally different, co-expressed PR forms; A and B (Kastner et al 1990). The

PR-A isoform can repress PR-B by sequestering P; leaving less available to bind PR-B (Wei 1997). A further form of PR, PR-C is an N-terminally truncated and lacks the DNA binding domain which means this form of PR is unable to bind DNA but is able to bind P_4 (Wei et al 1997). Similarly PR-C can bind to PR-B reducing PR-B availability to bind to PR (Wei et al 1997). An altered PR-C/PR-B ratio reduces capacity of PR-B to maintain quiescence (Condon et al 2006). PR-C expression is increased in human fundal myometrium at term as a result of NF-κB activation (Condon et al 2006).

The Zinc finger E-box binding homeobox proteins ZEB-1 and ZEB-2, inhibit the expression of contractile associated protein (CAP) genes *OXTR* (Oxytocin receptor), and *CXN-43* (connexin-43) (Renthal et al 2010). ZEB-1 is up-regulated by P_4/PR by binding of the PR to ZEB-1 promoter. As PR declines there is a reduction in ZEB-1 expression leading to up-regulation of miR-200 family which in turn repress the expression of ZEB-1 allowing expression of CAP genes. (Renthal et al 2010) The contribution of this pathway to contractility is further supported by the finding that the miRNA family miR-200 is increased at term (Renthal et al 2010).

Estrogen receptors do not increase steadily toward term, but an increase in responsiveness to estrogen in labour, is due to an increased expression of ERα and ERβ (Mesiano 2002). P_4 inhibits ERα expression, preventing estrogen-stimulated production of contractile associated protiens.

There appears to be a fine balance between P4, estrogen and PR forms, with varying ratios resulting in functional progesterone withdrawal and estrogen activation at the onset of labour.

1.6.1.2 Inflammation and labour onset

In observations made by stimulating myometrial inflammation through activation of NF-κB by IL-1β; IL-1β inhibited PR expression and possibly increased PR gene silencing (Lee et al 2012) highlighting the influence of inflammation on P_4 action. PR was associated genes concerned with cellular development, growth and proliferation but not on contraction associated proteins, failing to support a significant anti-inflammatory role of progesterone via PR and suggesting that activation of inflammatory mediators is more likely driven by NF-κB rather than P_4 withdrawal (Lee et al 2012).

Pro-inflammatory cytokines IL-1β induce calcium entry via increased expression of sacroplasmic reticulum calcium ATPase (SERCA) 2b (Tribe et al 2003). Increased cell excitability enhanced increased basal calcium entry were observed, indicating a role for inflammatory cytokines in the modulation of the electrophysiological thresholds of myometrium towards term (Tribe et al 2003).

There is some indication that inflammatory pathways may depend on location within the uterus. NF-κB activation in term human myometrium occurs in the fundus but not in the lower segment (Condon et al 2006) and pro-inflammatory cytokines IL-1b, IL-6, CXCL-8 are increased in the lower segment during term labour (Shynlova et al 2013). The myometrial stimulator prostaglandin F2 alpha

(PGF$_2\alpha$) receptor is reduced by P$_4$; IL-1β increases PGF$_2\alpha$ receptor expression via

PKC and NF-κB (Liang et al 2008)

A number of additional agents have been associated with the onset of human labour. For example, labour is associated with a reduction in the G protein alpha subunit (Gαs) levels in myometrium. Gαs activates adenylyl cyclase and the cAMP-dependent pathway to relaxation. Gαs expression was found to be reduced in women in spontaneous labour (Europe-Finner 1994). A summary of signalling/effecting molecules involved in maintaining quiescence and promoting contractility is found in *Table 1.3*.

	Activation	Relaxation	Source
Stretch	Activates COX-2 via AP-1 system Action supressed by P$_4$		Sooranna et al (2004) Shynlova et al (2008)
Fetal hormones (SP-A)	Stimulates microphage migration and NF-kB p65 and IL-1β		Reini and England (2015) Mendelson & Condon (2005)
NF-κB	Increases expression of contractile activating proteins PGF$_2\alpha$ receptor CX-43 COX-2 (promotes) prostagladins OXTR Inhibited PR expression		Lindeström and Bennett (2005) Lee et al (2012)
Chemokines/cytokines	IL-1β (Ca^{2+} activation) IL-6 CXCL-8 activation of NF-κB		Tribe et al (2003) Lee et al (2012)
cAMP		Via PKA Reduces NF-κB activity Enhances P$_4$ activity to drive gene expression	Chen et al (2014)
Gαs	Decreased expression leads to decreased relaxatory effect of	Increased expression leads to agonist induced cAMP	Europe-Finner et al (1994)

	cAMP	formation via adenylyl cyclase	
PKA	Reverses P_4 induced suppression of inflammation		Chen et al (2014)
G-protein coupled receptor	Lutenising hormone (LH) Human corionic gonadotrophin (hCG) Cortico-trophin releasing hormone (CRH) Relaxin (RLX) Prostaglandin E2 (PGE_2)	Promotion of Gαs cAMP PKA	Gellerson & Brosens (2003) Europe-Finner (1994)
20 α-HSD		Maintains P_4	Ishida et al (2007) Piekorz et al (2005)
	Activation	**Relaxation**	**Source**
STAT5b	Suppress 20a-HSD which regulates mi-R200a which is a regulator of ZEB1 & 2 which promote OXTR & CX-43		Williams et al (2012)
Estradiol (E_2)	Increase towards term promotes pro-inflammatory events; induce influx of macrophages and neutrophils, Enhances transcription of contractile activating proteins. Reduced PR function		Mesiano et al (2002)

Table 1.3: Summary of some of the signalling/effecting molecules involved in maintaining uterine quiescence/promoting contractility

1.6.2 Pathways to contractility.

The uterus is a uniquely adaptive organ with gradual transition from quiescence, whilst harbouring the developing fetus, to contractions during labour followed by a rapid remodelling in the immediate post partum period (Young, 2007). There is evidence that the initiating hormonal cascades of parturition are prone to redundancy (Blanks & Thornton, 2007). When the Oxytocin gene is deleted in the knockout mouse (OT -/-) parturition continues within normal timings and leads to delivery of live pups (Nishimori et al 1996, Young et al 1996). However, pups fail to thrive postpartum due to failure of maternal milk ejection suggesting that in mice, Oxytocin is essential to successful lactation but non-essential in labour and birth (Nishimori et al 1996, Young et al 1996). Also, manipulation of the cytosolic phospholipase A_2 (cPLA$_2$)and cyclooxygenase-1 (COX-1) pathways prevents the formation of PGF$_{2\alpha}$, a rise in which usually brings about luteolysis and the subsequent onset of labour in mice; prevents normal parturition (Uozumi et al 1997, Gross et al 1998). The non-labour phenotype can be rescued with the addition of PGF$_{2\alpha}$, and normal parturition is restored (Uozumi et al 1997, Gross et al 1998). In the COX-1/OT knockout mouse, luteolysis occurs on the normal day of gestation but labour is prolonged and pups do not survive (Gross et al 1998). As luteolysis fails in COX-1 (-/-) knockout mice, this suggests that Oxytocin has an influence on luteolysis. This points to a compensatory mechanism where more than one agent can influence pathways to contractility. These described pathways activate G-protein coupled receptors, ultimately leading to an increase in $[Ca^{2+}]_i$ and opening of voltage gated calcium channels (VGCC). Further evidence of this dual pathway is the effectiveness of tocolysis

that targets both simultaneously. When preterm labour is induced in sheep using glucocorticosteroids, dual treatment with the prostaglandin synthase type-2 inhibitor nimesulide, and the Oxytocin receptor antagonist atosiban, delivery within the 48 hour treatment phase is completely prevented (Scott et al 2001).

Pathways to contractility are complex and compete with the pathways that maintain quiescence. This means that targeted inhibition of one pathway (i.e. Oxytocin or prostaglandins) can be bypassed in the medium term (24-48hrs) by alternative stimulatory cascades. As a consequence of this fact it is the contention of this thesis that targeting of proteins, downstream of the stimulatory cascade, will demonstrate efficacy regardless of the activating mechanism. The therapies proposed herein rely on targeting voltage-gated calcium entry during a contraction, which is the key point at which contractility will occur.

1.6.3 *Role of Calcium in contractility*

Contractility is initiated by an increase in intracellular calcium ($[Ca^{2+}]_i$) from two possible sources; i) entry of extracellular Ca^{2+} ($[Ca^{2+}]_o$) into the myocyte through voltage gated calcium channels and TRP Ca^{2+} channels and ii) the release of $[Ca^{2+}]_i$ from the sarcoplasmic reticulum (SR) (Parkington et al, 1999, Shmygol et al, 2007, Babich et al, 2004). Influx of $[Ca^{2+}]_o$ is essential for the propagation of action potentials and the generation of contractile force (Shmygol et al, 2007). In rat myometrial tissue, Ca^{2+} spikes resulted in Nifedipine sensitive action potentials with varied initiation sites, spatial spread and frequency (Burdyga et al, 2009). The role of Ca^{2+} release from the SR may not play as significant a role

in activating the contractile machinery as influx of $[Ca^{2+}]_o$, but there are indications of a role for the SR in contraction regulation by moderating available $[Ca^{2+}]_i$ (Wray & Shmygol, 2007) *(Figure 1.7).* Spatially and localised Ca^{2+} signals may target specific signal transduction pathways such as the activation of Ca^{2+} activated K^+ channels thus initiating hyperpolarisation (Wray & Shmygol, 2007). The gestational difference in expression in SR components (RyR's and BK channels) also point to a function of the SR in the regulation of quiescence (Wray & Shmygol, 2007).

The increase in $[Ca^{2+}]_i$ activates, via calmodulin binding, the intracellular kinase myosin light chain kinase that phosphorylates myosin light chains allowing the interaction of myosin and actin resulting in contraction. Myosin light chains are phosphorylated at its binding site by Ca^{2+}-calmodulin dependant protein kinase II. This drives the ATP dependent cross bridge cycling interaction between actin and myosin. The excitation-contraction cycle is maintained by calcium entry during the length of the complex action potential thus creating the phasic contraction. Myometrial relaxation is preceded by a repolarisation of the plasma membrane to resting membrane potential and a decrease in $[Ca^{2+}]_i$ (McKillen et al, 1999, Parkington et al, 1999).
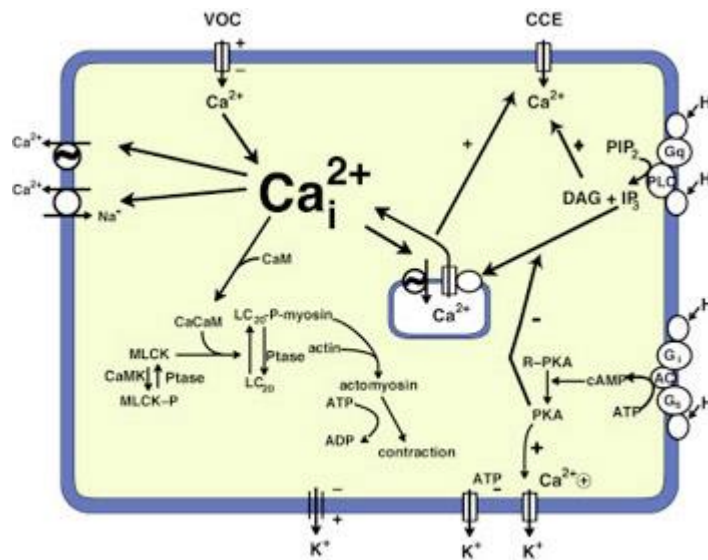
Figure 1.7: Mechanisms involved in smooth muscle calcium regulation ƒrom

Sanborn, B found at: http://csu-

cvmbs.colostate.edu/academics/bms/Pages/barbara-sanborn.aspx

1.6.4 Role of potassium in contractility

Potassium is essential in all cell types in the control and maintenance of the membrane potential. Membrane potential is the difference that arises from the unequal distribution of ions either side of the membrane (Sanborn, 2000). Selective permeability of the plasma membrane and active transport of ions provide a mechanism by which the electro-chemical gradients are prevented from reaching equilibrium, generating a trans-membrane potential (Khan et al, 2001). The net direction of flux of a given ion is determined by the concentration gradient and the electromotive force (Sanborn, 2000) *(Figure 1.8)*. The net flux of a given ion is described by the Nernst equation where an ion reaches equilibrium (E_x) when the electromotive force exactly counters the force of the

concentration gradient. If the membrane potential is more positive than the equilibrium potential for a specific ion, efflux of that ion from the cell is favoured (Sanborn, 2000). Under these biophysical constraints potassium is responsible for setting the negative membrane potential in most excitable cell types. This is a result of the increased permeability of the plasma membrane at rest to potassium when compared to the other important intracellular ions calcium, sodium and chloride. In addition to the higher permeability to potassium of the plasma membrane, ionic pumps move potassium into the cell under active transport creating a trans-membrane gradient of high intracellular and low extracellular potassium. Thus under resting conditions potassium ions constantly leak out of the cell down their chemical gradient shifting the trans-membrane potential towards the reversal potential for potassium E_k.

The permeability of the plasma membrane to potassium at rest is conferred by a small subgroup of K^+ channels that are active at negative potentials or are activated by $[Ca^{2+}]_i$. It is the contention of this thesis that manipulation of these channels, specific to myometrial smooth muscle, will hyperpolarise the myometrial cell membrane and render it less likely to stimulation and hence a lower probability that L-type calcium entry will be triggered.

Na⁺ (137 mM) Ca²⁺ (1.5mM) K⁺ (6mM) Cl⁻ (134mM)

Na⁺ (40mM) Ca²⁺ (0.13µM) K⁺ (169mM) Cl⁻ (65mM)

E_{Na} = 32.88mV E_{Ca} = 124.93mV E_{K} = -89.17mV E_{Cl} = -19.33mV

Nearnst Equation for membrane potential: $E_x = \frac{RT}{zF} \ln \frac{[X_o]}{[X_i]}$
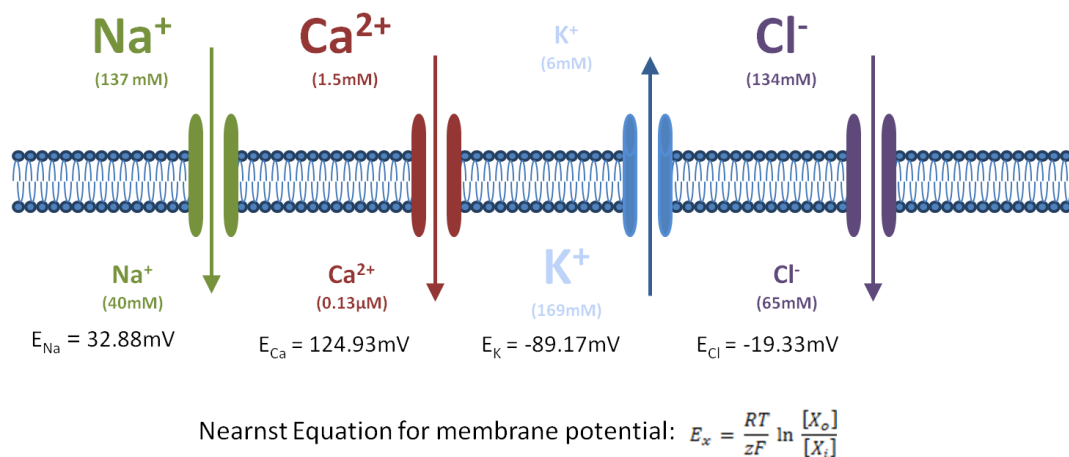
Figure 1.8: Electro-chemical gradient and membrane potentials for each ion in myometrial smooth muscle as calculated using the Nernst equation. Extracellular and intracellular volume data from Sanborn (2000)

1.7. L-type calcium entry and the actions of Nifedipine

The voltage gated L-type calcium channel (VGLCC) was first characterised by its binding to dihydropyridines (Kanngiesser 1988). Several other types of voltage gated calcium channels have been identified through electrophysiology and are described as L,N,P/Q, R and T type channels. The T type channel has been identified in human myometrium and when inhibited, contractile force is reduced (Young & Zhang, 2005). There is no difference in the expression of this channel across gestation or with labour but it appears to be important in determining contractile frequency (Blanks et al, 2007). The VGLCC allows for a large flux of calcium ions through the plasma membrane when activated by transmembrane voltage change. Ca²⁺ channel α subunits share a common

57

topology with four homologous domains, each of which is composed of six transmembrane segments (S1-S6) and two short segments that line the extracellular pore (SS1 and SS2) situated between S5 and S6 (Hockerman et al, 1997). The S4 segments are the site of voltage activation sensors. Dihydropyridines can act as agonists or antagonists of the channel depending on the drug structure and specificity of action (Hockerman et al, 1997). Antagonists favour inactivated state binding, stabilising a non-conducting blocked state that mimics a channel with a single Ca^{2+} ion in the filter (Hockerman et al, 1997, Wang et al, 2004). The VGLCC is highly selective to Ca^{2+} and this is probably due to its size and charge (Boda et al, 2009). This theory of dual selectivity is referred to as charged/space competition or CSC. Selectivity results from both the size of the cation and its carried charge. In the case of the VGLCC it attracts divalent over monovalent selecting Ca^{2+} over Na^{+} when the two are of approximately similar size. All other cations are excluded on the basis of size (Boda et al, 2009).

Extracellular Ca^{2+} entry during a contraction is dependent on the opening of the VGLCC as confirmed by the action of Nifedipine *in vitro* on myometrium, whereby spontaneous or augmented contraction can be abolished by its administration (Shmigol et al, 1998, Brown et al, 2007). Expression of the VGLCC modestly increases during pregnancy and labour (Mershon et al, 1994). There is however a change in binding of Nifedipine with gestation and in labour in rat myometrium suggesting an isoform change of physiological significance (Mershon et al, 1994). This suggests higher doses of Nifedipine may be necessary to inhibit contractions in preterm labour. Further to blocking extracellular influx of Ca^{2+}, Nifedipine also depletes $[Ca^{2+}]_i$ stores resulting in less

available calcium in the plasmalemmel space opening Ca^{2+} activated K^+ channels (Young et al, 2001). The action of Nifedipine is not specific to the uterus bringing about a simultaneous relaxatory effect on vascular smooth muscle through the VGLCC. Nifedipine was shown to progressively dilate depolarisation-induced constrictions in rat mesenteric arteries but not veins despite similar expression of VGLCC in both tissues (Thakali et al, 2010). This 'silencing' of VGLCC in venous smooth muscle suggests a mechanism by which Nifedipine reduces cardiac overload but not preload resulting in a relaxatory effect on arterial but not venous tone (Thakali et al, 2010). Venous sensitivity to Nifedipine was increased when intracellular stores were depleted indicating a role for $[Ca^{2+}]_i$ in providing negative feedback to VGLCCs supported by the proximity of the sarcoplasmic reticulum to the plasma membrane (Thakali et al, 2010). Nifedipine was first introduced as a tocolytic in 1977 and despite never having been licensed for this purpose it continues to be used today (Keirse, 2003). Nifedipine has commonly been used as an antihypertensive. Advances in newer treatments for hypertension have led to this group of drugs becoming limited

1.8. K^+ channel openers in myometrial smooth muscle and vascular smooth muscle

Prior to the establishing of this project, work undertaken in our laboratory identified a number of potassium channels that demonstrated differential expression between vascular and myometrial smooth muscle. In a screen of laser captured material the expression levels of all known potassium channels in

the current build of the human genome were compared between vascular and myometrial smooth muscle. A number of promising candidates were revealed in the screen and compounds were identified that might target these differentially expressed targets. The hypothesis being that specific activation of potassium channels expressed in myometrial but not vascular smooth muscle would specifically hyperpolarise myometrial cells. It is hypothesised that by allowing K+ efflux the membrane potential is kept below the level at which VGCC activation occurs in addition to the therapeutically tolerated dose of Nifedipine, it is anticipated that the combined effect would be greater on maintaining hyperpolarisation than Nifedipine alone. These compounds are summarised below.

1.8.1 Pinacidil

Pinacidil is an effective K^+ channel opener functioning on the K_{ATP} channel and is used therapeutically as an antihypertensive. K_{ATP} channels are regulated by the metabolic messenger adenosine triphosphate (ATP) which couples energy metabolism to the electrical activity of the plasma membrane (Proks & Ashcroft, 2009). This protective function is important in many organ systems, including seizure protection in neurological cells, response to cardiac stress and ischemic preconditioning (Proks & Ashcroft, 2009). This is best illustrated in pancreatic β-cells where K_{ATP} function links plasma glucose levels with insulin secretion (Khan et al, 2001). An increase in extracellular glucose and intracellular ATP metabolism results in the channel closing, allowing depolarisation via VGLCC and insulin secretion. A fall in glucose, decreases ATP metabolism, opens the

channel bringing about hyperpolarisation and no insulin release (Proks & Ashcroft, 2009). The K_{ATP} channel is made up of the K_{IR} 6 pore forming subunit and the sulphonylurea receptor (SUR) subunits. This channel is an octomer made up of 4 K_{IR} 6 subunits surrounded by 4 SUR subunits (Berridge, 2010). The SUR subunits are responsible for trafficking, pharmacology and ATP sensitivity. The binding of the SUR subunit allows translocation of the channel to the plasma membrane as signalling from SUR is required for release from the ER/Golgi (Burke et al, 2008). The SUR subunit is also important in regulation of channel function as demonstrated by the use of sulphonylureas as anti-diabetic drugs increasing insulin secretion by modulating channel function (Burke et al, 2008). In the rat uterus under hypoxic conditions which diminish ATP, an increase in K^+ efflux is observed that is inhibited by the K_{ATP} blocker glibenclamide (Heaton et al, 1993). This may be a protective mechanism to preserve myometrial integrity during labour (Heaton et al, 1993). Investigations into the effect of Pinacidil on human myometrium have shown that Pinacidil reduces spontaneous and augmented contractility in both pregnant term and non-pregnant myometrium exerting a more potent relaxatory effect on term non-labouring tissue vs. preterm and labouring tissue (Morrison et al, 1993, Kostrzewska et al, 1996). Pinacidil has a endothelium-independent relaxatory effect on vascular smooth muscle by opening of K_{ATP} channels (Stojnic et al, 2007). This is evidenced by reversal of this effect seen when the K_{ATP} channel blocker glibenclamide is added (Stojnic et al, 2007, Quast, 1993). Since Pinacidil should work on both vascular and myometrial smooth muscle we utilised this as a positive control.

1.8.2 Riluzole

Riluzole is a drug currently prescribed for the treatment of amyotrophic lateral sclerosis (ALS). This progressive neurodegenerative disease results in muscle atrophy from motor neurone damage. Riluzole treatment has been shown to delay the time of death for ALS patients however the exact mechanisms of its neuroprotective effects are not fully understood (*Table 1.4*). Some studies have shown that Riluzole potently reduces Na^+ current particularly in neuronal tissues (Mohammadi et al, 2002, Hebert et al, 1994, Wang et al, 2008, Noh et al, 2000). Riluzole elicited a selective block of inactivated Na^+ channels in rat brain tissue (Hebert et al, 1994). Binding of Riluzole to closed and inactivated states preferentially blocks depolarised, hyperactive neurons and this targeting of abnormally active cells points to the neuroprotective effects of Riluzole and its usefulness as an anti seizure drug (Hebert et al, 1994). Riluzole directly inhibits protein kinase C (PKC) by inhibition of the PKM catalytic domain rather than the ATP or DAG binding domains (Noh et al, 2000). Inhibition by Riluzole of glutamate release has led to the investigation of its potential therapeutic use in psychological disorders, anxiety and depression as well as treatment for addictions (Besheer et al, 2009, Sofuoglu et al, 2008). In a small scale study into the possible interactions with d-amphetamines, 100 mg Riluzole was administered to healthy volunteers with no effect on blood pressure, heart rate or cortisol levels and reporting of a mild sedative effect (Sofuoglu et al, 2008). Riluzole may also have an effect of calcium currents. Investigation into the effect of Riluzole on ionic currents in rat ganglion neurons showed an inhibition of P/Q and N type Ca^{2+} currents with no effect shown on L type currents (Huang et al,

1997). This finding was supported by Siniscalchi et al (1997), who found that Riluzole increases the threshold for the generation of Ca^{2+} spikes, and thus limits the influx of Ca^{2+} ions.

The dual action of Riluzole on the K_{2P} channel TREK 1 channel results in rapid stimulation followed by a decline in activation and a strong inhibition of K^+ current (Duprat et al, 2000). This effect is also associated with an increase in intracellular cAMP which could be brought about by activation of the PKA pathway. TREK 1 is expressed in human myometrium at term (Bai et al, 2005) and was described in our screen.

A further channel identified in our screen and targeted by Riluzole is the SK_3 channel. The small conductance calcium activated potassium channel SK_3 is known to be expressed in myometrium (Brown et al, 2007). Gating of the channel is dependent on calmodulin binding to Ca^{2+} which is constitutively bound to the channel (Nolting et al, 2007). Calmodulin is also involved in trafficking the cell to the plasma membrane (Brainard et al, 2007). SK channels are expressed in many sensory systems as well as in the heart, liver, skeletal muscle and urinary bladder. They are widely expressed throughout the central nervous system (CNS) with activation by Ca^{2+} in these tissues having a neuroprotective effect (Dilly et al, 2005). SK channels can be blocked by the bee venom toxin Apamin which assists in functional investigation (Nolting et al, 2007). The SK_3 channel is activated by Riluzole at concentrations at 3 µM and above (Grunnet et al, 2001). Brown et al (2007) describe the constitutive association with calmodulin and the SK_3 channels sensitivity to $[Ca^{2+}]_i$ levels provide an effective negative feedback

mechanism to regulate $[Ca^{2+}]_i$. In non pregnant murine myometrial strips from mice genetically modified to either under or over express SK_3 contractility was altered (Brown et al, 2007). Strips from mice with an overexpression of SK_3 often failed to contract or produced contractions with less amplitude and force. Contractions were completely inhibited by Nifedipine and there was impaired response to Oxytocin. Strips from mice with a reduced expression of SK_3 produced larger contractions when compared to wild type (Brown et al, 2007). Pierce et al (2008) confirmed that overexpression of SK_3 reduced contractility *in vitro* strips but additionally mice in which preterm labour was induced failed to labour effectively and often suffered dystocia of pups in birth canal. Wild type mice and mice with reduced expression of SK_3 delivered normally. Work in similarly modified mice has been conducted with the aim of investigating arterial tone and blood pressure (Taylor et al, 2003). SK_3 was expressed in the endothelium but not in vascular smooth muscle. The suppression of SK_3 resulted in an elevation of arterial tone and blood pressure. Similar results were seen in Burnham et al's (2002) work into porcine coronary arteries where a SK_3 activator, substance P, brought about hyperpolarisation in endothelial cells. The only study into the SK_3 activity in human pregnant myometrium was conducted by Gillham et al (2007). The authors found that the SK_3 blocker Apamin inhibited endothelium-derived hyperpolarising factor (EDHF) mediated relaxation in myometrial vasculature. The authors suggest there may be some significance in this mechanism in pre-eclampsia or intrauterine growth restriction where there is evidence of abnormal EDHF mediated relaxation. Further investigation into SK3 function in myometrial and vascular smooth muscle is warranted

Possible mechanisms of action of Riluzole
Reduction of voltage gated Na channels
Inhibition of protein kinase C
Inhibition/attenuation of arachidonic acid release
Inhibition of glutamate release
Inhibition of P/Q & N Ca^{2+} channels
Opening of TREK-1 (via AA)-Screen positive
Opening of SK_3 channels-Screen positive

Table 1.4: Summary of possible mechanisms of action of Riluzole derived from the

literature.

1.9 Maternal vascular adaptations to pregnancy; VSMC and endothelial function

From the start of pregnancy the cardiovascular system goes through adaptations to provide increased blood supply required for the development and growth of the fetus. The main cardiovascular change is a significant increase in circulating blood volume, which is initiated by increased blood flow to the uterus (Palmer et al 1992). These vital cardiac adaptations happen early in pregnancy with over 70% of the increase in cardiac output and 85% of the decrease in systemic vascular resistance occurring by the 16th week (Clap & Capeless 1997). Cardiac output was seen to increase significantly by week 5 from a mean of 4.88 l/min from conception to a maximum of 7.21 l/min by 32 weeks (Robson et al 1989). Total peripheral vascular resistance falls during the first 20 weeks (Robson et al 1989), and persists in normal (non-hypertensive) pregnancies throughout and into the postnatal period, with 23% of the increase in cardiac output and 30% of the decrease in systemic vascular resistance being still present 1 year postpartum (Clap & Capeless 1997). The observation that the drop from pre-pregnancy to post-partum mean arterial pressure was more significant in women who had a pregnancy compared to non-pregnant women over the same time scale, suggests that pregnancy-associated cardiovascular remodelling can be beneficial and may persist to subsequent pregnancies (Morris et al 2015). Palmer et al (1992) observed through ultrasound scan that the uterine artery diameter doubled by week 21 from 1.4 ± 0.1 to 2.8 ± 0.2mm, did not change from 21-30 and increased between 30-36 to 3.4±0.2mm. As well as increased diameter there was a significant increase in uterine artery mean flow velocity from early to late

pregnancy from - 8.4±2.2 to 61.4 ±3.0cm/second. Their observation was that in early pregnancy the increase in uterine flow was due to changes in uterine artery diameter and mean flow but in late pregnancy the rise in flow was mainly attributable to faster velocity (Palmer 1992).

This increase in flow to the uterus leads to a compensatory increase in heart rate and activation of volume-restoring mechanisms (Duvecot 1993). Cardiac output then increases due to a rise in stroke volume resulting from an increase in circulating blood volume. Appropriate and successful vascular remodelling is vital for fetal wellbeing and a good pregnancy outcome, as well as having implications for long term maternal health.

In a large UK cohort study (129,920 subjects), women who had a baby with birth weight on the lowest quintile for gestational age, a preterm delivery or pre-eclampsia were at increased risk of ischemic heart disease related admission or death (hazard ratio 1.9 (CI 1.5-2.4), 1.8 (CI 1.3-2.5), 2.0 (1.5-2.5) respectively) (Smith et al 2001). These associations were additive in that women with all three risk factors had a seven times increased risk of ischemic heart disease related admission or death (CI 3.3-14.5) (Smith et al 2001).

Women who had a preterm birth, regardless of any hypertension during pregnancy, had higher blood pressure after pregnancy when compared with women who had term births (Catov et al 2013). Data from the World Health Organization Calcium Supplementation for the Prevention of Preeclampsia Trial showed that in 5,167 singleton pregnancies, a rise in systolic pressure of over 30 mmHg or diastolic pressure over 15 mmHg was associated

with a 2 – 3-fold increase in risk of spontaneous preterm birth (Zhang et al 2007). These finding suggest a possible role of impaired endothelial function and adaptation to pregnancy in the pathology of preterm birth.

As in myometrial smooth muscle, vascular smooth muscle contractions can be stimulated by mechanical, electrical and chemical stimuli. Passive stretch brings about a myogenic response, membrane potential changes open VGCC allowing Ca^{2+} efflux raising $[Ca^{2+}]_i$ and facilitating the contractile mechanism. Chemical stimuli include norephinephrine, angiotensin II, vasopressin, endothelin-1 and thromboxane A_2, which all bind to specific receptors (Klabunde 2014). The end result in each case is a rise in $[Ca^{2+}]_i$ allowing for binding with calmodulin, activating MLCK, which in turn phosphorylates myosin light chains allowing for cross-bridge formation of the myosin head with actin filaments and contracting the cell (Klabunde 2014).

At the small artery level, vasoconstriction and vasodilatation is controlled by communication between VSMC and the endothelial layer, which in turn regulates vascular homeostasis. Bayliss (1902) first identified that regulation of arterial tone occurred independently of central nervous system involvement and was both peripheral and myogenic in nature, demonstrating an effect both *in vivo* and *in vitro*. Endothelial cells are in contact with blood, and blood borne components, and are responsive to signalling to bring about constriction and relaxation. The endothelium is considered an endocrine organ and is made up of a monolayer of endothelial cells which line the entire vascular system. Versatile and multifunctional, the endothelium regulates thrombosis and thrombolysis,

platelet adherence, modulation of vascular tone and blood flow, and regulation

of immune and inflammatory responses by controlling leucocyte monocyte and

lymphocyte interactions with the vessel wall (Sumpio et al 2002) (*Figure 1.9*).

Endothelial cells produce and release a variety of vasoactive substances such as

prostacyclin and nitric oxide (NO), which inhibit platelet aggregation and cause

vasodilation (Sumpio et al 2002). Endothelial cells from different locations, vessel

types and individuals can differ in response to stimuli. Endothelial cells are

exposed to a variety of microenvironments, when removed from native tissue for

growth in tissue culture they undergo phenotypic changes making it very difficult

to study cultured endothelial cells (Aird 2012).

Matrix Products
fibronectin
laminin
collagen I, II, III, IV, VIII, XVIII
proteoglycans
proteases

Antithrombotic factors
PGI$_2$
thrombomodulin
AT III
tPA
heparin sulfate

Procoagulant factors
vWF
TXA$_2$
thromboplastin
Factor V
PAF
PAI-1, PAI-2

Lipid Metabolism
LDL-receptor
lipoprotein lipase

Inflammatory Mediators
IL-1, 6, 8
LTB$_4$, C$_4$, D$_4$, E$_4$
MCP-1, MCP-2
MHC II
CAM

Vasomotor factors
-relaxing   -constricting
NO   ACE
PGI$_2$/E$_2$ TXA$_2$/F$_{2a}$
EDHF   EDCF
                   leukotrienes
                   free radicals
                   endothelin

Growth factors
PDGF
EDGF
FGF
IGF
TGF-b
GM-CSF
G-CSF

Figure 1.9: Known secretory/expression products of endothelial cells relating to

vessel physiology; from: Sumpio et al 2002

Endothelial-derived vasodilation is regulated by NO. The enzyme endothelial nitric oxide synthase (eNOS) generates NO from L-arginine and O_2 using nicotinamide adenine dinucleotide phosphate (NAPH$^+$). NO stimulates soluble guanylyl cyclase, increasing cyclic guanosine monophosphate (cGMP), which brings about relaxation of the VSMC (Förstermann & Münzel 2006). NO can also generate superoxide anions (O_2^-) in conditions of oxidative stress, this reacts with NO to form peroxynitrate and vascular protection diminishes (Förstermann & Münzel 2006). Peroxynitrate interacts with lipids, DNA and proteins via direct and indirect oxidative reactions resulting in oxidative injury, necrosis or apoptosis (Pacher et al 2007). L-arginine availability is important for eNOS production of NO. Arginase II is an enzyme involved in the urea cycle in which the body eliminates harmful amonia which also utilises L-arginine as a common substrate and so competes for availability. Lower levels of L-arginine are found in pre-eclamptic placenta compared to normotensive, with levels of arginase II higher (Noris et al 2004). This suggests raised arginase II in pre-eclampsia leads to a deficiency in available L-arginine impeding NO production. NO is important in placental angiogensis and regulation of vascular tone and maladaption of endothelial cells to pregnancy is a contributing factor to the development of pregnancy related conditions such as pre-eclampsia and IUGR. In animal studies the long-term inhibition of eNOS can elicit a pre-eclampsia type phenotype(Salas et al 1999).

Furchgott & Zawaszki (1980) found the vasodilator effect of acetylcholine is dependent on the presence of endothelium. They proposed that acetylcholine stimulated the release of unknown substance(s), which bring about a relaxation

of VSMC, and this factor still remains to be identified. This unknown form of communication was named as endothelial dependant hyperpolarisation factor (EDHF). There have been many theories relating to the nature of what EDHF may be. Adaptations of the NO pathway, endothelial-vascular gap junctions as well as Ca^{2+} signalling and K^+ channels have all been identified as possible candidates for EDHF or play a contributing role in EDHF. Although prostacyclin and NO were the earliest identified endothelium dependant vasodilation factors, it was found that vasodilation persisted with suppression of these factors and K^+ channels were proposed as having a role in EDHF (Taylor and Weston 1988). Possible mechanism includes a spatial increase in $[Ca^{2+}]_i$ which activates Ca^{2+} dependent K^+ channels, leading to K^+ efflux, and hyperpolarisation; or synthesis of a substance or generation of electrical signals which are capable of moving through membranes or myoendothelial gap junctions to influence VSMC (Luksha et al 2009). EDGF represents an additional layer of pathway to vasodilation. It has been proposed that EDHF may step in when the NO pathway is compromised (Luksha et al 2009) .It appears that the smaller the vessel , the larger the effect of EDHF (Taylor and Weston 1988). In human gastroepiploic arteries the role of EDHF is significantly greater in microvessels than in large arteries, with NO and EDHF having equal effect in large arteries and EDHF having most effect in microvessels (Urakami-Harasawa et al 1996). This may be because EDHF more easily diffuses to VSMC in microvessels due to less tissue density or perhaps increased myoendothelial gap junctions.

SK_3 and IK_3 have been identified as candidates for a role in EDHF and are located within endothelial cells and not in the VSMC (Luksha et al 2009), which is in

agreement with our own screen of VSMC's. While Ca^{2+} activated K^+ channels, and SK_3 in particular, are implicated as having a role in EDHF it is unclear how much this single protein contributes to the overall regulation of vascular tone (Luksah et al 2009, Lin et al 2012). While our initial screen differentiated channel expression between VSMC and MSMC the role these channels play in contributing to EDHF and therefore affect vascular tone is less clear and would require functional experiments to observe.

Riluzole, which has been used therapeutically and has been the subject of long term use in clinical trials does not have cardiovascular side effects within its profile (Bensimon et al 1994)

1.10 Inwardly rectifying potassium channel K_{ir} 7.1

A previous screen of K^+ channel expression in myometrium identified the inwardly rectifying potassium channel K_{ir} 7.1 as a channel of interest. An 8-fold increase in the gene encoding K_{ir} 7.1 KCNJ13 mRNA was seen in laser capture selected human myometrial smooth muscle compared to whole biopsy tissue at term. An increase in KCNJ13 mRNA expression was also observed in murine myometrium, which peaked at D15 predicting a functional increase in expression towards term *(Figure 1.10)*.

Inwardly rectifying K^+ channels demonstrate a greater flow into the cell rather than out as would be predicted by the Nernst equation and were previously described as anomalous rectifier K currents. The behaviour of these channels is clearly different from K_v channels and instead of voltage-dependant activation

they rely on an electrochemical gradient minus equilibrium potential (E_m-E_K) (Hibino *et al.*, 2010) *(Figure 1.11).*

Under correct physiological conditions K_{ir} channels generate large inward K^+ conductance at potentials negative to E_K and small outward currents when positive to E_K (Nakamura *et al.*, 1999). Cells that express a large K_{ir} conductance are expected to have a resting membrane potential close to E_K and generally have no spontaneous activity. The voltage independent nature of the channels allows K_{ir} to play a key role in setting and maintaining resting potential and action potential duration. This is demonstrated in vascular smooth muscle cells where K_{ir} 2 influences K^+ conductance at near resting potential modulated by hypoxia, hypo-osmotic stress and agonists. (Park *et al.*, 2008). The myometrial specific and gestational dependant increased expression of this channel observed by our group suggests K_{ir} 7.1 may be involved in gestational dependant depolarisation of resting membrane potential in myometrium towards term.

On studying K_{ir}7.1 unique pore properties were observed (Krapivinsky *et al.*, 1998). Widely expressed in the purjinke layer of the cerebellum and pyramidial cells of the hippocampus both cells that demonstrate electrical excitability and where K^+ channels play a role in the timing and duration of action potentials. A high expression was shown in kidney and small intestine but a low expression was found in the heart, placenta and ovary *(Figure 1.12).* In thyroid follicular cells K_{ir} 7.1 was found to co-localise with the Na^+K^+ATPase (Nakamura *et al.*, 1999). A low single channel conductance was suggested to support a

hypothetical functional coupling, with K_{ir} 7.1 providing K^+ recycling required for Na$^+$ pumping and maintaining resting membrane potential.

K_{ir} 7.1 was seen to be only 38% identical to its closest relative in the ROMK family K_{ir} 1.3. Among the unique features of K_{ir} 7.1 is its low estimated single channel conductance ~50fS and a low sensitivity to Ba^{2+}, Cs and Mg^{2+} block which is characteristic of ROMK. The amino acids in the pore sequence differ from all other K_{ir} channel proteins. The channel consists of 360 amino acids with 95% identity rat- human. The amino acid sequence of the pore region reveals 3 differences in conserved locations when compared to homologous sequences of other K_{ir} subunits. Novel sequences at Ser-111, Met-125 and Gly-129 appear to be responsible for the channels unique properties and its voltage independent nature(Krapivinsky *et al.*, 1998) *(Figure 1.13)*.

The specific function of Kir 7.1 has not been previously studied in myometrium. Study in other tissues was impeded by a lack of a specific pharmacological blocker. The small molecule intracellular blocker of Kir 7.1 VU590 has been described by investigators searching for inhibitors of ROMK (Lewis *et al.*, 2009) (*Figure 1.14*). They showed that at 10µM VU590 would elicit a 60% inhibition of K_{ir} 7.1 current at 120mV. The availability of a pharmacological blocker provided an opportunity to investigate the functional contribution of K_{ir} 7.1 to myometrial contractility. Predicted enhanced contractility may be of clinical interest in the development of therapies for PPH.
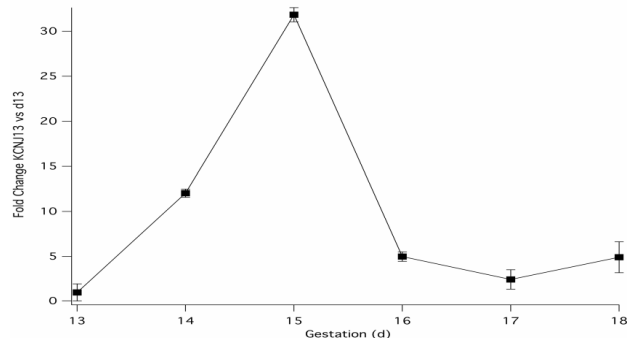
Figure 1.10: Fold change in mRNA expression of KCNJ13 in cDNA generated from laser captured mouse myometrial smooth muscle mRNA. Individual points denote mean±SE for 5 mice.
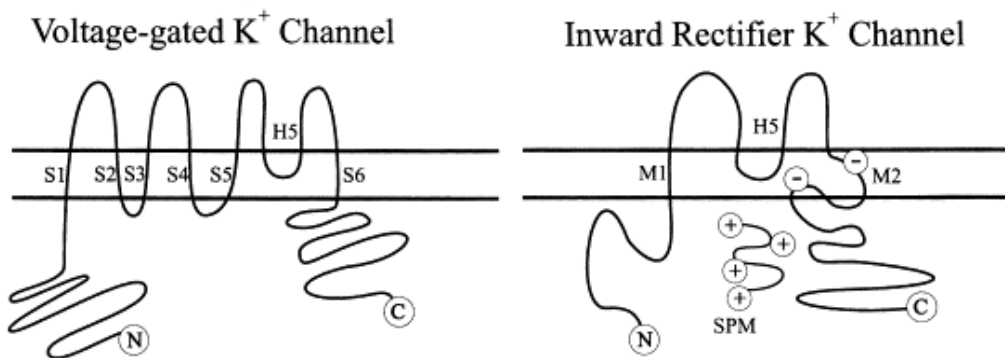
Figure 1.11: Topology of voltage gated and inward rectifier K$^+$ channels. K$_{ir}$ channels have 2 hydrophobic membrane spanning domains whereas K$_V$ channels have 6. From (Brown, 1997)
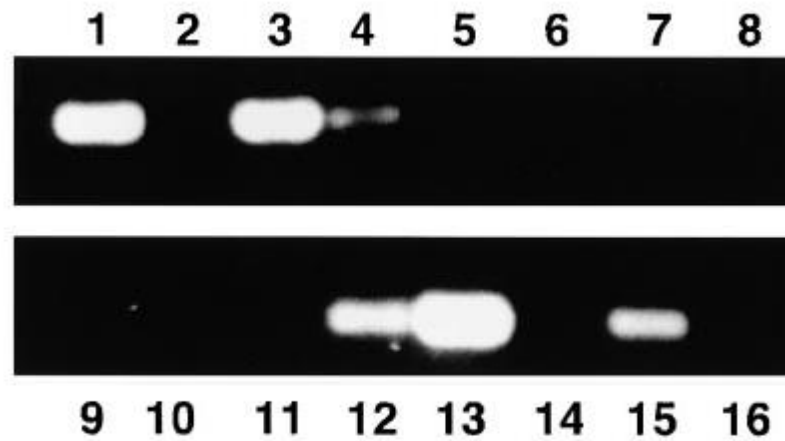
Figure 1.12: Human multiple tissue cDNA panel PCR screening demonstrates expression of Kir7.1 in kidney, brain, and intestine and to a lesser extent in testis, liver, and prostate: (1) brain, (2) heart, (3) kidney, (4) liver, (5) lung, (6) pancreas, (7) placenta, (8) skeletal muscle, (9) colon, (10) ovary, (11) peripheral blood leucocyte, (12) prostate, (13) small intestine, (14) spleen, (15) testis, and (16) thymus. From (Krapivinsky et al., 1998)

```
hKir7.1                                    MDSSNCKVIAPLLSQRYR---RMVTKDGHSTLQMDG  33
hKir1.2                            MTSVAKVYYSQTTQTESRPLMGPGIRRR---RVLTKDGRSNVRMEH  43
hKir2.1     MGSVRTNRYSIVSSEEDGMKLATMAVANGFGNGKSKVHTRQQCRSRFVKRDGHCNVQFIN  60
hKir3.3                   MAQENAAFSPGQEE-PPRRRGRQRYVERDGRCNVQQGN  37
rKir5.1         MSYYGSSYRIVNVDSKYPGYPPEHA-IAEKRRARRRLLHKDGSCNVYFKH  49
hKir6.2         MLSRKGIIPEEYVLTRLAEDPAEPRYRARQR-RARFVSKKGNCNVAHKN  48

                                                    ┌──────── M1 ────────┐
hKir7.1  -AQRGLAYLRDAWGTLMDMRWRWMMLVFSASFVVHWLVFAVLWYVLAEMNGDLELDHDAP  92
hKir1.2  IADKRFLYLKDLWTTFIDMQWRYKLLLFSATFAGTWFLFGVVWYLVAVAHGDLLELDPELD-E  101
hKir2.1  VGEKGQRYLADIFTTCVDIRWRWMLVIFCLAFVLSWLFFGCVFWLIALLHGDLDASKEG-  119
hKir3.3  VRET-YRYLTDLFTTLVDLQWRLSLLFFVLAYALTWLFFGAIWWLIAYGRGDLEHLEDTA  96
rKir5.1  IFGEWGSYMVDIFTTLVDTKWRHMFVVFSLSYILSWLIFGSIFWLIALHHGDLLSSPDIT  109
hKir6.2  IREQG-RFLQDVFTTLVDLKWPHTLLIFTMSFLCSWLLFAMAWWLIAFAHGDLAPSEGTA  107

                                ┌─── H5 ───┐              ┌──────── M2 ────────┐
hKir7.1  PENIITICVKYITSFTAAFSFSLETQLTIGYGTMFPSGDCPSAIALLAIQMLLGLMLEAFI  152
hKir1.2  EANHTPCVVQVHTLTGATLFSLFSQTTIGYGFRYISEECPLAIVLLIAQLVLTTILEIFI  161
hKir2.1  ----KACVSEVNSFTAAFLFSIETQTTIGYGFRCVTDECPIAVFMVVFQSIVGCIIDAFI  175
hKir3.3  ---WTPCVNNLNGFVAAFLFSIETETTIGYGHRVITDQCPEGIVLLLLQAILGSMVNAFM  153
rKir5.1  -----PCVDNVHSFTAAFLFSLETQTTIGYGYRCVTEECSVAVLTVILQSILSCIINTFI  164
hKir6.2  ----EPCVTSIHSFSSAFLFSIEVQVTIGFGGRMVTEECPLAILSLIVQNIVGLMINAIM  163

hKir7.1  TGAFVAKIARPKNRAFSLRHTDTAVVAHMDGKPNLIFQVANTRESPLTSVRVSAVLYQER  212
hKir1.2  TGIFLAKIARPKKRAETLRFSQHAVVASHNGKPCLMIRVANMRKSLLIGCQVTGKLLQTH  221
hKir2.1  IGAVMAKMAKPKKRNETLVFSHNAVIAMRDGKLCLMWRVGNLRKSHLVEAHVRAQLLKSR  235
hKir3.3  VGCMFVKISQPNKRAATLVFSSHAVVSLRDGRLCLMFRVGDLRSSHIVEASIRAKLIRSR  213
rKir5.1  IGAALAKMATARKRAQTLRFSYFALIGMRDGKLCLMWRIGDFRENHVVEGTVRAQLLRYS  224
hKir6.2  LGCIFMKTAQAHRRAETLIFSKHAVIALRHGRLCFMLRVGDLRKSMIISATIHMQVVRKT  223

hKir7.1  -----ENGKLYQTSVDFHLDGISSDEQPFFIFPLTYYHSITPSSPIATLQHE--NPSHP  265
hKir1.2  QTKEGENIRLNQVNVTFQVD--TASDSPFLILPLTFYHVVDETSPLKDLPLRS--GEGDF  277
hKir2.1  ITSEGEYIPLDQIDINVGFD--SGIDRIFLVSPITIVHEIDEDSPLYDLSKQDIDN-ADF  292
hKir3.3  QTLEGEFIPLHQTDLSVGFD--TGDDRLFLVSPLVISHEIDAASPFWEASRRALER-DDF  270
rKir5.1  EDSEGR-MTMAFKDLKLV------NDQIILVTPVTIVHEIDHESPLYALDRKAVAK-DNF  276
hKir6.2  TSPEGEVVPLHQVDIPMENG--VGGNSIFLVAPLIIYHVIDANSPLYDLAPSDLHHHQDL  281

hKir7.1  ELVVFLSAMQRGTGEICCRRTSYLPSEIMLHHCFASLLTRGSKGEYQIKMENFDKTVP-E  324
hKir1.2  ELVLILSGTVESTSATCQVRTSYLPEEILWGYEFTPAISLSASGKYIADFSLFDQVVKVA  337
hKir2.1  EIVVILEGMVEATAMTTQCRSSYLANEILWGHRYEPVLFEE-KHYYKVDYSRFHKTYEVP  351
hKir3.3  EIVVILEGMVEATCMTQQARSSYLVDEVLWGHRFTSVLTLE-DGFYEVDYASFHETFEVP  329
rKir5.1  RILVTFIYTGDSTGTSHQSRSSYVEREILWGHRFHDVLEVK-RKYYKVNCLQPEGSVEV-  334
hKir6.2  EIIVILEGVVETTGITTQARTSYIADEILWGQRFVPIVAEE-DGRYSVDYSKFGNTIKVP  343

hKir7.1  -FPT----PLVSKS-----PNRTDLDIHINGQSI-DNFQISETGLTE            360
hKir1.2  -SPS----GLRDSTVRYGDPEKLKLEESLREQAEKEGSALSV-----------------  374
hKir2.1  NTPLCSARDLAEKKYILSNANSFCYENEVALTSKEEDDSENGVPESTSTDTPPDIDLHNQ  411
hKir3.3  -TPSCSARELAEAAARLDAH--LYWSIPSRLDEKVEEE---GVGEGAGGEAGADKE--QN  381
rKir5.1  YAPFCSAKQLDWKDQQLNNL----------------EKTSPAPRILHLGHQHQEEILQR  377
hKir6.2  -TPLCTARQLDEDHSLLEA---------LTLASARGPLRKRSVPMAKAKPKFSISPDSLS  390


hKir1.2  ----------RISNV  379
hKir2.1  ASVPLEPRPLRRESEI  427
hKir3.3  GCLP----PPESESKV  393
rKir5.1  SCHGEQL          384
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Figure 1.13: Comparison of the amino acid sequences of K$_{ir}$7.1 with representative subunits of other K$_{ir}$ subfamilies. The predicted 360 amino acids of human K$_{ir}$7.1 (single-letter code) are shown aligned with sequences of human K$_{ir}$1.2 (GenBank accession number U73192), human K$_{ir}$2.1 (U12507), human K$_{ir}$3.3 (U52152), rat K$_{ir}$5.1 (X83581), and human K$_{ir}$6.2 (D50582). Residues are shaded in black in instances in which other subunits are identical to K$_{ir}$ 7.1; asterisks denote residues conserved in all known K$_{ir}$ channels, and boxed residues (arrowheads) indicate where K$_{ir}$7.1 is different from the consensus of all other K$_{ir}$ channels. Outlined also are the transmembrane segments M1 and M2 and the pore-forming P-region (H5). Amino acid gaps within the alignment are indicated by short bars. The GenBank accession number for the human and rat K$_{ir}$7.1 sequences are AJ006128 and AJ006129, respectively. From (Döring et al., 1998)
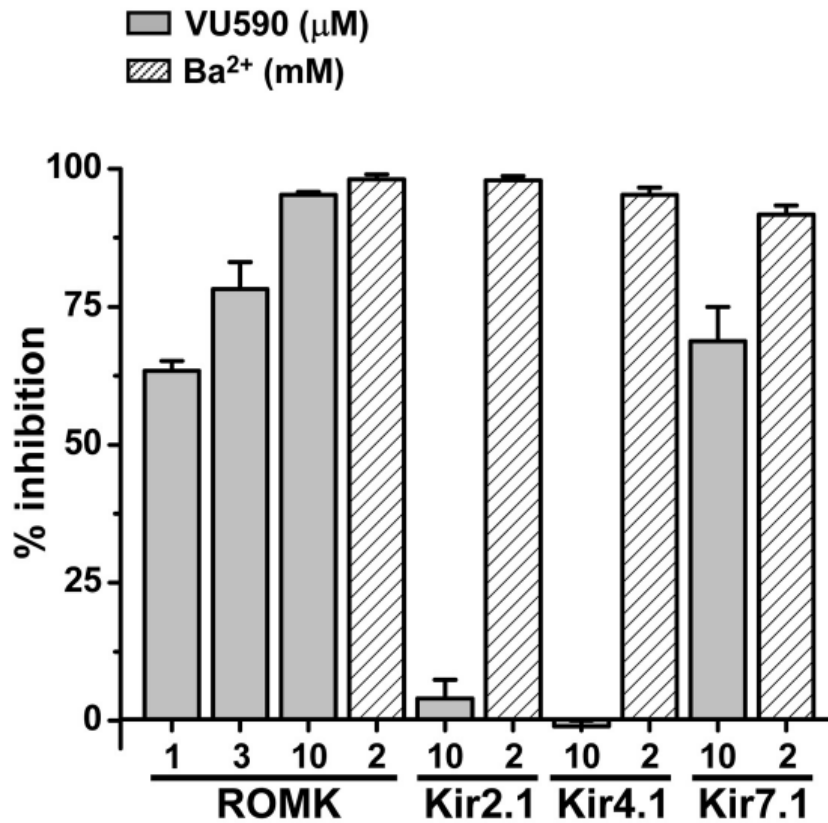
Figure 1.14: Inhibition of K⁺ channels with novel small molecule intracellular channel blocker VU590. A 60% inhibition of K_{ir} 7.1 current at 120mV is seen with 10 μM VU590 From: Lewis et al 2009.

1.12 Summary

In a previous screen carried out within our group a number of K⁺ channels were identified of interest due to the level of expression, gestational dependent nature of expression or relative abundance when compared with vascular smooth muscle. This has led to the prospect of further examination of the function of these ion channels in myometrium with a view their potential as therapeutic targets. Crucially, in the contractility pathway, modulation of K⁺

channel function has the potential to significantly impact on myometrial membrane resting potential and action potential, directly modifying contractility downstream of chemical stimuli or signalling (*Figure 1.15*).

1.11.1 *K^+ channel openers combined with Nifedipine as potential targets for tocolysis*

The problem of preterm labour has not diminished in recent years. There is a choice of tocolytic drugs available to clinicians for the treatment of preterm labour however none have demonstrated improved neonatal outcomes and efficacy is limited. Nifedipine is a widely chosen tocolytic due to a relatively good safety profile at tentative doses but full tocolytic effect cannot be elicited without inducing maternal hypotension. The use of K^+ channel openers in combination with Nifedipine in myometrium may be a way to enhance the effect of Nifedipine as a tocolytic by modulating membrane potential and preventing opening of VGCC (*Figure 1.15b*). Most importantly, in order to avoid exacerbation of the associated side effect of hypotension K^+ channels targeted should show preferential expression and function in myometrial tissue over vascular smooth muscle. Selective targeting of myometrial specific K^+ channels by specific K^+ channel openers, in combination with Nifedipine, could provide a useful clinical tocolytic therapy. The first part of this thesis includes experiments aimed at confirming this theoretical possibility by examining the effects of select K^+ channel openers, and thus K^+ channel function; together with Nifedipine in human myometrium and myometrial small arteries.

1.12.2 K$^+$ channel blockers as potential therapeutic target for PPH

PPH also continues to be an obstetric problem that threatens morbidity and mortality of women globally. Current prevention and management strategies rely heavily on the use of Oxytocin, which in the case of uterine atony is not always effective often leading to a further cascade of mechanical and chemical rescue measures. Morbidity and mortality can heavily depend on maternal location and level of skill and resources available. A uterotonic that would provide reliable contractile force would be of great benefit. The K$^+$ channel K$_{ir}$ 7.1 was identified as being expressed in myometrial smooth muscle. Theoretically it is possible that this channel contributes to maintaining resting membrane potential (*Figure 1.15c*). The action of K$_{ir}$ 7.1 has not previously been studied in myometrium, but this is now possible due to the availability of the selective K$_{ir}$ 7.1 blocker, VU590. The second part of this thesis aims to examine the effects of VU590, and thus the function of K$_{ir}$ 7.1 in murine and human myometrium with a view to a therapeutic potential for PPH.
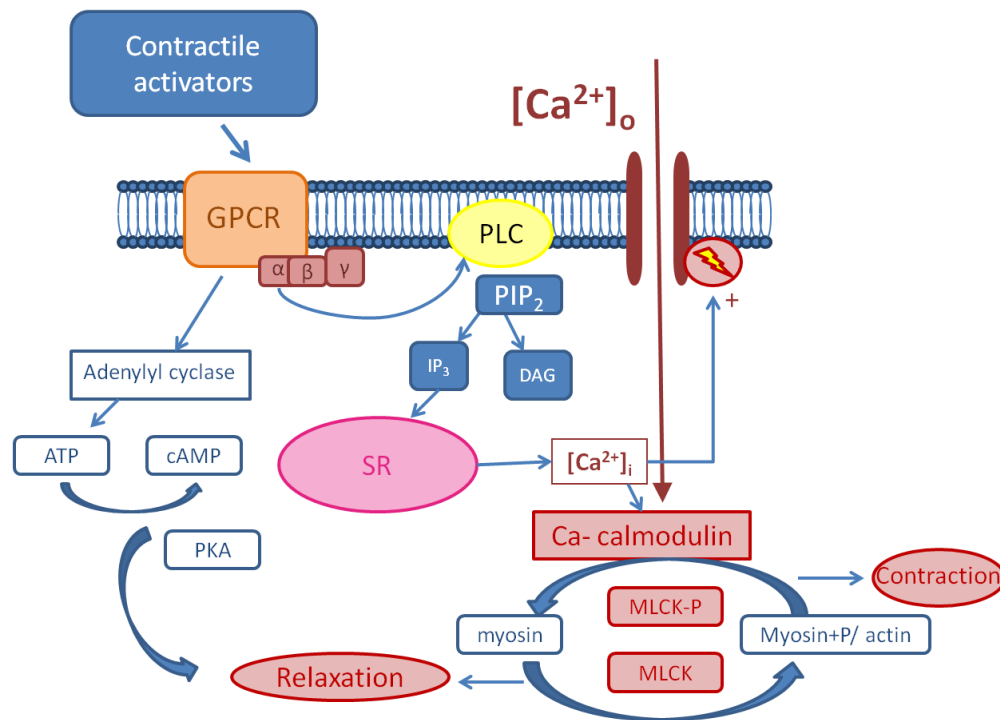
*Figure 1.15a: Exogenous activators of contractile action (such as Oxytocin, PGF$_2$α)
provide exogenous activation of the G-protein coupled receptors (GPCR) on the
cell membrane. Intracellular signalling of the alpha subunit Gαs activates
phospholipase C (PLC) which cleaves the phospholipid phosphatidylinositol 4,5-
biphosphate (PIP$_2$) into diacyl glycerol (DAG) and inositol 1, 4, 5-triphopshate
(IP$_3$). IP$_3$ activates calcium channels located in the sacroplasmic reticulum (SR), to
release Ca^{2+} from stores into the cytoplasm. This increases the internal voltage of
the cell, depolarising the cell membrane, allowing voltage-activated gating of the
L-type calcium channel and Ca^{2+} entry. [Ca^{2+}]$_i$ binds to calmodulin and the Ca-
calmodulin complex binds to myosin light chain kinase and phosphorylates
myosin light chains. This allows binding with actin and ATP dependent cross-
bridge cycling, leading to contraction of the smooth muscle cell. This continues as
long as [Ca^{2+}]$_i$ is available.*
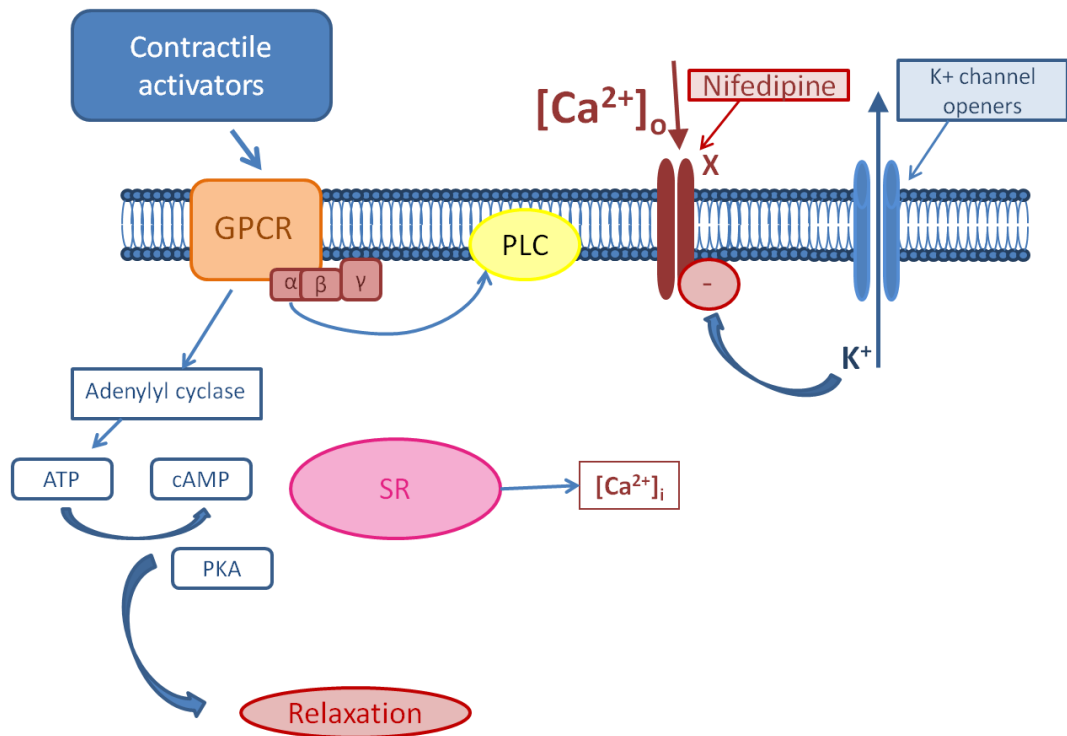
Figure 15.1b: Application the Ca^{2+} channel blocker Nifedipine provides an external block L-type voltage gated calcium channel (LVGCC), reducing Ca^{2+} influx via a chemical block. The hypothesis is that application of K^+ channel openers will allow K^+ efflux from the myometrial smooth muscle cell, maintaining a polarised state within the cell which prevents internal voltage-activation of the LVGCC, further suppressing the ability of Ca^{2+} and thus reducing the ability of the cell to contract. Concerns about maternal side-effects limits the dose of Nifedipine that can be safely administered as a tocolytic in current obstetric practice. The addition of a K^+ channel opener that targets a myometrial specific K^+ channel, could increase the potency of the effect of Nifedipine.
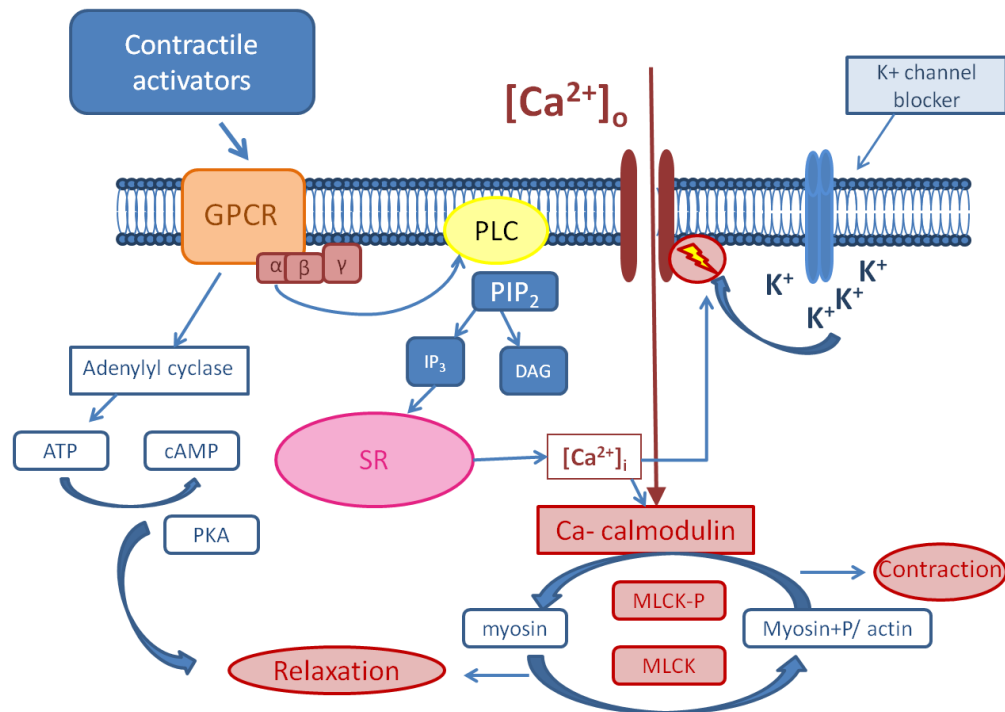
Figure 15.1c: The compound VU590 has been shown to inhibit the inwardly rectifying K^+ channel K_{ir} 7.1 in other tissues but has not previously been studied in myometrium. The hypothesised effect of a chemical block of K_{ir} 7.1 in myometrial smooth muscle cells will lead to an accumulation of $[K^+]_i$ and sustained depolarisation. This will allow continued voltage-gated activation of the VGLCC and sustained Ca^{2+} entry, this enhancing the action potential and increasing contractility.

2. Materials and Methods

2.1 Contractility

2.1.1 General laboratory reagents

Krebs TES: NaCl 125.72 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM, D^+-glucose 10 mM, KCL 4.68 mM, $NaHCO_3$ 4.16 mM, sucrose 2.9 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.5 mM, KH_2PO_4 0.44 mM, $MgSO_4$ 0.4 mM and K_2HPO_4 0.34 mM, pH 7.4

Krebs-Henseleit solution (KHS): NaCl 118 mM, $NaHCO_3$ 25 mM, KCL 4.8 mM, KH_2PO_4 1.2 mM $MgSO_4$ 1.2 mM, glucose 11.1 mM and $CaCl_2$ 1.25 mM, pH 7.4

2.1.2 Reagents and drugs

Potassium channel openers, Dimethyl-sulphoxide (DMSO) and Nifedipine were obtained from Sigma Aldrich Chemical Co, MO, USA. Riluzole and Nifedipine were dissolved in DMSO, pinacidil in 0.1 M HCL and linoleic acid in 100% methanol and stored in aliquots at -20°C. Dosing concentrations were prepared by serial dilution from stock solution with fresh KHS immediately prior to addition to the organ bath (*Table 2.1*).

Riluzole	DMSO	Pinacidil	HCL (0.1M)	Linoleic Acid	Methanol	Nifedipine	DMSO
0.3 µM	0.0001%	0.3 µM	1 µM	3 µM	0.001%	3nM	0.003%
1 µM	0.0003%	1 µM	3.3 µM	10 µM	0.003%	10nM	0.01%
3 µM	0.001%	3 µM	10 µM	30 µM	0.01%	30nM	0.03%
10 µM	0.003%	10 µM	33.3 µM	100 µM	0.03%	100nM	0.1%
30 µM	0.01%	30 µM	0.1 µM	300 µM	1%		

Table 2.1: Table showing the doses of each drug used and their volume equivalent dilution in final concentration when added to 10 mls organ bath as vehicle controls.

2.1.3 Isolation of tissue

2.1.3.1 Mouse

Experiments were carried out on whole tissue from day 15 (D15) and 18 (D18) B6 CB F1 pregnant mice, killed by CO_2 asphyxiation at the University of Warwick. The uterus was removed, stored in ice cold Krebs–Henseleit (KHS) solution and transported to the Clinical Sciences Research Laboratories where it was dissected within 4 hours. Strips of myometrium from the longitudinal layer (2 x 2 x 20 mm) were dissected and used for isometric force recording as per the following description (2.1.4 *Measurement isometric tension*).

2.1.3.2 Human

Myometrial biopsies were obtained at elective caesarean section from women prior to the onset of labour. Biopsies were taken from the lower edge of the upper part of the incision, placed in Krebs TES solution, stored at 4˚C and used within 24 hours.

All participants were singleton pregnancies at term; defined as pregnancy at 37-41 completed weeks (*see Figure 2.1 & 2.2*). Local ethical committee approval was in place for the collection of myometrial biopsies and informed written consent was obtained from each participant (*see appendix*). Women were eligible for elective caesarean surgery due to breech presentation, previous caesarean section, low-lying placenta or unstable lie. There was one participant with gestational diabetes and all others had no significant medical conditions.
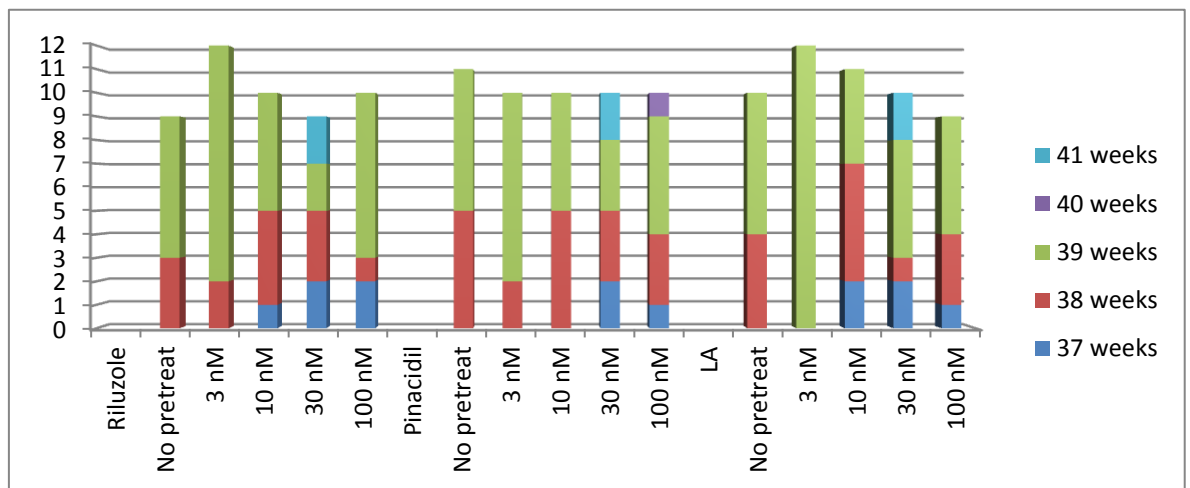
Figure 2.1: The distribution of gestation age of participants for dose response experiments. All participants were term; as defined by 37-41 completed weeks of pregnancy. The same biopsy was used for more than one experiment and so there is replication within the groups.
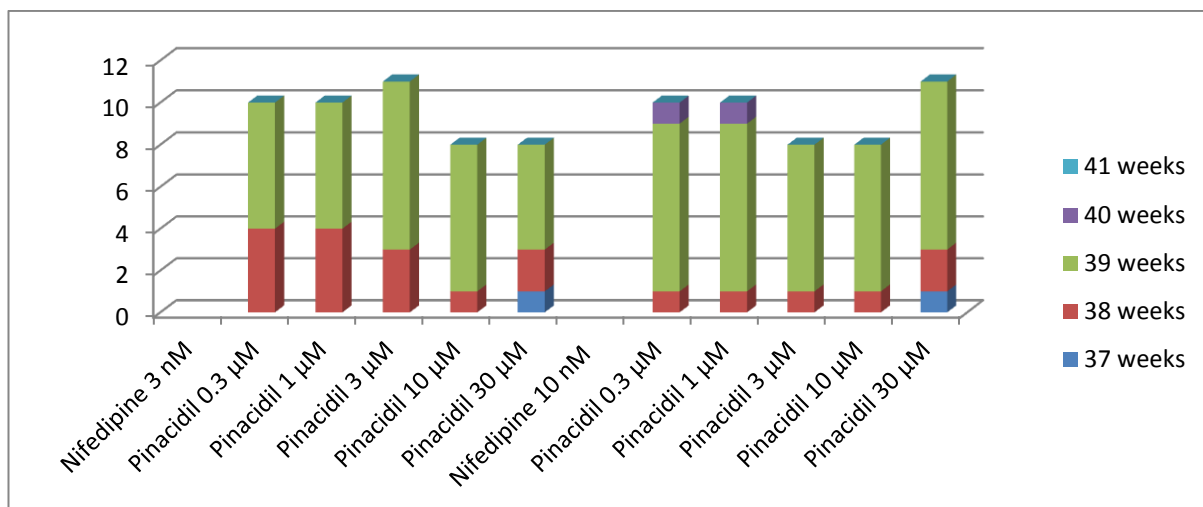
Figure 2.2: The distribution of gestation age of participants for combined dose experiments. All participants were term; as defined by 37-41 completed weeks of pregnancy. The same biopsy was used for more than one experiment and so there is replication within the groups

2.1.4 Measurement of isometric tension

Strips of myometrium (approx 10 x 2 x 2 mm) were mounted in 10ml organ bath chambers containing KHS at 37°C, perfused with 95% O_2/5% CO_2 to maintain pH 7.4, and under 2mN tension (LSI Letica automated organ bath, AD Instruments Ltd, Oxfordshire, UK) *(Figure 2.3)*. Isometric force was measured with ML TO201/D transducers and recorded digitally with PowerLab Chart software (AD Instruments Ltd, Oxfordshire, UK). Strips that failed to contract spontaneously within a 120 minute equilibration period were excluded. Approximately 1:10 strips would fail to contract. Baseline activity was recorded for 30 minutes before the addition of study drug. For dose response experiments drugs were added in a cumulative manner at

20 minute intervals following addition of Nifedipine (3nM, 10nM, 30nM, 100nM) whereas in combined dosing contractile activity was recorded for 40 min following dosing. Simultaneous recordings were made from vehicle-exposed strips taken from the same biopsy.
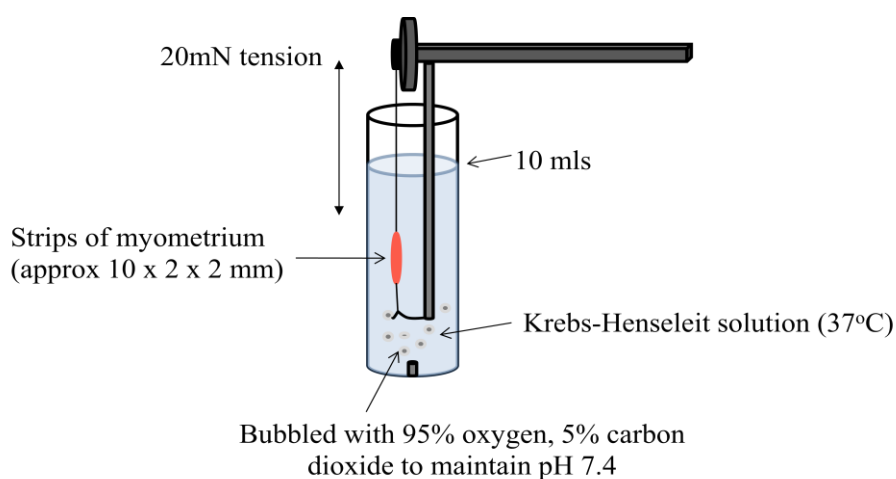
Figure 2.3: Diagramatic representation of tissue strip mounted in organ bath

2.1.5 Data analysis & Statistics

Each contractile phase was recorded using LabChart software and the following parameters were measured to quantify the phase plot of each contraction (*Figure 2.4*).
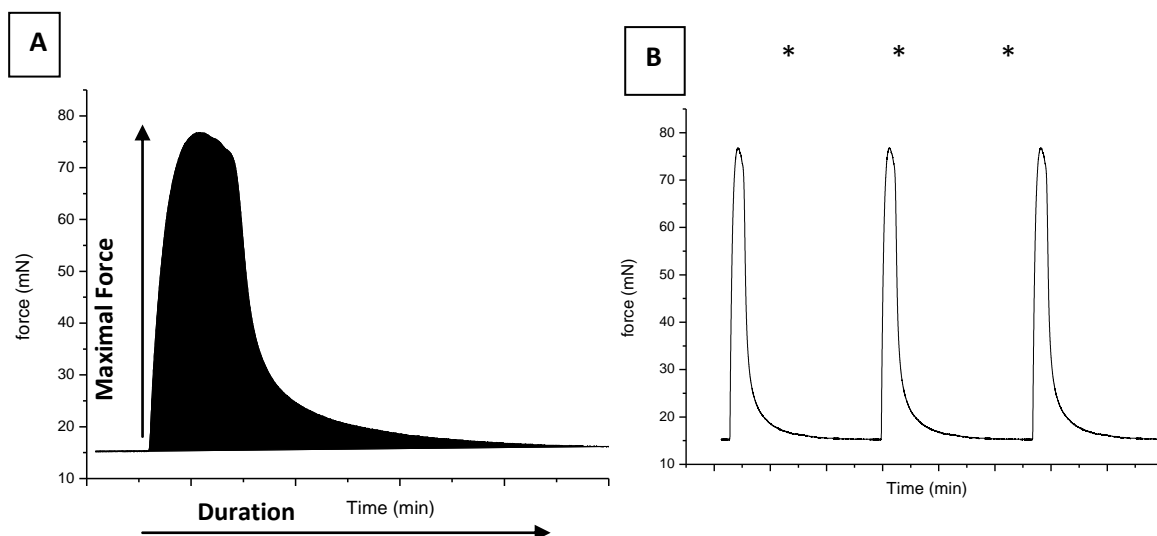
Figure 2.4: Parameters of each contraction phase plot used for analysis of contractility. A. Represents maximal force and duration, with activity integral representing the area under the curve. B. Represents frequency of contractions observed over a 20 min period.

Means of these parameters for pre-dose contractions (over 20 minutes) and contractions following each dose (per 20 min period) were calculated for both treated and paired vehicle control strips.

2.1.5.1 Dose response experiments:

Percentage inhibition from pre-dose period was compared to control for each dose period. Data was tested for normal distribution by Shapiro-Wilk normality test. Data did not fit a normal distribution and so the non-parametric Wilcoxon Signed rank test was selected for sample group comparison. Activity integral data was fitted to dose response curves for calculation of IC_{50}.

2.1.5.2 Combined dosing:

Data was analysed as described above. Percentage inhibition from pre-dose period was compared to control for each matched dose. Data was tested for normal distribution by Shapiro-Wilk normality test. All data did not fit a normal distribution and so the non parametric Wilcoxon Signed Rank test was selected for sample group comparison. There appeared to be a time dependent effect of riluzole combined with Nifedipine and so data at doses with significant results was further analysed in 4 separate 10 min periods from dosing. As the desired clinical outcome would be an immediate cessation of contractions combined dosing data was also tested for binomial distribution. A sign test with cessation of contractions within 40 minutes following dosing was considered a successful outcome verses continuation of contractions.

2.2 Electrophoresis

2.2.1 General Laboratory Reagents

RIPA Lysis buffer: (10x) 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA (Millipore, MA USA)

Complete, Mini, EDTA-free Protease Inhibitor Cocktail tablets: (Roche)

Laemmli Sample Buffer: 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue

Bio-Rad DC Protein Assay Kit: used as per manufacturer's instructions(Bio-Rad CA, USA)

Transfer buffer (1 litre): Glycine 2.93g, Tris 5.81g, SDS 0.375g, methanol 200 ml, ddH$_2$O 800ml. Solution made in the lab. Powder ingredients from Sigma Aldrich

10x Tris Buffered Saline (TBS): Tris HCL 24.2g (pH 7.6), NaCl 80g, ddH$_2$O 1L Solution made in the lab

Tris Buffered Solution-Tween (TBS-T): TBS x10 100 ml, ddH$_2$O 900 ml, Tween-20 1ml Solution made in the lab Tween-20 from Sigma Aldrich

Blocking buffer: TBS-T 100 ml, Marvel 5g (5%)

Primary antibody dilution buffer: TBS-T 20 ml, 1g Bovine Serum Albumin (BSA) (5%)

2.2.2. Isolation of tissue and sample preparation

Myometrial tissue samples were flash frozen in liquid nitrogen on retrieval from caesarean section and stored at -80 until use. Samples were selected that fell into 4 equal groups, pre-term labour, pre-term non-labour, term labour and term non-labour *(Table 2.2)*. Samples were kept frozen, weighed and crushed. Tissue was suspended in RIPA lysis buffer (RIPA Lysis buffer (10x) 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA, Millipore UK) containing protease inhibitor cocktail tablets (Complete, Mini, EDTA-free Protease Inhibitor Cocktail tablets, Roche) at a ratio of 3 mls per 1 g of tissue. Lysate was mechanically

homogenised and allowed to settle on ice, centrifuged at 12,000g for 15 mins at 4°C. The supernatant was aliquoted and frozen and the pellet discarded.

2.2.3 Protein quantification

Lysate protein quantification was performed by creating a range of protein standards. These were quantified by using the colorimetric Bio-Rad DC protein assay kits as per manufacturer's instructions.

2.2.4 Sample loading

An initial set of experiments was undertaken using a range of μg/μl loading of samples prepared in either reducing or non-reducing buffer to optimise results. Following this the appropriate volume of each sample to achieve 20 μg loading was added to 35μl of Laemmli buffer. Some antibodies required 'boiling' prior to electrophoresis at 95°C for 5 mins. Other proteins were found to aggregate at high temperatures and therefore did not separate during electrophoresis. For these experiments samples were incubated at 37°C for 30 mins prior to loading in the gels *(Table 2.3)*. All samples were settled on ice following heating and centrifuged at 13,000 rpm for 5 mins prior to loading.

2.2.5 Protein separation by SDS-PAGE

Novex© Tris-Glycine polyacrylamide gels from invitrogen were used in Invitrogen NuPage system tanks. Gels were run for 90-120 mins at 125V (constant) with Novex© Tris-glycine SDS running buffer (Novex©). Protein migration and transfer

was monitored with the use of Spectra Multicolor broad range protein ladder (Thermo Scientific, MA, USA).

Proteins were transferred onto Amersham ECL nitrocellulose membrane using Bio-Rad Trans Blot SD semi-dry blotting system. Transfer was achieved at 100mV for 50 mins. Successful transfer was assessed by staining using Poncaeu S and once visualised washed with 0.1nM NaOH prior to blocking.

Blocking of non-specific binding was achieved by washing in TBS-T blocking buffer solution for minimum of 4 hours at room temperature or overnight at 4°C. Following blocking, membranes were washed in TBS-T 3 times at 5 mins each wash prior to incubation with primary antibody.

2.2.6 Primary, Secondary antibodies and loading control

Incubation with primary antibody was at the appropriate dilution *(Tables 2.3 & 2.4)* in primary antibody dilution buffer for minimum of 2 hours at room temperature or overnight at 4°C. Membranes were then washed 3 times for 5 mins each wash prior to incubation with secondary antibodies. Secondary antibody used was Dako polyclonal goat anti-rabbit HRP antibody at a dilution of 1:500 and was incubated in TBS-T blocking buffer for 1 hour. Membranes were washed for 1 hour (10min, 20 min, 30 min wash) prior to electro-chemiluminescence.

Following electro-chemiluminescence membranes were washed in TBS-T overnight at 4°C. They were then exposed to anti-β actin antibody at a dilution of 1:50,000 for 1 hour before being washed in TBT-T 3 times for 5 mins each wash and then

incubated in anti- mouse secondary antibody for 30 mins, washed in TBS-T for 1 hour before electrochemiluminescence.

2.2.7 Electrochemiluminescence detection

Membranes were incubated for 5 mins in SuperSignal West Pico Substrate (Thermo Scientific, MA, USA) for detection of horseradish peroxidase (HRP). Membranes were then exposed to Kodak medical x-ray film (general purpose, blue) until bands were visible. Size approximation was made on films from ladder visible on the membrane.

2.2.8 Data analysis and statistics

Film images were scanned and band density was measured using Image J software and the analyze gels function for measurement of density of bands in individual lanes. The density of each lane is plotted and area under the curve measured. Ratio of value of band from antibody of interest was calculated against β-actin band value. Ratios from each sample group were statistically compared using ANOVA for significant difference.

Group	R number	Gest age	Indication for LSCS
PTL	R448	35	EMLSCS for premature labour in Twins
PTL	R385	35+3	EMLSCS previous LSCS plus Group B Strep infection
PTL	R611	35+3	EMLSCS Antepartum Haemorrhage
PTL	R525	35	EMLSCS failed induction for IUGR
PTL	R494	36+3	EMLSCS for Pre-eclampsia
PTL	R940	34	EMLSCS for fetal distress
PTNL	R456	35	EMLSCS for Pre-eclampsia (2x prev LSCS)
PTNL	R1010	34	ELLSCS for Twins plus hypertension
PTNL	R766	33+6	EMLSCS for IUGR (1 x prev LSCS)
PTNL	R529	29	EMLSCS for Antepartum Haemorrhage
PTNL	R582	34	EMLSCS for maternal hypertension
PTNL	R898	29	EMLSCS for Antepartum Haemorrhage
TL	R829	40+1	EMLSCS failure to progress in 1st stage
TL	R1032	40	EMLSCS failure to progress in 1st stage
TL	R814	39	EMLSCS failure to progress in 1st stage
TL	R858	40	EMLSCS failure to progress in 1st stage
TL	R886	40+3	EMLSCS sub optimal CTG plus poor blood gases
TL	R1017	40	EMLSCS failure to progress in 1st stage plus fetal distress

TNL	R971	41	ELLSCS breech presentation
TNL	R999	39	ELLSCS 1x previous LSCS
TNL	R981	39	ELLSCS breech
TNL	R1013	39	ELLSCS maternal cerebral palsy
TNL	R990	39+5	ELLSCS breech
TNL	R1302	39	ELLSCS 1 x previous LSCS

Table 2.2: Characteristics of frozen samples used in western blotting protocol. Split into groups of interest – Pre-Term Labour (PTL), Pre-Term non-Labour (PTNL), Term Labour (TL), Term non-Labour (TNL).

Antibody	dilution	kDa of protein	Sample preparation	Gel
Anti - Kv 2.1	1:200	96	37°C for 30 mins	8% gel
Anti - Kir 7.1	1:200	55	95°C for 5 mins	8% gel
Anti – SK 3	1:500	75	37°C for 30 mins	8% gel
Anti – Trek 1	1:250	55	95°C for 5 mins	8% gel

Table 2.3: preparation of samples, dilutions and gels used for each antibody plus expected kDa of proteins in western blotting protocol.

Antibody	Raised in	gene	Binding site	Sourced from
Anti - Kv 2.1	Rabbit polyclonal	KCNB1	Amino acid residues 755-804	Abcam PLC
Anti $K_{2P}2.1$ (TREK-1)	Rabbit polyclonal	KCNK2	Amino acid residues 8 -25	Alomone Labs UK
Anti – $K_{Ca}2.3$ (SK3)	Rabbit polyclonal	KCNN3	Amino acid residues 2 -21	Alomone Labs UK
Anti- K_{ir} 7.1	Rabbit polyclonal	KCNJ13	Amino acid residues 80-94 (rat, 13/15 residues identical in human)	Alomone Labs UK

Table 2.4: Details of antibodies used in western blotting protocol

2.5 Wire myography

Experiments on myometrial small arteries were performed at the Institute of Cellular Medicine' department of Reproductive and Vascular Research at Newcastle University under the supervision of Professor Mike Taggart and Dr Michele Sweeney.

2.5.1 General Laboratory Reagents

Physiological salt solution (PSS): NaCl 127 mM, KCl 4.7 mM, $MgSO_4.7H_2O$ 2.4 mM, $NaHCO_2$ 25 mM, KH_2PO_4 1.18 mM, EDTA 0.07 mM, Glucose 6.05 mM, $CaCl_2.2H_2O$ 1.6 at pH 7.4 gassed with 5% CO_2 in air

Physiological Salt solution – high potassium (KPSS): PSS plus 60mM KCL

2.5.2 Isolation of tissue

Myometrial biopsies were obtained with consent from women undergoing elective cesarean section at term (39-41 weeks gestation) and stored in ice-cold KHS. Small diameter arterial branches (<300 μM) were dissected from the surrounding connective tissue from the whole biopsy and placed in ice cold PSS. Artery branches were trimmed to approx. 2-3mm in length as measured using a calibrated eyepiece micrometer ahead of mounting.

Dissected arterial segments were threaded with 40μm wire as shown in *Figure 2.5*, and mounted in the M610-wire myograph (Danish Myotech, Aarhus, Denmark), one side attached to a force transducer and the other to a movable micrometer. Vessels were submerged in 6 mls of PSS pre-warmed to 37˚C and gassed with 5% CO_2 in air.
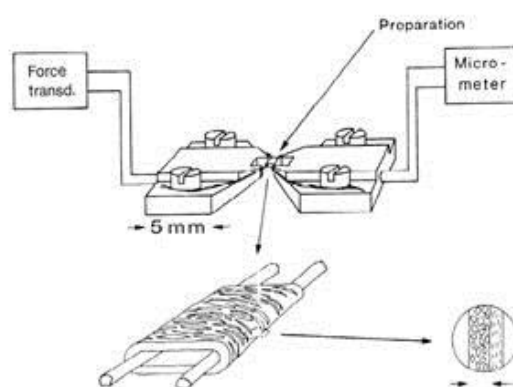
Figure 2.5: Small artery mounted within force transducer mechanism. Figure adapted from: Mulvany AND Aalkjkeir (1990) Structure and Function of Small Arteries The American Physiological Society Vol. 70, No. 4

2.5.3 Normalisation and measurement

Following the mounting procedure vessels were left to equilibrate for 10 mins. Vessels were then partially stretched by widening the wires and allowed to equilibrate again for a few minutes. This stretching and equilibrium cycle was repeated for approx. 6-7 stretches until the vessels reached beyond a passive tension of approx. 13.3 kPa. Measurement and normalisation software (Myodata, Danish Myotech) calculated internal vessel diameter and wire tension was adjusted accordingly to create a passive tension of equivalent of 100mmHg/13.3kPa for each vessel.

Following normalisation and equilibrium, vessels were assessed for tissue sample viability and constricted to maximal vessel tone using KPSS. A washout period of 30 minutes allowed full return to resting tension. A thromboxane mimetic and known vessel constrictor U46619 (1 µM) was added for a baseline contractile response. Following a further washout period and resumption to baseline equilibrium, the experimental protocol was commenced. Drugs were added either alone or in combination with some vessels exposed to vehicle only to act as controls. Any change to baseline tension was noted over a 30 minute incubation period before re-exposure to U46619 (1 µM). Further washout periods were commenced at the end of drug exposure completed by a final exposure to U46619 (1 µM) for each vessel.

2.5.4 Data analysis and statistics

Changes in maximal tension achieved by U46619 (1 µM) from pre, during and and post drug incubation exposure was measured using the Myodaq software (Version 2.02 M Danish Myotech). This was expressed as a ratio of the maximal tension achieved under pre-experiment exposure to U46619 (1 µM). Difference in kPa achieved was calculated using vessel diameter and difference of contraction achieved (maximal contraction – baseline tone).

3. Results

3.1 The effects of potassium channel openers on myometrial contractility

3.1.1 Clinical relevance & background

Calcium influx is essential for propagation of an action potential and also allows, via calmodulin, the interaction between actin and myosin that brings about contraction of the myocyte (Word et al, 1993). This mechanism of action is mirrored in vascular smooth muscle with myogenic tone being attenuated in calcium free media and increased with additional extracellular calcium (Davis & Hill, 1999). The resting potential for vascular smooth muscle ranges from -60 to -75 in unpressurised arteries and arterioles with a graded depolarization as pressure increases (Davis & Hill, 1999). This mechano-sensitive response is crucial for systemic blood pressure control. The L-type calcium channels are voltage gated (VGLCC) and as such open in response to de-polarisation of the membrane potential allowing calcium entry and further de-polarising of the smooth muscle cell. The activation threshold for the VGLCC is between -50 to -60 mV (Davis & Hill, 1999). In pregnancy the uterus is quiescent with resting potential of between -75 to -60 mV (Parkington et al, 1999). As contractile associated proteins increase there is a shift in balance towards excitability with resting potential reaching -55 mV (Parkington et al, 1999). In blocking calcium entry via the VGLCC, the dihydropyridine Nifedipine impedes contractility in both vascular smooth muscle and myometrium. It is this that leads to the unwanted side effect of hypotension when Nifedipine is used as a tocolytic and makes the

optimal therapeutic dose required to abolish contractions impossible to administer (*further detail found in introduction*).

Work carried out within our group, using laser capture micro-dissection, and PCR analysis has measured the expression of specific ion channels in myometrium in comparison to their expression in vascular smooth muscle. Channels identified as of interest were potassium channels, which demonstrated higher expression in myometrium over vascular smooth muscle. Potassium channels when open allow K^+ efflux resulting in membrane repolarisation. With the combined effect of less Ca^{2+} entry and increased K^+ efflux, membrane potential should be 'dampened' reducing the probability of the generation of action potentials and contractile activity *(Figure 3.1.1)*.

Our hypothesis was thereby developed that improving the potency of Nifedipine in the myometrium, but not vasculature, could be achieved by modulation of membrane potential via myometrial specific K^+ channels. K^+ channels identified in the work detailed above that had known openers available were selected as targets for investigation. Drugs chosen were Riluzole, a known activator of SK_3, TREK-1 and an inhibitor of voltage gated Na^+ channels; and linoleic acid, a known activator of $K_V2.1$. Pinacidil a known activator of K_{ATP} channels has been shown to reduce myometrial contractility and is used as a clinical anti-hypertensive. In offering no selectivity between myometrium and vasculature, Pinacidil was expected to act as a positive control for the other compounds.
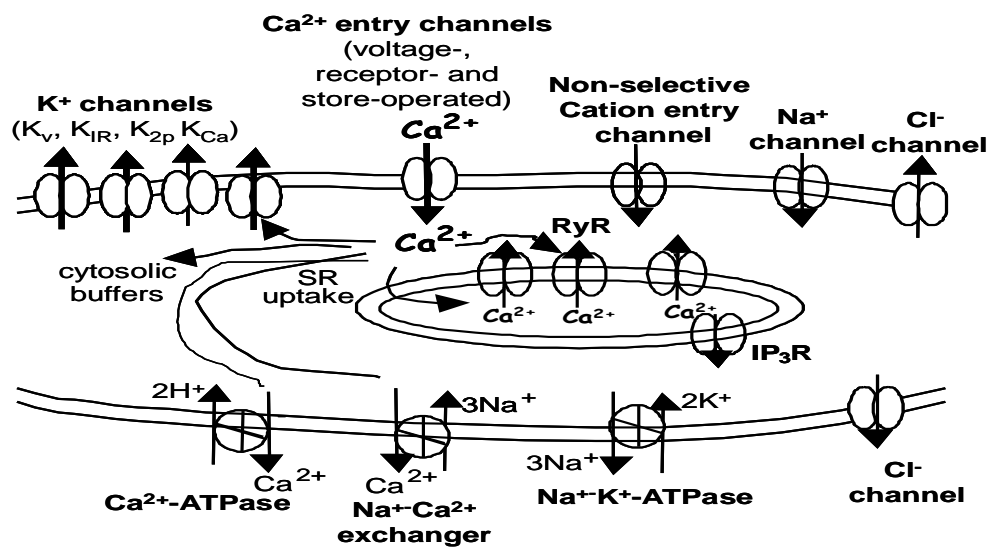
Figure 3.1.1: Diagram shows ion channels involved in modulation of membrane potential. Nifedipine blocks calcium entry through voltage gated calcium channels and potassium channel openers increase K^+ efflux resulting in repolarisation of membrane potential. Modulating membrane potential in this way should reduce the probability of the formation of action potentials and contractile activity (diagram drawn by Henggui Zhang).

3.1.2 Aims

3.1.2.1 Dose response experiments:

The first aim was to establish the dose response effect of the selected potassium channel openers on myometrial contractility. Following this, the same dose response was applied to strips pre-treated with a range of concentration of Nifedipine. Percentage inhibition from pre-dose period was compared to control for each dose period. Data was tested for normal distribution by Shapiro-Wilk normality test. Data did not fit a normal distribution and so the non-parametric Wilcoxon Signed rank test was selected for sample group comparison. Activity integral data was fitted to dose response curves to determine the IC_{50}.

3.1.2.2 Combined dosing:

In an extension to this investigation a combined dose protocol was added. It was envisaged that a dual dose regimen would be used in clinical application and so dual dosing was performed in the organ bath. Data was analysed as described above. Percentage inhibition from pre-dose period was compared to control for each matched dose. Data was tested for normal distribution by Shapiro-Wilk normality test. All data did not fit a normal distribution and so the non parametric Wilcoxon Signed Rank test was selected for sample group comparison. There appeared to be a time dependent effect of Riluzole combined with Nifedipine and so data at doses with significant results was further analysed in 4 separate 10 min periods from dosing. As the desired clinical

outcome would be an immediate cessation of contractions following combined dosing, data was also tested for binomial distribution and sign test with cessation of contractions within 40 minutes following dosing considered a successful outcome verses continuation of contractions.

3.1.3 Objective:

i) To determine the effect of selective K^+ channel openers, linoleic acid, Pinacidil and Riluzole, on the contractility of spontaneously contracting myometrial strips.

ii) To determine the effect of selective K^+ channel openers, linoleic acid, Pinacidil and Riluzole on the contractility of spontaneously contracting myometrial strips following pre-treatment with Nifedipine.

3.1.4 Results

3.1.4.1 Results - The effect of linoleic acid on myometrial contractility

Linoleic acid showed no significant effect on any of the measured parameters either alone or when combined with Nifedipine *(Figure 3.1.2).*
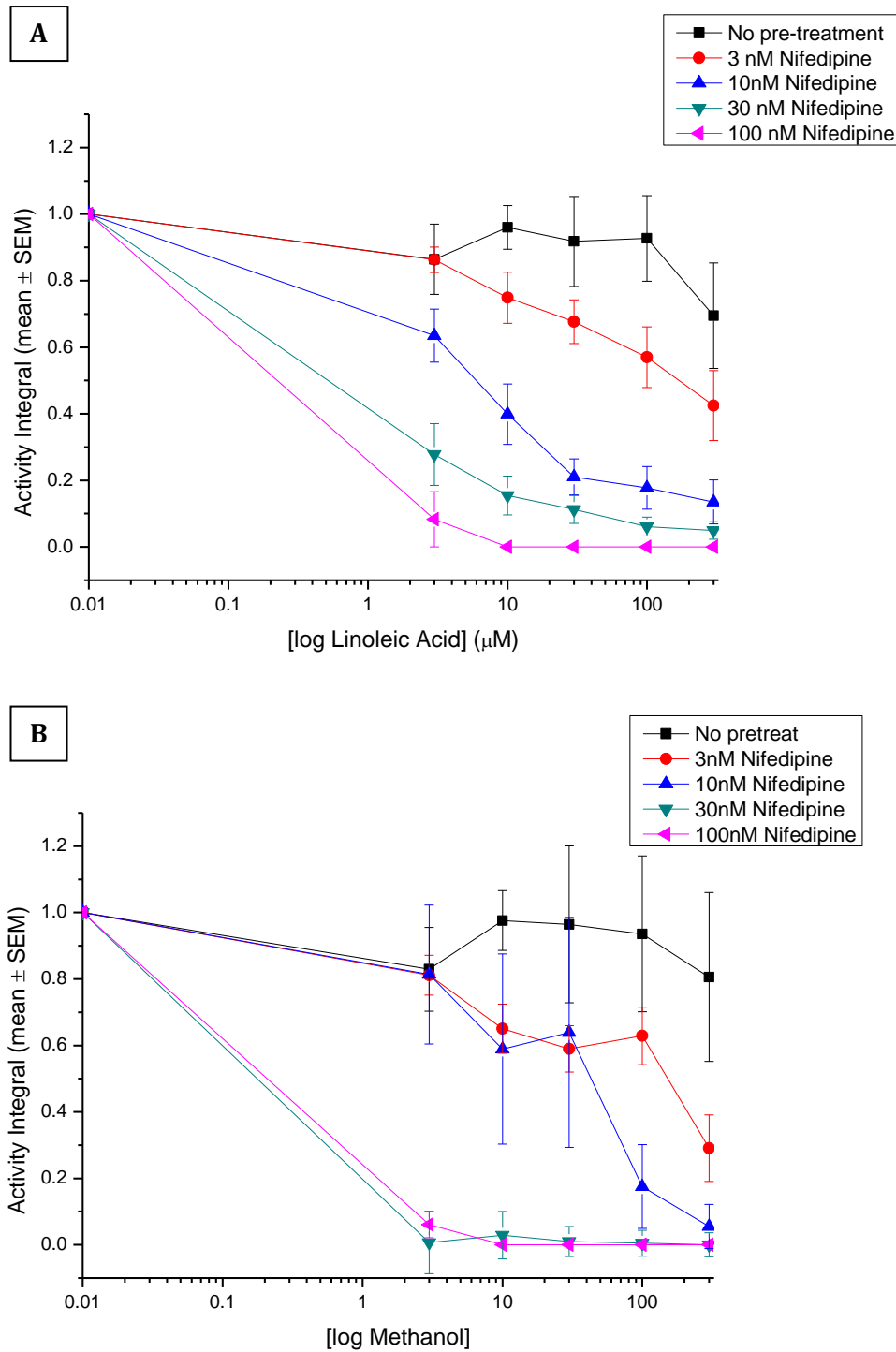
Figure 3.1.2: Linoleic acid: Graphs showing mean inhibition of activity integral ± SEM when (A) Linoleic acid added to spontaneously contracting strips either not pre-treated (n=10) or treated with Nifedipine (3 nM (n=12), 10 nM (n=11), 30 nM (n=9), 100 nM (n=8)); or (B) vehicle control equivalent. There was no significant difference in inhibition between Linoleic Acid and vehicle control.

3.1.4.2 Results – The effect of Pinacidil on myometrial contractility

Pinacidil reduced activity integral when added to spontaneously contracting strips alone at 3 μM (P=0.003) , 10 μM (P=0.0019) and 30 μM (P=0.0019) *(Figure 3.1.3)* (IC_{50} 1.85(± 0.03) μM) *(Table 2)*. When pre-treated with 3 nM Nifedipine activity integral was also reduced at 3 μM (P=0.007), 10 μM (P=0.015) and 30 μM (P=0.015) (IC_{50} 1.49(± 0.04) μM) *(Table 3.1)*. When pre-treated with 10 nM Nifedipine activity integral was reduced at 3 μM (P=0.005), 10 μM (P=0.0019) and 30 μM (P=0.003) (IC_{50} 0.52(± 0.12) μM) *(Table 3.1)*. Pinacidil either alone or combined with 3 nM or 10 nM Nifedipine had similarly significant reduction in maximal force, contraction duration and frequency *(Figures 3.1.3, 3.1.4, 3.1.5, 3.1.6, 3.1.7, 3.1.8, 3.1.9, 3.1.10)*. The lack of significant results in strips pre-treated with 30 nM Nifedipine or higher reflects the inhibitory effect of Nifedipine at these doses, which exceeds any discernible effect of Pinacidil over control strips. The time dependent effect of Nifedipine seen in the reduction in contractility in control strips may also explain the lessening power of significant results at higher combined doses.
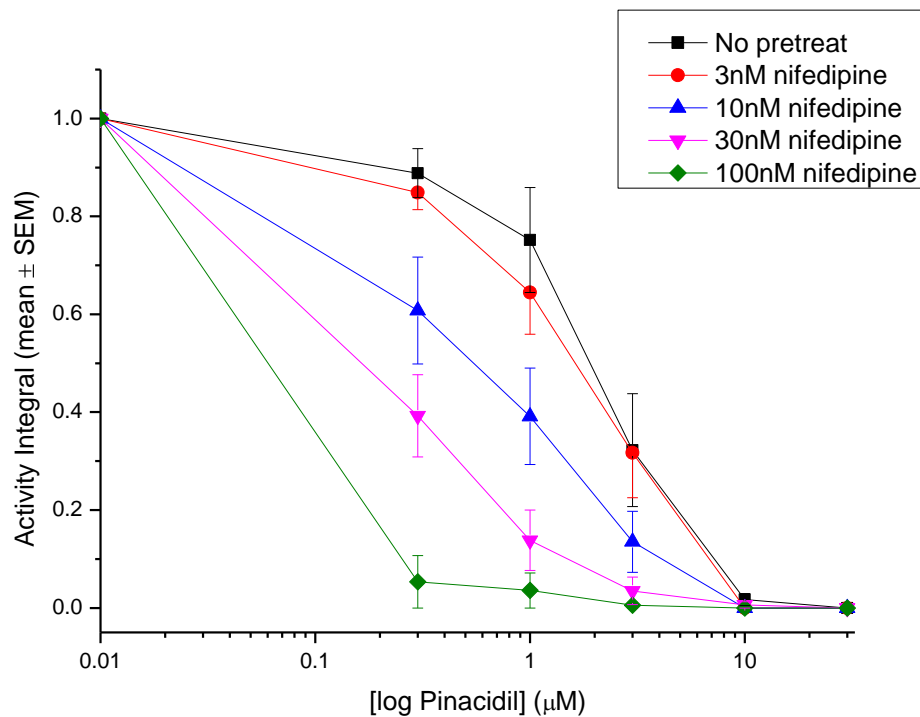
Figure 3.1.3: Mean inhibition of activity integral ± SEM when Pinacidil (0.3, 1, 3, 10 and 30 μM) added to spontaneously contracting strips either alone (n=11) or pre-treated with Nifedipine (3 nM (n=10), 10 nM (n=10), 30 nM (n=10), 100 nM (n=10)) . Activity integral is completely abolished at Pinacidil 10 μM alone.
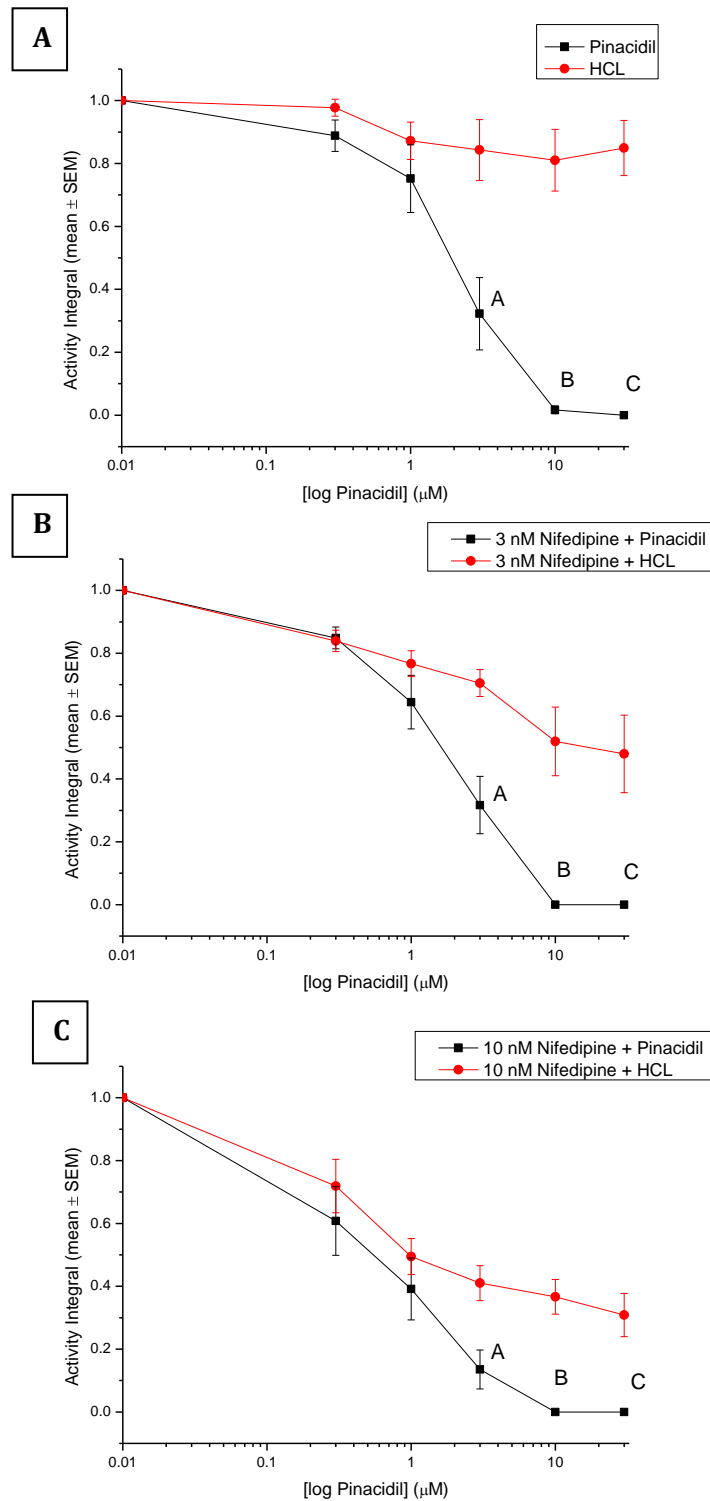
Figure 3.1.4: Significant inhibition of activity integral as seen when compared to vehicle control equivalent in (A) Pinacidil alone (A: 3 μM P=0.003, B: 10 μM P=0.0019, C: 30 μM P=0.0019 n=11) (B) Nifedipine 3 nM + Pinacidil (A: 3 μM P=0.007, B: 10 μM P=0.015, C: 30 μM P=0.015 n=10) and (C) Nifedipine 10 nM + Pinacidil (A: 3 μM P=0.005, B: 10 μM P=0.0019, C: 30 μM P=0.003 n= 10).
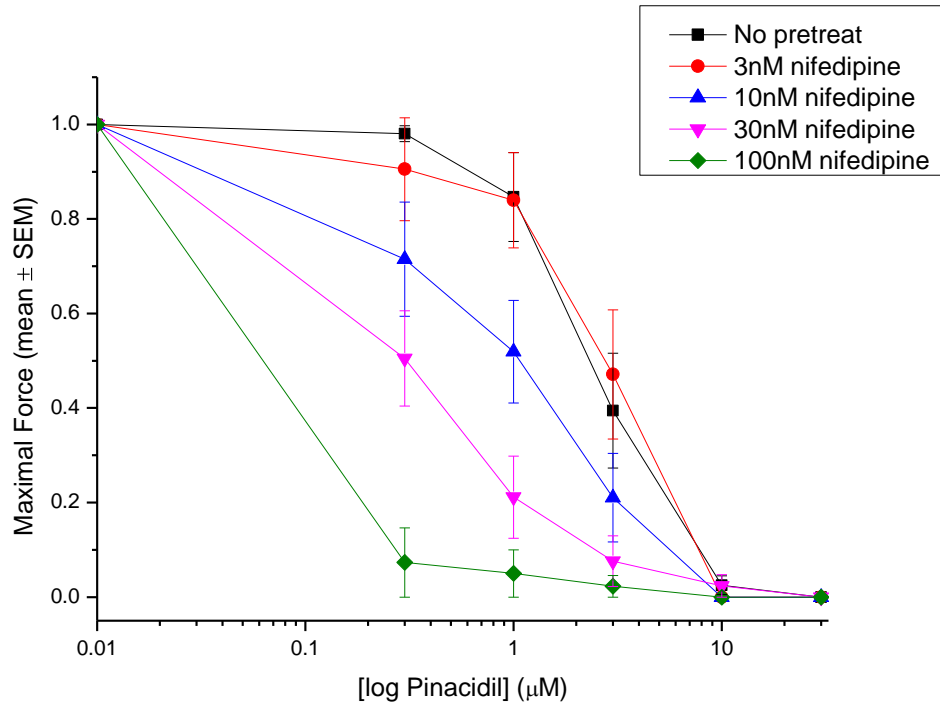
Figure 3.1.5: Mean inhibition of maximal force ± SEM after addition of Pinacidil

(0.3, 1, 3, 10 and 30 μM) to spontaneously contracting strips either alone (n=11)

or pre-treated with Nifedipine (3 nM (n=10), 10 nM (n=10), 30 nM (n=10), 100

nM (n=10)). Maximal Force is completely abolished at Pinacidil 10 μM alone.
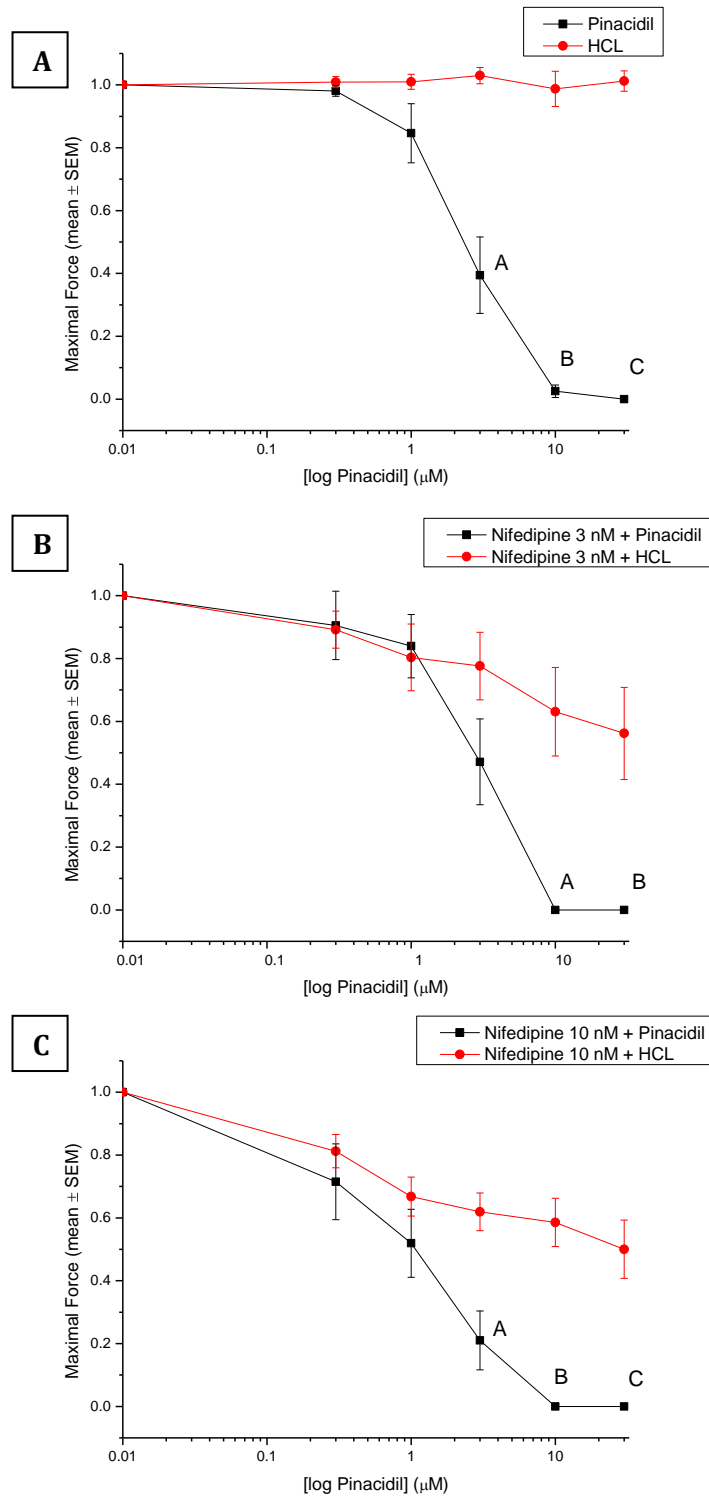
Figure 3.1.6: Significant inhibition of maximal force as seen when compared to vehicle control equivalent in (A) Pinacidil alone (A: 3 μM P=0.0009, B: 10 μM P=0.0009, C: 30 μM P=0.0009 n=11) (B) Nifedipine 3 nM + Pinacidil (A: 10 μM P=0.015, B: 30 μM P=0.015 n=10) and (C) Nifedipine 10 nM + Pinacidil (A: 3 μM P=0.013, B: 10 μM P=0.0019, C: 30 μM P=0.003 n= 10).
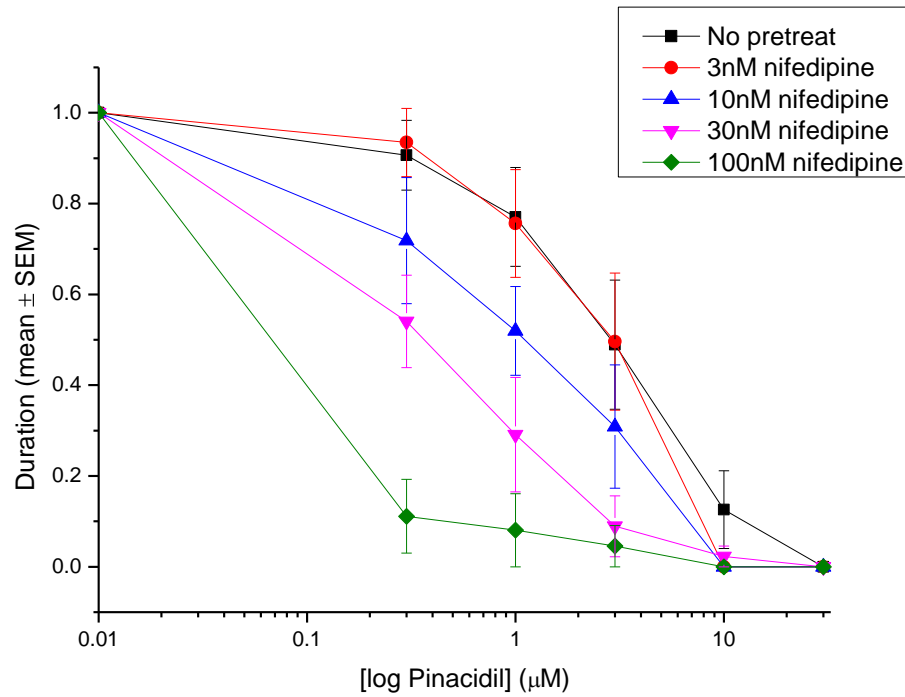
Figure 3.1.7: Mean inhibition of contraction duration ± SEM when Pinacidil (0.3, 1, 3, 10 and 30 μM) added to spontaneously contracting strips either alone (n=11) or pre-treated with Nifedipine (3 nM (n=10), 10 nM (n=10), 30 nM (n=10), 100 nM (n=10)). Contraction duration is at zero by Pinacidil 30 μM alone
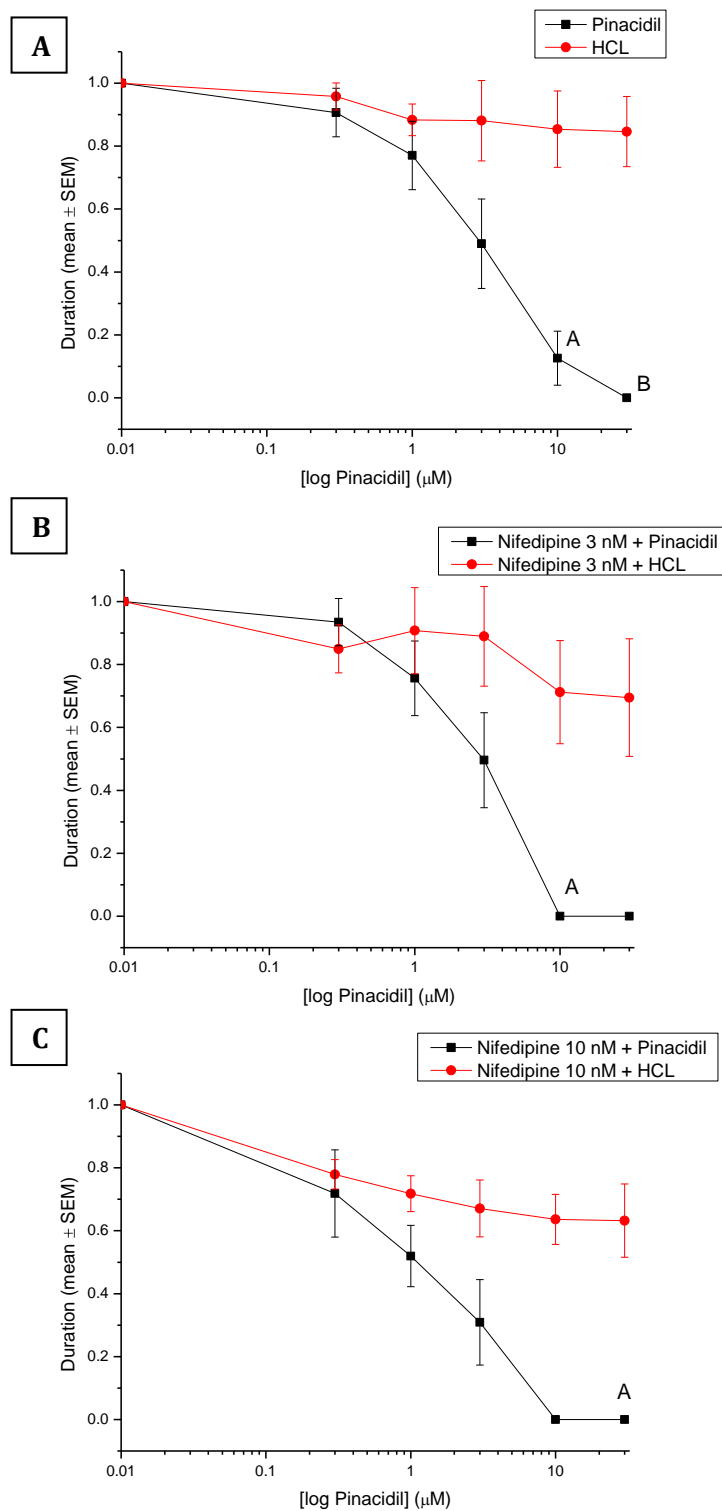
Figure 3.1.8: Significant inhibition of contraction duration as seen when

compared to vehicle control equivalent in (A) Pinacidil alone (A: 10 µM P=0.0019,

B: 30 µM P=0.0019 n=11) (B) Nifedipine 3 nM + Pinacidil (A: 10 µM P=0.015,

n=10) and (C) Nifedipine 10 nM + Pinacidil (A: 30 µM P=0.003 n= 10).
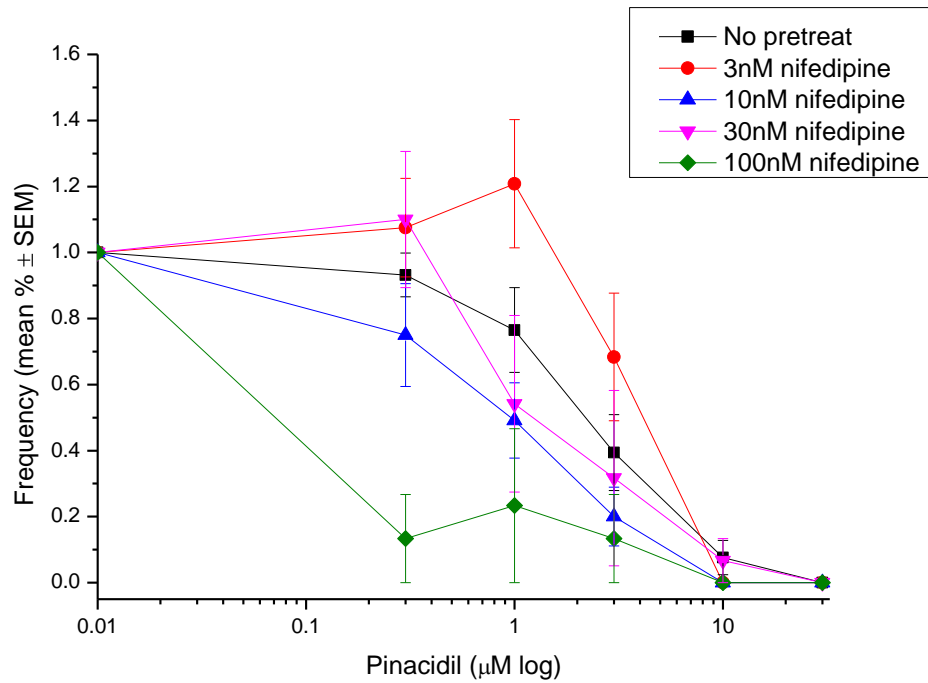
Figure 3.1.9: Mean inhibition of contraction frequency ± SEM when Pinacidil (0.3, 1, 3, 10 and 30 μM) added to spontaneously contracting strips either alone (n=11) or pre-treated with Nifedipine (3 nM (n=10), 10 nM (n=10), 30 nM (n=10), 100 nM (n=10)). Contraction frequency is at zero by Pinacidil 30 μM alone.
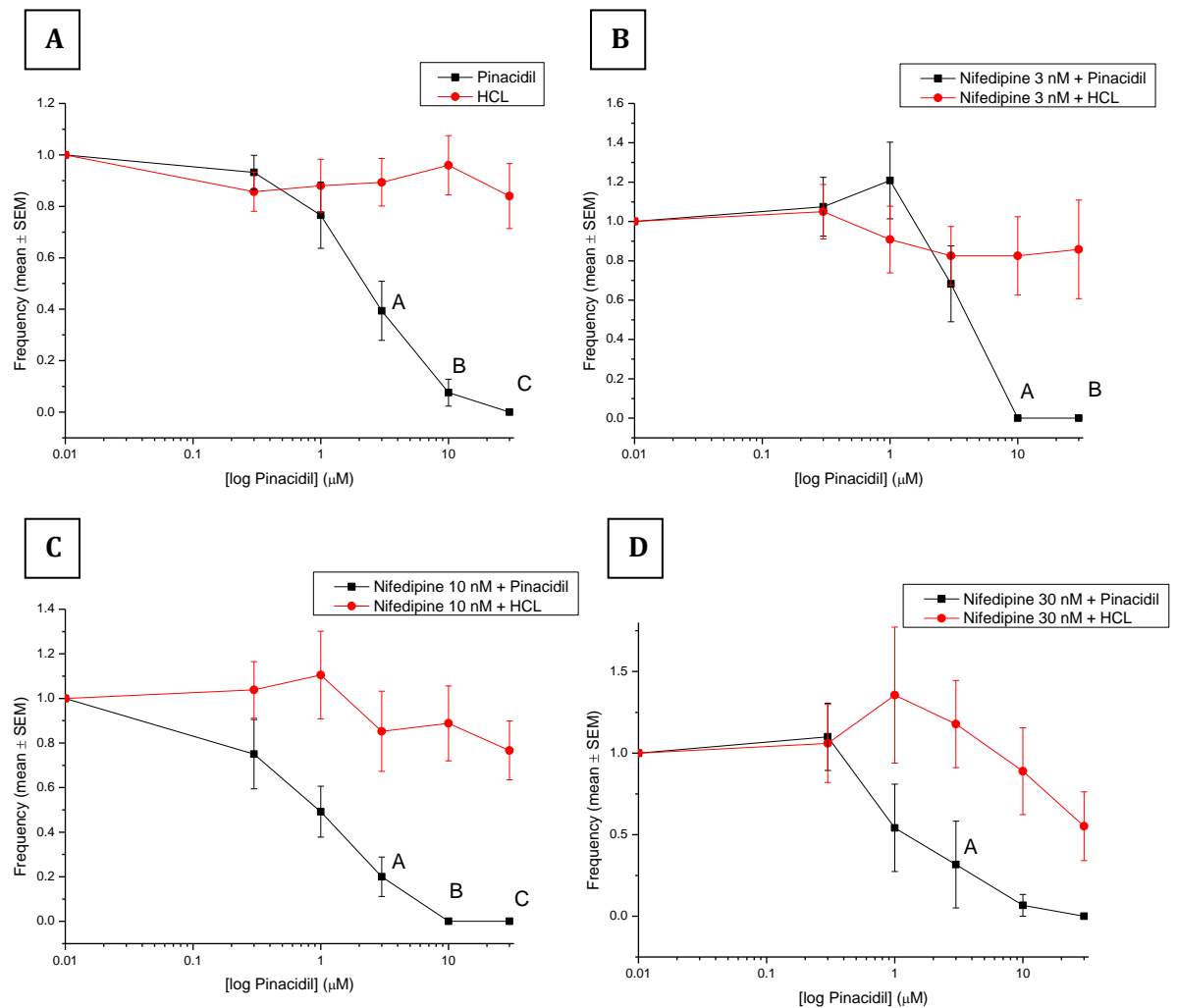
Figure 3.1.10: Significant inhibition of contraction frequency when compared to vehicle control equivalent in (A) Pinacidil alone (A: 3 µM P=0.03, B: 10 µM P=0.0019, C: 30 µM P=0.03 n=11) (B) Nifedipine 3 nM + Pinacidil (A: 10 µM P=0.006, B: 30 µM P=0.015 n=10) and (C) Nifedipine 10 nM + Pinacidil (A: 3 µM P=0.03, B: 10 µM P=0.0015, C: 30 µM P=0.03 n= 10) and (D) Nifedipine 30 nM + Pinacidil (A: 10 µM P=0.015 n=10).

	IC50	±SEM
No pre-treatment	1.85	0.03
Nifedipine 3 nM	1.49	0.04
Nifedipine 10 nM	0.52	0.12

Table 3.1: IC50 for Pinacidil either alone (n=11) or with Nifedipine pre-treatment

based on activity integral data (3 nM (n=10), 10 nM (n=10)). IC_{50}s not calculated

for higher doses of Nifedipine pre-treatment due to lack of significant results in

these series.

3.1.4.3 Results – The effect of Riluzole on myometrial contractility

Riluzole significantly reduced activity integral and maximal force of contraction at 30 µM (*P*= 0.0078) when administered alone *(Figure 3.1.11)* (IC$_{50}$ 26.4 (± 0.06) µM) *(Table 3.2)*. When combined with Nifedipine 3 nM activity integral was reduced at 3 µM (*P*=0.04) *(Figure 3.1.12)* (IC$_{50}$ 5.4 (± 0.04) µM) *(Table 3.2)* and maximal force reduced at 10 µM (*P*=0.041) and 30 µM (*P*=0.006) *(Figure 3.1.13,3.1.14)*. There was an increased duration at 10 µM when pre-treated with Nifedipine 30 nM (*P*=0.0019) *(Figure 3.1.15, 3.1.16)*. On further investigation, it was observed that the phase plot shape of contraction had altered at this dose *(Figure 3.1.17)*. The duration of the upstroke of contraction was increased at this dose *(Figure 3.1.17)* illustrating that the increased duration is due to a slower climb to maximal force. A similar change was evident in strips pre-treated with other doses of Nifedipine however analysis did not yield significant results in other strips. This may be down to variance of sample group response, particularly at higher doses where contractions were small and short by duration bringing down duration average. The lack of effect on frequency reflects the small short contractions that persisted at higher doses. Again, as with Pinacidil experiments, the lack of significant results in strips pre-treated with 30 nM Nifedipine or higher reflects the inhibitory effect of Nifedipine at these doses being equal to control.

117
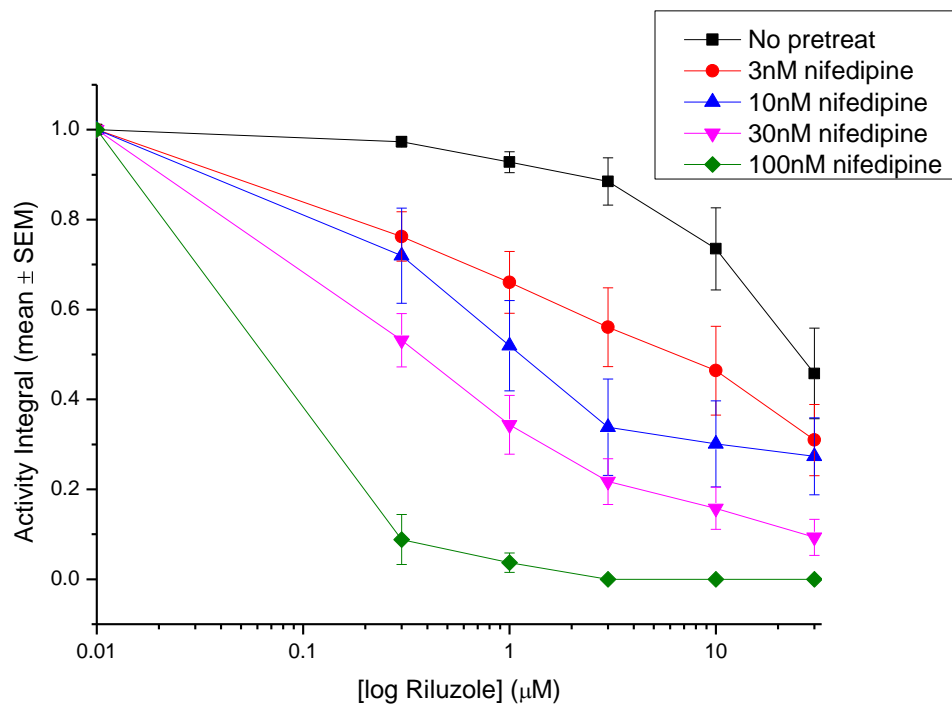
Figure 3.1.11: Mean inhibition of activity integral ± SEM when Riluzole (0.3, 1, 3, 10 and 30 μM) added to spontaneously contracting strips either alone (n=9) or pre-treated with Nifedipine (3 nM (n=12), 10 nM (n=10), 30 nM (n=9), 100 nM n=10)). Activity integral was never completely abolished by Riluzole alone.
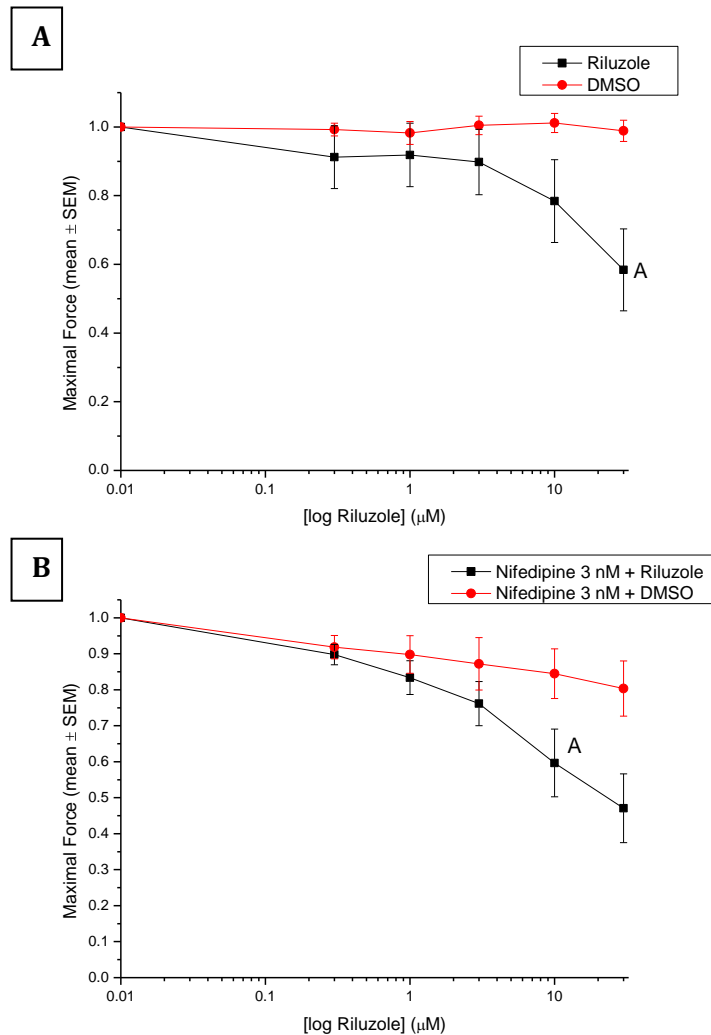
Figure 3.1.12: Significant inhibition of activity integral compared to vehicle

control equivalent in (A) Riluzole alone (A: 30 µM P=0.0078 n=9) and (B)

Nifedipine 3 nM + Riluzole (A: 30 µM P=0.04 n=12).
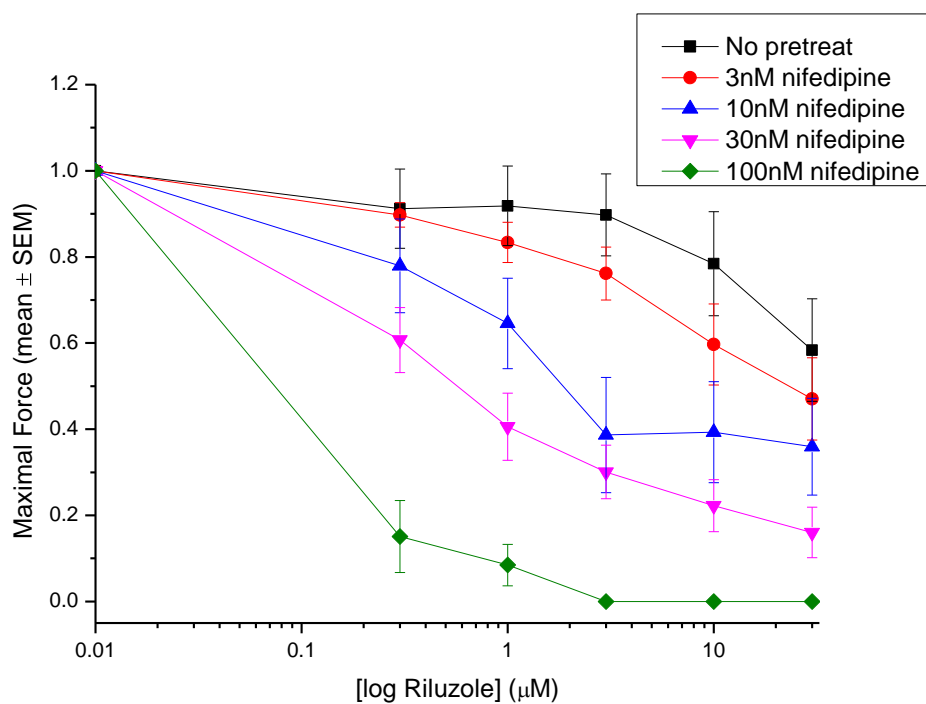
Figure 3.1.13: Mean inhibition of maximal force ± SEM when Riluzole (0.3, 1, 3, 10 and 30 μM) added to spontaneously contracting strips either alone (n=9) or pre-treated with Nifedipine (3 nM (n=12), 10 nM (n=10), 30 nM (n=9), 100 nM n=10)). Maximal force was never completely abolished by Riluzole alone.
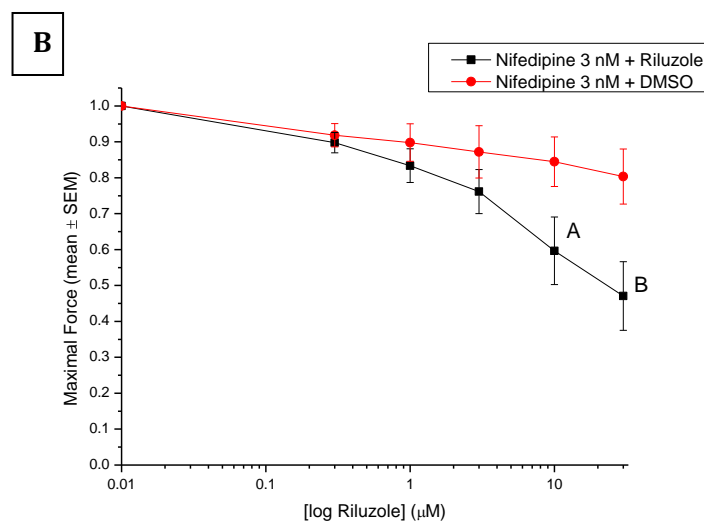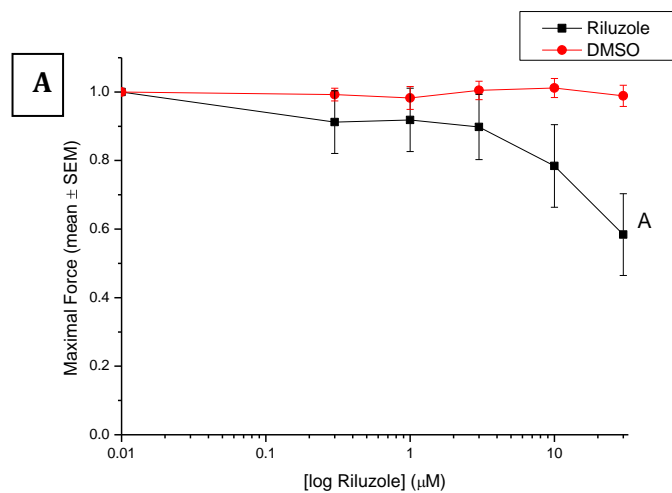
Figure 3.1.14 Significant inhibition of maximal force when compared to vehicle control equivalent in (A) Riluzole alone (A: 30 µM P=0.007 n=9), and (B) Nifedipine 3 nM + Riluzole (A: 10 µM P=0.04 n=10, and B: 30 µM P=0.006 n=12)
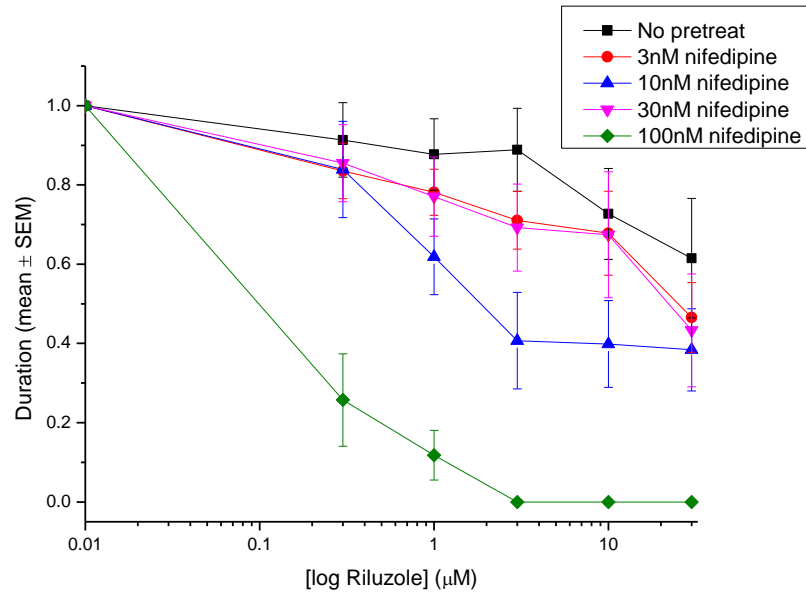
Figure 3.1.15: Mean inhibition of contraction duration ± SEM when Riluzole (0.3, 1, 3, 10 and 30 μM) added to spontaneously contracting strips either alone (n=9) or pre-treated with Nifedipine (3 nM (n=12), 10 nM (n=10), 30 nM (n=9), 100 nM n=10)).
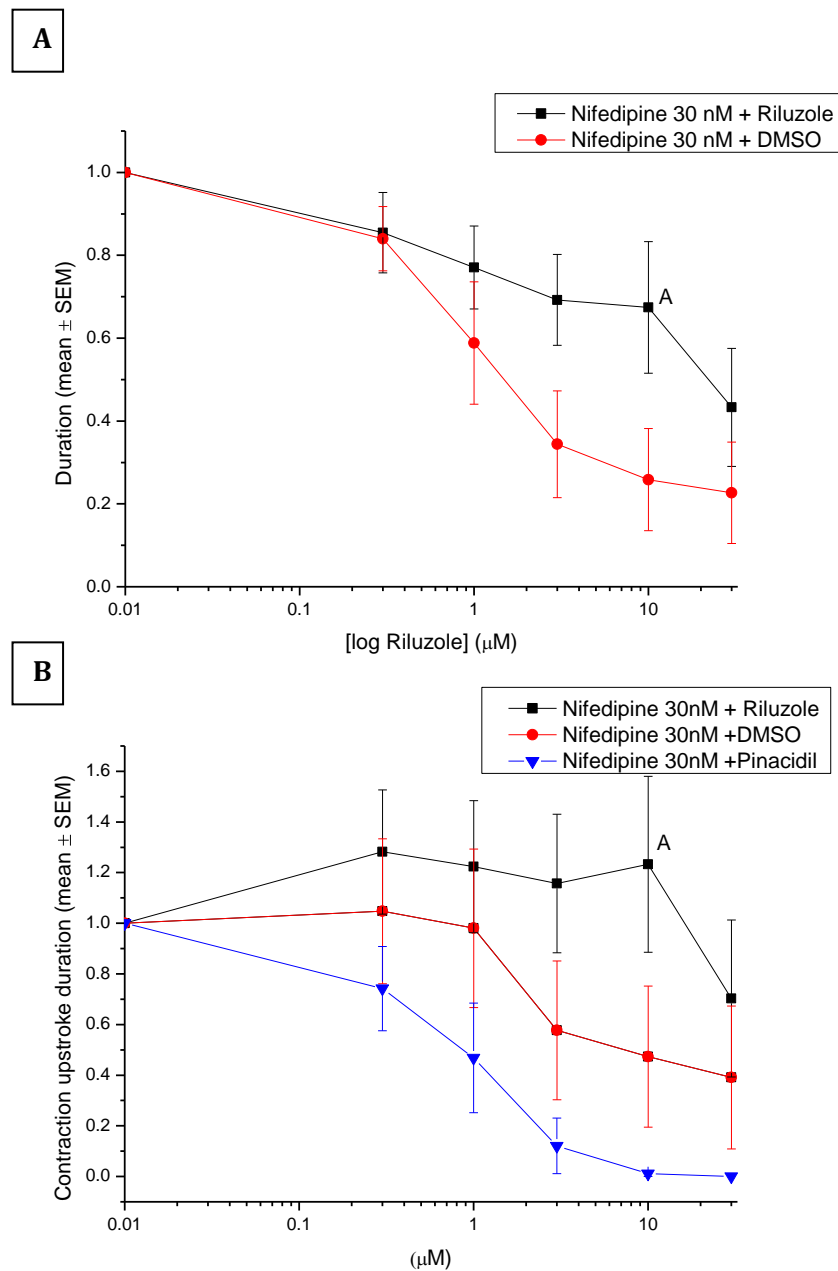
Figure 3.1.16: Significant increase in contraction duration in strips pre-treated with (A) Nifedipine 30 nM + Riluzole (A: 10 µM P=0.0019 n=9). Mean inhibition of contraction duration upstroke in (B) Riluzole ± SEM when compared with vehicle strip and Pinacidil at same dose (A: 10 µM P<0.05 n=11).
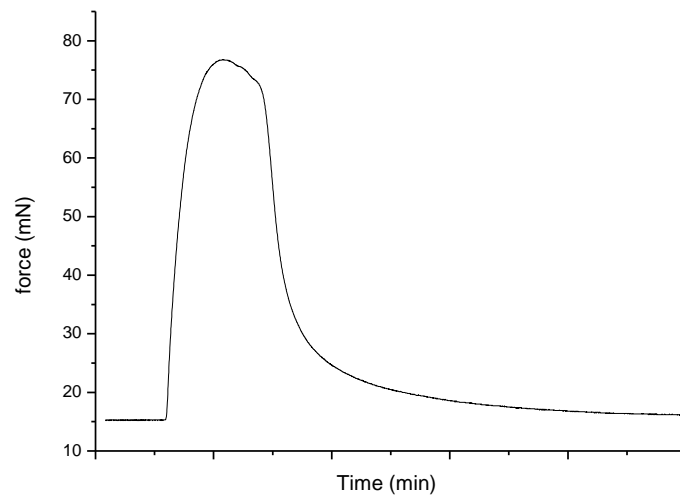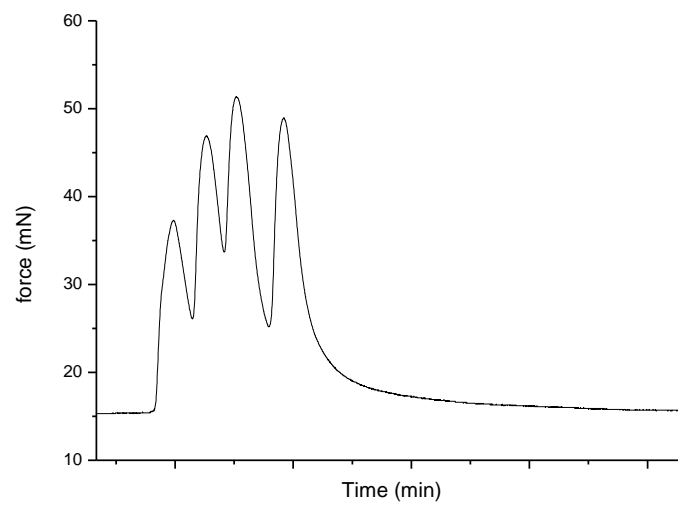
Figure 3.1.17: Phase plots showing (A) a typical 'plateau' shape contraction seen in spontaneous contractions and (B) a 'spike' shape contraction seen following treatment with Riluzole with interrupted upstroke phase leading to longer contraction duration.
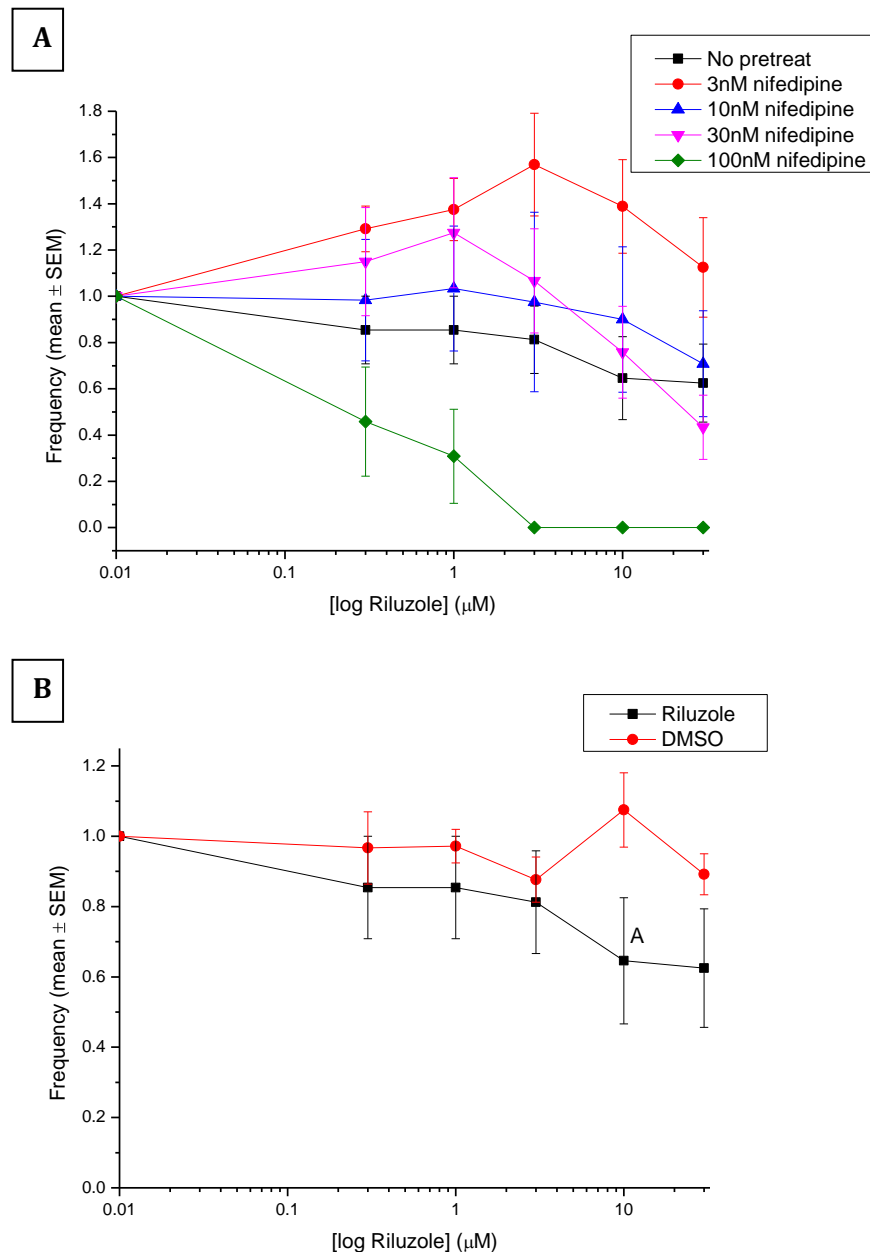
Figure 3.1.18: Mean inhibition of frequency of contractions ± SEM when (A) Riluzole added to spontaneously contracting strips either alone (n=10) or pre-treated with Nifedipine (3 nM (n=12), 10 nM (n=10), 30 nM (n=9), 100 nM (n= 10)). Significant inhibition when Riluzole compared to vehicle control equivalent (B). There was no significant change in frequency at any other doses and the significant result shown (10 μM P=0.04 n=10) may be due to uncharacteristic rise in frequency in control data.

	IC50	±SEM
No pre-treatment	26.4	0.06
Nifedipine 3 nM	5.4	0.04

Table 3.2: IC$_{50}$ for Riluzole either alone (n=9) or with Nifedipine pre-treatment (3 nM n=12) based on activity integral data. IC$_{50}$s not calculated for higher doses of Nifedipine pre-treatment due to lack of significant results in these series.

3.2 The effects of combined dose of Nifedipine with potassium channel openers on myometrial contractility

3.2.1 Clinical relevance and background

Following results from previous experimental series where Nifedipine was used as pre-treatment and cumulative doses of compounds added, further contractility work was undertaken to assess the impact on spontaneous contractions of a combination of Riluzole + Nifedipine and Pinacidil + Nifedipine when administered simultaneously. Due to the lack of effect shown with linoliec acid, it was omitted from further work. Larger doses of Nifedipine appeared to mask any combinatory effect in previous work and so combined doses of Nifedipine was limited to 3 nM and 10nM. A higher dose of Riluzole was added in this series as complete inhibition was not previously achieved at 30 μM. In order observe if the response Riluzole is mediated in the presence of physiologically occurring stimulators of contraction, combined doses were undertaken along with Oxytocin (1nM) and $PGF_2\alpha$ (10 μM). The dose of 1 nM of Oxytocin was selected as this has previously been shown to be the EC_{50} of Oxytocin and this dose has been used previously in our laboratories with sufficient effect on contractility to increase tone and prolong the phase-plot (Åkerlund et al 1999, Gullam et al 2009). The increase in activity integral of 150% of control produced here is similar to that seen by other published work at the same dose, although variation was noted between the response of term tissue

and preterm so sample gestation/time to onset of labour variation may influence the response (Åkerlund et al 1999, Gullam et al 2009).

Similarly, PGF$_2\alpha$ 10 μM, has been shown to produce enhanced activity integral ranging from 125% to 259% in other publications with variation seen between term, non-labouring and early or late stage labouring tissue at the same doses (Fischer et al 2008, Friel et al 2015).

3.2.2 Objective:

I. To determine the effect of selective K$^+$ channel openers, Pinacidil and Riluzole on the contractility of spontaneously contracting myometrial strips when administered simultaneously with 3 nM or 10 nM of Nifedipine.

II. To determine the effect of Riluzole alone and in combination with Nifedipine in the presence of Oxytocin and PGF$_2\alpha$.

3.2.3 Results

3.2.3.1 Nifedipine combined with Pinacidil

When Nifedipine 3 nM and 10 nM were combined with Pinacidil, contractions were frequently abolished from 3 μM dose and above. Simultaneous dosing of Pinacidil + Nifedipine at all dose intervals resulted in either complete inhibition or continuation of contractions. Strips that continued to contract did so with no significant difference from control *(Figure 3.2.1, 3.2.2)*. As the combined dosage of both compounds increased strips were more likely to discontinue contracting

altogether and all strips were inhibited by Pinacidil 30 µM combined with Nifedipine at 3 nM or 10 nM. Binomial sign test was applied to predict at what dose strips were significantly likely to have ceased 40 mins following administration of the dose *(Figure 3.2.3).* Strips were significantly likely to cease at a dose of, Nifedipine 10 nM + Pinacidil 3 µM (87% $P < 0.05$), 10 µM Pinacidil alone (80% $P < 0.05$), Nifedipine 10 nM + Pinacidil 10 µM (87% $P < 0.05$), Pinacidil 30 µM alone (100% $P < 0.001$), Nifedipine 3 nM + Pinacidil 30 µM (100% $P < 0.01$) and Nifedipine 10 nM + Pinacidil 30 µM (100% $P < 0.001$). Combined doses administered simultaneously brought about complete inhibition in a greater percentage of strips at doses of Pinacidil 0.3 µM – 3 µM than when strips were pre-treated with Nifedipine in the same combinations although there was no significance with binomial sign test most likely due to insufficient n numbers to power the test *(Figure 3.2.3).* Strips pre-treated with Nifedipine at either 3 nM or 10 nM with Pinacidil added at 10 µM from the experimental series presented in section 3.1.3.2 were significantly likely to result in complete inhibition (100% $P < 0.01$) whereas this was not the case for the same dose combination in simultaneous dosing *(Figure 3.2.4).*
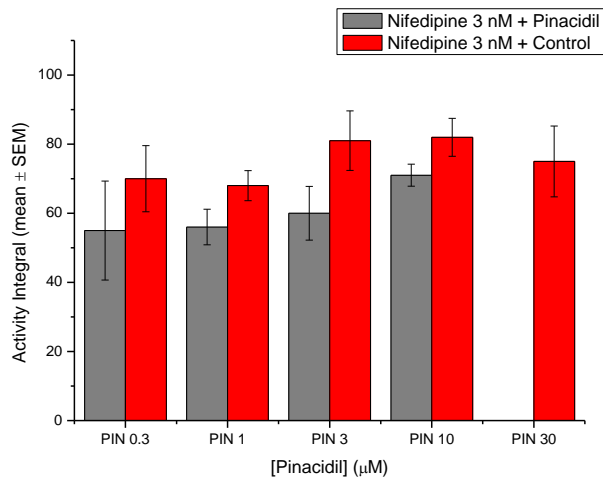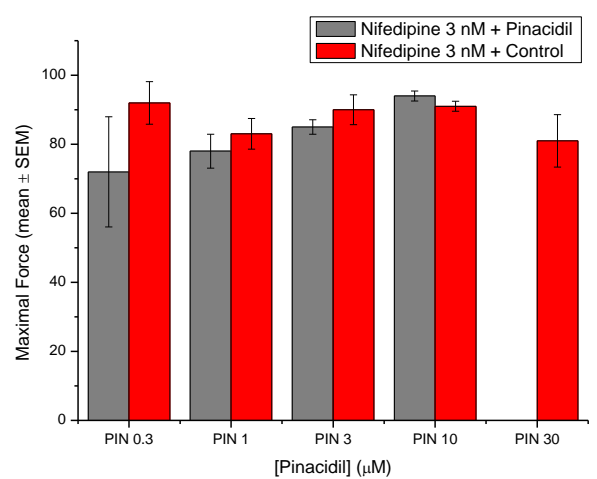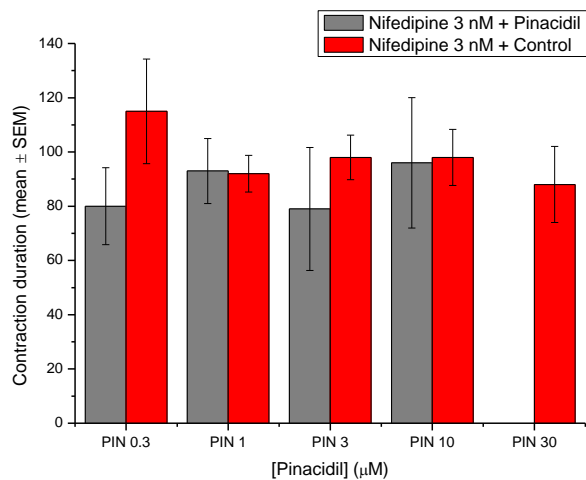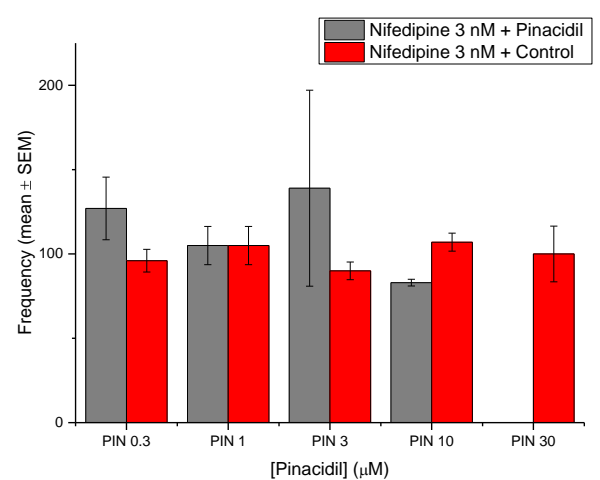
Figure 3.2.1: Inhibition of contraction activity integral (A), maximal force (B),

contraction duration (D) and frequency (D) with the dual administration of

Nifedipine 3 nM combined with either Pinacidil (0.3 µM (n=6/10), 1 µM (n=6/10),

3 µM (n=3/10), 10 µM (n=2/8), 30 µM n=0/8) or control. All contractions were

abolished at the Nifedipine 3nM + Pinacidil 30 µM dose hence no data shown.
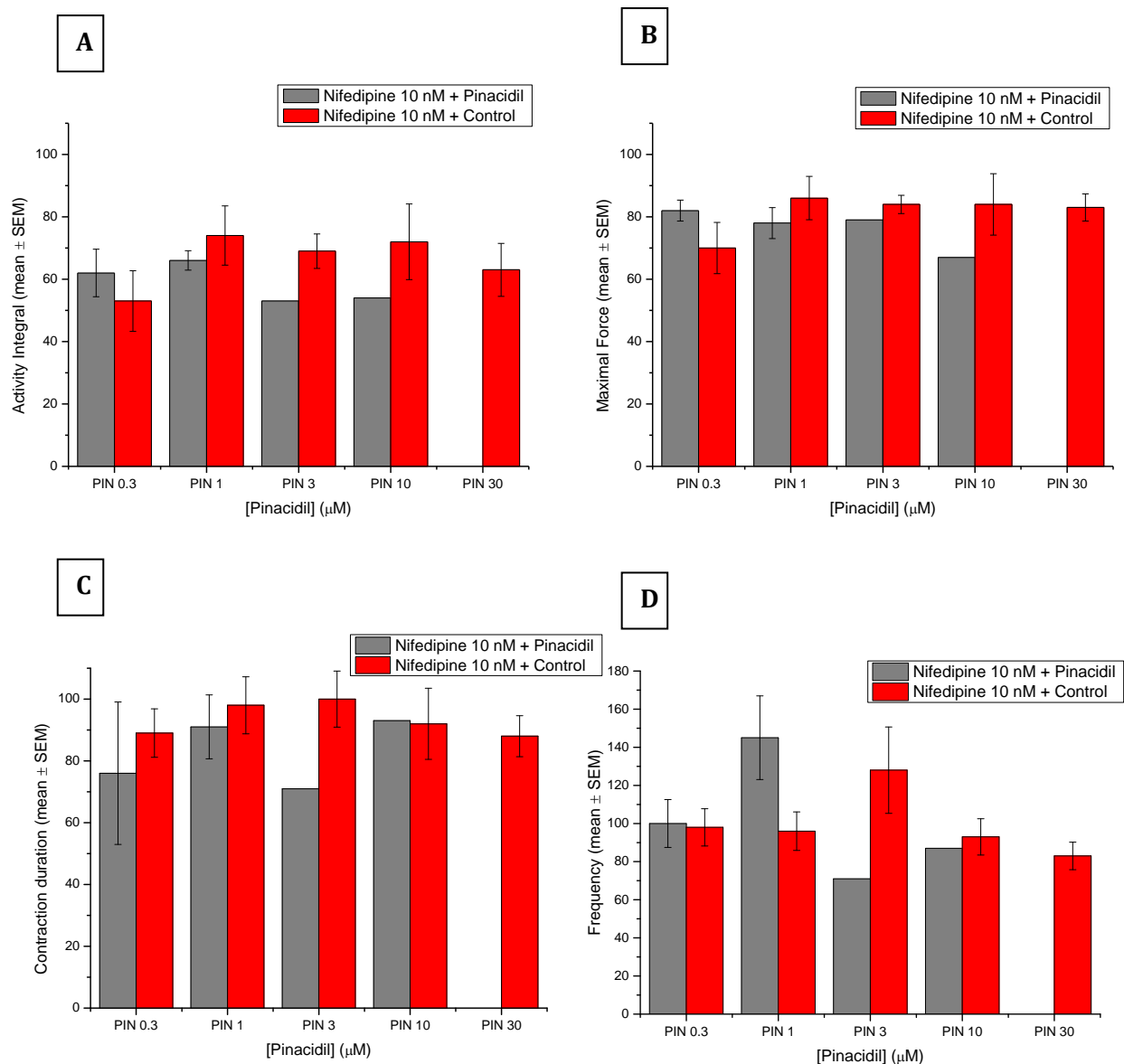
Figure 3.2.2: Inhibition of contraction activity integral (A), maximal force (B), contraction duration (D) and frequency (D) with the dual administration of Nifedipine 10 nM combined with either Pinacidil (0.3 μM (n=3/10), 1 μM (n=4/10), 3 μM (n=1/8), 10 μM (n=1/8), 30 μM n=0/11) or control. All contractions were abolished at the Nifedipine 10 nM + Pinacidil 30 μM dose hence no data shown.
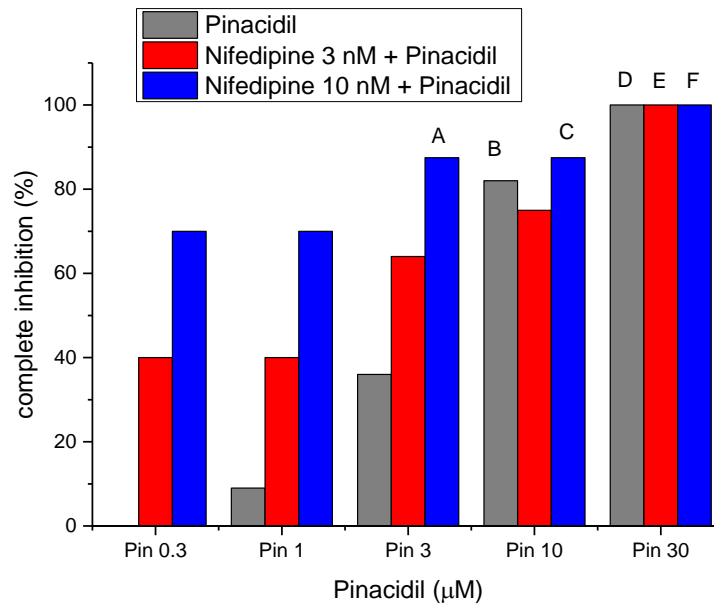
Figure 3.2.3: Graph showing the % of strips in each series that ceased to contract on addition of drug combinations. (A: Nifedipine 10 nM + Pinacidil 3 μM 87% P<0.05 n=8, B: Pinacidil 10 μM 80% P<0.05 n=10, C: Nifedipine 10 nM + Pinacidil 10 μM 87% P, 0.05 n=8, D: Pinacidil 30 μM 100% <0.001 n=10, E: Nifedipine 3 nM + Pinacidil 30 μM 100% <0.001 n=8, F: Nifedipine 10 nM + Pinacidil 30 μM 100% P<0.001 n=11) Statistical significance of likelihood to cease was calculated using binomial sign test.
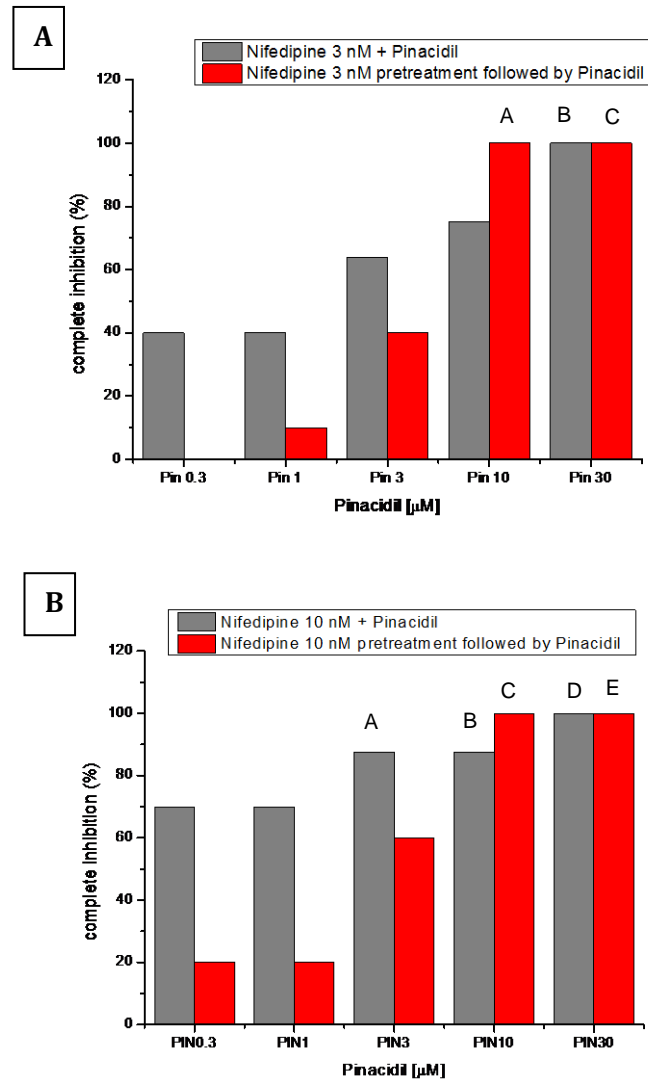
Figure 3.2.4: Graph showing the % of strips in each series that ceased to contract on addition of either simultaneously added drug combinations (grey) or where strips were pre-treated with (A) Nifedipine at 3 nM (A, B, C P,0.05) or (B) Nifedipine 10 nM (A, B: P<0.05, C: P< 0.01 D: P< 0.001, E: P<0.01) prior to the addition of Pinacidil (red). Statistical significance of likelihood to cease was calculated using binomial sign test.

3.2.3.2 Nifedipine combined with Riluzole

Data was initially analysed by averaging parameters over 40 min dosing period. When combined with 3 nM of Nifedipine, Riluzole reduced the maximal force of contraction at 3 µM, the maximal force, activity integral and contraction duration at 30 µM and the maximal force and activity integral at 100 µM. There was no effect on frequency at any dose. When combined with 10 nM Nifedipine Riluzole reduced the maximal force, activity integral and contraction duration at 100 µM only *(Figure 3.2.5 & 3.2.6).* When data from doses showing significant results was analysed in 4 separate 10 min periods from dosing the observed time dependent effect was confirmed. Significant results were seen when compared to control data at the same time interval following dosing *(Figure 3.2.7 & 3.2.8).* There is a more potent effect apparent when Nifedipine of either dose was combined with Riluzole 100 µM compared to Riluzole 30 µM.

When the shape of the phase plot is examined, the maximal force is reduced, contraction duration increased and time from baseline to maximal force or the upstroke period is increased *(Figure 3.2.9).* The activity integral will be of similar value explaining the lesser significance of reduction of activity integral seen compared reduction in maximal force *(Figure 3.2.10).*

The pattern seen with Pinacidil, where contractions were fully inhibited at certain combined doses was not seen in Riluzole with no combination likely to bring about total inhibition *(Figure 3.2.11).*
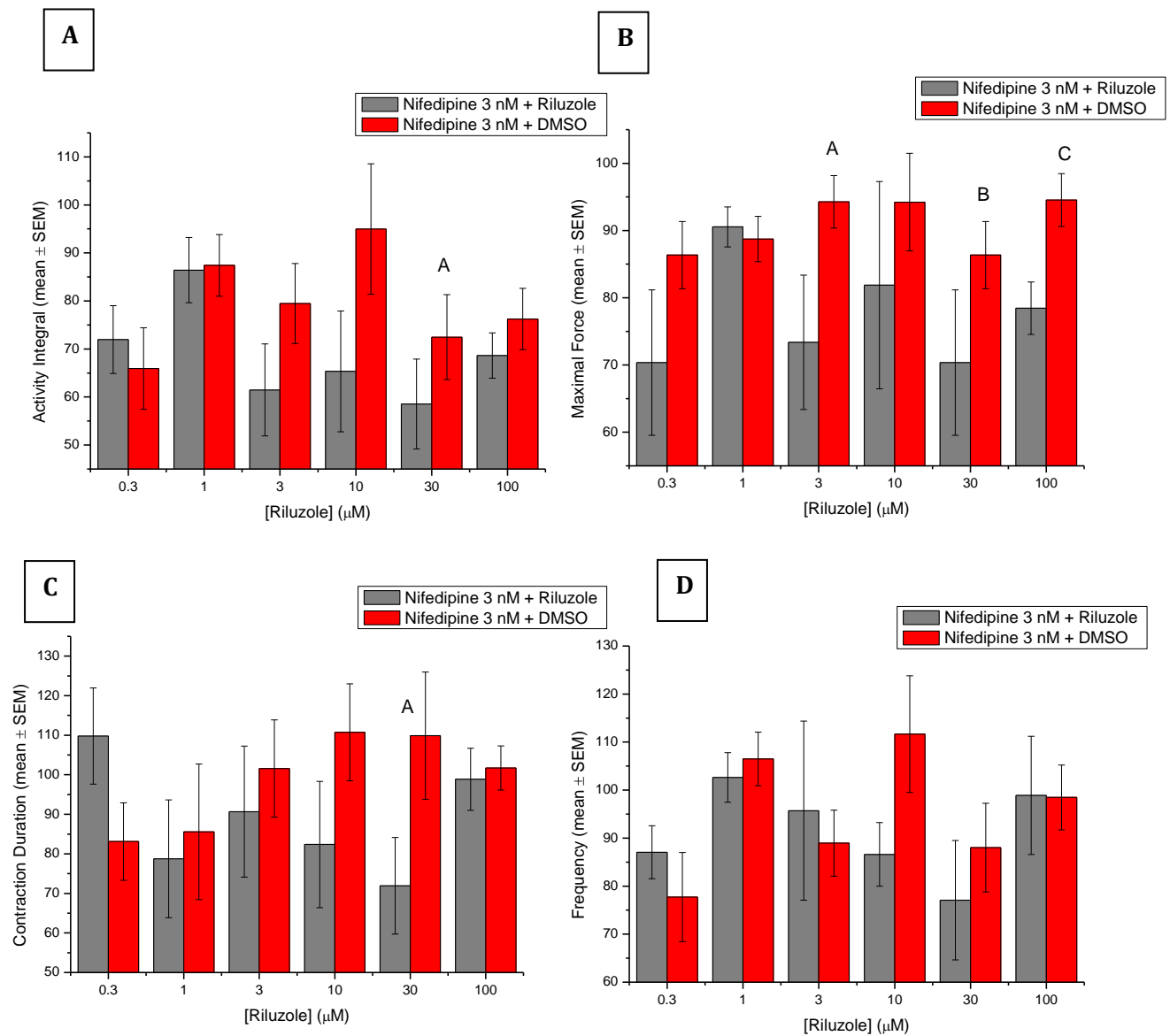
Figure 3.2.5: Mean inhibition of (A) activity integral (A: 30 µM P=0.03 n=11), (B)

maximal force (A: 3 µM P= 0.007 n=9, B: 30 µM P=0.02 n=11, C: 100 µM n=0.03

n=6) (C) contraction duration (A: 30 µM P=0.004 n=11) and (D) frequency ± SEM

when Riluzole added to spontaneously contracting strips in combination with 3

nM Nifedipine.
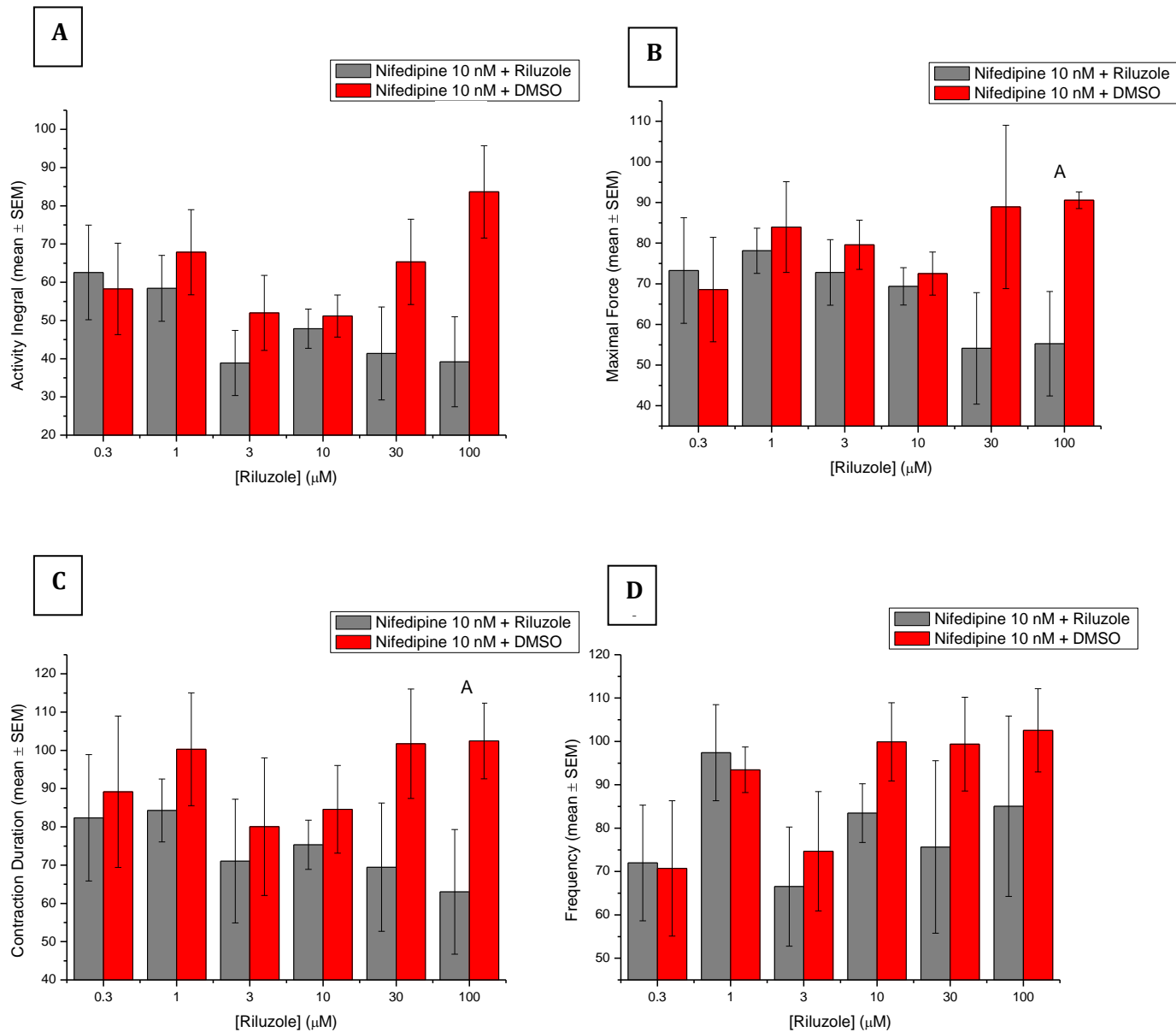
Figure 3.2.6: Mean (± SEM) inhibition of (A) activity integral (100 μM P=0.007 n=10), (B) maximal force (A: 100 μM P=0.007 n=10), (C) contraction duration (A: 100 μM P=0.015 n=10) and (D) frequency when Riluzole added to spontaneously contracting strips in combination with 10 nM Nifedipine.
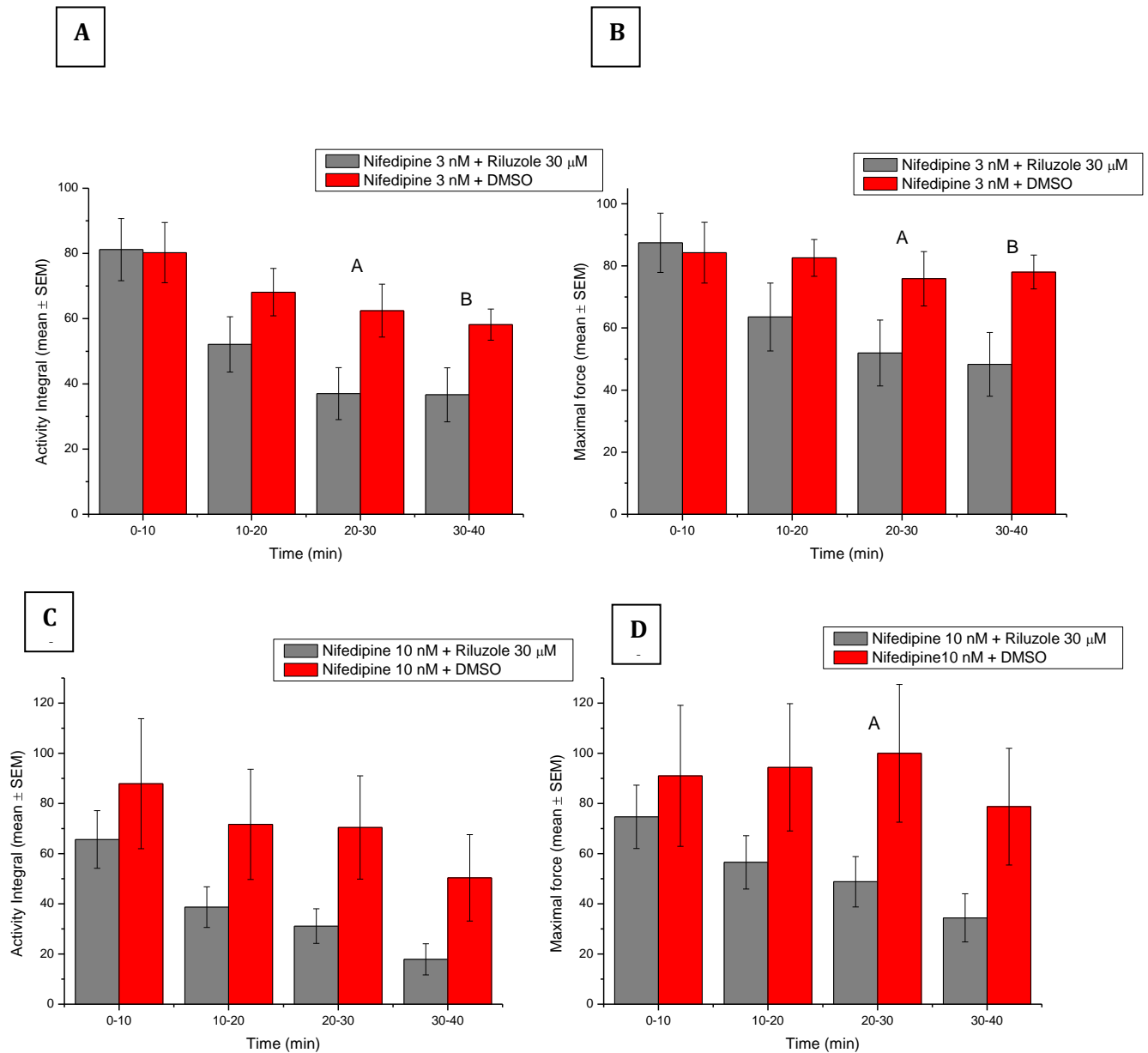
Figure 3.2.7: Mean (± SEM) inhibition of spontaneously contracting strips: (A) activity integral following addition of Nifedipine 3 nM + Riluzole 30 µM (A: 20-30 mins P=0.05 n11, B: 30-40 mins P=0.06 n=11), (B) maximal force following addition Nifedipine 3 nM + Riluzole 30 µM (A:20-30 mins P=0.04 n=11 & B: 30-40 mins P=0.02 n=11) (C) activity integral flowing addition of Nifedipine 10 nM + Riluzole 30 µM, (D) maximal force following addition Nifedipine 10 nM + Riluzole 30 µM (A: 20-30 mins P=0.03 n=11)

137
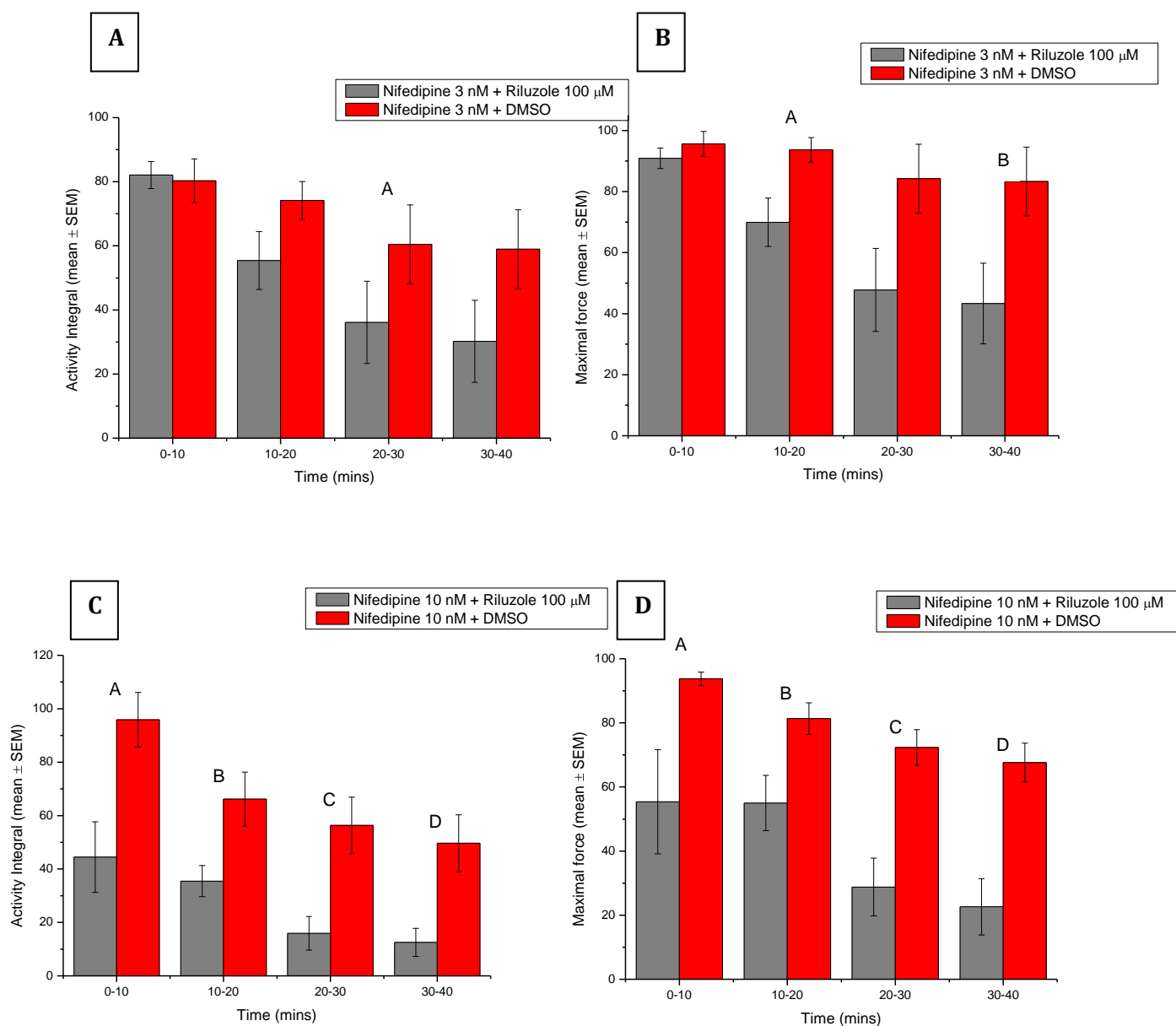
Figure 3.2.8: Mean (± SEM) inhibition of (A) activity integral when Nifedipine 3 nM added in combination with Riluzole 100 µM (A: 10-20 mins P=0.03 n=6), and (B) maximal force (A: 10-20 mins P=0.03 n=6 & B: 30-40 mins P=0.03 n=6), (C) activity integral when Nifedipine 10 nM added in combination with Riluzole 100 µM (A: 0-10 mins P=0.007, B: 10-20 mins P=0.03, C: 20-30 mins P=0.007, D:30-40 mins P=0.007 n=6), and (D) maximal force (A: 0-10 mins P=0.007, B: 10-20 mins P=0.007, C: 20-30 mins P=0.007, D: 30-40 mins P=0.007 n=6)
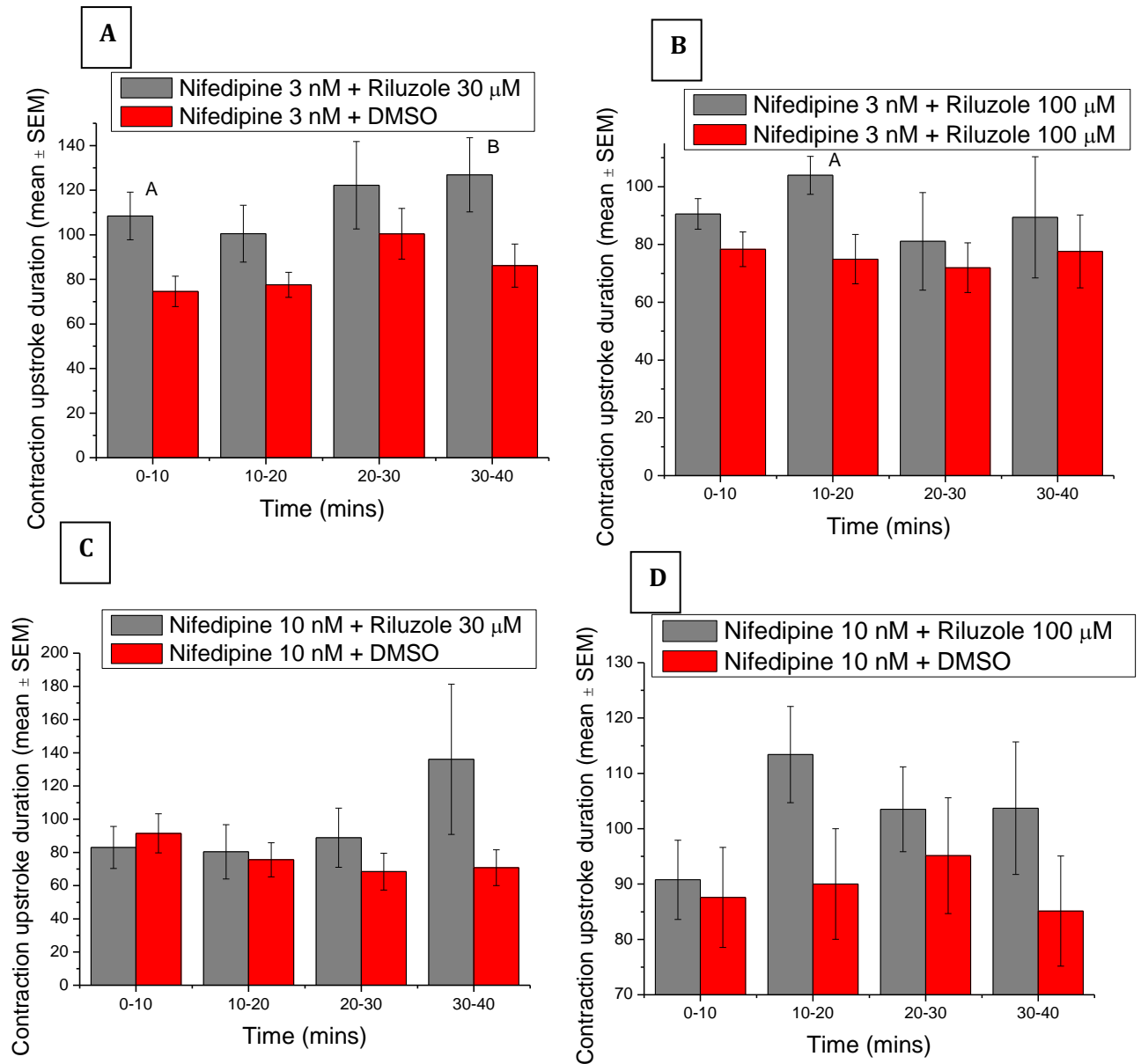
Figure 3.2.9: Mean (± SEM) inhibition of contraction upstroke in (A) Nifedipine 3

nM added in combination with Riluzole 30 μM (A: 0-10 mins & B: 30-40 mins

P=0.02 n=11), and (B) Nifedipine 3 nM added in combination with Riluzole 100

μM (A: 10-20 mins P=0.03 n=6), (C) Nifedipine 10 nM added in combination with

Riluzole 30 μM (n=11), and (D) Nifedipine 10 nM added in combination with

Riluzole 100 μM (n=10)
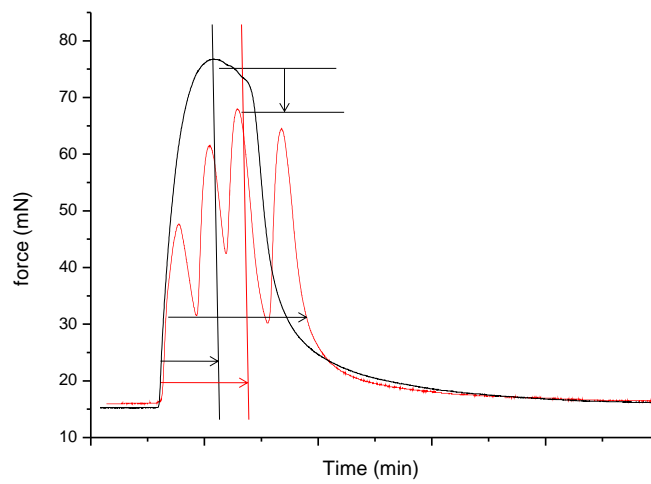
Figure 3.2.10: Changed shape of the phase plot when dosed with Riluzole.

Maximal force is reduced, contraction duration increased and time from baseline

to maximal force or the upstroke period is increased, but with a similar area

under the curve.
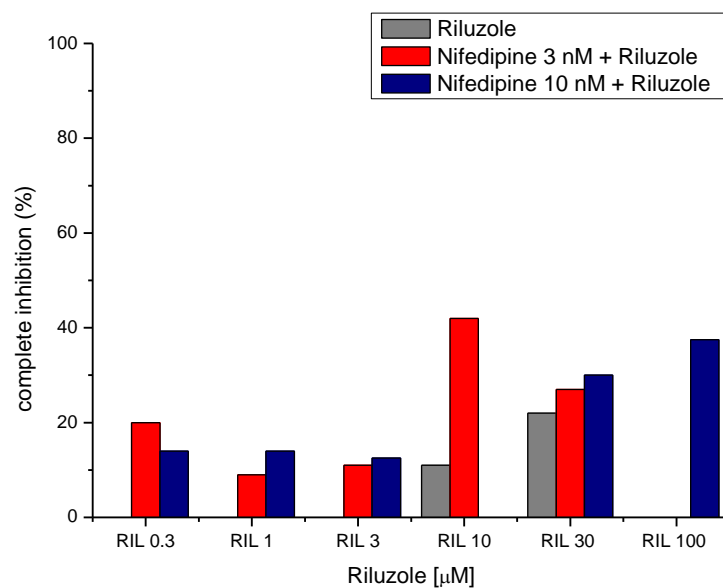
Figure 3.2.11: Graph showing the % of strips in each series that ceased to contract on addition of drug combinations. Statistical significance of likelihood to cease was calculated using binomial sign test. With Riluzole no combination was statistically likely to inhibit contractions.

3.2.3.3 Riluzole in combination with Nifedipine, PGF$_2$α or Oxytocin

Riuzole 100 µM alone, and dosed in combination with Nifedipine 3 nM was added to strips together with PGF$_2$α 10 µM or oxytocin 1 nM in order to measure if these physiologically available contractile agents would mediate any previously seen effect.

As expected, PGF$_2$α and oxytocin alone brought about an increase contraction parameters when added to strips alone *(Figure 3.2.12 & 3.2.13)*. When Riluzole 100 µM was added along with PGF$_2$α and oxytocin the contraction inhibition was similar to that seen without either PGF$_2$α or oxytocin being present. These contractile promoters do not seem to inhibit the effect of Riluzole *(Figure 3.2.12 & 3.2.13)*.
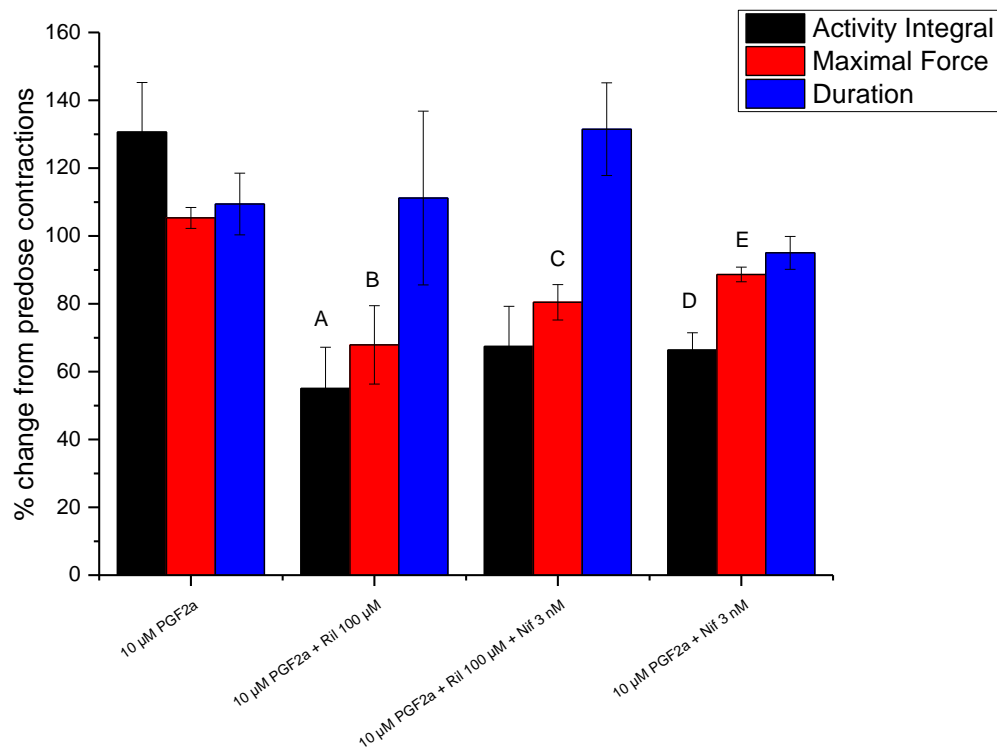
Figure 3.2.12: Mean (± SEM) inhibition of activity integral, maximal force, and contraction duration in strips dosed with either 10 μM PGF$_2$α (n=7), Riluzole 100 μM combined with 10 μM PGF$_2$α (A: AI P=0.01, B: MF P=0.01, n=7), Nifedipine 3 nM combined with Riluzole 100 μM and 10 μM PGF$_2$α (C: MF P=0.05 n=7), or Nifedipine 3 nM combined with 10 μM PGF$_2$α (D: AI P=0.01, E: MF P=0.01 n=7).
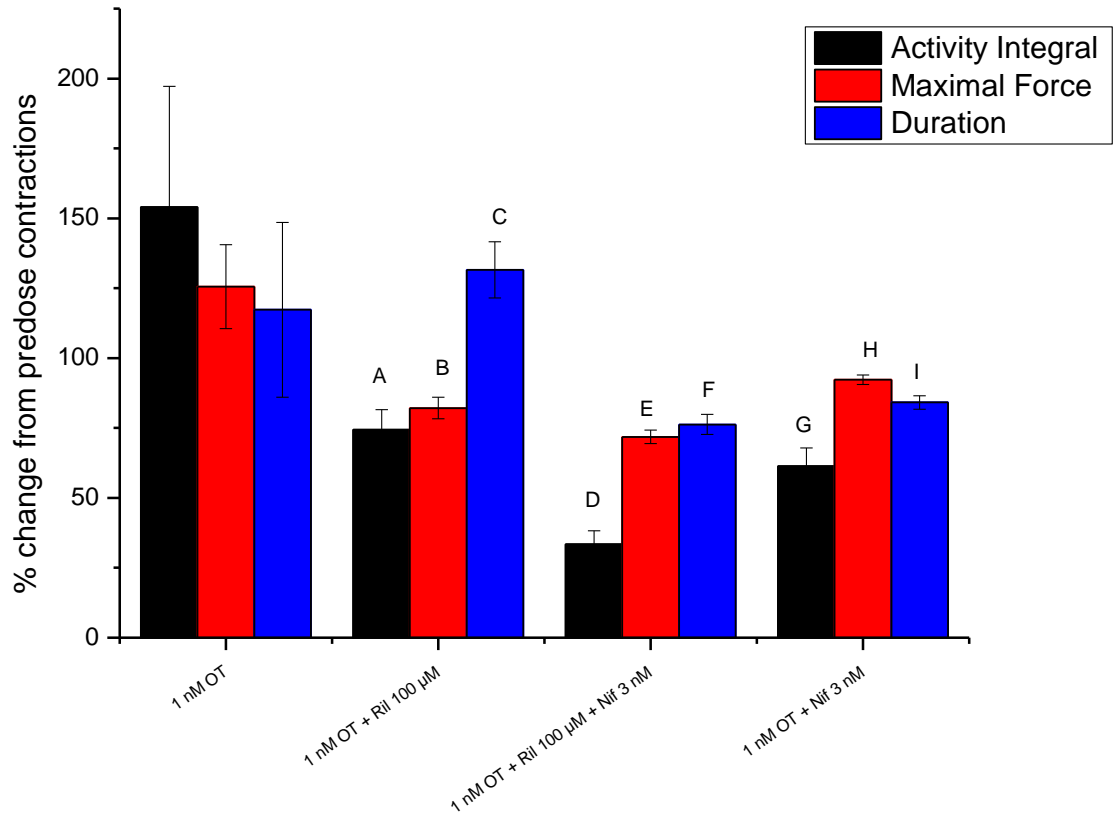
Figure 3.2.13: Mean (± SEM) inhibition of activity integral, maximal force, and contraction duration in strips dosed with either 1 nM oxytocin, 1 nM oxytocin and Riluzole 100 µM (A: AI P=0.03, B: MF P=0.01 C: Dur P=0.03 n=7), 1 nM oxytocin with Riluzole 100 µM and Nifedipine 3 nM (D: AI P=0.01, E: MF P=0.01, F: Dur P=0.01 n=7), and 1 nM oxytocin and Nifedipine 3 nM (G: AI P=0.01, H: MF P=0.01, I: Dur P=0.01 n=7).

3.2.3 Conclusion

There was no effect on contractility but linoloeic acid seen in these experiments. This could be because the target channel for this drug K_v 2.1 does not contribute to resting membrane potential. However it could be to functional issues with the substance, as LA is a fatty acid full permeation of the drug into the myometrial strips could have not been successful and so there was no cellular level action. It could be that the dose range selected was insufficient to have an effect in the organ bath strips. In order to verify the original hypothesis, other substances to target K_v 2.1 could be sought.

Pinacidil alone was effective at inhibiting contractions with total inhibition achievable within the dose range used. These findings support the hypothesis that by activating K_{ATP} channels in myometrium, a hyperpolarised state is maintained, preventing depolarisation and calcium entry via VGCC thus fully inhibiting contractions. The addition of Nifedipine further inhibits Ca^{2+} entry and critical threshold for action potential cannot be achieved at lower doses. Combination of Nifedipine with Pinacidil abolished contractions at a significantly lower dose than with Nifedipine alone (complete inhibition at Nifedipine 3 nM plus Pinacidil 10 µM, verses Nifedipine 30 nM alone).

Pinacidil is well tolerated as an antihypertensive with also a good safety profile but chances of side-effect are higher due to its hypertensive action and further work on vascular effects will be required to assess this risk fully. Riluzole alone inhibited contractions, but within the dose range used, total

inhibition was not achieved. The inhibitory effect of Riluzole was further enhanced when combined with Nifedipine in increasing doses. Riluzole at most concentrated combinations brought about a diminishment of contractions over time and changed the shape of the contraction. Whether *in vivo* this diminishment would be sufficient to delay preterm labour is hard to determine. Riluzole is currently used as a long term therapy for delaying the progression of ALS, is not associated with hypotension and has a good safety profile. Combination with Riluzole *in vivo* may reduce the dose strength of Nifedipine required to inhibit contractions in preterm labour without additional side-effects.

The mechanism of action underlying the effect of Riluzole on myometrial tissue is unknown. A number of possible mechanisms of actions that have been observed in other tissues points to a number of possibilities that would need further investigation. It may be that the inhibition seen is due to opening of potassium channel, either SK3 or TREK or a combination of these or other ion channels; and this is further explored in the discussion. If Riluzole is functioning on SK3 or TREK then perhaps a higher dose would be required to achieve sufficient K^+ efflux to maintain hyperpolarisation. The effect seen was not mediated by $PGF_2\alpha$ or oxytocin.

Prior to considering clinical use of a combination of potassium channel openers with Nifedipine, effects on vascular smooth muscle must be investigated. There needs to be strong indication that additional hypotensive effects will be avoided.

3.3 The effects of combined dose of Nifedipine with potassium channel openers on myometrial small arteries

3.3.1 Clinical relevance and background

The most commonly reported side effect associated with Nifedipine use as a tocolytic is hypotension, with concerns for fetal wellbeing as a result (de Heus et al 2009). The overall efficacy of Nifedipine in halting labour and reducing length of time form treatment to birth is similar to other tocolytics, some of which are associated with additional maternal and fetal side-effects (de Heus 2009). Often tocolytic compounds lack full clinical effectiveness and preterm birth is not prevented. This could be attributed to incomplete inhibition of the contractile pathway. In theory, calcium channel blockers will halt the contractile mechanism definitively but preventing large conductance calcium entry. As demonstrated in the previous experiments an optimal dose of Nifedipine will entirely inhibit contractions, but the real and clear barrier to achieving this *in vivo* is the fact that higher dose of Nifedipine would have more significant cardiovascular side-effects leading to poorer outcomes and as such the Nifedipine dose for tocolysis is limited (RCOG 2011). It has been the aim so far to establish the efficacy of a combined effect of Nifedipine with select potassium channel openers to enhance the tocolytic effect in the myometrium without additional cardiovascular impact. A previous screen has indicated that selected target K^+ channels are not as abundantly expressed in VSMC as in MSMC. This led to the hypothesis that adverse cardiovascular effects would be

avoided. This would be vital to establishing any possible clinical relevance of drug combinations and would need to take into consideration the influence of endothelial- mediated response to these agents. Previous experiments have demonstrated the combined effects of select potassium channel openers and Nifedipine on myometrium. Therefore we undertook the next set of experiments to explore the effect of these drug combinations on myometrial small arteries. Small arteries were dissected from myometrial biopsies obtained with consent at elective caesarean section at term. After a period of normalisation myometrial small arteries were exposed to the thromboxane A2 receptor agonist U46619 (1 μM), which also mimics the effect of vasopressin to bring about constriction of the artery. Following a washout period the selected drug combinations (or vehicle) were added for incubation to the myograph before a repeated exposure to U46619 (*Figure 3.3.1*). Data was analysed in terms of the ratio of pre-drug incubation constriction force/post-drug incubation constriction force (please see chapter 2 of this thesis for full details of methods used).

In addition we sought to quantify the expression of the target channels for these drugs, SK_3 and TREK-1 in myometrial small arteries compared to myometrial smooth muscle to demonstrate selectivity.
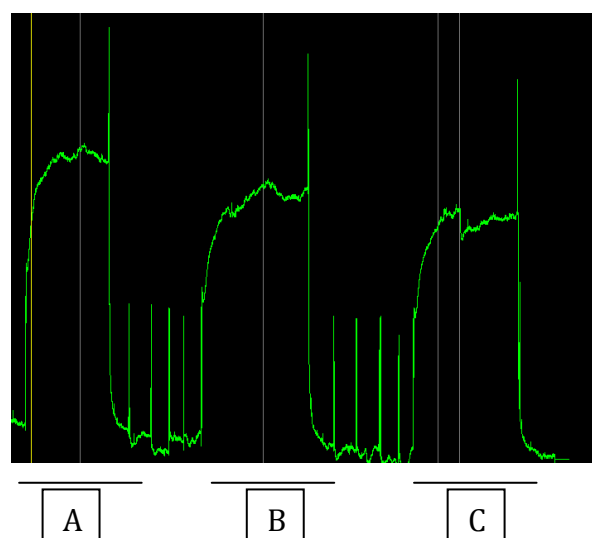
Figure 3.3.1: Typical image of screen recording of artery contractile response to (A) U46691 alone, (B) response to U46619 following incubation with drug of interest (in this example Riluzole 100 μM), and (C) response to U46619 following washout. (The spikes in between contractile phases are artefacts caused by organ bath washout and fluid replenishing at approx 20 min intervals). Recorded using Miodaq 2.0

3.3.2 Objective

I.   To determine the effect of selected combined doses of Nifedipine plus Pinacidil and Riluzole on the contractile responses of myometrial small arteries to U46619

II. To determine the expression of SK3 and TREK-1 in human pregnant myometrium and human pregnant myometrial small arteries.

3.3.3. Results

3.3.3.1 Results – the effect of Nifedipine on myometrial small arteries

Nifedipine at 10 nM brought about a reduction in baseline tension of -2.31 ± 0.72% from pre-incubation tone. When U46619 was added in the presence of Nifedipine 10 nM the maximal tone was significantly reduced at 56.84 ± 7.13% (P= 0.00006 n=8) of the non-treated response. Following a washout of the drug, re-exposure to U46619 elicited 89.04 ± 5.66% of the pre-treatment maximal tone (*Figure 3.3.2*).

3.3.3.2 Results – the effect of Pinacidil on myometral small arteries

Pinacidil at 3 µM brought about a mean reduction in baseline tension of -2.82 ± 0.72% from pre-incubation tone. Contractile response to U46619 in the presence of Pinacidil 3 µM significantly reduced at a mean of 54.15 ± 16.32% (P= 0.039 n=6) of the non-treated response to U46619. As with the myometrial smooth muscle experiments, there were some arteries that showed a greater response and some less at 13.52, 20, 20, 85.56, 89.53, 96.30% respectively. Following a washout of the drug, re-exposure to U46619 elicited a mean of 96.96 ± 13.18% of the pre-treatment maximal tone.

Pinacidil at 10 µM resulted in a slight increase of mean baseline tone of 0.27 ±1.05, and when U46619 was added a mean of 35.49 ± 24.6% (P= 0.054 n=4) of

non-treated response was achieved. As in other experiments there was a split in response with individual responses of 6.75, 7.10, 19.08, 108.73% hence non-significant results from the mean value.

Following drug washout, exposure to U46619 resulted in mean 97.84 ±5.93% of pre-treatment maximal tone (*Figure 3.3.2*).

3.3.3.3 *Results – the effect of Nifedipine combined with Pinacidil on myometrial small arteries*

Simultaneous pre-dose incubation of Nifedipine 10 nM and Pinacidil 3 µM resulted in a slight reduction in mean baseline tension of -1.70 ±1.05%. When U46619 was added, there was a significant reduction in mean maximal tone of 37.8 ±7.4% (P= 0.0005 n=6) of the pre-dose response. There was a less polarised response seen in this group with a range of 11.92-62.96%, hence the significant result. After the washout, a mean maximal tone achieved was 92.1 ±5.34% of the pre-dose response.

Nifedipine 10 nM and Pinacidil 10 µM resulted in a mean baseline tension of 6.2 ±4.59%. With the addition of U46619 a mean maximal tone of 53.48 ±19.6% (P=0.74 n=5) of the pre-dose response. Once again there was a polarised response with individual artery responses of 3.49, 10.82, 69.84 and 101.28% explaining the non-significant result from the mean response. Following drug washout there was a mean maximal tone of 72 ±19.78% of the pre-dose response (*Figure 3.3.2*).

3.3.3.4 *Results – the effect of Riluzole on myometrial small arteries*

Pre-dose incubation of Riluzole 100 µM resulted in a slight increase in mean baseline tone of 1.90 ± 1.15%. On addition of U46619, mean maximal tone achieved was 92 ±5.19 (P= 0.364 n=6) of the pre-dosed response. The response was more consistent than seen with Pinacidil with a range of 72.77-96.30%. Following drug washout the mean maximal tone was 92 ±6.48% (*Figure 3.3.2)*.

3.3.3.5 *Results – the effect of Nifedipine combined with Riluzole on myometrial small arteries*

Simultaneous pre-dose incubation of Nifedipine 10 nM and Riluzole 100 µM brought about a slight increase of baseline tension 0.75 ±0.56%. Following addition of U46619, mean maximal tone was 70.6 ±7.51% (P= 0.0102 n=5). After drug washout the mean baseline tone was 101.9 ±2.2% of the pre-dose tone (*Figure 3.3.2*).
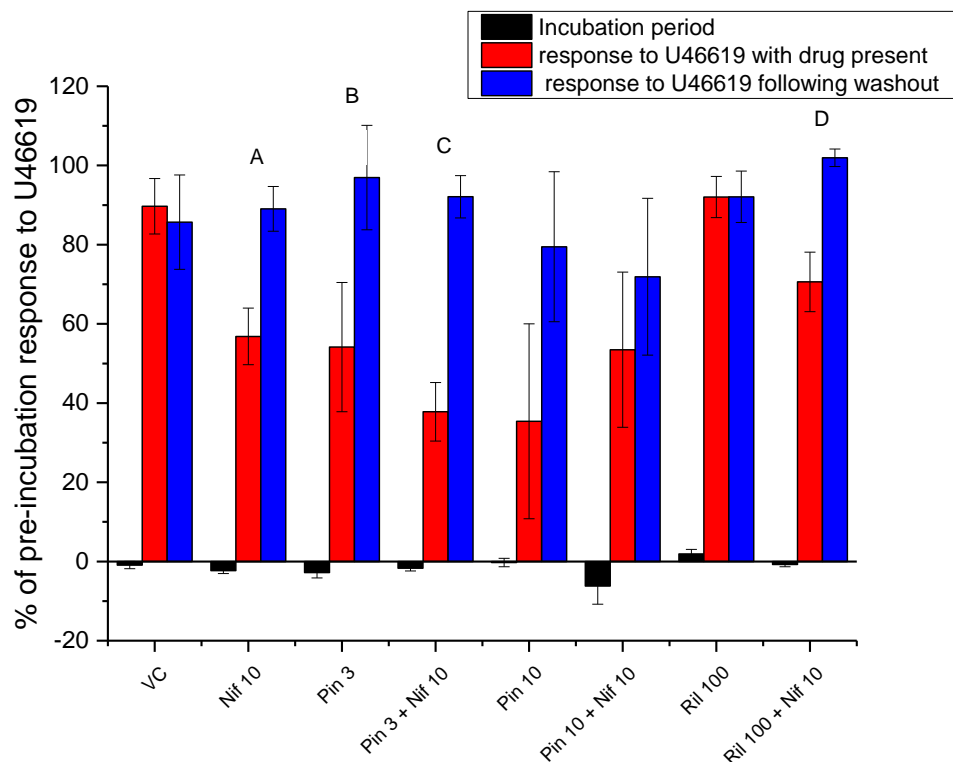
Figure 3.3.2: Graph to show % of pre-incubation response to U46619 following 20 minute incubation of the drug (black), response to U46619 while drug present (red) and response to U46619 following drug washout (blue) for the following drugs/combinations: Vehicle control (VC) (n=5), Nifedipine 10 nM (A: P=0.00006 n=8), Pinacidil 3 µM (B: P=0.039 n=6), Pinacidil 3 µM plus Nifedipine 10 nM (C: P=0.0005 n=6), Pinacidil 10 µM (n=4), Pinacidil 10 µM plus Nifedipine 10 nM (n=5), Riluzole 100 µM (n=6), Nifedipine 10 nM plus Riluzole 100 µM (D: P=0.0102 n=5).

Drug	Vehicle Control	NIF 10	PIN 3	PIN 3+ NIF 10	PIN 10	PIN 10 + NIF 10	RIL 100	RIL 100 + NIF 10
Drug incubation (% of pre-dosed maximal tone)	-0.9	-2.31	-2.82	-1.70	0.27	6.2	1.90	-0.75
± SEM (%)	0.911	0.72	1.32	0.7	1.05	4.59	1.15	0.56
With U46619 (% of pre-dosed maximal tone)	89.69	56.84	54.15	37.8	35.41	53.48	92	70.6
± SEM (%)	7	7.13	16.32	7.4	24.6	19.6	5.19	7.51
T-test P value	0.42232	0.00006***	0.039*	0.0005***	0.054	0.074	0.364	0.0102*
With U46619 following drug washout (% of pre-dosed maximal tone)	85.7	89.04	96.96	92.1	97.84	72	92	101.9
± SEM (%)	11.9	5.66	13.18	5.34	5.93	19.78	6.48	2.2
N	5	8	6	6	4	5	6	5

Table 3.3: Summary of results (mean values)

3.3.3.6 Results – the expression of SK$_3$ in human pregnant myometrium

Tissue samples consisted of 4 groups of myometrium samples, pre-term labour (PTL n=6), pre-term non-labour (PTNL n=6), term labour (TL n=6), term non-labour (TNL n=6) and quantified by western blotting. There was no significant difference in the expression of SK$_3$ by gestation in myometrium, and no significant difference between labour and non-labour samples (*Figure 3.3.3*).
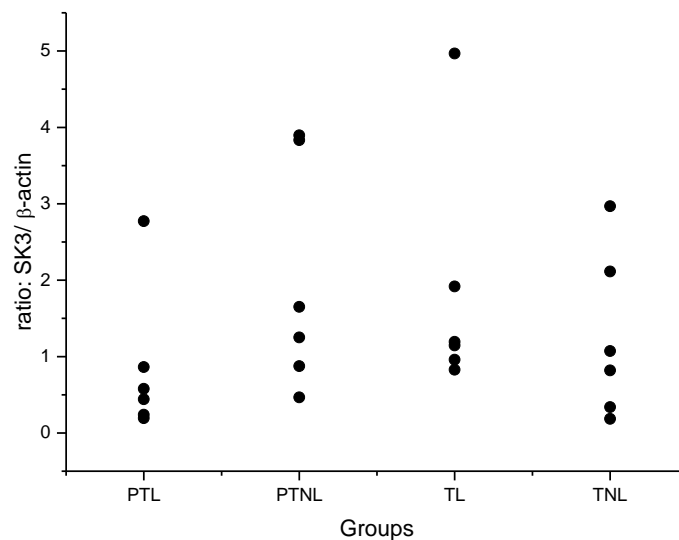
Figure 3.3.3: The expression of SK$_3$ in myometrial tissue (ratio SK$_3$/β-actin) from 4 groups of samples: pre-term labour (PTL n=6), pre-term non-labour (PTNL n=6), term labour (TL n=6), term non-labour (TNL n=6) quantified by western blot.

3.3.3.7 Results – the expression of SK$_3$ in human pregnant myometrial

small arteries compared to expression in myometrium

Myometrial small arteries were dissected from term non-labour (TNL

n=6) myometrial biopsies and quantified by western blotting along with

whole sample myometrium from additional term non-labour samples

(n=2) (*Figure 3.3.4*). There was a non-significant difference in expression

of SK$_3$ with a tendency to higher expression in myometrial tissue

compared to myometrial small arteries (*Figure 3.3.5).*

MA MA MA MYO MA MA MA MYO

SK$_3$ 96 kDa
β-actin   42 kDa
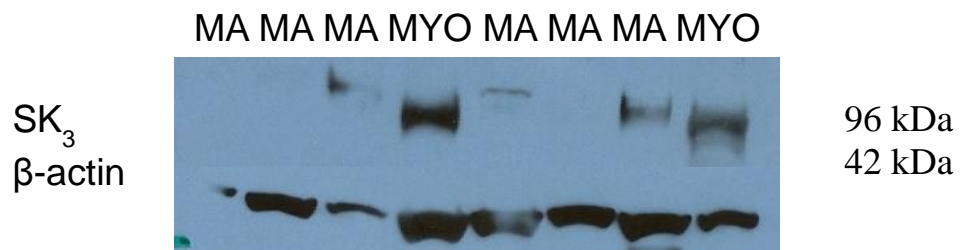
Figure 3.3.4: Image of exposed western blot film to show bands detected

for SK$_3$ in myometrial small arteries (MA; term non-labor; n=6) and

myometrial tissue (MYO; term non-labour; n=2) compared to the control

protein β-actin.
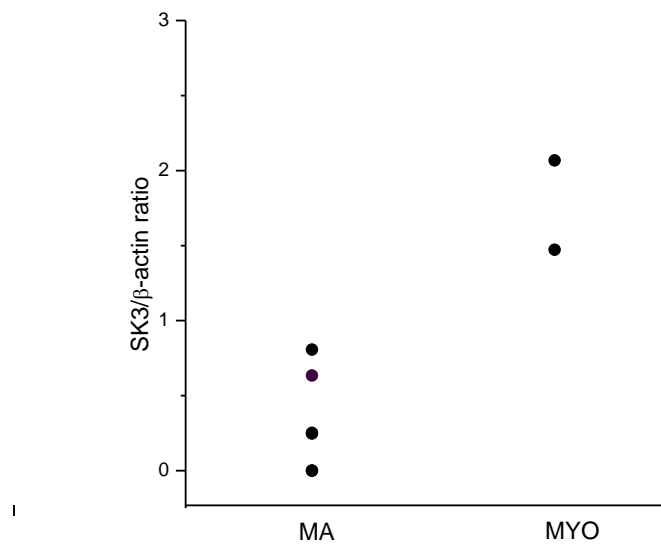
Figure 3.3.5: Expression of SK3 (ratio SK₃/β-actin) in myometrial small arteries (MA; term non-labour; n=6) and myometrial tissue (MYO; term non-labour; n=2).

3.3.3.8 *Results – the expression of TREK-1 in human pregnant myometrium*

Tissue samples consisted of 4 groups of myometrium samples, pre-term labour (PTL n=4), pre-term non-labour (PTNL n=4), term labour (TL n=4), term non-labour (TNL n=3, (1 outlier excluded)) and quantified by western blotting. There was no significant difference in the expression of TREK-1 by gestation in myometrium, and no significant difference between labour and non-labour samples (*Figure 3.3.6*).
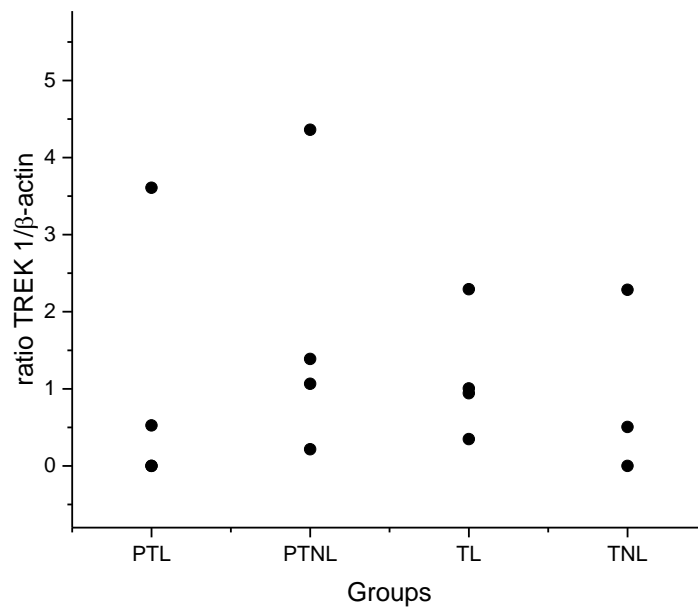
Figure 3.3.6: The expression of TREK-1 in myometrial tissue (ratio TREK-1/β-actin) from 4 groups of samples: pre-term labour (PTL n=4), pre-term non-labour (PTNL n=4), term labour (TL n=4), term non-labour (TNL n=3) quantified by western blot.

3.3.3.9 Results - the expression of TREK-1 in human pregnant myometrial small arteries compared to expression in myometrium

Myometrial small arteries were dissected from term non-labour (TNL n=6) myometrial biopsies and quantified by western blotting along with whole sample myometrium from additional term non-labour samples (n=2) (*Figure 3.3.7*). No TREK-1 was detected in myometrial small arteries, but was detected in myometrial whole tissue (*Figure 3.3.8).*

MA MA MA MYO MA MA MA MYO

TREK-1   55 kDa
β-actin   42 kDa

Figure 3.3.7: *Image of exposed western blot film to show bands detected for TREK-1 in myometrial small arteries (MA; term non-labor; n=6) and myometrial tissue (MYO; term non-labour; n=2) compared to the control protein β-actin.*
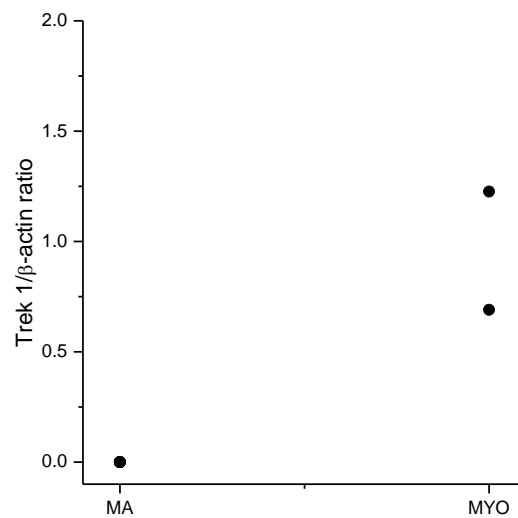
Figure 3.3.8: *Expression of TREK-1 (ratio TREK-1/β-actin) in myometrial small arteries (MA; term non-labour; n=6) and myometrial tissue (MYO; term non-labour; n=2).*

3.3.4 Conclusion

This group of experiments aimed to measure the effect of drug combinations on myometrial small arteries and the expression of select drug target K^+ channels in myometrial small arteries compared to whole myometrial tissue in order to speculate as to the possible clinical side effects that may be seen with this regime.

The myography experiments were conducted at Newcastle University's Institute of Cellular medicine under their guidance and the kind use of their equipment. There was a limited time available to conduct this work and so this is reflected in the low N in these experiments. Additional experiments with greater N would be beneficial to confirm the reliability of the results shown here and as such interpretation of findings is with caution.

Pinacidil showed a relaxatory effect on small artery function, which was anticipated. There were polarised responses to Pinacidil with some tissue samples, which could be classified as 'non-responders' which is a phenomenon observed in previous contractility experiments. Nifedipine alone had a significant effect at near halving the response to U46619 than seen before drug incubation. Combination with Pinacidil 3 µM showed a cumulative effect on further relaxation, a trend that may have been evident at the 10 µM dose were it not for the 'non-responders' to Pinacidil.

The reduced relaxatory effect on small arteries shown with Riluzole was initially reassuring as to its therapeutic potential. When combined with Nifedipine however, it appeared that the combination with Riluzole had a protective effect

on relaxation with less of a relaxatory response than when Nifedipine was dosed alone (70.6% vs 56.8%).

Crane & Garland (2004) found that U46619 blocked SK$_3$ channel in rat mesenteric artery, Riluzole (100 µM) evoked membrane hyperpolarisation from -55±2 mV to -71±3 mV (n=5) and this was reduced with repeated exposure to U46619 (incremental doses 1 nM to 0.1 µM) to 58±2 mV. Hyperpolarisation following application of levcromakalin (K$_{ATP}$ activator) was unaffected by repeat exposure to U46619.

Although this effect was only seen in repeated exposure to U46619 and in this set of experiments the dosing period was the second exposure to U46619 with drug pre-incubation preceding exposure (following a washout period) it is unclear to what extent there may have been a block of SK$_3$ channels from U46619 that may have inhibited the effect of Riluzole in these experiments.

Tissue samples used for protein quantification through Western blotting was obtained by dissecting myometrial small arteries from whole biopsies. This yielded a very small amount of tissue for sampling. Band variation in size and quality of blots mean that the results presented above are less than conclusive. Further, more accurate quantification of channel expression through techniques such as reverse transcription polymerase chain reaction (RT-PCR), or immunohistochemistry would be desirable before drawing any conclusions on difference in expression between myometrial smooth muscle and myometrial smooth arteries.

3.4. The effect of VU590 on myometrial contractility and expression of $K_{ir}7.1$ and $K_v2.1$ in human myometrium

3.4.1 Clinical relevance and background

3.4.1.1 Clinical relevance

Post-partum haemorrhage (PPH) is a significant blood loss (> 500 mls) during or after the 3rd stage of labour and can occur following a normal, assisted or surgical birth. Primary PPH is a significant blood loss in the 24 hours post birth with a secondary PPH occurring anywhere up to 6 weeks post-partum. Such a significant blood loss can lead to a rapid loss in haemodynamic stability, represents a risk to maternal morbidity and mortality; can often be unanticipated and is considered an obstetric emergency.

Uterine atony is the most common cause of PPH. Clinical strategies to manage PPH include mechanical methods such as catheterisation, manual compression, balloon tamponade, internal iliac ligation; or pharmacoloigcal.

Current pharmacological approaches include synthetic Oxytocin, ergot alkaloids, prostaglandins (PGF2α, prostaglandin E_1). All of these have different pharmacological mechanism of action but ultimately result in increased Ca^{2+} entry and therefore an increase in contractions. Often, more than one pharmacological agent or a mix of pharmacology and mechanical treatments are required to maintain uterine tone and haemostasis. If unsuccessful, then an obstetric hysterectomy is the only option.

3.4.1.2 Scientific background

The inwardly rectifying potassium channel K_{ir} 7.1 was identified as a channel of interest in a previous screen of myometrial specific K^+ channel expression.

In laser capture selected human myometrial smooth muscle, an 8-fold increase in the gene encoding K_{ir} 7.1 KCNJ13 mRNA was seen compared to whole biopsy tissue at term as well as increased expression observed in murine myometrium which peaked at D15.

K_{ir} channels function through electrochemical gradient and are voltage independent and therefore have a potential role in setting and maintaining resting potential and action potential duration. The gestational dependant decrease in expression towards term observed in murine tissue points to a possible role for K_{ir} 7.1 in the gestational-dependent depolarisation in resting membrane potential towards term in myometrium. The function of K_{ir} 7.1 had not previously been investigated in myometrium but the availability of the novel intracellular blocker of K_{ir} 7.1, VU590 has made investigation possible. Building on the previous work that identified expression of K_{ir} 7.1 in human myometrium it is yet to be demonstrated if there is a gestational variation in expression in human myometrium which was observed in murine myometrium.

We hypothesise that by blocking inwardly rectifying K^+ current via K_{ir} 7.1 would prevent repolarisation and prolong action potential by prolonging the voltage threshold for calcium entry via VGCCs.

3.4.2 <u>Objective</u>

I.   To assess the differential effect of the selective K_{ir} 7.1 blocker VU590 on spontaneous murine myometrial contractility at day 15 and day 18 gestation. To assess the combined impact of Oxytocin plus VU590 on spontaneous murine myometrial contractility at day 15 or day 18 gestation.

II. To assess the impact of VU590 on spontaneously contracting human myometrial strips. To assess the combined impact of Oxytocin plus VU590 on spontaneously contracting human myometrial strips.

III. To assess the expression of VU590 targets K_{ir} 7.1 and K_v 2.1 in human pregnant myometrium

3.4.3 Results – the effect of VU590 on contractility of murine myometrium

3.4.3.1 VU590 elicits a significant rise in baseline tone in murine myometrium with gestational dependent differences.

Dose series administration of VU590 in D18 murine myometrium elicited a significant increase in baseline tone at 30 µM (167±45% (P<0.001 n=4)) and 100 µM (343±113% (P<0.001 n=4)). No other parameters differed significantly from pre-dose values *(Figure 3.4.1 (A))*. VU590 10 µM elicited a baseline rise of 112±28% (n=6) in D18 murine myometrium whereas the same dose in D15 tissue saw an increased baseline rise of 180±59% (n=6) demonstrating a gestational dependant difference in response to VU590 *(Figure 3.4.1 (B&C))*.

3.4.3.2 VU590 combined with Oxytocin elicits a significant rise in baseline

In combination with Oxytocin a more significant rise in baseline was observed in D18 (466±161% (n=3)) myometrium than D15 (252±75% (P<0.001)) *(Figure 3.4.1 (B&C))*. The combination with Oxytocin had an effect that mimicked a 10 fold higher dose of VU590 alone in D18 tissue. There was again no significant effect on other parameters.
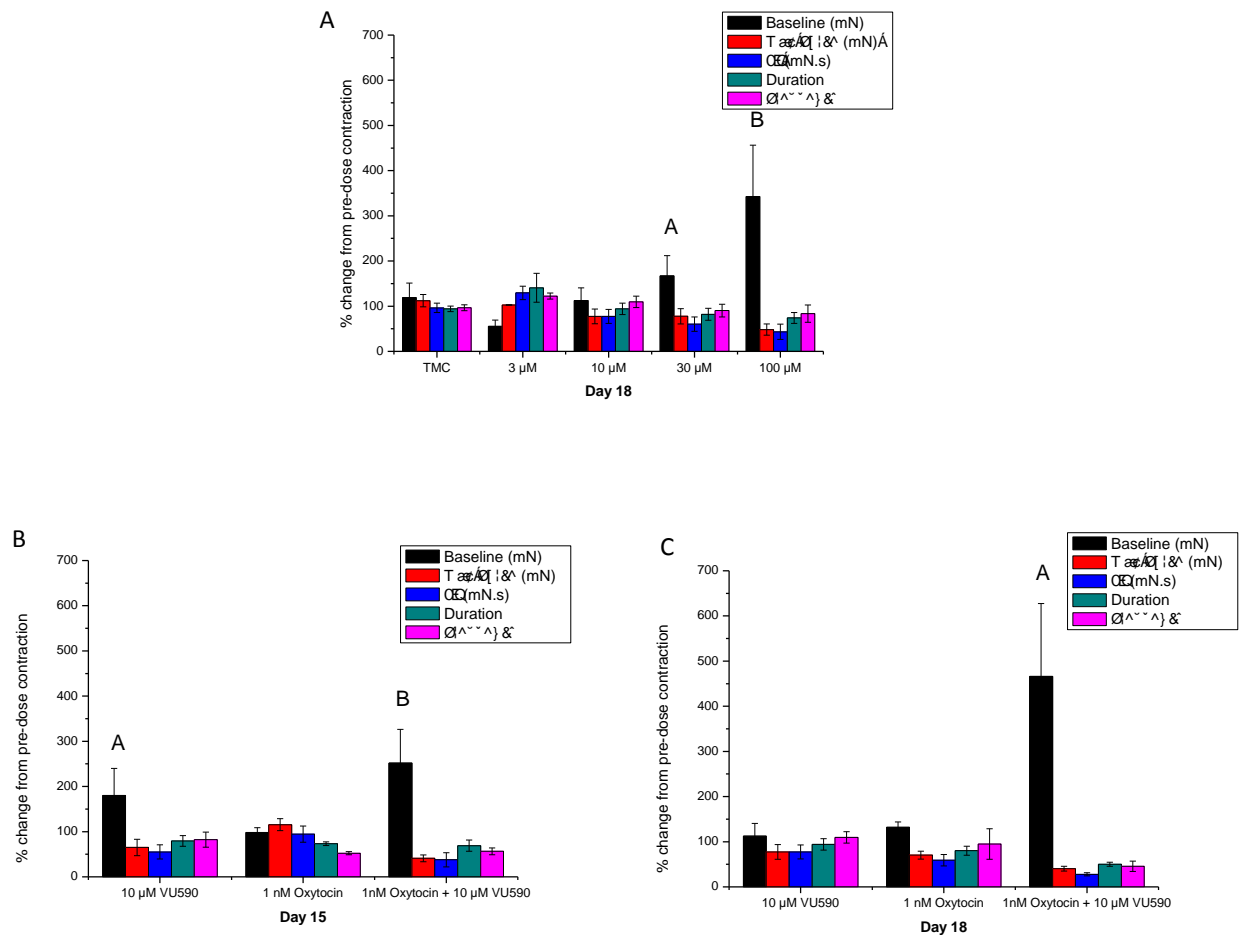
Figure 3.4.1: % change in measured parameters from pre-dose contractions with dose spread of VU590 alone in D18 murine myometrium (A) (A: P<0.001 n=4, B:P<0.001 n=6), and 1 nM Oxytocin, 10 µM VU590 with and without Oxytocin in D15 (n=5) (B) (A: P<0.001, B: P<0.001 n=5) and D18 murine myometrium (n=5) (C) (A: P<0.0001 n=3).

3.4.3.3 *VU590 alone and combined with Oxytocin elicits a significant rise in total activity integral in murine myometrium*

Such was the rise in baseline observed in the previously described experiments, with additional small contractile peaks above the baseline, re-analysis was undertaken where the whole time rise from baseline was recorded as activity integral and compared to pre-dose contraction activity integral. There was a dose-dependent and gestational differentiated increase in activity integral, rising to 1596±543% in day 18 myometrium and 1024±203% in day 15 myometrium following the highest dose of VU590 100 μM (*Figure 3.4.2*).

With the combination of Oxytocin 1 nM and VU590 10 μM there was a 17294±1457% increase in activity integral in day 15 and 8825±4310% in day 18 myometrium compared to an increase with VU590 10 μM alone of 935±429% at day 15 and 528±78% at day 18 (*Figure3.4.3*).
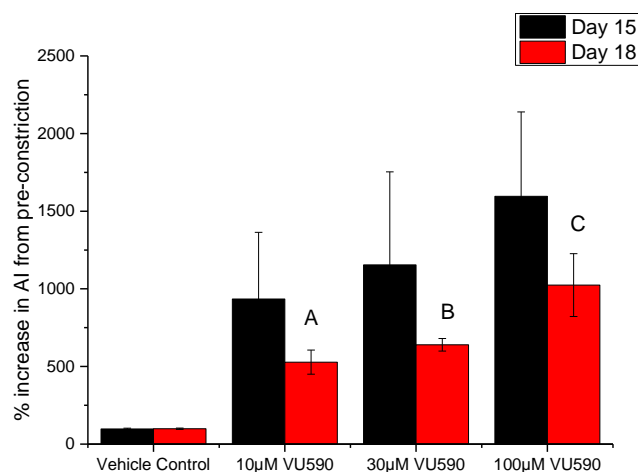
Figure 3.4.2: Gestation and dose dependent effect of VU590 on total activity integral as a % of pre-dose contractions, mean and SE (*D15 vs D18 A,B,C: P=<0.05 n=5).
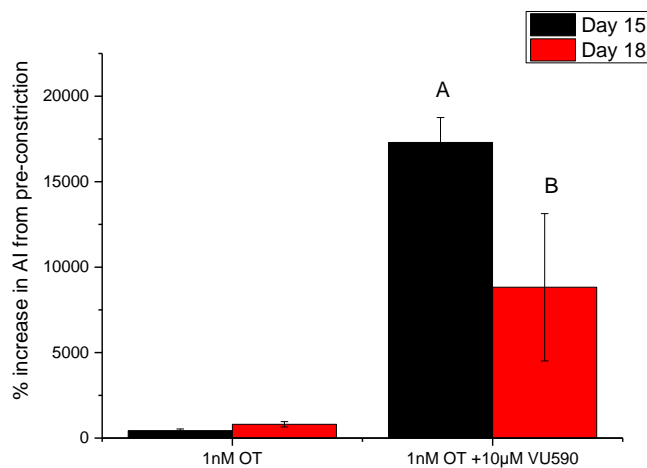
Figure 3.4.3: Gestation dependent effect of VU590 10µ M with and without Oxytocin 1 nM on total activity integral as a % of pre-dose contractions, mean and SE (*OT vs OT+VU590 A: P<0.05(D15) B:<0.05 (D18) n=5).

3.4.4 Results – the effect of VU590 on contractility of human myometrium

3.4.4.1 VU590 alone

Following administration of VU590 the effect on contractility differed from the immediate post-dose time period, through a prolonged contraction phase to remaining contractile activity. Based on this observation the overall effect was split into three phases in order to allow for more accurate quantification and comparison of effect (*Figure 3.4.4*). Analysis was split in 3 phases (1) first five contractions following dosing, (2) prolonged contraction phase and (3) remaining contractions *(Figure 3.4.4)*. For each phase the activity integral (AI), maximal force (MF) and contraction duration (CD) per contraction was measured and expressed as a percentage of mean baseline activity before dose. Results are expressed as ±SEM. Significance was determined by Wilcoxon signed-rank test.

3.4.4.2 VU590 augments spontaneous human myometrial contractions with prolonged contractile phase.

Phase (1) First 5 contractions

In the first 5 contractions AI increased on a contraction-by-contraction manner until reaching 167±22% (P=0.001 n=9) by contraction 5 following 1 µM VU590 *(Figure 3.4.5 (B))*. Following the first five contractions the 1 µM dose contractions continued at a regular rate and did not achieve the prolonged contraction phase seen in other doses.

There was also a dose dependent increase in AI at 3 µM (146±14% (P=0.05 n=10)) increase by contraction 5 and 10 µM (171±38% (n=6)) increase by contraction 5. There was minimal effect of CD and MF within the first 5 contractions. At 30 µM there was a slight reduction in all parameters and at 100 µM there was an increase in AI by contraction 5 where other parameters decreased *(Figure 3.4.5)*.

In strips dosed with either 30 µM or 100 µM, 40% and 60% respectively had already begun the prolonged contraction phase before the 5[th] contraction reducing the *n* numbers contributing to the data in the graphs.

Phase (2) Prolonged contraction phase

From 3 µM and higher a prolonged contraction was observed *(table 3.4.1)*. This contraction would start with a regular upstroke and continue for a prolonged but gradually diminishing contraction. Peak effect was at 10 µM VU590 with CD of 6.7±1.9 hrs (P= 0.001 n=7) *(Figure 3.4.6)* accompanied by increase in AI 1139±305% (P<0.001, n=7) *(Figure 3.4.7)*.

Phase (3) Remaining contractions

Of those experiments where contractions continued or resumed following prolonged contraction *(Table 3.4.1)* the remaining contractions were analysed *(Figure 3.4.8)*. Experiments were often left running overnight meaning that the timescale for each experiment was an average of 18 hours. If contractions resumed they would continue until the experiment was discontinued. Maximal increase in AI from pre-dose at 203±27% (P<0.05, n=9) was with 3 µM VU590. All doses saw an increased AI and SD with diminishing MF reflecting a change in

contraction shape. The shape changed from a typical plateau shape to a box
shape contraction with regular frequency *(Figure 3.4.9).*
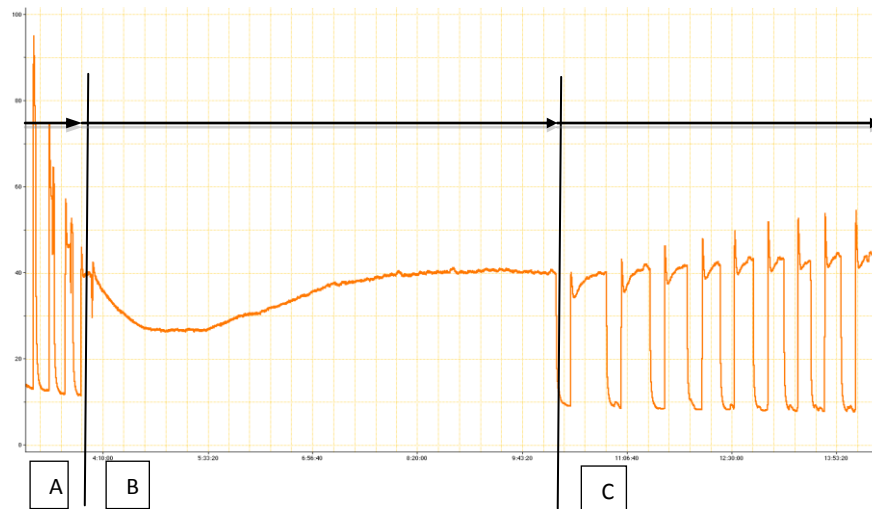
*Figure 4.3.4: An example of a dose response to VU590 in spontaneously
contracting human myometrium with illustration of divisions for analysis.*

*Phase (1) – the first 5 contractions following dose (A), Phase (2) – the prolonged
contraction phase (B), Phase (3) averaged remaining contractions (C).*
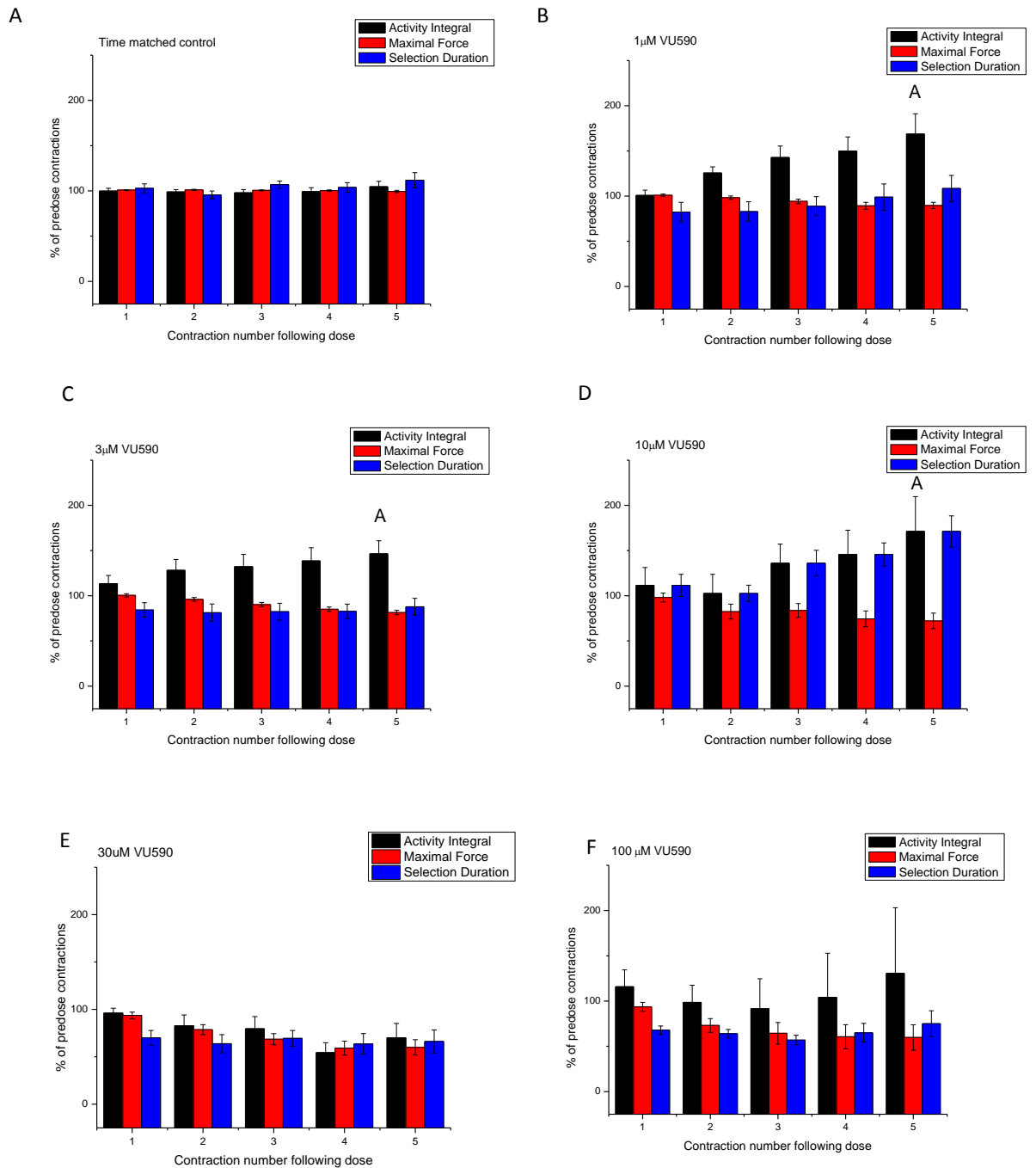
Figure 3.4.5: The percentage of activity integral, maximal force, and selection duration of pre-dose contractions of each of the first 5 contractions following dosing with either 1 μM (B)(A: P<0.001 n=9), 3 μM (A: P<0.001 n=10) (C), 10 μM (A: P<0.001 n=7) (D), 30 μM (n=6) (E), 100 μM (n=6)(F) VU590 or time matched control (A).

VU590 dose	Number of experiments with prolonged contraction	Number of experiments with prolonged contraction that continued contracting
3 µM	3/10	0
10 µM	7/10	2/7 (28.5%)
30 µM	8/10	6/8 (75%)
100 µM	10/10	3/10 (30%)

Table 3.4.1: Table showing the number of experiments with each dose that had a prolonged contraction phase and the number of those that continued to contract following the prolonged contraction.
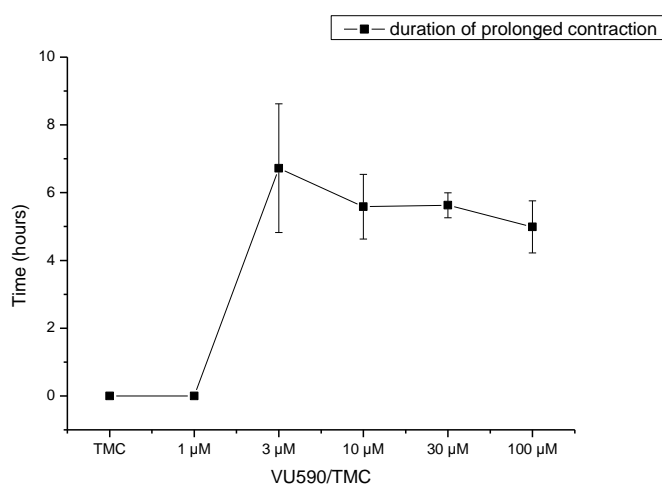
Figure 3.4.6: The average duration of the prolonged contraction phase in hours for each dose of VU590(n=7)
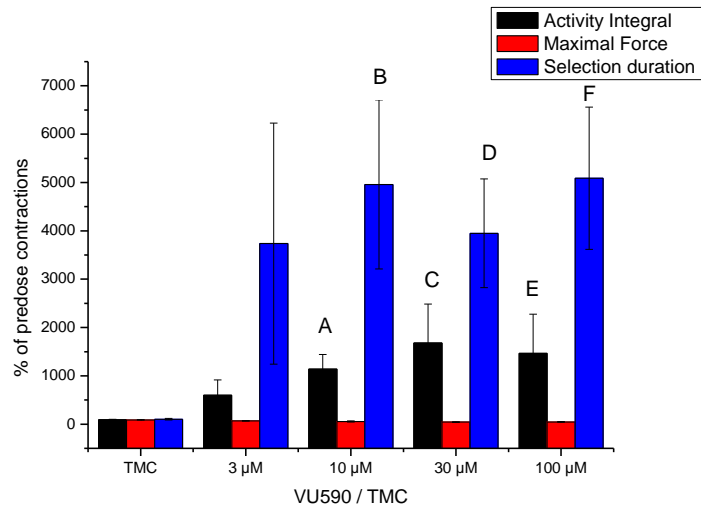
Figure 3.4.7: The percentage of activity integral, maximal force, and selection duration of pre-dose contractions of the prolonged contraction phase following dosing with VU590 vs a time match control (A,B,C,D,E,F: P <0.05)
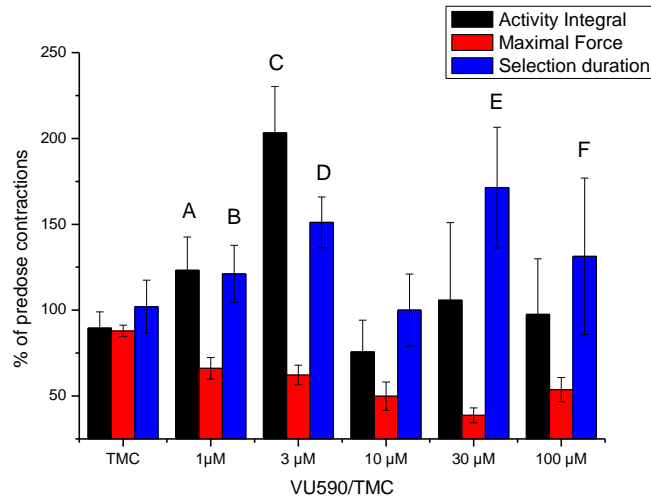
Figure 3.4.8: The percentage of activity integral, maximal force, and selection duration of pre-dose contractions of average of remaining contractions at 1 μM (A: P< 0.05, B: P<0.05 n=10), 3 μM (C: P<0.001, D: P<0.01 n=7), 10 μM (n=5), 30 μM (E: P< 0.01 n=8), 100 μM (F: P< 0.05 n=3).
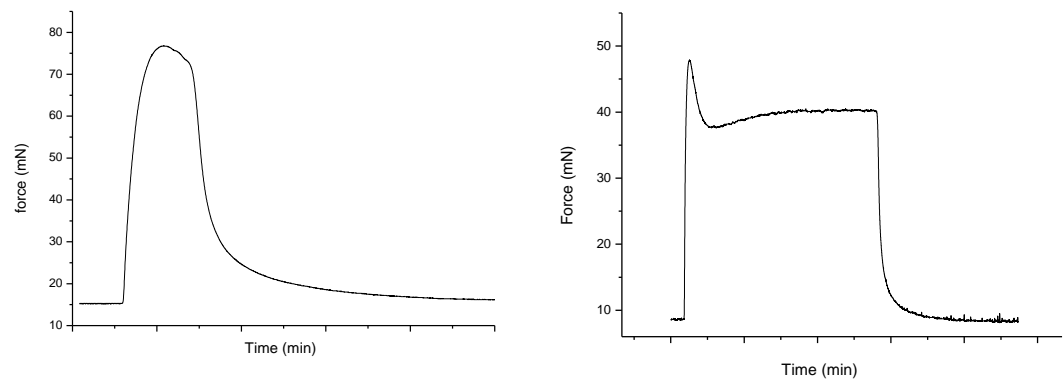
Figure 3.4.9: Examples of pre-dose contraction shape (A) and post-dose/recovery contraction shape (B) that was observed in all experiments with VU590 with Oxytocin or without.

3.4.5 VU590 combined with Oxytocin

The effect seen when VU590 was combined with VU590 was similarly suitable for division into three phases for analysis *(Figure 3.4.4)*.

VU590 combined with Oxytocin augments spontaneous human myometrial contractions with a lesser effect on prolonged contractile phase but increased AI at higher doses than when VU590 used alone.

Phase (1) First 5 contractions

With Oxytocin alone an initial increase in AI (160±29% (P<0.01 n=10)) is observed in the first contraction, which is mirrored in each dose combination to a similar value. This effect is not seen in the previous experiments where Oxytocin is not

175

present and so it is concluded that the immediate effect seen on the first contraction following dosing is due to the immediate effect of Oxytocin. When Oxytocin is combined with 1 µM VU590 there is a time dependant increase in activity integral similar to that seen with VU590 alone but with additive effects. At 1 µM VU590 + 1 nM Oxytocin AI has increased by 217±32 % ($P<0.001$ n=9) by contraction 5 whereas 1 nM OT alone reached 139±16% and VU590 alone reached 167±22% increase by contraction 5 *(Figure 3.4.11)*. This time dependant increase was less significant with rising doses and a combined dose of 1nM Oxytocin with 100 µM VU590 demonstrated a clear time dependant diminishing effect *(table 3.4.2)*.

Phase (2) Prolonged contraction phase

A prolonged contractile phase was seen in all doses including 1 µM VU590 + 1 nM Oxytocin where this was not observed with 1 µM VU590 alone *(table 3.4.2)*. The prolonged phase CD was maximal at 10 µM (3.3±1.2 hrs (n=5)), which was less than with 10 µM VU590 alone but demonstrated a greater increase in AI (2256±961 % ($P<0.001$, n=6)). *(Figures 3.4.12,13,14)*. MF was significantly reduced to 47±5.7% ($P<0.001$ n=7) average of predose contractions at 30 µM combined with 1 nM OT, with a similar reduction at 100 µM VU590 alone (46±6.5% ($P<0.001$ n=10)) *(Figure 3.4.13 B)*.

Phase (3) Remaining contractions

All the strips dosed with 1 µM and 3 µM VU590 + 1 nM Oxytocin resumed contractions following the prolonged contraction phase *(table 3.4.2)*. Maximal increase in AI was at 3 VU590 + 1 nM OT (281±33% ($P<0.05$, n=10)).A similar shape change was observed in remaining contractions with an increased activity

176

integral, selection duration and reduction in maximal force. When compared to VU590 alone, combination with 1 nM Oxytocin resulted in a further increase in activity integral and selection duration at all doses with the exception of 3 μM which showed a greater effect when dosed alone and 100μM which was similar alone or combined *(Figure 3.4.15)*.
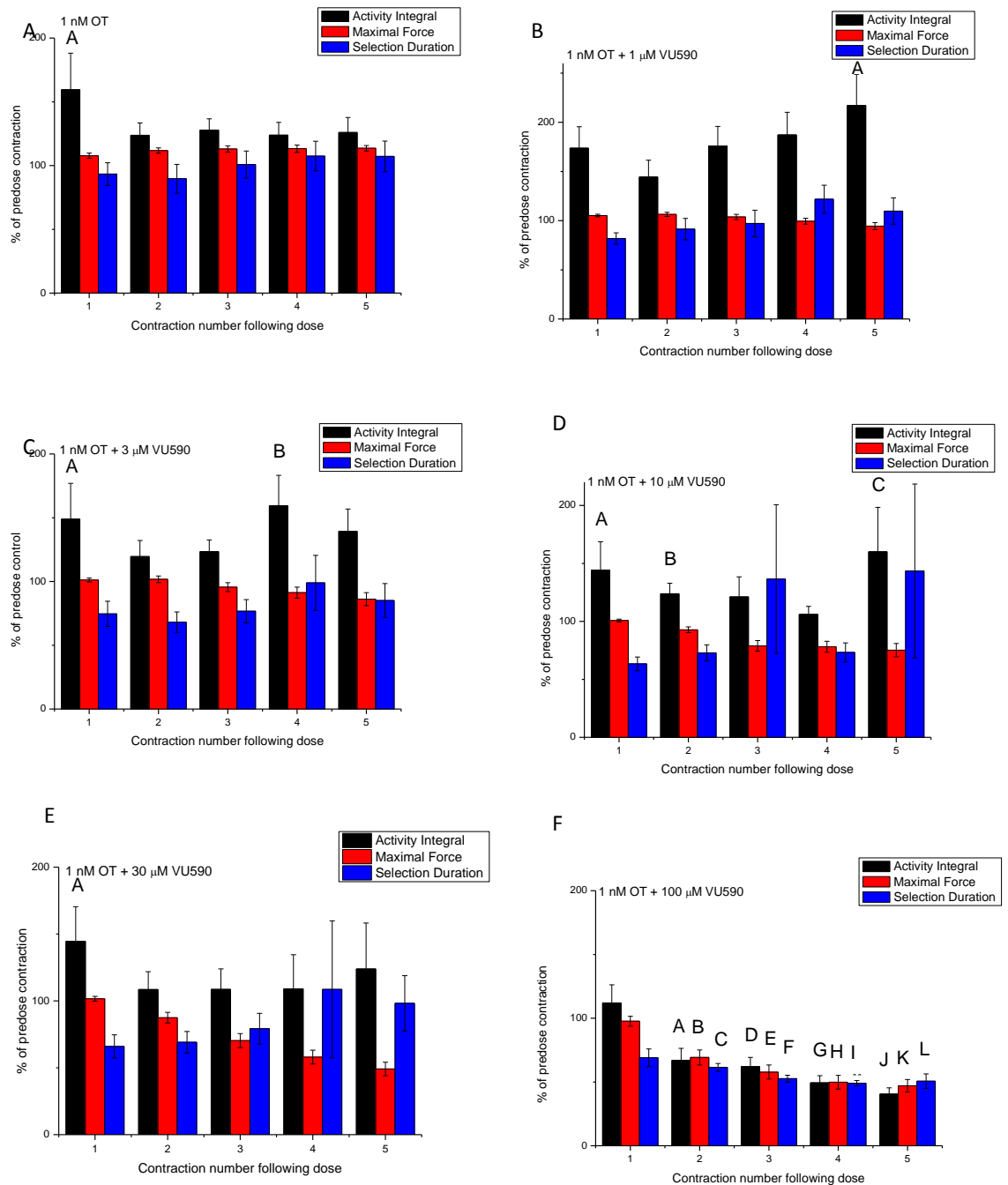
Figure 3.4.10: The percentage of activity integral, maximal force, and selection duration of pre-dose contractions of each of the first 5 contractions following dosing with 1 nM Oxytocin alone (A) (A: <0.01 n=10) or combined with either 1 μM (A: P<0.01, B,C,D,E: P<0.001n=9) (B), 3 μM (n=10) (C), 10 μM (A: P<0.01, B: P<0.05 n= 6) (D), 30 μM (A, B, C: P<0.01, n=7) (E), 100 μM (A: P<0.01 n=7) (F) VU590 (A-F P<0.01, G-L P<0.05 n=7).
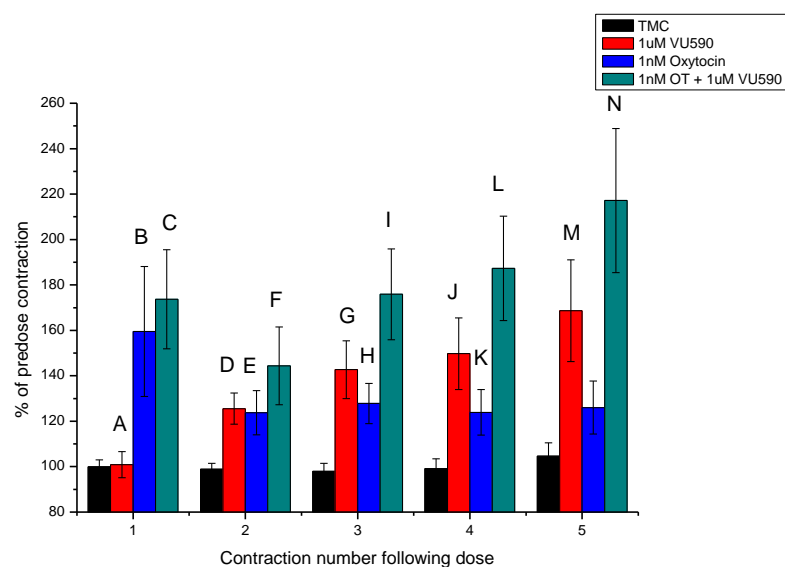
Figure 3.4.11: The percentage of pre-dose contractions of activity integral of each of the first 5 contractions following dosing with 1 nM Oxytocin (A, D,G,J,M P<0.01 n=10), 1 μM VU590 (B,E,H,K: P<0.01 n=7) and 1 μM VU590 combined with 1nM Oxytocin (C,F: P<0.05, I,L,N: P<0.0001 n=9).

VU590 dose	Number of experiments with prolonged contraction	Number of experiments with prolonged contraction that continued contracting
1 μM	3/10	3/3 (100%)
3 μM	7/10	7/7 (100%)
10μM	6/10	3/6 (50%)
30μM	9/10	6/9 (66.6%)
100 μM	7/10	3/7 (42.8%)

Table 3.4.2:Table showing the number of experiments with each dose combined with 1 nM Oxytocin that had a prolonged contraction phase and the number of those that continued to contract following the prolonged contraction.
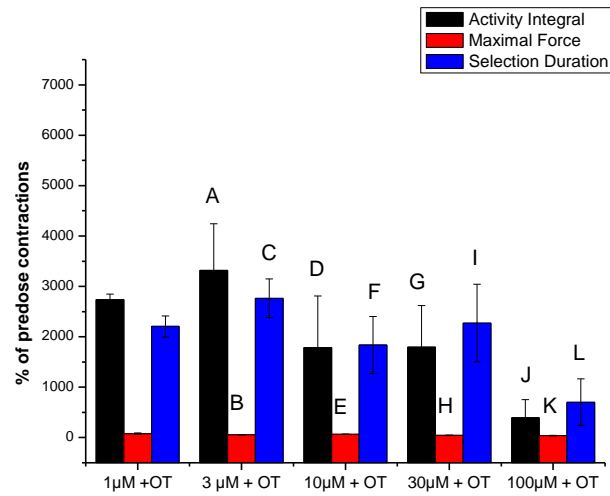
Figure 3.4.12:The percentage of activity integral, maximal force, and selection duration of pre-dose contractions of the prolonged contraction phase following dosing with 1 nM Oxytocin combined with VU590 1 μM (n=9), 3 μM (A,B,C: P<0.001 n=10), 10 μM (D,E,F: P<0.001 n= 6), 30 μM (G: P<0.01,H,I: P<0.001 n=7), and 100 μM (J,K,L: P<0.01 n=7).
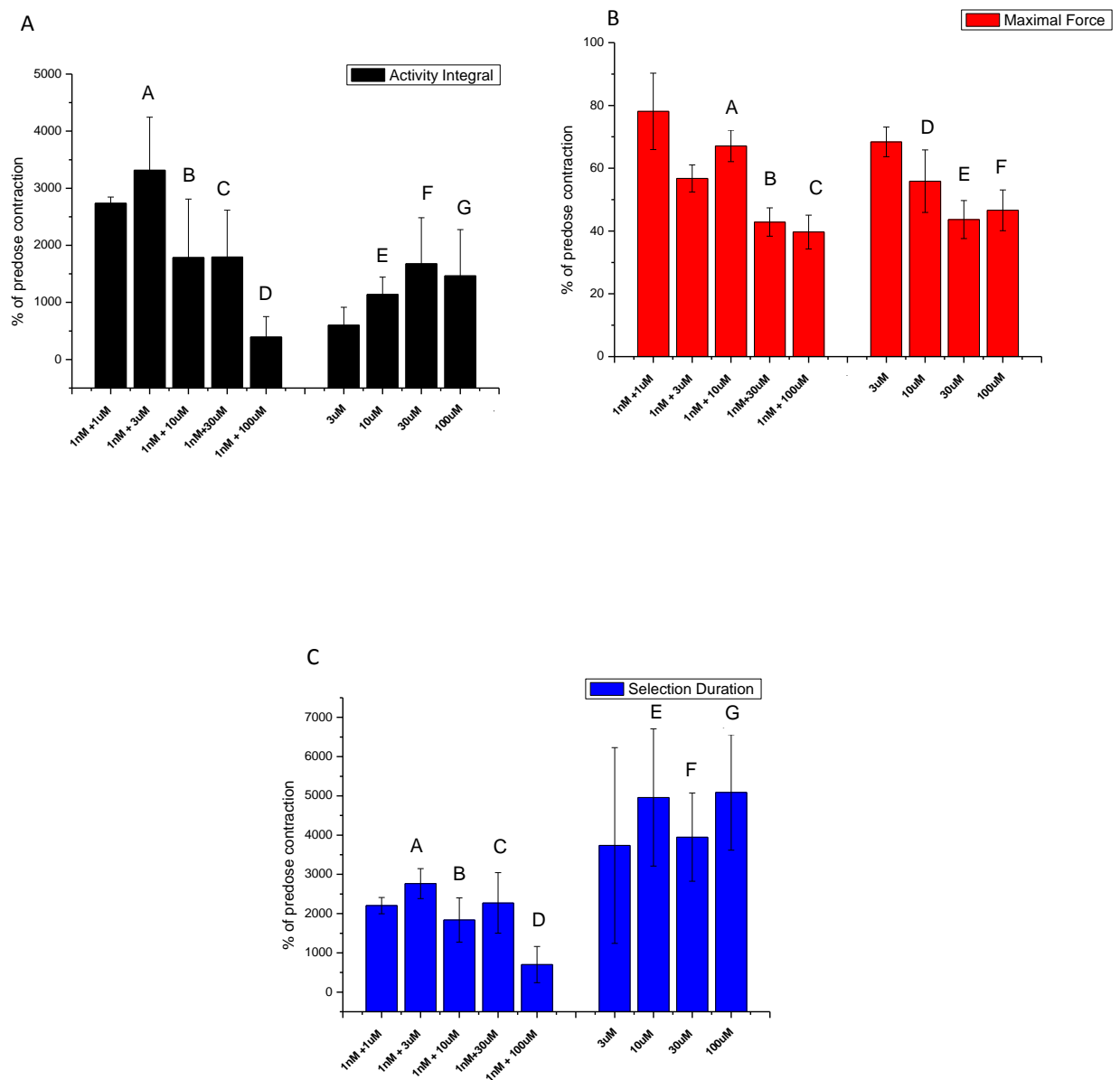
Figure 3.4.13:The percentage of activity integral (A) (A,B,C: P< 0.001, D: P<0.01, E,F: P< 0.001), maximal force (B)(A, B: P< 0.001,C,D: P<0.01, E<F: P<0.001) and selection duration (C) (A,B,C: P<0.001, D:P<0.01, E,F,G P<0.001) of pre-dose contractions of the prolonged contraction phase following dosing with 1 nM Oxytocin combined with VU590 (right) or VU590 alone (left).
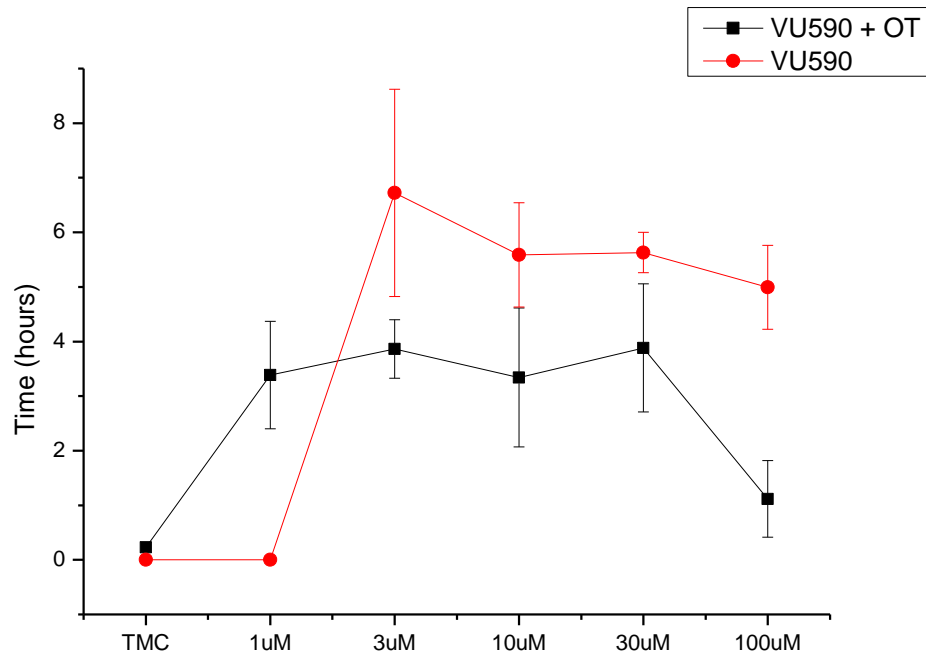
Figure 3.4.14: The average duration of the prolonged contraction phase in hours

for each dose of VU590 alone and when combined with 1 nM Oxytocin
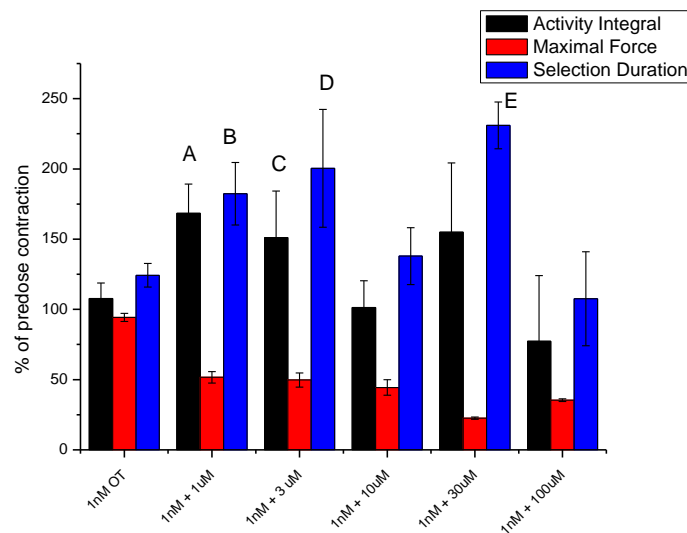
Figure 3.4.15: The percentage of activity integral, maximal force, and selection

duration of pre-dose contractions of remaining contractions with 1 nM OT and

combination with 1 μM (A: AI P<0.05, B: Dur P<0.05, n=10), 3 μM (C: AI P<0.05,

D: Dur P<0.05n=7), 10 μM (n=7), 30 μM (E: Dur P<0.05 n=7), 100 μM (n=6) VU590

3.4.6 Results - The expression of K_{ir} 7.1and K_{v}2.1 in human myometrium

3.4.6.1 The expression of K_{ir} 7.1 in human myometrium

Western blotting was performed on myometrial tissue samples flash frozen at caesarean section for quantification of the expression of the protein K_{ir}7.1. Samples used were from women grouped as being either in labour or not in labour at term or pre-term gestation (n=6 each group).

K_{ir} 7.1 expression in TL was significantly less than TNL (P=0.047), and expression in PTL was significantly less than TNL (P=0.049) (*Figure 3.4.16,17*). This is suggestive of a down-regulation of Kir 7.1 channel expression with the onset of labour.

In order to explore the gestation-dependant expression of K_{ir} 7.1 data was analysed on a gestational time-line for both labouring and non-labouring samples. The labour samples' expression was lower with a downward trend towards term, with the opposite of a rising trend towards term seen in the non-labour group (*Figure 3.4.18*).
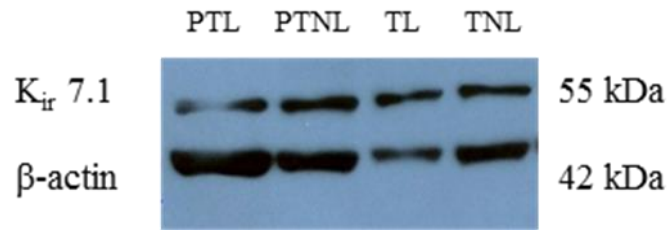
Figure 3.4.16: A typical film of a western blot quantifying the expression of K_{ir} 7.1 against the expression of the control protein β actin for pre-term labouring (PTL), pre-term non-labouring (PTNL), term labouring (TL), term non-labouring (TNL) samples of human myometrium.
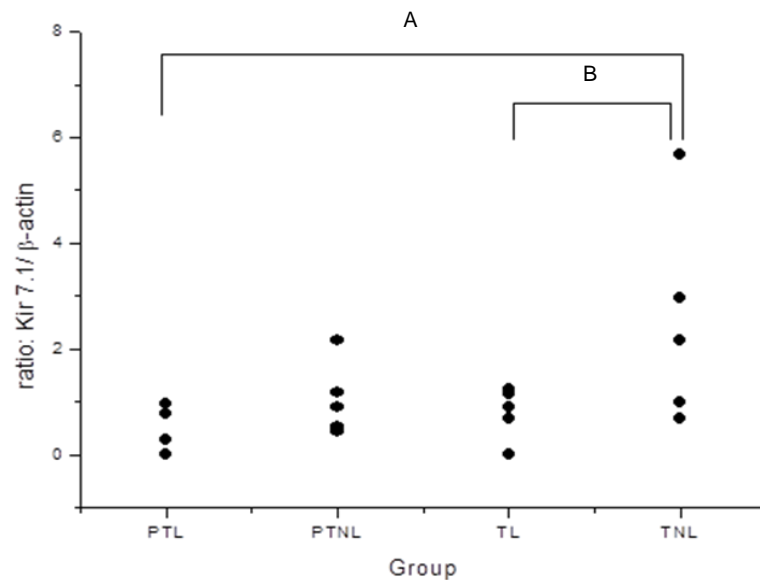
Figure 3.4.17: Graph showing individual K_{ir} 7.1/β-actin ratios for blots in each group of samples of human myometrium - pre-term labouring (PTL), pre-term non-labouring (PTNL), term labouring (TL), term non-labouring (TNL). K_{ir} 7.1 expression in the pre-term labouring (PTL) group was significantly less than the term non-labouring (TNL) group by ANOVA (A: P=0.049 PTL: n=6 TNL: n=5). K_{ir} 7.1 expression in the term labouring (TL) group was significantly less than the term non-labouring (TNL) group by ANOVA (B: P=0.047 TL: n=6 TNL: n=5).

184
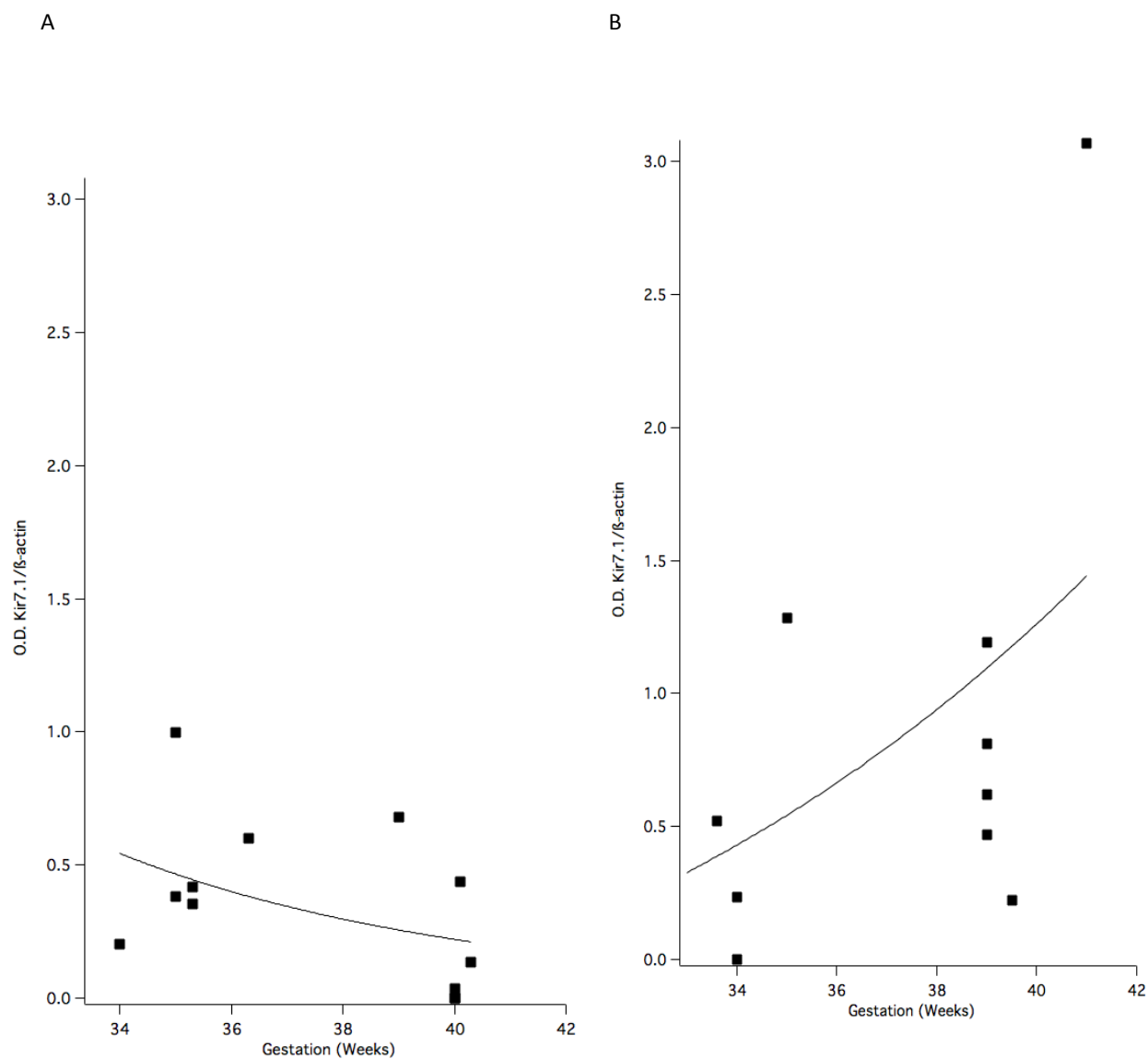
Figure 3.4.18: K_{ir} 7.1 expression plotted by gestational age at delivery with a lower expression and a downward trend towards term in labouring samples (A) and with a higher expression and an upward trend towards term in the non-labouring group (B)

3.4.6.2   The expression of K_v 2.1 in human myometrium

Western blotting was performed on the same myometrial tissue samples as
described above for quantification of the expression of the protein K_v 2.1.
There was no significant difference in expression of Kv2.1 seen between
either group of labouring or term or preterm samples (*Figure 3.4.19,20*).
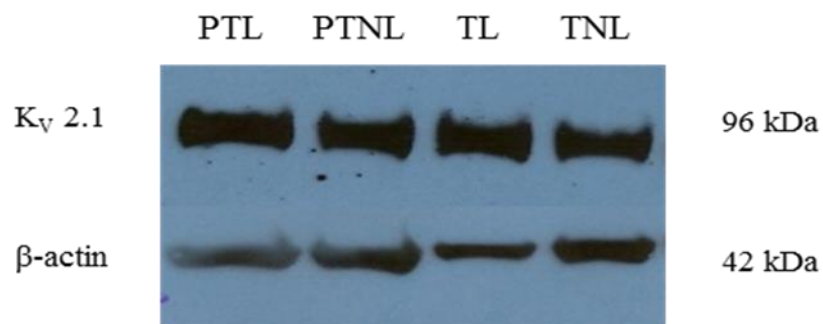
*Figure 3.4.19: A typical film of a western blot quantifying the expression of K_v2.1
against the expression of the control protein β actin for pre-term labouring (PTL),
pre-term non-labouring (PTNL), term labouring (TL), term non-labouring (TNL)
samples of human myometrium.*
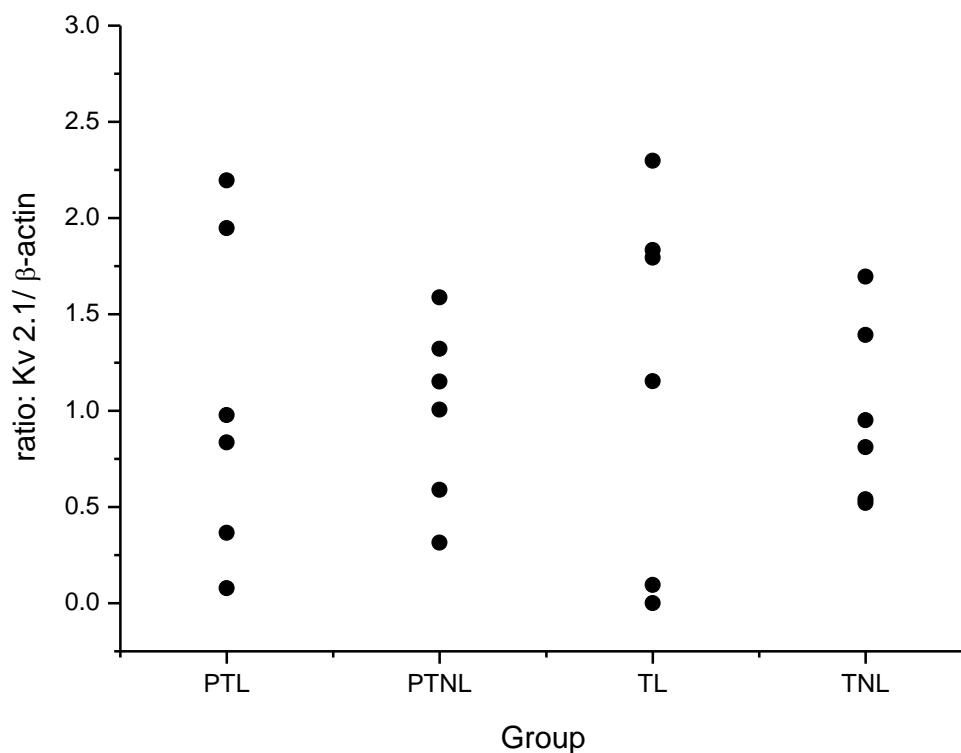
Figure 3.4.20: Graph showing individual K_v 2.1/β-actin ratios for blots in each group of samples of human myometrium - pre-term labouring (PTL)(n=6), pre-term non-labouring (PTNL) (n=6), term labouring (TL) (n=6), term non-labouring (TNL) (n=6). There was no significant difference between any of the groups by ANOVA.

3.4.7 Conclusion

VU590 increased baseline tone in murine myometrium. Gestational dependent effect was seen in dosing VU590 alone. Addition of Oxytocin increased the effect 10-fold in D18 mice.

In human myometrium VU590 alone or with Oxytocin brought about a significant augmentation of contractility at lower doses. This exceeded the effect of Oxytocin alone with a further doubling of effect by contraction 5 when Oxytocin was combined with 1 μM VU590. In higher doses a prolonged contraction phase was seen. VU590 combined with Oxytocin had a shorter CD in prolonged phase but a higher AI reflecting an improved maintenance of tone throughout the phase when compared to VU590 alone. A change in the shape of contractions reflects a pro-longed depolarised state and delay in repolarisation and contraction relaxation.

It appears that by blocking K_{ir} 7,1 with VU590 resting membrane potential is maintained in a depolarised state allowing for continued Ca^{2+} influx and sustained contraction. This suggests VU590 is a potent contractile agent and a potential therapeutic target for PPH.

4. Discussion

4.1 Potassium channel openers combined with Nifedipine as potential therapeutic targets for tocolysis

4.1.1 Linoleic Acid and Kv 2.1

With the exception of linoleic acid, the addition of potassium channel openers, independent of any other agents, to spontaneously contracting myometrial strips resulted in a reduction in contractility. The lack of effect seen by linoleic acid may be due to a lack of perfusion into the tissue strip so it was unable to act at a cellular level. Alternatively it may not act as expected on potassium channels, or the channels targeted may not be functional in reducing membrane potential. It is worth noting that in western blotting experiments presented in section 3.4 of this thesis, K_v 2.1 was not found to have a significant gestational-dependent expression, which may support the theory that it does not significantly contribute to membrane resting potential in human myometrium. In further work (unpublished data) conducted within our research group, transgenic expression data and mathematical modelling was used to predict the function and contribution of specific ion channels in myometrial contractility. Predicted function of K_v 2.1 was tested in voltage-clamp experiments in murine myometrium and an increased action potential frequency and amplitude was observed in response to the K_v 2.1 blocker stromatoxin. However there was little change in resting membrane potential. Although it is problematic to infer open state function from experimental work from blockers, this data (unpublished) suggests a role for K_v 2.1 in modulating contractility

frequency and amplitude but a limited role in contributing to the resting membrane potential.

Although LA and n-6 fatty acids appear to have a role in prostaglandin production and influence pregnancy duration (Wathes et al 2007, Allen & Harris 2001), it does not appear LA exerts a significant effect via membrane potential modulation.

4.1.2 Pinacidil and Riluzole combined with Nifedipine

Pinacidil resulted in a dose-dependent reduction of all parameters and achieved complete inhibition at higher doses. This result is consistent with the findings of Mandi et al (2005), Kostrzewska et al (1996) and Khan et al (1998). When Pinacidil was added following pre-treatment with Nifedipine, additional inhibition was observed. Riluzole showed less potency but still elicited a significant inhibition when administered alone which was enhanced when combined with Nifedipine. The effect of Riluzole and Nifedipine together on the slow climbing phase and shape of contractions points to a possible 'swinging' of action potentials due to unstable membrane potential across cells within the strip. This supports the hypothesis that the combination of potassium channel openers with a calcium channel blocker elicits a more potent inhibitory effect than calcium channel blockers alone. This effect is mediated by dose as seen at the higher doses of Nifedipine due to the potency of Nifedipine effect on control strips. In strips pre-treated with Nifedipine followed by Pinacidil, Riluzole and their controls, contractions were almost completely abolished at the 100 nM (0.1 µM) Nifedipine dose.

With a typical tocolytic dose regimen, steady state *in vivo* plasma levels of Nifedipine reach 0.195±0.082 µM (Papatsonis et al, 2007), however the optimal dose of Nifedipine has not been defined and the different release characteristics of the formulations available may affect the dosage required to abolish contractions. An increased dose would induce increased incidence of maternal and fetal side effects (RCOG 2011).

Pinacidil reduced contractility both on its own and inhibition became more potent when combined with Nifedipine. The effect of Pinacidil on myometrial smooth arteries was similar to that of Nifedipine in limiting arterial response to U46619 1 µM (56.84 ± 7.13% of pre-dose constriction with Nifedipine 10 nM and 54.15 ± 16.32% with Pinacidil 3 µM). This was not particularly surprising as Pinacidil has been used clinically as a hypertensive and has been shown to have a high binding affinity and potent effect on the KATP channel which elicits an endothelial-independent relaxatory effect on VSMC (Stojnic et al 2007, Atwal, K 1994). Pinacidil was selected to act as a positive control and performed as such within these experiments but supported the hypothesis that modulation of K^+ current would inhibit VGCC Ca^{2+} entry.

A surprising finding from this series of experiments was the apparent response or non-response to Pinacidil. Consistently across myometrial smooth muscle and artery experiments there was a sub-group of tissue samples that failed to respond to Pinacidil with others exhibiting total inhibition of contractile force. There has been some discussion in other published work about the possible existence of different receptor sub-types that may influence the effect of KATP openers(Atwal

1994). Inagaki et al (1996) identified a novel sulfonylurea receptor SUR2, which they found bound to glibenclamide with a lower affinity than SUR1(Inagaki et al 1996). They identified that SUR2 is co-expressed with Kir 6.2 which reconstitutes some KATP channels that appear to have properties distinct from the β-cell KATP. The SUR2-Kir6.2 sub-type is less sensitive to ATP, glibenclamide and is not activated by the KATP opener diazoxide (Inagaki et al 1996). A similar selective effect is described by Liss et al (1999) who identified a sub-population of substantia nigra (SN) neurons that were not affected by pre-incubation with the ATP blocker rotenone. Whereas some SN neurons exhibited a large KATP current and membrane hyperpolarisation with pre-incubation of rotenone 100 nM, others were only partially activated by doses of rotenone 1-10 μM (Liss et al 1999). Those that were sensitive to rotenone expressed the subunit combination SUR1+Kir 6.2 and those that were non-sensitive expressed the subunit combination SUR2B+ Kir 6.2 (Liss et al 1999). This suggests a metabolic sensitivity with particular subunit types. Further evidence of channel sensitivity comes from Nui et al (2011) who identified a sex specific response to Pinacidil in trigeminal ganglia in Sprague Dawley rats. All subunit components Kir 6.1, 6.2 and SUR1 and SUR2 were confirmed present in the tissue by PCR; but where 20 μg Pinacidil blocked capsaicin-induced mechanical sensitivity to pain in male rats, in females the maximum dose of 300 μ was only partially effective (Nui et al 2011). Some Type 2 diabetic patients fail to respond to sulfonylurea therapy while others appear over-sensitive with genetic polymorphisms possibly responsible for inter-individual variability (Aquilante 2010). With the burgeoning interest from drug companies into pharmacogenetics some of these variances and sensitivities will continued to be studied in future. Type 2

diabetes is a polygenetic disease with a complex aetiological pathway (Aquilante 2010). Polymorphisms that influence an individual's risk of developing the disease or which are altered in disease progression may underlie variance in response to sulfonylurea therapy. It must be taken into consideration the rise in the incidence of Type 2 diabetes and other metabolic syndromes in the general population and the childbearing population. Diabetes and metabolic conditions are known risk factors for poor pregnancy outcomes and continue to be of concern to obstetricians. In our sample population there was a very low number of known diabetics, but it cannot be known if there was underlying metabolic impairment or simply variance in genetics of the sample population that led to a proportion being resistant to Pinacidil. This warrants further laboratory investigation, perhaps into the different subtypes of SUR receptors expressed in a diverse population of women.

An additional factor to consider regarding variation in results and response to particular drugs, is the variability in the composition of myometrial muscle samples used. Within strip control for analysis should have diminished variability due to strip size and muscle fibre density. Patient to patient differences in myometrial composition and variances in gestation and time to onset of spontaneous labour may have influenced response. All samples included in this experimental work were obtained from the lower segment of the uterus. The uterus is made up of the upper uterine body and corpus, the lower segment and cervix; and the uterine wall consists of three layers the endometrium (inner layer) the perimetrium (outer layer) and the thickest layer between the two of the myometrium. The

myometrium consists mainly of smooth-muscle bundles and connective-tissue and is further structured within an external layer (stratum supravasculare), middle layer (stratum vasculare) and inner layer (stratum subvasculare) (Weiss et al 2005).

Muscle fibres of the inner myometrium have a predominantly circular orientation whereas the intermediate and outer layers have a longitudinal orientation (Naftalin and Jurkovic 2009). The endometrial-myometrial junction zone differs according to pregnant and non-pregnant state, as well as phase of menstrual cycle, with the subendometrial myometrium modulating uterine contractions throughout the menstrual cycle and implantation (Brosens et al 1995). In pregnancy, trophoblast invasion and vascular re-modelling reaches as far as the inner myometrium (Pijnenborg et al 2011). Myometrial zonal layers become less distinct in pregnancy and it is possible that in pregnancy there is endometrial infiltration into the myometrium, and there may be structural and functional differences in this layer (Turnbull et al 1995, Brosens et al 1995).

Viewed through high resolution magnetic resonance diffusion tensor imaging, the uterus appears to be an anisotropic organ, but with a muscle fibre architecture that is complex. Muscle fibres in the different areas and different layers of the uterus were seen to be overlapping and multi directional (Weiss et al 2005). Differences in gene expression before and after the onset of labour, between the fundus, lower segment and cervix have been demonstrated (Bukowski et al 2006, Romero et al 2006). As all samples used within this work came from the inner edge of the lower segment, it is possible that functional variability may exist in the tissue samples received which may be an explanation for variation in responses or non-response in some biopsies.

Despite this demonstrated spacial variation; work by Luckas and Wray (2000) provide reassurance in their observation that there was no difference in the contractile rate, force production and area under the curve produced by myometrium from the upper and lower segments. The authors conclude that for contactility, the use of lower segment biopsy is appropriate (Luckas and Wray 2000).

Riluzole reduced contractility on its own and inhibition became more potent when combined with Nifedipine but to a lesser effect than Pinacidil. Riluzole has a more promising safety profile which points to its suitability as a potential tocolytic when combined with Nifedipine. Riluzole is currently used as a long term drug therapy thought to be effective in delaying the progression of amyotrophic lateral sclerosis (ALS), is not associated with hypotension and has a good safety profile (Lipp et al, 2003, Bensimon & Doble, 2004). Combination of Nifedipine with Riluzole *in vivo* may reduce the dose of Nifedipine required to inhibit contractions in preterm labour without additional side-effects. It is envisaged that in clinical practice these drugs would be administered simultaneously. As the above experiments investigated dose response following pre-treatment, further work was indicated to investigate simultaneous dosing to identify an optimal combined dose.

Significant effects on contractility were observed with addition of Riluzole combined with Nifedipine at similar doses as was shown when doses were added incrementally. With Pinacidil there appears to be an immediate abolition of contraction whereas with Riluzole there appears to be a time-dependent effect. This could be due to a delay in this agent infiltrating the tissue strips or impedance in binding at a cellular level. This may also point to the fact that Nifedipine is more

potent than Riluzole when combined and so inhibition reflects that time dependent effect of Nifedipine. The faster response of Pinacidil and Nifedipine combined may point to a matched or increased contribution of Pinacidil into the combined effect.

Riluzole had less of an effect on myometrial small arteries which is reassuring regarding it's potential as a tocolytic. Combined with Nifedipine the relaxatory effect was less than when Nifedipine was added alone. This could be an artefact of the experiments due to low n numbers, but could also indicate an effect of Riluzole in VSMC that mediates the function or affinity of Nifedipine to VGCC. As a significant effect on myometrial arteries was not observed with Riluzole this suggests endothelial-mediated relaxation of VSMC via SK_3 was also not observed in these experiments. Previously, it has been demonstrated that suppression of SK_3 expression resulted in an elevation of arterial tone and blood pressure in modified mice, and in porcine arteries under exposure to the SK_3 activator substance P (Taylor et al 2003, Burnham et al 2002). In view of this it was reasonable to expect prior to experimentation that in opening SK_3 via Riluzole a more significant relaxatory effect may have been seen.

Crane & Garland (2004) found that U46619 blocked SK_3 channel in rat mesenteric artery, Riluzole (100 µM) evoked membrane hyperpolarisation from -55±2 mV to -71±3 mV (n=5) and this was reduced with repeated exposure to U46619 (incremental doses 1 nM to 0.1 µM) to 58±2 mV. Hyperpolarisation following application of levcromakalin (K_{ATP} activator) was unaffected by repeat exposure to U46619. Although this effect was only seen in repeated exposure to U46619 and in this set of experiments the dosing period was the second exposure to U46619 with

drug pre-incubation preceding exposure (following a washout period) it is unclear to what extent there may have been a block of SK3 channels from U46619 that may have inhibited the effect of Riluzole in these experiments. Repetition with a different vessel constricting agent may confirm whether this is the case. There appeared to be a non-significant reduction in the expression of SK_3 in myometrial small arteries compared with whole myometrium, and in this set of tissue, TREK-1 was absent from myometrial arteries. If Riluzole is predominantly targeting TREK-1 in this tissue then this could be an explanation for the reduced relaxatory effect seen in the myography experiments. Work by Duprat et al (2000) confirmed with patch-clamp experiments that Riluzole opens TREK-1 and allows K+ flow leading to hyperpolarisation. They observed that the current was not consistent, with a rapid stimulation followed by a decline and then inhibition of TREK-1 current (*figure 4.1* Duprat et al 2000). In the same set of experiments, it was also observed that TREK 1 response is sensitive to variation in cAMP. After inhibition with exposure to 8-(4-chlorophenylthio)(8CPT) cAMP 500 μM, the channel could not be re-activated by Riluzole. The authors conclude that Riluzole has an inhibitory effect on the PKA pathway, which they suggest is responsible for the inhibitory effect Riluzole on TREK-1 following initial activation (Duprat et al 2000). This described pattern of activation followed by inhibition of TREK-1 by Riluzole may present an explanation for the change in contraction shape seen in myometrial contractions following Riluzole. It may also explain why no dose brought about total inhibition. Whether the change in myometrial shape and strength with Riluzole and Nifedipine combined would be sufficient to decrease uterine tone in vivo requires further investigation.
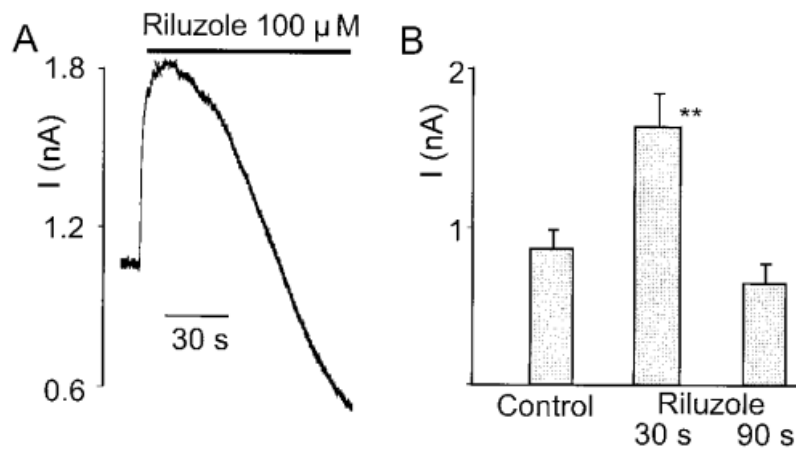
Figure 4.1: Reproduction of figure 2 from Duprat et al (2000) - Dual effect of Riluzole on TREK-1 current. A, example of transient stimulation of TREK-1 current followed by an inhibition, recorded at 0 mV, during 90 s perfusion with 100 mM of Riluzole. B, bar graph of TREK-1 mean currents, recorded at 150 mV, before perfusion with Riluzole (100 mM) (control) and after 30 s and 90 s of perfusion (n 5 29, P 5 .002).

4.2 The potassium channel blocker VU590 as potential therapeutic target for PPH

In human myometrium VU590 alone or with Oxytocin brought about a significant augmentation of contractility at lower doses. This exceeded the effect of Oxytocin alone with a doubling of effect by contraction 5 when Oxytocin was combined with 1 μM VU590. In higher doses a prolonged contraction phase was seen. VU590 combined with Oxytocin had a shorter CD in prolonged phase but a higher AI reflecting an improved maintenance of tone throughout the phase when compared to VU590 alone. A change in the shape of contractions reflects a pro-longed depolarised state and delay in repolarisation and contraction relaxation.

198

It is hypothesised that the effect seen by VU590 in these experiments is due to the blockade of K_{ir} 7.1. In order to confirm this some preliminary contractility experiments (full data not available for this thesis) were performed with VU591 a compound developed to specifically target ROMK with no block of K_{ir} 7.1. No effect on contractility was seen on addition of this compound at the same dose spread used in VU590. This added confidence to the effects seen being due to K_{ir} 7.1 blockade.

Other investigators have explored the effects of K^+ channel blockers in myometrium. BK_{Ca} blockers paxilline, iberiotoxin & penitrem A have not been shown to increase in contractility when dosed alone but have attenuated inhibitory effect of BK_{Ca} openers (Aaronson *et al.*, 2006; Doheny *et al.*, 2003). K_V channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP) have demonstrated an augmenting effect on myometrial contractility. TEA demonstrated a 20% increase in maximum contraction amplitude by 20% (5mM) and 4-AP demonstrating a 121% increase in maximal contraction amplitude in late pregnant rat tissue but with reduced potency on early pregnant tissue (Aaronson *et al.*, 2006). A similar effect of 4-AP was seen in non-pregnant murine myometrium with no effect in pregnant murine myometrium with an absence of expression of K_V 4.3 in pregnant tissue compared to non-pregnant revealed in western blotting. A difference in response between these tissues combined with the change of expression led to the conclusion that 4-AP targets K_V 4.3 (Smith *et al.*, 2007). To date no other K^+ channel blockers have elicited the degree of response seen with the application of VU590.

Effects seen in this thesis suggest that K_{ir} 7.1 plays a role in setting resting membrane potential in murine myometrium with blockade resulting in a rise in baseline tension that is gestation mediated. K_{ir} 7.1 appears to play an important role in the repolarisation of membrane potential and the termination of action potential in human myometrium with blockade resulting in prolonged plateau phase of contraction and increased force with lower doses and with increasing dose profound and prolonged contractile phase.

Together with results presented here and building on aforementioned work into K_{ir} 7.1 expression in myometrium, the results of additional study (and published paper) undertaken by our research team and collaborators support this hypothesis. Electrophysiology experiments on murine myometrial cells have demonstrated an inwardly rectifying potassium current was inhibited by VU590 under voltage-clamp conditions, and that this current was stronger at GD15 than GD18 in line with gestational decrease in expression of K_{ir} 7.1 *(Figure 4.2 from McCloskey et al 2014 Appendix 2).* This was further confirmed by modulation of expression of K_{ir} 7.1 in murine myometrial strips. Using Anti-Kir 7.1 or +Kir 7.1 lentiviral vectors the channel was either over-expressed or under-expressed before tissue was used in contractility (organ bath) or voltage-clamp experiments. When K_{ir} 7.1 is under-expressed there was a significant increase in activity integral, maximal force and contraction duration in contractility, as well as a depolarised resting membrane potential with extended periods of excitability (McCloskey et al 2014 (Appendix 2)). When Kir 7.1 was over-expressed there was a decrease in measured contractility parameters and an observed hyperpolarisation and suppression of excitability

(McCloskey et al 2014 (Appendix 2)). This is strong and novel evidence of the contribution made to resting membrane potential and myometrial contractility of K_{ir} 7.1.
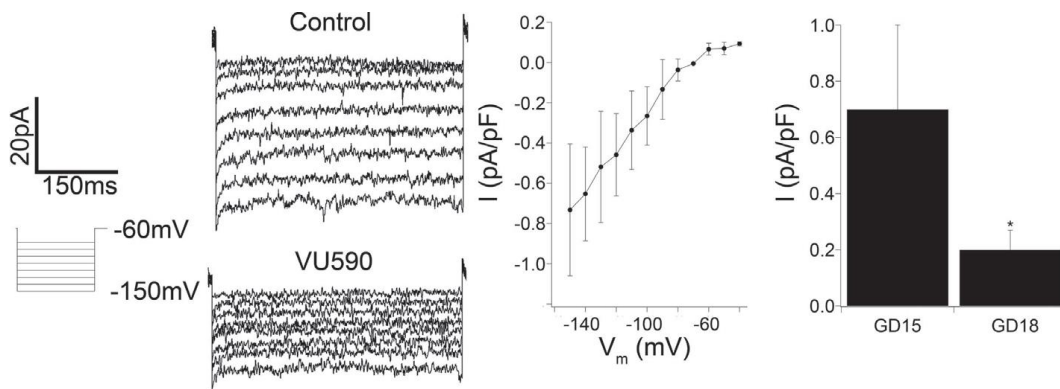
Figure 4.2: Results of electrophysiology experiments from our research group: Measurement of inwardly rectifying, VU590 sensitive current in GD15 murine myomentrial cells – voltage clamp recordings in the presesnce and absence of 10 µM of VU590 (A), Current-voltage relation of current density (VU590 subtracted from from control (vehicle alone) n=5; mean ±SD) (B), and current density (pA/pF) at -150 mV and 500 ms in murine myometrial cells from GD15 and GD18 (n=5; mean ±SD) *P<0.05 by Student's t test (C).

4.3 Overall conclusions

This thesis set out to build on findings of a previous screen carried out within our group where a number of K^+ channels were identified of interest. This led to further

examination of the function of these ion channels in myometrium with a view their potential as therapeutic targets.

Pinacidil proved a successful positive control and demonstrated that by activating K_{ATP} and allowing K^+ efflux myometrial contractility was diminished or abolished. This effect was further enhanced with the combination of Nifedipine bringing about abolition of contractions at a lower dose than with Nifedipine alone.

Riluzole diminished myometrial contractility but was not able to abolish with the range of doses used. This may not be down to lack of full tissue perfusion but may be down to the specific effect of Riluzole on smooth muscle. The specific K^+ channel targeted by Riluzole cannot be determined. Although Riluzole is a known activator of SK_3 some of the findings of experimental work presented here suggest that TREK-1 may be the predominant channel targeted by Riluzole. There is the possibility that Riluzole is activating both SK_3 and TREK-1. There was a cumulative relaxatory effect when Riluzole was dosed in combination with Nifedipine, again confirming our hypothesis that activation of K^+ channels hyperpolarises membrane potential sufficiently to block VGCC Ca^{2+} entry and improve the potency of Nifedipine. The effect seen appears to be myometrial specific due to the reassuring nature of the artery experiments, suggesting that in vivo additional cardiovascular side-effects may be avoided. This makes Riluzole in combination with Nifedipine of further consideration as a potential tocolytic therapy.

The K^+ channel K_{ir} 7.1 was identified as being expressed in myometrial smooth muscle and the availability of VU590 made experimental investigation of its action in MSMC possible. VU590 brought about significant and sustained myometrial

contraction in both human and myometrial smooth muscle. Further patch-clamp work has confirmed that VU590 is blocking K_{ir} 7.1 in human myometrium as hypothesised. Together with the contractility experiments present in 3.4 of this thesis there is compelling evidence that blockade of Kir 7.1 via VU590 has potential therapeutic value in the treatment of PPH and warrants further investigation.

This thesis has investigated how modulation of specific K^+ channels in murine and human myometrium can modulate contractility with a view to therapeutic targets. This represents a novel set of observations that will add to knowledge of myometrial function and will inform further work which has the potential to benefit pregnancy outcomes.

4.4 Future work

4.4.1 The combination of K^+ channel openers with Nifedipine for tocolysis

This thesis did not determine the mechanism through which Riluzole exerted its action on myometrial contractility. K^+ channel modulation was our hypothesised mode of action but which channel, or set of ion channels influenced by Riluzole is yet to be determined. Electrophysiology experiments to determine the effect of Riluzole on specific channels of interest would inform this further and advance understanding crucial for any prospective application of Riluzole as a tocolytic either on its own or in combination with Nifedipine.

Further consideration of either Pinacidil alone or the combination of Pinacidil and Nifedipine at low doses as tocolytics. Careful consideration should be given to

potential cardiovascular side-effects and whether these may be improved or increased over Nifedipine as used in current practice. Additional *in vitro* myogenic tone experiments (pressure-induced flow) may inform this further.

4.4.2 K$^+$ channel expression quantification in myometrial small arteries compared to myometrial smooth muscle.

The Western blots presented in this thesis did not provide data of sufficient quality to draw conclusions on variation of the relative expression of K$^+$ channels in myometrial small arteries compared to myometrial smooth muscle. Further work in RT-PCR or immunohistochemistry or channel gene expression in these tissues is required to evidence tissue selectivity in expression.

Further myometrial small arteries in functional response to drug combinations woul be beneficial including using pressure-flow technique. The *N* numbers used within this set of experiments was restricted by time demands and availability of equipment kindly supported by Newcastle University. Further work would strengthen the quality and reliability of the initial findings presented here.

4.4.3 The potential of VU590 as a stimulator of myometrial contractility

This thesis together with additional published work (McCloskey et al 2014 *Appendix*) builds a strong case for VU590 blockade of K$_{ir}$ 7.1 as potent promoter of myometrial contractility. The potential of this compound or variations of this compound are worth future consideration as therapeutic agents for treatment or prevention of PPH, or labour augmentation.

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26 October 2005

Professor Steve Thornton
Professor Obstetrics & Gynaecology/Associate Dean (Research)
University of Warwick & University Hospitals Coventry & Warwickshire NHS Trust
Warwick Medical School
University of Warwick
Coventry
CV4 7AL

Dear Professor Thornton

Full title of study:	**Laboratory Investigation of the Mechanism of Labour**
REC reference number:	**05/Q2802/107**

Thank you for your letter of 18 October 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chairman.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Application	Version 1	29 July 2005
Investigator CV	Prof. S Thornton	
Protocol	Version 2	01 March 2005
Covering Letter	Prof. Thornton	19 August 2005
Peer Review	Action	20 July 2005

		Medical Research Version 3 PTL121005	
Participant Information Sheet		Version 3 PTL121005	12 October 2005
Participant Consent Form		Version 3 PTL121005	12 October 2005
Response to Request for Further Information			18 October 2005

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q2802/107	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

**Mrs C C Wright
Chairman**

Email: pauline.pittaway@uhcw.nhs.uk

Enclosures:

Standard approval conditions SL-AC2
Site approval form

Copy to: R&D Dept. University Hospitals Coventry & Warwickshire NHS Trust

SF1 list of approved sites

Coventry Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	05/Q2802/107	Issue number:	1	Date of issue:	26 October 2005

Chief Investigator:	Professor Steve Thornton

Full title of study:	Laboratory Investigation of the Mechanism of Labour

This study was given a favourable ethical opinion by Coventry Research Ethics Committee on 25 October 2005. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	Post	Research site	Site assessor	Date of favourable opinion for this site	Notes [1]
Professor Steve Thornton	Professor of Obstetrics & Gynaecology	University Hospitals Coventry & Warwickshire NHS Trust	Coventry REC	26 October 2005	

Approved by the Chair on behalf of the REC:

.... (Signature of Co-ordinator)

...P Pittaway.. (Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

NRES Committee West Midlands - Coventry & Warwickshire

The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0115 8839311
Fax: 0115 8839294

31 December 2013

Isabella Petrie
University Hospitals Coventry R&D
Clifford Bridge Road
Walsgrave
CV2 9DX

Dear Isabella

Study title:	**Laboratory Investigation of the Mechanism of Labour**
REC reference:	**05/Q2802/107**
Amendment number:	
Amendment date:	**18 December 2013**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Notice of Substantial Amendment (non-CTIMPs)		18 December 2013
CV - Siobhan Quenby		19 October 2013

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

05/Q2802/107:	Please quote this number on all correspondence

Yours sincerely

**Dr Helen Brittain
Chair**

E-mail: nrescommittee.westmidlands-coventryandwarwick @nhs.net

Enclosures:	*List of names and professions of members who took part in the review*
Copy to:	
	Prof Steve Thornton, UHCW NHS Trust

University Hospitals Coventry and Warwickshire

NHS Trust

Clifford Bridge Road,
Coventry CV2 2DX

Tel 024 7696 4000
Fax 024 7696 6056

PARTICIPANT INFORMATION SHEET: October 2013　　Ref: PTL 011013

Investigation of the mechanism of labour

You are being asked to participate in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you.

What is the purpose of the study?

Pre-term labour, the onset of labour before 37 completed weeks of pregnancy, is a common complication, often resulting in premature birth. The main problem is that often no cause can be found. This is partly due to our poor understanding. This means that it has been difficult to develop successful treatments for preterm labour. At the Department of Obstetrics and Gynaecology, University of Warwick and UHCW, we are doing research on the causes of pre-term labour in the hope of providing better future care. Our research involves understanding the way in which cells contract and therefore labour starts. We are interested in hormones, investigating how these work, determining the controlling mechanisms and studying the structure of the uterus. This involves scientific investigations which we are happy to outline further if you wish. We need to study cells from non-pregnant and pregnant women. Those obtained in pregnancy are useful before or after labour and either preterm or term.

Why have I been chosen?

Patients who require emergency/ elective caesarean section or hysterectomy may be asked to participate.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form and be given this information sheet. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen if I choose to take part?

The research involves analysis of a small sample (biopsy) from the membranes, placenta and/or the lower/upper part of the womb (uterus). Samples are taken (a) at caesarean section after the baby has been delivered or (b) at hysterectomy following removal of the uterus (delete as appropriate). In order to make the best use of samples, more than one laboratory study may be undertaken on each sample. Samples may be stored prior to study and will represent a gift to the research team.

What are the possible disadvantages and risks to taking part?

This procedure has no effect on the speed at which you recover after operation nor does it influence the scar formation. It does not make a difference to the operation carried out. If you are having a caesarean section, the procedure does not influence future pregnancies. No particular precautions are required before, during or after involvement in the study. If something were to go wrong, indemnity against negligence is provided by the UHCW NHS Trust.

What are the possible benefits of taking part?

There is no direct benefit to you but knowledge will be gained to help patients in the future. The research could lead to commercial gain for the UHCW NHS Trust and/or collaborators.

Will my taking part be kept confidential?

The personal information obtained may be scrutinised by authorised persons, but information obtained will be treated as strictly confidential.

What will happen to the results of the research?

Results will be published in internationally recognised journals and/or may be shared with collaborators. No identifiable patient's details will be included.

We appreciate your assistance. In the event of any concerns, please contact:

Professor Siobhan Quenby, UHCW. 024 7696 4000

Complaints should be directed to Sharon Wyman UHCW NHS Trust, Coventry 02476 965198.

University Hospitals Coventry and Warwickshire

NHS Trust

Clifford Bridge Road,
Coventry CV2 2DX

Tel 024 7696 4000
Fax 024 7696 6056

CONSENT FORM

Title of Project: Investigation of the mechanism of labour

Principle Investigator: Professor S Quenby

Procedure: Tissue biopsy

Please Tick Box

1. I have read the above Information Sheet (Ref PTL011013) of the research study dated October 2013. I have been informed of the procedure and all my questions have been answered to my satisfaction. Furthermore, I have been assured that a member of the research team will answer any future questions that may arise.

2. I voluntarily agree to participate in this study and know that I can withdraw this consent to participate at any time and that and withdrawal would not affect my future medical care. I agree for the biopsy to be used for research in other areas of need, such as preeclampsia.

_____   _____   _____
Patient Name   Date   Signature

_____   _____   _____
Name of Person Taking Consent   Date   Signature

You will be given the information sheet to keep. You may also have a copy of the consent form if you wish.

The inwardly rectifying K⁺ channel KIR7.1 controls uterine excitability throughout pregnancy

Conor McCloskey[1], Cara Rada[2], Elizabeth Bailey[1], Samantha McCavera[1], Hugo A van den Berg[3], Jolene Atia[1], David A Rand[3], Anatoly Shmygol[1], Yi-Wah Chan[1], Siobhan Quenby[1], Jan J Brosens [1], Manu Vatish[1], Jie Zhang[1], Jerod S Denton[4], Michael J Taggart[5], Catherine Kettleborough[6], David Tickle[6], Jeff Jerman[6], Paul Wright[6], Timothy Dale[7], Srinivasan Kanumilli[7], Derek J Trezise[7], Steve Thornton[8], Pamela Brown[9], Roberto Catalano[9], Nan Lin[10], Sarah K England[2] & Andrew M Blanks[1,*]

Abstract

Abnormal uterine activity in pregnancy causes a range of important clinical disorders, including preterm birth, dysfunctional labour and post-partum haemorrhage. Uterine contractile patterns are controlled by the generation of complex electrical signals at the myometrial smooth muscle plasma membrane. To identify novel targets to treat conditions associated with uterine dysfunction, we undertook a genome-wide screen of potassium channels that are enriched in myometrial smooth muscle. Computational modelling identified Kir7.1 as potentially important in regulating uterine excitability during pregnancy. We demonstrate Kir7.1 current hyper-polarizes uterine myocytes and promotes quiescence during gestation. Labour is associated with a decline, but not loss, of Kir7.1 expression. Knockdown of Kir7.1 by lentiviral expression of miRNA was sufficient to increase uterine contractile force and duration significantly. Conversely, overexpression of Kir7.1 inhibited uterine contractility. Finally, we demonstrate that the Kir7.1 inhibitor VU590 as well as novel derivative compounds induces profound, long-lasting contractions in mouse and human myometrium; the activity of these inhibitors exceeds that of other uterotonic drugs. We conclude Kir7.1 regulates the transition from quiescence to contractions in the pregnant uterus and may be a target for therapies to control uterine contractility.

Introduction

At the end of pregnancy, the quiescent uterus must become highly contractile to mediate parturition. While the mechanisms initiating parturition in mammals are diverse (Smith, 2007), a final common pathway of uterine transition to a contractile phenotype appears to converge on the expression of a group of proteins, including the oxytocin receptor, prostaglandin endoperoxidase synthase 2, ion channels and connexin 43, that alter the uterine myometrial smooth muscle (MSM) cell from a state of low intrinsic excitability and refractory to stimulation, to a state that has high intrinsic excitability and is susceptible to stimulation (Garfield et al, 1977; Fuchs et al, 1982; Slater et al, 1995; Garfield & Maner, 2007). Control of stimulation is itself subject to complex gene-environment interactions, which act either independently of, or complimentary to, the underlying physiological changes of the uterus (Cha et al, 2013).

Irrespective of the underlying stimulus, the regulation of the duration and frequency of the myometrial contraction critically depends on control of calcium entry through voltage-gated L- and T-type calcium channels (Amedee et al, 1987; Blanks et al, 2007). Calcium is not only an important second messenger in the generation of force via calcium-calmodulin-dependent myosin light chain kinase, but also depolarizes the plasma membrane, allowing for activation of other voltage-dependent ion channels (Word et al, 1994; Brainard et al, 2007). This voltage-mediated control of uterine excitability is modulated in a gestation-dependent manner in all mammalian species. In particular, mid-gestation is characterized by a hyperpolarized membrane potential close to the reversal potential for potassium E_k (Casteels & Kuriyama, 1965). As pregnancy progresses towards term, the myometrium becomes increasingly

depolarized, to approximately −45 mV at parturition (Casteels & Kuriyama, 1965; Parkington et al, 1999). The mechanism underpinning this crucial, evolutionarily conserved process remains unknown.

A number of potassium channels have been demonstrated to play a role in shaping the myometrial action potential and modulating myometrial contractility (Brainard et al, 2007). The putative roles for the different channels are diverse and depend on the physiological environment. These roles range from voltage-dependent modulation of the action potential waveform (Knock et al, 1999), to modulating responses to intracellular calcium release through BK_{ca} and SK_3 (Khan et al, 1993; Pierce et al, 2008), intracellular ATP concentration through K_{ATP} (Khan et al, 1998) and uterine stretch through tandem pore channels (Tichenor et al, 2005).

Within the potassium channel super-family, inwardly rectifying potassium channels represents good candidates for the regulation of resting membrane potential. Kir7.1 is a member of the inwardly rectifying potassium channel sub-family and is only 38% identical to its closest relative, Kir4.2 (Doring et al, 1998; Krapivinsky et al, 1998; Partiseti et al, 1998). Kir7.1 is expressed in visceral tissues and some neurones within the CNS (Krapivinsky et al, 1998; Nakamura et al, 1999; Ookata et al, 2000; Shimura et al, 2001), although little is known about its functional role. Studies in retinal pigment epithelial cells indicate that the channel may play an integral role in setting resting membrane potential and modulating K^+ recycling (Shimura et al, 2001). A rare genetic mutation in Kir7.1 causes autosomal-dominant snowflake vitreoretinal degeneration characterized by congenital degeneration of ocular tissues including the vitreous (Hejtmancik et al, 2008). Kir7.1 has also been linked to developmental pathways. For example, the jaguar/obelix mutation in zebra fish renders the Kir7.1 homologue non-functional. As a result, melanophores fail to respond appropriately to external cues, leading to melanosome aggregation and the phenotype of a broader striping pattern (Iwashita et al, 2006). Kir7.1 may also be involved in palate formation in mice. Kcnj13 was identified as one of 8 genes whose mis-expression correlates with formation of cleft palate in TGF3beta knockouts, though the precise role of the channel remains to be described (Ozturk et al, 2013).

In this study, we demonstrate the crucial importance of Kir7.1 in modulating uterine contractility in mice and humans. We show that the physiological function of Kir7.1 is to maintain a hyperpolarized membrane potential during uterine quiescence and that removal of this hyperpolarizing drive renders the uterus more excitable. Furthermore, we show that Kir7.1 also modulates the action potential waveform, modifying the excitation-contraction cycle by participating in key stages of repolarization. Pharmacological manipulation of this normal physiological process could be an alternative strategy to treat an atonic uterus and obstetric haemorrhage.

Results

The aim of our study was to identify a potassium channel with the appropriate biophysical attributes to regulate the myometrial resting membrane potential during gestation. To identify candidates, we undertook a genome-wide qRT-PCR screen of all known K^+ channels and associated subunits in cDNA pools generated from laser-capture micro-dissected MSM and whole myometrial tissue. Of seven transcripts enriched in MSM (Supplementary Table S1), only KCNJ13 coded for a K^+ channel (Kir7.1) with appropriate biophysical attributes. Kcnj13 transcript levels increased markedly in the pregnant mouse uterus during mid-gestation, peaking on gestational day (GD) 15, and followed by a sharp decline towards term (C57BL/6J mice deliver in the morning of GD19; Fig 1A). KCNJ13 transcript levels (Fig 1B) were also significantly lower ($P < 0.05$) in samples taken from pregnant women in labour at term than in samples from women not in labour. Immunoblot of myometrial lysates from both labour and non-labour samples demonstrated a single immuno-reactive band at ~42 kDa. As positive and negative controls, we also tested human eye and adipose cell lysate, respectively (Fig 1C and Supplementary Fig S1). Furthermore, Kir7.1 immuno-reactivity was expressed in MSMs and was absent in the vasculature in both human and mouse myometrial samples (Fig 1D), supporting the specificity of the laser-capture screen.

To determine the functional significance of this K^+ channel in the uterus, we first investigated its electrophysiological properties in freshly dissociated mouse MSMs. Under voltage-clamp conditions, an inwardly rectifying potassium current (Fig 2A,B) was inhibited by VU590, a known Kir7.1 inhibitor (Lewis et al, 2009). Consistent with the finding that Kir7.1 expression was higher at GD15 than GD18, the VU590 sensitive current density at -150 mV was significantly greater ($P < 0.05$) on GD15 when compared to GD18 (Fig 2C).

To understand the function of Kir7.1 in the generation of the myometrial action potential, we modelled the potential impact of changes in Kir7.1 channel density on myometrial electrogenesis using a Hodgkin-Huxley type current summing model. In free-running simulations of membrane potential, increasing Kir7.1 channel density (within the range measured in our experimental data) hyperpolarized resting membrane potential and decreased calcium entry during the action potential (Fig 3). Furthermore, simulations predicted that overall membrane conductance during the excited phase of the action potential is so finely balanced that small changes in Kir7.1 current density exerted large effects on membrane potential. Given its expression profile and biophysical properties, we hypothesized that Kir7.1 is a key regulator of myometrial membrane potential during gestation.

To assess the role of Kir7.1 in regulating uterine contractility experimentally, we used lentiviral vectors expressing miRNA targeting Kcnj13 or the human Kir7.1 channel to inhibit and over-express the channel in murine MSM, both in vitro and in vivo. Knockdown of Kir7.1, in vitro, significantly increased the contractile activity integral (2.5-fold) (area under the time-force curve), duration (2.2-fold) and maximum force (1.3-fold) of phasic contractions when compared to scrambled miRNA control (Fig 4 and Supplementary Fig S2). Conversely, over-expression of Kir7.1 significantly decreased all three parameters. To determine whether these alterations in contractility were due to electrogenic effects, we used sharp microelectrodes to measure membrane potentials in the treated myometrial strips. Knockdown of Kir7.1 depolarized resting membrane potential with extended excited periods (Fig 5A,B), whereas over-expression of Kir7.1 hyperpolarized resting membrane potential and suppressed excitability (Fig 5C). Mean resting membrane potentials differed significantly between the two treatment groups (Fig 5D).
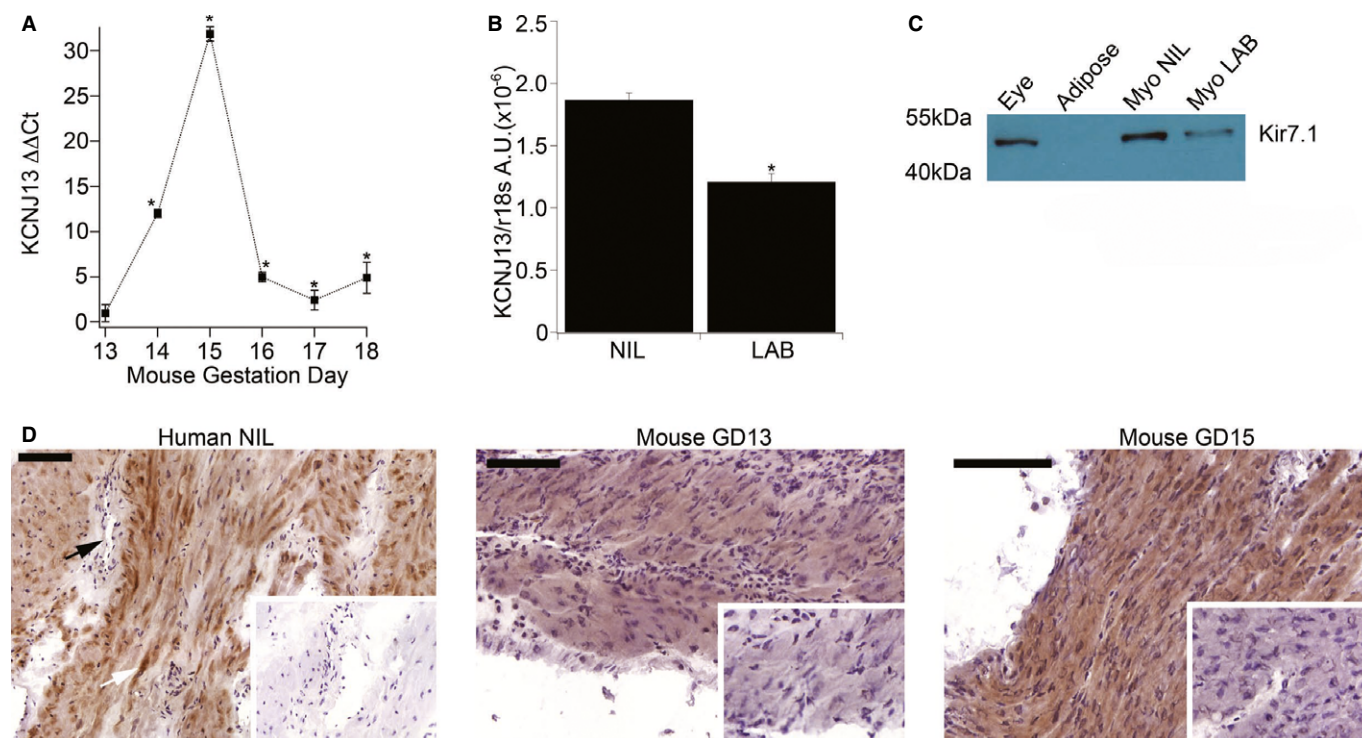
Figure 1. Kir7.1 is expressed in uterine myocytes and is regulated in pregnancy in mice and humans.

A   mRNA expression of *Kcnj13* in mice ($n = 5$; mean $\pm$ SD, per GD) normalized to GD13. *$P < 0.05$, Student's *t*-test.
B   mRNA expression of *KCNJ13* (plotted as arbitrary units relative to 18s rRNA) in human myometrial samples from women at term not in labour (NIL) and at term in labour (LAB) ($n = 8$; mean $\pm$ SD, per group). *$P < 0.05$, Student's *t*-test.
C   Immunoblot of pooled lysates from eye, adipose tissue, NIL and LAB myometrium ($n = 4$) probed with antibody to human Kir7.1 (full blot available in Supplementary Fig S1).
D   Immunohistochemistry for Kir7.1 in human NIL myometrium (left panel), GD13 murine myometrium (centre panel) and GD15 murine myometrium (right panel). Arrow indicates absence of staining in blood vessel. Inset panels show tissue treated with pre-absorbed primary antibody control counterstained with haematoxylin. Scale bar = 100 μm.
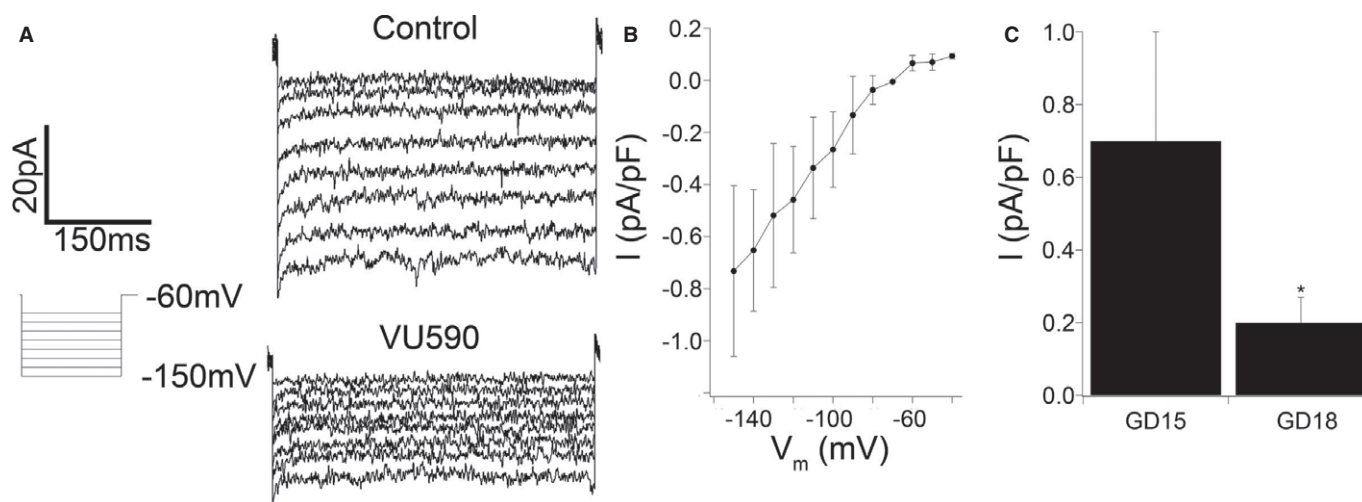
Figure 2. Kir7.1 current in uterine myocytes decreases from mid-pregnancy to term.

A   Measurement of inwardly rectifying, VU590-sensitive current in freshly dissociated GD15 murine myometrial cells. Shown are voltage-clamp recordings in the presence and absence of 10 μM VU590.
B   Current–voltage relation ($n = 5$; mean $\pm$ SD, per data point) of current density [VU590 subtracted from control (vehicle alone)].
C   Current density (pA/pF) at -150 mV and 500 ms in freshly dissociated murine myometrial cells from GD15 and GD18 ($n = 5$ mean $\pm$ SD, per GD). *$P < 0.05$, Student's *t*-test.
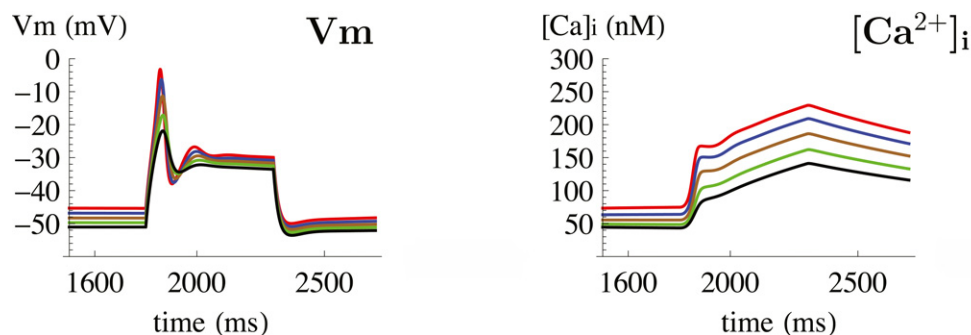
Figure 3. A free-running simulation of the effect on the myometrial action potential waveform of increasing densities of Kir7.1.

Time-dependent effect of increasing Kir7.1 channel densities on V_m (mV, left) and $[Ca]_i$ (nM, right). Increasing density of Kir7.1 within experimentally determined values hyperpolarizes resting membrane potential, whereas decreasing membrane excitability during depolarizing excursions in V_m leading to decreased calcium entry.

Ion channel function *in vivo* may differ to that observed *in vitro*, because of either effects of the sample preparation, or influence of *in vivo* factors not captured *in vitro*. To account for these potential experimental confounders and to assess the phenotype of targeting Kir7.1 during pregnancy, we injected mice uteri on GD9 with anti-Kir7.1 miRNA or scrambled miRNA control. To record uterine activity, we surgically implanted a pressure catheter with remote telemeter into the injected horn of both knockdown and control mice. Intra-uterine pressure was recorded continuously, and the animals were monitored by video. Mice in which Kir7.1 was knocked down had significantly increased intra-uterine pressure when compared to control mice (Fig 6 and Supplementary Fig S3). Consistent with our findings that Kir7.1 expression and channel activity were higher at GD15 than GD18, the effect on intrauterine pressure was more pronounced in mid-gestation.
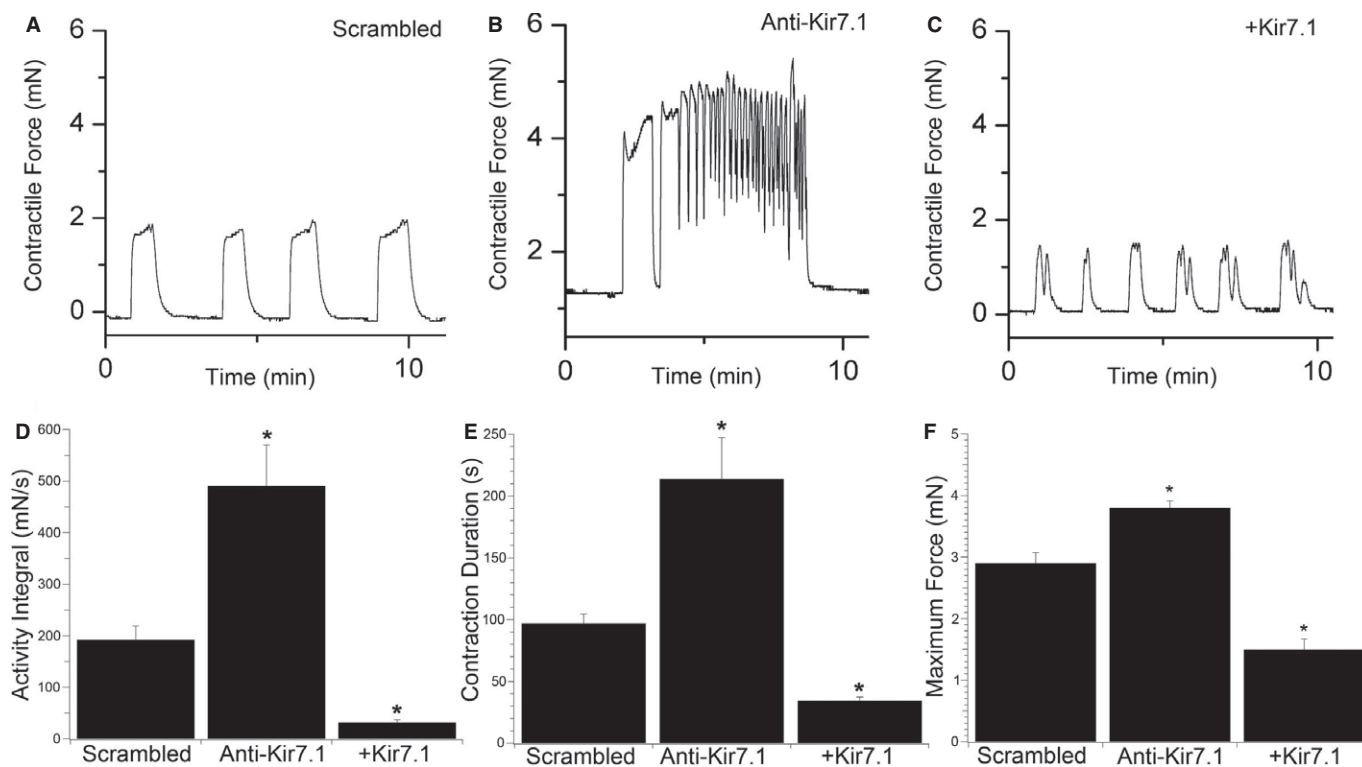
Figure 4. Knockdown of Kir7.1 *in vitro* increases myometrial activity and promotes tonic contractions.

A–C Representative time-force recordings of phasic contractions demonstrating (A) the effect of scrambled miRNA compared to (B) knockdown (Anti-Kir7.1) and (C) overexpression (+Kir7.1) of Kir7.1 on contractility in murine GD15 myometrial strips.

D–F Mean data are summarized ($n = 8$; mean ± SD, per group of experiments) as activity integral (area under the time-force curve) (D), contraction duration (E) and maximum force (F). *$P < 0.05$, compared to scrambled control by ANOVA with Tukey's *post hoc* test.
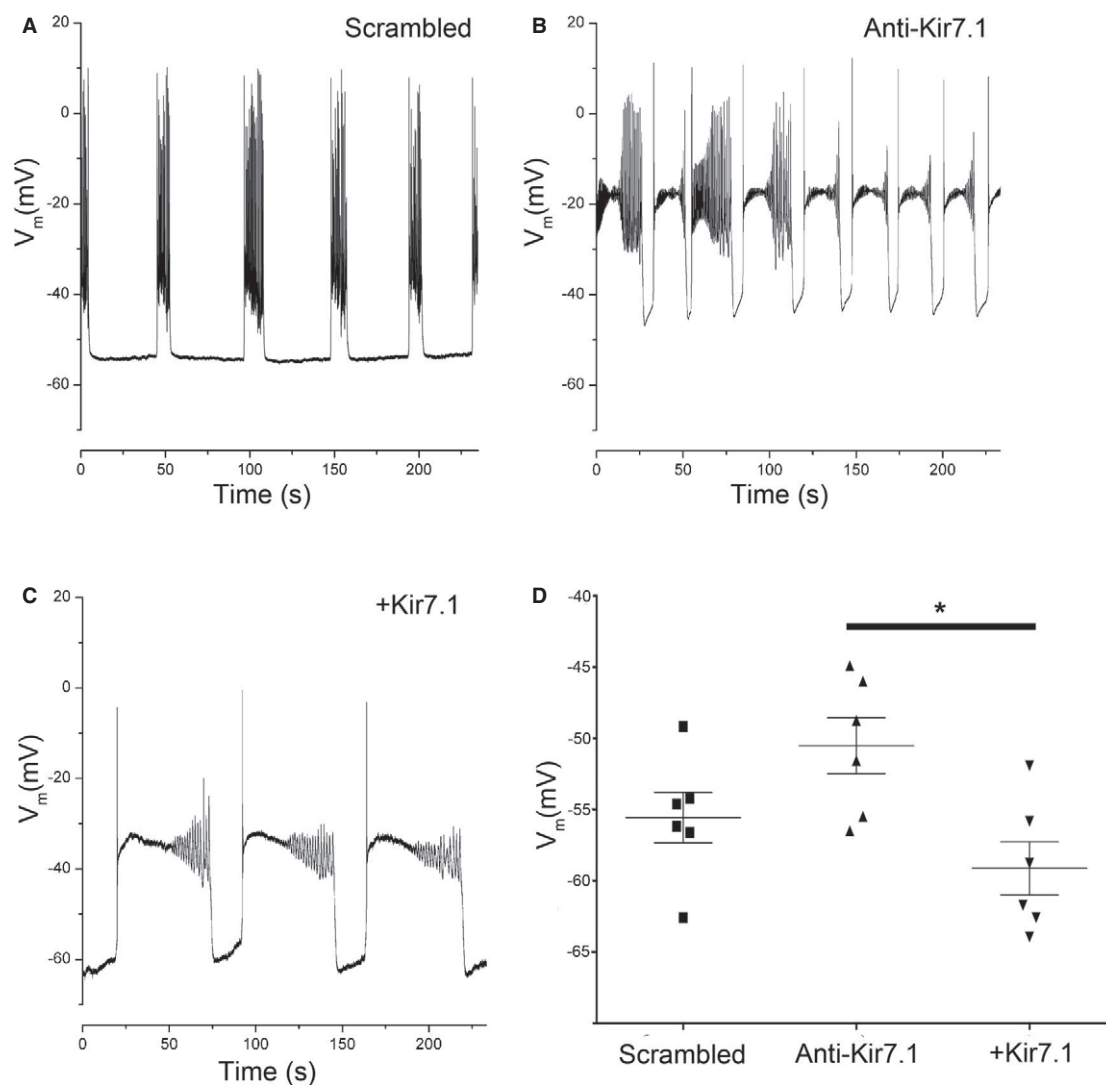
Figure 5. Knockdown of Kir7.1 *in vitro* depolarizes resting membrane potential.

A–C Representative membrane potential recordings in current clamp configuration from murine myometrial strips (A) treated with scrambled control miRNA (B) treated with Kir7.1 knockdown (Anti-Kir7.1) and (C) overexpressing Kir7.1 (+Kir7.1).

D Resting membrane potential in current clamp configuration from murine myometrial strips (*n* = 6; mean ± SD) from experiments depicted in (5A–C). *P < 0.05 by Student's *t*-test.

The increased contractility associated with Kir7.1 knockdown raised the possibility that pharmacological targeting of this ion channel could be of clinical value, for example in the management of severe post-partum haemorrhage. To assess this possibility, we first performed current clamp microelectrode recordings of spontaneous action potentials in isolated murine myometrial strips. We observed slow, inter-contraction, depolarization of resting membrane potential followed by transient complex action potentials (Fig 7). Administration of 10 μM VU590 rapidly depolarized resting membrane potential to threshold (Fig 7Ai), followed by a sustained plateau potential that was reversible on wash out (Fig 7Aii and Aiii). In myometrial strips from women, application of VU590 increased the activity integral, largely due to an increase in contraction duration (Fig 7B), with contractions lasting several hours observed.

To assess the potential therapeutic benefit of inhibiting Kir7.1, we compared the effect of VU590 with oxytocin, the established front-line treatment for post-partum haemorrhage. The effect of VU590 on contractile force in samples taken from GD15 and GD18 mice was dependent on dose and gestation (Fig 8A) and when used in combination with oxytocin, increased activity integral by 172 ± 14 fold and 90 ± 42 fold on GD15 and GD18, respectively, as compared to 4 ± 2 fold and 8 ± 3 fold for OXT treatment alone (Fig 8B). The effect of VU590 was similar in human term myometrium, which was also dose-dependent, with the observed increase in activity integral, largely due to an increase in contraction duration (Fig 8C,D). Importantly, sufficient channels remain at term to generate a significant phenotypic effect, underscoring the potential post-partum benefit of pharmacological intervention.
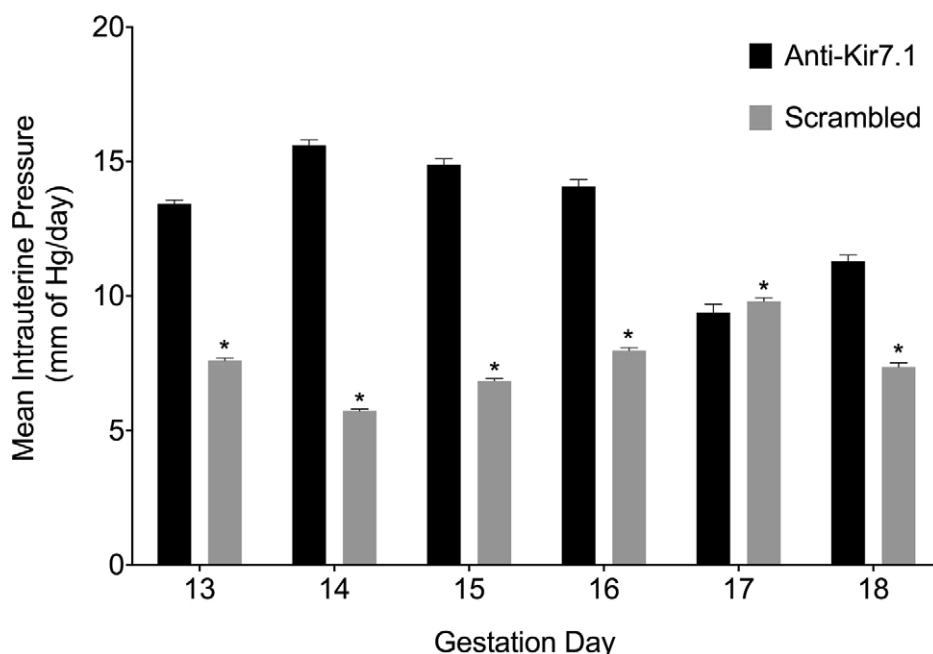
Figure 6.   Knockdown of Kir7.1 *in vivo* significantly increases intrauterine pressure.
Mice in which Kir7.1 was knocked down (Anti-Kir7.1) had significantly increased intrauterine pressure when compared to mice injected with scrambled miRNA lentivirus (scrambled) from GD13 to GD18 (Fig 6 and Supplementary Fig S3). (n = 6 per time point). *P < 0.05; see methods for statistical model.

To expand the pharmacological tools for Kir7.1 inhibition, we used population patch-clamp technology to screen a library of known ion channel inhibitors in a Chinese Hamster Ovary cell line expressing Kir7.1 (Supplementary Fig S4 and Supplementary Methods). We identified compounds with different structures than VU590 to assess whether the phenotype of Kir7.1 inhibition was similar (Fig 9A). We also manufactured a control compound 1,3-Bis (5-nitro-1H-benzo[d]imidazol-2-yl)propane (BNBI), which is known to inhibit the structurally related Kir1.1 (Renal outer medulla potassium channel) but does not inhibit Kir7.1 (Bhave *et al*, 2011). Administration of 100 μM BNBI had no detectable effect on uterine contractility in the GD15 mouse (Fig 9B), suggesting that loss of efficacy for Kir7.1 inhibition is associated with loss of pro-contractile activity. Finally, administration of 10 μM MRT2000769, a potent Kir7.1 inhibitor that is structurally unrelated to VU590, induced long-lasting contractions similar to those observed with VU590 (Fig 9C). The effectiveness of MRT2000769 further supported the correlation between effective Kir7.1 inhibition and the phenotype of long-lasting contractions (Supplementary Fig S5).

Discussion

Inwardly rectifying potassium channels are known to regulate diverse but important physiological processes such as insulin secretion in the pancreas, regulation of the cardiac action potential, para-sympathetic stimulation and potassium reuptake in the kidney (Hibino *et al*, 2010). In most cases, the channels act to hyperpolarize resting membrane potential by remaining persistently open, allowing the efflux of potassium. In excitable tissues, this dampens electrical activity, while in epithelial cells, the potassium gradient created in combination with energy-dependent ion pumps is used to transport other ions. In secretory cells, inhibition of the current causes depolarization and calcium entry leading to a secretion event (Liu *et al*, 2001; Ashcroft, 2005; Hebert *et al*, 2005). The important physiological roles of Kir channels are underscored by the many diseases that are associated with Kir channel malfunction, such as Bartter's syndrome, Anderson syndrome, short Q-T syndrome and neonatal diabetes (Derst *et al*, 1997; Andelfinger *et al*, 2002; Edghill *et al*, 2004; Priori *et al*, 2005; Ellard *et al*, 2007).

Here we present the novel finding that Kir7.1 is a crucial regulator of membrane potential in uterine myocytes during pregnancy in both mice and humans. In mid-gestation, high expression of Kir7.1 keeps the resting membrane potential close to the reversal potential for potassium, increasing the depolarizing drive required to initiate an action potential, calcium entry and subsequent contraction. At term, this damping of excitability is lost by reduction, but not complete loss, of Kir7.1. Our results also indicate that inhibition of Kir7.1 when combined with oxytocin administration is synergistic, a mechanism that may be related to the sensitivity of Kir7.1 to intracellular phosphatidylinositol 4,5-bisphosphate depletion (Pattnaik & Hughes, 2009). Such a mechanism, when acting in conjunction with a decreased gap junction density and decreased receptors to stimulatory ligands, could provide a robust means of maintaining uterine quiescence during gestation (Garfield *et al*, 1977; Fuchs *et al*, 1982; Smith, 2007).

Within the Kir superfamily, Kir7.1 displays several unique properties such as low sensitivity to Ba^{2+} and Cs^+, low single channel conductance, no internal block by Mg^{2+} ions, and a relative insensitivity to external K^+ concentration, tetraethylammonium (IC_{50} > 10 mM) or 4-aminopyridine (IC_{50} ~10 mM) (Krapivinsky *et al*, 1998). In *in vitro* expression systems, Kir7.1 exhibits rapid
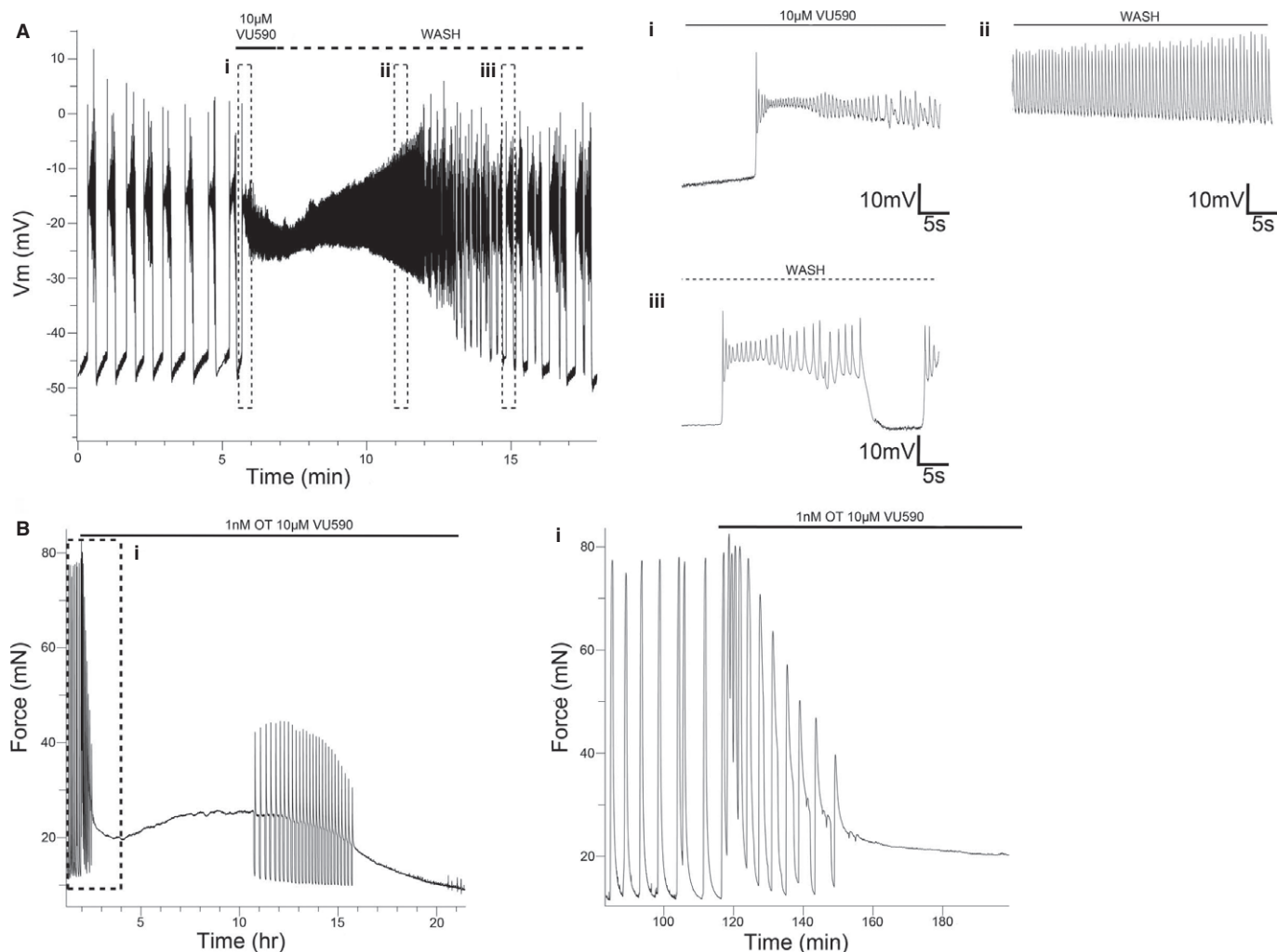
Figure 7. Pharmacological inhibition of Kir7.1 *in vitro* in human and murine myometrium induces membrane depolarization and long-lasting contractions.

A Representative membrane potential recording in current clamp configuration from a murine GD15 myometrial strip. (i) Addition of 10 μM VU590 depolarizes resting membrane potential (note slope change from resting potentials of preceding phasic bursts) and leads to a sustained plateau potential. (ii) Upon washout, spike potentials recover as plateau potential hyperpolarizes. (iii) On complete washout, phasic bursting resumes.

B Addition of 1 nM oxytocin and 10 μM VU590 to human myometrial strips stimulates long-lasting contractions. (i) Initial component of the response is phasic, followed by establishment of a tonic contraction.

activation kinetics and is essentially non-inactivating at potentials negative to 40 mV with a small single channel conductance in both recombinant and native cells (Doring *et al*, 1998; Krapivinsky *et al*, 1998; Shimura *et al*, 2001). Since the channel has a low conductance and little voltage or time dependence in physiological ranges, high expression of this channel gives rise to a stable and hyperpolarized resting membrane potential. Thus, the biophysics of Kir7.1 is ideally suited to regulating myometrial smooth muscle cells during mid-gestation quiescence.

Transgenic mouse models have demonstrated the critical nature of control of myometrial membrane potential during parturition. For example, mice overexpressing the small conductance potassium channel SK3 suffer acute uterine dystocia, and both mother and pups die during delivery (Bond *et al*, 2000). Similarly, mice harbouring a smooth muscle specific deletion of the important uterine gap junction protein connexin43 demonstrate a significant delay

in delivery and increased mortality of pups (Doring *et al*, 2006). These data, in conjunction with our *in vivo* observations demonstrating a significant increase in intrauterine pressure in the absence of endocrine changes, underline the importance of the development of uterine excitability during gestation to the overall delivery process. It is notable that the decrease in Kir7.1 expression precedes progesterone withdrawal in the mouse suggesting that modulation of excitability is, at least in part, independent of progesterone. These biophysical factors act in addition to, and in concert with, other endocrine/paracrine changes that alter uterine stimulants (Cha *et al*, 2013) and lead to uterine disorders such as preterm labour or dysfunctional labour. The overall role of membrane potential in the control of parturition in the context of other controlling factors is summarized in Fig 10.

In addition to a clear effect on resting membrane potential, our computer modelling predicted that increasing Kir7.1 activity
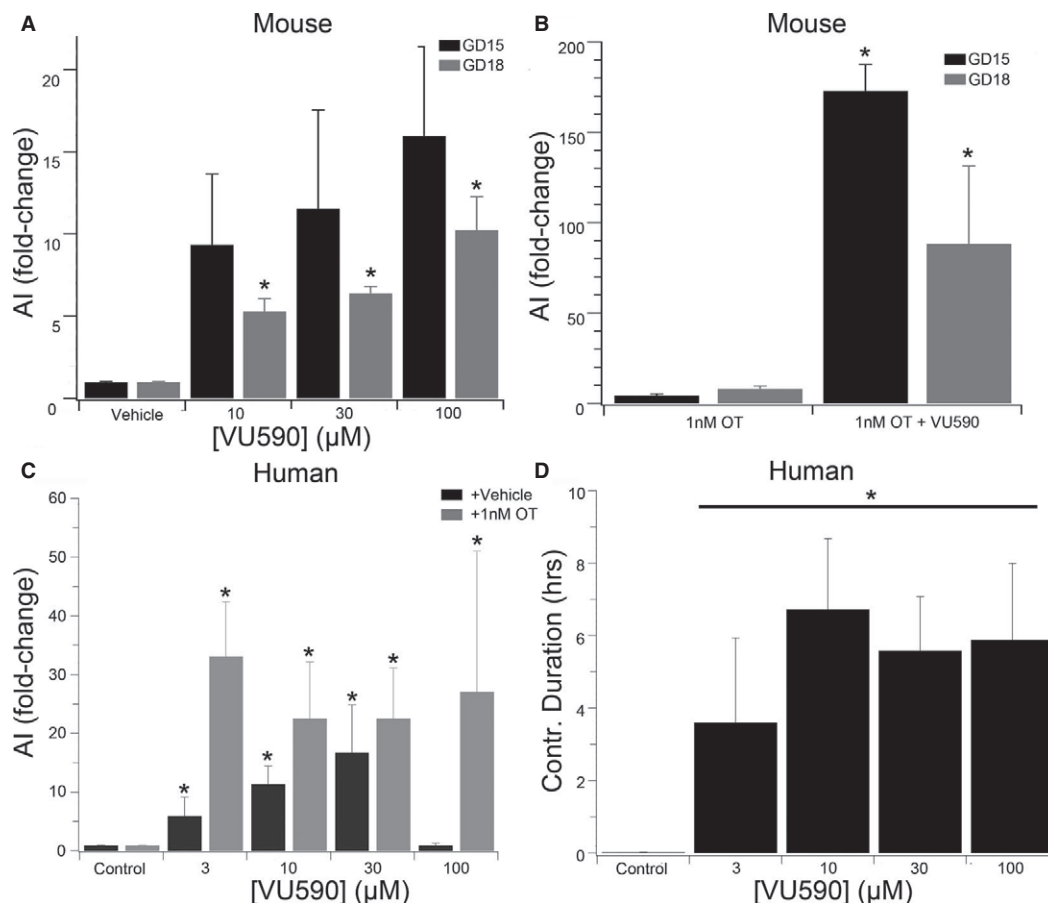
Figure 8. Pharmacological inhibition of Kir7.1 *in vitro* in human and murine myometrium stimulates longer-lasting contractions than oxytocin.

A   Dose- and gestation-dependent effect of VU590 on murine myometrial contractility [$n = 5$, activity integral expressed as a fold-change of pre-treatment contractions (mean $\pm$ SD, per GD)]. *$P < 0.05$ GD18 vs GD15 per dose by ANOVA with Tukey's *post hoc* test.

B   As in (A) but comparing the effect of 1 nM oxytocin with 1 nM oxytocin + 10 μM VU590. *$P < 0.05$ OT vs OT = VU590 per GD by Student's *t*-test.

C   Dose-dependent effect of VU590 (Black Bars) and VU590 + 1 nM oxytocin (grey bars) on human myometrial contractility ($n = 8$; activity integral expressed as fold-change over pre-treatment contractions, mean $\pm$ SD). *$P < 0.05$ compared to control by ANOVA with Tukey's *post hoc* test.

D   Dose-dependent effect of VU590 on contraction duration in human myometrial strips taken at term ($n = 8$, mean $\pm$ SD). *$P < 0.05$ compared to control by ANOVA with Tukey's *post hoc* test.

modulates calcium entry during the plateau phase of the complex action potential, resulting in a decrease in contractile force and inefficient contractions. Decreasing Kir7.1 has the effect of stressing the repolarizing drive such that increased contributions from other K^+ channels are required to ensure action potential repolarization (Supplementary Fig S6) (Fink *et al*, 2006; Greenwood *et al*, 2009; McCallum *et al*, 2011). These effects explain the longer action potentials observed during pharmacological inhibition and experimentally induced reduction in the expression of Kir7.1 protein. When Kir7.1 current density is reduced to very low levels, the myometrial smooth muscle effectively loses phasic behaviour and manifests a new tonic-like tone. The greatly increased contraction length induced by Kir7.1 inhibition may be useful for reducing blood loss in an atonic uterus. As a treatment for post-partum haemorrhage, Kir7.1 block would have the advantage of being more potent than current first line treatments. The mechanism of action also circumvents the agonist pathways (Fig 10) targeted by current treatments that are prone to desensitization during failed labour inductions.

Materials and Methods

Ethical approval

All animal procedures complied with the guidelines for the care and use of animals set forth by the National Institutes of Health. The Animal Studies Committee at Washington University in St. Louis approved all protocols (protocol number 20110138 to Sarah K. England). Adult C57BL/6J (Jackson Laboratory) female mice were mated at 8 weeks of age until 6 months of age. Mice were mated for 2-h time periods, and the presence of a copulatory plug was marked 0 days postcoitum (dpc). Animals were housed in Washington University School of Medicine vivarium in the BJCIH building, which is an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International)
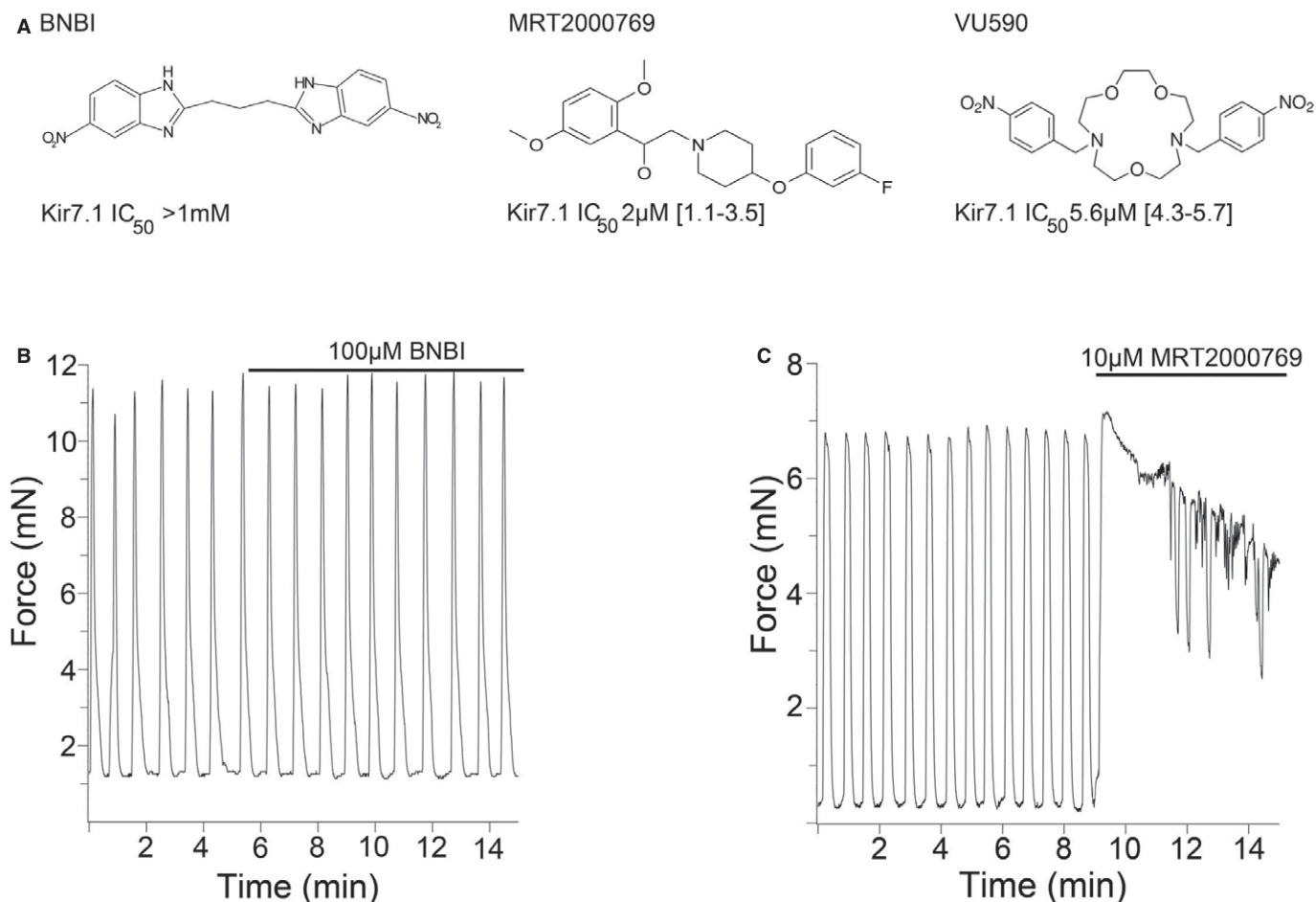
Figure 9. Stimulation of uterine contractions correlates with Kir7.1 inhibitory potency.

A   The structures of the three compounds tested in this study. BNBI, a potent Kir1.1 inhibitor does not inhibit Kir7.1. MRT2000769 is structurally unrelated to VU590 and was identified as inhibiting Kir7.1 in a high throughput electrophysiology screen. VU590 is the first described inhibitor of Kir7.1.

B, C   Effects of BNBI (B) and MRT2000769 (C) on murine GD15 myometrial contractility.

approved animal care facility. For *in vivo* intrauterine pressure measurements, eight pregnant mice were injected with scrambled miRNA and eight pregnant mice were injected with Kir7.1 miRNA.

Subject criteria and selection

Subjects were recruited into two groups, spontaneous labour (LAB) and elective caesarean section not in labour (NIL) between 38 and 40 weeks gestation. The LAB group was undergoing caesarean section for reasons of undiagnosed breech. LAB was defined as regular contractions (< 3 min apart), membrane rupture and cervical dilatation (> 2 cm) with no augmentation.

Sample collection

At caesarean section, samples were collected before syntocin administration by knife biopsy from the lower uterine segment incision. Samples were washed briefly in saline and flash-frozen in liquid nitrogen for mRNA, immunoblot or immunohistochemistry

analyses. Samples for cell isolation or contractility experiments were placed in ice-cold modified Krebs–Henseleit (m-KHS) solution and used the same day.

Laser capture screen

mRNA was extracted from 100 mg of frozen human myometrium using Trizol reagent (Invitrogen), and further column purified by RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA was quantified by spectrophotometer and further tested for quality and purity by bioanalyser (Agilent Technologies) according to the manufacturer's instructions. cDNA was generated from 100 ng of mRNA using Superscript III (Invitrogen) according to the manufacturer's instructions and stored at −80°C until qRT-PCR analysis.

Laser Capture Microdissection (LCM): All slides, LCM caps, Haematoxylin and Eosin (H&E) Staining Kit for LCM and solutions were obtained from Molecular Machines & Industries. Briefly, cryomold-mounted myometrial samples were transferred from −70°C and equilibrated in a pre-cooled cryostat (−30°C) for 10 min. Sections (8 µM) were cut and H&E stained according to

Figure 10. Model of the role of Kir7.1 in maintenance of uterine quiescence.

In normal labour in the human myometrium, changes take place over a number of weeks that increase both the intrinsic electrical excitability of the cell (blue box) and, by altering cellular receptors and contractile machinery, the susceptibility to stimulation. Alterations in stimulation either by infection or gene-environment interaction can precipitate preterm labour (Cha et al, 2013); however, changes in control of the myometrial membrane potential or interference with functional gap junctions can affect labour even under normal endocrine conditions (Bond et al, 2000; Doring et al, 2006). In this study, we demonstrate that alteration of the function of a single potassium channel profoundly alters uterine contractility. Loss of Kir7.1 function depolarizes the plasma membrane and promotes voltage-gated calcium entry. In addition, the duration of the depolarization is extended, preventing the normal phasic contractions. In this way, the process of excitation-contraction coupling in uterine myocytes overrides pharmaco-contraction coupling. This effect could be used for therapeutic benefit. (Key: CPI-17 = C-kinase potentiated protein phosphatase-1 inhibitor. CX26 = Connexin 26. CX43 = Connexin 43. CX45 = Connexin 45. GEFs = Guanine nucleotide exchange factors. GPCR = G-protein coupled receptors. IP3 = inositol 1,4,5-trisphosphate. IP3R = inositol 1,4,5-trisphosphate receptor. MLC = Myosin light chain. MLCp = Phosphorylated myosin light chain. PIP2 = Phosphatidylinositol 4,5-bisphosphate. PLC β = Phospholipase C. RHOgtp = Ras homologue gene family, member A. RHO-K = Rho-associated, coiled-coil containing protein kinase 1.)

the manufacturer's instructions. Slides were dehydrated in graded alcohols and xylene and placed in a desiccator until laser capture. At least 50,000 μm^2 of human uterine myocytes was captured for each gene. mRNA was extracted from each isolation by using the Picopure RNA isolation kit (Arcturus) according to the manufacturer's instructions. Reverse transcription was as previously described. PCR primer design and qRT-PCR are described in the Supplementary Methods.

Immunohistochemistry

Frozen sections (8 μM) were fixed in ice-cold acetone for 5 min prior to incubation in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by immersing the slides for 30 min in freshly prepared 0.3% hydrogen peroxide in PBS. Slides were pre-incubated in 1.5% non-immune goat serum in PBS for 30 min at room temperature and then in primary anti-Kir7.1 antibody (1:100; Alomone) overnight at 4°C in a humidified chamber. Primary

antibody pre-absorbed for 24 h with recombinant antigen served as negative control. Staining was visualized using the Vectastain Elite ABC rabbit IgG kit (Vector Laboratories) according to the manufacturer's instructions.

Immunoblot

After tissue was suspended in RIPA lysis buffer containing protease inhibitor cocktail tablets, mechanically homogenized and cleared by centrifugation, the supernatant was aliquoted and frozen. Whole tissue and protein lysates from adult human eye, adult mouse adipose and adult mouse brain were purchased from Novus Biologicals (Cambridge, UK) at a stock concentration of 5 mg/ml. All protein concentrations were confirmed with the BioRad assay (Bio-Rad laboratories, Hemel Hempstead, UK). A total of 40 μg of protein per sample (pooled from 4 biopsies each for NIL and LAB samples) was subjected to SDS-PAGE according to standard protocols. The membrane was blocked in 5% milk protein solution (Marvel, Lincs, UK) for 1 h at room temperature, incubated with primary rabbit polyclonal anti Kir7.1 antibody (1:200; Alomone Labs, Jerusalem) overnight at 4°C in blocking buffer and then incubated with polyclonal goat anti-rabbit HRP secondary antibody (1:100; Dako, Ely, UK). ECL Plus (GE Healthcare LTD, Amersham place, UK) was used to detect signal. To confirm equal loading, the blot was treated with Restore western blot stripping buffer (Thermo Fisher Scientific, Hemel Hempstead, UK) for 15 min at room temperature, washed, blocked and re-probed with an antibody to human β-actin.

Electrophysiology

Cell isolation

Strips of myometrium from the longitudinal layer ($2 \times 2 \times 20$ mm) of time-mated C57BL/6J mice were isolated and washed in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) at 37°C for 10, 20 and 30 min, respectively, followed by 45 min incubations in digestion solution (Roche Blendzyme 3) at 37°C according to the manufacturer's instructions. Digestion was terminated by several dilutions with fresh HBSS. Cells were dispersed by slow trituration through a wide-bore fire-polished glass pipette in HBSS. Single myometrial cells were filtered through a 200-μM gauze and stored in HBSS for use within six hours.

Voltage clamp

A drop of myometrial cell suspension was placed in a glass-bottomed Petri dish and mounted on the stage of an inverted microscope (IX51, Olympus). After settling (~ 10 min), cells were perfused with bath solution at a rate of 1–2 ml/min at 37°C. Patch pipettes were fabricated (Model P-87; Sutter Instruments, Novato, CA, USA) from 1.5-mm glass capillaries and had a resistance of 2.0–4.0 MΩ when filled with pipette solution (containing in mM: KCL 140; EGTA 1.1; $CaCl_2$ 0.06; Hepes 10; $MgCl_2$ 2; adjusted to pH 7.2 at 25°C with 5 m NaOH). Liquid junction potential was zeroed prior to seal formation. Transmembrane currents were recorded with an amplifier (Axopatch 700b; Axon Instruments) using the perforated patch configuration of the whole cell patch-clamp technique (Rae *et al*, 1991). The antibiotic amphotericin B (720 μg/ml) was used to perforate the cell membrane. Series resistance was compensated after membrane perforation. Currents were

elicited by stepping to a range of potentials between −150 mV and +80mV from a holding potential of −60 mV. To isolate currents that were sensitive to inhibition by drug application, difference currents were obtained by electronic subtraction of traces. Currents were filtered at 10 kHz and sampled at 5 kHz. Voltage protocols were delivered via a Digidata 1440a computer interface using pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA, USA).

Current clamp

Strips (5×10 mm) of murine myometrium from the longitudinal layer were pinned out on a sylgard base and perfused with m-KHS containing (in mM: NaCl, 133; KCl, 4.7; Tes, 10; glucose, 11.1; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; adjusted to pH 7.4 at 25°C with NaOH) at 37°C on an upright microscope (MVX10, Olympus). Tissue was incubated with 5 μM wortmannin (Sigma) to prevent spontaneous contractions from dislodging impalements. Smooth muscle cells were impaled with glass microelectrodes filled with 2 M KCl of resistance 80–120 MΩ. Transmembrane potentials were recorded with an amplifier (Axopatch 700b; Axon Instruments) and a Digidata 1440a computer interface running pCLAMP 9.0 software.

In vitro knockdown and contractility

Strips of GD15 or GD18 myometrium from the longitudinal layer ($2 \times 2 \times 20$ mm) were washed in sterile Ca^{2+} and Mg^{2+} free HBSS DMEM/F-12. Strips were placed (in triplicate) under 1.5× slack length tension in media containing 2% dextran-coated charcoal-treated foetal bovine serum with 0.5 mM 8-bromo-cAMP (Sigma), 10^{-6} M medroxyprogesterone acetate (Sigma) and pLenti6-cppt-CMV-mCherry-mouse 543A miRNA (Anti-Kir7.1), pLenti6-cppt-CMV-mCherry-neg miRNA (Scrambled), or pLenti6-cppt-CMV/TO-humKir7.1-IRES-mCherry-opre (+Kir7.1). Construction of lentiviral vectors is detailed in the Supplementary Methods.

On day five, strips were placed under 2mN tension in a four channel flatbed organ bath (DMT) in m-KHS solution. Isometric force was recorded on ADI Instruments LABCHART software. Activity integral was measured as the area under the time-force curve (mN/s) over a 20-min period. Contraction duration was determined as the mean duration (rise and fall to baseline) of contractions within a 20-min period. Maximum force was determined as the peak force measurement within a 20-min period. All measurements were made over the same time period for all strips.

In vivo intrauterine pressure measurements

C57BL/6J time-mated mice were anesthetized on GD8 to GD10 by intraperitoneal injection of ketamine (100 mg/kg) plus xylazine (10 mg/kg, IP), and a PhysioTel PA-C10 transmitter (Data Sciences International) was implanted in one horn of the pregnant uterus between the uterine wall and foetal sacs. At this time, 3.7×10^5 particles/ml of pLenti6-cppt-CMV-mCherry-Neg miRNA (scrambled) or pLenti6-cppt-CMV-mCherry-miRNA mouse Kir7.1 (Anti-Kir7.1) were injected into the uterine muscle of the implanted horn. Five days later, uterine pressures were continuously measured every 10 s at 500 Hz with Dataquest A.R.T. data acquisition system version 4.31 (DSI) for 4–8 days.

The paper explained

Problem

Abnormal uterine activity has profound health consequences for both mother and infant as well as health services and national wealth. Clinical conditions associated with abnormal uterine activity are preterm labour (PTL), dysfunctional labour and post-partum haemorrhage (PPH). Preterm birth is the biggest cause of neonatal mortality and morbidity. Dysfunctional labour leads to operative vaginal and abdominal delivery with its inherent maternal risks, and PPH is a major global cause of maternal morbidity and mortality accounting for around 25% of deaths in post-partum mothers in developing nations. The challenge is to understand both the heterogeneous aetiology of these conditions and to develop new methods to manipulate uterine function.

Results

Uterine contractions are fundamentally controlled by complex electrical signals that regulate calcium entry at the plasma membrane of the uterine myocytes. Our findings have identified Kir7.1 as a novel regulator of electrical activity of the uterus during gestation in both mice and humans. We found that the expression of Kir7.1 was regulated in pregnancy and that decreasing Kir7.1 activity *in vitro* and *in vivo* greatly increased uterine activity. Conversely, increasing Kir7.1 expression inhibited uterine contractions. We found that this effect was mediated by altering the uterine resting membrane potential and action potential. Finally, we identified novel pharmacological inhibitors of Kir7.1 that stimulated uterine contractions with greater efficacy than the currently used uterine stimulant oxytocin.

Impact

Our findings have identified Kir7.1 as a key regulator of uterine activity during gestation. The results of this study add to our understanding of the underlying mechanisms that control uterine activity during gestation. The development of new inhibitors to Kir7.1 may be a novel tool for manipulating uterine function for the treatment of dystocia and post-partum haemorrhage.

Data analysis and statistics for telemetry studies

Longitudinal changes in intrauterine pressure after infection with pLenti6-cppt-CMV-mCherry-Neg miRNA (scrambled) or pLenti6-cppt-CMV-mCherry-miRNA mouse Kir7.1 (Anti-Kir7.1) were analysed by using a linear-mixed effect (LME) model implemented by PROC MIXED in SAS 9.3. Before analysis, data were first temporally aligned according to gestational days. The dependent (response) variable was taken as the hourly average intrauterine pressure. Fixed effects were treatment group (scrambled vs miRNA), gestation day and their interaction. Random intercepts were also used to incorporate mouse-specific effects. In addition, a first-order autoregressive correlation structure was used to account for the repeated measurements over time, which implies that the temporal correlation among repeated measures decays as a power function of the time lag. Residuals from initial LME analysis displayed a skewed distribution, and a square-root transformation of the response variable was found to be effective to correct the non-normality in the residuals. A few mice had negative hourly average pressures in certain hours, and 58 such observations (around 3% of the entire data set and split equally between the scrambled and miRNA group) were excluded from the analysis.

Compound screening

Cell line generation, cell culture, and Kir7.1 automated electrophysiology assay and data analysis are all described in the Supplementary Methods. The MRCT ion channel focused compound file was selected in collaboration with the Dundee Hit Finding Unit and consisting of ~ 4,000 compounds. The set covered 119 bioactive templates from nine categories of ion channel targets and molecular weights of the screening compounds ranged between 150 and 450.

Computational simulations

Computational simulations are described in detail the Supplementary data.

Supplementary information for this article is available online: http://embomolmed.embopress.org

Author contributions

HAB, DAR, SKE, JSD, MJT, CK and AMB designed the research; CM, CR, EB, SM, JA, AS, YC, JZ, DT, JJ, PW, TD, SK, DJT, PB, RC and AMB carried out the research; SQ, MV and ST, phenotyped the subjects and provided the samples; HAB, CM, NL, SKE and AMB analysed the data; and AS, HAB, MJT, SKE, JJB and AMB wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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